

EFFECTS OF OBESITY AND TYPE 2 DIABETES ON MOUSE PLATELET AND  
PMN LEUKOCYTE MARKERS OF ACTIVATION

by

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## ABSTRACT

The prevalence of obesity and type 2 diabetes continues to rise. People with obesity and diabetes are at risk of developing ischemic vascular disease due to chronic activation of thrombotic (platelet) and inflammatory (polymorphonuclear [PMN] leukocyte) blood cells. However, little research has examined these blood cell changes in mouse models. Therefore, the purpose of this dissertation was to examine the changes in thrombotic and inflammatory blood markers in response to obesity and diabetes in mice and to explore the impact of these blood changes on ischemic stroke and reperfusion. We studied *db/db*, *ob/ob*, high fat fed, and high fat+streptozotocin (HF+STZ) mouse models of obesity and type 2 diabetes. Using flow cytometry and several measures of platelet aggregation, we did not find significant evidence of platelet or PMN activation in the *db/db*, *ob/ob*, or high fat fed models. However, we found that the HF+STZ mouse expressed increased levels of PMN CD11b and may develop platelet activation with prolonged disease. We found that mice fed a high fat diet develop lower PMN counts in response to hyperglycemia. To our knowledge, this is the first study that examined platelet and PMN activation after middle cerebral artery occlusion (MCAO) in obese, diabetic mice. MCAO in mice is associated with a high mortality. No diabetic mice survived MCAO. However, of the nondiabetic mice that survived MCAO, there was an increase in platelet and PMN activation at 24 hours of reperfusion. Our results indicate that platelet and leukocyte activation in the mouse models we examined do not reflect the blood activation observed in humans with obesity and type 2 diabetes. The HF+STZ model does demonstrate some aspects of platelet and PMN activation and may be the

most suitable for studying these disease processes. Technical difficulties in performing experimental stroke in mice suggest that it may be necessary to examine these blood processes in other animal models or in human studies of stroke and reperfusion. The results of this research may lead to the development of novel biomarkers and treatments for obese, diabetic patients who are at risk for ischemic vascular disease.

## CHAPTER 1

### INTRODUCTION AND LITERATURE REVIEW

#### Significance of the Problem

In 2002, the American Diabetes Association predicted that the number of people diagnosed with type 2 diabetes would reach 14.5 million people in the U.S. by 2010. In only 3 years, this prediction was surpassed, with 14.6 million people diagnosed with type 2 diabetes in 2005 and an estimated 6.2 million that were undiagnosed (CDC, 2005a). This rapid increase in the prevalence of type 2 diabetes is associated with the increasing weight of people in this country (Fox, Pencina, Meigs, Vasan, Levitsky, & D'Agostino, 2006). In 1995, the prevalence of obesity (body mass index (BMI) > 30 kg/m<sup>2</sup>) in the U.S. was 15%, and this number has increased dramatically to 24% in 2005 (CDC, 2005b). In addition, nearly two of every three people in the U.S. are currently overweight (BMI > 25 kg/m<sup>2</sup>) (CDC, 2005b) and it is estimated that nearly 54 million people have pre-diabetes (ADA, n.d.) and are at risk of developing type 2 diabetes in 5-10 years.

The economic cost of diabetes is significant. In 2002, the total cost of diabetes was estimated to be \$132 billion in medical expenditures and lost productivity (ADA, 2002). The resulting economic loss to the US economy was estimated to be \$40 billion (ADA, 2002), which is equivalent to the entire gross domestic product of Cuba (2005 estimate- \$40.06 billion, CIA, 2006). With the continued rise in obesity and type 2 diabetes, the economic burden of treating this disease could be considerable in the next few years. The excessive costs associated with treating type 2 diabetes occur because of

the multi-faceted treatment it requires (medications, supplies, intensive outpatient therapy, home health care, etc) and the increased morbidity (vascular complications) and mortality related to the disease (Williams, van Gaal, & Lucioni, 2002; ADA, 2002). The presence of vascular complications in diabetes increases medical costs 3.5-fold over patients with diabetes without complications, creating hospitalization costs by a total of 450% (Williams, et al., 2002).

People with diabetes are at an increased risk of developing several long term complications due to its complex pathophysiology (Bonow, & Gheorghide, 2004). The complications incurred in type 2 diabetes are due, in part, to prolonged vascular damage leading to chronic micro- and macrovascular disorders, including stroke. Type 2 diabetes mellitus and obesity/pre-diabetes are known independent risk factors for acute ischemic stroke and other cardiovascular disease (Lee, et al., 2001; Adachi, Hirai, Tsuruta, Fujiura, & Imaizumi, 2001, Hu, et al., 2002; Kernan, et al., 2003; Folsom, et al., 1999) and are associated with higher morbidity and mortality from stroke compared to non-obese, non-diabetic subjects (Almdal, Scharling, Jensen, & Vestergaard, 2004; Megherbi, et al., 2003). The relative risk of stroke in people with diabetes mellitus is 1.5-3 fold compared to the general population (Idiris, Thomson, & Sharma, 2006; Folsom, et al., 1999), and increases the risk of stroke 10-fold in people under the age of 55 (You, McNeil, O'Malley, Davis, Thrift, & Donnan, 1997). The cost of stroke related to diabetes is approximately 15.5% of total stroke expenditure (Currie, et al., 1997). Mortality one year after a stroke is twice as high in men and eight times higher in women with diabetes

compared to those without diabetes (Tuomilehto, Rastenyte, Jousilahti, Sarti, & Vartianinen, 1996).

The mechanisms that increase the risk, morbidity, and mortality of stroke in obese and diabetic patients are not completely clear. Obesity and type 2 diabetes are characterized by chronic low level inflammatory and thrombotic pathologies. Recent studies indicate that chronic blood cell activation, both platelet (McDonagh, et al., 2003) and polymorphonuclear leukocyte (Hokama, et al., 1997; Ohmori, et al., 2000; Hand, Hand, & Vasquez, 2006), occurs in people with diabetes. A study by Engstrom and colleagues, (2003) demonstrated that obese, type 2 diabetic patients exhibit elevated levels of proinflammatory mediators compared to non-obese type 2 diabetic patients, and that the increased level of inflammatory mediators was significantly correlated with a higher risk of developing stroke and myocardial infarction, and higher mortality after the event. In addition, pro-thrombotic diseases, such as thrombophilias and atherosclerosis are known to demonstrate a higher risk of stroke (Levine, 2005), and the prothrombotic condition in diabetes is hypothesized to be related to the elevated risk of developing stroke (Idris, Thomson, & Sharma, 2006), but no studies were found analyzing this relationship.

The implications of the rise in the incidence of obesity and diabetes are profound. Not only do obesity and diabetes place a burden on our medical system, but it decreases our economy through loss of work and productivity. The pathogenesis of obesity and type 2 diabetes is complex and affects most physiologic systems creating severe long-term complications. Prolonged vascular damage secondary to low grade activated

thrombotic and inflammatory processes, leads to many of these complications, including cerebral ischemia; and increases the cost, morbidity, and mortality. The mechanisms of chronic blood cell activation related to obesity and type 2 diabetes, however, are still to be completely elucidated. Animal models, particularly rodent models of obesity and type 2 diabetes represent a means by which the cellular mechanisms of the thrombotic and inflammatory changes associated with these diseases can be fully explored. Further, these models can be used to explore the effects of these blood cell changes on the outcomes of stroke and reperfusion. However, little research has been conducted characterizing the thrombotic and blood cell changes in obese and diabetic animal models or how the blood cell changes impact stroke and reperfusion.

### Significance of the Project

The purpose of this dissertation project was to examine the changes in thrombotic and inflammatory blood markers in response to obesity and type 2 diabetes in mice and to explore the impact of these blood changes on the outcomes of ischemic stroke and reperfusion injury. The understanding of the blood changes in mouse models of obesity and type 2 diabetes is necessary in order to understand if the changes are applicable to humans with obesity, diabetes and stroke. The results of this dissertation project may have implications for the future use of blood cell biomarkers of inflammation to predict the early stages and progression of type 2 diabetes that may lead to increased risk for ischemic vascular events. In addition, the result of this work may help to predict the outcomes of diabetic people who experience stroke. Finally, the results of this work may

lead to the development of novel therapeutic targets to reduce thrombotic and inflammatory complications in people with diabetes and ischemic events such as stroke.

### Pathogenesis of Obesity and Type 2 Diabetes

The metabolism of glucose and other macronutrients for normal energy balance and homeostasis are complex and beyond the scope of this work. The following paragraphs describe the functions of two hormones involved in metabolism, insulin and leptin, and their role in developing obesity, insulin resistance, and type 2 diabetes.

#### *Insulin*

Insulin is produced in pancreatic  $\beta$ -cells and is secreted in response to rising levels of blood sugar, amino acids, and hormones released by the gastrointestinal system after a meal (Sonksen, & Sonksen, 2000). Insulin's primary 'excitatory' metabolic functions are the stimulation of glucose uptake by skeletal muscle and adipocytes and lipid synthesis by adipocytes. In addition, insulin's 'inhibitory' metabolic functions include inhibiting lipolysis, proteolysis, glycogenolysis, gluconeogenesis, and ketogenesis. (Sonksen, & Sonksen, 2000). All of these actions, in concert, maintain blood glucose at a tightly regulated range throughout fasting and non-fasting states (70-100mg/dl) (Sonksen, & Sonksen, 2000). The secretion of insulin by the pancreas is dynamic and highly regulated to meet these metabolic needs. For example, after eating, insulin levels rise to meet the demands of rising glucose and then return to baseline as blood sugar drops. Additionally, during fasting, insulin and glucagons regulate hepatic

glucose release at the exact rate of glucose uptake, to maintain blood glucose until the next meal.

Insulin accomplishes its function through interaction with its receptor expressed primarily on liver, adipose, and skeletal muscle cells (Mlinar, Marc, Janez, & Pfeifer, 2006), but is also found on leukocytes and platelets. The insulin receptor is a heterotetramer, that autophosphorylates on tyrosine residues and tyrosine phosphorylates insulin receptor substrate-1 and other substrates upon binding with insulin (Mlinar, et al., 2006). The phosphorylation of the initial insulin receptor substrates provides the foundation for several downstream signaling pathways that propagate insulin's many functions (Mlinar, et al., 2006). One of insulin's functions is to increase glucose transporter protein 4 (GLUT 4) translocation, located in adipose, skeletal muscle, and cardiac myocytes. In the basal state, GLUT 4 resides in intracellular vesicles where it slowly cycles between the plasma membrane and intracellular compartments. After insulin signaling, the vesicles move to the cell membrane, increasing the expression of the transporter, allowing circulating glucose to passively diffuse into the cell (Mlinar, et al., 2006).

In addition to its effects in intermediary metabolism, insulin is known to regulate cell growth and differentiation, activation/repression of transcription, and immune and thrombotic processes (Saltiel, & Pessin, 2003). In a recent study, insulin demonstrated regulation of polymorphonuclear leukocyte functions, increasing adhesion molecule expression (including the integrin CD11b) and promoting chemotaxis (Waland, Guillet, Boire, and Vasson, 2004). Additionally, insulin is known to inhibit platelet aggregation

through reduction in intracellular  $\text{Ca}^{2+}$  mobilization (Ferreira, Eybrechts, Mocking, Kroner, & Akkerman, 2004). These 'non-metabolic' functions of insulin, however, although not as well understood, demonstrate the interwoven complexity between intermediary metabolism and immune and thrombotic processes.

### *Insulin Resistance*

Insulin resistance is a pathologic state in which the body can no longer respond to normal insulin levels, resulting in increased circulating levels of insulin to maintain normoglycemia (Mlinar, Marc, Janez, & Pfeifer, 2006). Insulin resistance is related to increasing levels of obesity (Reaven, Abbasi, & McLaughlin, 2004), particularly visceral adiposity (Kahn, & Flier, 2000) and is improved with weight loss (Dandona, Weinstock, Thusu, Abdel-Rahman, Aljada, & Wadden, 1998). The primary characteristics of insulin resistance are increased lipolysis, impaired glucose uptake by myocytes, and increased gluconeogenesis by the liver (Mlinar, et al., 2006).

Adipocytes, due to hyperplasia and hypertrophy, release proinflammatory cytokines, including TNF- $\alpha$  (Hotamisligil, Arner, Caro, Atkinson, & Spiegelman, 1995; Kern, et al., 1995; Dandona, et al., 1998), interleukin-6 (IL-6, Mohamed-Ali, et al., 1997, Vozarova, et al., 2001), and monocyte chemoattractant protein-1 (MCP-1, Sartipy, & Loskutoff, 2003; Kanda, et al., 2006). Increased levels of TNF- $\alpha$  increase release of free fatty acids (FFA) into the circulation from visceral adipocytes (Mlinar, et al., 2006) and downregulate gene expression for adiponectin (adipokine that improves insulin sensitivity), glucose transporter 4, insulin receptor substrates and decreased protein levels of insulin receptor and insulin receptor substrate-1 (Ruan, Hacoen, Golub, Parijs, &

Lodish, 2002). MCP-1 expression by adipocytes increases monocyte infiltration into adipose depots, increasing the inflammatory process (Kanda, et al., 2006). IL-6 expression is correlated with worsening insulin sensitivity, but the exact mechanisms of how it increases insulin resistance are not clear (Vozarova, 2001). In addition, the increased levels of circulating free fatty acids released from adipocytes, secondary to decreased inhibition by insulin and increased TNF-  $\alpha$ , are deposited in myocytes and other non-adipose tissue (cardiac cells, pancreatic  $\beta$ -cells) and are known to impair insulin signaling (Lewis, Carpenter, Adeli, & Giacca, 2002). In summary, it is clear that with worsening obesity, cellular changes in enlarged adipocytes increase production and secretion of pro-inflammatory cytokines and release FFAs into the circulation. The increase in these circulating cytokines and FFAs impairs insulin function by altering signaling pathways or decreasing genetic expression of proteins necessary for insulin function, inducing insulin resistance.

The cellular mechanisms of insulin resistance have not been completely elucidated, but are thought to be caused primarily by defects in the insulin receptor and post receptor signaling (Mlinar, Marc, Janez, & Pfeifer, 2006). Insulin receptor tyrosine phosphorylation has been demonstrated to be decreased in obese patients compared to lean patients, leading to defective signaling of insulin in skeletal muscle (Goodyear, Giorgino, Sherman, Carey, Smith, & Dohm, 1995). Increased levels of FFAs stimulate protein kinase C activity which can directly inhibit insulin signaling by serine-threonine phosphorylation of insulin receptor substrates and other intermediates of the insulin stimulated pathways (Shulman, 2000; Danielson, Liu, Hosomi, Shii, & Roth, 1995).

Insulin signaling can also be downregulated through internalization of the receptor and degradation of insulin receptor substrates through cytokine signaling (Rui, Yuan, Frantz, Sheolson, & White, 2002).

### *Leptin*

The hormone leptin is produced primarily by adipocytes, but also by gastrointestinal, placental, and skeletal muscle cells (Ahima, & Osei, 2004). Leptin circulates partially bound to plasma proteins (Houseknecht, et al., 1996) and stimulates its functions in glucose homeostasis through interaction with its receptor found in both central and peripheral tissues. There are six isoforms of the leptin receptor, and the long form (LEPRb) contains an intracellular domain that is necessary for activation of the leptin signal (Bahrenberg, et al., 2002). Leptin regulates body weight through signaling at the hypothalamus, initiating a ‘satiety’ signal decreasing food intake (Ahima, & Osei, 2004). Leptin and insulin secretion are dependent upon each other forming the ‘adipo-insular axis’ of glucose regulation (Seufert, 2004). Leptin secretion increases after a meal which is partially mediated by insulin’s effect on adipocyte production (Barr, Malide, Zarnowski, Taylor, & Cushman, 1997). In addition, leptin decreases pancreatic secretion of insulin (Seufert, 2004) and enhances insulin’s inhibition of hepatic glucose production *in vivo* (Rossetti, et al., 1997). Furthermore, leptin stimulates the oxidation of fatty acids and the uptake of glucose, preventing the accumulation of lipids in non-adipose tissue (Minokoshi, et al., 2002), and decreases insulin resistance and hyperglycemia in type 2 diabetic mice (non-leptin resistant) (Toyoshima, et al., 2005).

Besides its effects on glucose metabolism, leptin is now known to effect many other physiologic processes, including thrombosis and inflammation. The leptin receptor is expressed on platelets, although its function on platelets has demonstrated a varying degree of responsiveness, regardless of BMI (Giandomenico, Dellas, Czekay, Koschnick, & Loskutoff, 2005). In healthy individuals, leptin has demonstrated a dose dependent augmentation in ADP-induced platelet aggregation and platelet free calcium concentrations (Corsonello, et al., 2004; Elbatarny, & Maurice, 2005). Additionally, serum leptin levels increase in response to acute infections and proinflammatory cytokines and leptin treatment stimulates immune responses (Mito, Yoshino, Hosado, & Sato). The leptin receptor is expressed on all immune cells, and is now known to promote cytokine production and upregulation of phagocytosis by macrophages (Loffreda, et al., 1998) and leukocyte content in adipose tissue has demonstrated dependence on leptin levels (Casper-Bauguil, et al., 2006). Leptin has also demonstrated a function in regulating the respiratory burst in neutrophils (Caldefie-Chezet, Poulin, Tridon, Sion, & Vasson, 2001). A fall in leptin triggers immune system suppression, and is thought to be an evolutionary protective mechanism against starvation (Ahima, & Oesi, 2004).

### *Leptin Resistance*

Serum leptin levels increase in relation to increasing adiposity. The increasing levels of leptin occurring with obesity have demonstrated an increase in the resistance to leptin signaling (Ahima, & Oesi, 2004). The complete mechanisms of how leptin resistance develops and the functional consequences of it are not completely understood.

The decreased response to leptin by hypothalamic 'satiety center' may increase hunger in already obese people, leading to further weight gain (Ahima, & Osei, 2004). Increased triglyceride levels in obese people have demonstrated leptin resistance by the blood brain barrier (Banks, et al., 2004) and the soluble leptin receptor (LEPRe) is decreased in the obese state (necessary for transport of leptin across the blood brain barrier) (Mlinar, et al., 2006), are both potential mechanisms for decreased central responsiveness to leptin in leptin resistant state. Hyperleptinemic, obese mice have demonstrated decreased hypothalamic response to *in vivo* leptin administration, which appear to be related to alterations in postreceptor signaling (El-Haschimi, Pierroz, Hileman, Bjorbak, & Flier, 2000). Additionally, mice fed a high fat diet and hyperleptinemic did not respond to peripheral leptin administration, whereas lean mice injected with leptin decreased food intake and lost weight (van Heek, et al., 1997). Resistance to leptin signaling has also demonstrated increased FFA accumulation in skeletal and cardiac myocytes, liver, and pancreatic  $\beta$ -cells, similar to that seen with insulin resistance (Unger, & Orei, 2000). In addition to the metabolic consequences of leptin resistance, obese, leptin resistant people have demonstrated a decrease in ADP-stimulated platelet aggregation in response to leptin compared to lean individuals (Corsonello, et al., 2003).

### *Type 2 Diabetes Mellitus*

Type 2 diabetes is a consequence of prolonged insulin resistance. Type 2 diabetes is diagnosed when fasting blood sugar rises above 126 mg/dl or a random or 2 hour post-oral glucose tolerance test blood sugar is greater than 200 mg/dl (ADA, 2006). Progression to frank type 2 diabetes from insulin resistance occurs over time. Initially,

the pancreas is able to compensate for rising levels of glucose and maintain normoglycemia by increasing the release of insulin (Weir, & Bonner-Weir, 2004) through hyperplasia of the beta cell mass (Ferrannini, et al., 2004). When pancreatic beta cells can no longer produce enough insulin to compensate for rising blood sugar, type 2 diabetes ensues (Kasuga, 2006). The failure of pancreatic  $\beta$ -cells occurs with prolonged cellular damage incurred by increased levels of fatty acids, inflammatory cytokines, adipokines, and mitochondrial dysfunction (Stumvoll, Goldstein, & van Haeften, 2005). Although fatty acids are necessary for normal insulin secretion by pancreatic beta cells, the chronic elevation in FFA accumulation in pancreatic  $\beta$ -cells leads to dysfunction and subsequent diminished insulin secretion (Yaney, & Corkey, 2003). Chronically elevated intracellular FFA levels inhibit insulin biosynthesis and secretion and decreases expression of GLUT2 (glucose transporter 2, important for glucose stimulated insulin secretion) (Yaney, & Corkey, 2003). In addition, with worsening hyperglycemia, mitochondrial overproduction of ROS in pancreatic  $\beta$ -cells occurs, with subsequent irreversible damage of cellular components leading to further dysfunction (Evans, Goldfine, Maddux, & Grodsky, 2003; Robertson, Harmon, Tran, Tanaka, & Takahashi, 2003). With prolonged disease, type 2 diabetics, who initially had excessive amounts of insulin will develop substantial insulin deficiency and require insulin therapy to manage the illness (Stumvoll, Goldstein, & van Haeften, 2005).

## Blood Cell Functions and Alterations in Obesity and Type 2 Diabetes

### *Platelet Function*

Hemostasis maintains blood in a fluid state under normal physiologic conditions, through inhibition of coagulation and promotion of fibrinolysis. When the vasculature is compromised, the hemostatic system constricts the surrounding vessels to reduce blood flow (minimizing loss), activates platelets, and initiates coagulation forming a platelet 'plug' and subsequent clot that stops blood loss (Pierce, Razzuk, Razzuk, & Hoover, 1999).

Platelets are the smallest circulating blood cells, and are released as anuclear fragments of megakaryocytes into the circulation (Jurk, & Kehrel, 2005; Gachet, 2006). Platelets are central in hemostasis and thrombosis, but also have functions in the innate immune system and mediate inflammatory processes (Jurk, & Kehrel, 2005). Under normal conditions, platelets circulate freely in the blood and do not adhere to the vascular endothelium or other cells. Intact endothelial cells produce prostacyclin and nitric oxide that inhibit platelet adhesion. During early hemostasis after vascular injury, platelets become activated by interaction with vascular collagen that is exposed on subendothelial tissue (Pierce, Razzuk, Razzuk, & Hoover, 1999). The platelets initially roll and slow down through interactions of endothelial von Willibrand factor and platelet receptor GPIb, and then form tight adhesions with vascular exposed collagen and platelet collagen receptor integrin  $\alpha 2\beta 1$  (Jurk, & Kehrel, 2005). Platelet activation occurs through multiple agonists released by the damaged endothelium, including ADP and collagen, that result in positive feedback loops, potentiating the cycle (Jurk, & Kehrel, 2005).

Upon the initial adhesion of platelets to damaged vasculature, platelets bind fibrinogen to the activated GPIIb/IIIa (CD41/CD61) receptor that in turn binds several platelets forming a platelet 'plug' (Parise, 1999; Sims, Ginsberg, Plow, & Shattil, 1991). The P2Y1 receptors amplify the platelet response to collagen and ADP and are responsible for the initial aggregation of platelets and initiate platelet shape change and further release of ADP. ADP then further stimulates P2Y1 and P2Y12, which is responsible for completing platelet aggregation in response to ADP (Gachet, 2006; Packham, & Mustard, 2005).

In addition to aggregation, upon activation platelets release microparticles and express adhesion molecules, including CD62P (P-selectin) (Celi, Lorenzet, Furie, & Furie, 2004). Platelets and other blood cells form microparticles on activation by shedding these small membrane vesicles through a process similar to apoptosis regulated by caspases (Cohen, Gonzales, Davis-Gorman, Copeland, & McDonagh, 2002). Platelet derived microparticles are highly thrombogenic and express binding sites for factor VIII, and tissue factor (Diamont, Neiuwland, Pablo, Sturk, Smit, & Radder, 2002), and express P-selectin and other pro-thrombotic adhesion molecules (van der Zee, et al., 2006). Platelet microparticles are also known to upregulate endothelial and leukocyte production of proinflammatory cytokines (Celi, et al., 2004). The selectin CD62P is expressed on platelets and endothelial cells and is a key adhesion molecule in these cell types. Platelet P-selectin is normally stored in  $\alpha$ -granules with minimal surface expression and is translocated to the membrane during platelet activation (Jurk, & Kehrel, 2005). P-selectin mediates platelet-leukocyte conjugation and platelet-endothelial adhesion (Celi,

Lorenzet, Furie, & Furie, 2004), and is shed readily from the platelet's surface during activation becoming a soluble mediator of inflammation and thrombosis (Jurk, & Kehrel, 2005).

#### *Altered Platelet Function in Obesity and Type 2 Diabetes*

Obesity and diabetes induce several alterations in platelet function, coagulation, and fibrinolysis, initiating a state of chronic platelet activation and hyperaggregability (Schneider, 2005). Most components of the coagulation pathways and platelet function are compromised in obese and diabetic states (Carr, 2000; Schneider, 2005; Yazbek, Bapat, & Kleiman, 2003). Several coagulation factors are known to be elevated in type 2 diabetes, including factors I (fibrinogen) (Coppola, et al., 2006; Coca, Cucuianu, & Hancu, 2005; Erem, et al., 2005), VII, (Coca, et al., 2005; Ludwig, et al., 2005), VIII, (Coca, et al.), and endothelial surface antigen von Willebrand factor (Coca, et al.; Erem, et al.). Platelet aggregation is enhanced with ADP stimulation in obese and diabetic people (Corsonello, et al., 2003; Ferreira, et al., 2006) compared to platelets from non-obese, non-diabetic subjects. Furthermore, platelets from people with diabetes are larger (increased mean platelet volume, an indicator of activation) (Tschoepe, et al., 1990; Coban, Ozdogan, Yazicioglu, & Akcit, 2005), demonstrate increased platelet expression of P-selectin (Nomura, et al, 1995; Tan, Tayebjee, Lim, & Lip, 2005; McDonagh, et al., 2003), and form increased levels of circulating platelet derived microparticles (PMPs) (Koga, et al., 2006; Tan, et al., 2005; Diamont, Nieuwland, Pablo, Sturk, Smit, & Radder, 2002; Nomura, et al., 1995) compared to non-diabetics.

The coagulation and thrombotic inhibitory pathways are also altered in obesity and diabetes. Obese subjects have demonstrated an impaired synthesis and reaction to the platelet anti-aggregatory effects of both nitric oxide and prostacyclin (Anfossi, et al., 2004). Diabetic blood demonstrates significantly slower fibrinolysis and lower plasmin generation than blood from lean non-diabetic people (Dunn, Philippou, Ariens, & Grant, 2006) resulting from post-translational modification of fibrinogen that impaired the fibrinolytic process. Additionally, diabetic platelets do not demonstrate aggregation inhibition in response to insulin (insulin resistance) (Westerbacka, et al., 2002; Ferreira, et al., 2006).

The hypercoagulable state observed in obese and type 2 diabetic patients has several implications. The hypercoagulable state and chronic activation of platelets observed in obese and type 2 diabetic patients is associated with development of vascular complications, including ischemic heart disease (McDonagh, et al., 2002) and atherosclerosis (Tan, Tayebjee, Lim, & Lip, 2005). Obese and type 2 diabetic patients are at higher risk of developing other ischemic vascular diseases, such as stroke.

#### *Polymorphonuclear Leukocyte Function*

Leukocytes, or white blood cells, are dynamic cells that interact with platelets, endothelial cells, other leukocytes, and inflammatory mediators (cytokines, chemokines, complement) to generate responses to foreign invaders or host damage (Patarroyo, et al., 1990). The innate immune response is a rapid, acute, first line defense signaled by inflammatory mediators in response to infection, tissue injury, and malignancy (Pickup, & Crook, 1998). With chronic production of proinflammatory mediators, however, this

typically 'acute' response can become prolonged and produce dysfunction in various 'normal' tissues (Pickup, & Crook, 1998). This chronic state of activation of the innate immune system is observed in several pathologic conditions, such as rheumatoid arthritis, gout, etc, (Roos, van Bruggen, & Meischl, 2003) and is now appreciated to be the central, underlying pathology in obesity, type 2 diabetes, and vascular diseases (including stroke and cardiovascular disease (Duncan, et al., 2003; Dandona, Aljada, Chaudhuri, Mohanty, 2004; Huang, Upadhyay, Tamargo, 2006).

Polymorphonuclear (PMN) leukocytes are a crucial component of the innate immune system and primarily respond to fungi, protozoa, and bacterial infection (Moraes, & Downey, 2003). The PMN mediated inflammatory response is a complex process initiated by rolling and adhesion of the PMN to vascular endothelial cells. Rolling of PMNs along the vascular wall slows the cell down and is mediated by interactions of endothelial P-selectin and PMN P-selectin glycoprotein ligand-1 (Sperandio, 2005). PMN L-selectin binds to its endothelial receptor for secondary slowing and its adhesion stimulates CD11b/CD18 integrin expression that leads to firm venular adhesion and extravasation in to the extravascular space (Sperandio, 2005). Upon entering inflamed tissue, PMNs form pseudopods (Zhelev, Alteraifi, & Chodniewicz, 2004), that assist in movement toward and engulfing the pathogen exposing them to toxic mediators (Roos, van Bruggen, & Meischl, 2003; Faurschou, & Borregaard, 2003). Because of their toxic nature, PMNs are tightly regulated to minimize the risk of self-inflicted damage, and they undergo apoptosis immediately following their

function and are disposed by macrophages (Ottonello, Furmento, Arduino, Dapino, Tortolina, & Dallegri, 2001).

The integrin CD11b is the  $\alpha$  chain portion of the leukocyte  $\beta$ 2-integrin Mac-1 (CD11b/CD18 complex). CD11b is expressed primarily on PMNs and monocytes and on a subset of activated CD8 T cells and NK cells (Mazzone, & Ricevuti). In PMNs the CD11b integrin is expressed on the surface membrane in low numbers, and is primarily stored in peroxidase negative granules and secretory vesicles that rapidly bring the integrin to the surface in response to inflammatory signals (Faurischou, & Borregaard, 2003). The Mac-1 complex is also involved in other inflammatory and thrombotic processes, and is a receptor for complement factor 3bi, fibrinogen, and other inflammatory mediators (such as LPS) (Mazzone, & Ricevuti). In addition to increasing surface expression of CD11b during activation, PMNs increase their oxygen consumption through activity of NADPH-oxidase (located in the cell surface and granule membranes), which produces superoxide anion and hydrogen peroxide (Dahlgren, & Karlsson, 1999). These reactive oxidative species then destroy bacterial invaders, but may also damage and destroy surrounding tissue (Dahlgren, & Karlsson).

#### *Altered PMN Function in Obesity and Type 2 Diabetes*

The role of PMNs in the chronic inflammatory process observed in type 2 diabetes, though, is not fully elucidated. Historically, it was believed that decreased PMN function in diabetes mellitus was the cause for the increased incidence in bacterial infection in people with the disease (McManus, Bloodworth, Prihoda, Blodgett, & Pinckard, 2001). Total leukocyte counts and the PMN fraction of the total leukocyte

count are known to be higher in type 2 diabetics (Shurtz-Swirski, et al., 2001; Veronelli, et al., 2004, van Oostrom, van Wijk, Sijmonsma, Rabelink, & Cabezas, 2004). PMNs demonstrate chronic activation in humans with diabetes, exhibiting an increase in the expression of the integrin CD11b (van Oostrom, et al., 2004; Advani, Marshall, & Thomas, 2002). Additionally, diabetic PMNs have demonstrated increased adhesion and aggregation compared to non-diabetics, and this increases with worsening disease (Ohmori, et al., 2000).

Diabetic PMNs have also demonstrated an increase in respiratory oxidative burst (Hand, Hand, & Vasquez, 2006; Ohmori, et al., 2000) and enzymatic activity of cathepsin B and D compared to non-diabetics (Llorente, et al., 2000). Diabetic PMNs demonstrate an increased production of ROS (Hokama, et al. 1997, Shurtz-Swirski, et al., 2001; Evans, Goldfine, Maddux, & Grodsky, 2003), that not only damages endothelium and other cells (Brownlee, 2001), but increases DNA oxidative damage in the PMNs themselves (Pitozzi, Giovannelli, Bardini, Rotella, & Dolara, 2003). The over expression of PMN CD11b and increased respiratory burst observed in diabetes is implicated in the increase in vascular adhesion of PMNs that leads to vascular endothelial and surrounding tissue damage (Advani, 2002) and exacerbates reperfusion injury after myocardial ischemia (Hokama, Ritter, Davis-Gorman, Cimetta, Copeland, & McDonagh, 2000).

Pathogenesis of Ischemic Stroke and Reperfusion Injury  
in Obesity and Type 2 Diabetes

The brain is highly vascular, characterized by collateral circulations that protect against a potential decrease in blood flow. Hypoperfusion, even a reduction of 65% in blood flow, can alter brain functioning and cause ischemia (Huang, Urvashi, Upadhyay, & Tamargo, 2006). A stroke occurs when a region of the brain's blood supply is transiently or permanently disrupted, caused by an embolus, thrombosis, or hemodynamic alterations leading to a decrease in blood flow (Tegos, Kalodiki, Daskalopoulou, & Nicolaides, 2000). A prolonged interruption in blood flow results in damage and necrosis (infarct) of the effected cerebral tissues, leading to extended neurologic deficits and chronic disability (Tegos, et al., 2000).

*Temporal Pathophysiology of Ischemic Stroke*

The brain is dependent on both glucose and oxygen for viability and function, and within minutes of a disruption in blood supply, changes in cerebral cell metabolism occur, leading to subsequent cell death (Dirnagl, Iadecola, & Moskowitz, 1999). Without glucose and oxygen, the membrane potential of neurons is diminished, secondary to a decrease in ATP production (Dirnagl, Iadecola, & Moskowitz, 1999). The initial depolarization of neurons releases  $K^+$  and glutamate into the extracellular space, causing rapid neuronal depolarization in the peri-infarct region, which leads to further consumption of glucose and oxygen in the ischemic area and increased damage (Hossmann, 1996). Additionally, as a result of this neuronal excitability,  $Na^+$ ,  $Cl^-$ , and  $Ca^{2+}$  enter cells, followed by water, leading to cellular edema (Dirnagl, 1999).

The increase in intracellular  $\text{Ca}^{2+}$  initiates a series of intracellular cascades leading to overproduction of oxidative radicals initiating cell injury and apoptosis (Chan, 2001) and increase the expression of pro-inflammatory genes (O'Neill, & Kaltschmidt, 1997). Consequently, the vascular endothelium induces expression of adhesion molecules, including ICAM-1, (Lindberg, Carpen, Paetau, Karjalainen-Lindsberg, & Kaste, 1996), P-selectin, and E-selectin (Zhang, Chopp, Zhang, Jiang, & Powers, 1998). The increased expression of vascular adhesion molecules and expression of pro-inflammatory cytokines recruits leukocytes to the site of injury after reperfusion of the disrupted vessel (Dirnagl, et al., 1999).

#### *Reperfusion Injury*

Return of blood to the ischemic region after stroke is crucial for limiting cell death due to lack of oxygen and glucose. However, the return of blood brings platelets and leukocytes to the ischemic region exacerbating tissue damage (del Zoppo, Becker, & Hallenbeck, 2001; Wang, Chen, Yang, & Zhou, 2004). Rolling and adhesion of leukocytes, particularly PMNs, initiate inflammation during ischemia and the first minutes of reperfusion by adhering to vascular endothelium (Ritter, Orozco, Coull, & McDonagh, 2000) and transmigrating into neural tissue. Activated platelets and PMNs accumulate in cerebral vessels (Ishikawa, Arumugam, Zhang, Nanda, & Granger, 2004; Ritter, Orozco, Coull, & McDonagh, 2000) and occlude the cerebral microvasculature leading to the prolonged ischemia (del Zoppo, et al., 1991). The adhesion of platelets to post-ischemic vessels appears to be due to fibrinogen deposition in the vessels during ischemia (Massberg, et al., 1999). The activation and accumulation of platelets and

leukocytes in the cerebral microvasculature exacerbates neuronal and endothelial damage by further release of ROS and continued activation of inflammatory cells (del Zoppo, 2001).

#### *Altered Platelet Function during Ischemic Stroke and Reperfusion*

Platelets are significantly activated in subjects with acute ischemic stroke (Smith, Pathansali, & Bath, 1999), although, not all research confirms this conclusion. Early studies demonstrated that after acute cerebral ischemia patients demonstrated significant increases in beta thromboglobulin and platelet factor 4 (markers of platelet activation) (Shah, Beamer, & Coull, 1985), and increased circulating platelet activating factor and von Willebrand factor (Uchiyama, et al., 1983). Additionally, circulating platelets increase expression of P-selectin (CD62P) (McCabe, et al., 2004; Marquardt, et al., 2002) after stroke and during reperfusion, that mediates adhesion to leukocytes forming circulating platelet-monocyte (McCabe, 2004) and platelet-leukocyte conjugates (Ritter, Stempel, Coull, & McDonagh, 2005).

The results of platelet aggregation studies after stroke have not been as clear and have demonstrated increased, no change, or decreased aggregation after ischemic stroke (Smith, Pathansali, & Bath, 1999). For example, Uchiyama, et al., (1983) demonstrated that after ischemic stroke ADP induced platelet aggregation *ex vivo* was increased compared to non-stroked individuals. Additionally, shear-induced platelet aggregation *in vitro* has been demonstrated to be increased after atherothrombotic stroke, but not after cardioembolic or lacunar strokes (Uchiyama, et al., 1994). However, platelet aggregation was demonstrated to be decreased with collagen stimulation and was further decreased in

the presence of monocytes and PMNs after ischemic stroke (Grau, Sigmund, & Hacke, 1994). Another study, demonstrated that after stroke in patients not treated with anti-platelet therapy, there was no difference in whole blood aggregation after stroke, but stroke patients demonstrated an increase in ATP release to collagen compared to non-stroke patients (Joseph, D'Andrea, Oster, & Welch, 1989).

#### *Altered Leukocyte Function during Ischemic Stroke and Reperfusion*

Although the mechanisms of local brain ischemic cell death and inflammatory processes are well known in cerebral ischemia, few studies have examined the response of peripheral blood inflammatory cells after cerebral ischemia and reperfusion. Patients demonstrate elevated white blood counts after stroke, specifically an increase in circulating polymorphonuclear leukocytes (PMNs) (Beamer, Coull, Clark, Briley, Wynn, & Sexton, 1998; Suzuki, Kelley, Reyes-Iglesias, Alfonso, & Dietrich, 1995, McCabe, et al., 2004) and monocytes (McCabe, 2004). Several proinflammatory cytokines (Offner, Subramanian, Parker, Afentoulis, Vandenbark, & Hurn, 2005), and circulating platelet-leukocyte conjugates (Ritter, Stempel, Coull, & McDonagh, 2005) have also been demonstrated to be increased after experimental stroke, but few other studies have been done examining the changes in peripheral blood cell inflammation after stroke.

#### *Type 2 Diabetes and Ischemic Stroke and Reperfusion Injury*

The increased tendency for thrombosis and chronic inflammation observed in patients with obesity and type 2 diabetes are known to increase the risk of developing ischemic stroke (Mankovsky, & Ziegler, 2004; Idris, Thomson, & Sharma, 2005; Engstrom, et al., 2003) and cause increased mortality and morbidity after stroke

(Engstrom, 2003). Type 1 diabetic rats have demonstrated an increased level of oxidation in the cerebral microvasculature that exacerbated cerebral and reperfusion injury (Wei, Huang, & Quast, 1997). Additionally, during reperfusion, leukocytes have demonstrated increased adhesion in type 1 diabetic rat mesentery (Salas, et al., 1998) and coronary circulations (Hokama, et al., 1997). Examining type 2 diabetes, our laboratory demonstrated also that leukocyte accumulation was increased in the cerebral microcirculation after stroke in *db/db* mice (Ritter, Davis-Gorman, Maes, Davidson, & McDonagh, 2005). Few other studies were found, however, that examined type 2 diabetes in experimental stroke and reperfusion.

#### Animal Models of Obesity and Type 2 Diabetes

The increasing incidence of obesity and type 2 diabetes has become a global health crisis and is projected to reach even higher rates in the future. Appropriate animal models are used to explore the cellular mechanisms, pathogenesis, and development of vascular and other complications associated with these diseases. Mice provide a tool for disease research because of the generally low cost and the many transgenic animals available. Other models of obesity and type 2 diabetes are also available, such as genetically diabetic rats (Zucker diabetic fatty (*fa/fa*), Goto-Kakizaki, Otsuka Long-Evans Tokushima fatty, Chen, & Wang, 2004) and diet induced obese and diabetic rats (Rees, & Alcolado, 2005). Mouse models of obesity and type 2 diabetes were used in this dissertation project, and will be described in detail below.

*db/db Mouse*

The *db/db* mouse was the result of a spontaneous mutation of the *db* gene, producing a phenotype similar to human type 2 diabetes. The exact molecular changes incurred by the mutation were not discovered for several decades, but is now known to be an insertion of a premature stop codon in the long form of the leptin receptor mRNA transcript, resulting in the production of a nonfunctional short form of the receptor lacking an intracellular domain (Lee et al., 1996). Without leptin signaling, the mouse becomes hyperphagic and develops severe obesity and diabetes (Coleman & Hummel, 1967). The mouse displays visual deposition of axillary and inguinal fat and intrabdominal mesenteric and gonadal fat pads at 3 to 4 weeks of age and reaches a plateau of weight gain at 10 weeks with an average weight of 40 to 45 gm (normal mouse- 20-30 gm) (Hummel, Dickie, & Coleman, 1966; Vannucci, et al., 1997). Hyperglycemia increases gradually from 5-12 weeks of age with blood sugars ranging 153-530 mg/100 mL (Hummel, et al., 1966; Coleman & Hummel, 1967; Vannucci, et al., 1997). The mice also demonstrate polyphagia, polydipsia, polyuria, and glucosuria (Hummel, et al., 1966; Hummel, Coleman, & Lane, 1972), and marked hyperinsulinemia as early as 10 days of age, which peaks to 6-10 times the normal mouse by 2-3 months (Coleman, 1982). In later life, insulin levels drop with worsening pancreatic function that is evident with morphologic changes, including severe hyperplasia and hypertrophy of  $\beta$ -cells (Coleman, 1978; 1982; Hummel, et al., 1966).

The *db/db* mouse develops complications of long term type 2 diabetes, similar to humans. Peripheral neuropathies (Hanker, Ambrose, Yates, Koch, & Carson, 1980; Sima

& Robertson, 1978), disturbances of the sympathetic nervous system (Giachetti, 1978), and microvascular lesions (Bohlen & Niggli, 1979) are characteristic in the *db/db* adult mice with chronic diabetes. Kidney disturbances, including basement membrane thickening, glomerular filtration abnormalities, and deposition of immune complexes have also been identified in these mice (Like, Lavine, Poffenbarger, & Chick, 1972).

#### *ob/ob Mouse*

The *ob/ob* mouse model of insulin resistance was maintained by Jackson Laboratories in Bar Harbor, MA after a spontaneous mutation on chromosome 6 (Malik, & Young, 1996) that resulted in a truncated leptin hormone that is not secreted (Zhang, et al., 1994). The mouse model is characterized by marked obesity, hyperphagia, hyperinsulinemia, and insulin resistance most of its adult life (Garthwaite, Martinson, Tseng, Hagen, & Mehan, 1980; Dubac, 1976; Menhan, 1983; Tomita, Doull, Pollock, & Krizsan, 1992), but only develops transient hyperglycemia from age 4 weeks to 14-20 weeks (Tomita, et al., 1992; Garthwaite, et al., 1980; Menhan, 1983). The mice are obese from an early age, weighing approximately twice their lean match controls by 10 weeks (Menhan, 1983; Garthwaite, 1980). *Ob/ob* mice have demonstrated 2-3 fold increase in total cholesterol synthesis compared to lean controls (Feingold, Lear, & Moser, 1984) and an increase in liver triglyceride level 20 times greater (Menhan, 1983). Similar to *db/db* mice, *ob/ob* mice demonstrate pancreatic  $\beta$ -cell degranulation and pancreatic hypertrophy (Tomita, Doull, Pollock, & Krizsan, 1992).

*High Fat Fed Obese and Diabetic Mouse*

The C57BL/6J mouse strain develops obesity and type 2 diabetes when fed a diet high in animal fat and demonstrates many metabolic disturbances similar to humans. Other strains of mice do not demonstrate the extent of weight gain and insulin resistance and subsequent hyperglycemia observed in the C57BL/6J mouse, but the genetic explanation for the protection in some mouse strains is not completely understood (Surwit, et al., 1995; Surwit, Seldin, Kuhn, Cochrane, & Feinglos, 1991; Collins, Martin, Surwit, & Robidoux, 2004). The C57BL/6J mice gain weight (Surwit, Kuhn, Cochrane, McCubbin, & Feinglos, 1988; Collins, Martin, Surwit, & Robidoux, 2004) and demonstrates impaired glucose tolerance after only one week on the high fat diet (Winzell, & Ahren, 2004). The weight gain is primarily in the mesentery and inguinal depots, similar to abdominal obesity observed in diabetic people (West, Boozer, Moody, & Atkinson, 1992; Surwit, Feinglos, Rodin, Sutherland, Petro, Opara, et al., 1995; Rebuffe-Scrive, Surwit, Feinglos, Kuhn, & Rodin, 1993). These mice develop hyperglycemia, impaired second phase insulin excursion (Wencel, et al., 1995), hyperinsulinemia (Surwit, et al., 1988) and hyperleptinemia (Surwit, Petro, Parekh, & Collins, 1997) after 4 months on the high fat diet. Triglyceride levels remain normal, but total plasma cholesterol is increased compared to normal chow fed mice (Libinaki, et al., 1999). These mice, however, do not demonstrate severe hyperglycemia and represent a disease state similar to obesity and early type 2 diabetes mellitus (Winzell, & Ahren, 2004)

The high fat fed mouse also demonstrates cellular changes resulting in insulin and leptin resistance, similar to obese/diabetic people. Insulin resistance is first noted in cardiac myocytes after only 1.5 weeks on the diet characterized by blunted insulin signaling of GLUT 4 translocation, and progresses to whole body insulin resistance after 3 weeks (Park, et al., 2005). In addition, these mice develop hypothalamic leptin resistance secondary to post receptor signaling defects (El-Haschimi, Pierroz, Hileman, Bjorbaek, & Flier, 2000), and this resistance alters their ability to limit food intake. The high fat fed mice develop peripheral leptin resistance characterized by a lack of food intake decrease in response to leptin injections (van Heek, et al., 1997). Additionally, after 12 months on the high fat diet, these mice develop pancreatic beta cell loss secondary to increased oxidative stress (Sone, & Kagawa, 2005). These metabolic disturbances were completely reversible in the obese diet induced diabetic mice with weight loss and return to a normal diet (Parekh, Petro, Tiller, Feinglos, & Surwit, 1998).

In addition to the metabolic disturbances observed in the high fat fed obese and diabetic mice, these mice develop several vascular complications of these disease processes similar to humans. The diet induced obese and diabetic mice demonstrate hypertension (Mills, Kuhn, Feinglos, & Surwit, 1993) and increased renal arterial pressure secondary to increased sympathetic action (Rahmouni, Morgan, Morgan, Mark, & Haynes, 2005). Endothelial dysfunction occurs after only 10 weeks on the diet, (Molnar, et al., 2005), and they develop mild atherosclerosis after 14 weeks (Schreyer, Wilson, & LeBouf, 1998). However, these mice do not appear to demonstrate altered

renal function, a common complication in human type 2 diabetics (Noonan, & Banks, 2000).

*High Fat Fed Obese and Diabetic Mouse Injected with Low dose Streptozotocin*

A model of later stage type 2 diabetes, when the pancreas is severely damaged and is no longer able to produce sufficient insulin, has also been described. In this model, C57BL/6J mice are fed a high fat diet (45% beef lard) for a shorter time period (1mo), injected with a dose of streptozotocin (STZ) that is lower than that used to induce type 1 diabetes (100mg/kg), and then fed the high fat diet for another month (Luo, et al., 1998). To induce type 1 diabetes, mice are either injected intraperitoneally with 40 ug/g STZ for 5 days (Botolin, et al., 2005), or injected intraperitoneally with one high dose STZ (250mg/kg) (Inukai, et al., 2005). These mice suffer severe damage to pancreatic beta cells due to overproduction of free radical nitric oxide (Haluzik, & Nedvidkova, 2000) and become severely hypoinsulinemic. Nicotinamide partially protects against the damaging effects of STZ, and has been given in addition to a high dose of STZ to produce a different model of type 2 diabetes in the mouse (Kobayashi, Taguchi, Yasuhiro, Matsumo, & Kamata, 2004). These mice (STZ+nicotinamide) demonstrate mild hyperglycemia that decreases in response to tolbutamide (an insulin secretagogue) but are not obese, which is similar to some, but not the majority of people with type 2 diabetes. In addition, a rat model of type 2 diabetes, in which the animals are fed a high fat diet (58% beef lard) for 2 weeks, then injected with a low dose STZ (35mg/kg) and fed the diet for another week (3 weeks total on the diet), has been recently described and demonstrates similar metabolic characteristics to the mouse model (high fat+STZ), in

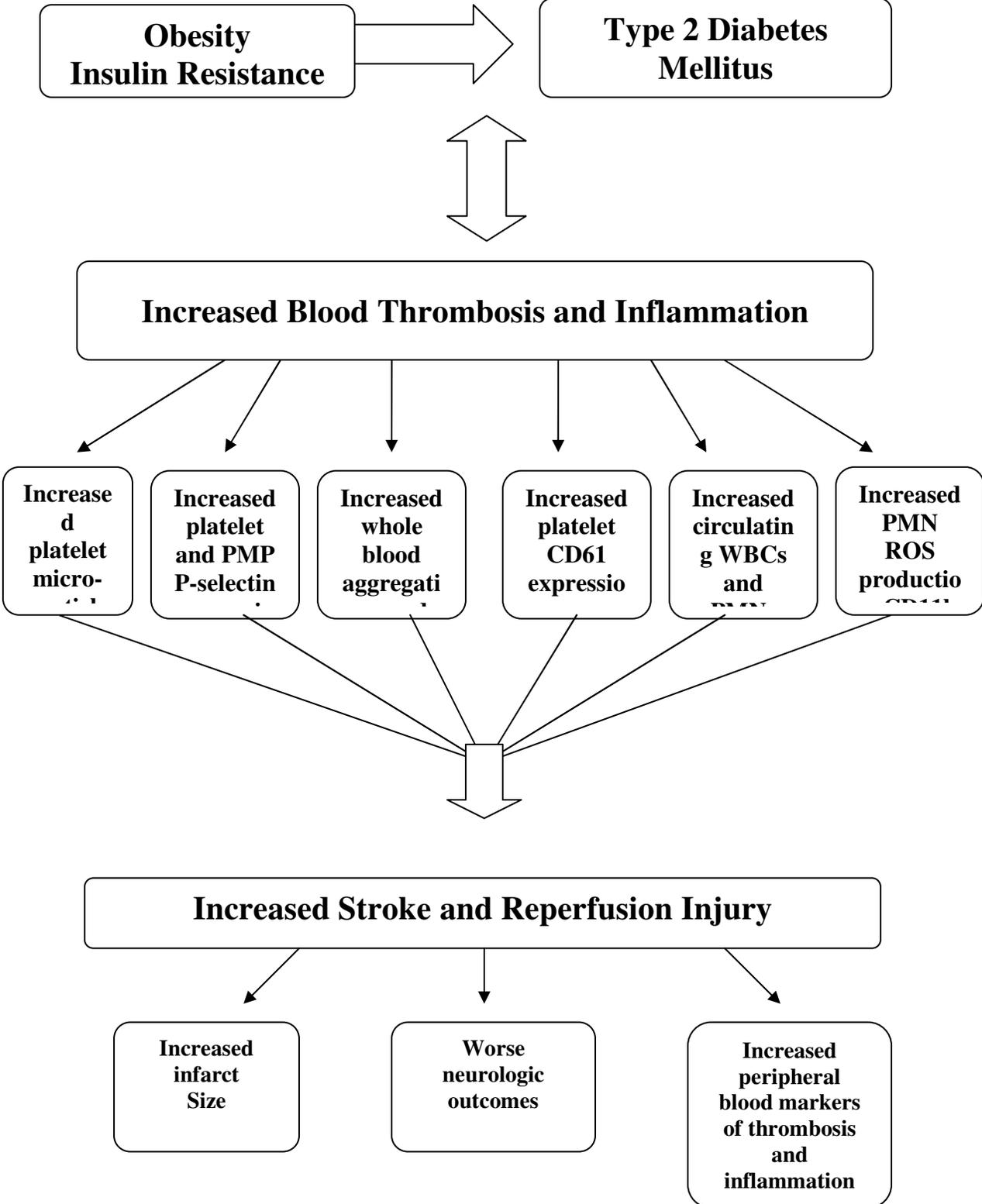
which the rats are obese and hyperglycemic (Srinivasan, Viswanad, Asrat, & Ramarao, 2005).

There are few studies, though, that have examined the metabolic characteristics of mice fed a high fat diet and injected with low dose STZ. It is known that they demonstrate significant obesity and hyperglycemia after the STZ injection, whereas mice fed a chow diet and injected with STZ do not develop these metabolic characteristics (Luo et al., 1998). Additionally, these mice respond to tolbutamide, indicating that they have not lost all pancreatic function (type 1 diabetic model) and can increase insulin production to a stimulus.

### Theoretical Framework

Figure 1.1 describes the physiologic framework that was used to support this research. It is well established that obesity and type 2 diabetes are associated with increased blood thrombotic and inflammatory states compared to lean, non-diabetic individuals (Schneider, 2005; Carr, 2001; Duncan, et al., 2003; Dandona, Aljada, Chaudhuri, & Mohanty, 2004). Diabetics are known to demonstrate increased platelet aggregation (Corsonello, et al., 2003; Ferreira, et al., 2006), coagulation (Dunn, Philippou, Ariens, & Grant, 2006), and platelet microparticle formation and P-selectin expression (Nomura, et al, 1995; Tan, Tayebjee, Lim, & Lip, 2005; McDonagh, et al., 2003).

Figure 1.1 Theoretical framework



Additionally, diabetics demonstrate a chronic, low level inflammatory state that is characterized by elevated levels of white blood cells, particularly PMNs (Shurtz-Swirski, et al., 2001; Veronelli, et al., 2004, van Oostrom, van Wijk, Sijmonsma, Rabelink, & Cabezas, 2004). Diabetic PMNs are chronically activated demonstrating elevated levels of CD11b expression (van Oostrom, et al., 2004; Advani, Marshall, & Thomas, 2002) and elevated respiratory burst activity (Hand, Hand, & Vasquez, 2006; Ohmori, et al., 2000). However, little is known about these processes in mouse models of obesity and type 2 diabetes. Furthermore, obese and diabetic patients are at a higher risk of developing stroke (Lee, et al., 2001; Adachi, Hirai, Tsuruta, Fujiura, & Imaizumi, 2001, Hu, et al., 2002; Kernan, et al., 2003; Folsom, et al., 1999), and demonstrate a higher morbidity and mortality after stroke (Almdal, Scharling, Jensen, & Vestergaard, 2004; Megherbi, et al., 2003).

The elevated chronic baseline pro-thrombotic and pro-inflammatory condition observed in obese and type 2 diabetic patients could explain the increased severity of stroke observed in humans, but this process has not been fully examined. In addition, patients with ischemic stroke are known to incur further cerebral tissue damage as a consequence of the increased thrombotic and inflammatory response during reperfusion in normal experimental animal models (Ritter, Orozco, Coull, & McDonagh, 2000; Soriano, et al., 1999), but little is known about the effects of diabetes on this process during cerebral ischemia. Diabetes has been demonstrated to increase cardiac ischemia and reperfusion injury secondary to an increased inflammatory response (McDonagh, Hokama, Copeland, & Reynolds, 1997; Hokama, et al., 2000), but no studies have been

conducted in mouse models of diabetes and stroke analyzing the effects of thrombosis or inflammation.

### Research Aims

The central hypothesis of this dissertation is that obesity and type 2 diabetes produces an overall hypercoaguable and proinflammatory state in mice and that these processes exacerbate stroke and reperfusion injury.

Aim 1: Develop a reliable flow cytometry methodology to measure platelet and PMN markers of activation in mouse whole blood.

Hypothesis 1: Flow cytometry method will be a reliable method for measuring platelet and PMN markers of activation in mouse whole blood.

Aim 2: To determine *in vitro* coagulation, whole blood aggregation, and platelet markers of activation in mouse models of obesity and type 2 diabetes and lean, non-diabetic mice.

Hypothesis 2: Obese and type 2 diabetic mice will demonstrate a hypercoaguable state and increased circulating levels of platelet markers of activation compared to lean, non-diabetic mice.

Aim 3: To determine *in vitro* PMN inflammatory markers of activation, CD11b expression and ROS production, in mouse models of obesity and type 2 diabetes and lean, non-diabetic mice.

Hypothesis 3: Obese and type 2 diabetic mice will demonstrate increased expression of PMN CD11b and ROS production compared to lean, non-diabetic mice.

Aim 4: To determine cerebral infarction and neurologic deficits in obese and type 2 diabetic mice compared to lean, non-diabetic mice after experimental stroke and 24 hours reperfusion.

Hypothesis 4: Obese and type 2 diabetic mice will have larger infarcts and worse neurologic deficits compared to lean, non-diabetic mice.

Aim 5: To determine platelet and PMN markers of activation after experimental stroke and 24 hours reperfusion in obese, type 2 diabetic mice compared to lean, non-diabetic mice.

Hypothesis 5: Circulating platelet and PMN markers of activation will be significantly increased in obese/diabetic mice compared to lean, non-diabetic mice after stroke and reperfusion.

## CHAPTER 2

## EVALUATION OF SAMPLE FIXATION AND COMPARISON OF LDS-751 OR ANTI-CD45 FOR LEUKOCYTE IDENTIFICATION IN MOUSE WHOLE BLOOD USING FLOW CYTOMETRY

## Abstract

Flow cytometry methods used to measure leukocyte function often entail sample preparation procedures that cause artifactual cell activation. To avoid leukocyte activation by isolation techniques, some preparation methods use fluorescent markers to discriminate leukocytes from erythrocytes in whole blood. One of these markers, laser dye styryl-751(LDS-751), has been used to distinguish leukocytes by staining nucleic acid, but has been found to stain other blood cells and dead cells indiscriminately. Thus, LDS-751 may not be an appropriate reagent for leukocyte identification in whole blood. Fixing samples with formaldehydes increases cell permeability and causes surface protein cross-linking that may alter staining of both intra- and extracellular markers. The degree of this sample alteration by formaldehyde fixation, however, remains in question. In addition, little is known about flow cytometry and sample preparation methods in mouse whole blood. The purpose of this study was to determine if labeling leukocytes with a monoclonal antibody specific to leukocyte common antigen (CD45) was superior to labeling with LDS-751 and to determine the effect of sample fixation on a mouse whole blood preparation for flow cytometry. Samples were incubated with CD16/CD32 Fc receptor blocker, and either 10 µg/ml LDS-751 or phosphate buffered saline (PBS). The samples were then fixed with paraformaldehyde or diluted with PBS followed by incubation with 5ug/ml PerCP-conjugated anti-CD45, 5ug/ml FITC-conjugated anti-

CD11b, or 80  $\mu$ M dichlorofluorescein diacetate. We found that samples labeled with LDS-751 demonstrated decreased fluorescence intensity for granulocyte CD11b expression and ROS production compared to samples labeled with anti-CD45. In addition, sample fixation decreased mean fluorescence intensity in samples labeled with either LDS-751 or anti-CD45. We conclude that labeling leukocytes with monoclonal antibody CD45 in a mouse whole blood preparation is preferable, as it provides improved measurement of leukocyte indices compared to LDS-751. Also, while sample fixation prior to antibody staining caused a decrease in overall fluorescence; it can be used to successfully identify extra-cellular markers.

### Introduction

Flow cytometry is a widely used and important method for both research and clinical applications (Marti, Stetler-Stevenson, Bleesing, and Fleisher, 2001), but few studies describe the effects of sample preparation on results. Erythrocyte lysing, sample fixation, and other sample preparation techniques used in flow cytometry are known to activate blood cells, particularly leukocytes, leading to an alteration in surface glycoprotein expression and intracellular processes (Macey, et al., 1995; Hageberg, and Lyberg, 2000; Alvarez, Toll, Rivas, and Estella, 2005).

Erythrocyte lysing is a commonly used sample preparation technique for cytometry, because it allows for rapid identification of leukocytes by the flow cytometer. However, studies indicate that erythrocyte lysing reagents cause increased leukocyte expression of glycoprotein CD11b and shedding of granulocyte glycoprotein L-selectin (McCarthy,

Macey, Cahill, and Newland, 1994; Macey, et al., 1995; Macey, et al., 1999; Alvarez-Larran, Toll, Rivas, and Estella, 2005). Erythrocyte lysing also causes other blood cells, including leukocytes and platelets, to lyse (Terstappen, Meiners, and Loken, 1989; Alvarez-Larran, et al., 2005), creating cell microparticles and debris that interact with and activate leukocytes (Repo, Jansson, and Leirisalo-Repo, 1993). Therefore, an improved sample preparation method is needed to discern leukocytes from other cells in whole blood samples for flow cytometry and minimize experimental artifact.

Without erythrocyte lysing, discrimination of leukocytes from other cells in whole blood samples requires labeling leukocytes with a fluorescent marker, either by nuclear staining or labeling a leukocyte specific protein. An example of a nuclear stain used historically for flow cytometry to label leukocytes is Laser dye styryl -751 (LDS-751) (Hokama, et al., 2000; McDonagh, Hokama, Copeland, and Reynolds, 1997; McCarthy, Macey, Cahill, and Newland, 1994; Macey, et al., 1999; Simon, Chambers, and Sklar, 1990; Terstappen, et al., 1991; Terstappen, Meiners, and Loken, 1989; Repo, Jansson, and Leirisalo-Repo, 1993; Himmelfarb, Hakim, Holbrook, Leeber, and Ault, 1992). Although LDS-751 has been used extensively to discriminate leukocytes in whole blood samples, it has been reported to indiscriminately stain both live and dead cells (O'Brien, and Bolton, 1995) and has been used to identify platelets and nucleated erythrocytes in whole blood (Terstappen, 1991). Consequently, the use of LDS-751 may not be the optimal method for discriminating leukocytes in whole blood samples for flow cytometry.

Cell surface antigen staining is another approach for leukocyte discrimination in whole blood samples. A leukocyte specific, surface protein antibody that has achieved wide use for flow cytometry in recent years is the monoclonal antibody to cell surface glycoprotein CD45 (leukocyte common antigen) (Hageberg, and Lyberg, 2000; Fugimoto, et al., 2000). CD45 is a receptor-like protein tyrosine phosphatase, and is a critical regulator of leukocyte signaling (Hermiston, Xu, and Wiess, 2003; Takeda, Matsuda, Paul, and Yaseen, 2004). To date, no studies have described an artifactual change in leukocyte function with the use of a monoclonal antibody to CD45 to identify leukocytes in whole blood.

In addition to erythrocyte lysing, the sample preparation process of cell fixation changes cell function and structure, making implementation of this method controversial for flow cytometry. Sample fixation increases cell permeability, causes cell surface protein cross-linking, (Shapiro, 2003), and produces cell surface aldehyde groups that can bind antibodies. Studies that have examined fixation on sample preparation have produced conflicting results. A number of studies determined that cell fixation decreases the mean fluorescence of several surface antigens, including leukocyte CD11b and CD18, regardless of erythrocyte lysing agents, anticoagulant, and time of fixation (McCarthy, Macey, Cahill, and Newland, 1994; Macey, McCarthy, Milne, Cavanaugh, and Newland, 1999). Conversely, other groups demonstrate that granulocyte CD11b expression and platelet-leukocyte conjugate formation are increased with sample fixation after antibody staining (Repo, Jansson, and Leirisalo-Repo, 1993). Because of this, Hageberg and Lyberg (2000), fixed samples *before* antibody staining and demonstrated a reduction in

platelet-leukocyte conjugation, which they determined was closer to *in vivo* levels. Even though the use of sample fixation is controversial, it is often necessary, because cell interactions and surface antigen expression change over time (Macey, McCarthy, Vordermeier, Newland, and Brown, 1995; Hageberg, and Lyberg, 2000). Therefore, understanding the degree of cellular change caused by fixation during sample preparation and its effect on results is imperative to the development of flow cytometric methods with the least amount of experimental artifact possible.

Mice are commonly used to examine immune function in disease models, however, little is known about the effects of various flow cytometry methods on mouse blood. In order to address the methodologic issues of leukocyte identification and cell fixation in mouse blood, the purpose of these experiments was to compare the effects of 1) LDS-751 and anti-CD45 monoclonal antibody for identification of leukocytes and 2) fixed and unfixed samples in a mouse whole blood preparation on intra- and extracellular staining methods for flow cytometry. Two protocols were compared: whole blood samples stained with 10 µg/ml LDS-751 or a fluorescently labeled monoclonal antibody to CD45 specific for mouse leukocyte common antigen. Both protocols were then compared with and without cell fixation with paraformaldehyde prior to antibody staining. Granulocyte CD11b glycoprotein expression and reactive oxidative species production were measured to evaluate changes on cell function (artifactual activation) and the effects on extra- and intra-cellular staining by use of LDS-751 and/or paraformaldehyde fixation. We found that samples labeled with LDS-751 demonstrated decreased fluorescence intensity for granulocyte CD11b expression and ROS production in fixed and unfixed samples

compared to anti-CD45. In addition, sample fixation decreased mean fluorescence in samples labeled with the extracellular marker CD11b, but not with the intracellular marker for ROS.

## Materials and Methods

### *Animal Model*

All animal experiments were conducted in accordance with the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* (OLAW, 2002) after IACUC approval of all researchers. C57BKS/J mice (Jackson Laboratories, Inc., Bar Harbor, MA), age 12-16 weeks, 5-7 mice per group, were transported to the laboratory the day prior to blood acquisition in order to decrease stress-induced changes in leukocyte protein expression. Mice were allowed free access to food and water and were housed in a temperature controlled, quiet environment.

### *Blood Acquisition*

Mice were anesthetized in a chamber of 3.5% isoflurane (JD Medical Distributing Co., Inc) and oxygen (Medical Grade Oxygen) at 0.8 L/minute flow until unresponsive. The mice were then mask-ventilated with 1.5% isoflurane and 0.8L/minute flow oxygen for maintenance anesthesia. Eight hundred to 900  $\mu$ L of blood was withdrawn from the ascending vena cava with a 23-gauge needle/1 mL syringe containing 0.14 mL of undiluted citrate-phosphate-dextrose (Sigma, Cat #C7165) (Leino, and Sorvajarvi, 1992; Repo, Jansson, and Leirisalo-Repo, 1995; Macey, et al., 1995; Peter, et al., 1999).

### *Whole Blood Staining Procedure*

Whole mouse blood was kept at room temperature in 1.5 mL amber tubes and covered with aluminum foil to protect it from light during all incubations. Whole blood (WB) samples were first incubated with 0.5  $\mu\text{g}/100\mu\text{l}$  WB/PBS purified rat anti-mouse CD16/CD32 Fc $\gamma$  III/II receptor blocking monoclonal antibody (PharMingen, Clone 2.4G2) for 15 minutes at room temperature to decrease non-specific binding of antibodies to leukocyte Fc receptors (BD Biosciences, 2005). All incubations were performed at room temperature to diminish changes in leukocyte surface antigen expression with cooling and rewarming of samples (Forsyth and Levinsky, 1990; Repo, Jansson, and Leirisalo-Repo, 1995). During the 15 minute incubation with the Fc receptor blocker, samples were also stained with 10  $\mu\text{g}/\text{mL}$  LDS-751 at a 1:1 LDS-751:WB concentration, or with an equal volume of PBS (final volume-100  $\mu\text{L}$ ).

Granulocyte positive control samples were incubated for 30 minutes with either lipopolysaccharide (LPS, Sigma #2680, diluted in PBS, final concentration 10  $\mu\text{g}/0.1\text{mL}$ ) for CD11b expression (Repo, Jansson, and Leirisalo-Repo, 1995), or with phorbol myristate acetate (PMA, (Sigma # P-148) diluted in dimethyl sulfoxide (Sigma #D-5879), final concentration 16  $\mu\text{M}$ ) for ROS production (Himmelfarb, Hakim, Holbrook, Leeber, and Ault, 1992; Vowells, et al., 1995; Alvarez-Larran, Toll, Rives, and Estella, 2005). After incubation with agonists or PBS, samples were fixed with paraformaldehyde (Sigma, catalog # P6148, final concentration of 0.5%) or diluted with PBS for 20 minutes at room temperature.

Monoclonal antibodies or isotype controls (Becton-Dickenson, San Jose, CA) were added to each sample after fixation and incubated for 15 minutes. Ten  $\mu\text{L}$  diluted (1:9 concentration with filtered PBS) peridinin chlorophyll-*a* protein (PerCP)-conjugated rat anti-mouse CD45 (leukocyte common antigen, Ly-5) monoclonal antibody (catalog # 557235, clone 30-F11) were added to samples not labeled with LDS-751. For CD11b measurement, 6.25  $\mu\text{L}$  (1:9 concentration with filtered PBS) fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD11b (integrin  $\alpha_m$  chain, Mac-1  $\alpha$  chain) monoclonal antibody (catalog # 557396, clone M1/70) and FITC-conjugated rat IgG<sub>2b</sub>,  $\kappa$  monoclonal immunoglobulin isotype control (catalog # 553988, clone A95-1) were added to their respective samples. For granulocyte reactive oxidative species (ROS) analysis, cells were incubated for 15 minutes with 10  $\mu\text{L}$  2',7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Cat #D399) (diluted with filtered PBS, 80  $\mu\text{M}$  final concentration, stored at  $-80^\circ$  Celsius) (Bass, et al., 1983; Himmelfarb, Hakim, Holbrook, Leeber, and Ault, 1992; McDonagh, Hokama, Copeland, and Reynolds, 1997). After incubation with the monoclonal antibodies, all samples were diluted with 100  $\mu\text{L}$  1% cold paraformaldehyde or PBS, and placed on ice (refer to Table 2.1 for a summary of whole blood staining procedure).

Table 2.1 Summary of Flow Cytometry Whole Blood Staining Procedure

	<b>LDS-751/ Fc block stain</b>	<b>x 15 min</b>	<b>Agonist addition</b>	<b>x 30 min</b>	<b>Fixation</b>	<b>x 15 min</b>	<b>Antibody, DCF-DA stain</b>	<b>x 15 min</b>	<b>Final dilution</b>
10 µg/ml LDS-751	+ Fc block +LDS-751		+ LPS, PMA, or PBS		+ PBS or PFA		+anti-CD11b, or DCF-DA		+ PBS or PFA
anti-CD45	+ Fc block		+ LPS, PMA, or PBS		+ PBS or PFA		+anti-CD11b, anti-CD45 or DCF-DA		+ PBS or PFA

+ is the addition of reagent/solution, please refer to Methods section for amount and concentration. Times indicate incubation periods between reagent additions.

#### *Flow Cytometry Data Acquisition*

Sample data were acquired by flow cytometry (FACScalibur, 488 nm argon laser, Becton Dickinson, San Jose, CA) within 3 hours of blood acquisition to decrease cellular changes that occur with time. CD11b expression is known to increase 3 hours from blood acquisition when unfixed samples are kept at 4 C° (McCarthy, & Macey, 1993) and ROS production increases after 3.5 hours when samples are kept on ice (Himmelfarb, et al., 1992). Calibration of the flow cytometer was performed daily prior to each experiment using Calibrite Beads and FACsComp software (Becton Dickinson). All samples were acquired on the low flow setting to avoid multi-cell triggering of the flow cytometer (Himmelfarb, et al., 1992; Hageberg, and Lyberg, 2000) after adjusting PMT voltages to align negative control samples <math>10^1</math> on a log scale.

### *Flow Cytometry Analysis*

FCS Express v2.0 (De Novo Software, Inc., Ontario, Canada) was used for all flow cytometry analyses. Spectral compensation was performed for each day's experiments by using negative and positive control samples. A dot plot of linear forward and side scatter properties of FL3 threshold events was used to identify and electronically gate the granulocyte population as demonstrated previously (Hageberg and Lyberg, 2000; Alvarez-Larran, Toll, Rivas, and Estella, 2005; Horn, et al., 2005). Each sample's fluorescence emission within the gated region was then analyzed with histograms for each fluorescent channel. The mean fluorescence intensity (MFI) and number of positive events are reported for CD11b measurement of 5000 gated granulocytes, after background fluorescence subtraction ( $<10^1$  on a log scale). Mean fluorescence intensity of all 5000 gated granulocytes is reported ROS production.

### *Statistical Analysis*

Three different group comparisons were made using t-test (*t*), or Mann-Whitney Rank Sum Test (*T*) (non-parametric data) using SigmaStat 3.1 software (Systat Software, Inc., Point Richmond, CA). The group comparisons were 1) LDS-751 compared to anti-CD45 (i.e. unstimulated anti-CD45 vs. unstimulated LDS-751); statistically significant results denoted with #, 2) paraformaldehyde fixed compared to unfixed samples (i.e. anti-CD45 fixed vs. anti-CD45 unfixed); significant results denoted with +, and 3) unstimulated samples compared to samples stimulated with LPS (i.e. anti-CD45 unstimulated vs. anti-CD45 stimulated); significant results denoted with \*. Results are

represented as mean  $\pm$  SEM. An a priori  $\alpha$  of  $p \leq 0.05$  was considered statistically significant.

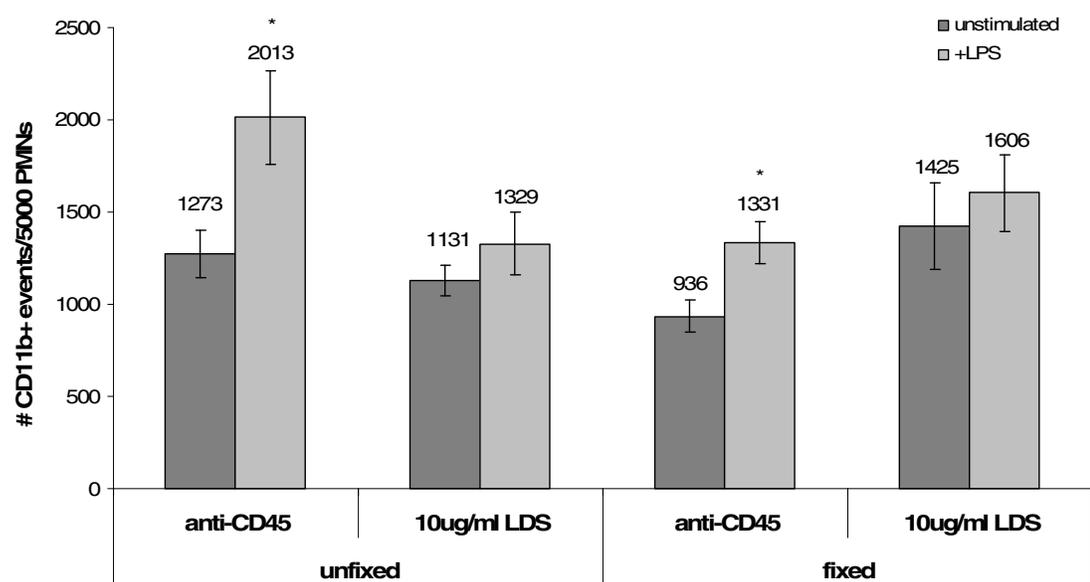
## Results

### *Granulocyte CD11b Expression*

There was no statistical difference in the number of CD11b positive events between samples labeled with anti-CD45 or LDS-751 (unfixed, unstimulated samples- anti-CD45  $1273 \pm 128$ , LDS-751  $1131 \pm 83$ ,  $t(10) = -0.84$ ,  $p = 0.42$ ; unfixed, stimulated samples- anti-CD45  $2013 \pm 253$ , LDS-751  $1329 \pm 168$ ,  $t(10) = -2.05$ ,  $p = 0.07$ ; fixed, unstimulated samples- anti-CD45  $936 \pm 86$ , LDS-751  $1425 \pm 232$ ,  $t(9) = 1.84$ ,  $p = 0.10$ ; fixed, stimulated samples- anti-CD45  $1331 \pm 114$ , LDS-751  $1606 \pm 208$ ,  $t(9) = 1.11$ ,  $p = 0.30$ , Figure 2.1). Additionally, fixation of the samples with paraformaldehyde did not significantly change the number of events in either samples labeled with anti-CD45 or LDS-751 (anti-CD45 unstimulated,  $t(10) = 2.03$ ,  $p = 0.07$ ; stimulated,  $t(10) = 2.16$ ,  $p = 0.06$ ; LDS-751 unstimulated,  $t(9) = -1.10$ ,  $p = 0.30$ ; stimulated,  $t(9) = -1.00$ ,  $p = 0.34$ ). The number of CD11b positive granulocytes increased with LPS stimulation in samples labeled with anti-CD45 (unfixed,  $t(12) = -2.61$ ,  $p = 0.02$ ; fixed,  $t(8) = -3.09$ ,  $p = 0.02$ ), but not with LDS-751 (unfixed,  $t(8) = -1.06$ ,  $p = 0.32$ ; fixed,  $t(10) = -0.58$ ,  $p = 0.57$ ).

Figure 2.1 Number of granulocytes expressing CD11b with and without LPS stimulation in whole blood samples labeled with anti-CD45 or 10  $\mu\text{g/ml}$  LDS-751. Number of events expressing CD11b per 5000 PMNs in both unfixed samples and samples fixed with paraformaldehyde. Results are mean  $\pm$  *SEM*. \* $p < 0.05$ , difference from unstimulated samples.

Figure 2.1



Granulocyte CD11b mean fluorescence intensity was decreased in all samples labeled with LDS-751 compared to anti-CD45 (unfixed samples- unstimulated, anti-CD45  $44.6 \pm 3.5$ , LDS-751  $34.7 \pm 1.3$ ,  $T= 21.500$ ,  $p= 0.07$ , stimulated, anti-CD45  $112.6 \pm 26.3$ , LDS-751  $56.6 \pm 9.0$ ,  $T= 20.00$ ,  $p= 0.05$ ; fixed samples- unstimulated anti-CD45  $31.2 \pm 3.6$ , LDS-751  $19.7 \pm 0.8$ ,  $T= 44.00$ ,  $p< 0.01$ , stimulated anti-CD45  $65.9 \pm 13.6$ , LDS-751  $29.3 \pm 1.5$ ,  $T= 42.00$ ,  $p= 0.03$ , Figure 2.2). Sample fixation with paraformaldehyde also decreased CD11b mean fluorescence intensity in all samples (anti-CD45, unstimulated  $t(10)= 2.73$ ,  $p= 0.02$ , stimulated  $t(10)= 1.41$ ,  $p= 0.19$ ; LDS-751, unstimulated  $t(9)= 10.37$ ,  $p< 0.001$ , stimulated  $T= 45.00$ ,  $p< 0.01$ , Figure 2.3). Despite the overall decrease in MFI, all fixed samples demonstrated a significant increase CD11b mean fluorescence with LPS stimulation (anti-CD45, unfixed  $T= 31.00$ ,  $p= 0.004$ , fixed,  $t(8)= -2.75$ ,  $p= 0.03$ ; LDS-751, unfixed  $T= 15.00$ ,  $p< 0.01$ , fixed  $t(10)= -5.65$ ,  $p< 0.001$ ).

Figure 2.2 Mean fluorescence intensity of granulocyte CD11b expression in whole blood samples labeled with 10  $\mu\text{g/ml}$  LDS-751 or anti-CD45. Mean fluorescence (arbitrary units) of 5000 PMNs in both unfixed samples and samples fixed with paraformaldehyde. Results are mean  $\pm$  *SEM*. \* $p < 0.05$ , difference from unstimulated sample. # $p < 0.05$ , difference from anti-CD45 sample. + $p < 0.05$ , difference from unfixed samples.

Figure 2.2

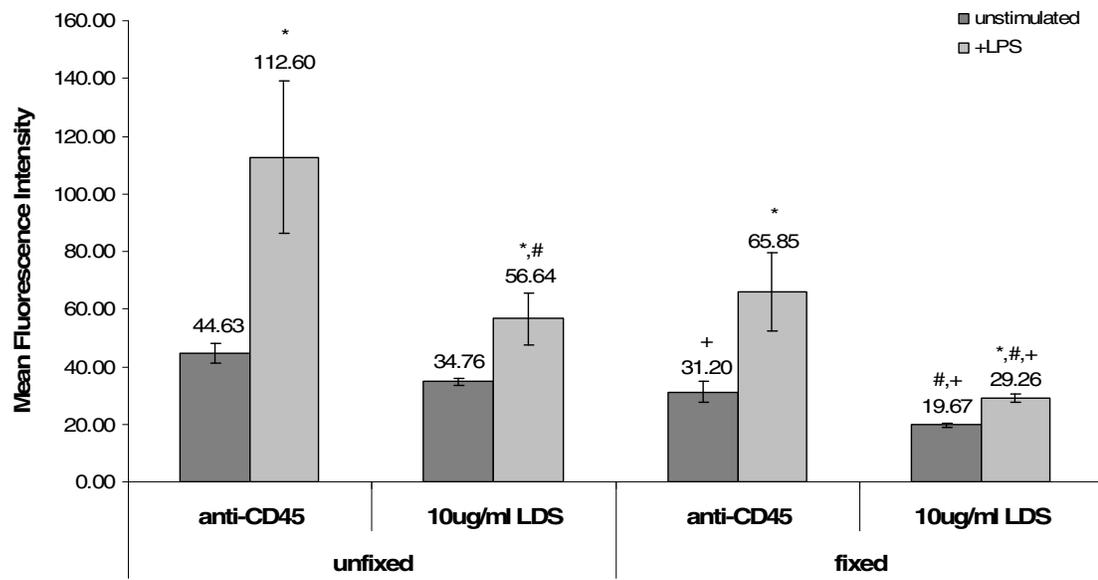
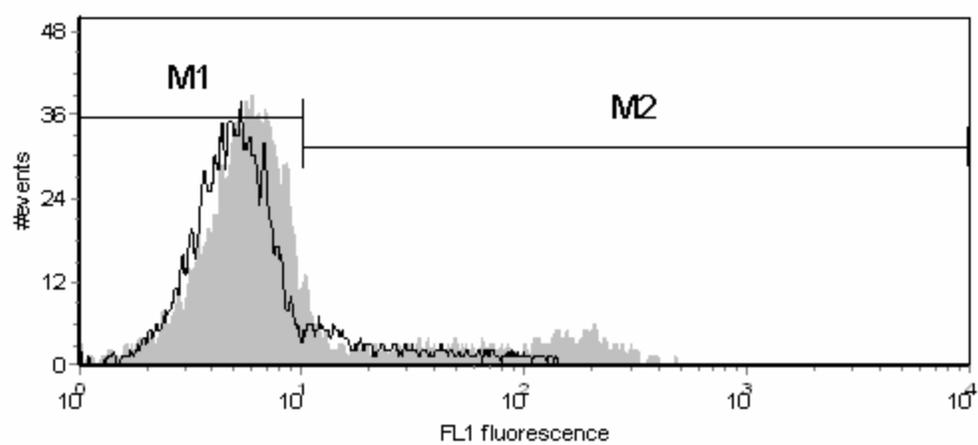


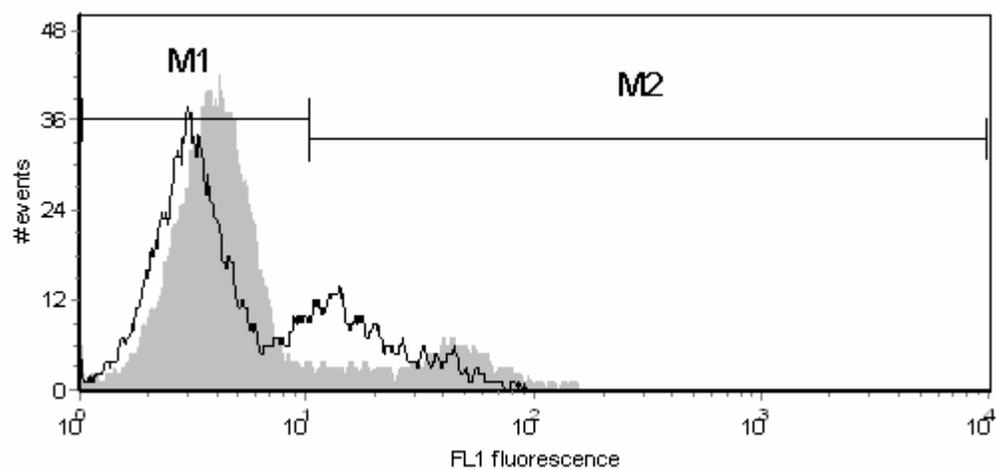
Figure 2.3 Histogram representation of granulocyte CD11b expression in fixed and unfixed samples. Shaded histograms are unfixed samples, open histograms are samples fixed with paraformaldehyde. (A) anti-CD45 (B) 10  $\mu$ g/ml LDS-751.

Figure 2.3

A.



B.

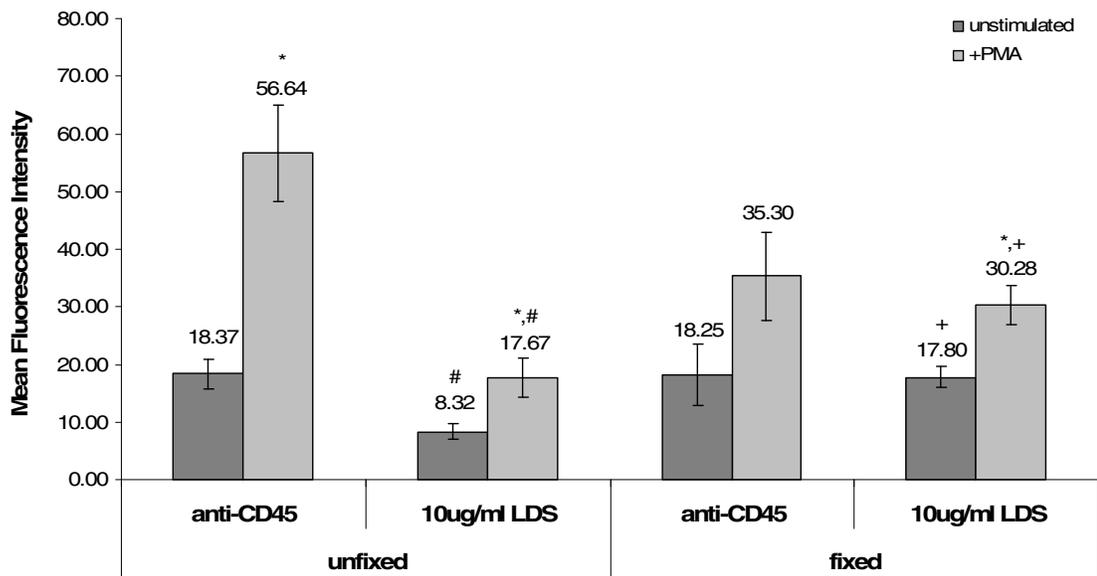


*Granulocyte Reactive Oxidative Species Production*

Granulocyte reactive oxidative species production was measured by the mean fluorescence intensity (MFI) of all 5000 granulocytes gated in each sample. The measurement of ROS in unfixed samples was significantly decreased with LDS-751 staining (unstimulated samples- anti-CD45  $18.4 \pm 2.5$ , LDS-751  $8.3 \pm 1.4$ ,  $T = 23.00$ ,  $p < 0.01$ ; stimulated samples- anti-CD45  $56.6 \pm 8.4$ , LDS-751  $17.7 \pm 3.4$ ,  $T = 22.00$ ,  $p = 0.004$ ), but not in samples fixed with paraformaldehyde (unstimulated samples- anti-CD45  $18.3 \pm 5.3$ , LDS-751  $17.8 \pm 1.9$ ,  $t(9) = -0.10$ ,  $p = 0.92$ , stimulated samples- anti-CD45  $35.3 \pm 7.6$ , LDS-751  $30.3 \pm 3.5$ ,  $T = 33.00$ ,  $p = 0.66$ , Figure 2.4). Fixation did not change ROS MFI in anti-CD45 samples (unstimulated,  $T = 25.00$ ,  $p = 0.43$ ; stimulated,  $t(9) = 1.63$ ,  $p = 0.14$ ), and increased MFI in samples labeled with LDS-751 (unstimulated,  $t(10) = -4.12$ ,  $p < 0.01$ , stimulated,  $t(10) = -2.59$ ,  $p = 0.03$ ). Similar to CD11b MFI, all samples demonstrated an increase in ROS MFI with PMA stimulation (anti-CD45 unfixed,  $T = 23.00$ ,  $p = 0.009$ , fixed,  $t(8) = -2.06$ ,  $p = 0.07$ ; LDS-751 unfixed,  $T = 24.00$ ,  $p = 0.02$ , fixed,  $t(10) = -3.20$ ,  $p = 0.01$ ).

Figure 2.4 Mean fluorescence intensity of granulocyte reactive oxidative species production. Mean fluorescence (arbitrary units) of 5000 PMNs in both unfixed samples and samples fixed with paraformaldehyde. Results are mean  $\pm$  SEM. \* $p$  < 0.05, difference from unstimulated sample. # $p$  < 0.05, difference from anti-CD45 sample. + $p$  < 0.05, difference from unfixed samples.

Figure 2.4



## Discussion

Our experiments demonstrate that the use of LDS-751 to delineate leukocytes in mouse whole blood underestimates mean fluorescence measurement of granulocyte markers. Compared to labeling leukocytes with anti-CD45, leukocytes labeled with LDS-751 demonstrated a general decrease in mean fluorescence intensity in samples measuring PMN CD11b expression and ROS production. The reason for the decrease in fluorescence, though, is not clearly understood. The labeling of samples with the intracellular dye, LDS-751, may produce functional changes to the cell that inhibit cell surface marker expression (demonstrated by an inability of the LDS-751 group to increase the number of CD11b positive cells with stimulation) and intracellular production of oxidative species. Another potential explanation for the results is that LDS-751 altered the fluorescent properties of the cells labeled with these FITC stains. In other experiments using a lower dose of LDS-751 (1  $\mu\text{g/ml}$ ), we observed similar findings as the 10  $\mu\text{g/ml}$  LDS-751 group, but to a lesser degree, indicating that the decrease in fluorescence is dose dependent (unpublished observation). In any case, our experiments demonstrate that LDS-751 caused an overall decrease in fluorescence of FITC labeled cells for both intra- and extra-cellular stains and should be used with caution when analyzing granulocytes from mice using flow cytometry.

The process of fixing samples with formaldehydes produces protein cross-linking, increases number of cell surface aldehyde groups, and increases cell permeability (Shapiro, 2003). Thus, its use has been controversial for flow cytometric cell analysis because it could potentially alter monoclonal antibody adherence and allow outward

diffusion of intracellular probes. McCarthy, et al., (1994) compared the effects of formaldehyde fixation before and after antibody staining and found that overall, fixation underestimated the mean fluorescence intensity of each antigen stained. Our findings are consistent with theirs, as we found that samples fixed with paraformaldehyde before staining demonstrated lower mean fluorescence than unfixed samples. Interestingly, samples labeled with LDS-751 demonstrated a significant increase in ROS measurement when fixed, in both the unstimulated and stimulated groups.

The timing of fixation is an important consideration in sample preparation for flow cytometry. Fixation before antibody addition may inhibit binding of fluorescent monoclonal antibodies to surface antigens. In this study, we fixed prior to antibody staining, similar to the methods used in other studies (Hageberg, and Lyberg, 2000; Repo, Jansson, Leirisalo-Repo, 1993). McCarthy, et al., (1994) found an overall decrease in mean fluorescence intensity with fixation before or after antibody staining. Because of this finding, we did not include a group that was fixed after sample staining. This is a limitation to the study, as the addition of a group fixed after sample staining would have provided further information on the effects of fixation on sample characteristics.

Few studies describe flow cytometry methods in whole mouse blood. It is important to note that in mouse blood, in contrast to humans, we found a consistently lower level of CD11b positive granulocytes. Human studies demonstrate constitutive expression of CD11b on granulocytes (80-90%) (Repo, Jansson, Leirisalo-Repo, 1993; 1995; Alvarez-Larran, Toll, Rivas, and Estella, 2005; McCarthy, Macey, Cahill, and Newland; 1994). We obtained 15-30% positive CD11b granulocytes in unstimulated

samples and 20-50% in stimulated samples. These results may be due to differences between species or other experimental artifacts. For example, mouse blood does not demonstrate well defined leukocyte populations on forward scatter/side scatter (FSC/SSC) dot plots as seen in human studies (unpublished observation). Thus, the granulocyte gate may be contaminated with lymphocytes that only express CD11b on a subset of cells. The choice of anticoagulant may also have underestimated the CD11b results. Citrate-phosphate-dextrose is a divalent cation chelator, and because CD11b is a Ca-dependent integrin (Leino, and Sorvajarvi, 1992), use of citrated anticoagulants may have reduced CD11b expression and its ability to bind with the fluorescent antibodies.

Development of reliable, valid methods for flow cytometry to analyze blood is challenging because blood cells interact and respond to their environment. There is a need to develop methods that closely reflect *in-vivo* conditions and that respond to positive controls. In addition, flow cytometry procedures in whole mouse blood have not been widely reported. The results of our experiments indicate that mouse leukocyte studies for flow cytometry should be conducted in whole blood with use of anti-CD45 for leukocyte identification. Also, while sample fixation prior to staining caused a decrease in overall fluorescence; it can still be used to successfully identify extra-cellular markers, but should be used with caution for measurement of ROS or other intracellular stains.

## CHAPTER 3

WHOLE BLOOD AGGREGATION AND COAGULATION IN *db/db* AND *ob/ob*  
MOUSE MODELS OF TYPE 2 DIABETES

## Abstract

Type 2 diabetes is associated with a significant hypercoagulable state. Diabetes produces alterations in platelet structure and function, initiating a state of chronic activation and hyperaggregability. The mechanisms by which diabetes causes changes in platelet function and hypercoaguability and the effects of these processes on stroke and cardiovascular disease are not completely understood. The spontaneous genetic mutations in *db/db* and *ob/ob* mice produce metabolic abnormalities similar to obesity and type 2 diabetes and could be used to study these disease processes, but little is known about their platelet or coagulation properties. The purpose of this study was to examine whole blood aggregation, coagulation, and platelet CD61 expression in the *db/db* and *ob/ob* mouse models to determine the degree of platelet alteration induced by diabetes. We also examined two known inhibitors of platelet aggregation, aspirin and fucoidan, on the ADP-induced whole blood aggregation response in these genetic mouse models. We found that both *db/db* and *ob/ob* mice demonstrate significantly less whole blood aggregation after stimulation with ADP compared to control mice ( $p < 0.001$  *db*;  $p < 0.01$  *ob*). The rate and extent of whole blood aggregation were significantly inhibited with aspirin in all groups; however, fucoidan only inhibited aggregation in control mice. *db/db* and *ob/ob* mice also demonstrated significantly less maximal clot strength compared to control mice measured by thromboelastography ( $p < 0.01$ ). In addition, *ob/ob* mice demonstrated early onset

fibrinolysis unlike the *db/db* or their controls. The decrease in whole blood aggregation and coagulation do not appear to be mediated by differences in platelet CD61 expression, but may be influenced by a significantly lower platelet count found in the *db/db* and *ob/ob* mice. These results are considerably different from studies that demonstrate a hypercoagulable state in humans with diabetes. We conclude that the *db/db* and *ob/ob* mouse models of type 2 diabetes may not be ideal for studying platelet aggregation and coagulability and their effects on stroke or cardiovascular disease.

### Introduction

Type 2 diabetes is associated with a severe hypercoagulable state (Carr, 2001). Most deaths associated with diabetes are secondary to thrombotic events, such as ischemic stroke, caused by platelet and coagulation factor abnormalities (Carr, 2001). Diabetes induces alterations in platelet structure and function, initiating a state of chronic activation and hyperaggregability. Platelets from people with diabetes demonstrate increased platelet expression of P-selectin (Nomura, et al., 1995; McDonagh, et al., 2003) and increased levels of circulating platelet microparticles (PMPs, Koga, et al., 2006; Diamont, Nieuwland, Pablo, Sturk, Smit, & Radder, 2002; Nomura, et al., 1995). Diabetic platelets also aggregate more readily to collagen, ADP (Ferreira, et al., 2006), and thrombin (Redondo, et al., 2005) compared to platelets from those without diabetes. Several coagulation factors are also known to be elevated in human type 2 diabetes, including factors I (fibrinogen) (Coppola, et al., 2006; Coca, Cucuianu, & Hancu, 2005; Erem, et al., 2005), VII, (Coca, et al., 2005; Ludwig, et al., 2005), VIII, (Coca, et al.), and endothelial surface antigen von

Willebrand factor (Coca, et al.; Erem, et al.). In addition, diabetics demonstrate significantly slower fibrinolysis (Dunn, Philippou, Ariens, & Grant, 2006) than people without diabetes. Together, these defects lead to an overall state of hypercoaguability in people with type 2 diabetes. However, the mechanisms of the hypercoaguable state are not completely understood. Animal models that represent the metabolic characteristics of type 2 diabetes could be utilized to further explore the contribution of altered platelet function and coagulation on the diabetic complications of thrombotic and vascular disease.

Two mouse models that are often used to study type 2 diabetes are the *db/db* *ob/ob* mouse. As a result of spontaneous genetic mutations, the *db/db* and *ob/ob* mice demonstrate metabolic disturbances that are similar to type 2 diabetes in humans. The *db* mutation is characterized by an insertion of a premature stop codon in the long form of the leptin receptor mRNA transcript, resulting in the production of a nonfunctional short form of the receptor that lacks an intracellular domain (Lee et al., 1996). The *ob* mutation results in a truncated leptin hormone that is not secreted (Zhang, et al., 1994). Both mutations cause a change in leptin function, which normally induces satiety and weight maintenance. Because these mutations cause a decrease in leptin function (Coleman, 1973), the mice become hyperphagic and subsequently develop obesity, hyperinsulinemia, and hyperglycemia (although the *ob* mutation causes only transient hyperglycemia) (Coleman, & Hummel, 1967; Dubac, 1976). Since few studies examining platelet function have been conducted in these animal models, it is unknown if the platelet response to diabetes is similar to that seen in humans. The aim of the present study was therefore, to investigate the effects of type 2 diabetes on mouse platelet

function by measuring whole blood aggregation, coagulation, and platelet CD61 expression in the *db/db* and *ob/ob* mouse models of type 2 diabetes. We found that the *db/db* and *ob/ob* mice demonstrated significantly less whole blood aggregation and coagulation compared to control mice.

### Materials and Methods

All animal experiments were conducted in accordance with the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* (OLAW, 2002) after IACUC approval of all researchers. C57BKS/Cgm<sup>+</sup>/+Lepr<sup>db</sup>, C57BL/6J B6.V-Lep<sup>ob</sup>, and their respective controls, (*db/db* control- C57BLKs/J; *ob/ob* control- C57BL/6J) (Jackson Laboratories, Inc., Bar Harbor, MA), age 8-16 weeks, were transported to the laboratory the day prior to blood acquisition. Mice were allowed free access to food and water and were housed in a temperature controlled, quiet environment.

#### *Blood Acquisition*

Mice were anesthetized in a chamber of 5.0% isoflurane (JD Medical Distributing Co., Inc) and 1.0 L/minute oxygen (Medical Grade Oxygen) until unresponsive, then mask-ventilated with 5.0% isoflurane and 1.0 L/minute oxygen for maintenance anesthesia. Seven hundred  $\mu$ L of blood was withdrawn from the ascending vena cava with a 23-gauge needle/1 mL syringe containing 0.17 mL of 70 units/mL heparin sodium (1000 USP units/ml, diluted with PBS, Baxter Healthcare Corp., Deerfield, IL.) for aggregometry and flow cytometry experiments, 0.17 mL of undiluted sodium citrate (Sigma, Cat #S1804) for

selected aggregometry experiments, or 0.17 mL buffered 0.105 M 3.2 % sodium citrate (BD Vacutainer Systems, Franklin Lakes, NJ) for TEG® experiments.

#### *Blood Smear Analysis of Platelet Microaggregation*

Peripheral blood smears were made for all experiments using standard methods on glass microscope slides (VWR, West Chester, PA, #16004-368) (Brown, 1980). Eight  $\mu\text{l}$  anticoagulated blood was smeared across a glass slide, air-dried, and stained with Diff-Quik (Dade Behring, VWR #47733-152). The monolayer region on the top and the bottom of the slides was used for analysis. The number of platelet microaggregates (defined as 3 or more adhered platelets) in 20 visual fields (10 top/10 bottom) and the area of 10 random aggregates were quantified with an ocular micrometer at 100X (Koplitz, Scott, & Cohn, 2001). The percentage of visual fields with platelet microaggregates, the average number of platelet microaggregates per visual field, and the average area of 10 platelet microaggregates were calculated for analysis.

#### *Whole Blood Aggregometry*

Whole blood aggregometry was performed in heparinized samples diluted 1:1 per a standard protocol (Goldenberg, Veriabo, & Soslau, 2001) with 0.9% saline (Associated Medical, Philadelphia, PA, #101310), or diluted with saline to a standardized platelet count of  $315 \times 10^3/\mu\text{l} \pm 10\%$  to determine the effect of differing platelet counts. In a second set of experiments, whole blood was diluted to a standardized platelet count and anticoagulated with sodium citrate to determine the effects of the anticoagulant on whole blood aggregation. Nine hundred seventy  $\mu\text{l}$  of blood were placed in 2 ml cuvettes and incubated for 6 minutes at 37°C in the Chrono-Log Whole Blood Lumi-Ionized Calcium

Aggregometer (Goldenberg, Veriabo, & Soslau, 2001). The cuvettes were then placed into the test chamber and incubated with the impedance electrode at 37°C while stirring at 900 rpm. After 2 minutes, 30µL of 1µM adenosine diphosphate (ADP) (Chrono-Log Corp., Havertown, PA), and 15 µL .2 mM CaCl<sub>2</sub> (to samples anticoagulated with sodium citrate) were added and aggregation (ohms of electrical impedance) was recorded for 6 minutes. Whole blood aggregation inhibitors aspirin (4 µl 0.5M diluted with 100% ethanol, Sigma-Aldrich, #A5376) and fucoidan (*fucus vesiculosus*) (50 µl, 20 mg/mL, Sigma-Aldrich, #F5631-1G) were added to the heparinized samples diluted 1:1 with saline during incubation in the test chamber. All whole blood aggregometry tests were performed within 3 hours of blood acquisition.

#### *Thromboelastography*

An aliquot of 340 µL of sodium citrated whole blood recalcified with 20 µL 0.2M CaCl<sub>2</sub> (Haemoscope Corp., Skokie, IL, #7003) was added to 37°C pre-warmed cuvettes (Haemoscope, #6211) in the thromboelastograph (Thromboelastograph Coagulation Analyzer 5000, Haemoscope Corp.) (Bowbrick, Mikhailidis, & Stansby, 2001; Salooja, & Perry, 2001) and coagulation was recorded for 30 minutes. Parameters measured and used for analysis were the maximal amplitude (MA, in mm) of clot strength; R (reaction time, in secs), the time to initiation of clot formation; K (kinetic time, in secs), the amount of time from the beginning of clot formation until the amplitude reaches 20mm; α-angle (° degree), a measure of clot strengthening speed; G (dyn/cm<sup>2</sup>), the value of clot firmness (calculated as  $G=5000MA/(100-MA)$ ); and LY30 (lysis 30min), the percent fibrinolysis at 30 minutes (Salooja, & Perry, 2001).

### *Flow Cytometry*

Twelve microliters of heparinized whole blood (WB) was diluted in 1 ml Dulbecco's phosphate-buffered saline with 0.2% bovine serum albumin and 0.09% sodium azide (pH 7.4) (Pharmingen Stain Buffer, BD Biosciences Pharmingen, San Jose, CA, #554657). Twenty  $\mu\text{L}$  of 50  $\mu\text{M}$  calcimycin A23187 (Sigma-Aldrich Co., St. Louis, MO, #C7522) was added to the positive control samples and incubated at room temperature for 10 minutes. One hundred  $\mu\text{L}$  of the stain buffer/WB mixture was then added to amber microcentrifuge tubes filled with 31.25  $\mu\text{L}$  of 1:10 dilution of fluorescent monoclonal antibody (BD Biosciences Pharmingen) R-phycoerythrin (PE)-conjugated hamster anti-mouse CD61 (Clone 2C9.G2). The samples were covered with aluminum foil and incubated at room temperature for 10 minutes, then fixed with 1 mL 1% paraformaldehyde (Sigma-Aldrich Co., #P6148-500G) and placed on ice. Data were acquired on a FACScalibur flow cytometer (488 nm argon laser, Becton Dickinson, San Jose, CA). The flow cytometer was calibrated daily prior to each experiment using Calibrite Beads (Becton Dickinson, #340486) and FACsComp software (Becton Dickinson). Acquisition threshold was set on FL2 to capture only CD61 positive events. A dot plot of logarithmic forward and side scatter properties were used to distinguish platelets. Data were analyzed with FCS Express v.3.0 (De Novo Software, Thornhill, Ontario).

### *Metabolic Characteristic Measurements*

Weight in grams was obtained prior to each experiment (Triple Beam Balance, Ohaus, Columbia, MD). Blood sugar was measured (AccuCheck Advantage, Roche, Inc., Mannheim, Germany) with anticoagulated blood obtained during venipuncture. The

glucometer was calibrated weekly with high and low controls (Roche). White blood cell count, hematocrit, and platelet count were measured by a whole blood analyzer (Coulter A<sup>c</sup>T™ 5 diff Hematology Analyzer, Beckman Coulter, Fullerton, CA), which was calibrated daily. Triglycerides and total cholesterol were measured by the Trinder's Method using reflective photometry (CardioChek, model #542165, Polymer Technologies Systems (PTS), Inc., Indianapolis, IN). The CardioChek unit was tested for monthly for reliability with high and low controls (PTS, Inc.).

#### *Statistical Analysis*

Data were expressed as mean  $\pm$  SEM. Groups were compared using unpaired t-test ( $t$ ), Mann-Whitney Rank Sum Test ( $T$ ) (non-parametric data), One-way ANOVA ( $F$ ) or Kruskal-Wallis ANOVA on Ranks ( $H$ ) (non-parametric data). ANOVA post hoc testing was done with either Holm-Sidak or Dunn's Method (non-parametric). For comparison between samples within the same group, a paired t-test ( $t$ ) or Wilcoxon Signed Rank test ( $T$ ) was performed (i.e. stimulated vs. unstimulated samples). The flow cytometry experiments were performed in duplicate or triplicate (depending upon the amount of blood obtained from the venipuncture) and the coefficient of variation was determined by dividing the standard deviation by the mean. All experiments with a variation of >10% were removed from the analysis (Waltz, Strickland, & Lenz, 1991). Statistical analysis was completed using SigmaStat for Windows version 3.10 (Systat Software, Inc., Point Richmond, CA). An a priori  $\alpha$  of  $p \leq 0.05$  was considered statistically significant.

## Results

### *Metabolic Characteristics*

Table 3.1 describes the metabolic characteristics of mice from the whole blood aggregometry experiments, in which heparinized samples were diluted 1:1 with 0.9% saline. *Db/db* and *ob/ob* mice were obese (C57BLKS/J (*db* control) ( $n= 23$ )  $20.7 \pm 0.4$ , *db/db* ( $n= 23$ )  $39.0 \pm 1.1$  gm  $T= 805.00$ ,  $p < 0.001$ ; C57BL6/J (*ob* control) ( $n= 21$ )  $23.2 \pm 0.5$ , *ob/ob* ( $n= 21$ )  $51.1 \pm 1.2$  gm,  $T= 630.00$ ,  $p < 0.001$ ) and hyperglycemic (C57BLKS/J  $155.6 \pm 8.9$ , *db/db*  $354.2 \pm 30.5$  mg/dl,  $T= 596.50$ ,  $p < 0.001$ ; C57BL6/J  $190.2 \pm 8.6$ , *ob/ob*  $298.8 \pm 25.6$  mg/dl,  $T= 223.50$ ,  $p < 0.01$ ) compared to control mice. Additionally, *db/db* and *ob/ob* mice demonstrated altered lipid profiles compared to control mice, exhibiting significant hypertriglyceridemia (C57BLKS/J  $80.3 \pm 8.0$ , *db/db*  $188.3 \pm 23.2$  mg/dl,  $T= 177.50$ ,  $p < 0.001$ ; C57BL6/J  $62.6 \pm 5.8$ , *ob/ob*  $134.2 \pm 18.7$  mg/dl,  $T= 124.00$ ,  $p < 0.01$ ) and hypercholesterolemia (C57BLKS/J  $< 100.0$ , *db/db*  $130.3 \pm 7.3$  mg/dl,  $T= 186.00$ ,  $p < 0.001$ ; C57BL6/J  $102.0 \pm 1.2$ , *ob/ob*  $154.9 \pm 8.5$  mg/dl,  $T= 99.00$ ,  $p < 0.001$ ).

### *Whole Blood Cell Counts*

To account for variations in blood dilution by the anticoagulant, platelet and total white blood counts (WBC) were standardized to a hematocrit of 45% (normal mouse hematocrit is 40-50% (Everds, 2004; Table 2). Platelet counts in samples anticoagulated in either heparin or sodium citrate and used for aggregometry experiments were significantly lower in both *db/db* and *ob/ob* mice compared to controls (C57BLKS/J  $818.7 \pm 25.8$ , *db/db*  $617.1 \pm 12.3$   $10^3/\mu\text{l}$ ,  $T= 304.00$ ,  $p < .001$ ; C57BL6/J  $23.2 \pm 0.5$ , *ob/ob*  $51.1 \pm 1.2$   $10^3/\mu\text{l}$ ,  $t(40)= -6.93$ ,  $p < .001$ ). The WBC was significantly lower in the *db/db* mice compared to

their controls (C57BLKS/J  $8.8 \pm 0.3$ , *db/db*  $7.9 \pm 0.4 \times 10^3/\mu\text{l}$ ,  $t(44)=-1.99$ ,  $p=0.05$ ).

However, the *ob/ob* mice had a significantly higher WBC compared to their controls (C57BL6/J  $6.1 \pm 0.6$ , *ob/ob*  $8.6 \pm 0.5 \times 10^3/\mu\text{l}$ ,  $t(40)=3.34$ ,  $p<0.01$ ). These WBC counts, however, were still within normal range for all groups (Everds, 2004). The use of sodium citrate was not associated with a change in the platelet (C57BLKS/J ( $n=5$ ) heparin-  $826.7 \pm 13.7$ , citrate-  $774.1 \pm 15.6 \times 10^3/\mu\text{l}$   $t(8)=2.5$ ,  $p=0.04$ ; *db/db* heparin ( $n=6$ )  $618.5 \pm 9.1$ , citrate ( $n=4$ )  $588.3 \pm 34.3 \times 10^3/\mu\text{l}$ ,  $T=16.00$ ,  $p=0.26$ ; C57BL6/J heparin ( $n=5$ )  $751.8 \pm 14.7$ , citrate ( $n=8$ )  $696.7 \pm 32.7 \times 10^3/\mu\text{l}$ ,  $t(11)=1.26$ ,  $p=0.23$ ; *ob/ob* heparin ( $n=5$ )  $532.3 \pm 13.9$ , citrate ( $n=4$ )  $482.0 \pm 45.7 \times 10^3/\mu\text{l}$ ,  $t(7)=1.17$ ,  $p=0.28$ ) or total WBC counts (C57BLKS/J heparin-  $9.1 \pm 0.5$ , citrate-  $10.0 \pm 0.5 \times 10^3/\mu\text{l}$ ,  $t(8)=-1.23$ ,  $p=0.25$ ; *db/db* heparin  $6.4 \pm 0.3$ , citrate  $8.7 \pm 1.9 \times 10^3/\mu\text{l}$ ,  $T=29.00$ ,  $p=0.17$ ; C57BL6/J heparin-  $7.1 \pm 0.6$ , citrate-  $7.1 \pm 0.7 \times 10^3/\mu\text{l}$ ,  $t(11)=0.04$ ,  $p=0.97$ ; *ob/ob* heparin-  $9.2 \pm 1.3$ , citrate  $8.4 \pm 1.0 \times 10^3/\mu\text{l}$ ,  $t(7)=0.44$ ,  $p=0.68$ ), except in the C57BLKS/J group, which had a significantly lower platelet count in samples anticoagulated with heparin.

Table 3.1 Metabolic Characteristics and Whole Blood Counts of *db/db*, *ob/ob*, and Control Mice from Whole Blood Aggregometry Experiments

Strain (n)	Weight (g)	Blood Sugar (mg/dl)	Trig. (mg/dl)	Total Cholesterol (mg/dl)	Platelet count (10 <sup>3</sup> /μl)	WBC count (10 <sup>3</sup> /μl)
C57BLKS/J (23)	20.7 ± 0.4	155.6 ± 8.9	80.3 ± 8.0	<100 ± 0.0	818.7 ± 25.8	8.8 ± 0.3
<i>db/db</i> (23)	39.0 ± 1.1***	354.2 ± 30.5***	188.3 ± 23.2***	130.3 ± 7.3***	617.1 ± 1 2.3***	7.9 ± 0.4
C57BL/6J (21)	23.2 ± 0.5	190.2 ± 8.6	62.6 ± 5.8**	102.0 ± 1.2	710.8 ± 21.4	6.1 ± 0.6
<i>ob/ob</i> (21)	51.1 ± 1.2***	298.8 ± 25.6*	134.2 ± 18.7**	154.9 ± 8.5***	544.1 ± 11.1***	8.6 ± 0.5**

Data represented as mean ± SEM. Trig.- Triglycerides Platelet and white blood cell (WBC) count standardized to hematocrit of 45%. \*significantly different from control, \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

To determine if the lower platelet counts in the diabetic mice were a result of differences in the degree of artifactual platelet microaggregation after venipuncture, we analyzed the peripheral smear on a subset of experiments anticoagulated with heparin. In general, there was no difference in the three indices of platelet microaggregation between *db/db* or *ob/ob* and their controls, indicating that the counts were not significantly affected by differences in microaggregation (%visual fields with platelet microaggregates- C57BLKS/J 56.0 ± 6.0, *db/db* 61.0 ± 6.0%,  $t(7) = -0.57$ ,  $p = 0.59$ ; C57BL6/J 63.0 ± 4.0, *ob/ob* 49.0 ± 6.0%,  $t(7) = -2.05$ ,  $p = 0.08$ ; average platelet microaggregates/visual field- C57BLKS/J 0.8 ± 0.1, *db/db* 1.0 ± 0.1,  $t(7) = -1.0$ ,  $p = 0.34$ ; C57BL6/J 1.0 ± 0.1, *ob/ob* 0.7 ± 0.1,  $T = 13.00$ ,  $p = 0.11$ ; average platelet microaggregate area (μm<sup>2</sup>)- C57BLKS/J 98.4 ±

3.5, *db/db*  $165.2 \pm 31.3 \mu\text{m}^2$ ,  $T= 14.00$ ,  $p= 0.19$ ; C57BL6/J  $175.6 \pm 51.4$ , *ob/ob*  $107.6 \pm 22.8 \mu\text{m}^2$ ,  $T= 15.00$ ,  $p= 0.29$ , Table 3.2).

Table 3.2 Platelet Microaggregate Formation in Heparinized Whole Blood Samples

Strain ( <i>n</i> )	% visual fields with microaggregates	Avg number of microaggregates/visual field	Avg area of microaggregates ( $\mu\text{m}^2$ )
C57BLKS/J (4)	$56.0 \pm 6.0$	$0.8 \pm 0.1$	$98.4 \pm 3.5$
<i>db/db</i> (5)	$61.0 \pm 4.0$	$1.0 \pm 0.1$	$165.2 \pm 31.3$
C57BL/6J (5)	$63.0 \pm 4.0$	$1.0 \pm 0.1$	$175.6 \pm 51.4$
<i>ob/ob</i> (4)	$49.0 \pm 6.0$	$0.7 \pm 0.1$	$107.6 \pm 22.8$

Data represented as mean  $\pm$  SEM.

The metabolic characteristics of mice from the thromboelastography experiments in samples anticoagulated with sodium citrate are summarized in Table 3.3. Both *db/db* and *ob/ob* mice were obese compared to controls, (C57BLKS/J ( $n= 7$ )  $23.4 \pm 0.4$ , *db/db* ( $n= 7$ )  $41.5 \pm 2.1$  gm,  $T= 28.00$ ,  $p< 0.001$ ; C57BL6/J ( $n= 6$ )  $26.1 \pm 1.0$ , *ob/ob* ( $n= 6$ )  $55.1 \pm 1.1$  gm,  $t(10)= -19.49$ ,  $p< 0.001$ ), but in these experiments, only the *db/db* were hyperglycemic (C57BLKS/J  $146.0 \pm 6.3$ , *db/db*  $389.7 \pm 60.1$  mg/dl,  $T= 63.00$ ,  $p= 0.001$ ; C57BL6/J  $153.7 \pm 12.2$ , *ob/ob*  $180.0 \pm 19.5$  mg/dl,  $t(10)= -1.15$ ,  $p= 0.28$ ). The total white blood counts were no different between *db/db* their controls in sodium citrated blood (C57BLKS/J  $9.1 \pm 1.2$ , *db/db*  $7.1 \pm 1.1$ ,  $t(12)= 1.28$ ,  $p= 0.23$ ), but in this set of experiments the *ob/ob* mice had a significantly lower WBC than controls (C57BL6/J  $7.4 \pm 0.7$ , *ob/ob*  $6.8 \pm 0.5$ ,  $t(10)= 0.70$ ,  $p= 0.50$ , Table 3.3). Similar to the heparinized and sodium citrated blood used for whole

blood aggregometry, both the *db/db* and *ob/ob* mice had significantly lower platelet counts than their controls (C57BLKS/J  $795.1 \pm 19.1$ , *db/db*  $641.3 \pm 17.1$  mg/dl,  $t(12)= 6.00$ ,  $p < 0.001$ ; C57BL6/J  $710.1 \pm 19.7$ , *ob/ob*  $510.0 \pm 17.7$  mg/dl,  $T= 57.00$ ,  $p < 0.01$ ).

Table 3.3 Metabolic Characteristics and Whole Blood Counts of *db/db*, *ob/ob*, and Control Mice from Thromboelastography Experiments

Strain (n)	Weight (g)	Blood Sugar (mg/dl)	Platelet count ( $10^3/\mu\text{l}$ )	WBC count ( $10^3/\mu\text{l}$ )
C57BLKS/J (7)	$23.4 \pm 0.7$	$146.0 \pm 6.3$	$795.1 \pm 19.1$	$9.1 \pm 1.2$
<i>db/db</i> (7)	$41.5 \pm 2.0^{***}$	$389.7 \pm 60.1^{***}$	$641.3 \pm 17.1^{***}$	$7.1 \pm 1.1$
C57BL/6J (6)	$26.1 \pm 1.0$	$153.7 \pm 12.2$	$710.14 \pm 19.7$	$7.4 \pm 0.7$
<i>ob/ob</i> (6)	$55.1 \pm 1.1^{***}$	$180.0 \pm 19.5$	$510.0 \pm 17.7^{**}$	$6.8 \pm 0.5$

Data represented as mean  $\pm$  SEM. Platelet and white blood cell (WBC) count standardized to hematocrit of 45%. \*significantly different from control, \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

#### Whole Blood Aggregation

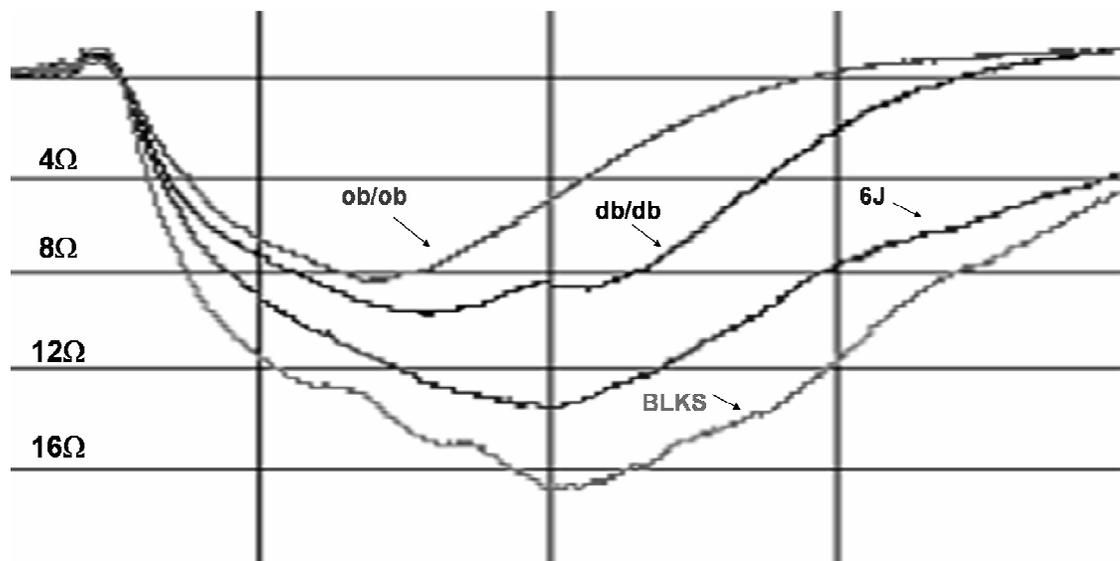
Whole blood aggregometry was performed in samples diluted 1:1 with 0.9% saline per a standard protocol (Goldenberg, Veriabo, & Soslau, 2001) or diluted to a standardized platelet count of  $315 \times 10^3/\mu\text{l} \pm 10\%$  to determine the effect of platelet count on the results. Additional experiments in samples with standardized platelet counts were completed with sodium citrate anticoagulation to determine the effects of anticoagulant on whole blood aggregation. In blood samples diluted 1:1 with saline, both *db/db* and *ob/ob* mice demonstrated significantly less ADP stimulated whole blood aggregation compared to

controls (C57BLKS/J ( $n=6$ )  $20.2 \pm 0.5$  ohms ( $\Omega$ ), *db/db* ( $n=6$ )  $13.2 \pm 1.0$   $\Omega$ ,  $t(10)=-6.42$ ,  $p<0.001$ ; C57BL6/J ( $n=5$ )  $14.8 \pm 1.2$   $\Omega$ , *ob/ob* ( $n=5$ )  $10.0 \pm 0.6$   $\Omega$ ,  $t(8)=-3.54$ ,  $p<0.01$ ; Figure 3.1A/B), but no significant difference in the rate of aggregation (slope) (Figure 3.1C, C57BLKS/J  $19.8 \pm 1.6$ , *db/db*  $17.7 \pm 1.0$   $\Omega/\text{sec}$ ,  $T=28.00$ ,  $p=0.09$ ; C57BL6/J  $15.0 \pm 2.2$ , *ob/ob*  $11.4 \pm 0.7$   $\Omega/\text{sec}$ ,  $t(8)=-1.57$ ,  $p=0.16$ ). However, when samples were diluted to a standardized platelet count, the *db/db* mice no longer demonstrated less ADP induced whole blood aggregation (C57BLKS/J ( $n=5$ )  $14.4 \pm 1.7$   $\Omega$ , *db/db* ( $n=6$ )  $13.6 \pm 1.6$   $\Omega$ ,  $t(9)=0.31$ ,  $p=0.76$ ) and no difference in the rate of aggregation in heparinized samples (C57BLKS/J  $7.4 \pm 1.4$   $\Omega/\text{sec}$ , *db/db*  $11.2 \pm 1.4$   $\Omega/\text{sec}$ ,  $t(9)=-1.89$ ,  $p=0.09$ ), but did demonstrate significantly less aggregation (C57BLKS/J ( $n=5$ )  $12.8 \pm 0.5$   $\Omega$ , *db/db* ( $n=4$ )  $8.3 \pm 0.9$   $\Omega$ ,  $t(7)=4.88$ ,  $p<0.01$ ) and a decreased rate of aggregation in citrated samples (C57BLKS/J  $19.0 \pm 0.4$   $\Omega/\text{sec}$ , *db/db*  $11.5 \pm 1.0$   $\Omega/\text{sec}$ ,  $t(7)=7.64$ ,  $p<0.001$ , Figure 3.2).

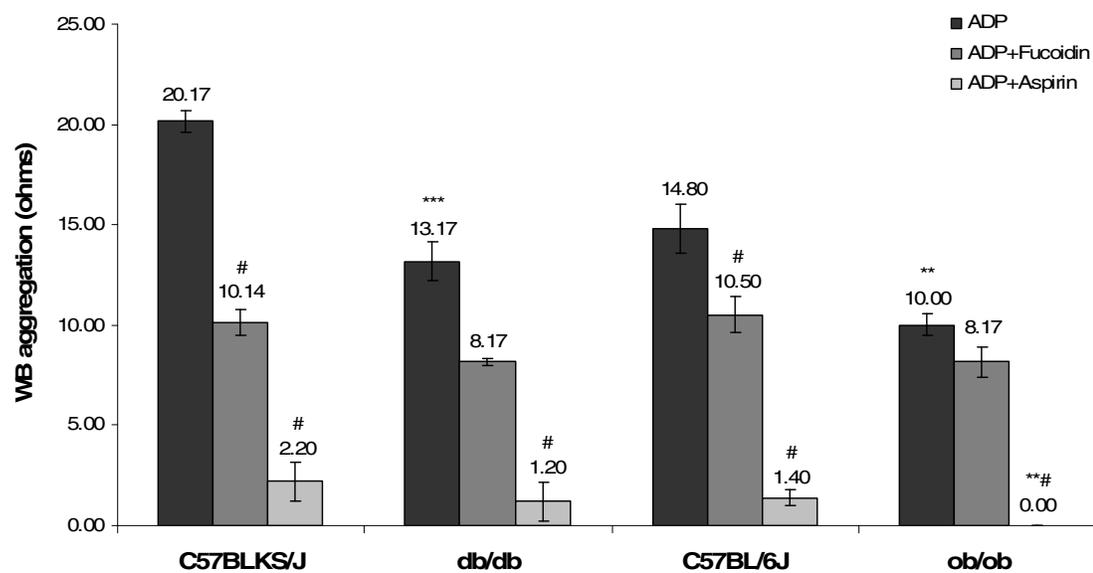
Figure 3.1 Whole blood aggregation in *db/db*, *ob/ob*, and control mice in heparinized samples diluted 1:1 with 0.9% saline. (A) Representative tracings of whole blood aggregometry. 6J-C57BL/6J *ob/ob* control, BLKS-C57BLKS/J *db/db* control. (B) Summative data of ADP-induced whole blood aggregation in Ohms ( $\Omega$ ). ( $n= 5-6/\text{group}$ ). (C) Summative data of the rate of aggregation ( $\Omega/\text{sec}$ ) ( $n= 5-6/\text{group}$ ). Data represented as mean  $\pm$  SEM. \*significantly different from control, \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . #significantly different from aggregation without inhibitor, # $p < 0.05$ , ###  $p < 0.001$ . +significantly different from aggregation with fucoidan, + $p < 0.05$ .

Figure 3.1

A.



B.



C.

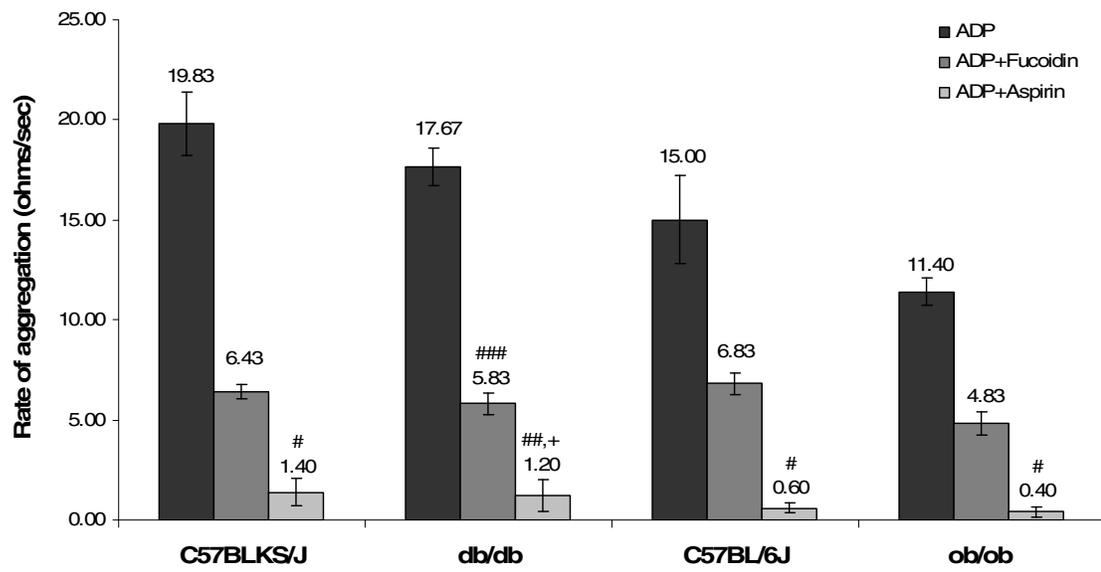
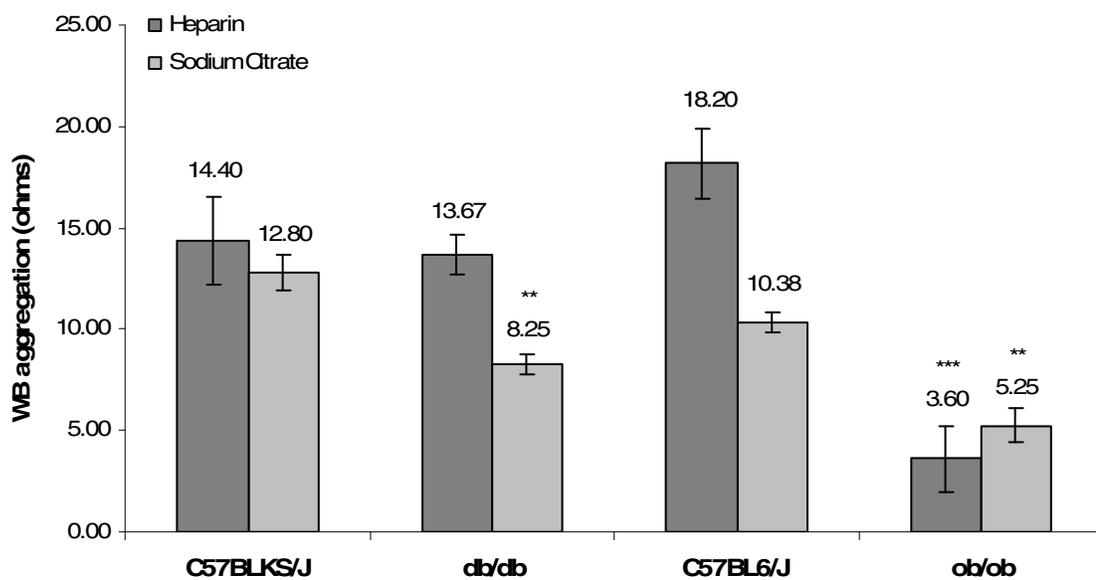


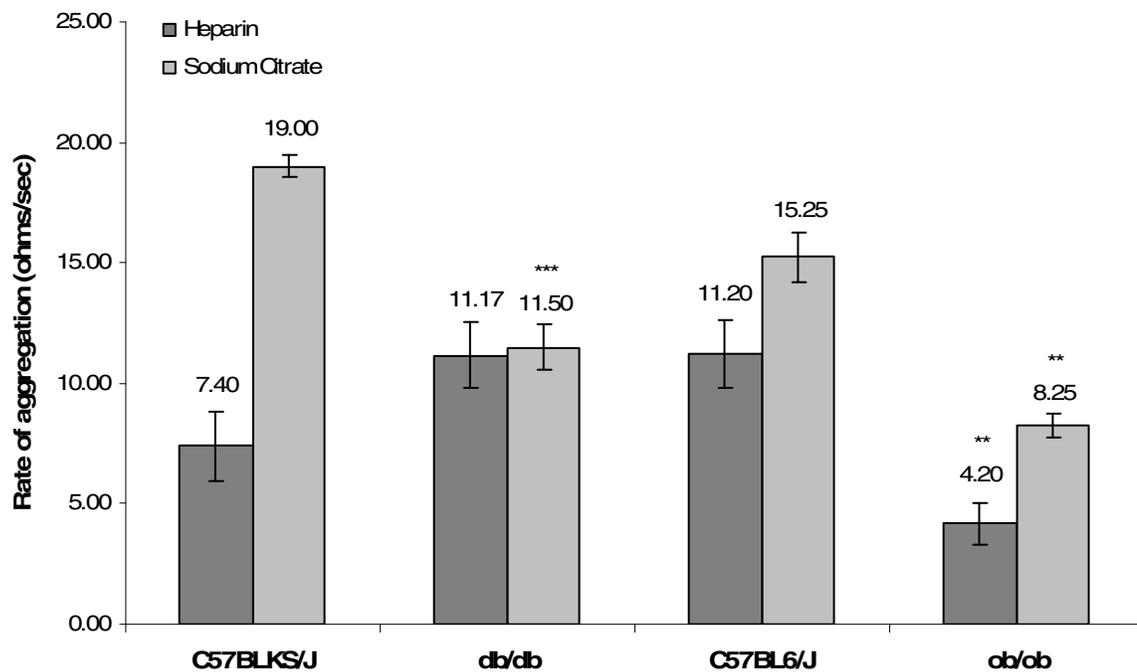
Figure 3.2 Whole blood aggregation of *db/db*, *ob/ob*, and control mice in samples anticoagulated with heparin and citrate and diluted with 0.9% saline to a standardized platelet count. C57BL/6J-control strain for *ob/ob* mice. C57BLKS/J-control strain for *db/db* mice. (A) Summative data of ADP-induced aggregation in ohms of aggregation ( $n=4-5/\text{group}$ ). (B) Summative data of the rate of aggregation ( $\Omega/\text{sec}$ ). Data presented as mean  $\pm$  SEM. \*significantly different from control mice, \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

Figure 3.2

A.



B.



The *ob/ob* mice continued to demonstrate a significant decrease in whole blood aggregation with a standardized platelet count compared to controls in either heparinized (C57BL6/J ( $n= 5$ )  $18.2 \pm 2.2$ , *ob/ob* ( $n= 5$ )  $3.6 \pm 1.0 \Omega$ ,  $t(8)= 6.17$ ,  $p < 0.001$ ) or citrated whole blood samples (C57BL6/J ( $n= 8$ )  $10.4 \pm 1.0$ , *ob/ob* ( $n= 4$ )  $5.3 \pm 0.5 \Omega$ ,  $t(10)= 3.80$ ,  $p < 0.01$ ).

In contrast to the samples without a standardized count, in the samples with a standardized platelet count, the *ob/ob* mice demonstrated a decrease in the rate of aggregation in both heparinized (C57BL6/J  $11.2 \pm 1.4$ , *ob/ob*  $4.2 \pm 0.5 \Omega/\text{sec}$ ,  $t(8)= 4.28$ ,  $p < 0.01$ ) and citrated samples (C57BL6/J  $15.2 \pm 1.0$ , *ob/ob*  $8.2 \pm 0.5 \Omega/\text{sec}$ ,  $t(10)= 4.58$ ,  $p = 0.001$ ).

In addition to baseline ADP-induced whole blood aggregation; we also assessed the response of *db/db* and *ob/ob* mice to 2 known platelet inhibitors, aspirin and fucoidan, in samples diluted per the standard protocol (1:1 dilution with 0.9% saline). All groups had a significant decrease in whole blood aggregation with aspirin (C57BLKS/J ( $n= 5$ )  $2.2 \pm 1.0 \Omega$   $F(2,15)= 150.89$ ,  $p < 0.001$ ; *db/db* ( $n=5$ )  $1.2 \pm 1.0 \Omega$ ,  $H=14.69$ ,  $df= 2$ ,  $p < 0.001$ ; C57BL6/J ( $n= 5$ )  $1.4 \pm 0.4 \Omega$   $F(2,13)= 53.18$ ,  $p < 0.001$ , *ob/ob* ( $n= 5$ )  $0.0 \pm 0.0 \Omega$ ,  $H= 11.61$   $df= 2$ ,  $p < 0.01$ , Figure 3.1B). Additionally, whole blood aggregation in the *ob/ob* mice was completely abolished with aspirin and these mice had a significantly greater response to aspirin compared to their controls ( $T= 17.50$ ,  $p= 0.03$ ). *db/db* and *ob/ob* mice, however, did not have a significant decrease in whole blood aggregation in response to fucoidan, unlike their respective control mice (post hoc analyses- C57BLKS/J ( $n= 7$ )  $10.1 \pm 0.6 \Omega$   $p < 0.001$ ; *db/db* ( $n= 6$ )  $8.2 \pm 0.2 \Omega$ ,  $p > 0.05$ ; C57BL6/J ( $n= 6$ )  $10.5 \pm 0.9 \Omega$   $p < 0.001$ , *ob/ob* ( $n= 6$ )  $8.2 \pm 0.8 \Omega$ ,  $p > 0.05$ , Figure 3.1B). All groups had a significant decrease in the rate of ADP stimulated aggregation inhibited with aspirin (C57BLKS/J  $1.4 \pm 0.7 \Omega/\text{sec}$   $H=$

15.26  $df=2$ ,  $p < 0.001$ ;  $db/db$   $1.2 \pm 0.8$   $\Omega/\text{sec}$ ,  $F(2,14)=116.21$ ,  $p < 0.001$ ; C57BL6/J  $0.6 \pm 0.2$   $\Omega/\text{sec}$   $H=13.57$   $df=2$ ,  $p=0.001$ ,  $ob/ob$   $0.4 \pm 0.2$   $\Omega/\text{sec}$ ,  $H=13.21$   $df=2$ ,  $p=0.001$ ).

Fucoidan did not decrease the rate of aggregation in any group except in the  $db/db$  mice (post hoc analyses- C57BLKS/J  $6.4 \pm 0.4$   $\Omega/\text{sec}$   $p > 0.05$ ;  $db/db$   $5.8 \pm 0.5$   $\Omega/\text{sec}$ ,  $p < 0.001$ ; C57BL6/J  $6.8 \pm 0.5$   $\Omega/\text{sec}$   $p > 0.05$ ,  $ob/ob$   $4.8 \pm 0.6$   $\Omega/\text{sec}$ ,  $p > 0.05$ , Figure 3.1C).

### *Thromboelastography*

The  $db/db$  ( $n=7$ ) and  $ob/ob$  ( $n=6$ ) mice demonstrated a decreased MA (maximal amplitude) (C57BLKS/J  $59.7 \pm 3.1$ ,  $db/db$   $46.6 \pm 3.9$  mm,  $t(12)=2.75$ ,  $p=0.02$ ; C57BL6/J  $59.7 \pm 1.1$ ,  $ob/ob$   $36.3 \pm 6.3$  mm,  $T=55.00$ ,  $p < 0.01$ ) and G value, (C57BLKS/J  $7.8 \pm 0.9$ ,  $db/db$   $4.6 \pm 0.7$   $\text{dyn}/\text{cm}^2$ ,  $t(12)=2.81$ ,  $p=0.02$ ; C57BL6/J  $7.5 \pm 0.3$ ,  $ob/ob$   $3.3 \pm 0.9$   $\text{dyn}/\text{cm}^2$ ,  $t(10)=4.33$ ,  $p < 0.01$ ), both indicators of clot formation and strength (Table 3.4). The  $db/db$ , but not the  $ob/ob$  mice, had a significant decrease in time to the beginning of clot formation (R time) (C57BLKS/J  $18.2 \pm 1.8$ ,  $db/db$   $12.3 \pm 1.5$  min,  $t(12)=2.51$ ,  $p=0.03$ ; C57BL6/J  $14.0 \pm 3.1$ ,  $ob/ob$   $6.6 \pm 1.6$  min,  $t(10)=2.09$ ,  $p=0.06$ ), indicating an increase in the rapidity to initial clot development. But once clot formation began, there were no significant differences in the kinetics (K time) of clot formation between  $db/db$ ,  $ob/ob$  and their controls (C57BLKS/J  $7.9 \pm 1.3$ ,  $db/db$   $9.5 \pm 2.1$  min,  $t(12)=-0.62$ ,  $p=0.55$ ; C57BL6/J  $4.1 \pm 0.6$ ,  $ob/ob$   $4.5 \pm 0.9$  min,  $t(9)=-0.36$ ,  $p=0.73$ ). The  $ob/ob$  mice demonstrated a significant amount of fibrinolysis before the assay was completed (C57BL6/J, 0.0%,  $ob/ob$   $20.5 \pm 8.6\%$  lysis at 30 minutes,  $T=24.50$ ,  $p=0.02$ , Table 3.4). Figure 3.3A is a representative tracing of an  $ob/ob$  mouse clot undergoing lysis (black tracing) compared to a control mouse (grey tracing), demonstrated as a gradual decrease in

MA over time. None of the *db/db* or their controls had any fibrinolysis before the end of the testing (Table 3.4 and Figure 3.3B).

Table 3.4 Thromboelastograph Coagulation Parameters of *db/db*, *ob/ob*, and Control Mice

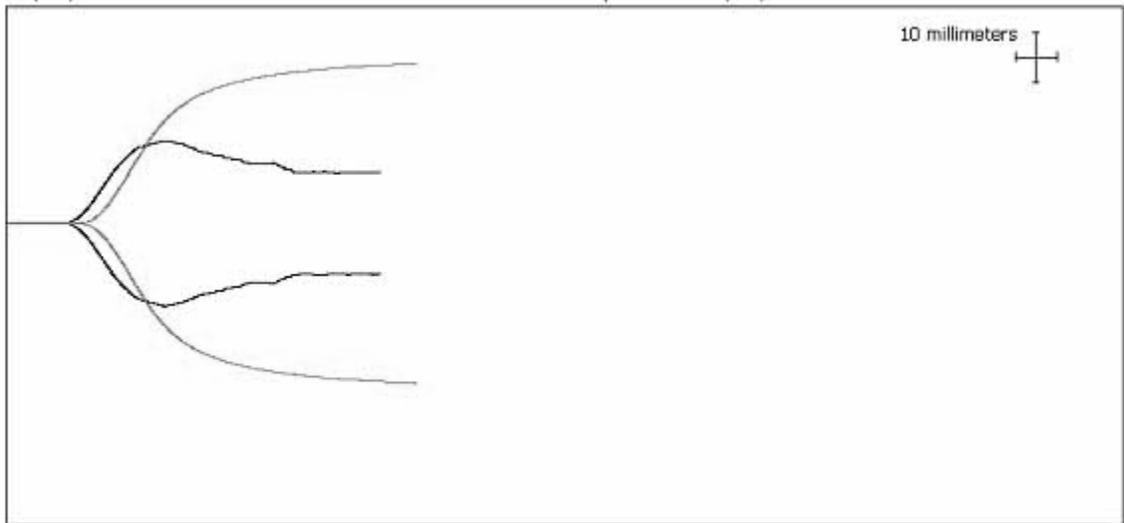
Strain ( <i>n</i> )	MA (mm)	R time (min)	K time (min)	$\alpha$ angle (°)	G value (dyn/cm <sup>2</sup> )	LY30 (%)
C57BLKS/J (7)	59.7 ± 3.1	18.2 ± 1.8	7.9 ± 1.3	29.4 ± 4.3	7.8 ± 0.9	0.1 ± 0.1
<i>db/db</i> (7)	46.6 ± 3.9**	12.3 ± 1.5**	9.5 ± 2.1	27.4 ± 3.6	4.6 ± 0.7**	0.1 ± 0.1
C57BL/6J (6)	59.7 ± 1.1	14.0 ± 3.1	4.1 ± 0.6	46.4 ± 3.9	7.5 ± 0.3	0.0 ± 0.0
<i>ob/ob</i> (6)	36.3 ± 6.3**	6.6 ± 1.6**	4.5 ± 0.9	48.6 ± 6.1	3.3 ± 0.9**	20.5 ± 8.6**

MA (Maximum amplitude), R (Reaction)-time to beginning clot formation, K (Kinetic)-time of clot formation once clotting starts,  $\alpha$  angle- rapidity of clot formation, G value- a value of clot firmness, LY30-(Lysis 30 minutes)- %fibrinolysis at 30 minutes. Data represented as mean ± *SEM*. \*significantly different from control, \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

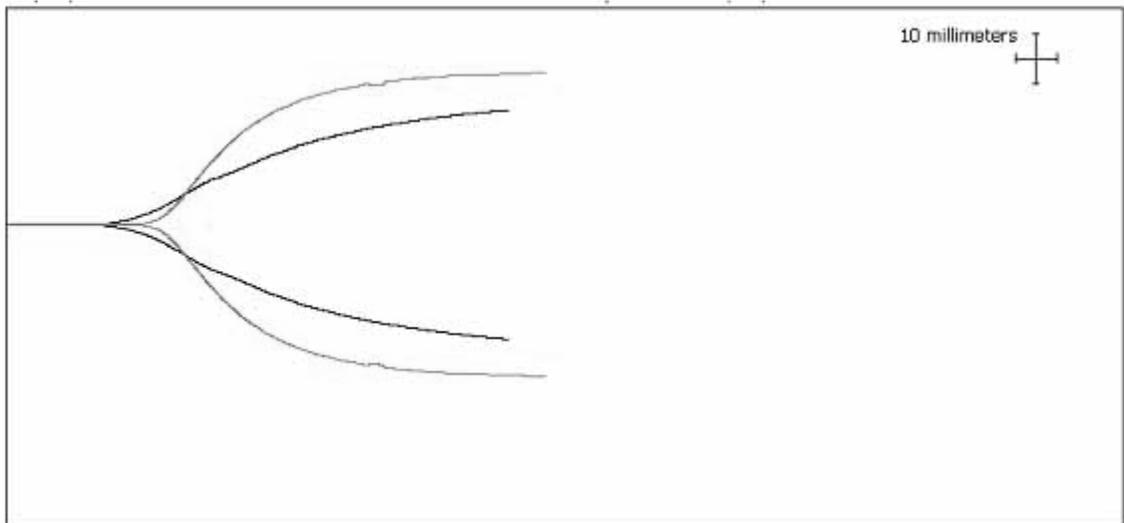
Figure 3.3 Representative coagulation tracings from the thromboelastograph in *db/db*, *ob/ob*, and control mice. (A) C57BL6J (grey) and *ob/ob* (black). (B) C57BLKS/J (grey) and *db/db* (black).

Figure 3.3

A.



B.

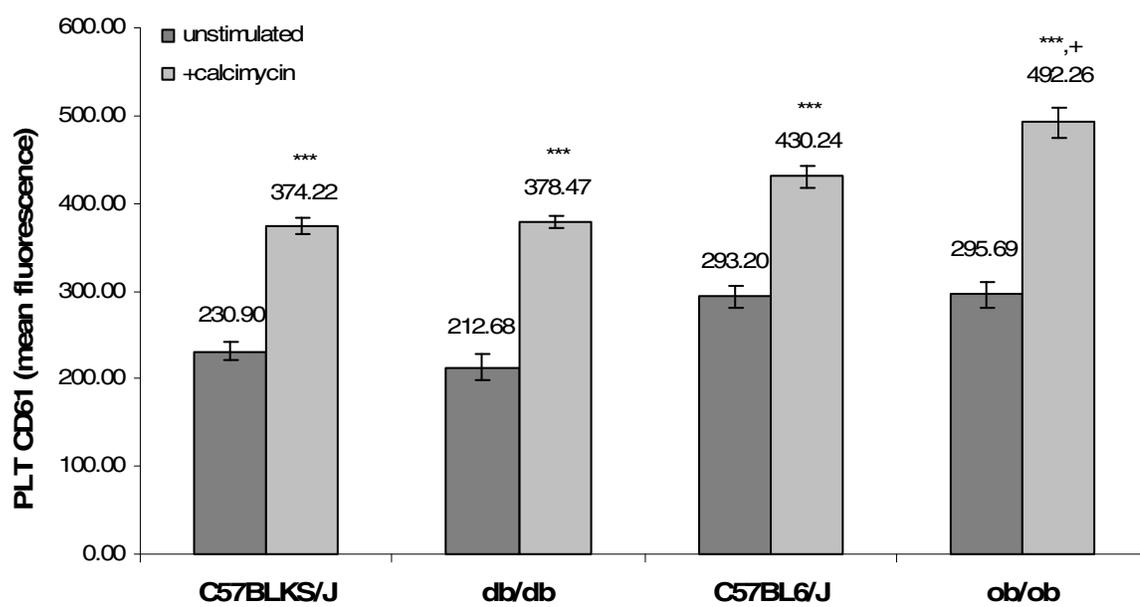


### *Flow Cytometry*

Platelet CD61 (GPIIb/IIIa, fibrinogen receptor) expression was measured by flow cytometry to determine the effect of this adhesion molecule on platelet aggregation and clot formation. There was no difference in CD61 expression between *db/db* or *ob/ob* and their controls at baseline (C57BLKS/J ( $n=7$ )  $230.9 \pm 10.4$ , *db/db* ( $n=4$ )  $212.7 \pm 15.2$  mean fluorescence intensity (MFI),  $t(9)=1.02$ ,  $p=0.34$ ; C57BL6/J ( $n=5$ )  $293.2 \pm 12.6$ , *ob/ob* ( $n=6$ )  $295.7 \pm 14.3$  MFI,  $t(9)=-0.13$ ,  $p=0.90$ , Figure 3.4). There was a significant increase in CD61 expression in all groups after stimulation with calcimycin (paired t-test, C57BLKS/J  $t(6)=-9.47$ ,  $p<0.001$ ; *db/db*  $212.7 \pm 15.2$  MFI,  $t(3)=-13.55$ ,  $p<0.001$ ; C57BL6/J  $293.2 \pm 12.6$   $t(4)=-18.30$ ,  $p<0.001$ ; *ob/ob*  $295.7 \pm 14.3$  MFI,  $t(5)=-15.93$ ,  $p<0.001$ ). Unlike the *db/db* mice, the *ob/ob* mice demonstrated a significant increase in CD61 expression after calcimycin stimulation compared to their control (C57BLKS/J  $374.2 \pm 9.3$ , *db/db*  $378.5 \pm 7.1$  MFI,  $t(9)=-0.31$ ,  $p=0.76$ ; C57BL6/J  $430.2 \pm 11.9$ , *ob/ob*  $492.3 \pm 17.2$  MFI,  $t(9)=-2.84$ ,  $p=0.02$ , Figure 3.4).

Figure 3.4 Platelet CD61 expression in *db/db*, *ob/ob*, and control mice. Data presented as mean  $\pm$  SEM. Mean fluorescence intensity is arbitrary fluorescent units. \* significant difference from unstimulated sample \*\*\* $p < 0.001$ ; + significant difference from control mouse, + $p < 0.05$ .

Figure 3.4



## Discussion

Type 2 diabetes in humans causes platelet abnormalities, alterations in coagulation factors, and disturbances in endothelial cells that produces a hypercoaguable state. People with diabetes die prematurely from thrombotic events and subsequent vascular disease secondary to this hypercoaguable state (Carr, 2001). The effects of these changes in coagulation and platelet function on stroke and cardiovascular disease have been studied, but are not completely understood. Mouse models that reflect the metabolic characteristics of type 2 diabetes are currently available, and could potentially be utilized to further explore these mechanisms, but little is known about their platelet function and coagulation. Therefore, the purpose of this study was to determine the effects of type 2 diabetes on mouse platelet function by measuring whole blood aggregation, coagulation, and platelet CD61 expression in the *db/db* and *ob/ob* genetic mouse models of type 2 diabetes.

Few studies have examined platelet function or coagulation in *db/db* or *ob/ob* mice. *db/db* and *ob/ob* mice have elevated circulating levels of plasminogen activator inhibitor type 1 (PAI-1), an inhibitor of fibrinolysis (Samad, Pandey, Bell, & Loskutoff, 2000), and an increase in tissue factor gene expression (Samad, Pandey, & Loskutoff, 2001). While an increase in these proteins may contribute to a hypercoaguable state in these models, evidence from other studies, and from the current study, do not support this hypothesis. In 1981, Rosenblum, El-Sabban, and Loria, demonstrated in an *in vivo* model of mesenteric aggregation, that *db/db* mice, but not *ob/ob* mice, had a decreased level of aggregation compared to control mice. Additionally, Konstantinides, et al. (2001), demonstrated that *db/db* and *ob/ob* mice exhibited a delayed time to and unstable thrombus formation using

an *in vivo* model of FeCl<sub>2</sub> arterial injury. They found that the addition of leptin normalized the time to thrombus formation and stabilized clot formation in *ob/ob*, but not *db/db* mice. The addition of leptin also enhanced ADP stimulated *in vitro* platelet rich plasma platelet aggregation in *ob/ob*, but not *db/db mice*. Recent studies have begun to examine the influence of leptin on platelet function in humans, and have identified the importance of this hormone on normal platelet aggregation (Elbatarny, & Maurice, 2005; Nakata, Maruyama, & Yada, 2005; Giandomenico, Dellas, Czekay, Koschnick, & Loskutoff, 2005; Corsonello, et al., 2004).

The previous studies on *db/db* and *ob/ob* mouse hemostatic function were performed with *in vivo* methodologies or with isolated platelet *in vitro* analyses. However, *in vivo* methodologies are difficult to reproduce and do not correlate directly with hematology assays used in human studies. In addition, isolation of platelets used for *in vitro* platelet function assays are known to cause artifactual platelet activation (Vollmar, Slotta, Nickels, Wenzel, & Menger, 2003; Walkowiak, et al., 2000). In this study, therefore, we examined the effects of platelet function in the *db/db* and *ob/ob* mouse using whole blood methods in order to decrease potential artifactual activation of platelets with isolating procedures.

The results of our study are consistent with earlier studies, demonstrating that both the *db/db* and *ob/ob* mouse models of type 2 diabetes have significantly reduced aggregation and thrombosis compared to their controls. While other studies did not find a difference in *in vitro* platelet aggregation in *ob/ob* mice compared to control; using whole blood methodologies, we found that there was a significant and severe decrease in

aggregation and coagulation these mice. Our results may be explained by an inherent difference in platelet aggregation and coagulation due to either the lack of leptin or its receptor, as indicated in the Konstantinides (2001) study.

Another potential explanation for the significant decrease in aggregation and coagulation seen in the *db/db* and *ob/ob* mice in our study is the significantly lower platelet counts observed in the diabetic animals. Studies have demonstrated that platelet counts directly correspond with the maximal amplitude (MA) of clot formation on TEG® (Bowbrick, Mikhailidis, & Stansby, 2003) and that the MA is correlated with optical platelet aggregation (Craft, et al., 2004), suggesting that platelet count also directly corresponds with aggregometry results. Thus, a lower platelet count could decrease both whole blood aggregation and TEG® indices. In this study, both the *db/db* and *ob/ob* mice exhibited significantly lower platelet counts than their controls in both heparin and sodium citrate anticoagulated samples. Heparin is known to activate the GPIIb/IIIa complex, increasing platelet-fibrinogen binding, (Sobal, et al., 2001), which may have lowered the platelet count secondary to microaggregate formation. Platelet microaggregation was noted in all heparinized samples, but did not differ between groups. In contrast to our study, a previous report did not demonstrate a difference in platelet count in *db/db* mice compared to control mice in EDTA anticoagulated blood (Jones, et al., 1999). No study was found that reported platelet counts in *ob/ob* mice. EDTA anticoagulation is standard for determining whole blood counts, but platelet functional assays such as aggregometry and TEG cannot be performed in EDTA anticoagulated samples because it inhibits platelet aggregation (Golanski, Pietrucha, Baj, Greger, & Watala, 1996). Because we did not do

our platelet counts in EDTA, the platelet counts we obtained probably do not reflect the true platelet count in these animals (normal mouse platelet count-  $900-1700 \times 10^3/\mu\text{l}$  (Tsakiris, et al., 1999)), but give us valuable information on the actual sample platelet count used for each assay.

To discern the effect of platelet count and any differential effects of heparin anticoagulation on whole blood aggregation, experiments standardizing the platelet count were conducted in both heparin and sodium citrate anticoagulated samples. When whole blood samples were diluted with saline to a standardized platelet count, *db/db* mice demonstrated no difference in aggregation from controls in heparinized blood, but exhibited less aggregation in citrated blood; similar to unstandardized whole blood samples. Additionally, standardizing the platelet count in the *ob/ob* mice, with either anticoagulant, had no effect on their whole blood aggregation, and they consistently demonstrated less aggregation than their controls. In summary, these experiments demonstrate that in these genetic mouse models of type 2 diabetes, effects of platelet count and choice of anticoagulant had little effect on whole blood aggregation as they consistently demonstrated less aggregation than their controls.

In addition to decreased whole blood aggregation in the diabetic models, we also found a decrease in whole blood coagulation, measured by TEG®. In these experiments the *db/db* and *ob/ob* mice demonstrated less maximal clot strength. Platelet function is the primary contributor to the measurement of clot strength, with a small contribution from fibrin (Salooja, & Perry, 2000), indicating that the overall decrease in coagulation and aggregation observed in these genetic mouse models of type 2 diabetes is probably due to

an inherent defect in platelet function. In contrast, even though they had a decrease in clot formation, the *db/db* mice had a significantly shortened time to the beginning of clot development compared to controls. This indice is influenced by clotting factors and initial fibrin formation, suggesting that the *db/db* mice may have some hypercoagulable changes, but because their platelet function is abnormal they are unable to develop normal aggregates or hemostasis. Additionally, the decrease in clot strength over time observed in the *ob/ob* mice, (Figure 3A) is consistent with early fibrinolysis or degradation in clot firmness. Konstantinides (2001) also observed unstable thrombus formation in *ob/ob* mice that was corrected with *in vivo* administration of leptin. These results suggest that the early clot dissolution seen in our experiments is likely due to a lack of leptin, altering platelet function, clot firmness and stability. The hypocoaguability observed in the *db/db* and *ob/ob* mice, though, does not appear to be related to the expression of the GPIIb/IIIa complex (fibrinogen receptor) (measured by CD61 expression), as there was no difference in expression in this adhesion molecule in any of the mice.

Diabetic patients are often treated with platelet inhibitors to decrease the risk of developing thrombotic events (Mehta, Silver, Aaronson, Abrahamson, & Goldfine, 2005). One of these agents is aspirin, which decreases platelet aggregation by inhibiting cyclooxygenase (COX)-1 and -2 and decreasing thromboxane A<sub>2</sub> production (a potent mediator of platelet aggregation) (Awtry, & Loscalzo, 2000). There is considerable variation in the response to aspirin in people with type 2 diabetes and the metabolic syndrome, in which some people display a decreased platelet inhibitory response to the drug (Mehta, et al., 2005; Tamminen, Lassila, Westerbacka, Vehkavaara, & Yki-Jarvinen,

2003). The metabolic basis of aspirin resistance is not completely understood, but has been associated with insulin resistance (Tamminen, et al., 2003; Ferreira, et al., 2006). Although we did not measure insulin levels, *db/db* and *ob/ob* mice are known to be hyperinsulinemic and insulin resistant (Dubac, 1976; Takeshita, et al., 2001; Wyse, & Dulin, 1970). We found that both types of mice responded robustly to aspirin, and, interestingly, the *ob/ob* mice had a significantly greater response to aspirin than their controls.

To our knowledge this is the first study that analyzed inhibition of ADP induced whole blood aggregation with fucoidan in diabetes. Fucoidan is a polysaccharide isolated from brown algae that has known anti-thrombotic and selectin inhibitory functions (Bertau, & Mulloy, 2003). Fucoidan has demonstrated inhibition of thrombin generation and thrombin induced platelet aggregation and thrombosis in rabbit platelets (Trento, Cattaneo, Pescador, Porta, & Ferro, 2001), and inhibition of *in vivo* thrombus formation, but not platelet-endothelial adhesion, in a mouse cremaster arterial injury preparation (Thorlacius, Vollmer, Seyfert, Vestweber, & Menger, 2000). Previously, we found that fucoidan significantly decreased whole blood aggregation in non-diabetic rats (Davidson, McDonagh, Nolan, & Ritter, 2005). Similar to what we found in non-diabetic rats, fucoidan significantly decreased aggregation in control mice compared to ADP alone; however, there was a non-significant decrease in diabetic mice compared to ADP alone and there was no difference in *db/db* or *ob/ob* mice and their controls. The lack of a significant platelet inhibition by fucoidan in the diabetic mice is not completely clear, but may be related to a difference in the aggregatory response to ADP in these animals that lack leptin or its receptor.

The results of our study demonstrate that both the *db/db* and *ob/ob* mouse have significantly diminished aggregation and coagulation properties compared to their genetic controls. These results are considerably different from studies that demonstrate a platelet-mediated hypercoagulable state in humans with diabetes. We conclude that the *db/db* and *ob/ob* mouse models of diabetes are not be ideal for studying platelet aggregation and coagulability. Further investigation of these processes in animal models of diabetes is warranted, as it may lead to a greater understanding of the complexities of diabetes and adverse vascular events.

## CHAPTER 4

WHOLE BLOOD AGGREGATION, COAGULATION, AND PLATELET MARKERS  
OF ACTIVATION IN DIET INDUCED OBESE AND DIABETIC MICE

## Abstract

Obesity and type 2 diabetes produce alterations in platelet function, coagulation, and fibrinolysis, initiating a state of chronic activation and hyperaggregability. However, the mechanisms by which these changes in platelet function and coagulation occur and the effects of these processes on stroke and cardiovascular disease are not completely understood. Mouse models that reflect the metabolic characteristics of type 2 diabetes are currently available, but few studies have been conducted examining platelet function and coagulation in these animals. The purpose of this study was to examine whole blood aggregation, coagulation, platelet CD61 and CD62P expression, and microparticle formation in mice fed standard chow (CF) and in two mouse models of diet induced obesity and diabetes: 1) mice fed a high fat (HF, 60% beef lard) diet for 4 months and 6 months producing a moderate form of type 2 diabetes (groups- CF/HF at 4 months and 6 months) and 2) mice fed a HF diet for 2 months and injected with a low dose of streptozotocin (100mg/kg) producing a more severe form of type 2 diabetes (groups- CF+vehicle, CF+STZ, HF+vehicle, HF+STZ). HF mice in both models became obese, hyperglycemic, hyperinsulinemic ( $p < 0.01$ ), and insulin resistant (HOMA-IR scores  $> 5$ ) compared to chow fed (CF) mice. We found that the HF fed mice for 4 months had a significantly lower platelet count and a lower agonist stimulated platelet CD61 expression compared to mice fed a standard chow diet. The 4 month HF mice also demonstrated significantly less ADP-

induced whole blood aggregation ( $p < 0.05$ ) and maximal clot strength ( $p < 0.01$ ) compared to CF mice, that was positively correlated with the low platelet count (aggregometry  $r = 0.73$ ,  $p = 0.01$ ; TEG  $r = 0.58$ ,  $p = 0.03$ ). The 6 month HF mice did not demonstrate any difference in WB aggregation or CD61 expression compared to CF mice. CD62P expression and microparticle formation did not differ between HF fed mice for 4 and 6 months and CF mice. HF+STZ mice demonstrated a more severe state of diabetes than HF mice. The HF+STZ mice did not demonstrate any difference in platelet count, WB aggregation, or any platelet markers of activation from mice fed the diet alone or CF mice. We conclude that in our study, diet induced obese and diabetic mice did not demonstrate a hypercoagulable state. However, more studies need to be conducted to examine platelet function and coagulation in these obese diabetic mice.

## Introduction

Obesity and type 2 diabetes are associated with increased mortality from thrombotic events (Carr, 2001). Obesity and diabetes induce alterations in platelet function, coagulation, and fibrinolysis, initiating a state of chronic platelet activation and hyperaggregability (Schneider, 2005). Platelets from people with diabetes demonstrate increased platelet expression of P-selectin (Nomura, et al, 1995; Tan, Tayebjee, Lim, & Lip, 2005; McDonagh, et al., 2003), and increased levels of circulating total (Sabatier, et al., 2002) and platelet derived microparticles (PMPs) (Koga, et al., 2006; Tan, et al., 2005; Diamont, Nieuwland, Pablo, Sturk, Smit, & Radder, 2002; Nomura, et al., 1995).

Platelets from obese and diabetic people aggregate more readily to ADP (Corsonello, et al., 2003; Ferreira, et al., 2006) compared to platelets from non-obese, non-diabetic subjects. Several coagulation factors are also known to be elevated in type 2 diabetes, including factors I (fibrinogen) (Coppola, et al., 2006; Coca, Cucuianu, & Hancu, 2005; Erem, et al., 2005), VII, (Coca, et al., 2005; Ludwig, et al., 2005), VIII, (Coca, et al.), and endothelial surface antigen von Willebrand factor (Coca, et al.; Erem, et al.). In addition, platelets from obese and diabetic subjects are known to have larger mean volumes (a marker for platelet activation) (Tschoepe, et al., 1990; Coban, Ozdogan, Yazicioglu, & Akcit, 2005), diminished response to insulin's inhibitory effects on aggregation (Westerbacka, et al., 2002; Ferreira, et al., 2006), and demonstrate significantly slower fibrinolysis (Dunn, Philippou, Ariens, & Grant, 2006) than platelets from healthy subjects. Together, these defects lead to an overall state of hypercoaguability in people with obesity and type 2 diabetes. However, the mechanisms of the hypercoaguable state observed in these individuals are not completely understood. Animal models that represent the metabolic characteristics of insulin resistance and type 2 diabetes are therefore needed to further explore the contribution of altered platelet function and coagulation on the complications of vascular disease.

Several mouse models of obesity and diabetes are currently available, including genetic mutations that cause obesity and diabetes in mice. The *ob/ob* and *db/db* mouse models of obesity and diabetes demonstrate many metabolic similarities to humans with these disorders, including hyperinsulinemia, hyperglycemia, and obesity (Dubac, 1976; Wyse, & Dulin, 1970). Both models develop obesity and diabetes (only transient in the

*ob/ob* mouse) through the lack functional leptin (*ob/ob*) or its receptor (*db/db*), which causes hyperphagia and subsequent weight gain. These mouse models, however, have demonstrated less platelet activation and aggregation than control mice (Konstantinides, Schafer, Koschnick, & Loskutoff, 2001). We recently corroborated these studies and found that the *db/db* and *ob/ob* mice also demonstrated less whole blood aggregation and coagulation (Chapter 3). The decrease in platelet function noted in these animals is due to their lack of functional leptin, which is now known to be important in normal platelet aggregation (Corsonello, et al., 2004; Elbatarny, & Maurice, 2005; Giandomenico, Dellas, Czekay, Koschnick, & Loskutoff, 2005; Nakata, Maruyama, & Yada, 2005). In light of these findings, the *db/db* and *ob/ob* mice may not be optimal mouse models to explore the contributions of platelet-induced thrombosis on vascular disease in obesity and type 2 diabetes.

The C57BL/6J mouse strain develops obesity and type 2 diabetes when fed a diet high in fat. Several different types (from animal and vegetable sources) and amount (20-60% total calories) of fat are used to induce obesity and diabetes in these mice. In general, after 1 week on a high fat diet, C57BL/6J mice exhibit significant weight gain compared to mice fed a standard chow diet, and double their weight after 4 months (Surwit, Kuhn, Cochrane, McCubbin, & Feinglos, 1988; Collins, Martin, Surwit, & Robidoux, 2004). The high fat diet produces adiposity primarily in the mesentery and inguinal depots, similar to the metabolic syndrome of abdominal obesity in humans (West, Boozer, Moody, & Atkinson, 1992; Surwit, Feinglos, Rodin, Sutherland, Petro, Opara, et al., 1995). In addition, these mice develop hyperglycemia, hyperinsulinemia

(Surwit, et al., 1988), and hyperleptinemia (van Heek, M., et al., 1997; Surwit, Petro, Parekh, & Collins, 1997) after 4 months on the diet. They also develop peripheral leptin resistance (Van Heek, Compton, France, Tedesco, Fawzi, Graziano, et al., 1997), hypertension (Mills, Kuhn, Feinglos, & Surwit, 1993), and atherosclerosis (Schreyer, Wilson, & LeBoeuf, 1998) similar to people with obesity and type 2 diabetes. However, the extent of hyperglycemia in these mice is not severe, and blood sugar levels are more typical of early human type 2 diabetes with obesity.

A model of later stage type 2 diabetes, when the pancreas is severely damaged and is no longer able to produce sufficient insulin, has also been described. In this model, C57BL/6J mice are fed a high fat diet (45% beef lard) for a shorter time period (1mo), injected with a dose of streptozotocin (STZ) that is lower than that used to induce type 1 diabetes (100mg/kg), and then fed the high fat diet for another month (Luo, et al., 1998). These mice (HF+STZ) develop a more severe state of diabetes than the mice fed the high fat diet alone. There are few studies, though, that have examined the metabolic characteristics of HF+STZ mice. It is known that they demonstrate significant obesity and hyperglycemia after the STZ injection, whereas mice fed a chow diet and injected with STZ do not develop these metabolic characteristics (Luo).

There are few studies that examine platelet function and coagulation in either the HF or HF+STZ mouse models of obesity and type 2 diabetes, and it is unknown if their platelet function exhibits characteristics similar to humans. The purpose of these experiments was, therefore, to examine the whole blood aggregation, coagulation, and platelet expression of CD61 (fibrinogen receptor), CD62P (P-selectin), and total and

platelet microparticles in chow fed mice and 2 mouse models of obesity and type 2 diabetes, 1) mice fed a high fat (HF, 60% beef lard) diet for 4 months and 6 months producing a moderate form of type 2 diabetes (groups- CF/HF at 4 months and 6 months) and 2) mice fed a HF diet for 2 months and injected with a low dose of streptozotocin (100mg/kg) producing a more severe form of type 2 diabetes (groups- CF+vehicle, CF+STZ, HF+vehicle, HF+STZ). Our results indicate that mice fed a diet rich in animal fat, with or without an injection of STZ, do not exhibit the increased platelet aggregation, coagulation, microparticle formation, or platelet structural changes found in humans with obesity and type 2 diabetes.

## Materials and Methods

### *Animal Model*

All animal experiments were conducted in accordance with the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* (OLAW, 2002) after IACUC approval of all researchers. All C57BL/6J mice (Jackson Laboratories, Inc., Bar Harbor, MA), were randomized to a standard rodent chow (chow fed-CF), (6% fat, NIH-31 Modified Mouse/Rat sterilizable diet 7013, Harlan Teklad, Madison, WI), or a diet enriched with 60% beef lard (high fat fed-HF) (Research Diets, New Brunswick, New Jersey, diet no. D12492) at 4 weeks of age. Mice were kept on the high fat or chow diets for 4 and 6 months (Surwit, Kuhn, Cochrane, McCubbin, & Feinglos, 1988). The second set of mice was injected intraperitoneally with a low dose of streptozotocin (STZ) (Sigma # S0130, St. Louis, MO) or vehicle (citric acid, pH 4.0) after eating the high fat or chow diets

for 4 weeks (groups- CF+vehicle, CF+STZ, HF+vehicle, HF+STZ). These mice were then fed the diet for another 4 weeks (Luo, et al., 1998). Mice were allowed free access to food and water and were housed in a temperature controlled, quiet environment.

#### *Blood Acquisition*

Mice were transported to the laboratory the day prior to blood acquisition and fasted for 4 hours the day of the experiment (0600-1000). For venipuncture, mice were anesthetized in a chamber and then mask ventilated with 5.0% isoflurane (JD Medical Distributing Co., Inc) and 1.0 L/minute oxygen. Approximately, seven hundred  $\mu$ L of blood was withdrawn from the ascending vena cava with a 23-gauge needle/1 mL syringe containing 0.05 mL of 70 units/mL heparin sodium (1000 USP units/ml, diluted with PBS, Baxter Healthcare Corp., Deerfield, IL.) for aggregometry, 0.05 mL buffered 0.105 M 3.2 % sodium citrate (BD Vacutainer Systems, Franklin Lakes, NJ) for TEG®, or 0.05 mL sodium citrate (Sigma-Aldrich Cat #S1804) for flow cytometry experiments.

#### *Blood Smear Analysis*

Blood smears were made for all experiments using standard methods (Brown, 1980). Eight  $\mu$ l of anticoagulated blood was smeared across a glass slide (VWR, West Chester, PA, #16004-368), air-dried, and stained with Diff-Quik (Dade Behring, VWR #47733-152). The number of platelet microaggregates (defined as 3 or more adhered platelets) in 20 visual fields (10 top/10 bottom) and the area of 10 random aggregates in the monolayer region on the slide were quantified with an ocular micrometer at 100X (Koplitz, Scott, & Cohn, 2001). The percentage of visual fields with platelet microaggregates, the

average number of platelet microaggregates per visual field, and the average area of 10 platelet microaggregates were calculated for analysis.

#### *Bleeding Time*

The bleeding time protocol was adapted from methods described by Ma et al. (2001) and Tsakiris et al. (1999). Mice were mask ventilated with 5% isoflurane and 1.0 L/min O<sub>2</sub> and immobilized on a surgical stage with their tails hanging dependent over the side of a platform. Using micro-scissors, tails were clipped 2mm from the tip and immediately placed in 37° C 0.9% saline (Associated Medical, Philadelphia, PA, #101310). The time (secs) to cessation of bleeding was observed and recorded.

#### *Whole Blood Aggregometry*

Nine hundred seventy µl of heparinized blood samples (whole blood diluted with 0.9% NaCl to a standardized platelet count of 350 X10<sup>3</sup>/uL) were placed in 2 ml cuvettes, and incubated for 6 minutes at 37°C in the Chrono-Log Whole Blood Lumi-Ionized Calcium Aggregometer (Goldenberg, Veriabo, & Soslau, 2001). The cuvettes were placed into the test chamber and incubated with the impedance electrode at 37°C while stirring at 900 rpm. After 2 minutes, 30µL of 1µM adenosine diphosphate (ADP) (Chrono-Log Corp., Havertown, PA) was added and aggregation (ohms of electrical impedance) was recorded for 6 minutes. All whole blood aggregometry tests were performed within 3 hours of blood acquisition.

#### *Thromboelastography*

An aliquot of 340 µL of sodium citrated whole blood recalcified with 20 µL 0.2M CaCl<sub>2</sub> (Haemoscope Corp., Skokie, IL, #7003) was added to 37°C pre-warmed cuvettes

(Haemoscope, #6211) in the thromboelastograph (Thromboelastograph Coagulation Analyzer 5000, Haemoscope Corp.) (Bowbrick, Mikhailidis, & Stansby, 2001; Salooja, & Perry, 2001) and coagulation was recorded for 30 minutes. Parameters measured and used for analysis were the maximal amplitude (MA, in mm) measurement of clot strength; R (reaction), the time (secs) to initiation of clot formation; K (kinetic), the amount of time from the beginning of clot formation until the amplitude reaches 20mm;  $\alpha$ -angle ( $^{\circ}$  degree), a measure of clot strengthening speed; G ( $\text{dyn}/\text{cm}^2$ ), value of clot firmness (calculated as  $G=5000\text{MA}/(100-\text{MA})$ ); and LY30 (lysis 30min), the percent fibrinolysis at 30 minutes (Salooja, & Perry, 2001).

#### *Flow Cytometry*

Twelve microliters of heparinized whole blood (WB) was diluted in 1 ml Dulbecco's phosphate-buffered saline with 0.2% bovine serum albumin and 0.09% sodium azide (pH 7.4) (Pharming Stain Buffer, BD Biosciences Pharmingen, San Jose, CA, #554657). Twenty  $\mu\text{L}$  of 50  $\mu\text{M}$  calcimycin A23187 (Sigma-Aldrich Co., St. Louis, MO, #C7522) was added to positive control samples and incubated at room temperature for 10 minutes. One hundred  $\mu\text{L}$  of the stain buffer/WB mixture was then added to amber microcentrifuge tubes filled with 50.0  $\mu\text{L}$  of 1:10 dilution of fluorescent monoclonal antibody (BD Biosciences Pharmingen) R-phycoerythrin (PE)-conjugated hamster anti-mouse CD61 (Clone 2C9.G2), 20  $\mu\text{L}$  of 1:10 dilution of fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD62P (P-selectin) monoclonal antibody (clone RB40.34), or 20  $\mu\text{L}$  of 1:10 FITC-conjugated rat IgG $_{1,\lambda}$  monoclonal immunoglobulin isotype control (clone A110-1). The samples were covered with aluminum foil and incubated at room

temperature for 10 minutes, then fixed with 1 mL 1% paraformaldehyde (Sigma-Aldrich Co., #P6148-500G) and placed on ice. Data were acquired on a FACScalibur flow cytometer (488 nm argon laser, Becton Dickinson, San Jose, CA). The flow cytometer was calibrated daily prior to each experiment using Calibrite Beads (Becton Dickinson, #340486) and FACsComp software (Becton Dickinson). Acquisition threshold was set on FL2 to capture only CD61 positive events. For the experiments in the mice fed a HF diet for 4 and 6 months, data were collected to 50000 total events, for all other experiments data were collected to 50,000 total CD61 positive events. A dot plot of logarithmic forward and side scatter properties were used to distinguish the platelet and microparticle populations. Data were analyzed with FCS Express v.3.0 (De Novo Software, Thornhill, Ontario).

#### *Plasma Insulin Levels and HOMA-IR Calculation*

Plasma insulin levels were measured with a mouse ultrasensitive ELISA (American Laboratory Products Company (ALPCO), Windham, NH # 08-10-1150-01) or a Insulin Rat/Mouse ELISA Kit (Linco Research, St. Charles, MO #EZRMI-13K). Both assays were performed per manufacturer protocol using an absorbance microplate reader (SpectraMax M5 Microplate Reader, Molecular Devices, Sunnyvale, CA). HOMA-IR (Homeostasis Model Assessment Insulin Resistance score) was calculated by: fasting blood glucose (mmol/L) X fasting plasma insulin ( $\mu$ U/mL) / 22.5 (adapted from Bonora, et al., 2002).

#### *Metabolic Characteristics Measurements*

Weight (gms) was monitored weekly and prior to each experiment (Triple Beam Balance, Ohaus, Columbia, MD). Blood sugar was measured with a glucometer

(AccuCheck Advantage, Roche, Inc., Mannheim, Germany) that was tested monthly for reliability using high and low controls (Roche). White blood cell count, hematocrit, and platelet counts were measured by a bench top whole blood analyzer (Coulter A<sup>c</sup>T™ 5 diff Hematology Analyzer, Beckman Coulter, Fullerton, CA), which was tested daily for reliability with high, low, and normal control samples (Beckman Coulter). Triglyceride and total cholesterol levels were measured by the Trinder's method using reflective photometry (CardioChek, model #542165, Polymer Technologies Systems (PTS), Inc., Indianapolis, IN). The CardioChek unit was tested for monthly for reliability with high and low controls (PTS, Inc.).

#### *Statistical Analysis*

Data were expressed as mean  $\pm$  SEM. Groups were compared using t-test (*t*), Mann-Whitney Rank Sum Test (*T*) (non-parametric data), One-way ANOVA (*F*) or Kruskal-Wallis ANOVA on Ranks (*H*) (non-parametric data). ANOVA post hoc testing was done with either Holm-Sidak or Dunn's Method (non-parametric). For comparison between samples within the same group, a paired t-test (*t*) or Wilcoxon Signed Rank test (*T*) was performed (i.e. stimulated v unstimulated samples). Linear regression was used to assess the relationship between variables and expressed with the *r* correlation coefficient. The flow cytometry experiments were performed in duplicate or triplicate (depending upon the amount of blood obtained from the venipuncture) and the coefficient of variation determined by dividing the standard deviation/mean. All experiments with a variation of >10% were removed from analysis or noted in the results section. SigmaStat

for Windows version 3.10 (Systat Software, Inc., Point Richmond, CA) was used for statistical analyses. An a priori  $\alpha$  of  $p \leq 0.05$  was considered statistically significant.

## Results

### *High Fat and Chow Fed Mice for 4 and 6 Months*

#### *Metabolic characteristics*

After eating the diet for 4 or 6 months, the high fat (HF) fed mice were significantly obese (4 mo-chow fed (CF) ( $n=20$ )  $32.1 \pm 1.0$ , high fat (HF) ( $n=19$ )  $49.4 \pm 0.9$  gm,  $t(37) = -12.77$ ,  $p < 0.001$ ; 6 mo- CF ( $n=11$ )  $33.4 \pm 1.2$ , HF ( $n=10$ )  $52.1 \pm 0.6$  gm,  $T = 165.00$ ,  $p < 0.001$ , Table 4.1), hyperglycemic (4 mo- CF  $182.7 \pm 8.5$ , HF  $233.8 \pm 7.5$  mg/dl,  $t(37) = -4.49$ ,  $p < 0.001$ ; 6 mo- CF  $145.2 \pm 9.9$ , HF  $171.6 \pm 4.0$  mg/dl,  $T = 141.00$ ,  $p = 0.03$ ), hyperinsulinemic (4 mo- CF ( $n=9$ )  $63.2 \pm 20.5$ , HF ( $n=7$ )  $333.7 \pm 108.7$  pM,  $T = 84.00$ ,  $p = 0.01$ ; 6 mo- CF ( $n=3$ )  $101.6 \pm 43.4$ , HF ( $n=2$ )  $718.0 \pm 116.0$  pM,  $t(3) = -5.99$ ,  $p < 0.01$ ), and insulin resistant (HOMA-IR scores- 4mo-CF  $3.9 \pm 1.2$ , HF  $27.6 \pm 9.1$ ,  $T = 84.00$ ,  $p = 0.01$ ; 6mo- CF  $4.7 \pm 1.5$ , HF  $44.9 \pm 7.0$ ,  $T = 9.00$ ,  $p = 0.20$ ) compared to control mice fed a standard chow diet. Weight was significantly correlated with blood sugar (4mo-  $r = 0.52$ ,  $p < 0.001$ ; 6mo-  $r = 0.53$ ,  $p = 0.01$ ) and insulin levels (4mo-  $r = 0.64$ ,  $p < 0.01$ ; 6mo-  $r = 0.93$ ,  $p = 0.02$ ). The mice fed the HF diet for 4 and 6 months were also hypercholesterolemic (4 mo-CF  $101.6 \pm 1.6$ , HF  $158.7 \pm 8.1$  mg/dl,  $T = 197.00$ ,  $p < 0.001$ ; 6mo- CF  $< 100$ , HF  $190.0 \pm 10.3$  mg/dl,  $T = 144.00$ ,  $p < 0.001$ ), but not hypertriglyceridemic (4mo- CF  $58.2 \pm 0.8$ , HF  $57.5 \pm 0.8$  mg/dl,  $T = 193.00$ ,  $p = 0.38$ ; 6mo- CF  $50.1 \pm 0.1$ , HF  $55.6 \pm 5.4$  mg/dl,  $T =$

101.50,  $p=0.62$ , Table 4.1). The weight of the mice was also significantly correlated with increasing total cholesterol levels (4mo-  $r=0.90$ ,  $p<0.001$ ; 6mo-  $r=0.81$ ,  $p<0.001$ ).

#### *Whole blood cell counts*

The whole blood counts were standardized to a hematocrit of 45% to eliminate the effects of anticoagulant dilution. The platelet counts were significantly lower at both time points in the HF fed mice compared to chow fed mice (4mo- CF  $844.4 \pm 24.4$ , HF  $697.3 \pm 8.1 \times 10^3/\mu\text{l}$ ,  $t(36)=4.93$ ,  $p<0.001$ ; 6 mo- CF  $882.8 \pm 25.4$ , HF  $736.3 \pm 23.6 \times 10^3/\mu\text{l}$ ,  $t(19)=4.21$ ,  $p<0.001$ , Table 4.1), and were significantly correlated with weight (4mo-  $r=0.67$ ,  $p<0.001$ ; 6mo-  $r=0.75$ ,  $p<0.001$ ), and blood sugar (4mo-  $r=0.33$ ,  $p=0.04$ ; 6mo-  $r=0.49$ ,  $p=0.03$ ), but not insulin levels (4mo-  $r=0.48$ ,  $p=0.07$ ; 6mo-  $r=0.87$ ,  $p=0.06$ ).

#### *Whole blood aggregation*

Significantly less whole blood aggregation was observed in mice fed the HF diet for 4 months compared to chow fed mice, but this difference was eliminated after 6 months (4 mo- CF  $15.4 \pm 0.7$ , HF  $13.0 \pm 0.8 \Omega$ ,  $t(9)=2.28$ ,  $p<0.05$ ; 6 mo- CF  $14.6 \pm 0.7$ , HF  $13.0 \pm 1.5 \Omega$ ,  $t(7)=1.01$ ,  $p=0.35$ , Figure 4.1A). The lower platelet count in the HF fed mice at 4 months was significantly correlated with lower whole blood aggregation ( $r=0.73$ ,  $p=0.01$ , Figure 4.1B). There was no significant correlation between weight or blood sugar and aggregation. The rate of whole blood aggregation of the HF fed mice was no different than the CF mice at either time point (4 mo- CF  $15.0 \pm 1.6$ , HF  $16.8 \pm 1.2 \Omega/\text{sec}$ ,  $t(9)=-0.93$ ,  $p=0.38$ ; 6 mo- CF  $12.0 \pm 1.4$ , HF  $13.8 \pm 2.3 \Omega/\text{sec}$ ,  $t(7)=-0.68$ ,  $p=0.52$ , Figure 4.1C).

Table 4.1 Metabolic Characteristics and Platelet Counts of Mice Fed a Standard Chow or High Fat Diet for 4 and 6 Months

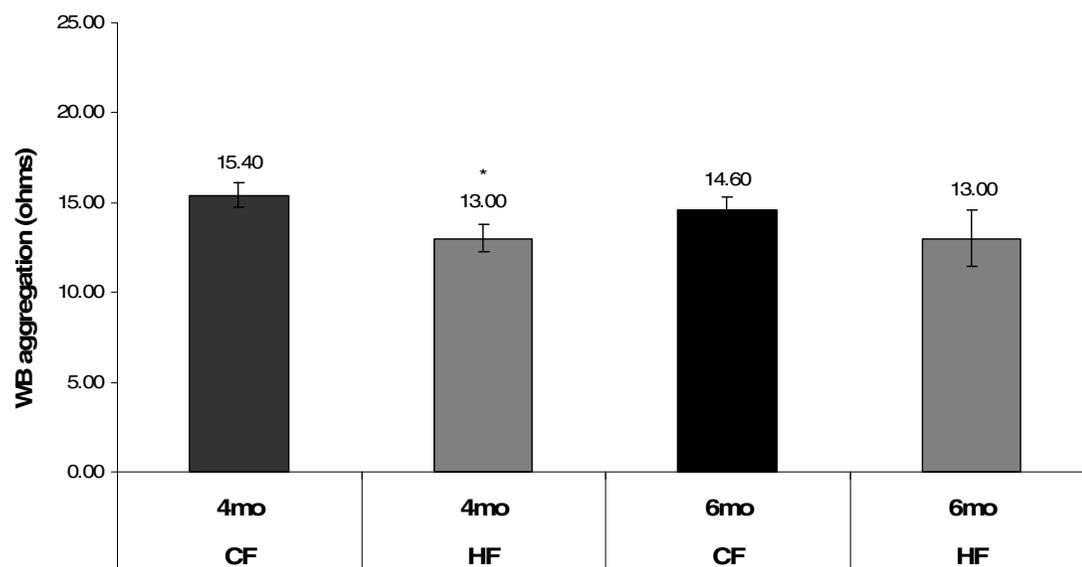
Strain (n) <sup>^</sup>	Weight (g)	Blood Sugar (mg/dl)	Trig (mg/dl)	Total Chol (mg/dl)	Platelet count (10 <sup>3</sup> /μl)	Plasma Insulin (pM) <sup>^</sup>	HOMA- IR score <sup>^</sup>
CF x 4mo (20)	32.1± 1.0	182.7± 8.5	58.2± 0.8	101.6± 1.6	844.4± 24.4	63.2± 20.5	3.9± 1.2
HF x 4mo (19)	49.4± 0.9***	233.8± 7.5***	57.5± 0.8	158.7± 8.1***	697.3± 15.9***	333.7± 108.7**	27.6± 9.1**
CF x 6mo (11)	33.4± 1.2	145.2± 9.9	50.1± 0.1	<100.0	882.8± 25.4	101.6± 43.4	4.7± 1.5
HF x 6mo (10)	52.1± 0.6***	171.6± 4.0*	55.6± 5.4	190.0± 10.3***	736.3± 23.6***	718.0± 116.0**	44.9± 7.0

CF-chow fed, HF-High fat fed, Trig- Triglycerides, Total Chol- Total Cholesterol, HOMA-IR- Homeostasis Model Assessment Insulin Resistance Score. All measurements were made after 4 hour fast except weights. Platelet counts were standardized to a hematocrit of 45%. <sup>^</sup>See results text for (n) of each measurement. Data presented as mean ± SEM. \*significantly different from CF mice, \* $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\* $p < 0.001$ .

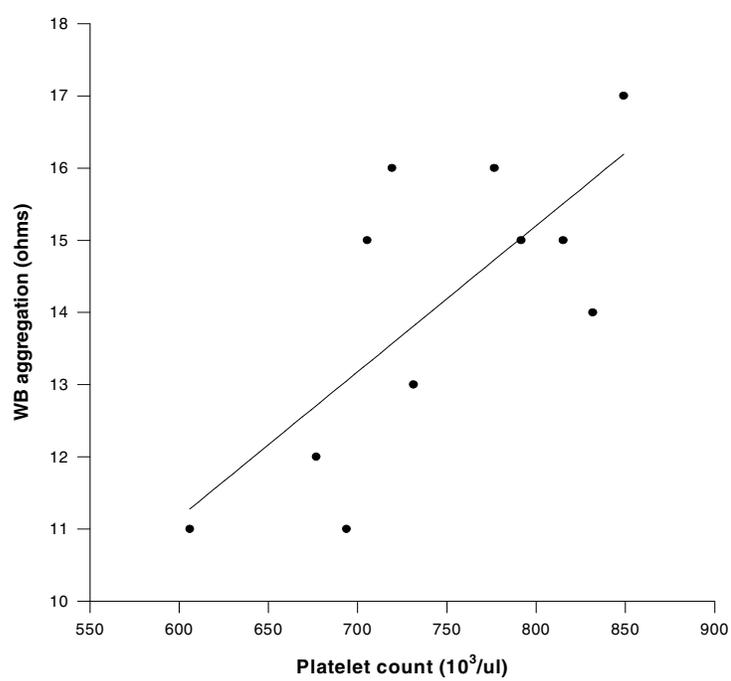
Figure 4.1 Whole blood aggregation in mice fed a standard chow or high fat diet for 4 and 6 months. (A) Summative data for ADP-induced whole blood aggregation (ohms ( $\Omega$ )) (4 months-CF  $n= 5$ ; HF  $n= 6$ ; 6 months- CF  $n= 5$ ; HF  $n= 4$ ). (B) Correlation between standardized platelet count and whole blood aggregation in HF and CF mice at 4 months ( $r= 0.73$ ,  $p= 0.01$ ). (C) Summative data for the rate of ADP-induced whole blood aggregation ( $\Omega/\text{sec}$ ). Data presented as mean  $\pm$  SEM. \*significantly different from CF mice,  $*p < 0.01$ .

Figure 4.1

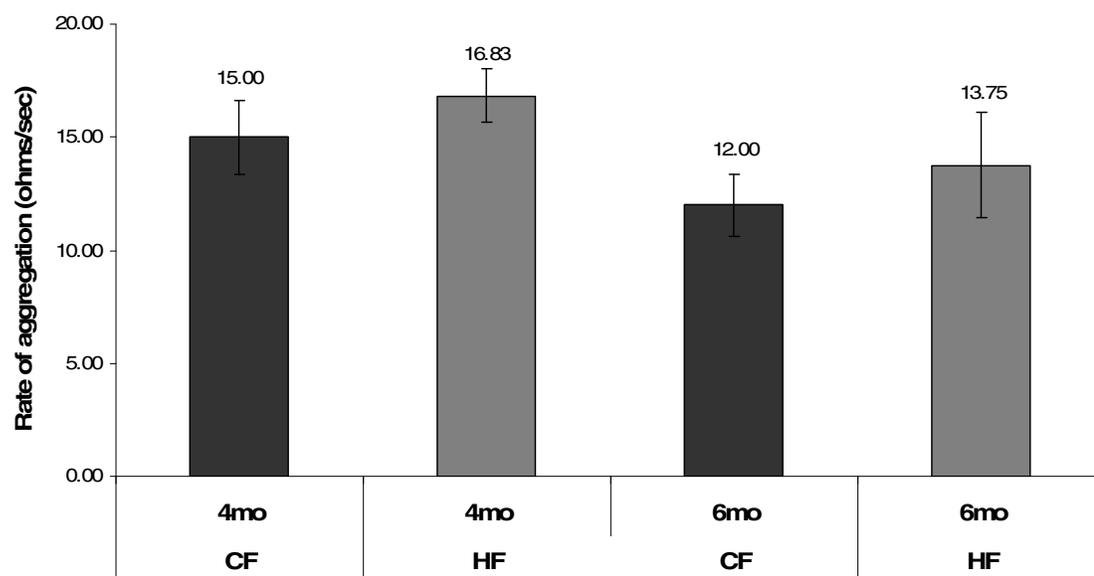
A.



B.



C.



### Thromboelastography

To further explore platelet function in the mice fed a HF diet for 4 months, coagulation was measured by whole blood thromboelastography. The HF mice did not demonstrate any differences in coagulation (Table 4.2) except a decrease in MA ( $66.8 \pm 1.2$  vs.  $54.1 \pm 3.3$  mm,  $t(12)= 3.61$ ,  $p < 0.01$ ) and G values ( $10.1 \pm 0.5$ ,  $6.3 \pm 1.0$  dyn/cm<sup>2</sup>,  $t(12)= 3.42$ ,  $p < 0.01$ ) compared to chow fed mice. As weight ( $r = 0.77$ ,  $p < 0.001$ , Figure 4.2A) and insulin levels ( $r = 0.86$ ,  $p = 0.03$ , Figure 4.2B) increased the MA decreased. There was a positive correlation with platelet count and MA ( $r = 0.58$ ,  $p = 0.03$ , Figure 4.2C). There was no observed correlation with blood sugar and MA values ( $r = 0.18$ ,  $p = 0.55$ , Figure 4.2D).

Table 4.2 Thromboelastograph Coagulation Parameters in Mice Fed a Standard Chow or High Fat Diet for 4 Months

Strain (n)	MA (mm)	R time (min)	K time (min)	$\alpha$ angle ( $^{\circ}$ )	G value (dyn/cm <sup>2</sup> )	LY30 (%)
CF (7)	$66.8 \pm 1.2$	$6.0 \pm 0.6$	$1.5 \pm 0.1$	$69.6 \pm 1.7$	$10.2 \pm 0.5$	$0.6 \pm 0.6$
HF diet (7)	$54.1 \pm 3.3^{**}$	$6.4 \pm 0.7$	$1.9 \pm 0.3$	$62.7 \pm 4.0$	$6.3 \pm 1.0^{**}$	$0.7 \pm 0.5$

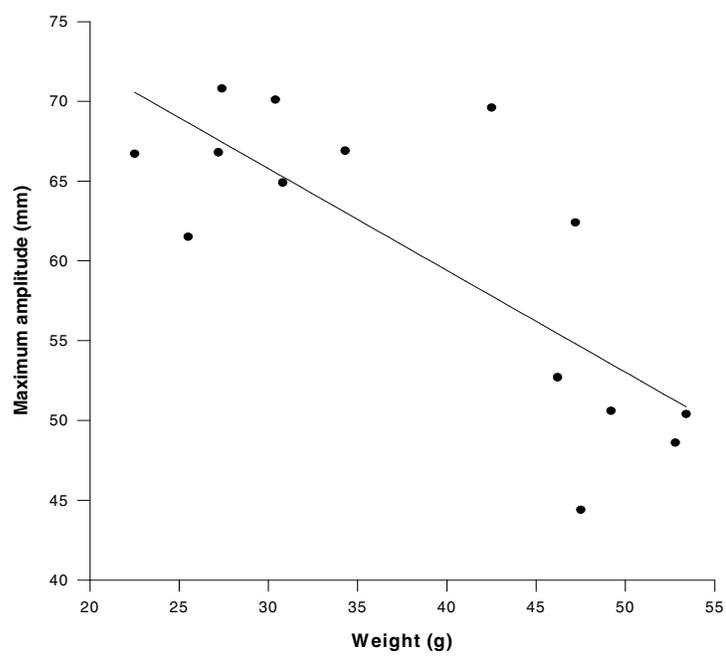
CF-chow fed, HF-high fat, MA-Maximum amplitude, R (Reaction)- time to beginning clot formation, K (Kinetic)- time of clot formation once clotting starts,  $\alpha$  angle (rapidity of clot formation), G value, LY30-%fibrinolysis 30 minutes. Data presented as mean  $\pm$  SEM.

\*significantly different from CF mice, \*\* $p < 0.01$ .

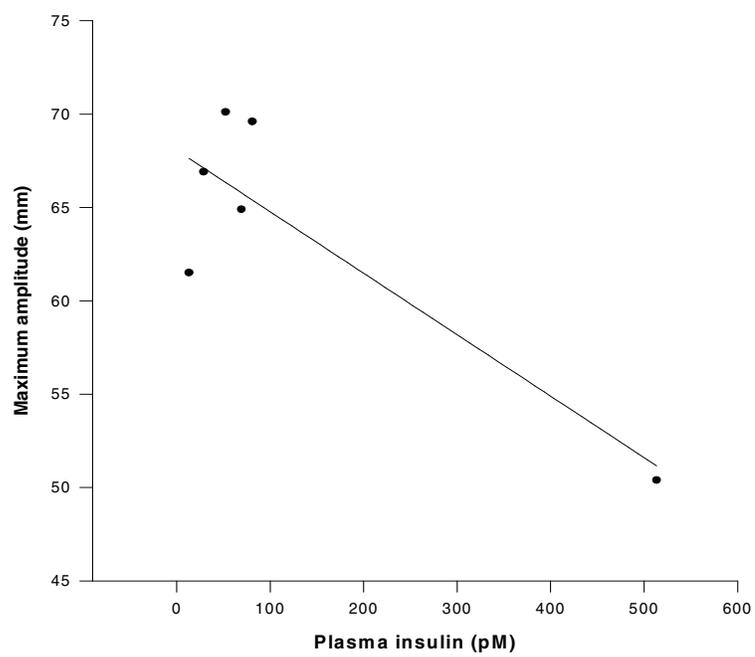
Figure 4.2 Correlation between weight, insulin levels, platelet count, and blood sugar on thromboelastography parameter of clot strength in mice fed a standard chow or high fat diet for 4 months. (A) weight ( $r= 0.77, p< 0.001$ ), (B) insulin level ( $r= 0.89, p= 0.03$ ), (C) platelet count ( $r= 0.58, p= 0.03$ ), and (D) blood sugar ( $r= 0.18, p= 0.55$ ).

Figure 4.2

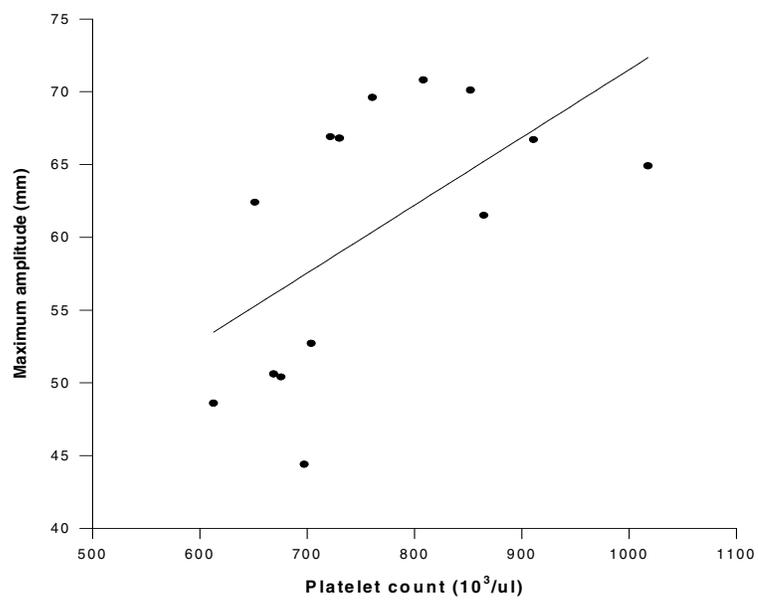
A.



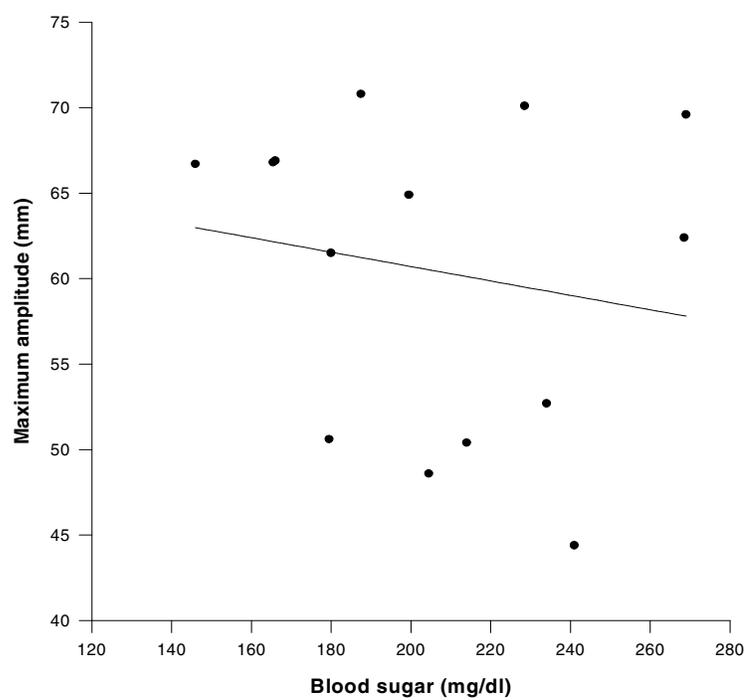
B.



C.



D.



### *Bleeding times*

Tail bleeding times were conducted in order to obtain an *in vivo* measurement of coagulation. The HF mice did not demonstrate any clot formation; consequently the experiments were stopped at 25 minutes to minimize blood loss. Three of four CF mice had clot formation before 25 minutes, but the results were highly variable. There was no significant difference in bleeding times between CF and HF mice ( $T= 12.00$ ,  $p= 0.11$ , Figure 4.3).

### *Flow cytometry*

*Microparticle formation.* Total and platelet specific microparticle formation was measured to examine any changes that might occur with diet induced obesity and diabetes. The baseline levels of total microparticles were no different between HF mice at 4 months (CF ( $n= 3$ )  $0.0032 \pm 0.0003$ , HF ( $n= 5$ )  $0.0029 \pm 0.0004$  %total events,  $t(6)= 0.44$ ,  $p= 0.67$ ) or 6 months (CF ( $n= 5$ )  $0.0067 \pm 0.0073$ , HF ( $n= 4$ )  $0.0069 \pm 0.0071$  %total events,  $T= 22.00$ ,  $p= 0.73$ ) and CF mice. The CF and HF mice after 6 months on the diet, but not after 4 months, demonstrated a significant increase in total microparticle formation after stimulation with calcimycin (calcium ionophore) (paired t-tests- 4mo- CF  $t(2)= -3.21$ ,  $p= 0.09$ , HF  $t(4)= -2.45$ ,  $p= 0.07$ ; 6mo CF  $t(4)= -5.64$ ,  $p< 0.01$ , HF  $t(3)= -5.56$ ,  $p= 0.01$ ). After stimulation, there was no difference in total microparticle formation at 4 months between HF and CF mice (CF  $0.0235 \pm 0.0063$ , HF  $0.0203 \pm 0.0072$  %total events,  $t(6)= 0.30$ ,  $p= 0.78$ ), but at 6 months the HF fed mice demonstrated less total microparticle formation compared to CF (CF  $0.0387 \pm 0.0050$ , HF  $0.0217 \pm 0.0026$  %total events,  $t(7)= 2.77$ ,  $p= 0.03$ , Figure 4.4A).

Figure 4.3 Tail bleeding times in mice fed a standard chow or high fat diet for 4 months.

Time measured in seconds ( $n= 4$  each group).

Figure 4.3

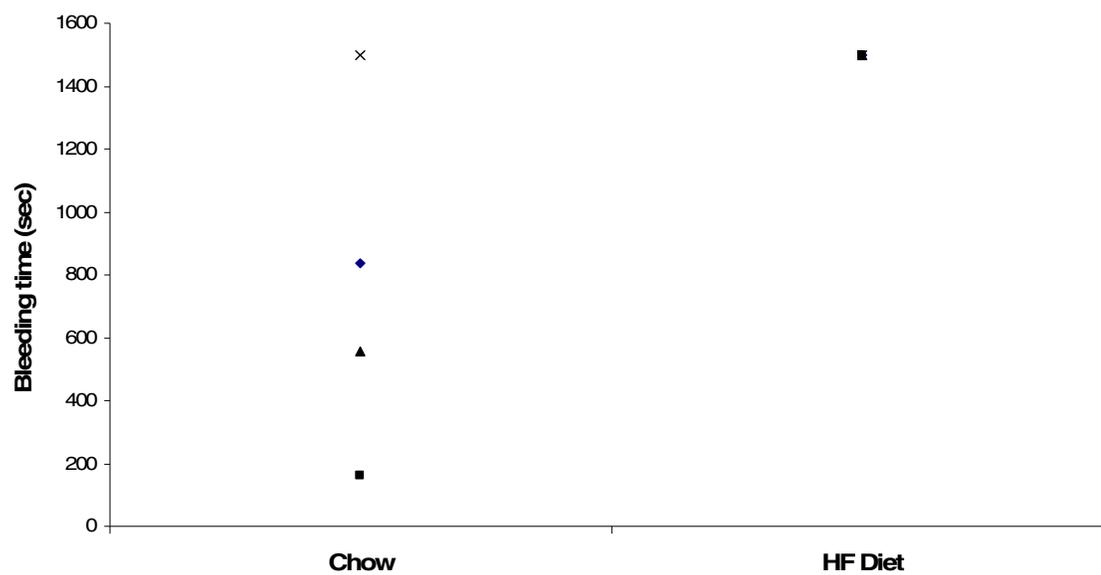
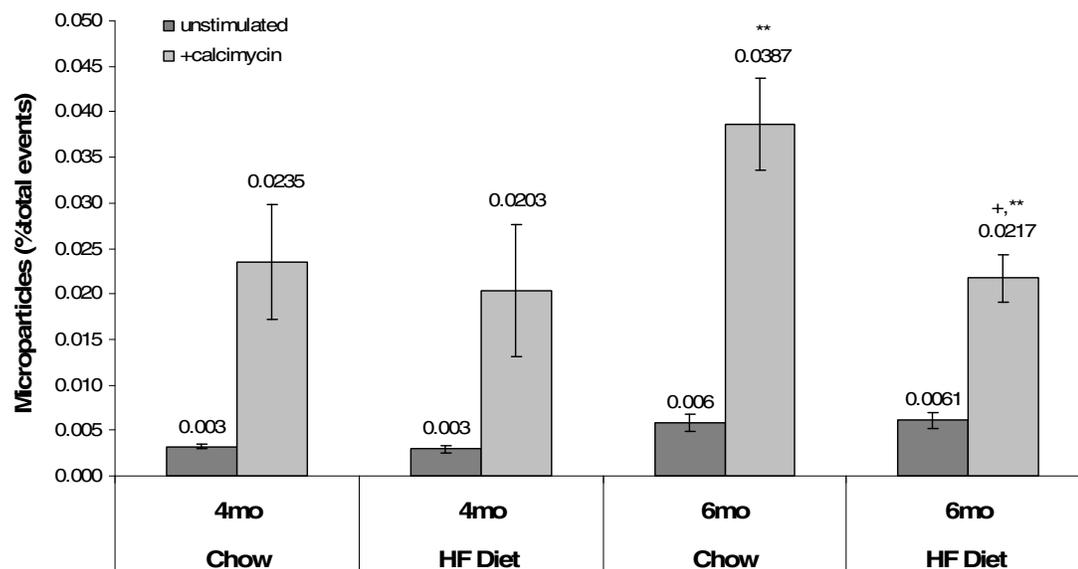


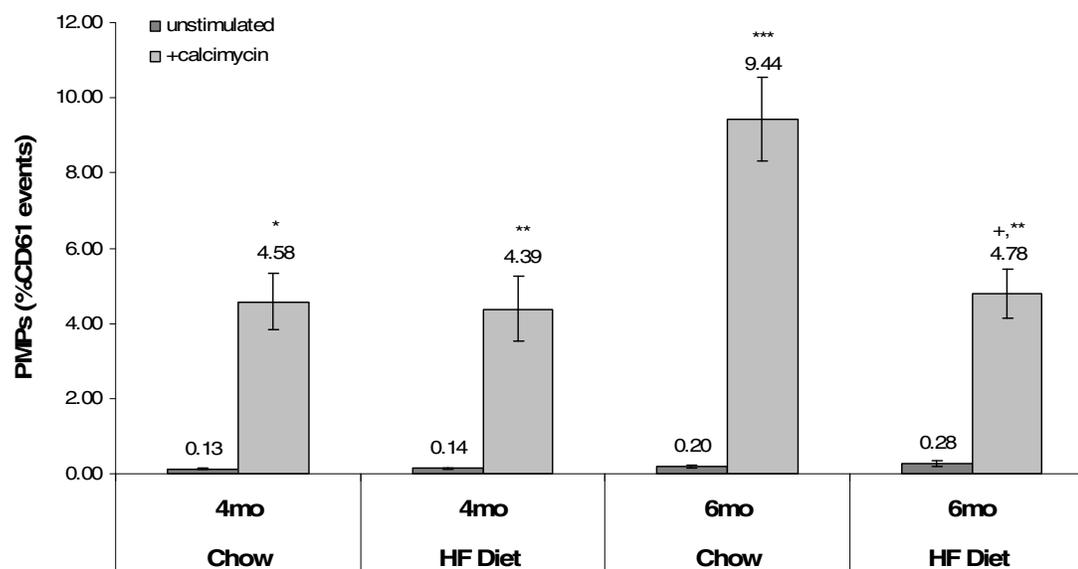
Figure 4.4 Total and platelet microparticle formation in mice fed a standard chow or high fat diet for 4 and 6 months. (A) Summative data of total microparticle formation (%total acquired events) (4 mo- CF  $n= 3$ , HF  $n= 5$ ; 6 mo- CF  $n= 5$ ; HF  $n= 4$ ). (B) Summative data of platelet microparticle formation (%CD61 positive events). Data presented as mean  $\pm$  SEM. \*significantly different from unstimulated sample,  $*p < 0.05$ ,  $** p < 0.01$ ;  $***p < 0.001$ , +significantly different from CF mice  $+p < 0.05$ .

Figure 4.4

A.



B.



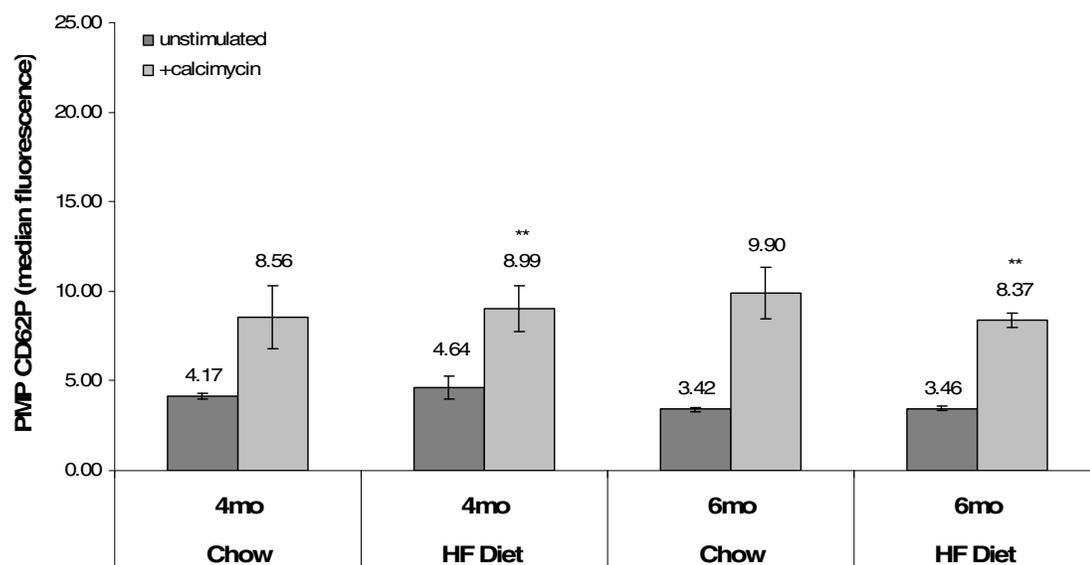
The baseline levels of platelet microparticles (PMPs) were also no different between HF fed mice at 4 months (CF  $0.126 \pm 0.013$ , HF  $0.142 \pm 0.018$  %CD61 positive events,  $t(6) = -0.62$ ,  $p = 0.56$ ) or 6 months compared to CF mice (CF  $0.199 \pm 0.047$ , HF  $0.276 \pm 0.066$  %CD61+ events,  $t(7) = -0.98$ ,  $p = 0.36$ ). All groups demonstrated a significant increase in PMPs after stimulation with calcimycin (4mo- CF  $t(2) = -5.92$ ,  $p = 0.03$ , HF  $t(4) = -4.98$ ,  $p < 0.01$ ; 6mo CF  $t(4) = -8.04$ ,  $p = 0.001$ , HF  $t(3) = -6.99$ ,  $p < 0.01$ ). After stimulation, there was no difference in PMP formation at 4 months between HF and CF mice (CF  $4.6 \pm 0.7$ , HF  $4.4 \pm 0.9$  % CD61+ events,  $t(6) = 0.15$ ,  $p = 0.88$ ), but at 6 months the HF fed mice demonstrated less PMP formation compared to CF mice (CF  $9.4 \pm 1.1$ , HF  $4.8 \pm 0.6$  % CD61+ events,  $t(7) = 3.38$ ,  $p = 0.01$ , Figure 4.4B). It should be noted that the majority of samples in the microparticle data demonstrated intersample variation greater than 20%.

*P-selectin expression.* We examined the expression of P-selectin on both platelet derived microparticles and platelets, to see if there were any changes in expression with diet induced obesity and diabetes. PMP P-selectin expression was no different at baseline or after calcimycin stimulation in both the 4 and 6 month HF and CF mice (baseline- 4 mo- CF  $4.2 \pm 0.2$ , HF  $4.6 \pm 0.7$  median fluorescence intensity (MDFI),  $t(6) = -0.52$ ,  $p = 0.62$ ; 6 mo- CF  $3.4 \pm 0.1$ , HF  $3.5 \pm 0.1$  MDFI,  $t(7) = -0.23$ ,  $p = 0.82$ ; stimulated- 4 mo- CF  $8.6 \pm 1.7$ , HF  $9.0 \pm 1.3$  MDFI,  $T = 11.00$ ,  $p = 0.57$ ; 6 mo- CF  $9.9 \pm 1.5$ , HF  $8.4 \pm 0.4$  MDFI,  $T = 16.00$ ,  $p = 0.41$ ). Only mice fed a HF diet at either 4 or 6 months demonstrated an increase in PMP P-selectin expression with calcimycin stimulation (4mo- CF  $t(2) = -2.80$ ,  $p = 0.11$ , HF  $t(4) = -4.98$ ,  $p < 0.01$ ; 6mo CF  $T = 15.00$ ,  $p = 0.06$ , HF  $t(3) = -9.88$ ,  $p < 0.01$ ).

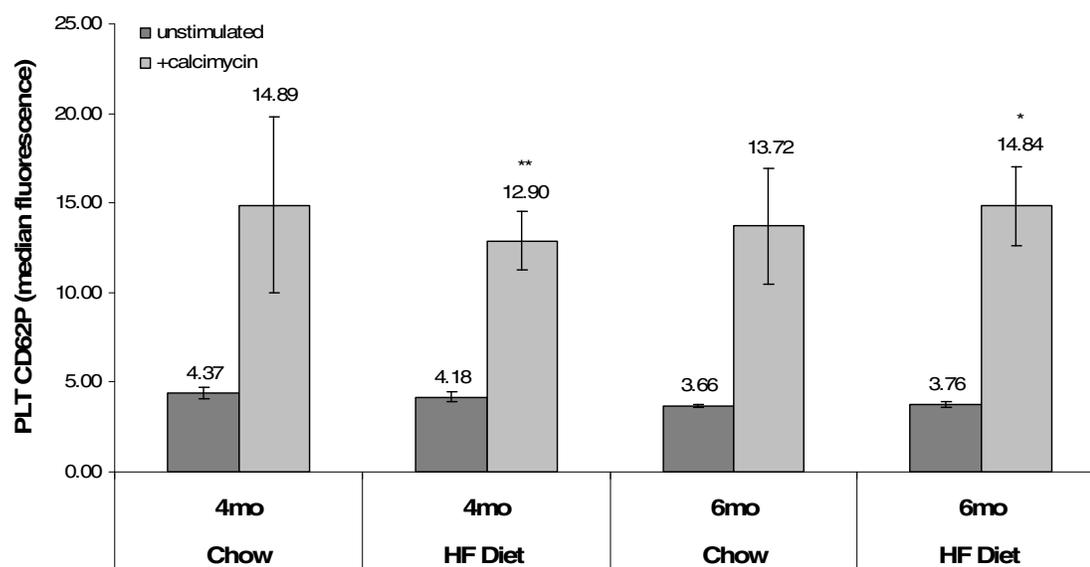
Figure 4.5 Platelet microparticle (PMP) and platelet CD62P (P-selectin) expression in mice fed a standard chow or high fat diet for 4 and 6 months. (A) Summative data of PMP CD62P expression (median fluorescence intensity, MDFI) (4 mo- CF  $n=3$ ; HF  $n=5$ ; 6 mo- CF  $n=5$ ; HF  $n=4$ ). (B) Summative data of platelet CD62P expression (MDFI). Data presented as mean  $\pm$  SEM. \*significantly different from unstimulated sample,  $*p < 0.05$ ,  $**p < 0.01$ .

Figure 4.5

A.



B.



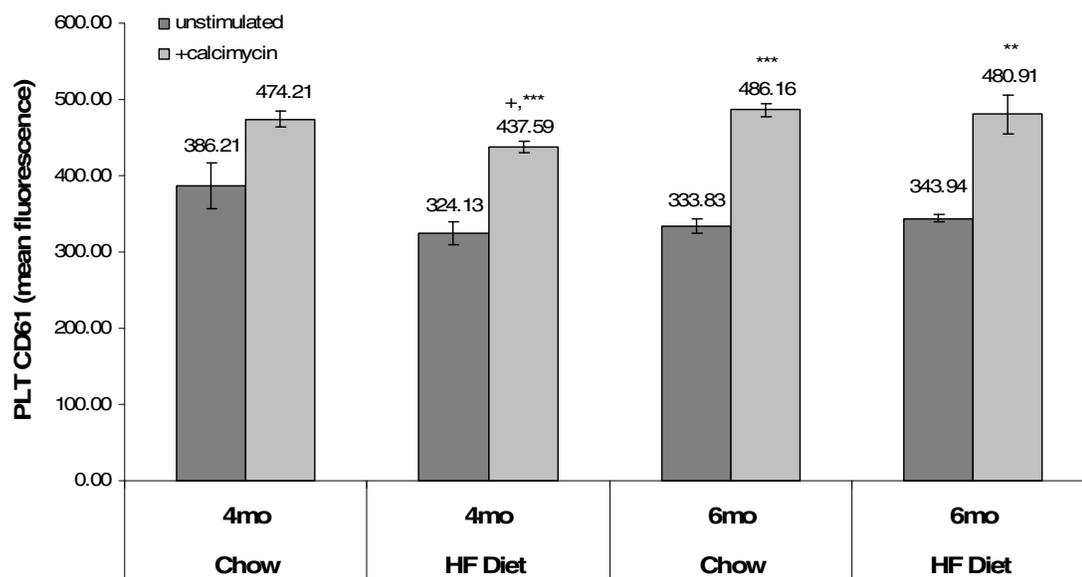
In addition, the platelet expression of P-selectin was no different between mice fed a HF or chow diet at either 4 or 6 months (4 mo- CF  $4.4 \pm 0.3$ , HF  $4.2 \pm 0.3$  MDFI,  $t(6) = 0.46$ ,  $p = 0.66$ ; 6 mo- CF  $3.7 \pm 0.1$ , HF  $3.8 \pm 0.1$  MDFI,  $t(7) = -0.61$ ,  $p = 0.56$ ), or with stimulation (4 mo- CF  $14.9 \pm 4.9$ , HF  $12.9 \pm 1.6$  MDFI,  $t(6) = 0.47$ ,  $p = 0.65$ ; 6 mo- CF  $13.7 \pm 3.2$ , HF  $14.8 \pm 2.2$  MDFI,  $T = 24.00$ ,  $p = 0.41$ ). Similar to PMP P-selectin expression, only mice fed a HF diet (both 4 and 6 months) demonstrated a significant increase in platelet p-selectin expression with stimulation (paired t-tests- 4mo- CF  $T = 15.00$ ,  $p = 0.06$ , HF  $t(4) = -6.26$ ,  $p < 0.01$ ; 6moCF  $T = 15.00$ ,  $p = 0.06$ , HF  $t(3) = -4.85$ ,  $p = 0.02$ , Figure 4.5B).

*Platelet CD61 expression.* There was no difference in baseline platelet CD61 expression between the HF and CF mice at 4 and 6 months (4 mo- CF  $386.2 \pm 30.0$ , HF  $324.1 \pm 14.8$  mean fluorescence intensity (MFI),  $t(6) = 2.11$ ,  $p = 0.08$ ; 6 mo- CF  $333.8 \pm 9.3$ , HF  $343.9 \pm 4.8$  MFI,  $t(7) = -0.89$ ,  $p = 0.40$ , Figure 4.6). Except in CF mice at 4 months, there was a significant increase in platelet CD61 expression in all groups after stimulation with calcimycin (paired t-tests- 4mo- CF  $t(2) = -4.02$ ,  $p = 0.06$ , HF  $t(4) = -10.60$ ,  $p < 0.001$ ; 6mo CF  $t(4) = -12.34$ ,  $p < 0.001$ , HF  $t(3) = -6.46$ ,  $p < 0.01$ ). The HF fed mice demonstrated a significantly less CD61 expression after calcimycin stimulation compared to CF mice at 4 months, but not at 6 months (4 mo- CF  $474.2 \pm 11.0$ , HF  $437.6 \pm 8.1$  MFI,  $t(6) = 2.72$ ,  $p = 0.04$ ; 6 mo- CF  $486.2 \pm 8.5$ , HF  $480.9 \pm 25.5$ ,  $T = 20.00$ ,  $p = 1.00$ , Figure 4.6A). The decrease in stimulated platelet CD61 expression after 4 months of HF feeding was significantly correlated with the decrease in platelet count noted in these mice ( $r = 0.84$ ,  $p = 0.04$ , Figure 4.6B).

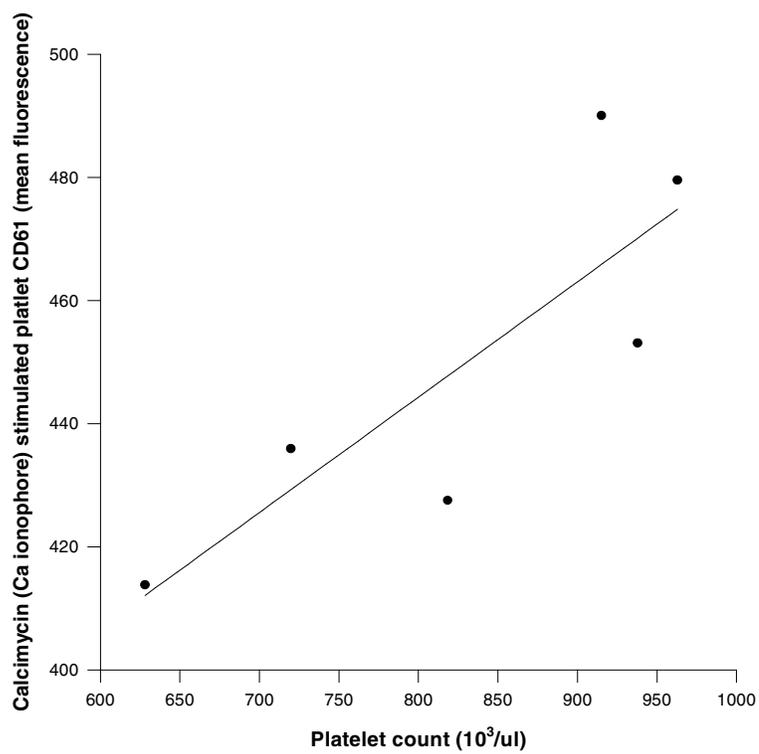
Figure 4.6 Platelet CD61 expression in mice fed a standard chow or high fat diet for 4 and 6 months. (A) Summative data of platelet CD61 expression (mean fluorescence intensity, MFI) (4 mo-CF  $n=3$ ; HF  $n=5$ ; 6 mo- CF  $n=5$ ; HF  $n=4$ ). Data presented as mean  $\pm$  SEM. (B) Correlation between platelet count and stimulated platelet CD61 expression in 4 month HF and CF mice ( $r=0.84$ ,  $p=0.04$ ). \*significantly different from unstimulated sample, \*\* $p<0.01$ , \*\*\* $p<0.001$ ; +significantly different from chow fed mice, +  $p<0.05$ .

Figure 4.6

A.



B.



*High Fat and Chow Fed Mice Injected with STZ*

The HF fed mouse model of obesity and type 2 diabetes does not demonstrate severe hyperglycemia, as seen with patients with long standing type 2 diabetes. Because we did not observe a significant hypercoagulable state in the HF fat mouse model, we decided to examine whole blood aggregation and platelet markers of activation in another set of experiments using the HF fed mouse with a low dose STZ, inducing a more severe form of type 2 diabetes.

*Metabolic characteristics*

After eating the diet for 2 months mice fed a HF diet with or without the STZ injection were significantly obese compared to mice fed a standard chow diet (CF +vehicle ( $n= 9$ )  $24.3 \pm 0.8$ , CF+STZ ( $n= 10$ )  $24.8 \pm 1.0$ , HF +vehicle ( $n= 10$ )  $31.6 \pm 0.9$ , HF +STZ ( $n= 11$ )  $35.6 \pm 1.2$  gm,  $H= 30.15$ ,  $df= 3$ ,  $p< 0.001$ ). Only the HF+STZ were significantly hyperglycemic (CF+vehicle  $195.6 \pm 9.8$ , CF+STZ  $197.2 \pm 8.2$ , HF+vehicle  $239.4 \pm 7.5$ , HF+STZ  $335.2 \pm 22.1$  mg/dl,  $H= 27.14$ ,  $df= 3$ ,  $p< 0.001$ ). There was no difference in plasma insulin levels (CF+vehicle ( $n= 5$ )  $195.6 \pm 9.8$ , CF+STZ ( $n= 5$ )  $197.2 \pm 8.2$ , HF +vehicle ( $n= 7$ )  $239.4 \pm 7.5$ , HF+STZ ( $n= 7$ )  $335.2 \pm 22.1$  pM,  $H= 6.43$ ,  $df= 3$ ,  $p= 0.09$ ) or in the HOMA-insulin resistance score (CF+vehicle ( $n= 3$ )  $2.6 \pm 1.4$ , CF+STZ ( $n= 2$ )  $1.3 \pm 0.2$ , HF+vehicle ( $n= 5$ )  $7.9 \pm 2.7$ , HF+STZ ( $n= 5$ )  $10.6 \pm 1.0$ ,  $F(3,11)= 3.88$ ,  $p= 0.04$ , no significant post hoc comparisons), although the mice fed the HF diet have HOMA scores consistent with insulin resistance. The weight of these mice was significantly correlated with blood sugar ( $r= 0.72$ ,  $p< 0.001$ ), but not with insulin levels ( $r= 0.46$ ,  $p= 0.08$ ). The HF mice with or without the STZ injection were also hypercholesterolemic (CF+vehicle

( $n=7$ )  $101.7 \pm 1.7$ , CF+STZ ( $n=10$ )  $101.8 \pm 1.3$ , HF+vehicle ( $n=10$ )  $148.8 \pm 7.8$ , HF+STZ ( $n=10$ )  $166.2 \pm 13.2$  mg/dl,  $H=26.68$ ,  $df=3$ ,  $p<0.001$ ), but not hypertriglyceridemic (CF+vehicle ( $n=5$ )  $59.8 \pm 1.0$ , CF+STZ ( $n=5$ )  $62.2 \pm 2.5$ , HF+vehicle ( $n=5$ )  $73.8 \pm 11.3$ , HF+STZ ( $n=6$ )  $68.5 \pm 4.4$  mg/dl,  $H=5.75$ ,  $df=3$ ,  $p=0.12$ , Table 4.3), similar to mice fed the HF diet for 4 and 6 months.

Table 4.3 Metabolic Characteristics and Platelet Count of Mice Fed Standard Chow or a High Fat Diet for 2 Months With or Without a Low Dose STZ Injection

Strain ( $n$ ) <sup>^</sup>	Weight (g)	Blood Sugar (mg/dl)	Trig. (mg/dl)	Total Chol (mg/dl)	Platelet count ( $10^3/\mu\text{l}$ )	Plasma Insulin (pM) <sup>^</sup>	HOMA-IR score <sup>^</sup>
CF+vehicle (11)	24.3 $\pm$ 0.8	195.6 $\pm$ 9.8	59.8 $\pm$ 1.0	101.7 $\pm$ 1.7	839.7 $\pm$ 32.3	66.1 $\pm$ 23.6	1.5 $\pm$ 1.0
CF+STZ (13)	24.8 $\pm$ 1.0	197.2 $\pm$ 8.2	62.2 $\pm$ 2.5	101.8 $\pm$ 1.3	808.6 $\pm$ 54.4	38.0 $\pm$ 10.0	1.3 $\pm$ 0.2
HF+vehicle (13)	31.6 $\pm$ 0.8* $\dagger$	239.4 $\pm$ 7.5	73.8 $\pm$ 11.3	148.8 $\pm$ 7.8* $\dagger$	741.2 $\pm$ 24.8	174.3 $\pm$ 50.7	7.9 $\pm$ 2.7
HF+STZ (13)	35.6 $\pm$ 1.2* $\dagger$	335.2 $\pm$ 22.1* $\dagger$	68.5 $\pm$ 4.4	166.2 $\pm$ 13.2* $\dagger$	696.7 $\pm$ 27.6	76.6 $\pm$ 12.8	8.9 $\pm$ 2.0

CF-chow fed, HF-high fat, Trig- Triglycerides, Total Chol- Total Cholesterol, HOMA-IR- Homeostasis Model Assessment Insulin Resistance Score. All measurements were made after 4 hour fast in whole blood unless otherwise specified. Platelet counts were standardized to a hematocrit of 45%. <sup>^</sup>See results text for ( $n$ ) of each measurement. Data presented as mean  $\pm$  SEM. \*significantly different from CF+vehicle mice,  $*p<0.05$ ,  $\dagger$ significantly different from CF+STZ mice,  $\dagger p<0.05$ .

### *Whole blood cell counts*

The whole blood counts were standardized to a hematocrit of 45% to eliminate the effects of anticoagulant dilution. In contrast to mice fed a high fat diet for 4 or 6 months, the platelet counts were no different between CF and HF mice at this 2 month time point (CF+vehicle ( $n= 7$ )  $839.7 \pm 32.3$ , CF+STZ ( $n= 10$ )  $808.6 \pm 54.4$ , HF+vehicle ( $n= 10$ )  $741.2 \pm 24.8$ , HF+STZ ( $n= 9$ )  $696.7 \pm 27.6$ ,  $F(3,32)= 2.67 \text{ } 10^3/\mu\text{l}$ ,  $p= 0.06$ , Table 4.3). Even though there was no statistical difference in platelet count, the platelet counts were lower in the mice fed a HF diet, and this was significantly correlated with weight ( $r= 0.53$ ,  $p< 0.001$ ) and blood sugar levels ( $r= 0.48$ ,  $p< 0.001$ ), but not insulin levels ( $r= 0.52$ ,  $p= 0.07$ ). To determine if the platelet count was affected by differences in the amount of platelet microaggregate formation (clumping) after to venipuncture, we analyzed the blood smears on a subset of experiments ( $n= 4$  each group). The HF+STZ mice demonstrated a significantly higher number of visual fields with platelet microaggregates than the CF mice (CF+vehicle  $25.0 \pm 0.1$ , CF+STZ  $32.5 \pm 0.05$ , HF+vehicle  $60.0 \pm 0.1$ , HF+STZ  $67.5 \pm 0.1$ ,  $F(3,12)= 5.94$ ,  $p= 0.01$ , Table 4.4). There was no significant difference in the average number of microaggregates per field (CF +vehicle  $0.3 \pm 0.1$ , CF+STZ  $0.5 \pm 0.1$ , HF+vehicle  $1.0 \pm 0.2$ , HF+STZ  $1.0 \pm 0.3$  avg/field,  $F(3,12)= 3.3$ ,  $p= 0.052$ ) or size of microaggregates (CF+vehicle  $119.4 \pm 45.4$ , CF+STZ  $96.5 \pm 33.6$ , HF+vehicle  $97.1 \pm 25.6$ , HF+STZ  $327.5 \pm 92.7 \mu\text{m}^3$ ,  $H= 6.15$ ,  $df= 3$ ,  $p= 0.10$ , Table 4.4).

Table 4.4 Platelet Microaggregate Formation in Mice Fed a Standard Chow or a High Fat Diet with or without STZ Injection.

Strain (n)	% visual fields with microaggregates	Avg number of microaggregates/ visual field	Avg area of microaggregates ( $\mu\text{m}^2$ )
CF			
+Vehicle(4)	25.0 $\pm$ 8.7	0.3 $\pm$ 0.1	119.4 $\pm$ 45.4
+STZ (4)	32.5 $\pm$ 4.8	0.5 $\pm$ 0.1	96.5 $\pm$ 33.6
HF			
+Vehicle (4)	60.0 $\pm$ 10.8	1.0 $\pm$ 0.2	97.1 $\pm$ 25.6
+STZ (4)	67.5 $\pm$ 8.5**	1.0 $\pm$ 0.3	327.5 $\pm$ 92.7

CF-chow fed, HF-high fat fed. Data presented as mean  $\pm$  SEM. \*significantly different from CF+vehicle mice, \*\*  $p < 0.01$ .

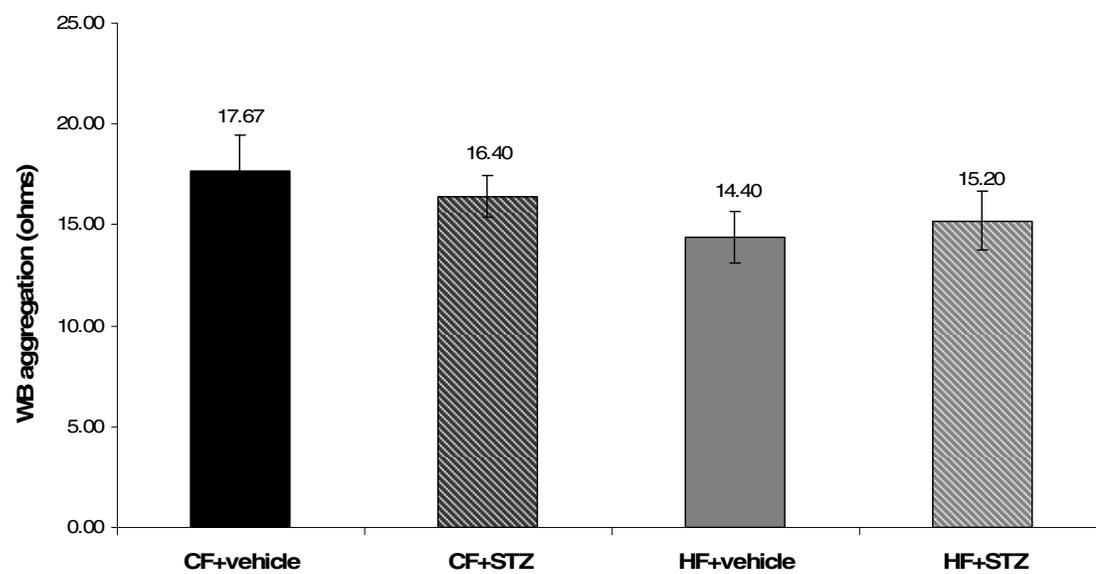
#### *Whole blood aggregation*

In contrast to the whole blood aggregometry results in mice fed a HF diet for 4 months, there was no significant difference in the extent (CF+vehicle  $17.7 \pm 1.7$ , CF+STZ  $16.4 \pm 1.0$ , HF+vehicle  $14.4 \pm 1.3$ , HF+STZ  $15.2 \pm 1.5$   $\Omega$ ,  $F(3,14) = 0.96$ ,  $p = 0.44$ , Figure 4.7A) or rate of aggregation (CF+vehicle  $9.7 \pm 1.2$ , CF+STZ  $9.8 \pm 1.6$ , HF+vehicle  $8.2 \pm 1.2$ , HF+STZ  $10.0 \pm 1.0$   $\Omega/\text{sec}$ ,  $F(3,14) = 0.432$ ,  $p = 0.73$ , Figure 4.7B) in mice fed a HF or chow diet with or without STZ injection.

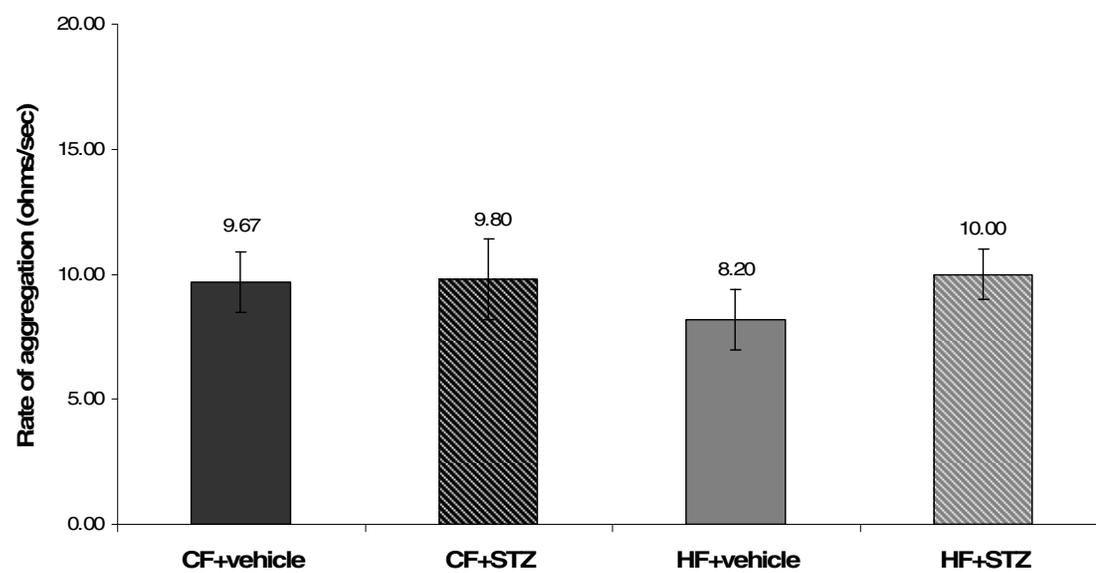
Figure 4.7 Whole blood aggregation in mice fed a standard chow or high fat diet with or without a low dose STZ injection. (A) Summative data for ADP-induced whole blood aggregation (ohms ( $\Omega$ )) (CF+vehicle ( $n= 3$ ), CF+STZ ( $n= 5$ ); HF +vehicle ( $n= 5$ ), HF +STZ ( $n= 5$ )). (B) Summative data for the rate of ADP-induced whole blood aggregation ( $\Omega$ /sec). Data presented as mean  $\pm$  *SEM*.

Figure 4.7

A.



B.



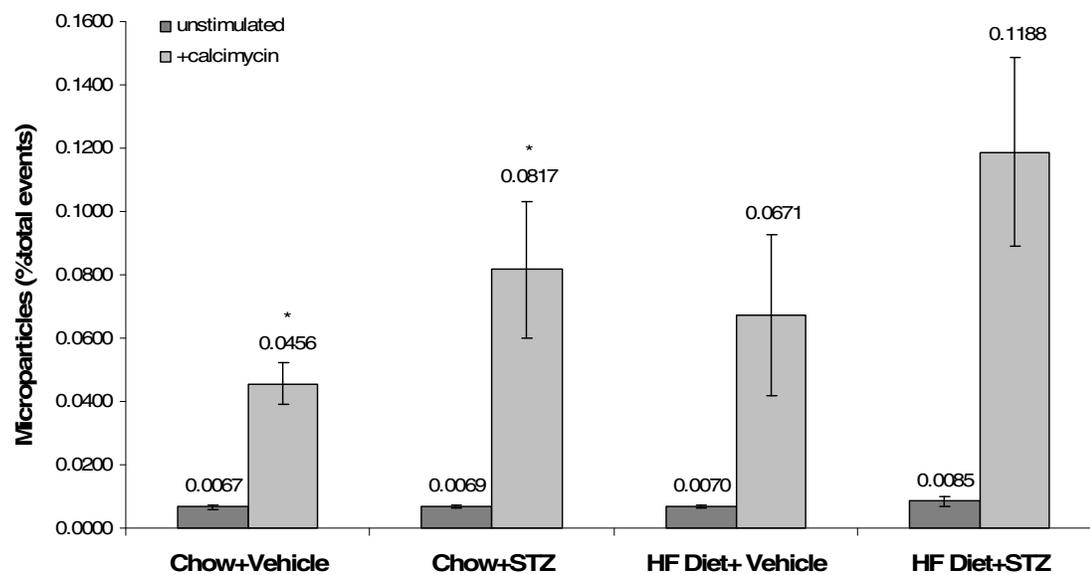
*Flow cytometry*

*Microparticle formation.* Similar to results obtained in mice fed a HF diet for 4 or 6 months, the levels of total microparticle formation was no different between HF or chow fed mice with or without STZ injection (CF+vehicle  $0.0067 \pm 0.007$ , CF+STZ  $0.0069 \pm 0.0003$ , HF+vehicle  $0.0070 \pm 0.0005$ , HF+STZ  $0.0085 \pm 0.0015$  %total events,  $H= 1.46$ ,  $df= 3$ ,  $p= 0.69$ ) or after calcimycin stimulation (CF+vehicle  $0.0456 \pm 0.0066$ , CF+STZ  $0.0817 \pm 0.0216$ , HF+vehicle  $0.0671 \pm 0.0255$ , HF+STZ  $0.119 \pm 0.02990$  %total events,  $F(3,10)= 1.52$ ,  $p= 0.27$ ). Only the CF groups demonstrated a significant increase in total microparticles after stimulation with calcimycin (paired t-tests, CF+vehicle  $t(2)= -5.47$ ,  $p= 0.03$ , CF+STZ  $t(3)= -3.45$ ,  $p= 0.04$ , HF+vehicle  $T=10.00$ ,  $p= 0.13$ , HF+STZ  $t(2)= -3.80$ ,  $p= 0.06$ , Figure 4.8A). The baseline levels of platelet derived microparticles (PMPs) were also no different between HF and CF mice at baseline (CF+vehicle  $0.356 \pm 0.041$ , CF+STZ  $0.319 \pm 0.038$ , HF+vehicle  $0.378 \pm 0.050$ , HF+STZ  $0.470 \pm 0.099$  %total CD61 positive events,  $F(3,15)= 1.16$ ,  $p= 0.36$ ) or after stimulation (CF+vehicle  $12.0 \pm 1.5$ , CF+STZ  $16.9 \pm 2.6$ , HF+vehicle  $13.1 \pm 1.6$ , HF+STZ  $20.2 \pm 3.1$  %CD61 positive events,  $F(3,10)= 2.43$ ,  $p= 0.13$ ). All groups demonstrated a significant increase in PMPs after stimulation with calcimycin (paired t-tests, CF+vehicle  $t(2)= -7.47$ ,  $p= 0.02$ , CF+STZ  $t(3)= -6.41$ ,  $p< 0.01$ , HF+vehicle  $t(3)= -7.80$ ,  $p< 0.01$ , HF+STZ  $t(2)= -6.53$ ,  $p= 0.02$ , Figure 4.8B).

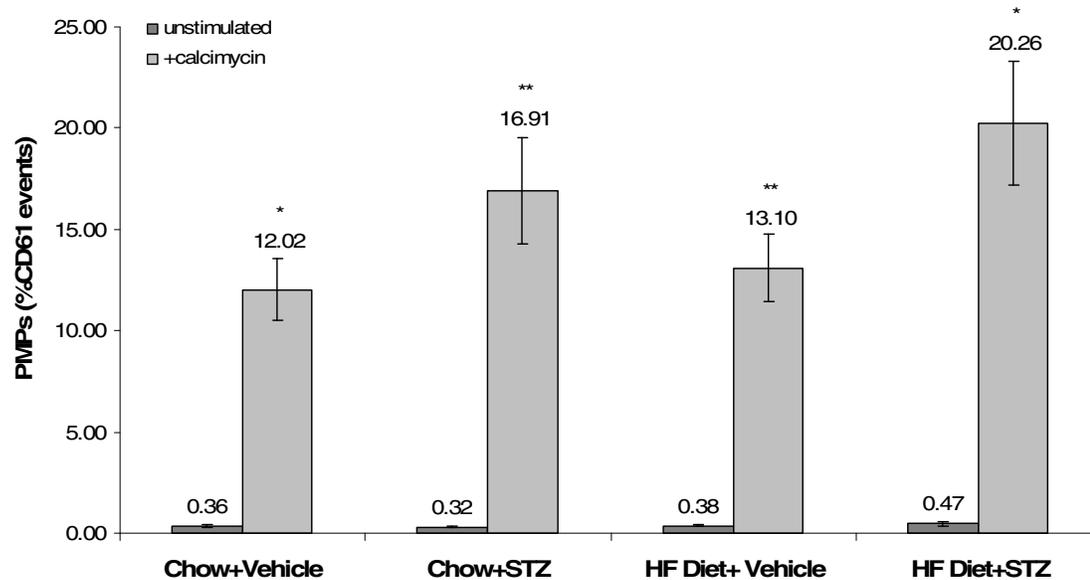
Figure 4.8 Total and platelet microparticle formation in mice fed a standard chow or high fat diet with or without a low dose STZ injection. (A) Summative data of total microparticle formation (%total acquired events) (CF+vehicle ( $n= 5$ ), CF+STZ ( $n= 5$ ); HF +vehicle ( $n= 5$ ), HF +STZ ( $n= 4$ )). (B) Summative data of platelet microparticle formation (%CD61 positive events). Data presented as mean  $\pm$  SEM. \*significantly different from unstimulated sample,  $*p < 0.05$ ,  $** p < 0.01$ .

Figure 4.8

A.



B.



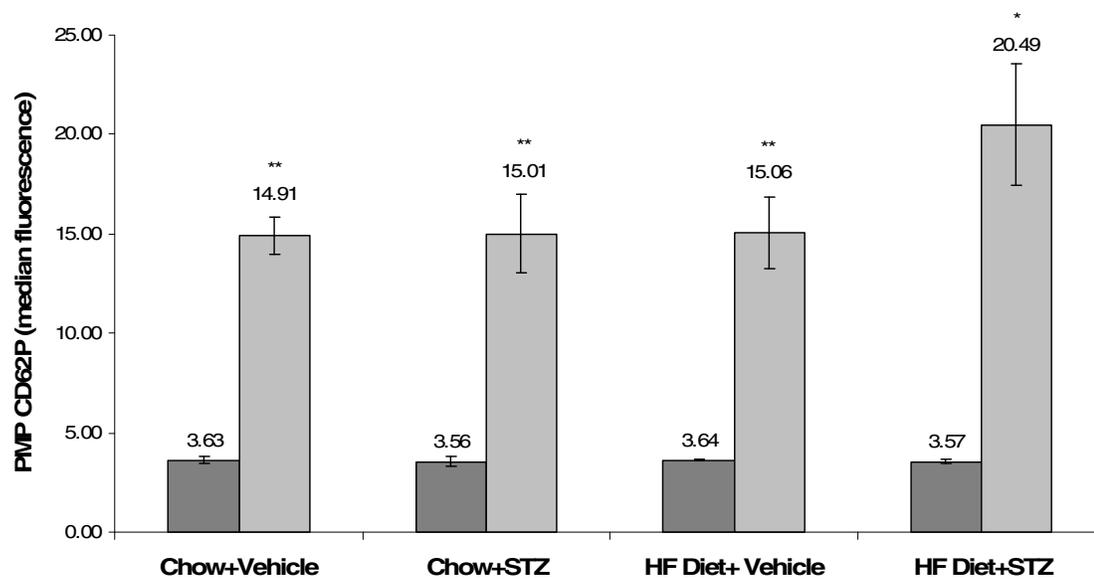
*P-selectin expression.* PMP P-selectin expression was no different at in any group at baseline (CF+vehicle  $3.6 \pm 0.2$ , CF+STZ  $3.6 \pm 0.2$ , HF+vehicle  $3.6 \pm 0.1$ , HF+STZ  $3.6 \pm 0.1$  MDFI,  $F(3,15)= 0.05$ ,  $p= 0.99$ ) or after stimulation (CF+vehicle  $14.9 \pm 1.0$ , CF+STZ  $15.0 \pm 1.8$ , HF+vehicle  $15.1 \pm 1.8$ , HF+STZ  $20.5 \pm 3.1$  MDFI,  $F(3,10)= 1.61$ ,  $p= 0.25$ ). All groups were able to significantly increase expression with stimulation (paired t-tests, CF+vehicle  $t(2)= -9.92$ ,  $p= 0.01$ , CF+STZ  $t(3)= -5.94$ ,  $p= 0.01$ , HF+vehicle  $t(3)= -6.55$ ,  $p< 0.01$ , HF+STZ  $t(2)= -5.87$ ,  $p= 0.03$ , Figure 4.9A). In addition, the platelet expression of P-selectin was no different between in any group (CF+vehicle  $3.9 \pm 0.1$ , CF+STZ  $3.8 \pm 0.1$ , HF+vehicle  $3.8 \pm 0.1$ , HF+STZ  $4.1 \pm 0.3$  MDFI,  $F(3,15)= 0.50$ ,  $p= 0.69$ ) or after stimulation (CF+vehicle  $20.8 \pm 1.2$ , CF+STZ  $23.1 \pm 5.2$ , HF+vehicle  $20.6 \pm 1.9$ , HF+STZ  $36.2 \pm 6.6$  MDFI,  $F(3,10)= 2.73$ ,  $p= 0.10$ ). All groups demonstrated significant increased platelet p-selectin expression with stimulation (paired t-tests, CF+vehicle  $t(2)= -14.06$ ,  $p< 0.01$ , CF+STZ  $t(3)= -3.74$ ,  $p= 0.03$ , HF+vehicle  $t(3)= -8.54$ ,  $p< 0.01$ , HF+STZ  $t(2)= -5.11$ ,  $p= 0.04$ , Figure 4.9B).

*Platelet CD61 expression.* There was no difference in baseline platelet CD61 expression (CF+vehicle  $310.6 \pm 4.3$ , CF+STZ  $299.2 \pm 5.5$ , HF+vehicle  $300.4 \pm 10.4$ , HF+STZ  $312.8 \pm 4.6$  MFI,  $F(3,15)= 0.98$ ,  $p= 0.43$ ) or after stimulation (CF+vehicle  $471.6 \pm 6.0$ , CF+STZ  $441.8 \pm 25.0$ , HF+vehicle  $433.7 \pm 19.4$ , HF+STZ  $434.4 \pm 6.1$  MFI,  $F(3,10)= 0.803$ ,  $p= 0.52$ ) in mice fed either diet. There was a significant increase in CD61 expression in all groups after stimulation ( $p< 0.05$ , (paired t-tests, CF+vehicle  $t(2)= -14.93$ ,  $p< 0.01$ , CF+STZ  $t(3)= -7.18$ ,  $p< 0.01$ , HF+vehicle  $t(3)= -14.60$ ,  $p< 0.001$ , HF+STZ  $t(2)= -17.16$ ,  $p< 0.01$ , Figure 4.10).

Figure 4.9 Platelet microparticle (PMP) and platelet CD62P (P-selectin) expression formation in mice fed a standard chow or high fat diet with or without a low dose STZ injection. (A) Summative data of PMP CD62P expression (MDFI) (CF+vehicle ( $n= 5$ ), CF+STZ ( $n= 5$ ); HF +vehicle ( $n= 5$ ), HF +STZ ( $n= 4$ )). (B) Summative data of platelet CD62P expression (MDFI). Data presented as mean  $\pm$  SEM. \*significantly different from unstimulated sample,  $*p < 0.05$ ,  $**p < 0.01$ .

Figure 4.9

A.



B.

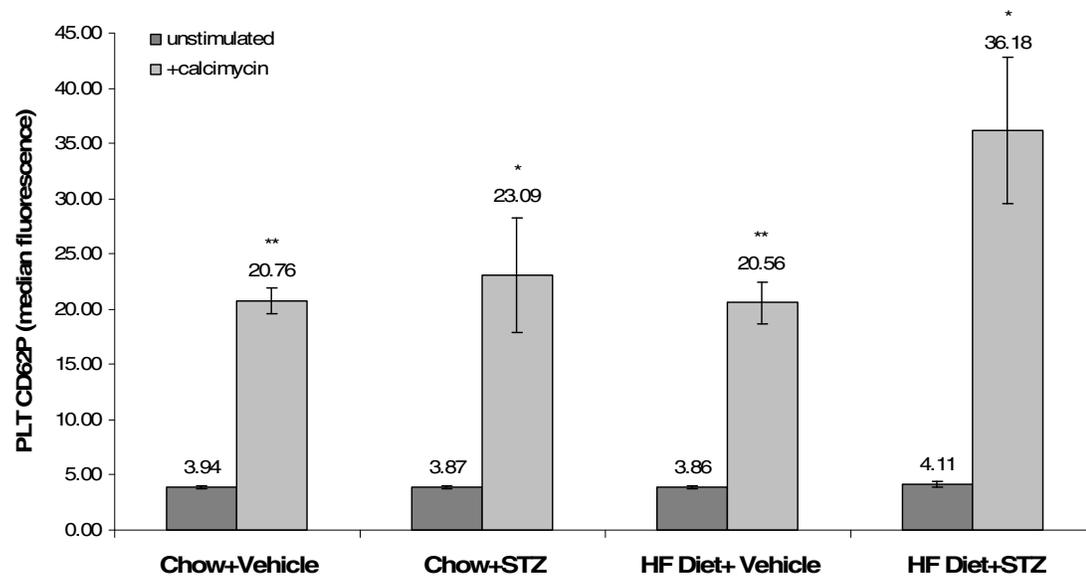
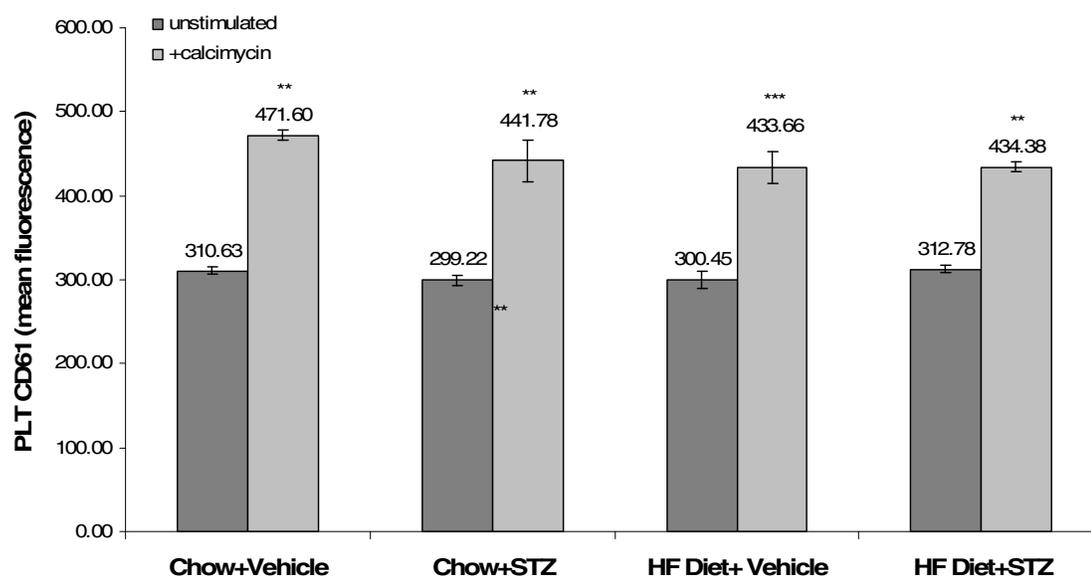


Figure 4.10 Platelet CD61 expression in mice fed a standard chow or high fat diet with or without a low dose STZ injection. Summative data of platelet CD61 expression (mean fluorescence intensity) (CF+vehicle ( $n= 5$ ), CF+STZ ( $n= 5$ ); HF +vehicle ( $n= 5$ ), HF +STZ ( $n= 4$ )). Data presented as mean  $\pm$  SEM. \*significantly different from unstimulated sample, \*\* $p < 0.01$ , \*\*\* $p < 0.001$

Figure 4.10



## Discussion

Obesity and type 2 diabetes in humans causes platelet abnormalities, alterations in coagulation factors, and disturbances in endothelial cells that lead to a hypercoaguable state. People with obesity and diabetes die prematurely from thrombotic events and subsequent vascular disease secondary to this hypercoaguable state (Carr, 2001; Schneider, 2005). The effects of these changes in coagulation and platelet function on stroke and cardiovascular disease have been studied, but are not completely understood. Therefore, examining animal models is necessary to further explore the contributions of diabetes on thrombotic and vascular complications.

The C57BL/6J mouse strain, fed a diet enriched with animal fat, has been used extensively for examining the effects of diet induced obesity and diabetes on many of the vascular complications associated with diabetes. This mouse model demonstrates many of the same metabolic attributes of humans with diabetes, including, predominant abdominal obesity, hyperglycemia, (Collins, Martin, Surwit, & Robidoux, 2004; Surwit, et al., 1988; Surwit, Petro, Parekh, & Collins, 1997), hypercholesterolemia (Libinaki, et al., 1999), and hyperleptinemia (Surwit, et al., 1997). Similar to previous findings, we found that mice fed a HF diet (60% beef lard) for 4 and 6 months demonstrated obesity, hyperglycemia, hyperinsulinemia, insulin resistance, and hypercholesterolemia. The mice fed a HF diet and injected with STZ after only 1 month on the diet also demonstrated these metabolic changes, but the extent of hyperglycemia and hypercholesterolemia was more severe than mice fed the chow diet or HF diet alone. The HF fed mice have also previously demonstrated many of the vascular complications seen in humans with diabetes, including

endothelial dysfunction (Molnar, et al., 2005), hypertension (Mills, et al., 1993), cardiac dysfunction (Thakker, et al., 2006; Park, et al., 2005), and atherosclerosis (Schreyer, Wilson, & LeBoeuf, 1998). Because it is known that platelet dysfunction and hypercoagulation are associated with these vascular complications in human diabetics, we hypothesized that the diet induced obese and diabetic mice would also demonstrate platelet dysfunction and hypercoagulation. Therefore, the aim of this study was to examine whole blood aggregation, coagulation, and platelet markers of activation in the diet induced obese and diabetic mouse.

Few studies have been done examining platelet function or coagulation in diet induced obese and diabetic mice. One study conducted by Mark et al. (1984) in mice fed an atherogenic diet (high fat and cholesterol content), developing high levels of serum cholesterol, demonstrated a significant decrease in vascular production of prostacyclin and an increased production of platelet thromboxane  $A_2$ . *Apo E*  $-/-$  mice fed a high fat diet have demonstrated enhanced thrombosis in an *in vivo* model of ferric chloride arterial injury, increased expression of arterial PAI-1 (Schafer, et al., 2003), and impaired flow mediated vasodilation and enhanced platelet reactivity (Aoki, et al., 2006). Another study evaluating thrombosis in *apo E*  $-/-$  and *LDL receptor*  $-/-$  mice demonstrated an increase in thrombogenesis with a He-Ne laser induced carotid artery injury in mice fed a high fat compared to a low fat diet (Ijiri, et al., 2002). These studies demonstrate that genetically modified mice with severe elevation in cholesterol levels and diabetes induced by diet demonstrate altered thrombosis and coagulation. No studies, however, were found

analyzing platelet function or coagulation in high fat (no cholesterol) diet induced obese and diabetic C57BL6/J mice (diet alone or with a low dose injection of STZ).

We found that the mice fed a HF diet for 4 months demonstrated significantly less whole blood aggregation and clot strength measured by thromboelastography compared to mice fed a standard chow diet. These results were significantly correlated with the lower platelet count. Because mice have high numbers of circulating platelets, the platelets aggregate (clump) readily after venipuncture, which decreases platelet counts that are obtained with standard hematology analyzers (Tsakaris, et al., 1999). No reports of normal platelet counts in this mouse model were found, and it is unknown if these mice normally demonstrate lower platelet counts than chow fed mice. To further explore coagulation and to attempt to eliminate the potential platelet microaggregate artifact in the *in vitro* whole blood experiments, we also looked at *in vivo* bleeding times. In contrast to our hypothesis, the mice fed a HF diet for 4 months did not demonstrate *any* cessation in bleeding and these experiments were stopped to minimize blood loss. Together, these results suggest that diet induced diabetic mice do not demonstrate hypercoaguability or whole blood aggregation, and may even demonstrate less coagulation than control mice.

In light of the results noted in the mice fed a HF diet for 4 months, we decided to examine a more severe state of diabetes on whole blood aggregation. We fed mice a HF diet for 4 weeks, injected them with a low dose of STZ, and then fed them the diet for another 4 weeks. These mice developed a much more severe state of diabetes than with the HF diet alone, with blood sugars well over 300 mg/dl. Interestingly, the mice fed a chow diet and injected with STZ did not develop hyperglycemia and only the HF mice that were

obese at the time of injection developed hyperglycemia (experimental observation), indicating that the mice needed to exhibit some degree of insulin resistance to develop diabetes. Also, the mice fed the HF diet with the STZ injection developed an even higher level of hypercholesterolemia than mice fed the HF diet alone, signifying a more severe state of diabetes and metabolic syndrome. To our knowledge, there have been no other studies reporting the use of this model beyond the original report describing its development. In our study, severely diabetic (HF+STZ) mice did not demonstrate differences in whole blood aggregation compared to chow fed mice. However, they demonstrated significantly more artifactual platelet microaggregation than chow fed mice, and the resultant lower platelet count may have erroneously decreased the aggregation results. These mice may have produced more whole blood aggregation than chow fed mice without this artifact. More experiments are needed to fully elucidate the state of coagulation and aggregation in these more severe diet induced diabetic mice.

Platelet microparticle formation, and platelet expression of CD61 (fibrinogen receptor) and CD62P (P-selectin) are established, sensitive indicators of platelet function, that are known to be altered in diabetic and obese subjects. We also examined these platelet markers of activation to determine the differences in expression, if any, caused by diabetes and the reactivity of diabetic platelets to a calcium ionophore (calcimycin) using whole blood flow cytometry analysis.

Microparticles are small membrane vesicles that arise from blood and endothelial cells upon activation or during programmed cell death (apoptosis). Microparticles are highly thrombogenic, as they expose negatively charged phospholipids

(phosphatidylserine), and other membrane bound proteins i.e. tissue factor, (Diamont, et al., 2002). Platelet microparticles also bind to and activate platelets, leukocytes, and endothelial cells, increasing thrombosis (Jurk, & Kehrel, 2005). Several studies document increased levels platelet derived microparticles in patients with atherosclerosis and diabetes (Nomura, et al., 1995; Koga, et al., 2006). In contrast, a study by Sabatier et al. (2002), did not find an increase in elevated levels of circulating platelet derived microparticles in type 2 diabetes, but did observe an elevation in the level of total microparticles. We, therefore, decided to analyze both total and platelet derived microparticles after inducing obesity and diabetes in mice. We found no difference in the number to total or platelet derived microparticles in diabetic mice, high fat fed for 4 and 6 months, compared to their controls. Mice fed the HF diet for 6 months demonstrated significantly less total and platelet derived microparticles after stimulation compared to control mice. These results indicate that after prolonged ingestion of the HF diet, the diabetic mice did not demonstrate the same reactivity to calcimycin as the chow fed, non-diabetic mice. The mice with more severely induced diabetes (HF+STZ) also did not demonstrate any significant difference in total or platelet derived microparticle formation from control mice. These results are significantly different from previous studies in humans and rats with type 2 diabetes (Cohen, Gonzales, Davis-Gorman, Copeland, & McDonagh, 2002) and do not support our hypothesis of a hypercoaguable state in the diet induced diabetic mouse.

The selectin CD62P (P-selectin) is expressed on platelets and endothelial cells and is a key adhesion molecule in these cell types. Platelet p-selectin is normally stored in  $\alpha$ -granules with minimal surface expression and is translocated to the membrane during

platelet activation (Jurk, & Kehrel, 2005). P-selectin mediates platelet-leukocyte conjugation and platelet-endothelial adhesion (Celi, Lorenzet, Furie, & Furie, 2004). P-selectin is shed readily from the platelet's surface during activation and becomes a soluble mediator of inflammation and thrombosis (Jurk, & Kehrel, 2005). Several studies have demonstrated that platelet P-selectin expression is elevated in patients with type 2 diabetes (McDonagh, et al., 2003; Tschoepe, et al., 1991). Platelet derived microparticles have also been demonstrated to express P-selectin, and it has been hypothesized that its expression causes these microparticles to be even more thrombogenic (Jurk, & Kehrel, 2005; van der Zee, et al., 2006), although this has not been examined in the setting of diabetes. In this study, we looked at the expression of P-selectin on both platelets and platelet microparticles. We found that there was no difference in platelet microparticle or platelet P-selectin expression in diabetic mice after either 4 or 6 months of the high fat diet compared to chow fed mice. The chow fed mice did not demonstrate a significant increase in PMP or platelet P-selectin expression after stimulation, but this may have been because of the high level of variability in the stimulated samples. High fat fed mice injected with STZ, also did not demonstrate a difference in PMP or platelet expression of P-selectin, but after stimulation with calcimycin the HF+STZ mice had higher levels of expression in both PMP and platelets, but this did not reach statistical significance. Perhaps with a more prolonged diabetic state (>1 mo) the expression level would have been statistically significant. Our results, though, are quite different from previous work in humans with diabetes, and do not demonstrate hypercoaguability in these animals.

The integrin CD61 (IIIa portion of the GPIIb/IIIa complex) is constitutively expressed on platelets, but is generally in an inactive state (Jurk, & Kehrel, 2005). Upon activation, the integrin undergoes a conformational change exposing its fibrinogen binding site and more receptors are brought to the membrane through exocytosis of  $\alpha$ -granules (Jurk, & Kehrel, Shattil, Kashiwagi, & Pampori, 1998). The binding of fibrinogen leads to platelet-fibrinogen bridging and subsequent platelet aggregation. Few studies have examined the expression of CD61 in diabetics. McDonagh and colleagues (2003) demonstrated that CD61 expression was higher in diabetic patients with ischemic heart disease than non-diabetics, although this difference was not significant. Tschoepe, et al. (1990) also demonstrated that platelet CD61 expression is elevated on diabetic platelets compared to healthy patients, and that this elevation is due to a genetic variation in the *PIA1/A2*-SNP gene (Tschoepe, et al., 2003). No studies were found analyzing CD61 expression in diabetic mice. We found no difference in the expression of platelet CD61 in any of our diet induced diabetic mice and mice fed a standard chow diet. However, mice fed the high fat diet for 4 months demonstrated significantly less platelet CD61 expression after stimulation with calcimycin. Stimulated platelet CD61 expression was positively correlated with the platelet count in these animals. The inability of the HF fed mice to increase CD61 expression after stimulation may indicate an inherent platelet dysfunction that could potentially explain the lower whole blood aggregation and prolonged bleeding times noted in this group. We found no difference in platelet CD61 expression in the HF group at 6 months, or in the group of mice injected with STZ after stimulation compared to control mice.

The study of platelet function and coagulation in type 2 diabetes is important, in order to explore the potentially harmful changes that occur during this complex disease process. Mice are valuable models of obesity and diabetes, but little is known about their platelet and coagulation function. We conclude that while the high fat fed mouse models may represent most metabolic characteristics of obesity and type 2 diabetes, they do not appear to demonstrate the hypercoaguable state observed in humans. After development of more severe disease with injection of STZ, HF fed mice have some indicators of hypercoagulation, but because of experimental artifact working with mouse platelet *ex vivo*, it is difficult to discern to extent of their aggreatory response to ADP. More studies are needed in these mouse models to fully understand their platelet function, coagulation, and aggregation in response to obesity and diabetes.

## CHAPTER 5

NEUTROPHIL CD11B EXPRESSION AND ROS PRODUCTION IN DIET INDUCED  
OBESE AND DIABETIC MICE

## Abstract

Obesity and type 2 diabetes are characterized by a low level chronic inflammatory state. Total leukocyte counts, predominantly polymorphonuclear (PMNs) leukocytes, are elevated in diabetes. In addition, PMNs demonstrate altered adhesion molecule expression and an increase in reactive oxidative species (ROS) production in diabetes. The mechanisms by which the changes in PMN function and structure and the effects of these processes on stroke and cardiovascular disease, though, are not completely understood. Few studies have been conducted examining PMN function in mice with obesity and type 2 diabetes. The purpose of this study was to examine PMN CD11b expression and ROS production in mice fed normal chow (CF) and in two mouse models of diet induced obesity and diabetes: 1) mice fed a high fat (HF, 60% beef lard) diet for 4 months and 6 months producing a moderate form of type 2 diabetes (groups- CF/HF at 4 months and 6 months) and 2) mice fed a HF diet for 2 months and injected with a low dose of streptozotocin (100mg/kg) producing a more severe form of type 2 diabetes (groups- CF+vehicle, CF+STZ, HF+vehicle, HF+STZ). Mice fed a HF diet in both models became obese, hyperglycemic, hyperinsulinemic ( $p < 0.01$ ), and insulin resistant (HOMA-IR scores  $>5$ ) compared to the CF mice. We found that mice fed a HF diet for 4 months demonstrated a significantly increased total white blood count (WBC) ( $p < 0.05$ ) that decreased after 6 months on the diet. The HF+STZ groups did not demonstrate a difference in WBC counts.

Mice fed a HF diet in either model demonstrated a decrease in the number of circulating PMNs (%WBC) that was inversely correlated with the increase in blood sugar. CD11b positive PMNs were lower in HF mice, but this was only significantly lower after eating the diet for 6 months. The HF mice fed for 4 and 6 months did not demonstrate a difference in PMN CD11b expression. In contrast, after 2 months of a high fat diet, the HF+STZ and HF+vehicle mice demonstrated a significant increase in PMN CD11b expression that was positively correlated with increasing blood sugar ( $p=0.01$ ) and HOMA-IR score ( $p<0.001$ ). Even though all groups in both models had a significant increase in LPS stimulated PMN CD11b expression, the HF fed mice did not demonstrate the same extent of the response compared to CF mice. The decreased expression of LPS stimulated PMN CD11b expression was positively correlated with the number of circulating PMNs (%WBC) ( $p<0.001$ ). The mice fed the HF diet for 6 months demonstrated a significant increase in PMA induced PMN ROS production compared to CF mice. No other groups demonstrated a difference in PMN ROS production. We conclude that mice fed a diet rich in animal fat demonstrated evidence of a PMN mediated low grade inflammatory response, particularly with a more severe diabetic state. The PMNs from diabetic mice, however, did not respond to LPS as robustly as the CF mice. In order to determine the applicability of these models in examining blood activation and ischemic cardiovascular disease, more studies are indicated to fully understand the PMN response to diabetes and obesity in these mouse models.

## Introduction

Obesity and type 2 diabetes are known to be associated with a low grade inflammatory state (Duncan, et al., 2003; Dandona, Aljada, Chaudhuri, & Mohanty, 2004). Studies have related an increase in systemic inflammatory markers (C - reactive protein, IL-6), which are strongly linked to increasing adiposity and overnutrition (Pickup, & Crook, 1998; Xu, et al., 2003), with the development of diabetes in humans (Schmidt, et al., 1999; Duncan, 2003; Hanely, et al., 2004). Several studies have identified that adipose tissue releases several pro-inflammatory mediators, including TNF- $\alpha$  (Hotamisligil, Arner, Caro, Atkinson, & Spiegelman, 1995; Kern, et al., 1995, Dandona, et al., 1998), MCP-1 (Sartipy, & Loskutoff, 2003), and IL-6 (Mohamed-ali, et al., 1997; Vozarova, et al., 2001), that then signal leukocytes, particularly monocytes, to become activated and infiltrate the adipose tissue. In this environment the macrophage/adipose tissue amplifies inflammation, contributing to worsening insulin resistance and eventually leading to type 2 diabetes (Xu, 2003).

The role of other leukocytes, particularly polymorphonuclear leukocytes (PMNs), in the pathogenesis of diabetes is not as clear. PMNs are a key component of the innate immune system and primarily respond to bacterial infection (Maraes, & Downey, 2003). The PMN mediated inflammatory response is a complex process initiated by adhesion of the PMN to vascular endothelial cells, followed by extravasation and migration of the cell to the site of inflammation, and then destruction of the pathogen through phagocytosis and release of reactive oxidative species (ROS) (Roos, van Bruggen, & Meischl, 2003) and proteases (Faurischou, & Borregaard, 2003). In addition to their role in infections,

PMNs participate in acute ischemia-reperfusion injury. PMNs adhere to the microcirculatory endothelium during reperfusion after myocardial (Ritter, & McDonagh, 1997; Ritter, Wilson, Williams, Copeland, & McDonagh, 1995) and cerebral (del Zoppo, Schmid-Schonbein, Mori, Copeland, & Chang, 1991; Ritter, Orozco, Coull, & McDonagh, 2000) ischemia, where they mediate an exaggerated inflammatory response, contributing to the vascular and tissue damage initiated by ischemia. It is known that diabetics experience worse outcomes after ischemic events (Almdal, Scharling, Jensen, & Vestergaard, 2004; Megherbi, et al., 2003); findings from our group suggest that there is a blood contribution, specifically a PMN contribution, to these worse outcomes (Hokama, Ritter, Davis-Gorman, Cimetta, Copeland, & McDonagh, 2000).

The role of PMNs in the chronic inflammatory process observed in type 2 diabetes, though, has not been fully elucidated. Historically, it was believed that decreased PMN function in diabetes mellitus was the cause for the increased incidence in bacterial infection in people with the disease (McManus, Bloodworth, Prihoda, Blodgett, & Pinckard, 2001). Total leukocyte counts and the PMN fraction of the total leukocyte count are known to be higher in type 2 diabetics (Shurtz-Swirski, et al., 2001; Veronelli, et al., 2004, van Oostrom, van Wijk, Sijmonsma, Rabelink, & Cabezas, 2004), but the exact implications of this are not understood. PMNs demonstrate chronic activation in humans with diabetes, exhibiting alterations in adhesion molecule expression (Caimi, et al., 2003), particularly an increase in the expression of the integrin CD11b (van Oostrom, et al., 2004; Advani, Marshall, & Thomas, 2002). The chronic over expression of PMN CD11b in diabetes is implicated in the observed increase in vascular adhesion of PMNs

that leads to vascular endothelial and surrounding tissue damage (Advani, 2002) and exacerbates reperfusion injury after myocardial ischemia (Hokama, Ritter, Davis-Gorman, Cimetta, Copeland, & McDonagh, 2000) . Diabetic PMNs in humans also demonstrate an increased production of ROS (Hokama, et al. 1997, Shurtz-Swirski, et al., 2001; Evans, Goldfine, Maddux, & Grodsky, 2003), that not only damages endothelium and other cells (Brownlee, 2001), but increases DNA oxidative damage in the PMNs themselves (Pitozzi, Giovannelli, Bardini, Rotella, & Dolara, 2003). Mouse models that demonstrate metabolic characteristics of obesity and diabetes are available, but few studies have examined the PMN alterations produced by the obese or diabetic state in these animals.

The *ob/ob* and *db/db* mouse models of obesity and type 2 diabetes demonstrate many metabolic similarities to humans with these disorders, including hyperinsulinemia, hyperglycemia, and obesity (Dubac, 1976; Wyse, & Dulin, 1970). Both models develop obesity and diabetes (only transient in the *ob/ob* mouse) through the lack of functional leptin (*ob/ob*) or its receptor (*db/db*), which causes hyperphagia and subsequent weight gain. However, these mice do not demonstrate similar blood inflammatory processes observed in humans with obesity and diabetes. This discrepancy may be related to a decrease in leptin function. *db/db* and *ob/ob* mice have demonstrated a decrease in macrophage phagocytic activity that was normalized in the *ob/ob* mice after leptin administration (Loffreda, et al., 1998). The *ob/ob* mice have also demonstrated decreased production of TNF- $\alpha$  and IL-6 after injection with LPS compared to controls (Loffreda). In another study, *db/db* and *ob/ob* mice demonstrated inhibited hepatic bacterial clearance

and decreased production of MCP-1 in response to infection with *Listeria monocytogenes* compared to non-diabetic mice. These defects were normalized in *ob/ob* mice, but not *db/db* mice, after injection with leptin (Ikejima, et al., 2005). Leptin is now known to be important in immune system regulation; its receptor is expressed on all leukocytes and is necessary for PMN ROS production (Caldefie-Chezet, Poulin, Tridon, Sion, & Vasson, 2001). The *ob/ob* and *db/db* mice, therefore, may not be the optimal mouse models to explore the contributions of inflammation and vascular disease in obesity and type 2 diabetes.

Another mouse model used to study obesity and type 2 diabetes is the C57BL/6J mouse fed a diet high in animal fat. These mice begin gaining weight after 1 week on a high fat (HF) diet compared to mice fed a standard chow diet and double their weight after 4 months (Surwit, Kuhn, Cochrane, McCubbin, & Feinglos, 1988; Collins, Martin, Surwit, & Robidoux, 2004). The high fat diet produces adiposity primarily in the mesentery and inguinal depots, similar to the metabolic syndrome of abdominal obesity in humans (West, Boozer, Moody, & Atkinson, 1992; Surwit, Feinglos, Rodin, Sutherland, Petro, Opara, et al., 1995). The mice develop hyperglycemia, hyperinsulinemia (Surwit, et al., 1988), and hyperleptinemia (van Heek, M., et al., 1997; Surwit, Petro, Parekh, & Collins, 1997) after 4 months on the diet. The high fat fed C57BL/6J mice also develop hypothalamic leptin resistance (El-Haschimi, Pierroz, Hileman, Bjorbaek, & Flier, 2000), peripheral leptin resistance (Van Heek, Compton, France, Tedesco, Fawzi, Graziano, et al., 1997), hypertension (Mills, Kuhn, Feinglos, & Surwit, 1993), and atherosclerosis (Schreyer, Wilson, & LeBoeuf, 1998) similar to people

with obesity and type 2 diabetes. The extent of hyperglycemia in these mice, though, is not severe, and is more typical of early type 2 diabetes and obesity, in which pancreatic function is still able to overcome insulin resistance.

A model of later stage type 2 diabetes, when the pancreas is no longer able to produce sufficient insulin, has also been described. In this model, C57BL/6J mice are fed a high fat diet (45% beef lard) for a shorter time period (1mo), injected with a dose of streptozotocin (STZ) that is lower than that used to induce type 1 diabetes (100mg/kg), and then fed the high fat diet for another month (Luo, et al., 1998). These mice (HF+STZ) develop a more severe state of diabetes than the mice fed the high fat diet alone. There are few studies, though, that have examined the metabolic characteristics of HF+STZ mice. It is known that they demonstrate significant obesity and hyperglycemia after the STZ injection compared to chow fed, lean mice injected with STZ, signifying the interaction between insulin resistance and decreased pancreatic function (from the STZ injection), similar to humans with long standing type 2 diabetes (Luo).

There are few studies that have examined PMNs in mouse models of diabetes. The aim of this study was therefore, to examine PMN CD11b expression and ROS production in chow fed mice and 2 mouse models of obesity and type 2 diabetes, 1) mice fed a high fat (HF, 60% beef lard) diet for 4 months and 6 months producing a moderate form of type 2 diabetes (groups- CF/HF at 4 months and 6 months) and 2) mice fed a HF diet for 2 months and injected with a low dose of streptozotocin (100mg/kg) producing a more severe form of type 2 diabetes (groups- CF+vehicle, CF+STZ, HF+vehicle, HF+STZ). Our results indicate that mice fed a diet rich in animal fat for 4 and 6 months

with moderate diabetes do not exhibit PMN changes similar to that seen in humans with type 2 diabetes. However, with more severe diabetes (HF + STZ) and shorter time period of eating the diet (HF + vehicle), there was a significant increase in CD11b expression. PMN CD11b expression was not as robustly increased with LPS stimulation in any group fed a HF diet compared to CF mice, and this was significantly correlated with a decrease in PMN counts observed in the obese/diabetic mice. Additional studies need to be conducted in mouse models of diet induced obesity and diabetes to completely understand the blood inflammatory changes, and specifically, the PMN changes that occur under these conditions.

## Materials and Methods

### *Animal Model*

All animal experiments were conducted in accordance with the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* (OLAW, 2002) after IACUC approval of all researchers. All C57BL/6J mice (Jackson Laboratories, Inc., Bar Harbor, MA), were randomized to a standard rodent chow (chow fed-CF), (6% fat, NIH-31 Modified Mouse/Rat sterilizable diet 7013, Harlan Teklad, Madison, WI), or a diet enriched with 60% beef lard (high fat fed-HF) (Research Diets, New Brunswick, New Jersey, diet no. D12492) at 4 weeks of age. Mice were kept on the HF or chow diets for 4 and 6 months (Surwit, Kuhn, Cochrane, McCubbin, & Feinglos, 1988). The second group of mice was injected intraperitoneally with a low dose of streptozotocin (STZ, 100mg/kg) (S0130, Sigma-Aldrich, St. Louis, MO) or vehicle (citric acid, pH 4.0) after eating the HF

or chow diets for 4 weeks (groups- CF+vehicle, CF+STZ, HF+vehicle, HF+STZ). These mice were then fed their respective diets for another 4 weeks (Luo, et al., 1998). Mice were allowed free access to food and water and were housed in a temperature controlled, quiet environment.

#### *Blood Acquisition*

All mice were transported to the laboratory the day prior to blood acquisition and fasted for 4 hours the day of the experiment (0600-1000). Mice were anesthetized in a chamber and then mask ventilated with 5.0% isoflurane (JD Medical Distributing Co., Inc) and 1.0 L/minute oxygen for venipuncture. Approximately, seven hundred  $\mu$ L of blood was withdrawn from the ascending vena cava with a 23-gauge needle/1 mL syringe containing 0.05 mL sodium citrate (S1804, Sigma-Aldrich, St. Louis, MO).

#### *Blood Smear Analysis*

Peripheral blood smears were made for all experiments using standard methods (Brown, 1980). Eight  $\mu$ l anticoagulated blood was smeared across a glass slide (VWR, West Chester, PA, #16004-368), air-dried, and stained with Diff-Quik (Dade Behring, VWR #47733-152). The white blood cell differential count was measured by counting 100 white blood cells on one slide three times, categorizing each cell type, and averaging the counts. The total number of circulating WBC subtypes was calculated as: percent differential count/ 100 X standardized WBC.

#### *Flow Cytometry*

Whole blood (WB) samples were initially diluted 1:1 with Dulbecco's phosphate-buffered saline with 0.2% bovine serum albumin and 0.09% sodium azide (pH 7.4)

(PharMingen Stain Buffer, BD Biosciences PharMingen, San Jose, CA, #554657) and then incubated with 0.5 ug/100ul WB/PBS purified rat anti-mouse CD16/CD32 Fc $\gamma$  III/II receptor blocking monoclonal antibody (PharMingen, Clone 2.4G2) for 15 minutes to decrease non-specific binding of antibodies to leukocyte Fc receptors (BD Biosciences, 2005). All incubations were performed at room temperature to diminish changes in leukocyte surface antigen expression with cooling and rewarming of samples (Forsyth and Levinsky, 1990; Repo, Jansson, and Leirisalo-Repo, 1995). Positive control samples were incubated for 30 minutes with either lipopolysaccharide (LPS, Sigma #2680, diluted in PBS, final concentration 10  $\mu$ g/0.1mL) for CD11b expression (Repo, Jansson, and Leirisalo-Repo, 1995), or with phorbol myristate acetate (PMA, (Sigma # P-148) diluted in dimethyl sulfoxide (Sigma #D-5879), final concentration 16  $\mu$ M) for ROS production (Himmelfarb, Hakim, Holbrook, Leeber, and Ault, 1992; Vowells, et al., 1995; Alvarez-Larran, Toll, Rives, and Estella, 2005). After agonist stimulation, monoclonal antibodies or isotype controls (Becton-Dickenson, San Jose, CA) were added to each sample and incubated for 15 minutes. Twenty  $\mu$ L diluted (1:9 concentration diluted with PharMingen Stain Buffer) peridinin chlorophyll-*a* protein (PerCP)-conjugated rat anti-mouse CD45 (leukocyte common antigen, Ly-5) monoclonal antibody (catalog # 557235, clone 30-F11) was added to all samples for leukocyte detection by the flow cytometer. For CD11b measurement, 20  $\mu$ L (1:99 concentration diluted with PharMingen Stain Buffer) fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD11b (integrin  $\alpha_m$  chain, Mac-1  $\alpha$  chain) monoclonal antibody (catalog # 557396, clone M1/70) and FITC-conjugated rat IgG<sub>2b</sub>,  $\kappa$  monoclonal immunoglobulin isotype control (catalog # 553988, clone A95-1)

were added to their respective samples. For PMN ROS analysis, cells were incubated for 15 minutes with 10  $\mu\text{L}$  2',7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Cat #D399) (diluted with filtered PBS, 80  $\mu\text{M}$  final concentration, stored at  $-80^{\circ}$  Celsius) (Bass, et al., 1983; Himmelfarb, Hakim, Holbrook, Leeber, and Ault, 1992; McDonagh, Hokama, Copeland, and Reynolds, 1997). The CD11b samples were then fixed with 100  $\mu\text{L}$  1% cold paraformaldehyde and the ROS samples were diluted with 100  $\mu\text{L}$  PBS. The samples were placed on ice until data acquisition.

Data was acquired by flow cytometry (FACScalibur, 488 nm argon laser, Becton Dickinson, San Jose, CA) within 3 hours of blood acquisition to decrease cellular changes that occur with time. Calibration of the flow cytometer was performed daily prior to each experiment using Calibrite Beads and FACsComp software (Becton Dickinson). Samples were acquired on the low flow setting to avoid multi-cell triggering of the flow cytometer (Himmelfarb, et al., 1992; Hageberg, and Lyberg, 2000). Acquisition threshold was set on FL3 to capture only CD45 positive events (leukocytes).

FCS Express v3.0 (De Novo Software, Inc., Ontario, Canada) was used for flow cytometry analyses. Spectral compensation was performed for each day's experiments by using negative and positive control samples. A dot plot of linear forward and side scatter properties of FL3 threshold events was used to identify and electronically gate the granulocyte population as demonstrated previously (Hageberg and Lyberg, 2000; Alvarez-Larran, Toll, Rivas, and Estella, 2005; Horn, et al., 2005). CD11b positive PMNs (%CD11b+PMN) and PMN expression of CD11b (median fluorescence, MDFI) for 5000 gated PMNs, after background fluorescence subtraction ( $<10^1$  on a log scale), are reported

for CD11b analyses. Median fluorescence intensity of all 5000 gated PMNs is reported for ROS production.

#### *Plasma Insulin Levels and HOMA-IR Calculation*

Plasma insulin levels in mice fed a HF or chow diet for 4 and 6 months were measured with a mouse ultrasensitive ELISA (American Laboratory Products Company (ALPCO), Windham, NH # 08-10-1150-01) or a Insulin Rat/Mouse ELISA Kit (Linco Research, St. Charles, MO #EZRMI-13K). Both assays were performed per manufacturer protocol using an absorbance microplate reader (SpectraMax M5 Microplate Reader, Molecular Devices, Sunnyvale, CA). HOMA-IR (Homeostasis Model Assessment Insulin Resistance score) was calculated by: fasting blood glucose (mmol/L) X fasting plasma insulin ( $\mu\text{U/mL}$ ) / 22.5 (adapted from Bonora, et al., 2002).

#### *Metabolic Characteristic Measurements*

Weight (gms) was monitored weekly and prior to each experiment (Triple Beam Balance, Ohaus, Columbia, MD). Blood sugar was measured with a glucometer (AccuCheck Advantage, Roche, Inc., Mannheim, Germany) that was tested monthly for reliability using high and low controls (Roche). White blood cell count, hematocrit, and platelet counts were measured by a bench top whole blood analyzer (Coulter A<sup>c</sup>T™ 5 diff Hematology Analyzer, Beckman Coulter, Fullerton, CA), which was tested daily for reliability with high, low, and normal control samples (Beckman Coulter). Triglyceride and total cholesterol levels were measured by the Trinder's method using reflective photometry (CardioChek, model #542165, Polymer Technologies Systems (PTS), Inc.,

Indianapolis, IN). The CardioChek unit was tested for monthly for reliability with high and low controls (PTS, Inc.).

### *Statistical Analysis*

Data were expressed as mean  $\pm$  SEM. Groups were compared using unpaired t-test (*t*), Mann-Whitney Rank Sum Test (*T*) (non-parametric data), ANOVA (*F*) or Kruskal-Wallis ANOVA on Ranks (*H*) (non-parametric data). ANOVA post hoc testing was done with either Holm-Sidak or Dunn's Method (non-parametric data). Within group comparisons were analyzed with paired t- tests (*t*) or Wilcoxon Signed Rank Test (*T*) (non-parametric data). Correlations were performed using linear regression and the data expressed with the *r* correlation coefficient. The flow cytometry experiments were performed in duplicate and the coefficient of variation determined by dividing the standard deviation by the mean. All experiments with a variation of >10% were removed from analysis or noted in the results section. SigmaStat for Windows version 3.10 (Systat Software, Inc., Point Richmond, CA) was used for statistical analyses. An a priori  $\alpha$  of  $p \leq 0.05$  was considered statistically significant.

## Results

### *High Fat and Chow Fed Mice for 4 and 6 Months*

#### *Metabolic characteristics*

After eating a HF diet for 4 and 6 months, mice were significantly obese (4 mo- chow fed (CF) ( $n= 4$ )  $32.7 \pm 0.5$ , HF ( $n= 5$ )  $47.2 \pm 1.3$  gm,  $T= 10.00$ ,  $p= 0.02$ ; 6 mo- CF ( $n= 5$ )  $34.8 \pm 1.4$ , HF ( $n= 4$ )  $53.2 \pm 0.6$  gm,  $T= 30.00$ ,  $p= 0.02$ ). The mice fed the HF diet

for 4 months were hyperglycemic, but this increase in blood sugar returned to normal levels after eating the diet for 6 months (4 mo- CF  $144.6 \pm 28.3$ , HF  $230.5 \pm 8.0$  mg/dl,  $t(7) = -3.25$ ,  $p = 0.01$ ; 6 mo- CF  $153.1 \pm 16.3$ , HF  $180.4 \pm 3.8$  mg/dl,  $T = 25.00$ ,  $p = 0.29$ , Table 5.1). Both groups of HF mice were significantly hyperinsulinemic (4 mo- CF ( $n = 3$ )  $20.6 \pm 3.0$ , HF ( $n = 4$ )  $116.6 \pm 38.8$  pM,  $T = 6.00$ ,  $p = 0.06$ ; 6 mo- CF ( $n = 3$ )  $101.6 \pm 43.4$ , HF ( $n = 2$ )  $718.0 \pm 116.0$  pM,  $t(3) = -5.99$ ,  $p < 0.01$ ), but not significantly insulin resistant compared to CF mice (HOMA-IR scores- 4mo-CF ( $n = 3$ )  $0.9 \pm 0.2$ , HF ( $n = 4$ )  $8.8 \pm 2.6$ ,  $T = 6.00$ ,  $p = 0.06$ ; 6mo- CF ( $n = 3$ )  $4.7 \pm 1.5$ , HF ( $n = 2$ )  $44.9 \pm 7.0$ ,  $T = 9.0$ ,  $p = 0.20$ ), although the HOMA-IR of the HF mice scores were consistent with insulin resistance. The insulin results in this set of experiments were calculated from a standard curve that was below accepted limits ( $r < 0.90$ ) and should be noted with caution. Weight was significantly correlated with blood sugar ( $r = 0.52$ ,  $p = 0.03$ , Figure 5.1A) and insulin levels ( $r = 0.69$ ,  $p = 0.01$ , Figure 5.1B). The mice fed the HF diet were also hypercholesterolemic (4 mo-CF  $< 100.0$ , HF  $161.0 \pm 9.8$  mg/dl,  $T = 10.00$ ,  $p = 0.02$ ; 6mo- CF  $< 100$ , HF  $219.0 \pm 10.7$  mg/dl,  $T = 30.00$ ,  $p = 0.02$ ), but not hypertriglyceridemic compared to CF mice (4mo- CF  $58.8 \pm 1.2$ , HF  $56.6 \pm 1.8$  mg/dl,  $t(7) = 0.93$ ,  $p = 0.39$ ; 6mo- CF  $50.2 \pm 0.2$ , HF  $50.3 \pm 0.3$  mg/dl,  $T = 20.50$ ,  $p = 0.91$ , Table 5.1).

Table 5.1 Metabolic Characteristics of Mice Fed a Standard Chow or High Fat Diet for 4 and 6 Months

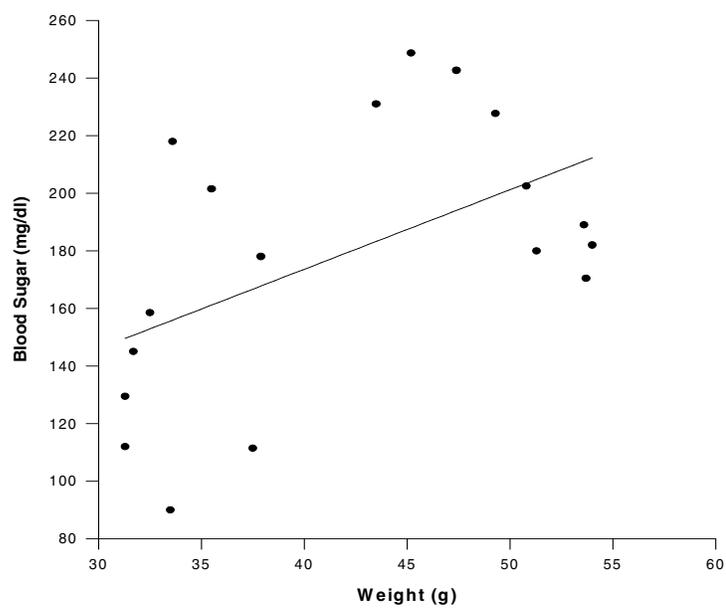
Strain (n) <sup>^</sup>	Weight (g)	Blood Sugar (mg/dl)	Triglycerides (mg/dl)	Total Cholesterol (mg/dl)	Plasma Insulin (pM)	HOMA- IR Score <sup>^</sup>
CF x 4mo (4)	32.7 ± 0.5	144.6 ± 28.3	58.8 ± 1.2	100.0 ± 0.0	20.6 ± 3.0	0.9 ± 0.2
HF x 4mo (5)	47.2 ± 1.3*	230.5 ± 8.0*	56.6 ± 1.8	161.0 ± 9.8*	116.6 ± 38.8	8.8 ± 2.6
CF x 6mo (5)	34.8 ± 1.4	153.1 ± 16.3	50.2 ± 0.2	100.0 ± 0.0	101.6 ± 43.4	4.7 ± 1.5
HF x 6mo (4)	53.2 ± 0.6*	180.4 ± 3.8	50.3 ± 0.3	219.0 ± 10.7*	718.0 ± 116.0***	44.9 ± 7.0

CF-Chow fed, HF-High fat fed, HOMA-IR- Homeostasis Model Assessment Insulin Resistance Score. All measurements except weight were made after 4 hour fast. Data presented as mean ± SEM. <sup>^</sup>See results text for (n) of each measurement. \*significantly different from CF mice, \* $p < 0.05$ , \*\*\* $p < 0.001$ .

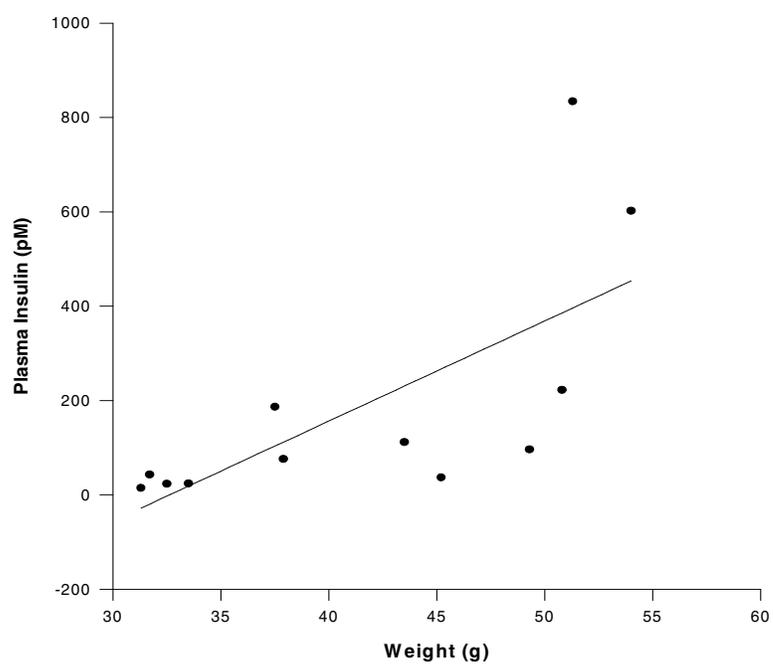
Figure 5.1 Blood sugar and insulin correlate with weight and PMN leukocyte counts (%WBC) in mice fed a standard chow or high fat diet for 4 and 6 months. Correlation of (A) weight and blood sugar ( $r= 0.52, p= 0.03$ ), (B), weight and insulin ( $r= 0.69, p= 0.01$ ), and (C) blood sugar and PMNs (%WBC) ( $-r= 0.68, p= 0.02$ ).

Figure 5.1

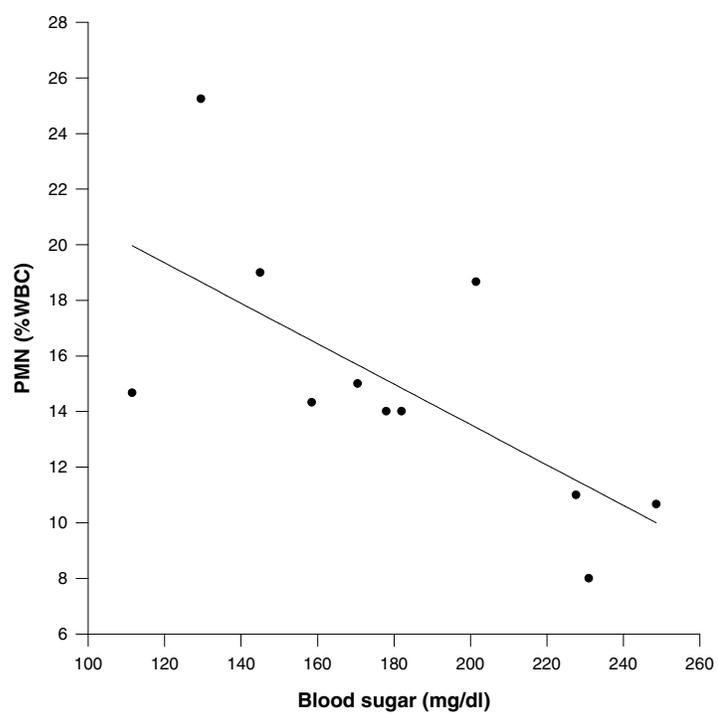
A.



B.



C.



*Whole blood cell counts*

Whole blood counts were standardized to a hematocrit of 45% to eliminate the effects of anticoagulant dilution. The standardized white blood count (WBC) was significantly higher in the HF fed mice after 4 months (CF ( $n=4$ )  $4.1 \pm 1.1$ , HF ( $n=5$ )  $7.7 \pm 0.8$   $10^3/\mu\text{l}$ ,  $t(6)=-2.81$ ,  $p=0.03$ ), but not after 6 months compared to CF mice (CF  $7.6 \pm 0.5$ , HF  $6.0 \pm 0.5$   $10^3/\mu\text{l}$ ,  $t(7)=2.27$ ,  $p=0.06$ , Table 5.2). There was no significant difference in WBC differential between HF and CF mice; PMNs (4mo- CF ( $n=1$ ) 14.3, HF ( $n=3$ )  $9.9 \pm 1.0\%$  (%WBC); 6 mo- CF ( $n=5$ )  $18.3 \pm 2.0$ , HF ( $n=2$ )  $14.5 \pm 0.5\%$ ,  $t(5)=1.13$ ,  $p=0.31$ ), lymphocytes (4mo- CF 85.3, HF  $88.4 \pm 1.3\%$ ; 6 mo- CF  $79.8 \pm 1.8$ , HF  $83.2 \pm 0.8\%$ ,  $t(5)=-1.09$ ,  $p=0.32$ ), or monocytes (4mo- CF 0.3, HF  $1.8 \pm 0.5\%$ ; 6 mo- CF  $2.3 \pm 0.6$ , HF  $2.3 \pm 1.3\%$ ,  $t(5)=0.01$ ,  $p=0.99$ , Table 5.2). Even though there was no difference between groups, the PMN fraction of the WBC (%WBC) was inversely correlated with blood sugar ( $-r=0.68$ ,  $p=0.02$ , Figure 5.1C). The circulating numbers of PMNs (4mo- CF ( $n=1$ ) 0.8, HF ( $n=3$ )  $0.8 \pm 0.1$   $10^3/\mu\text{l}$ ; 6 mo- CF ( $n=5$ )  $1.4 \pm 0.1$ , HF ( $n=2$ )  $0.8 \pm 0.1$   $10^3/\mu\text{l}$ ,  $t(5)=2.45$ ,  $p=0.06$ ), lymphocytes (4mo- CF 4.7, HF  $7.4 \pm 0.6$   $10^3/\mu\text{l}$ ; 6 mo- CF  $6.1 \pm 0.5$ , HF  $4.6 \pm 0.3$   $10^3/\mu\text{l}$ ,  $t(5)=1.81$ ,  $p=0.13$ ), and monocytes (4mo- CF 0.0, HF  $0.1 \pm 0.0$   $10^3/\mu\text{l}$ ; 6 mo- CF  $0.2 \pm 0.0$ , HF  $0.1 \pm 0.1$   $10^3/\mu\text{l}$ ,  $t(5)=0.64$ ,  $p=0.55$ , Table 5.2) were no different between groups.

Table 5.2 White Blood Cell (WBC) Count and WBC differential of Mice Fed a Standard Chow or High Fat Diet for 4 and 6 Months

Strain (n) <sup>^</sup>	WBC count (10 <sup>3</sup> /μl)	PMN (%)	Lymph (%)	Monocyte (%)	PMN (10 <sup>3</sup> /μl)	Lymph (10 <sup>3</sup> /μl)	Monocyte (10 <sup>3</sup> /μl)
CF x 4mo (4)	4.1 ± 1.1	14.3 ± 0.0	85.3 ± 0.0	0.3 ± 0.0	0.8 ± 0.0	4.7 ± 0.0	0.0 ± 0.0
HF x 4mo (5)	7.7 ± 0.8*	9.9 ± 1.0	88.4 ± 1.3	1.8 ± 0.5	0.8 ± 0.1	7.4 ± 0.6	0.1 ± 0.0
CF x 6mo (5)	7.6 ± 0.5	18.3 ± 2.0	79.8 ± 1.8	2.3 ± 0.6	1.4 ± 0.1	6.1 ± 0.5	0.2 ± 0.0
HF x 6mo (4)	6.0 ± 0.5	14.5 ± 0.5	83.2 ± 0.8	2.3 ± 1.3	0.8 ± 0.1	4.6 ± 0.3	0.1 ± 0.1

CF-Chow fed, HF-High fat fed, WBC-White Blood Count, PMN-polymorphonuclear leukocyte. Differential counts are % of total white blood count. Absolute differential counts were calculated by %differential count / 100 X standardized WBC. WBC counts were standardized to a hematocrit of 45%. <sup>^</sup>See results text for (n) of each measurement. Data presented as mean ± SEM. \*significantly different from CF mice, \**p* < 0.05.

#### Flow cytometry

*CD11b positive PMNs.* The percentage of CD11b positive PMNs was non-significantly lower in the HF mice than CF at 4 months (CF (n=4) 55.9 ± 12.7, HF (n=5) 29.2 ± 2.1 % CD11b+ PMNs, *T* = 27.00, *p* = 0.11), and was significantly lower at 6 months (CF (n=5) 38.4 ± 3.2, HF (n=4) 29.4 ± 0.7 % CD11b+ PMNs, *t*(7) = -2.43, *p* < 0.05). The percentage of CD11b positive PMNs did not increase with LPS stimulation in any group (paired t-test, 4 mo- CF *t*(3) = -2.91, *p* = 0.06, HF *t*(4) = -0.05, *p* = 0.96; 6 mo- CF *t*(4) = 0.12, *p* = 0.91, HF *t*(3) = -1.30, *p* = 0.29). After stimulation, there was significantly less CD11b positive PMNs at 4 months between HF and CF mice (CF 58.5 ± 11.9, HF 29.1 ± 2.7

%CD11b+PMNs,  $T= 29.00$ ,  $p= 0.03$ ), but not at 6 months (CF  $38.0 \pm 3.0$ , HF  $31.5 \pm 1.2$  %CD11b+PMNs,  $t(7)= -1.80$ ,  $p= 0.12$ , Figure 5.2A).

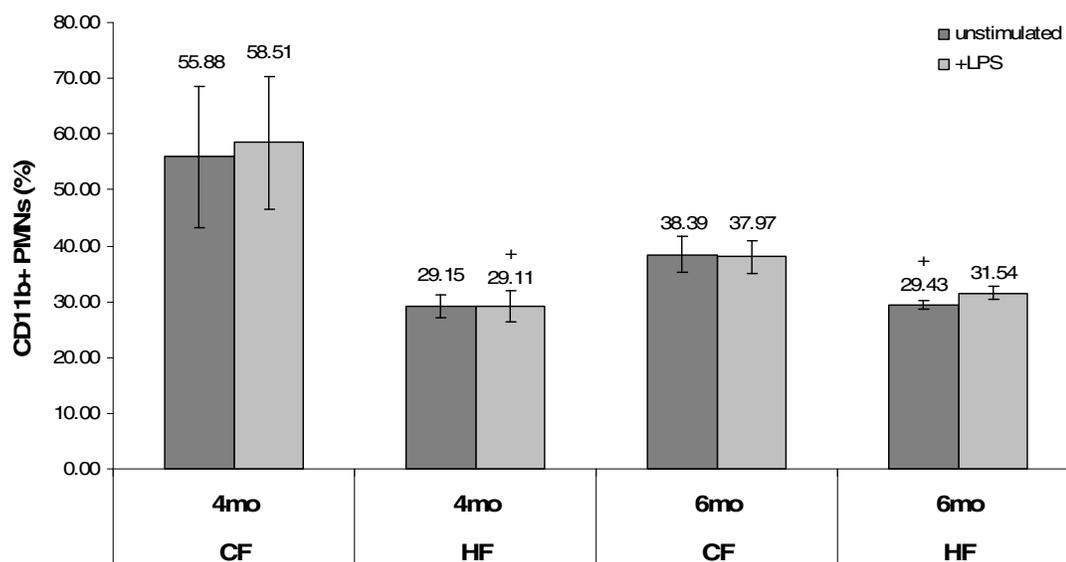
*PMN CD11b expression.* The PMN expression of CD11b was no different in HF or CF mice at 4 months (CF  $36.1 \pm 6.4$ , HF  $36.0 \pm 1.7$  median fluorescence intensity (MDFI),  $t(7)= -0.01$ ,  $p= 0.99$ ) or 6 months (CF  $26.2 \pm 2.0$ , HF  $27.8 \pm 3.9$  MDFI,  $t(7)= 0.39$ ,  $p= 0.71$ ). All groups demonstrated a significant increase in PMN CD11b expression after stimulation with LPS (4 mo- CF  $t(3)= -8.12$ ,  $p< 0.01$ , HF  $t(4)= -6.04$ ,  $p< 0.01$ ; 6 mo- CF  $t(4)= -12.20$ ,  $p< 0.001$ , HF  $t(3)= -9.36$ ,  $p= 0.99$ ). After stimulation, there was a significant decrease in PMN CD11b expression between HF and CF mice at 4 months (CF  $103.0 \pm 13.0$ , HF  $71.4 \pm 6.7$  MDFI,  $t(7)= -2.32$ ,  $p= 0.05$ , Figure 5.2B) that was inversely correlated with an increase in blood sugar ( $-r= 0.85$ ,  $p< 0.01$ , Figure 5.2C) and HOMA-IR score ( $-r= 0.80$ ,  $p= 0.03$ , Figure 5.2D). There was no difference in PMN CD11b expression at 6 months (CF  $92.8 \pm 6.7$ , HF  $85.6 \pm 6.8$  MDFI,  $t(7)= -.075$ ,  $p= 0.48$ ).

*PMN ROS production.* There was no difference in baseline ROS production between the HF and CF mice at 4 and 6 months (4 mo- CF  $7.5 \pm 1.4$  HF  $5.3 \pm 0.9$  MDFI,  $t(7)= -1.39$ ,  $p=0.21$ ; 6 mo- CF  $20.3 \pm 4.1$ , HF  $26.7 \pm 6.3$  MDFI,  $t(7)= 0.89$ ,  $p= 0.40$ , Figure 5.3). There was a significant increase in PMA stimulated PMN ROS production in both CF and HF mice at 6 months, but not those fed for 4 months (paired t-test- 4 mo- CF  $t(2)= -2.58$ ,  $p= 0.12$ , HF  $t(4)= -2.05$ ,  $p= 0.11$ ; 6 mo- CF  $t(4)= -7.38$ ,  $p< 0.01$ , HF  $t(3)= -7.11$ ,  $p< 0.01$ ). HF mice demonstrated significantly more PMN ROS production after stimulation at 6 months, but not at 4 months (4 mo- CF  $23.1 \pm 8.0$ , HF  $18.9 \pm 7.4$  MDFI,  $T= 24.00$ ,  $p= 0.41$ ; 6 mo- CF  $200.0 \pm 26.5$ , HF  $346.2 \pm 45.7$  MDFI,  $t(7)= 2.92$ ,  $p= 0.02$ , Figure 5.3).

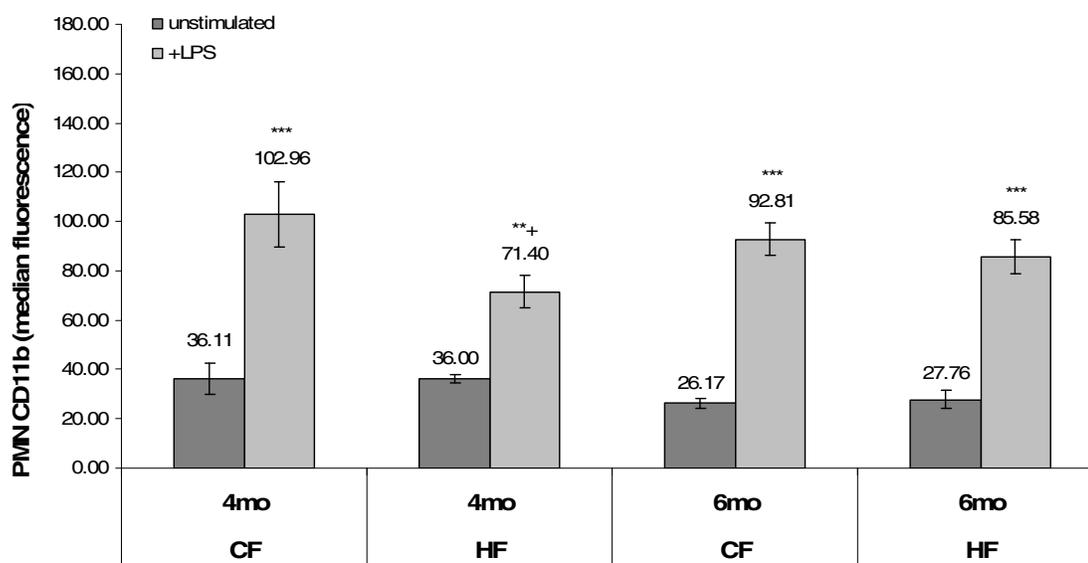
Figure 5.2 PMN CD11b expression in mice fed a standard chow or high fat diet for 4 and 6 months. (A) CD11b positive PMNs (% CD11b+ PMNs) (B) PMN CD11b expression (median fluorescence intensity (MDFI) in arbitrary units). Data presented as mean  $\pm$  SEM. Correlation of (C) blood sugar ( $-r= 0.85$ ,  $p< 0.01$ ) and (D) HOMA-IR ( $-r= 0.80$ ,  $p= 0.03$ ) score on LPS stimulated PMN CD11b expression. \*significantly different from unstimulated sample,  $*p< 0.05$ ,  $**p< 0.01$ ,  $*** p< 0.001$ ; +significantly different from CF mice  $+p< 0.05$ .

Figure 5.2

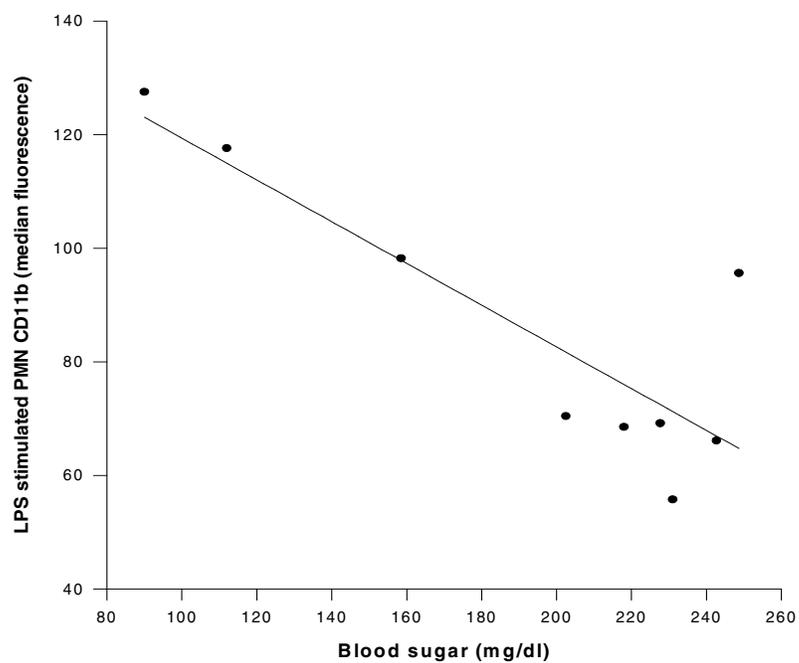
A.



B.



C.



D.

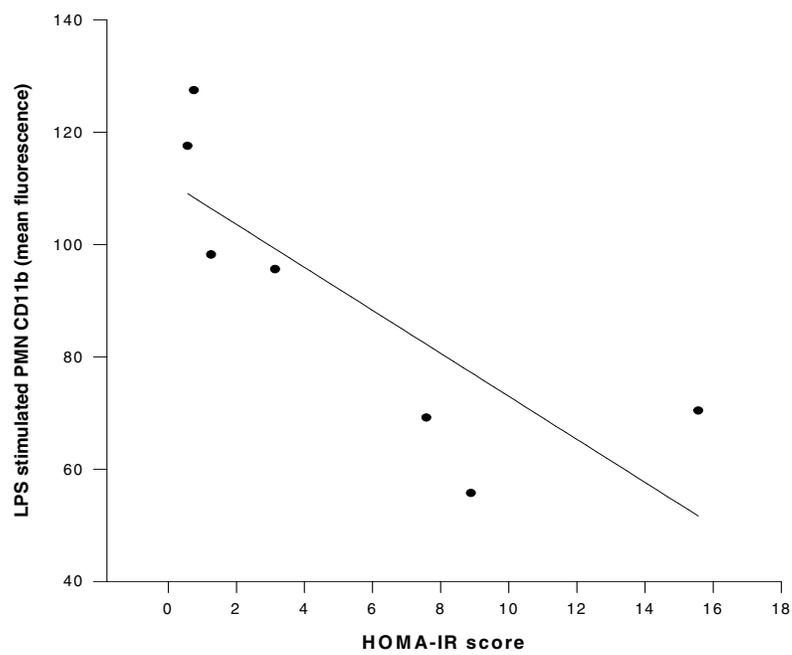
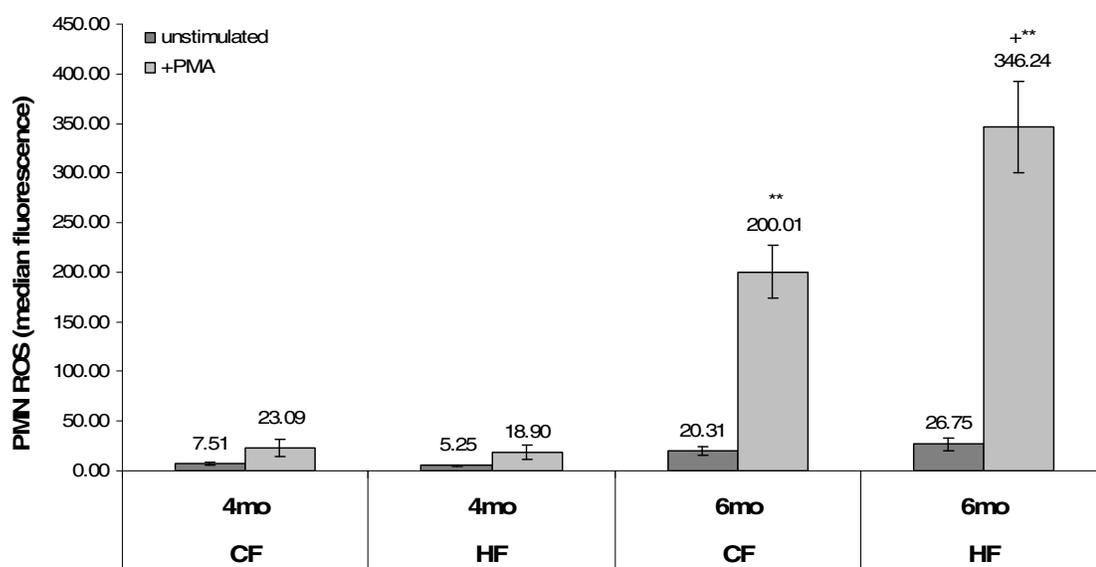


Figure 5.3 PMN ROS production in mice fed a standard chow or high fat diet for 4 and 6 months. (MDFI, in arbitrary units). Data presented as mean  $\pm$  SEM. \*significantly different from unstimulated sample, \*\* $p < 0.01$ , +significantly different from CF mice + $p < 0.05$ .

Figure 5.3



*High Fat and Chow Fed Mice Injected with STZ*

*Metabolic characteristics*

Mice fed a HF diet for 2 months and injected with vehicle or with low dose STZ were significantly obese compared to mice fed a standard chow diet (CF +vehicle ( $n= 5$ )  $24.2 \pm 1.2$ , CF+STZ ( $n= 5$ )  $22.5 \pm 1.0$ , HF +vehicle ( $n= 5$ )  $30.5 \pm 0.8$ , HF +STZ ( $n= 4$ )  $35.2 \pm 1.6$ ,  $F(3,15)= 26.03$ ,  $p < 0.001$ , Table 5.3). Only the HF+STZ were significantly hyperglycemic (CF+vehicle  $217.1 \pm 5.3$ , CF+STZ  $208.9 \pm 13.3$ , HF+vehicle  $224.6 \pm 8.2$ , HF+STZ  $384.3 \pm 26.0$ ,  $F(3,15)= 32.83$ ,  $p < 0.001$ ). There was no difference in plasma insulin levels (CF+vehicle ( $n= 3$ )  $77.1 \pm 34.8$ , CF+STZ ( $n= 4$ )  $29.5 \pm 6.9$ , HF +vehicle ( $n= 8$ )  $174.3 \pm 50.7$ , HF+STZ ( $n= 4$ )  $76.9 \pm 17.1$  pM,  $H= 6.34$ ,  $df= 3$ ,  $p = 0.10$ ) or in the HOMA-insulin resistance score between HF and CF mice (CF+vehicle ( $n= 2$ )  $3.5 \pm 1.8$ , CF+STZ ( $n= 2$ )  $1.3 \pm 0.2$ , HF+vehicle ( $n= 5$ )  $7.9 \pm 2.7$ , HF+STZ ( $n= 3$ )  $11.3 \pm 1.2$ ,  $F(3,8)= 2.40$ ,  $p= 0.14$ ), although the mice fed the HF diet have HOMA scores consistent with insulin resistance. The weight of these mice was significantly correlated with blood sugar ( $r= 0.80$ ,  $p < 0.001$ , Figure 5.4A). The HF mice with or without the STZ injection were not significantly hypercholesterolemic (CF+vehicle ( $n= 3$ )  $104.0 \pm 4.0$ , CF+STZ ( $n= 5$ )  $103.6 \pm 2.4$ , HF+vehicle ( $n= 5$ )  $158.2 \pm 14.7$ , HF+STZ ( $n= 4$ )  $204.0 \pm 13.7$ ,  $H= 11.93$ ,  $df= 3$ ,  $p < 0.01$ , no significant post hoc comparisons  $p > 0.05$ ), and were not hypertriglyceridemic compared to chow fed mice (CF+vehicle  $59.7 \pm 1.5$ , CF+STZ  $62.0 \pm 2.5$ , HF+vehicle  $73.8 \pm 11.3$ , HF+STZ  $72.5 \pm 5.5$ ,  $H= 6.78$ ,  $df= 3$ ,  $p= 0.08$ , Table 5.3).

Table 5.3 Metabolic Characteristics of Mice Fed Standard Chow or a High Fat Diet for 2 Months With or Without a Low Dose STZ Injection.

Strain (n) <sup>^</sup>	Weight (g)	Blood Sugar (mg/dl)	Trig. (mg/dl)	Total Cholesterol (mg/dl)	Plasma Insulin (pM) <sup>^</sup>	HOMA-IR Score
CF+vehicle (5)	24.2± 1.2	217.1± 5.3	59.7± 1.5	104.0 ± 4.0	77.1 ± 34.8	3.5 ± 1.8
CF+STZ (5)	22.5± 1.0	208.9± 13.3	62.0 ± 2.5	103.6 ± 2.4	29.5 ± 6.9	1.3 ± 0.2
HF+vehicle (5)	30.5± 0.8**†††	224.6 ± 8.2	73.8 ± 11.3	158.2 ± 14.7	174.3 ± 50.7	7.9 ± 2.7
HF+STZ (4)	35.2 ± 1.6*** †††#	384.3 ± 26.0*** †††###	72.5 ± 5.5	204.0 ± 13.7	76.9 ± 17.1	11.3 ± 1.2

CF-Chow fed, HF-High fat, Trig- Triglycerides, HOMA- IR- Homeostasis Model Assessment Insulin Resistance Score. All measurements except weight were made after 4 hour fast. Data presented as mean ± SEM. <sup>^</sup>See results text for (n) of each measurement. \*significantly different from CF+vehicle mice, \*\*p< 0.01, \*\*\*p< 0.001, †significantly different from CF+STZ mice, †††p< 0.001, # significantly different from HF+vehicle mice # p< 0.05, ### p< 0.001.

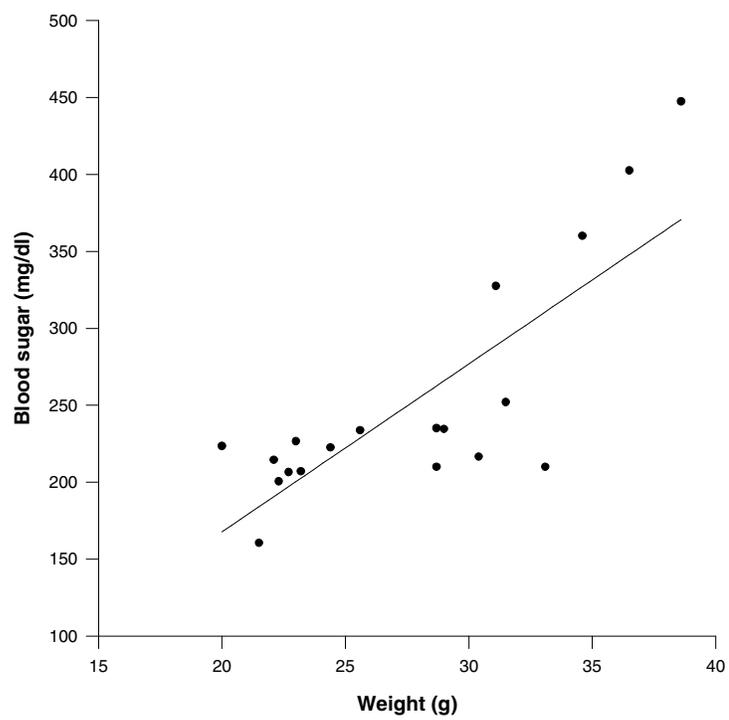
#### Whole blood cell counts

The whole blood counts in these experiments were also standardized to a hematocrit of 45% to eliminate the effects of anticoagulant dilution. The standardized white blood count (WBC) was no different between CF and HF mice (CF+vehicle (n= 3) 3.4 ± 0.6, CF+STZ (n= 5) 5.0 ± 0.8, HF+vehicle (n= 5) 5.5 ± 0.9, HF+STZ (n= 4) 5.3 ± 0.8 10<sup>3</sup>/μl, F(3,13)= 1.03, p= 0.41).

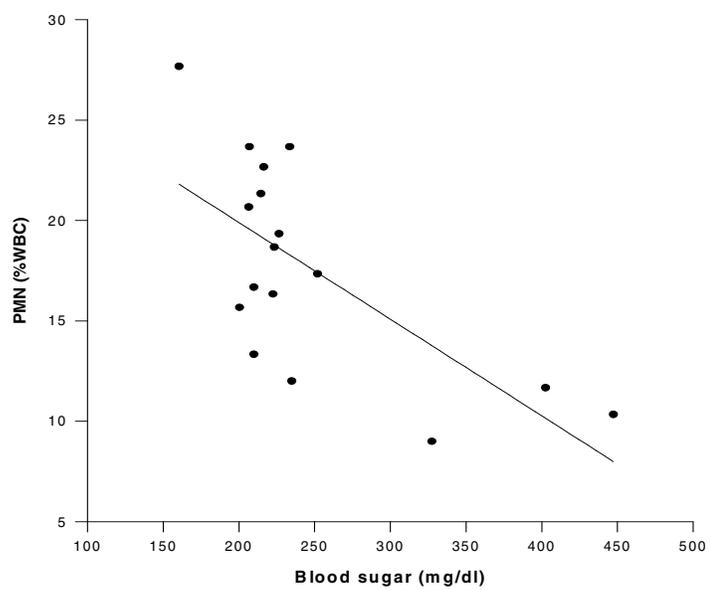
Figure 5.4 Blood sugar correlates with weight and PMN leukocyte counts (%WBC) in mice fed a standard chow or high fat diet with or without a low dose STZ injection. Correlation of (A) weight and blood sugar ( $r= 0.80, p< 0.001$ ), and (B) blood sugar and PMN (%WBC) ( $-r= 0.69, p< 0.01$ ).

Figure 5.4

A.



B.



In the WBC differential analysis, HF+STZ mice demonstrated a significant decrease in the percentage of PMNs compared to CF+STZ (CF+vehicle ( $n=5$ )  $18.8 \pm 2.1$ , CF+STZ ( $n=5$ )  $21.0 \pm 2.1$ , HF+vehicle ( $n=4$ )  $17.5 \pm 1.9$ , HF+STZ ( $n=3$ )  $10.3 \pm 0.8\%$  (%WBC),  $F(3,13)=4.35$ ,  $p=0.03$ ), and an increase in lymphocytes (CF+vehicle  $79.2 \pm 2.4$ , CF+STZ  $77.5 \pm 2.2$ , HF+vehicle  $80.5 \pm 2.0$ , HF+STZ  $88.0 \pm 1.2\%$ ,  $F(3,13)=3.61$ ,  $p=0.04$ ), but no difference in monocytes (CF+vehicle  $2.0 \pm 0.4$ , CF+STZ  $1.7 \pm 0.3$ , HF+vehicle  $2.0 \pm 0.2$ , HF+STZ  $1.7 \pm 0.4$ ,  $F(3,13)=0.26$ ,  $p=0.85$ , Table 5.4). There was a significant correlation between blood sugar and the decrease in PMN count (%WBC) ( $-r=0.69$ ,  $p<0.01$ , Figure 5.4B). Even though the percentage of PMNs per 100 WBC was lower, there was no significant difference in circulating numbers of PMNs (CF+vehicle ( $n=3$ )  $0.6 \pm 0.0$ , CF+STZ ( $n=5$ )  $1.0 \pm 0.2$ , HF+vehicle ( $n=4$ )  $1.1 \pm 0.2$ , HF+STZ ( $n=3$ )  $0.5 \pm 0.1$   $10^3/\mu\text{l}$ ,  $F(3,11)=2.33$ ,  $p=0.13$ ), lymphocytes (CF+vehicle  $2.8 \pm 0.7$ , CF+STZ  $3.9 \pm 0.7$ , HF+vehicle  $5.0 \pm 0.5$ , HF+STZ  $4.6 \pm 1.0$   $10^3/\mu\text{l}$ ,  $F(3,11)=1.69$ ,  $p=0.23$ ), or monocytes (CF+vehicle  $0.1 \pm 0.0$ , CF+STZ  $0.1 \pm 0.0$ , HF+vehicle  $0.1 \pm 0.1$ , HF+STZ  $0.1 \pm 0.0$   $10^3/\mu\text{l}$ ,  $F(3,11)=1.52$ ,  $p=0.26$ , Table 5.4) within any group.

Table 5.4 White Blood Cell (WBC) Counts and WBC Differential of Mice Fed Standard Chow or a High Fat Diet for 2 Months With or Without a Low Dose STZ Injection

Strain (n)	WBC count (10 <sup>3</sup> /μl)	PMN (%)	Lymph (%)	Monocyte (%)	PMN (10 <sup>3</sup> /μl)	Lymph (10 <sup>3</sup> /μl)	Monocyte (10 <sup>3</sup> /μl)
CF+vehicle (5)	3.4± 0.6	18.8 ± 2.1	79.2 ± 2.4	2.0 ± 0.4	0.6 ± 0.0	2.8 ± 0.7	0.5 ± 0.0
CF+STZ (5)	5.0 ± 0.8	21.0 ± 2.1	77.5 ± 2.2	1.7 ± 0.3	1.0 ± 0.2	3.9 ± 0.7	0.1 ± 0.0
HF+vehicle (5)	5.5 ± 0.9	17.5 ± 1.9	80.5 ± 2.0	2.0 ± 0.2	1.1 ± 0.2	5.0 ± 0.5	0.1 ± 0.0
HF+STZ (4)	5.3 ± 0.8	10.3 ± 0.8***	88.0 ± 1.2***	1.7 ± 0.4	0.5 ± 0.1	4.6 ± 1.0	0.1 ± 0.0

CF-Chow fed, HF-High fat fed, STZ- streptozotocin, PMN-polymorphonuclear leukocyte. Differential counts are % of total white blood count. Absolute differential counts were calculated by %differential count / 100 X standardized WBC. WBC counts were standardized to a hematocrit of 45%. Data presented as mean ± SEM. \*significantly different from CF mice, \*\*\*p< 0.001.

#### Flow cytometry

*CD11b positive PMNs.* There was no difference in the percentage of CD11b positive PMNs in the HF and CF groups (CF+vehicle (n= 5) 39.0 ± 4.9, CF+STZ (n= 5) 49.4 ± 7.0, HF+vehicle (n= 5) 44.1 ± 4.7, HF+STZ (n= 4) 39.1 ± 6.0% CD11b+PMNs,  $F(3,15)= 0.75, p= 0.54$ ) or after LPS stimulation (CF+vehicle (n= 3) 55.6 ± 5.8, CF+STZ (n= 4) 54.7 ± 6.0, HF+vehicle (n= 5) 45.9 ± 4.7, HF+STZ (n= 4) 50.4 ± 13.1% CD11b+PMNs,  $F(3,12)=0.32, p= 0.81$ ). No group demonstrated an increase in the percentage of CD11b positive PMNs after LPS stimulation (paired t-tests, CF+vehicle  $t(3)=$

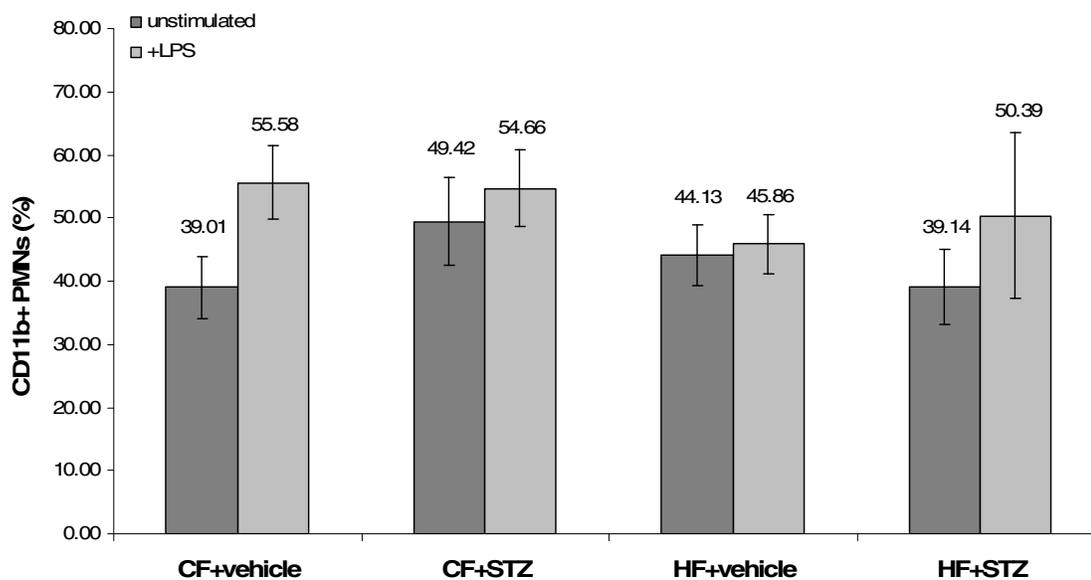
-0.62,  $p= 0.58$ , CF+STZ  $t(3)= -2.06$ ,  $p= 0.13$ , HF+vehicle  $t(4)= -1.13$ ,  $p= 0.32$ , HF+STZ  $t(3)= -0.62$ ,  $p= 0.58$ , Figure 5.5A).

*PMN CD11b expression.* The PMN expression of CD11b was significantly increased in the HF+vehicle and HF+STZ compared to CF mice (CF+vehicle  $16.0 \pm 1.3$ , CF+STZ  $17.8 \pm 0.8$ , HF+vehicle  $20.8 \pm 1.4$ , HF+STZ  $23.6 \pm 1.3$  MDFI,  $F(3,15)= 98$ ,  $p< 0.01$ , Figure 5.5B). The increase in CD11b expression was positively correlated with blood sugar ( $r= 0.56$ ,  $p= 0.01$ , Figure 5.5C) and HOMA-IR scores ( $r= 0.84$ ,  $p< 0.001$ , Figure 5.5D). There was no significant difference in LPS stimulated CD11b expression between HF and CF mice (CF+vehicle  $96.2 \pm 19.9$ , CF+STZ  $135.7 \pm 24.6$ , HF+vehicle  $114.1 \pm 6.0$ , HF+STZ  $63.6 \pm 22.9$  MDFI,  $F(3,12)= 2.70$ ,  $p= 0.09$ ). Only the CF and HF groups injected with the vehicle demonstrated a significant increase in PMN CD11b expression after stimulation with LPS (paired t-tests, CF+vehicle  $t(2)= -4.51$ ,  $p< 0.05$ , CF+STZ  $T= 10.00$ ,  $p= 0.13$ , HF+vehicle  $t(4)= -16.12$ ,  $p< 0.001$ , HF+STZ  $t(3)= -1.76$ ,  $p= 0.18$ ). The HF+vehicle and HF+STZ groups had less LPS induced PMN CD11b expression than CF mice, and this observation was inversely correlated with blood sugar ( $r= 0.60$ ,  $p= 0.02$ , Figure 5.5E) and positively correlated with a decrease in PMN% ( $r= 0.78$ ,  $p< 0.001$ , Figure 5.5F).

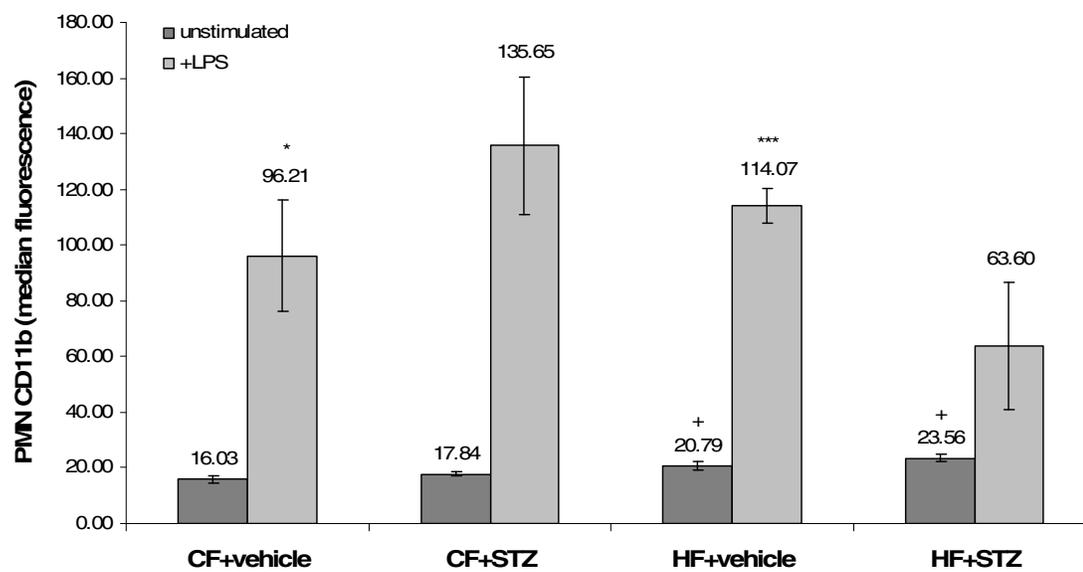
Figure 5.5 PMN CD11b expression in mice fed a standard chow or high fat diet with or without a low dose STZ injection. (A) CD11b positive PMNs (% CD11b+ PMNs) (B) PMN CD11b expression (MDFI). Correlation of (C) blood sugar ( $r= 0.56, p= 0.01$ ), and (D) HOMA-IR ( $r= 0.84, p< 0.001$ ) score on PMN CD11b expression. Correlation of (E) blood sugar ( $-r= 0.60, p= 0.02$ ) and (F) PMN (%WBC) ( $r= 0.78, p< 0.001$ ) on LPS stimulated PMN CD11b expression. \*significantly different from unstimulated sample, \* $p< 0.05$ , \*\* $p< 0.01$ , \*\*\*  $p< 0.001$ ; +significantly different from CF mice + $p< 0.05$ .

Figure 5.5

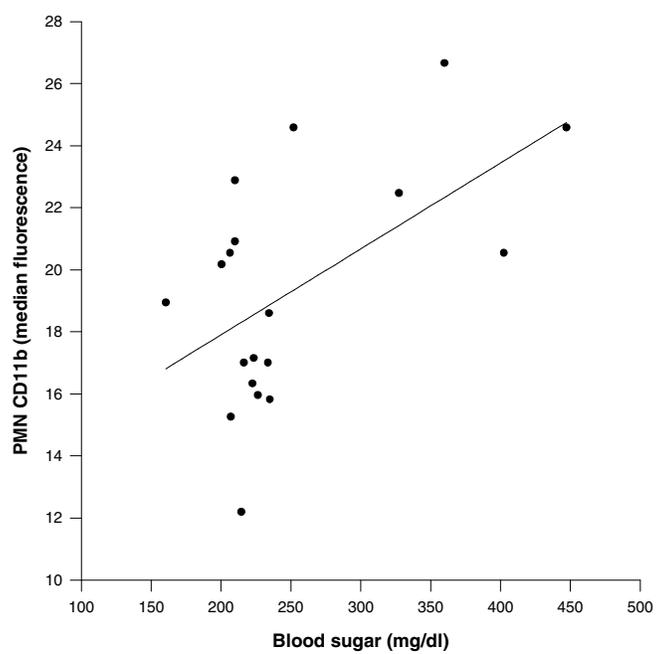
A.



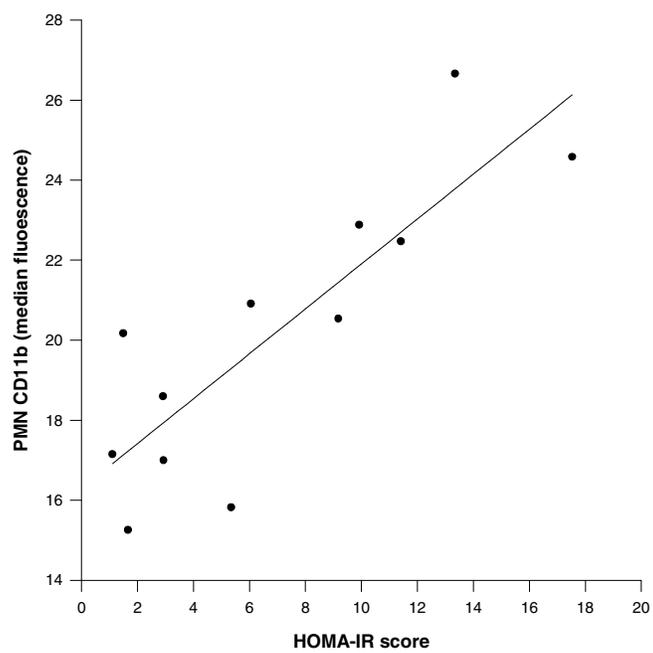
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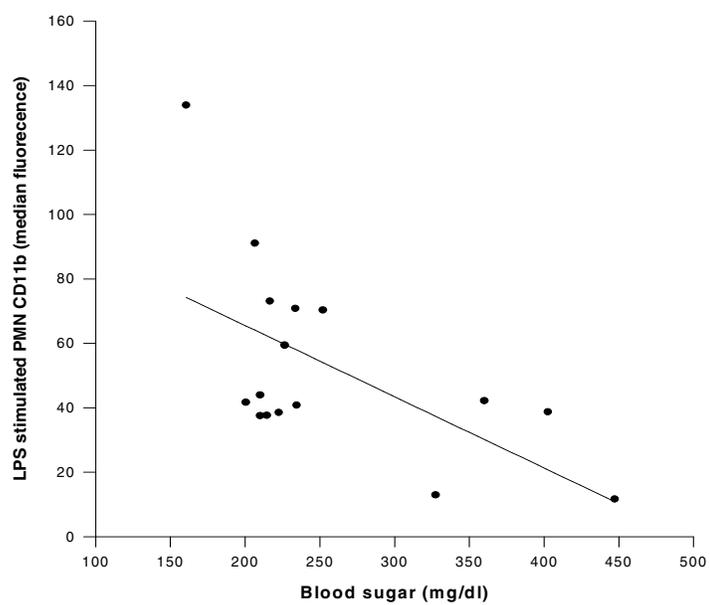
C.



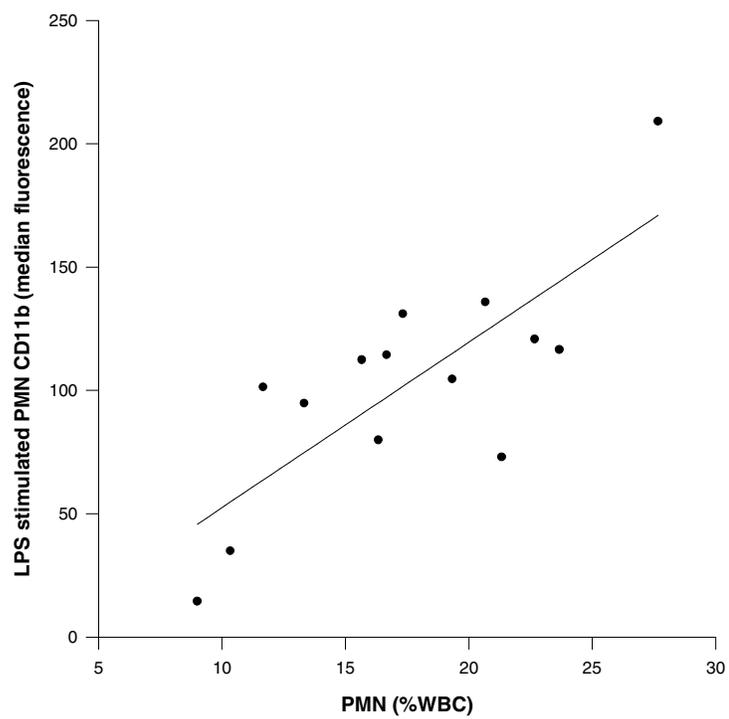
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E.



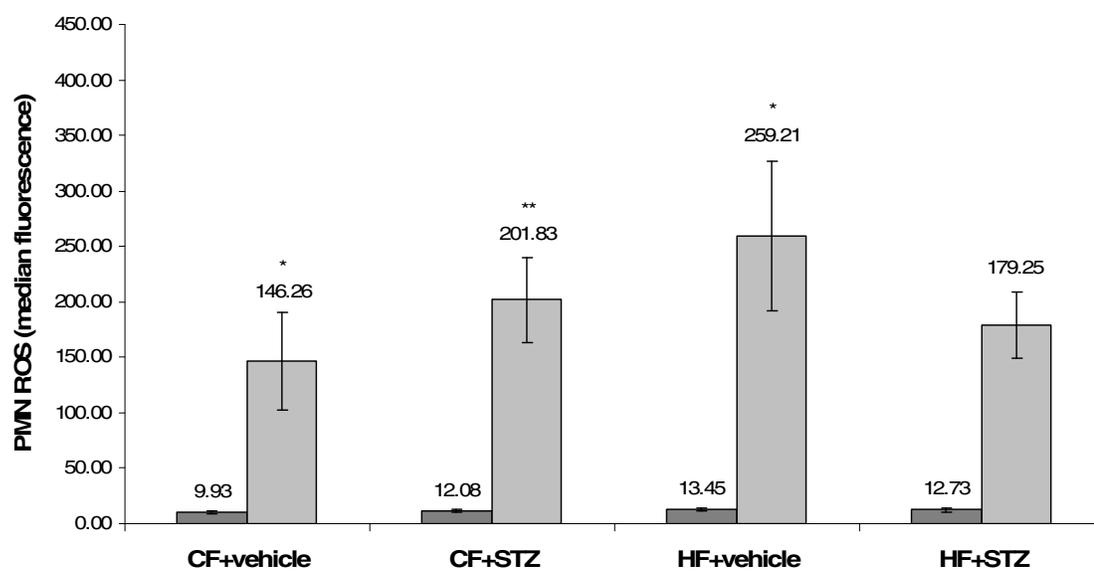
F.



*PMN ROS production.* PMN ROS production was no different in any group before (CF+vehicle  $9.9 \pm 1.4$ , CF+STZ  $12.1 \pm 1.5$ , HF+vehicle  $13.5 \pm 1.2$ , HF+STZ  $12.7 \pm 1.8$  MDFI,  $F(3,15)= 1.14$ ,  $p= 0.37$ ) or after PMA stimulation (CF+vehicle  $146.3 \pm 44.0$ , CF+STZ  $201.8 \pm 38.4$ , HF+vehicle  $259.2 \pm 67.3$ , HF+STZ  $179.2 \pm 30.1$  MDFI,  $F(3,15)= 0.99$ ,  $p= 0.42$ ). All groups except the HF+STZ group demonstrated a significant increase ROS production with PMA stimulation (paired t-test- CF+vehicle  $t(4)= -3.16$ ,  $p= 0.03$ , CF+STZ  $t(4)= -5.12$ ,  $p< 0.01$ , HF+vehicle  $t(4)= -3.70$ ,  $p= 0.02$ , HF+STZ  $t(3)= -5.81$ ,  $p= 0.37$ , Figure 5.6).

Figure 5.6 PMN ROS production in mice fed a standard chow or high fat diet with or without a low dose STZ injection. (MDFI, in arbitrary units). Data presented as mean  $\pm$  *SEM*. \*significantly different from unstimulated sample, \* $p < 0.05$ , \*\* $p < 0.01$ .

Figure 5.6



## Discussion

Systemic inflammation is known to be predictive of developing type 2 diabetes (Schmidt, et al., 1999) and increases the risk of death from vascular disease. The inflammatory state appears to begin with increasing adiposity that attracts infiltration of monocytes. The macrophage/adipose complex is known to secrete several inflammatory cytokines (Xu, et al., 2003), which in turn signal other immune cells, including leukocytes, to become activated. The activation of leukocytes in diabetes leads to worsened reperfusion injury after myocardial infarction (Hokama, et al., 2000) and leads to increased complications such as nephropathy (Chung, Tsai, Chang, Shin, & Lee, 2005). The complete mechanisms underlying the complex relationship of the immune system and obesity and diabetes, particularly the role of leukocytes and PMNs, are not understood. Animal models that reflect the metabolic disturbances in diabetes are necessary to further explore the relationships between inflammation and vascular disease in the setting of diabetes. Therefore, the aim of our study was to examine PMN activation, specifically, PMN CD11b expression and ROS production, in 2 mouse models of diet induced obesity and type 2 diabetes.

Few studies have examined the contribution of blood cell activation as a marker of chronic inflammation in mouse models of obesity or diabetes. In the C57BL6/J mouse fed a high fat diet, studies have found that increased mesenteric adiposity in obese and diabetic mice leads to an infiltration of monocytes/macrophages to the adipose tissue (Kanda, et al., 2006; Yu, Kim, Kwon, & Kawada, 2006; Chen, et al., 2005), increasing plasma MCP-1 (Takahashi, et al., 2003; Chen, 2005), adipocyte MCP-1 (Kanda, 2006; Yu, 2006; Chen,

2005), and adipocyte TNF-  $\alpha$  (Mito, Hosoda, Kato, & Sato, 2000). Adipocyte ICAM-1, IL-6, and MCP-1 mRNA are also increased in high fat fed mice after only 3 weeks on the diet (Brake, Smith, Mersmann, Smith, & Robker, 2006). The infiltration of monocytes in adipose tissue is mediated through increasing levels of monocyte CD11b expression in response to increased levels of MCP-1 (Takahashi, 2003). In addition, after myocardial infarction, mice fed a high fat diet have demonstrated an increase in myocardial MCP-1 and IL-6 expression and monocyte infiltration compared to chow fed mice (Thakker, et al., 2006). Atherosclerotic plaque development, however, was not mediated by leukocyte CD11b in a mouse model of severe atherosclerosis (Kubo, et al., 2000). Together these studies indicate that diet induced obesity and diabetes in mice produces a low level inflammatory state, primarily in adipocytes and monocytes, which affects increased organ damage after insult (myocardial infarction), similar to that seen in humans with diabetes. No studies, however, were found that examined the role of PMN leukocytes in this mouse model of obesity and type 2 diabetes.

The role of PMNs in the pathogenesis of diabetes is not clear. Previous work demonstrated that PMN function was impaired in the setting of diabetes, and this was believed to be in part why diabetics are more susceptible to and have more severe infections (McManus, et al., 2001). PMNs in diabetics have been observed to have decreased endothelial adhesion molecule expression and phagocytosis (Alba-Loureiro, Hirabara, Mendonca, Curi, & Curi, 2006). In contrast, other studies have demonstrated that PMNs in type 2 diabetics appear to demonstrate a low level chronic activation that leads to increased vascular adhesion and tissue damage (van Oostrom, et al., 2004; Shurtz-Swirski,

et al., 2001). Insulin is known to be a regulator of PMN function, including increasing surface expression of CD11b, enhancing chemotaxis, and phagocytosis (Walrand, Buillet, Boirie, & Vasson), but the effect of insulin resistance on PMNs has not been studied. The questions raised by the inconsistency of these research findings indicates the need for further research on the role of PMNs in the setting of obesity and diabetes.

Elevated total leukocyte (white blood cell (WBC)) counts are known to be a marker of chronic inflammation. In humans, elevated total leukocyte counts are predictive of developing the metabolic syndrome (Lohsoonthorn, Dhanamum, & Williams, 2006) and type 2 diabetes (Ohshita, et al., 2004; Schmidt, et al., 1999); are associated with an increased risk of stroke (Ohira, et al., 2006), endothelial dysfunction (Elkind, et al., 2005), and atherosclerosis (Elkind, et al., 2001), and are associated with more severe diabetic complications, such as nephropathy, (Chung, Tsai, Chang, Shin, & Lee, 2005). Crevel (1992) did not find any difference in total or differential WBC counts between high fat and chow fed mice. No other report of WBC counts was found in diet induced obese/diabetic mice. In our study, we found that mice fed the HF diet for 4 months demonstrated an increase in the total WBC compared to CF mice, consistent with reports from diabetic patients. This is different from the study by Crevel and colleagues, (1992), but the difference could be due to the type of fat used in the study. Crevel (1992), used diets enriched with plant fatty acids (coconut oil) and these mice were not obese compared to chow fed mice. In contrast, we used a diet enriched with animal fat (beef lard) which is known to cause obesity, and may cause the other metabolic changes that occur with obesity and diabetes. The HF+STZ mice demonstrated WBC counts that were higher than chow

fed mice, although this did not reach statistical significance. These mice, though, were only diabetic for one month and may have demonstrated an increase in WBC with prolonged exposure to the disease. In contrast, the mice fed the diet for 6 months, demonstrated a lower WBC than CF mice. Many of these mice developed skin lesions, lost weight, were no longer hyperglycemic, and demonstrated an increase in mortality (unpublished observations). In addition, PMN ROS production was increased in these mice, which may have caused increased damage and destruction of PMN and other leukocytes (Shurtz-Swirski, et al., 2001; Pitozzi, et al., 2003). Taken together, the changes in this group may have resulted in a decrease in the total leukocyte number.

The balance of lymphocytes and neutrophils in mice and humans is quite different. Mice are normally lymphocyte rich and have decreased PMN leukocyte counts compared to humans, (mice-75-90% lymphocytes, 10-25% PMNs, humans-30-50% lymphocytes, 50-70% PMNs) and it is not known if the difference in total PMNs results in functional differences between the two species (Mestas, & Hughes, 2004). The PMN fraction of the WBC differential is increased in diabetic patients (van Oostrom, et al., 2004; Shurtz-Swirski, et al., 2001), but the physiologic implication of the increase in circulating PMNs is not clear. No studies were found that reported PMN counts in obese and diabetic mice. A study of myocardial infarction by Thakker, et al., (2006) found a decrease in myocardial PMN infiltration during reperfusion after ischemia in HF fed mice compared to chow fed mice, demonstrating a decrease in PMN activity in diet induced obese and diabetic mice. In this study, we found that mice fed a high fat diet demonstrated a decrease in the percentage of circulating PMNs. Interestingly, the decrease in PMNs was associated with

more severe diabetes and was correlated with increasing blood sugar and insulin resistance levels. In addition, the decrease in PMNs was also correlated with a less robust LPS stimulated PMN CD11b expression compared to chow fed mice, demonstrating that the PMNs that are present are not able to maximize their response to a stimulus. These results are quite different from studies in human diabetics and probably demonstrate a difference in species PMN response to obesity and diabetes.

The integrin CD11b is the  $\alpha$  chain portion of the leukocyte  $\beta$ 2-integrin MAC-1 (CD11b/CD18 complex). CD11b is expressed primarily on PMNs and monocytes and on a subset of activated CD8 T cells and NK cells (Mazzone, & Ricevuti). In PMNs the CD11b integrin is expressed on the surface membrane in low numbers, and is primarily stored in peroxidase negative granules and secretory vesicles that rapidly bring the integrin to the surface in response to inflammatory signals (Fauschou, & Borregaard, 2003). The Mac-1 complex is also involved in other inflammatory and thrombotic processes, and is a receptor for complement factor 3bi, fibrinogen, and other inflammatory mediators (such as LPS) (Mazzone, & Ricevuti). Several studies have identified that PMN CD11b expression is amplified in diabetic patients (van Oostrom, et al., 2004; Advani, Marshall, & Thomas, 2002; Senior, Marshall, & Thomas, 1999) and type 2 diabetic rats (Zucker Diabetic Fatty, ZDF) (Miller, et al., 2005). Studies in diet induced obese and diabetic mice demonstrate that monocyte CD11b is increased, leading to increased infiltration of monocytes in to the adipose tissue (Takakashi, et al., 2003). The elevated expression of PMN CD11b is implicated as a mechanism for the increase in vascular damage associated with diabetes, as it increases vascular adhesion of PMNs and their subsequent release of toxic mediators.

Our results are consistent with previous studies. We found that PMN CD11b expression was increased with more severe diabetes (HF+STZ) and this increased expression was correlated with increasing insulin resistance and blood sugar levels. However, PMN CD11b expression in response to a stimulus, LPS, was lower in the HF mice, with or without STZ. These results are similar to a study by McManus, et al. (2001), who demonstrated that PMNs from type 2 diabetics produced less lysosomal enzyme secretion after stimulation with both fMLP and platelet activating factor that was significantly correlated with the extent of hyperglycemia in these patients. They propose that diabetic PMNs may have agonist selective decreased responsiveness that contributes to patient's increase risk of infection. Our results may reflect this observation, and indicate that mice with diet induced diabetes are at risk of infection. Consistent with this hypothesis, several of our mice fed the HF diet for longer than 5 months developed skin lesions and died.

In addition to increasing surface expression of CD11b during phagocytosis, PMNs increase their oxygen consumption through activity of NAPDH-oxidase (located in the cell surface and granule membranes), which produces superoxide anion and hydrogen peroxide (Dahlgren, & Karlsson, 1999). These reactive oxidative species then destroy bacterial invaders, but may also damage and destroy surrounding tissue (Dahlgren, & Karlsson). The increase in ROS production by PMNs is hypothesized to be, in part, what leads to the increased organ injury in diabetic patients and during reperfusion. Previous studies have demonstrated that in humans, PMNs have an increase in ROS production leading to an increased overall oxidative state and increased self-necrosis of the PMNs (Shurtz-Swirski, 2001). Another study by Pitozzi and colleagues (2003), demonstrated that PMNs were

more susceptible to DNA damage from ROS than other leukocytes in diabetic patients compared to non-diabetics. Additionally, our group has demonstrated an increase in PMN ROS production in both diabetic patients (Hokama, et al. 1997) and type 2 diabetic ZDF rats (Miller, et al., 2005). We also previously found that in type 1 diabetic rats PMN ROS production was increased compared to non-diabetics (McDonagh, Hokama, Copeland, & Reynolds, 1997). A more recent study in type 1 diabetic rats, however, demonstrated a decrease in PMN phagocytosis and PMA stimulated ROS production compared to nondiabetic rats (Alba-Loureiro, Hirabara, Mendonca, Curi, & Curi, 2006). In the current study, we found no difference in PMN ROS production in mice fed a HF diet for 4 months or after STZ injection. With prolonged exposure to the diet, though, the mice fed a HF diet for 6 months demonstrated a significant increase in PMA stimulated ROS production similar to previous reports.

The role of PMN leukocytes in the pathogenesis of obesity and type 2 diabetes is not well understood. In this study we investigated the PMN response to obesity and type 2 diabetes in 2 mouse models. We were interested in examining the PMN response in mice with both moderate and severe type 2 diabetes, to observe changes with disease progression. The diet induced obese and diabetic mouse, which is fed a high fat diet for 4 months, has been well characterized and is used as a model of obesity and diabetes seen in humans with moderate blood sugar levels. Typically, people receive treatment before they become severely diabetic, and this mouse model has been utilized extensively because it closely represents the human condition. There have been no studies examining the effects of a more severe obese and diabetic state in mice. Our findings indicate that diet induced

obesity and diabetes in mice produces different PMN activation characteristics than in humans. Diet induced obese and diabetic mice demonstrated some evidence of a PMN mediated low grade inflammatory response, particularly with a more severe diabetic state. The PMNs from diabetic mice, however, did not respond to LPS as robustly as the CF mice, which appears to be related to the decreased number of circulating PMNs and with increasing severity of diabetes and insulin resistance. Our results indicate that mice fed a high fat diet and injected with a low dose STZ more closely resemble the inflammatory state of humans with obesity and type 2 diabetes. More studies are clearly indicated to completely understand the PMN response to obesity and diabetes in mice to determine the applicability of studying the cellular mechanisms of vascular and other complications of human disease in animal models.

## CHAPTER 6

ELEVATION OF PLATELET AND PMN MARKERS OF ACTIVATION AFTER  
ISCHEMIC STROKE AND REPERFUSION IN MICE

## Abstract

Hypercoaguability and inflammation are implicated in the development and pathogenesis of stroke. Obesity and type 2 diabetes are known independent risk factors for acute ischemic stroke and cardiovascular disease and are associated with higher morbidity and mortality from stroke compared to non-obese, non-diabetic patients. The role of thrombosis and inflammation in the development and subsequent injury from stroke and reperfusion in the setting of diabetes, though, has not been clearly elucidated. The purpose of our study was to examine activation of platelets and polymorphonuclear leukocytes (PMNs) in peripheral blood after experimental stroke and reperfusion in mice fed a standard chow (CF) and a high fat diet (60% beef lard, a model of obesity and type 2 diabetes) for 6 months. We measured total and platelet derived microparticles, platelet microparticle and platelet p-selectin expression, platelet CD61 expression, and PMN CD11b expression and ROS production after one hour middle cerebral artery occlusion (MCAO) and 24 hour reperfusion and non-surgical and SHAM surgical controls. There was a high mortality rate associated with the MCAO procedure in all groups. All diabetic mice died either intraoperatively or during reperfusion. The surviving non-obese, non-diabetic mice in 3 groups were used for analysis, 1) non-surgical controls ( $n=5$ ), 2) surgical SHAMs ( $n=2$ ), and 3) after MCAO and reperfusion ( $n=2$ ). We found that PMP P-selectin expression was significantly increased after MCAO and reperfusion compared

to non-surgical controls ( $p < 0.01$ ), but not compared to surgical SHAM animals. The expression of platelet CD61 was no different between groups, but after agonist stimulation, the mice that underwent MCAO and reperfusion and surgical SHAM animals demonstrated significantly less expression than non-surgical controls ( $p < 0.01$ ). Additionally, total WBC counts were significantly lower after MCAO and SHAM surgery and reperfusion ( $p < 0.05$ ) compared to non-surgical controls, but no different than surgical SHAMS. The number of circulating PMNs, however, was significantly increased after MCAO and 24 hour reperfusion compared to both non-surgical controls ( $p < 0.001$ ) and SHAMs ( $p < 0.05$ ). The percentage of PMNs expressing CD11b was also significantly increased after MCAO and reperfusion compared to both SHAM ( $p < 0.001$ ) and non-surgical control animals ( $p < 0.001$ ), although the PMN expression of CD11b and PMN ROS production were no different between groups. In summary, we were unable to compare the changes in peripheral platelet and PMN activation markers between non-obese, non-diabetic (chow fed) and obese, diabetic mice because of the high mortality rate. In the chow fed mice, we did find evidence of activated peripheral thrombotic and inflammatory processes, but because of the small number of experiments we were unable to determine the extent of these changes. Further experiments are clearly indicated to completely understand the changes in peripheral platelet and PMN activation in the setting of obesity and diabetes during cerebral ischemia and reperfusion.

### Introduction

Cerebral ischemia, or stroke, continues to be the third leading cause of death and a major cause of disability in the developed world (Thom, et al., 2006). Hypercoagulability

and inflammation are known to be implicated in the development and pathogenesis of stroke. Hypercoagulable states, such as genetic thrombophilias, atrial fibrillation, and other coagulation defects are known risk factors for stroke (Levine, 2005). Additionally, people with chronic inflammation, evidenced by elevated white blood cell counts (WBC) (Noto, et al., 2001; Ohira, Shahar, Chambless, Rosamond, Mosley, & Folsom, 2006) and elevated circulating levels of C-reactive protein (Rost, et al., 2001) are at an increased risk to develop stroke. Accordingly, pro-thrombotic, pro-inflammatory conditions, such as obesity and type 2 diabetes, are also known independent risk factors for acute ischemic stroke and cardiovascular disease (Lee, et al., 2001; Adachi, Hirai, Tsuruta, Fujiura, & Imaizumi, 2001, Hu, et al., 2002; Kernan, et al., 2003; Folsom, et al., 1999) and are associated with higher morbidity and mortality from stroke compared to non-obese, non-diabetic patients (Almdal, Scharling, Jensen, & Vestergaard, 2004; Megherbi, et al., 2003). The role of thrombosis and inflammation in the development and subsequent injury from stroke and reperfusion in the setting of diabetes, though, has not been clearly elucidated.

Previous studies have established that local cerebral tissue damage during ischemia is due to changes in cellular metabolism secondary to a lack of oxygen and glucose that leads to generation of reactive oxidation species, death of ischemic tissue (Dirnagl, Iadecola, & Moskowitz, 1999), and local inflammation brought about by return of platelets and leukocytes during reperfusion (del Zoppo, Becker, & Hallenbeck, 2001; Wang, Chen, Yang, & Zhou, 2004). Rolling and adhesion of leukocytes, particularly PMNs, initiate inflammation during ischemia by adhering to vascular endothelium and

transmigrating into neural tissue (Ritter, Orozco, Coull, & McDonagh, 2000). Activated platelets and PMNs accumulate in cerebral vessels (Ishikawa, Arumugam, Zhang, Nanda, & Granger, 2004) and occlude the cerebral microvasculature leading to the 'no-reflow' phenomenon seen after clot lysis (del Zoppo, et al., 1991). This process exacerbates neuronal and endothelial damage by further release of ROS and continued activation of inflammatory cells (del Zoppo, 2001).

Although the mechanisms of local ischemic cell death and inflammatory processes are well known in cerebral ischemia, few studies have examined the response of peripheral blood thrombotic and inflammatory cells after cerebral ischemia and reperfusion. Human platelets have demonstrated increased expression of platelet P-selectin (CD62P) (McCabe, et al., 2004) after stroke and during reperfusion. These activated platelets adhere to leukocytes forming circulating platelet-monocyte (McCabe, 2004) and platelet-leukocyte conjugates (Ritter, Stempel, Coull, & McDonagh, 2005). In addition, patients demonstrate elevated white blood counts after stroke, specifically an increase in circulating polymorphonuclear leukocytes (PMNs) (Beamer, Coull, Clark, Briley, Wynn, & Sexton, 1998; Suzuki, Kelley, Reyes-Iglesias, Alfonso, & Dietrich, 1995, McCabe, et al., 2004) and monocytes (McCabe, 2004). Several proinflammatory cytokines have also been demonstrated to be increased after experimental stroke (Offner, Subramanian, Parker, Afentoulis, Vandenbark, & Hurn, 2005). Few other studies have examined changes in the peripheral thrombotic and inflammatory pathways that occur after stroke and reperfusion.

Subjects with diabetes have an earlier onset and more severe strokes than those without diabetes (Idris, Thompson, & Sharma, 2006), but the mechanisms contributing to

this are not clearly understood. Previous research has clearly demonstrated that obesity and type 2 diabetes in humans are associated with a state of chronic platelet activation and hyperaggregability (Schneider, 2005) and chronic low grade inflammation (Tataranni, & Ortega, 2005). Few studies, however, have examined the effects of thrombosis and inflammation on cerebral ischemia and reperfusion injury in type 2 diabetes. Type 1 diabetic rats have demonstrated an increased level of oxidation in the cerebral microvasculature that exacerbated cerebral and reperfusion injury (Wei, Huang, & Quast, 1997). Additionally, during reperfusion, leukocytes have demonstrated increased adhesion in type 1 diabetic rat mesentery (Salas, et al., 1998) and coronary circulations (Hokama, et al., 1997). No studies were found examining the relationship of platelet and PMN activation in type 2 diabetes and stroke and reperfusion injury.

The purpose of this study was to examine to peripheral activation of platelets and PMNs after experimental stroke (middle cerebral artery occlusion, MCAO) and 24 hours reperfusion in a diet induced mouse model of obesity and type 2 diabetes. Unfortunately, there was a high level of mortality from the MCAO procedure. All of the diabetic mice and 40% of the chow fed mice died intra- or postoperatively. While the low number of experiments limits the conclusions that can be drawn from this study, we found that PMP P-selectin expression was significantly increased and that agonist stimulated platelet CD61 was decreased after ischemic stroke and reperfusion. Additionally, total WBC counts were significantly lower after stroke and reperfusion; however the number of circulating PMNs and the percentage of PMNs expressing CD11b were significantly

increased after stroke and reperfusion. There was no difference in PMN expression of CD11b or ROS production after stroke and reperfusion compared to non-stroke animals. Taken together, our results suggest that some level of platelet and PMN activation occurs in the peripheral circulation, but more experiments are needed to fully appreciate the changes that occur after stroke and reperfusion.

## Materials and Methods

### *Animal Model*

All animal experiments were conducted in accordance with the *Public Health Service Policy on Humane Care and Use of Laboratory Animals* (OLAW, 2002) after IACUC approval of all researchers. C57BL/6J mice (Jackson Laboratories, Inc., Bar Harbor, MA), were randomized to a standard rodent chow (chow fed-CF), (6% fat, NIH-31 Modified Mouse/Rat sterilizable diet 7013, Harlan Teklad, Madison, WI), or a diet enriched with 60% beef lard (high fat fed-HF) (Research Diets, New Brunswick, New Jersey, diet no. D12492) at 4 weeks of age and kept on their respective diet for 6 months (Surwit, Kuhn, Cochrane, McCubbin, & Feinglos, 1988). Mice were allowed free access to food and water and were housed in a temperature controlled, quiet environment.

### *Middle Cerebral Artery Occlusion and Reperfusion*

Induction anesthesia was obtained by placing the mice in a chamber with 1.0 L/min O<sub>2</sub> and 3.5 % isoflurane (Halocarbon Laboratories, River Edge, NJ) for 3 minutes. Mice were then mask ventilated with 2.5%-2.0% isoflurane and a mixture of 70% N<sub>2</sub>O/30% O<sub>2</sub> for the remaining surgery. Body temperature was monitored with a rectal probe

(TCAT-2AC controller, Physitemp Instruments Inc., Clifton, NJ) and maintained with a heating pad (TP500, Gaymar Industries, Inc., Orchard Park, NY) and heat lamp throughout the surgical procedure. To measure cerebral blood flow, adhesive (Ross Products, Inc., Columbus OH) was used to fix a flexible laser Doppler probe (Periflux system 5000, PeriMed, Inc. Järfälla, Sweden) to the skull externally over a superficial branch of the MCA (Hata, et al, 1998). A midline neck incision was made and the fatty tissue separated to isolate the right carotid artery. Under a dissecting microscope, the right carotid bifurcation was exposed and the external carotid artery was cauterized distal to the bifurcation. A 7-0 nylon silicone coated monofilament was inserted through the external carotid artery stump and advanced approximately 10 mm to occlude the origin of the middle cerebral artery (Migliati, et al., 2003; Clark, Lessov, Dixon, & Eckenstein, 1997) and verified with a decrease in cerebral blood flow 70% from baseline. Animals that did not demonstrate occlusion after placement of the filament were not included in the study. The surgical area was covered and kept moist with sterile PBS during 60 minutes of ischemia. After ischemia, the filament was withdrawn, initiating reperfusion, and the neck wound was sutured. The mice were subcutaneously injected with 1.5 ml/gm sterile filtered PBS to prevent dehydration and placed in cages to recover at room temperature for 24 hours (Migliati, Wilson, Coull, McDonagh, & Ritter, 2003; Ritter, Copeland, & McDonagh, 1998; Ritter, Orozco, Coull, & McDonagh, 2000). SHAM surgeries were performed as above without introduction of a filament.

### *Measurement of Neurologic Function*

Neurologic scores were obtained 2 and 23 hours after the initiation of reperfusion. The mice were scored on three scales; 1) a composite neurologic score (Hattori, et al., 2000) and 2) general and 3) focal neurological deficit scales (Clark, et al., 1997). The general and focal scoring tools are based on a total score from 0-28 (reported correlation between scores and infarct size of  $r = 0.766$  (general) and  $r = 0.788$  (focal) (Clark, 1997; Migliati, et al., 2003) (See Appendix A for scoring system for each scale).

### *Measurement of Infarct Size*

Mice were deeply anesthetized with isoflurane, euthanized, and their brains removed. Each brain was placed in ice cold PBS and placed in  $-80^{\circ}\text{C}$  freezer for 5 minutes until firm. The brains were sliced into four 2-mm slices using the olfactory lobe and brainstem as landmarks (Migliati, et al., 2003; Clark, Lessov, Dixon, & Eckenstein, 1997). Each section was placed in wells containing 2% 2,3,5-triphenyltetrazolium chloride (T8877, Sigma-Aldrich, St. Louis, MO) in 0.9% PBS, incubated for 30 minutes at  $37^{\circ}\text{C}$ , and fixed in 10% formalin over night (Migliati, et al., 2003; Ritter, et al., 1998; 2000; Clark, 1997). Serial sections were scanned (HP Scanjet 5370c) on each side using HP scanning software (HP Image Zone Software, version 5.3) and saved as JPEG images. The area of non- infarcted (red) and infarcted tissue (white) on the front and back of each slice was quantified in square millimeters ( $\text{mm}^2$ ) using National Institutes of Health (NIH) Image 1.32J software. The volume ( $\text{mm}^3$ ) of non-infarcted and infarcted tissue was quantified by summation of all the measurements. Cerebral infarction volume is expressed as a percentage of the ipsilateral and contralateral hemispheres.

### *Blood Acquisition*

Venipuncture was performed on the non-surgical control animals after a four hour fast (0600-1000) and on the surgical SHAM and MCAO animals 24 hours after the procedure. Mice were anesthetized in a chamber and then mask ventilated with 5.0% isoflurane and 1.0 L/minute oxygen for venipuncture. Approximately, seven hundred  $\mu\text{L}$  blood was withdrawn from the ascending vena cava with a 23-gauge needle/1 mL syringe containing 0.05 mL sodium citrate (S1804, Sigma-Aldrich, St. Louis, MO).

### *Blood Smear Analysis*

Peripheral blood smears were made for all experiments using standard methods on glass microscope slides (VWR, West Chester, PA, #16004-368) (Brown, 1980). Eight  $\mu\text{L}$  anticoagulated blood was smeared across a glass slide, air-dried, and stained with Diff-Quik (Dade Behring, VWR #47733-152). The white blood cell differential count was measured by counting 100 white blood cells on one slide three times, categorizing each cell type, and averaging the counts.

### *Flow Cytometry*

*Platelet analysis.* Twelve microliters of heparinized whole blood (WB) was diluted in 1 ml Dulbecco's phosphate-buffered saline with 0.2% bovine serum albumin and 0.09% sodium azide (pH 7.4) (Pharmingen Stain Buffer, BD Biosciences Pharmingen, San Jose, CA, #554657). Twenty  $\mu\text{L}$  of 50  $\mu\text{M}$  calcimycin A23187 (Sigma-Aldrich Co., St. Louis, MO, #C7522) was added to positive control samples and incubated at room temperature for 10 minutes. One hundred  $\mu\text{L}$  of the stain buffer/WB mixture was then added to amber microcentrifuge tubes filled with 50  $\mu\text{L}$  of 1:10 dilution of fluorescent monoclonal

antibody (BD Biosciences Pharmingen) R-phycoerythrin (PE)-conjugated hamster anti-mouse CD61 (Clone 2C9.G2), 20  $\mu$ L of 1:10 dilution of fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD62P (p-selectin) monoclonal antibody (clone RB40.34), or 20  $\mu$ L of 1:10 FITC-conjugated rat IgG<sub>1</sub>, $\lambda$  monoclonal immunoglobulin isotype control (clone A110-1). The samples were covered with aluminum foil and incubated at room temperature for 10 minutes, then fixed with 1 mL 1% paraformaldehyde (Sigma-Aldrich Co., #P6148-500G) and placed on ice.

*PMN analysis.* Whole blood (WB) samples were initially diluted 1:1 with Dulbecco's phosphate-buffered saline with 0.2% bovine serum albumin and 0.09% sodium azide (pH 7.4) (Pharmingen Stain Buffer, BD Biosciences Pharmingen, San Jose, CA, #554657) and then incubated with 0.5  $\mu$ g/100 $\mu$ L WB/PBS purified rat anti-mouse CD16/CD32 Fc $\gamma$  III/II receptor blocking monoclonal antibody (PharMingen, Clone 2.4G2) for 15 minutes to decrease non-specific binding of antibodies to leukocyte Fc receptors (BD Biosciences, 2005). All incubations were performed at room temperature to diminish changes in leukocyte surface antigen expression with cooling and rewarming of samples (Forsyth and Levinsky, 1990; Repo, Jansson, and Leirisalo-Repo, 1995). Positive control samples were incubated for 30 minutes with either lipopolysaccharide (LPS, Sigma #2680, diluted in PBS, final concentration 10  $\mu$ g/0.1mL) for CD11b expression (Repo, Jansson, and Leirisalo-Repo, 1995), or with phorbol myristate acetate (PMA, (Sigma # P-148) diluted in dimethyl sulfoxide (Sigma #D-5879), final concentration 16  $\mu$ M) for ROS production (Himmelfarb, Hakim, Holbrook, Leeber, and Ault, 1992; Vowells, et al., 1995; Alvarez-Larran, Toll, Rives, and Estella, 2005). After agonist stimulation, monoclonal

antibodies or isotype controls (Becton-Dickenson, San Jose, CA) were added to each sample and incubated for 15 minutes. Twenty  $\mu\text{L}$  diluted (1:9 concentration diluted with Pharmingen Stain Buffer) peridinin chlorophyll-*a* protein (PerCP)-conjugated rat anti-mouse CD45 (leukocyte common antigen, Ly-5) monoclonal antibody (catalog # 557235, clone 30-F11) was added to all samples for leukocyte detection by the flow cytometer. For CD11b measurement, 20  $\mu\text{L}$  (1:99 concentration diluted with Pharmingen Stain Buffer) fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD11b (integrin  $\alpha_m$  chain, Mac-1  $\alpha$  chain) monoclonal antibody (catalog # 557396, clone M1/70) and FITC-conjugated rat IgG<sub>2b</sub>,  $\kappa$  monoclonal immunoglobulin isotype control (catalog # 553988, clone A95-1) were added to their respective samples. For PMN ROS analysis, cells were incubated for 15 minutes with 10  $\mu\text{L}$  2',7'-dichlorofluorescein diacetate (DCF-DA, Molecular Probes, Cat #D399) (diluted with filtered PBS, 80  $\mu\text{M}$  final concentration, stored at  $-80^\circ$  Celsius) (Bass, et al., 1983; Himmelfarb, Hakim, Holbrook, Leeber, and Ault, 1992; McDonagh, Hokama, Copeland, and Reynolds, 1997). The CD11b samples were then fixed with 100  $\mu\text{L}$  1% cold paraformaldehyde and the ROS samples were diluted with 100  $\mu\text{L}$  PBS. The samples were placed on ice until data acquisition.

Data were acquired by flow cytometry (FACScalibur, 488 nm argon laser, Becton Dickinson, San Jose, CA) within 3 hours of blood acquisition to decrease cellular changes that occur with time and acquired on the low flow setting to avoid multi-cell triggering of the flow cytometer (Himmelfarb, et al., 1992; Hageberg, and Lyberg, 2000). The flow cytometer was calibrated daily prior to each experiment using Calibrite Beads (Becton Dickinson, #340486) and FACsComp software (Becton Dickinson). Acquisition threshold

was set on FL2 to capture only CD61 events for platelet analysis and FL3 to capture only CD45 positive events (leukocytes) for PMN analysis.

FCS Express v3.0 (De Novo Software, Inc., Ontario, Canada) was used for flow cytometry analyses. Spectral compensation was performed for each day's experiments by using negative and positive control samples. A dot plot of logarithmic forward and side scatter properties of all cells and of only CD61 positive events was used to distinguish the platelet and microparticle populations. For PMN analysis, a dot plot of linear forward and side scatter properties of FL3 threshold events was used to identify and electronically gate the granulocyte population (Hageberg and Lyberg, 2000; Alvarez-Larran, Toll, Rivas, and Estella, 2005; Horn, et al., 2005).

#### *Metabolic Characteristic Measurements*

One week prior to MCAO or SHAM surgeries, mice were fasted for 4 hours (0600-1000) for measurement of blood sugar via tail nip under anesthesia using a glucometer (AccuCheck Advantage, Roche, Inc., Mannheim, Germany). The glucometer was tested monthly for reliability using high and low controls (Roche). Additionally, blood sugar was measured after reperfusion during blood acquisition for flow cytometry experiments. Weight (gms) was monitored weekly and prior to each experiment (Triple Beam Balance, Ohaus, Columbia, MD). White blood cell count, hematocrit, and platelet counts were measured by a bench top whole blood analyzer (Coulter A<sup>c</sup>T™ 5 diff Hematology Analyzer, Beckman Coulter, Fullerton, CA), which was tested daily for reliability with high, low, and normal control samples (Beckman Coulter).

### *Statistical Analysis*

Data were expressed as mean  $\pm$  SEM. Groups were compared using t-test (*t*), Mann-Whitney Rank Sum Test (*T*) (non-parametric data), one- way ANOVA (*F*) or Kruskal-Wallis ANOVA on Ranks (*H*) (non-parametric data). ANOVA post hoc testing was done with either Holm-Sidak or Dunn's Method (non-parametric). Within group comparisons were analyzed with paired t- tests (*t*) or Wilcoxon Signed Rank Test (*T*) (non-parametric data). The flow cytometry experiments were performed in duplicate and the coefficient of variation determined by dividing the standard deviation by the mean. All experiments with a variation of >10% were removed from analysis or noted in the results section. SigmaStat for Windows version 3.10 (Systat Software, Inc., Point Richmond, CA) was used for statistical analyses. An a priori  $\alpha$  of  $p \leq 0.05$  was considered statistically significant.

## Results

### *Mortality*

Four of the five obese, diabetic mice died intraoperatively, secondary to difficulties maintaining anesthesia or isolating the external carotid. The vessel walls in these animals were friable compared to chow fed mice and appeared to develop clots during the procedure. The one diabetic mouse that survived the MCAO procedure died during reperfusion. All of the chow fed mice survived MCAO, but two of the five died postoperatively. Of the three that survived reperfusion, only 2 had measurable infarcts, and

their data are presented below. Five SHAM surgeries were attempted in chow fed mice, but only 2 survived.

#### *Metabolic Characteristics*

One week prior to MCAO or SHAM surgeries, mice were weighed and fasted for 4 hours to measure fasting blood sugar. The HF fed mice were significantly obese (chow fed (CF) ( $n=9$ )  $32.1 \pm 1.3$ , HF ( $n=9$ )  $54.3 \pm 1.3$  gm,  $t(16) = -12.32$ ,  $p < 0.001$ ) and hyperglycemic compared to chow fed mice (CF  $142.5 \pm 12.3$ , HF  $177.6 \pm 8.4$  mg/dl,  $t(16) = -2.35$ ,  $p = 0.03$ ).

For the remaining portion of the results section, the chow fed mice are referred to as control mice and three groups are presented- chow fed (non-surgical control), chow fed after SHAM surgery (SHAM), and chow fed after MCAO and reperfusion (MCAO).

After MCAO or SHAM surgery, there was no difference in the weight (control ( $n=5$ )  $34.8 \pm 1.4$ , SHAM ( $n=2$ )  $33.7 \pm 0.3$ , MCAO ( $n=2$ )  $29.7 \pm 1.0$  gm,  $H = 4.29$ ,  $df = 2$ ,  $p = 0.12$ ) or blood sugar (control  $153.1 \pm 16.3$ , SHAM  $111.8 \pm 51.3$ , MCAO  $87.8 \pm 5.6$  mg/dl,  $H = 3.24$ ,  $df = 2$ ,  $p = 0.24$ ) compared to non-surgical control mice. It should be noted that after MCAO mice are unable to eat and their blood sugars were much lower than surgical SHAM or non-surgical control mice. Additionally, there was no noted difference in body temperature (maintained at  $37.0^\circ\text{C}$ ) or length of surgery in mice undergoing MCAO or SHAM.

#### *Whole Blood Cell Counts*

Whole blood counts were standardized to a hematocrit of 45% to eliminate the effects of anticoagulant dilution. There were no difference in the standardized platelet

counts between groups (control ( $n= 5$ )  $881.3 \pm 44.37$ , SHAM ( $n= 2$ )  $891.72 \pm 67.36$ , MCAO ( $n= 2$ )  $964.82 \pm 53.1$   $10^3/\text{ul}$ ,  $F(2,6)= 0.57$ ,  $p= 0.59$ , Table 6.1). The standardized white blood count (WBC) was significantly lower in mice undergoing SHAM or MCAO compared to non-surgical control mice (control  $7.6 \pm 0.5$ , SHAM  $3.9 \pm 0.4$ , MCAO  $5.1 \pm 0.2$   $10^3/\text{ul}$ ,  $F(2,6)=13.94$ ,  $p= 0.12$ ). Even though the total WBC was lower in mice undergoing MCAO or SHAM surgeries, these mice had significantly increased percentage of PMNs after reperfusion compared to controls (control  $18.3 \pm 2.0$ , SHAM  $34.3 \pm 8.7$ , MCAO  $75.5 \pm 0.2$  %WBC,  $H= 6.53$ ,  $df= 2$ ,  $p< 0.01$ ). Only after MCAO and reperfusion mice demonstrated significantly lower levels of lymphocytes compared to non-surgical control mice (control  $79.8 \pm 0.1$ , SHAM  $56.3 \pm 0.4$ , MCAO  $21.7 \pm 1.3$  %WBC,  $H= 6.53$ ,  $df= 2$ ,  $p< 0.01$ ), but there was no difference between groups in the differential percentage of monocytes (control  $2.3 \pm 0.6$ , SHAM  $3.6 \pm 1.1$ , MCAO  $2.7 \pm 1.3$  %WBC,  $F(2,6)= 0.48$ ,  $p= 0.64$ ). In addition to an increase in the fraction of the WBC as PMNs in the MCAO and SHAM groups, the absolute number of PMNs was elevated after MCAO and reperfusion compared to both SHAM and control mice (control  $1.4 \pm 0.1$ , SHAM  $1.3 \pm 0.2$ , MCAO  $3.8 \pm 0.2$   $10^3/\text{ul}$ ,  $F(2,6)= 52.84$ ,  $p< 0.001$ ). There was a significantly lower number of circulating lymphocytes in the MCAO mice (control  $6.1 \pm 0.5$ , SHAM  $2.2 \pm 0.8$ , MCAO  $1.1 \pm 0.0$   $10^3/\text{ul}$ ,  $H= 6.53$ ,  $df= 2$ ,  $p< 0.01$ ), but no difference in monocytes (control  $0.2 \pm 0.0$ , SHAM  $0.1 \pm 0.1$ , MCAO  $0.1 \pm 0.1$   $10^3/\text{ul}$ ,  $F(2,6)= 0.13$ ,  $p= 0.88$ ) between groups.

Table 6.1 Whole Blood Counts of Non-Surgical or SHAM-surgical Control Mice and After MCAO and 24 Hours Reperfusion

Strain (n)	Platelet count (10 <sup>3</sup> /μl)	WBC count (10 <sup>3</sup> /μl)	PMN (%)	Lymph (%)	Mono (%)	PMN (10 <sup>3</sup> /μl)	Lymph (10 <sup>3</sup> /μl)	Mono (10 <sup>3</sup> /μl)
Control (5)	881.3 ± 44.3	7.6 ± 0.5	18.3 ± 2.0	79.8 ± 0.1	2.3 ± 0.6	1.4 ± 0.1	6.1 ± 0.5	0.2 ± 0.0
SHAM (2)	891.7 ± 67.4	3.9 ± 0.4*	34.3 ± 8.7*	56.3 ± 0.4	3.6 ± 0.4	1.3 ± 0.2	2.2 ± 0.8	0.1 ± 0.1
MCAO (2)	964.8 ± 53.1	5.1 ± 0.3**	75.5 ± 0.2*	21.7 ± 1.3*	2.7 ± 1.3	3.8 ± 0.2***#	1.1 ± 0.0*	0.1 ± 0.1

PMN-polymorphonuclear leukocyte, Lymph-lymphocyte, Mono- monocyte. WBC counts were standardized to a hematocrit of 45%. Differential counts are % of total white blood count. Absolute numbers of circulating leukocytes were calculated as %differential count / 100 X standardized WBC. Data presented as mean ± SEM. \*significantly different from control, \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ , # significantly different from SHAM, # $p < 0.05$ .

#### *Infarct Size*

Two mice had measurable infarcts after MCAO and 24 hours reperfusion. The mean infarct volumes was  $7.5 \pm 1.1$  % (individual measures were 8.59 and 6.38 %) of the contralateral cerebral hemisphere, and  $7.0 \pm 1.1$  % (8.10 and 5.88 %) of the ipsilateral cerebral hemisphere. All mice undergoing SHAM procedure had infarct volumes of 0 %.

#### *Neurological Scores*

##### *Composite neurologic score*

The two mice that had measurable infarcts had neurological scores of 3.5 and 3.0 after 2 hours of reperfusion that improved to 2.0 after 23 hours of reperfusion. Interestingly, the mouse that underwent MCAO, but did not have a visible infarct, demonstrated a neurological score of 4.5, which represents worsened neurological outcome

than the 2 mice that had measurable infarcts. After 23 hours of reperfusion, this mouse improved to a score of 2.0, similar to the 2 mice with measurable infarcts. All mice undergoing SHAM surgery had neurological scores of 0 at both time points.

#### *General deficits*

The general deficits neurological scores were 13.0 and 11.0 after MCAO that improved to 6.0 and 5.0 after 23 hours reperfusion. The mouse that underwent MCAO without a measurable infarct initially had a score of 14.5 that was worse than the 2 mice with measurable infarcts, and did not have the extent of improvement after 23 hours seen with the other mice (score of 9.0 after 23 hours reperfusion). The SHAM mice had scores of 1.5 and 4.0 that improved to 1.5 or 1.0 respectively after 23 hours.

#### *Focal deficits*

After MCAO the mice with measurable infarcts had scores of 27.0 and 20.0 that improved to 11 and 9 respectively with 23 hours reperfusion. The mouse that underwent MCAO but had no appreciable infarct had an initial focal deficit score of 28 that improved to 10.5 after 23 hours reperfusion. The SHAM mice demonstrated very little focal neuro deficits, and had scores of 1.5 and 2.0 that were completely normal after 23 hours.

#### *Flow Cytometry*

##### *Platelet markers*

*Microparticle formation.* The baseline (control  $0.0058 \pm 0.0009$ , SHAM  $0.0051 \pm 0.0004$ , MCAO  $0.0059 \pm 0.0009$  %total events,  $F(2,6)= 0.13$ ,  $p= 0.88$ ) or stimulated levels of total microparticles (control  $0.0387 \pm 0.0050$ , SHAM  $0.0500 \pm 0.0227$ , MCAO  $0.0241 \pm 0.0070$  %total events,  $F(2,6)= 1.24$ ,  $p= 0.35$ ) were no different between non-surgical

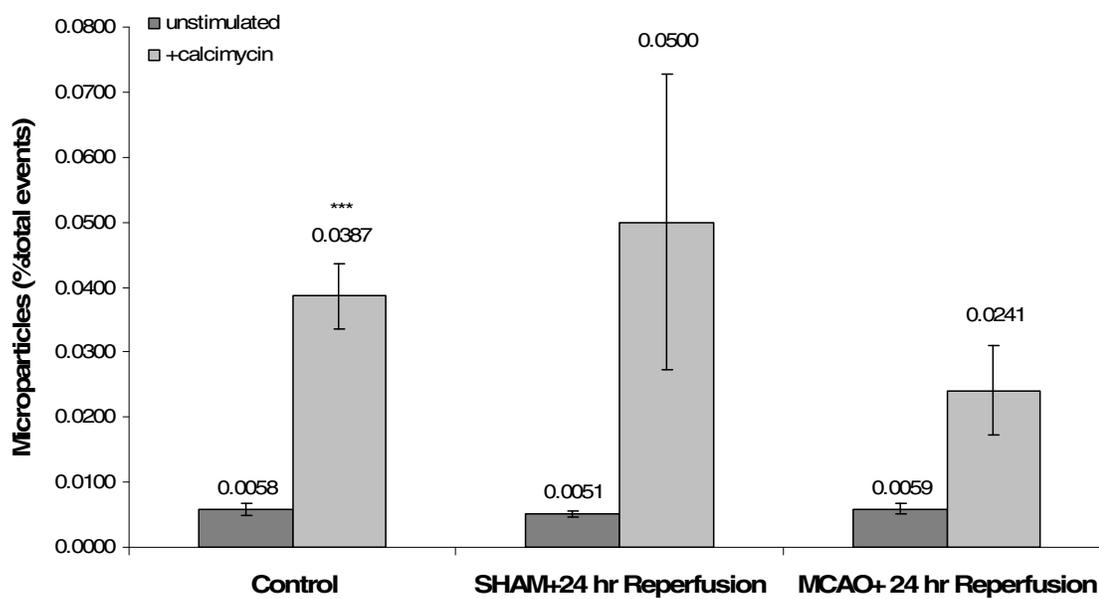
controls, surgical-SHAMs, or after MCAO and reperfusion. Only the non-surgical control mice demonstrated a significant increase in total microparticle formation after stimulation with calcimycin (calcium ionophore) (paired t-test, control  $t(4) = -5.64$ ,  $p < 0.01$ , SHAM  $t(1) = -1.94$ ,  $p = 0.30$ , MCAO  $t(1) = -2.98$ ,  $p = 0.21$ , Figure 6.1A). The baseline levels of platelet microparticles (PMPs) were also no different between groups (control  $0.20 \pm 0.05$ , SHAM  $0.30 \pm 0.02$ , MCAO  $0.25 \pm 0.05$  %CD61+ events,  $F(2,6) = 0.92$ ,  $p = 0.45$ ) or after stimulation (control  $9.44 \pm 1.11$ , SHAM  $6.79 \pm 0.69$ , MCAO  $5.46 \pm 1.21$  %CD61+ events,  $F(2,6) = 2.77$ ,  $p = 0.14$ ). Only the non-surgical control mice demonstrated a significant increase in PMP formation after stimulation with calcimycin (paired t-test, control  $t(4) = -8.04$ ,  $p < 0.001$ , SHAM  $t(1) = -9.14$ ,  $p = 0.07$ , MCAO  $t(1) = -4.49$ ,  $p = 0.14$ , Figure 6.1B).

*P-selectin expression.* PMP P-selectin expression was significantly higher after MCAO and reperfusion compared to non-surgical controls, but not compared to surgical SHAMs (control  $3.4 \pm 0.1$ , SHAM  $3.9 \pm 0.3$ , MCAO  $4.4 \pm 0.3$  (MDFI),  $F(2,6) = 8.50$ ,  $p = 0.02$ ). There was no difference in PMP P-selectin expression after stimulation with calcimycin (control  $9.9 \pm 1.5$ , SHAM  $10.4 \pm 1.5$ , MCAO  $13.3 \pm 1.5$  MDFI,  $F(2,6) = 0.96$ ,  $p = 0.44$ ). No group demonstrated an increased PMP P-selectin expression after stimulation (paired t-test, control  $T = 15.00$ ,  $p = 0.06$ , SHAM  $t(1) = -4.15$ ,  $p = 0.15$ , MCAO  $t(1) = -5.20$ ,  $p = 0.12$ ).

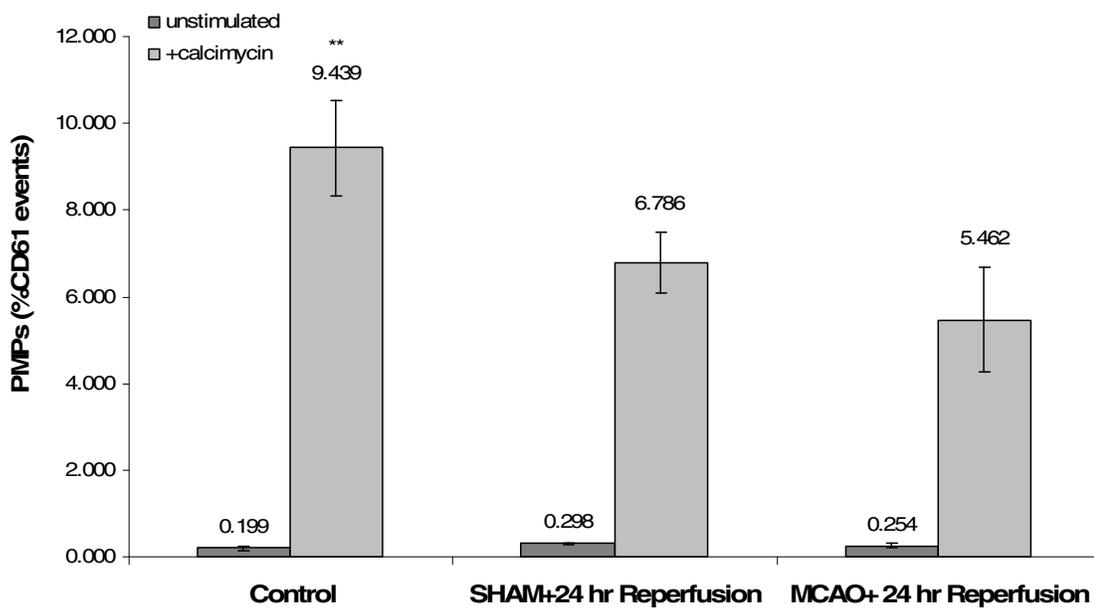
Figure 6.1 Total and platelet microparticle formation in control mice and in mice after SHAM or MCAO and 24 hours reperfusion. (A) Summative data of total microparticle formation (%total acquired events) (Control  $n= 5$ , SHAM  $n= 2$ ; MCAO  $n= 2$ ). (B) Summative data of platelet microparticle formation (%CD61 positive events). Data presented as mean  $\pm$  SEM. \*significantly different from unstimulated sample, \*\* $p < 0.01$ , \*\*\*  $p < 0.001$ .

Figure 6.1

A.



B.



Platelet P-selectin expression was no different between groups before (control  $3.7 \pm 0.1$ , SHAM  $4.4 \pm 0.5$ , MCAO  $4.1 \pm 0.0$  MDFI,  $H= 6.00$ ,  $df= 2$ ,  $p= 0.02$ ), or after stimulation with calcimycin (control  $13.7 \pm 3.2$ , SHAM  $15.0 \pm 1.5$ , MCAO  $20.9 \pm 2.6$  MDFI,  $F(2,6)= 0.99$ ,  $p= 0.42$ ). No group demonstrated an increased platelet p-selectin expression with agonist stimulation (paired t-test, control  $T= 15.00$ ,  $p= 0.06$ , SHAM  $t(1)= -5.37$ ,  $p= 0.12$ , MCAO  $t(1)= -6.61$ ,  $p= 0.09$ , Figure 6.2B).

*Platelet CD61 expression.* There was no difference in baseline platelet CD61 expression between non-surgical controls, surgical SHAM, or after MCAO and reperfusion (control  $333.8 \pm 9.3$ , SHAM  $287.4 \pm 13.8$ , MCAO  $298.5 \pm 22.9$  mean fluorescence intensity (MFI),  $F(2,6)= 3.63$ ,  $p= 0.09$ ). After stimulation with calcimycin, surgical SHAM and after MCAO and 24 hours reperfusion demonstrated significantly less platelet CD61 expression compared to non-surgical control mice (control  $486.2 \pm 8.5$ , SHAM  $395.1 \pm 20.8$ , MCAO  $417.6 \pm 8.9$  MFI,  $F(2,6)= 17.94$ ,  $p< 0.01$ ). There was a significant increase in platelet CD61 expression in non-surgical control mice, but not in surgical SHAMs or after MCAO and reperfusion with stimulation with calcimycin (control  $t(4)= -12.34$ ,  $p< 0.001$ , SHAM  $t(1)= -3.11$ ,  $p= 0.20$ , MCAO  $t(1)= -8.51$ ,  $p= 0.08$ , Figure 6.3).

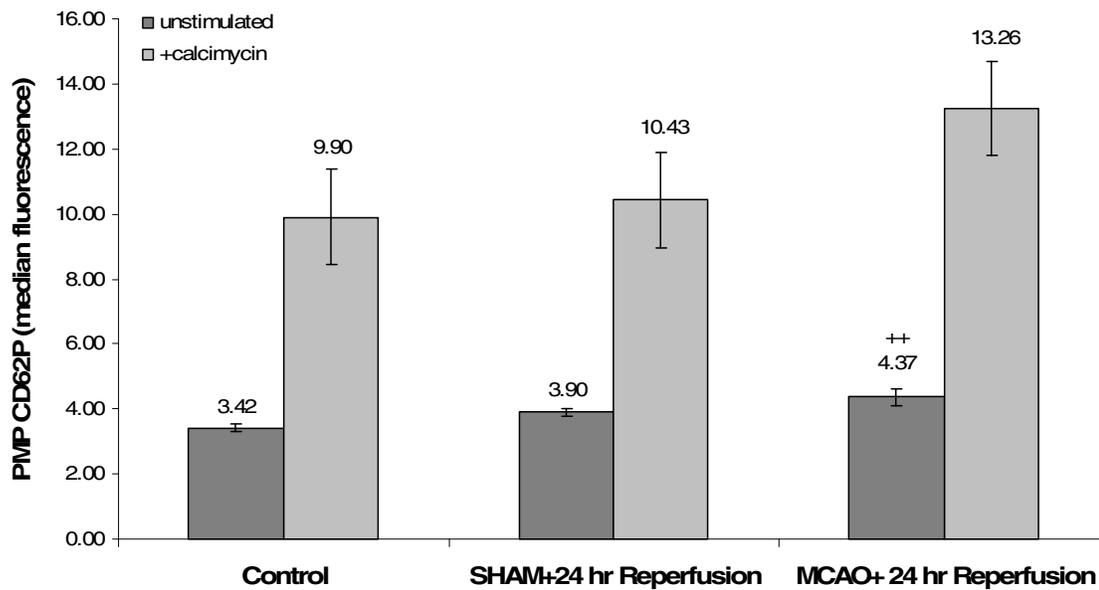
#### *PMN markers*

*CD11b positive PMNs.* The percentage of CD11b positive PMNs was significantly higher after MCAO and reperfusion compared to surgical SHAM and non-surgical control mice (control  $38.4 \pm 3.2$ , SHAM  $46.1 \pm 3.9$ , MCAO  $86.6 \pm 0.8$  % CD11b+ PMNs,  $F(2,6)= 42.67$ ,  $p< 0.001$ ).

Figure 6.2 Platelet microparticle (PMP) and platelet CD62P (P-selectin) expression in control mice and in mice after SHAM or MCAO and 24 hours reperfusion. (A) Summative data of PMP CD62P expression (median fluorescence intensity, MDFI) (Control  $n= 5$ , SHAM  $n= 2$ ; MCAO  $n= 2$ ). (B) Summative data of platelet CD62P expression (MDFI). Data presented as mean  $\pm$  SEM. \*significantly different from unstimulated sample, \*\*  $p<0.001$ ; +significantly different from control mice, ++ $p< 0.01$ .

Figure 6.2

A.



B.

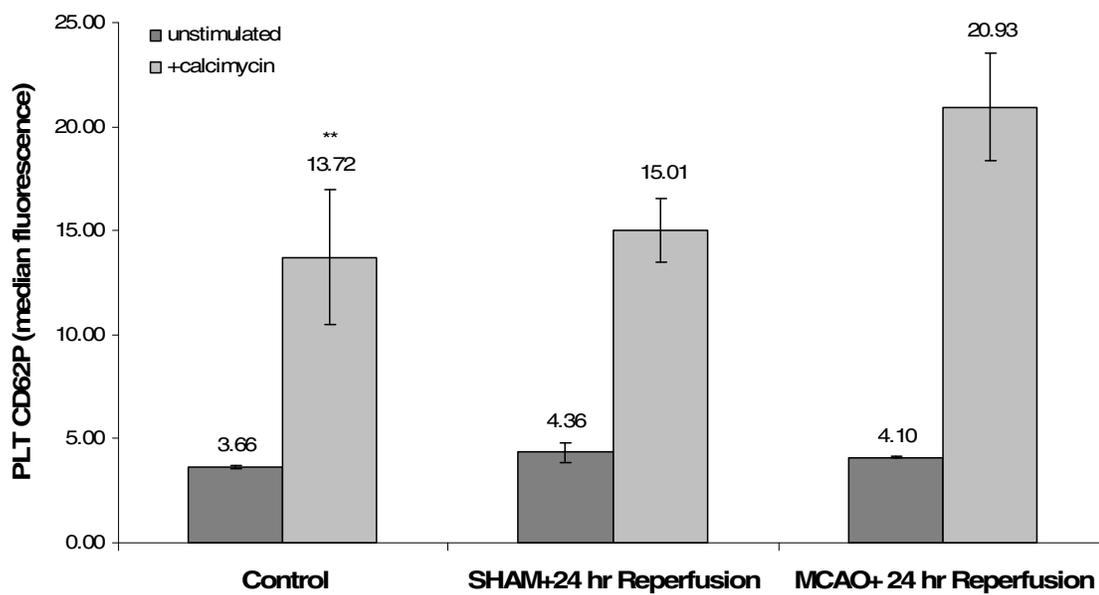
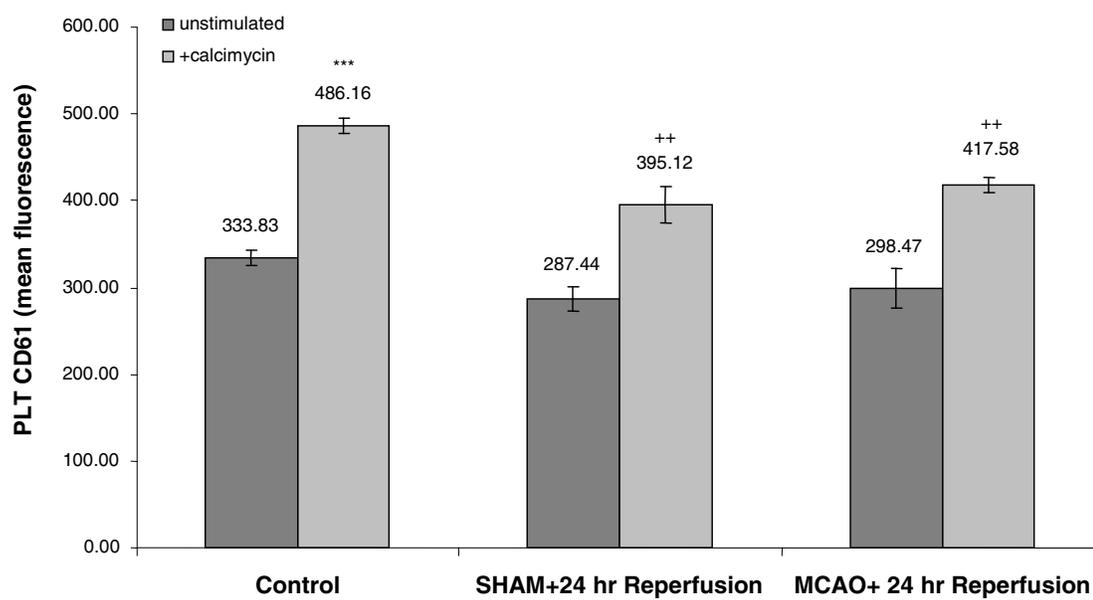


Figure 6.3 Platelet CD61 expression in control mice and in mice after SHAM or MCAO and 24 hours reperfusion. Summative data of platelet CD61 expression (mean fluorescence intensity, MFI) (Control  $n= 5$ , SHAM  $n= 2$ ; MCAO  $n= 2$ ). Data presented as mean  $\pm$  SEM. \*significantly different from unstimulated sample, \*\*\*  $p < 0.001$ ; ++significantly different from non-surgical control mice, ++  $p < 0.01$ .

Figure 6.3



Compared to the non-surgical control mice, both surgical SHAMs and mice after MCAO and reperfusion demonstrated a significantly higher number of CD11b positive PMNs with LPS stimulation (control  $38.0 \pm 3.0$ , SHAM  $62.7 \pm 10.7$ , MCAO  $88.5 \pm 0.2$  % CD11b+ PMNs,  $F(2,6) = 27.82$ ,  $p < 0.001$ ). The percentage of CD11b positive PMNs did not increase with LPS stimulation in any group (paired t-test, control  $t(4) = 0.12$ ,  $p = 0.91$ , SHAM  $t(1) = -2.45$ ,  $p = 0.25$ , MCAO  $t(1) = -2.02$ ,  $p = 0.29$ , Figure 6.4A).

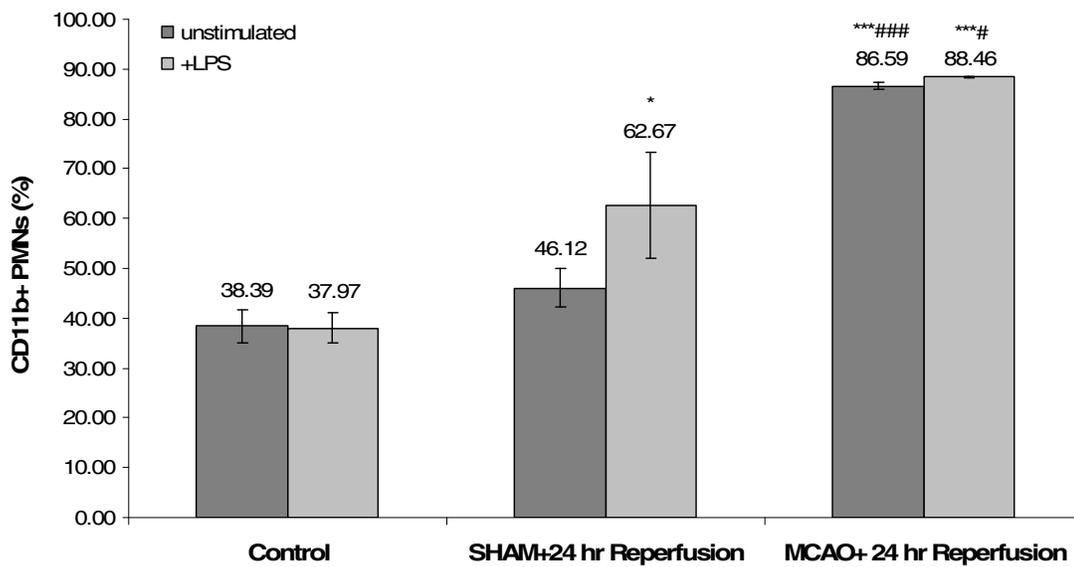
*PMN CD11b expression.* The PMN expression of CD11b was lower in mice after surgical SHAM and after MCAO compared to non-surgical control mice (control  $26.2 \pm 2.0$ , SHAM  $16.7 \pm 1.9$ , MCAO  $23.2 \pm 1.4$  MDFI,  $F(2,6) = 4.46$ ,  $p = 0.07$ ), but not after LPS stimulation (control  $92.8 \pm 6.7$ , SHAM  $88.1 \pm 7.5$ , MCAO  $95.0 \pm 1.5$  MDFI,  $F(2,6) = 0.15$ ,  $p = 0.86$ ). All groups demonstrated a significant increase in PMN CD11b expression after stimulation with LPS (paired t-test, control  $t(4) = -12.20$ ,  $p < 0.001$ , SHAM  $t(1) = -12.66$ ,  $p = 0.05$ , MCAO  $t(1) = -24.66$ ,  $p = 0.03$ , Figure 4.4B).

*PMN ROS production.* There was no difference between groups in baseline ROS production (control  $20.3 \pm 4.1$ , SHAM  $26.4 \pm 11.8$ , MCAO  $96.1 \pm 66.4$ , MDFI,  $H = 2.69$ ,  $df = 2$ ,  $p = 0.32$ ), or after PMA stimulation (control  $200.0 \pm 26.5$ , SHAM  $319.1 \pm 36.3$ , MCAO  $858.3 \pm 524.1$  MDFI,  $H = 6.13$ ,  $df = 2$ ,  $p = 0.01$ ). Only the non-surgical control mice demonstrated a significant increase in PMN ROS production after stimulation with PMA (control  $t(4) = -7.38$ ,  $p < 0.01$ , SHAM  $t(1) = -6.09$ ,  $p = 0.10$ , MCAO  $t(1) = -1.67$ ,  $p = 0.34$ , Figure 6.5).

Figure 6.4 PMN CD11b expression in control mice and in mice after SHAM or MCAO and 24 hours reperfusion. (A) CD11b positive PMNs (% gated PMNs) (B) PMN CD11b expression (median fluorescence intensity (MDFI) in arbitrary units). Data presented as mean  $\pm$  SEM. \*significantly different from unstimulated sample, \*  $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; +significantly different from non-surgical control mice, + $p < 0.05$ , +++ $p < 0.001$ ; #significantly different from surgical SHAM, # $p < 0.05$ , ### $p < 0.001$ .

Figure 6.4

A.



B.

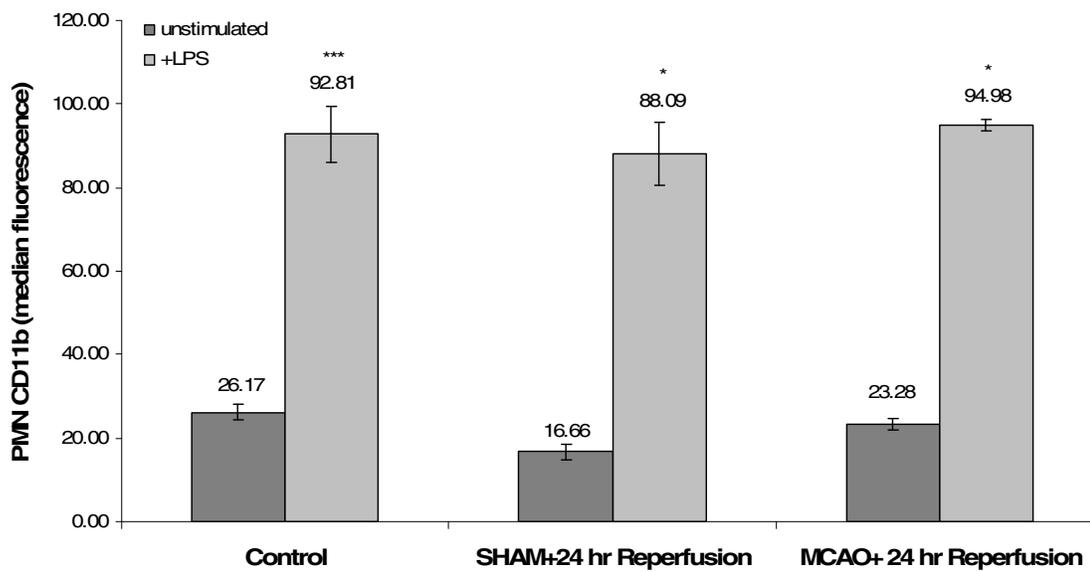
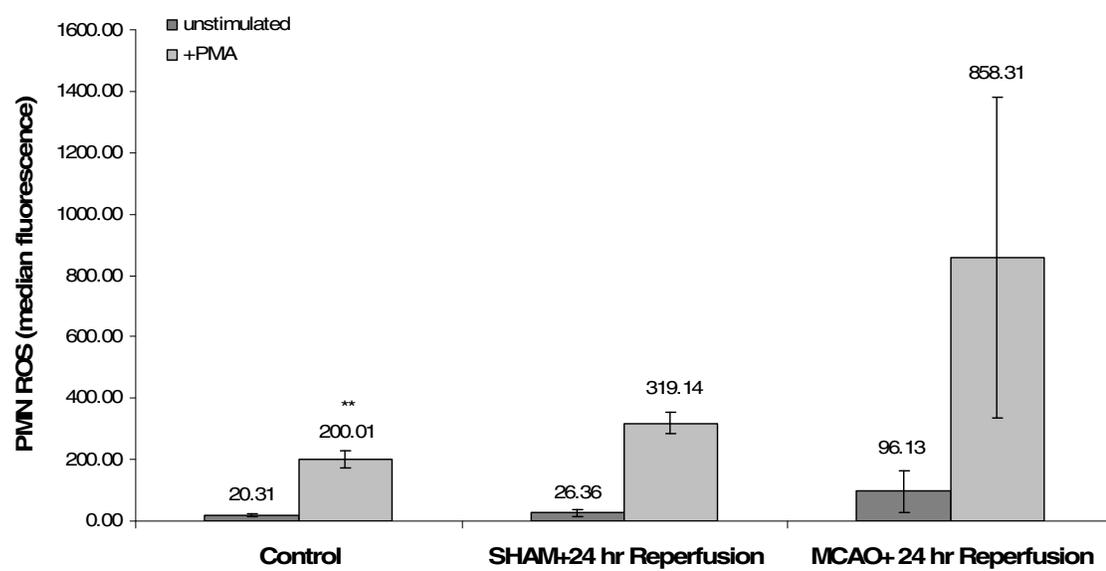


Figure 6.5 PMN ROS production in control mice and in mice after SHAM or MCAO and 24 hours reperfusion. (MDFI, in arbitrary units). Data presented as mean  $\pm$  SEM.

\*significantly different from unstimulated sample, \*\* $p < 0.01$

Figure 5

A.



## Discussion

Ischemic stroke is known to be mediated by local inflammatory changes that recruit platelets and leukocytes during reperfusion to the ischemic site. The reperfusion of ischemic regions worsens the cerebral tissue damage through activation of platelets and leukocytes that release damaging toxic mediators and occlude microvessels. Although these mechanisms of local ischemic cell death and inflammatory processes are well known in cerebral ischemia, little is known about the effects of stroke and reperfusion on peripheral thrombotic and inflammatory responses and the effects of obesity and type 2 diabetes on these processes. The purpose of this study was therefore, to examine peripheral blood platelet and PMN markers of activation in control (chow fed) and a diet induced mouse model of type 2 diabetes.

To our knowledge, this is the first study that attempted to examine peripheral platelet and PMN activation after middle cerebral artery occlusion in a mouse model of diet induced obesity and type 2 diabetes. Unfortunately, we experienced a high mortality rate and had no successful experiments in the obese/diabetic mice. The MCAO procedure in mice is known to have a mortality rates as high as 80% (Duckworth, Butler, Mesquita, Collier, Collier, & Pennypacker, 2005) and high interexperimental variability (Connolly, Winfree, Stern, Soloman, & Pinsky, 1996). All five non-obese, non-diabetic mice demonstrated successful MCAO (reduction of cerebral blood flow by 70%) but only three of the five survived the 24 hours of reperfusion. Of the three that survived, only two demonstrated measurable infarcts, but interestingly, the mouse that did not have measurable infarct with TTC staining had worse neurological outcomes (measured by a

composite neurologic score, and general and focal deficit scores) than the two mice with measurable infarcts. TTC stains viable mitochondria red and thus infarcted, necrotic tissue does not stain and remains white. TTC staining may miss neural degeneration that leads to delayed cell death (Duckworth, et al., 2005) and this mouse may have incurred an ischemic event, but the cellular necrosis was delayed past the time point of measurement.

The contribution of circulating platelets in cerebral damage after ischemia and reperfusion has not been well characterized. Platelets in humans after ischemic stroke have demonstrated an increase in CD62P (P-selectin), and a decrease in PAC-1 (CD61, GP IIb/IIIa) expression during the convalescent phase (>3mo) after stroke (McCabe, et al., 2004). In addition, CD62P expression was positively correlated with an increase in circulating levels of both monocyte- and PMN-platelet conjugates in patients after stroke compared to non-stroke patients (McCabe, 2004). These findings are similar to the results of this study. We found that after experimental stroke and reperfusion, platelet microparticles (but not platelets) in non-obese, non-diabetic mice demonstrated an increased level of P-selectin expression. We did not observe any other significant elevation in platelet activation markers, but this may be explained by the low number of experiments in our study and the small infarct sizes noted in these mice. No studies were found examining the levels of PMPs after stroke, and although we did not find significant differences, the levels of platelet microparticles were decreased after experimental stroke and reperfusion. We hypothesize that the PMPs may have become activated and adhered to circulating leukocytes, other platelets, or endothelial cells, lowering their overall levels. We also observed a decreased expression in platelet CD61 expression compared to non-

surgical controls. McCabe, et al., (2004) suggested that the decreased expression of CD61 observed in their study may have occurred because the activated platelets after stroke had fibrinogen bound to the CD61 receptor, inhibiting binding of the monoclonal antibody. This reasoning may also explain our results. Taken together, our results suggest that some level of platelet activation occurs in the peripheral circulation, but more experiments are needed to fully appreciate the changes that occur with stroke and reperfusion.

After stroke, patients continue to demonstrate elevated total leukocyte counts, particularly an increase in circulating polymorphonuclear leukocytes (Beamer, Coull, Clark, Briley, Wynn, & Sexton, 1998; Suzuki, Kelley, Reyes-Iglesias, Alfonso, & Dietrich, 1995, McCabe, et al., 2004). After experimental stroke and reperfusion or SHAM surgeries in mice, we found a significant decrease in the total leukocyte count compared to non-surgical controls. The number of circulating PMNs, however, was significantly higher after experimental stroke and reperfusion compared to both non-surgical and surgical SHAM experiments, similar to that seen in humans after stroke. We found no studies that examined circulating PMN function after stroke. We found a significant increase in the number of PMNs that expressed CD11b after stroke and reperfusion compared to SHAM and non-surgical controls. We found no difference, however, in the PMN expression of CD11b or ROS production, and again this may have been because of the small number of experiments in our study and the generally small infarcts in our 2 stroked mice. Our results suggest that after experimental stroke, mice demonstrate an acute PMN inflammatory response, but more experiments are needed to fully understand this process.

As stated previously, the results of our flow cytometry experiments may underestimate the effects of stroke on platelet and PMN activation because we observed small infarct sizes after MCAO in this study. Earlier studies have demonstrated infarct sizes of 48 % (Stevens, Bao, Hollis, Lessov, Clark, & Stenzel-Poore, 2002) and 30 % (Connolly, Winfree, Stern, Solomon, & Pinsky, 1996) of the ipsilateral hemisphere in the C57BL6 mouse. In our study the average infarct was 7.0 % of the ipsilateral hemisphere. The study by Connolly, et al., (1996) used a larger filament than used in this study. Although the study by Stevens, et al., used a smaller filament than used in this study, the mice in their study were considerably smaller (20-25 gm) than those used in our study (avg wgt- 30gm). It has been demonstrated in another study that the size of the filament should be increased with the increasing weight of mice (Hata, et al., 1997) to produce consistent infarct sizes. We may have used filaments that were too small for the weight of the mice and did not completely occlude the vessel. The mice in our study may not have acquired the peripheral blood changes that occur with more severe stroke and reperfusion because of the relatively small strokes observed in these mice.

Little is known about the effects of ischemic stroke and reperfusion on peripheral platelet and PMN activation, particularly in the patients with obesity and diabetes. Although we were unable to examine the effects of experimental stroke and reperfusion in obese and diabetic mice, we were able to demonstrate activation of platelet and PMN markers in mice, similar to patients after ischemic stroke. Unfortunately, because of the difficulty performing MCAO in obese, diabetic mice and the high mortality observed with these experiments, it may not be feasible to examine these processes in mice. Other models

of stroke and reperfusion (i. e. rat models), that have higher success rates, may be necessary to explore peripheral blood changes in obesity and diabetes after stroke and reperfusion.

## CHAPTER 7

### DISCUSSION AND CONCLUSIONS

The incidence of obesity and type 2 diabetes continues to rise. The prevalence of obesity in the U.S. has increased almost 10% from 1995 to 2005, and the number of people overweight has increased to every 2 out of 3 Americans (CDC, 2005b). The rise in obesity is directly correlated with the increased incidence of type 2 diabetes (Fox, et al., 2006) and over 20 million people in the U. S. was estimated to have type 2 diabetes in 2005 (CDC, 2005a). The economic burden of these diseases is significant, and has been estimated to cost the US economy \$40 billion per year (est on 2002 numbers, ADA, 2002) in lost wages and productivity. Obesity and type 2 diabetes are also associated with considerable medical costs due to the multi-faceted treatment regimen needed to care for these patients that was estimated in 2002 to be over \$92 billion (ADA, 2002).

Obese and type 2 diabetic patients develop long-term vascular complications, secondary to chronic activation of thrombotic and inflammatory processes (Carr, 2000; Schneider, 2005; Duncan, et al., 2003; Dandona, Aljada, Chaudhuri, & Mohanty, 2004). Recent studies indicate that obese, diabetic patients demonstrate alterations in both platelet (McDonagh, et al., 2003) and polymorphonuclear leukocyte function (Hokama, et al., 1997; Ohmori, et al., 2000; Hand, Hand, & Vasquez, 2006) indicative of a state of chronic activation. The cellular mechanisms of how these chronic processes lead to the development of vascular disease has been studied, but is still not completely understood because of the complex nature of these disorders.

One such vascular complication of obesity and type 2 diabetes is ischemic stroke. Type 2 diabetes mellitus and obesity are known independent risk factors for acute ischemic stroke (Lee, et al., 2001; Adachi, Hirai, Tsuruta, Fujiura, & Imaizumi, 2001, Hu, et al., 2002; Kernan, et al., 2003; Folsom, et al., 1999) and are associated with higher morbidity and mortality from stroke compared to non-obese, non-diabetic subjects (Almdal, Scharling, Jensen, & Vestergaard, 2004; Megherbi, et al., 2003). The higher risk of developing and mortality after stroke is partially due to chronic inflammation (Engstrom et al., 2003). In addition, pro-thrombotic diseases, such as atherosclerosis, are known to incur a higher risk of developing stroke (Levine, 2005), and the prothrombotic condition in diabetes is hypothesized to be related to the elevated risk of developing stroke (Idris, Thomson, & Sharma, 2006), but no studies were found analyzing this relationship.

To fully explore the contribution of chronic thrombotic and inflammatory activation on the exacerbated injury from vascular complications in obesity and type 2 diabetes, particularly ischemic stroke, it is necessary to develop animal models demonstrating these altered blood processes. However, little research has been conducted in obese, type 2 diabetic mice to characterize their thrombotic and inflammatory state. Therefore, the purpose of this dissertation project was to examine the changes in thrombotic and inflammatory markers of activation in response to obesity and type 2 diabetes in mice and to explore the impact of these blood changes on the outcomes of ischemic stroke and reperfusion injury. The understanding of these processes in mouse models of obesity and type 2 diabetes is important for future study of the cellular

mechanisms underlying vascular injury in these disease processes and for potential use of blood cell markers to predict the development of vascular events.

The central hypothesis of this dissertation was that obesity and type 2 diabetes produces an overall hypercoaguable and proinflammatory state in mice and that these processes exacerbate stroke and reperfusion injury. The specific aims of this dissertation project were: 1) to develop a reliable flow cytometry methodology to measure platelet and PMN markers of activation in mouse whole blood, 2) to measure *in vitro* coagulation, whole blood aggregation, and platelet markers of activation in mouse models of obesity and type 2 diabetes and lean, non-diabetic mice, 3) to measure *in vitro* PMN inflammatory markers of activation, CD11b expression and ROS production, in mouse models of obesity and type 2 diabetes and lean, non-diabetic mice, 4) to measure cerebral infarction and neurologic deficits in obese and type 2 diabetic mice and lean, non-diabetic mice after experimental stroke and 24 hours reperfusion, and 5) to measure platelet and PMN markers of activation after experimental stroke and 24 hours reperfusion in obese, type 2 diabetic mice and lean, non-diabetic mice.

#### Development of a Reliable Flow Cytometry Method

In previous experiments (Maes, 2002), we found that the routine flow cytometry method used in rat and human blood in our laboratory did not produce consistent, reliable results when performed in mouse blood. This finding led to a thorough literature review and analysis of each reagent, timing, etc., of the sample preparation technique used for

this methodology. Chapter 2 outlines two methodologic questions that we addressed while developing the flow cytometry protocol.

Several sample preparation techniques, particularly erythrocyte lysing, are known to create artifactual cell activation (Macey, et al., 1995; Hageberg, and Lyberg, 2000; Alvarez, Toll, Rivas, and Estella, 2005). Erythrocyte lysing is known to lyse other blood cells, including white blood cells and platelets (Terstappen, Meiners, and Loken, 1989; Alvarez-Larran, et al., 2005), creating microparticles and debris that activate leukocytes (Repo, Jansson, and Leirisalo-Repo, 1993). Additionally, use of erythrocyte lysing agents have demonstrated to increase leukocyte activation, including increased expression of CD11b and shedding of L-selectin (McCarthy, Macey, Cahill, and Newland, 1994; Macey, et al., 1995; Macey, et al., 1999; Alvarez-Larran, Toll, Rivas, and Estella, 2005). Use of erythrocyte lysing provides an easier sample preparation and flow cytometry analysis, but its use is questionable when examining leukocyte and platelet activation markers.

Another approach to leukocyte identification by flow cytometry is to fluorescently label white blood cells. The flow cytometer can then identify leukocytes in the presence of red blood cells and isolate them for analysis. Two fluorescent markers were compared in this study, a nuclear stain, LDS-751 (Hokama, et al., 2000; McDonagh, Hokama, Copeland, and Reynolds, 1997; McCarthy, Macey, Cahill, and Newland, 1994; Macey, et al., 1999), and a fluorescently labeled monoclonal antibody to CD45 (leukocyte common antigen, Hageberg, and Lyberg, 2000; Fugimoto, et al., 2000). The use of LDS-751 was in question, however, as it had been demonstrated in earlier studies to label both live and

dead cells (O'Brien, and Bolton, 1995), had been used to identify platelets and nucleated erythrocytes (Terstappen, 1991). Our experiments demonstrated that use of LDS-751 decreased the fluorescence intensity of both intra- and extracellular labeling with other fluorescently labeled probes in a dose dependent manner, compared to labeling leukocytes with the monoclonal antibody for CD45. The explanation for this decrease is unknown, and may be due to intracellular changes that occur in response to LDS-751. As a result, we determined that use of the leukocyte specific monoclonal antibody to CD45 was the preferred method for labeling leukocytes for flow cytometry analysis in mouse whole blood and this method was used for the experiments in this project.

In addition to erythrocyte lysing, the sample preparation process of cell fixation changes cell function and structure, increasing cell permeability and surface protein-crosslinking (Shapiro, 2003) and produces surface aldehyde groups, making implementation of this method controversial for flow cytometry. Studies have demonstrated that cell fixation decreases fluorescence intensity of antibodies bound to cell surface antigens, including CD11b and CD18 in human blood (McCarthy, Macey, Cahill, and Newland, 1994; Macey, McCarthy, Milne, Cavanaugh, and Newland, 1999). A pivotal study by Hageberg and Lyberg (2000) demonstrated that sample fixation prior to antibody staining produced lower levels of platelet-leukocyte conjugation, that they postulated was more representative of *in vivo* levels. They proposed that during the time needed for cell staining, platelets and leukocytes had time to interact and conjugate, explaining why most studies analyzing platelet-leukocyte conjugation demonstrated levels greater than 50%. The authors of this study felt that these levels of conjugates

were artifactually high and that fixation of samples immediately postvenipuncture demonstrated the 'true' level of *in vivo* platelet leukocyte conjugation.

Because of the results and theoretical implications of the study by Hageberg and Lyberg (2000), we tested fixation prior to antibody staining compared to non-fixed samples. In our study (chapter 2), we found that fixation decreased fluorescence of both intracellular (ROS) and extracellular (CD11b) stains. The decreased fluorescence in samples stained with DCF-DA for ROS measurement was postulated to be due to diffusion of the intracellular stain out of the cell with increased cell permeabilization. We concluded that sample fixation was not appropriate for ROS measurement, because of the loss of the intracellular probe and because ROS measurement requires healthy, viable cells. Additionally, the decrease in CD11b fluorescence was hypothesized to be due to crosslinking of the surface antigen, inhibiting antibody binding. Although, fixation prior to antibody staining still allows for measurement of extracellular markers, we felt that this measurement may be underestimated. Additionally, flow cytometry sample preparation methods generally fix samples *after* antibody staining because of this potential complication. In light of this information, the sample preparation procedure was finalized with the fixation step occurring *after* antibody staining, to be consistent with current literature and to decrease fluorescence underestimation of extracellular stains (in this case, CD11b).

A reanalysis of the variation between duplicate samples in the finalized sample preparation and flow cytometry protocol for leukocytes demonstrated that the improved method decreased the coefficient of variation to less than 10%. The results from the

platelet samples, however, demonstrated a large amount of variability in microparticle formation, as was noted in chapter 4 of this dissertation . This variation was reduced with acquisition of more events, and the HF+STZ groups did not demonstrate the same degree of variation as the previous groups.

### Thrombotic and Inflammatory Changes in Mouse Models of Obesity and Type 2 Diabetes

Mouse models that demonstrate the changes in platelet and leukocyte function observed in obese/diabetic humans are necessary to fully explore the cellular dysfunction that leads to complications of vascular disease, particularly stroke and reperfusion injury. Little research has been conducted examining these processes in obese, type 2 diabetic mouse models. Two of the aims of this dissertation project were to measure the response of 1) platelets and 2) leukocytes to obesity and diabetes prior to stroke and reperfusion injury. During this project, we examined these blood cell functions in four mouse models of obesity and diabetes in order to determine the model that most closely resembles human disease. The next five sections summarize the findings from Chapters 3 through 5; the summary includes platelet and leukocyte markers of activation from each mouse model.

#### *db/db Mouse*

The *db/db* mouse has been used as a representative model of human obesity and type 2 diabetes. The model develops severe obesity and metabolic changes secondary to a spontaneous mutation on the *db* gene that produces an alteration in the long form of the

leptin receptor (LepRb) (Lee, et al., 1996). These mice subsequently become hyperphagic, obese, hyperinsulinemic, and hyperglycemic at an early age (Coleman, & Hummel, 1967). Previous studies examining the coagulation and aggregation response in the *db/db* mouse were conflicting. Earlier studies demonstrated that the *db/db* mice expressed higher levels of circulating plasminogen activator inhibitor type-1, which inhibits fibrinolysis (Samad, Pandey, Bell, & Loskutoff, 2000), and an increase in tissue factor expression (Samad, Pandey, & Loskutoff, 2001). Other studies, however, demonstrated that because of the lack of a functional leptin receptor, these mice developed alterations in thrombosis and inflammation that were not consistent with the human forms of obesity and type 2 diabetes (except rare genetic mutations that produce alterations in leptin signaling (Ahima, & Osei, 2004)). An aim of this dissertation was to determine the thrombotic state in these mice using whole blood methods in order to examine the applicability of the model to human obese and diabetes.

We found that the *db/db* mice developed similar metabolic characteristics as obese and diabetic humans. The mice were significantly obese, hyperglycemic, hypertriglyceridemic, and hypercholesterolemic. Contrary to previous studies (Jones, et al., 1999), the *db/db* mice demonstrated a significant decrease in platelet count that did not appear to be related to differences in platelet clumping after venipuncture (microaggregation) compared to control mice. These mice demonstrated no difference in total white blood count.

Similar to isolated platelet aggregation and *in vivo* arterial injury platelet aggregation studies (Konstantinides, et al., 2001; Rosenblum, El-Sabban, & Loria, 1981),

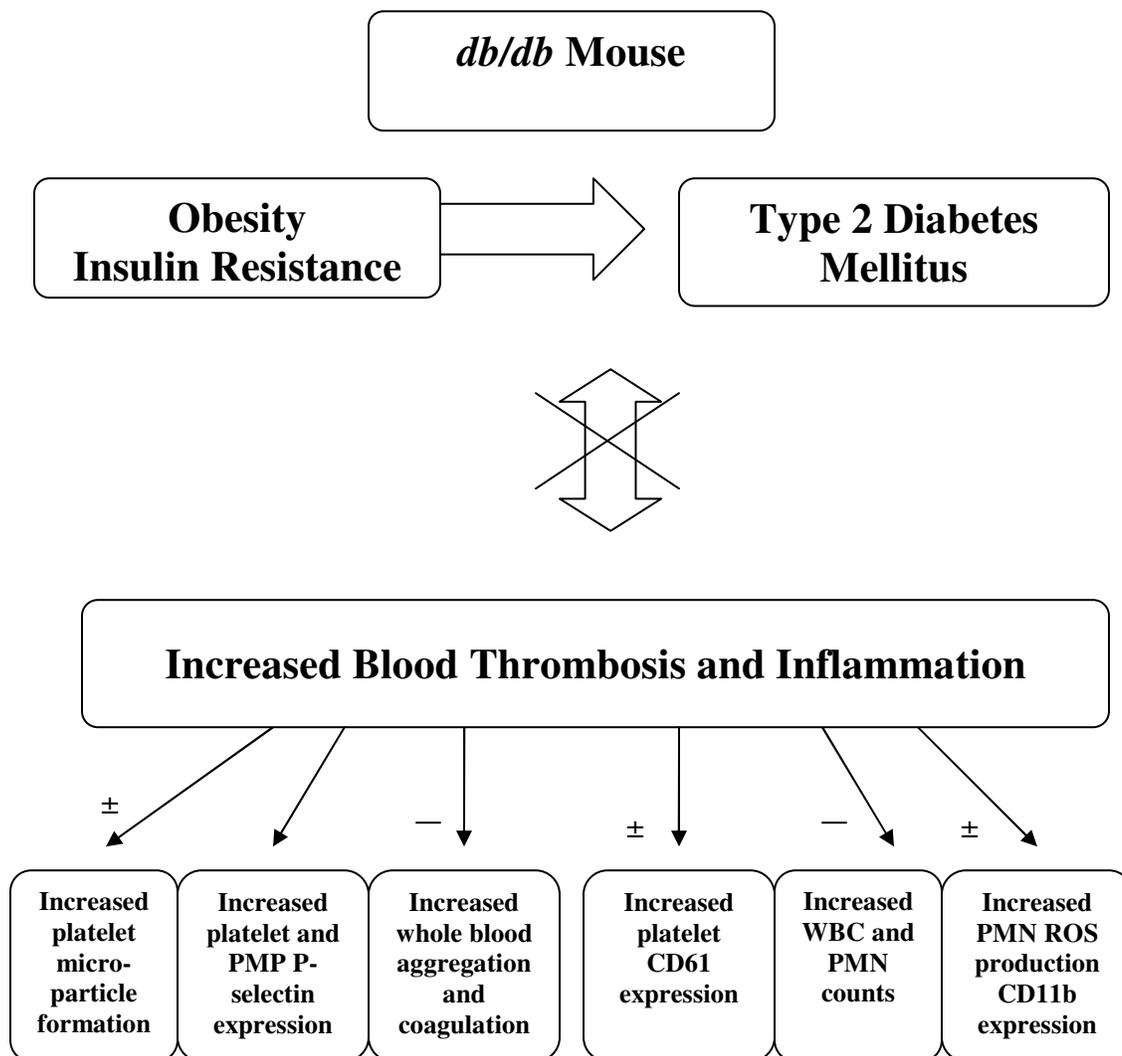
the *db/db* mice in this study demonstrated significantly less whole blood aggregation and coagulation compared to non-diabetic, control mice. The decreased aggregation did not appear to be related to differences in platelet CD61 expression (fibrinogen receptor, important for platelet aggregation), but may have been effected by the lower platelet count in these mice (similar to that seen in the diet induced diabetic mice in chapter 4). Additionally, in previous experiments (master's thesis, Maes, 2002), the *db/db* mice did not demonstrate a difference in PMN CD11b or ROS production compared to non-diabetic mice (data not presented).

In conclusion, the *db/db* mouse model of obesity and type 2 diabetes demonstrated no difference in PMN or inflammatory markers (increase total WBC count) and demonstrated significantly altered whole blood aggregation and coagulation compared to control mice (Figure 7.1). These results may be due to functional changes in platelet and leukocyte function due to the spontaneous mutation in the *db* gene. However, these mice, although severely hyperglycemic and obese, do not represent the human changes in thrombosis and inflammation that occur with obesity and type 2 diabetes and are not well suited to be animal models for examining these processes.

#### *ob/ob Mouse*

Another mouse model often used to examine cellular pathology in obesity and type 2 diabetes is the *ob/ob* mouse. This mouse develops obesity secondary to hyperphagia due to a lack of secreted leptin (Zhang, et al., 1994). The mouse becomes severely obese, and develops hyperinsulinemia and transient hyperglycemia (Dubac, 1976).

Figure 7.1 The effects of obesity and type 2 diabetes in *db/db* mice on thrombotic and inflammatory markers of activation. '+' signifies an increase, '±' no difference, and '-' a decrease in the measurement from non-obese, non-diabetic, control mice. Indices without a symbol were not tested.



Similar to the *db/db* mouse, previous studies have demonstrated that because of the lack of a functional leptin, these mice develop alterations in thrombosis and inflammation that were not consistent to the human forms of obesity and type 2 diabetes (Ahima, & Osei, 2004). As with the *db/db* model, we determined the thrombotic state in the *ob/ob* mice using whole blood methods in order to assess the applicability of the model to human obesity and diabetes.

Similar to the *db/db* mice, the *ob/ob* mice demonstrated metabolic disturbances that are similar to humans with obesity and type 2 diabetes. The mice were obese, hypertriglyceridemic, and hypercholesterolemic. Comparable to previous studies, the mice demonstrated inconsistent elevation in blood sugar; the *ob/ob* mice used for whole blood aggregometry experiments were hyperglycemic, whereas the *ob/ob* mice used for the TEG® experiments were not. The *ob/ob* mice also demonstrated lower platelet counts in all experiments that did not appear to be related to platelet clumping (microaggregation).

The *ob/ob* mice demonstrated significantly less whole blood aggregation and coagulation than control mice. Conversely, the *ob/ob* mice demonstrated an increase in platelet CD61 expression with calcimycin stimulation compared to controls, but this overexpression did not appear to effect aggregation or coagulation. The only inflammatory marker measured in these mice was the total white blood count, which was elevated in the group of mice used for whole blood aggregometry experiments, but not the group used for TEG®. No studies were found reporting WBC counts and it is

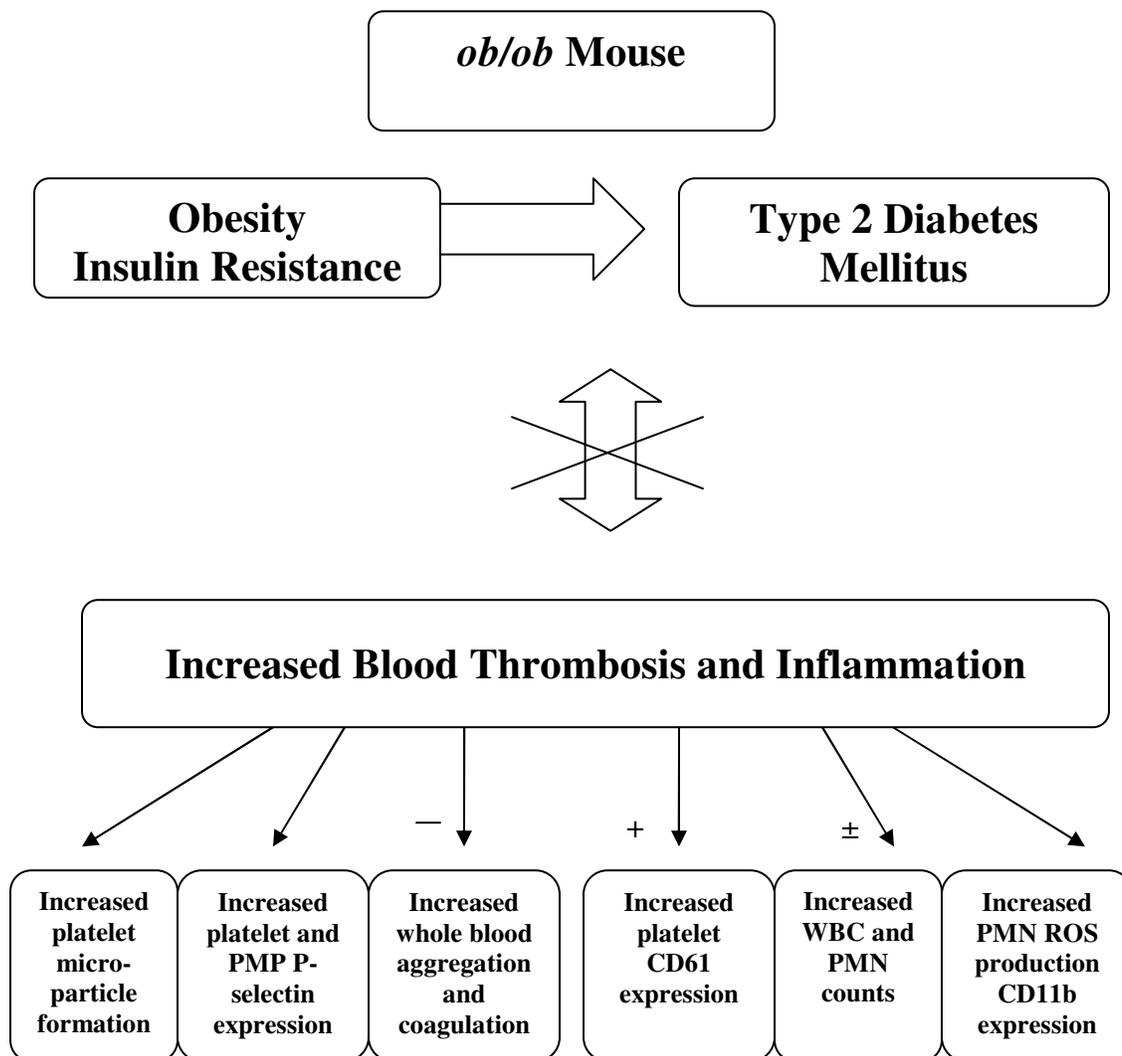
unknown if *ob/ob* mice generally have elevated WBC counts or if the mice used in the aggregation study were ill or stressed.

In conclusion, the *ob/ob* mouse model appears to demonstrate some markers of chronic platelet and leukocyte activation (elevated platelet CD61 expression and increased WBC). However, because of the severe alteration in whole blood aggregation and coagulation, and in light of previous studies that also demonstrated a decrease in platelet function due to leptin deficiency, we conclude that these mice are not suitable models for studying the effects of thrombotic and inflammatory changes in obesity and type 2 diabetes (Figure 7.2).

#### *High Fat Fed Mouse for 4 and 6 Months*

Taking into consideration the findings of the thrombotic properties of *db/db* and *ob/ob* mice, another mouse model of obesity and type 2 diabetes was examined to determine the thrombotic and inflammatory processes in non-genetic obesity and type 2 diabetes in mice. The C57BL/6J strain of mice also develops significant weight gain and subsequent obesity and metabolic characteristics similar to humans with obesity/insulin resistance and early type 2 diabetes when fed a diet enriched in animal fat (Surwit, Kuhn, Cochrane, McCubbin, & Feinglos, 1988; Collins, Martin, Surwit, & Robidoux, 2004). These mice develop hyperleptinemia and leptin resistance, similar to humans, and it was hypothesized that these animals would demonstrate hypercoaguability and increased platelet/PMN activation markers that were decreased in the *db/db* and *ob/ob* mice because of their lack of leptin function.

Figure 7.2 The effects of obesity and type 2 diabetes in *ob/ob* mice on thrombotic and inflammatory markers of activation. '+' signifies an increase, '±' no difference, and '-' a decrease in the measurement from non-obese, non-diabetic, control mice. Indices without a symbol were not tested.



In these experiments (presented in detail in chapters 4 and 5), mice were fed a diet enriched with beef lard (60% total calories) for 4 and 6 months to induce the obese and diabetic state. After four months on the diet, these mice demonstrated similar metabolic characteristics of obese, diabetic humans; displaying significant weight gain, hyperglycemia, hypercholesterolemia, and hyperinsulinemia. After 6 months on the diet, these mice continued to demonstrate similar metabolic changes similar to the mice after 4 months on the diet, although the degree of weight gain and hyperglycemia had begun to decline. The decline in weight could be explained by a decrease in tolerance to the diet at this time point, observed by several of the mice developing skin lesions and dying (this strain of mouse is prone to lesions with high fat diets, Dr. M. Rand, Associate Director, University of Arizona Animal Care, personal communication, June, 2005).

Similar to the *db/db* and *ob/ob* mice, the mice fed the high fat diet for 4 and 6 months also demonstrated significantly lower platelet counts than mice fed a standard chow diet. In later experiments, platelet clumping was found to be significantly higher in mice fed a high fat diet for 2 months (see chapter 4), and it could be postulated that this occurred in these experiments as well. Because of the potential for higher platelet clumping in these mice, it was difficult to determine if the decreased whole blood aggregation was due to this experimental artifact or if these mice demonstrate a true decrease in platelet aggregation compared to chow fed, non-diabetic mice.

To further explore the thrombotic process in these mice, we examined an *in vivo* methodology of coagulation by measuring tail bleeding times. Even though there was no statistical difference, the high fat fed mice did not demonstrate any clotting during the

experiment, whereas, all but one of the chow fed mice had bleeding cessation during the 25 minute experiment time. Additionally, we measured whole blood coagulation parameters with TEG®. The TEG® results corroborated the results from the whole blood aggregometry and tail bleeding times and demonstrated that the high fat fed mice exhibited a decrease in clot formation size and strength. These results were correlated with the lower platelet count in the high fat fed mice, but were also correlated with increased insulin resistance (significantly correlated with weight and insulin levels).

Other platelet parameters (platelet microparticle formation, P-selectin expression) were no different between mice fed a high fat diet for 4 and 6 months and their respective controls. The mice fed a high fat diet for 4 months demonstrated significantly less platelet CD61 expression after stimulation compared to control mice, and this may have also effected the results from the whole blood aggregometry and TEG coagulation experiments. In conclusion, the results from these experiments suggest that high fat fed obese, type 2 diabetic mice do not develop a prothrombotic state similar to humans with these diseases (Figure 7.3 and 7.4). Interestingly, these results suggest that the high fat fed, obese and diabetic mice appear to become hypocoaguable and less thrombotic as insulin resistance and obesity increase. However, the mechanisms of this response are not clear.

The high fat fed mice for 4 and 6 months demonstrated some aspects of a chronic inflammatory state, but impairment of PMN function appears to be related to worsening hyperglycemia and insulin resistance.

Figure 7.3 The effects of obesity and type 2 diabetes in high fat fed mice for 4 months on thrombotic and inflammatory markers of activation. '+' signifies an increase, '±' no difference, and '-' a decrease in the measurement from non-obese, non-diabetic, control mice.

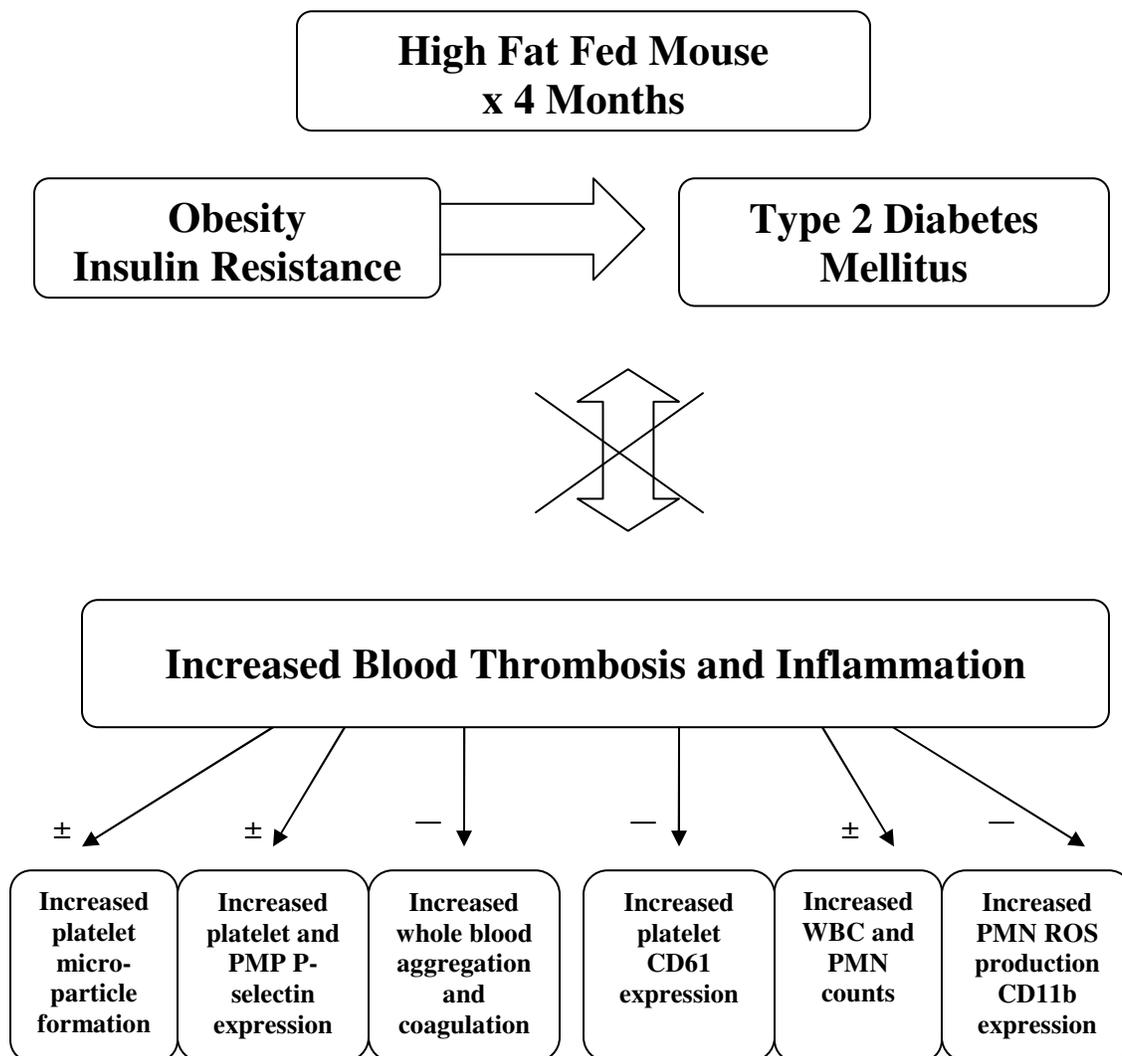
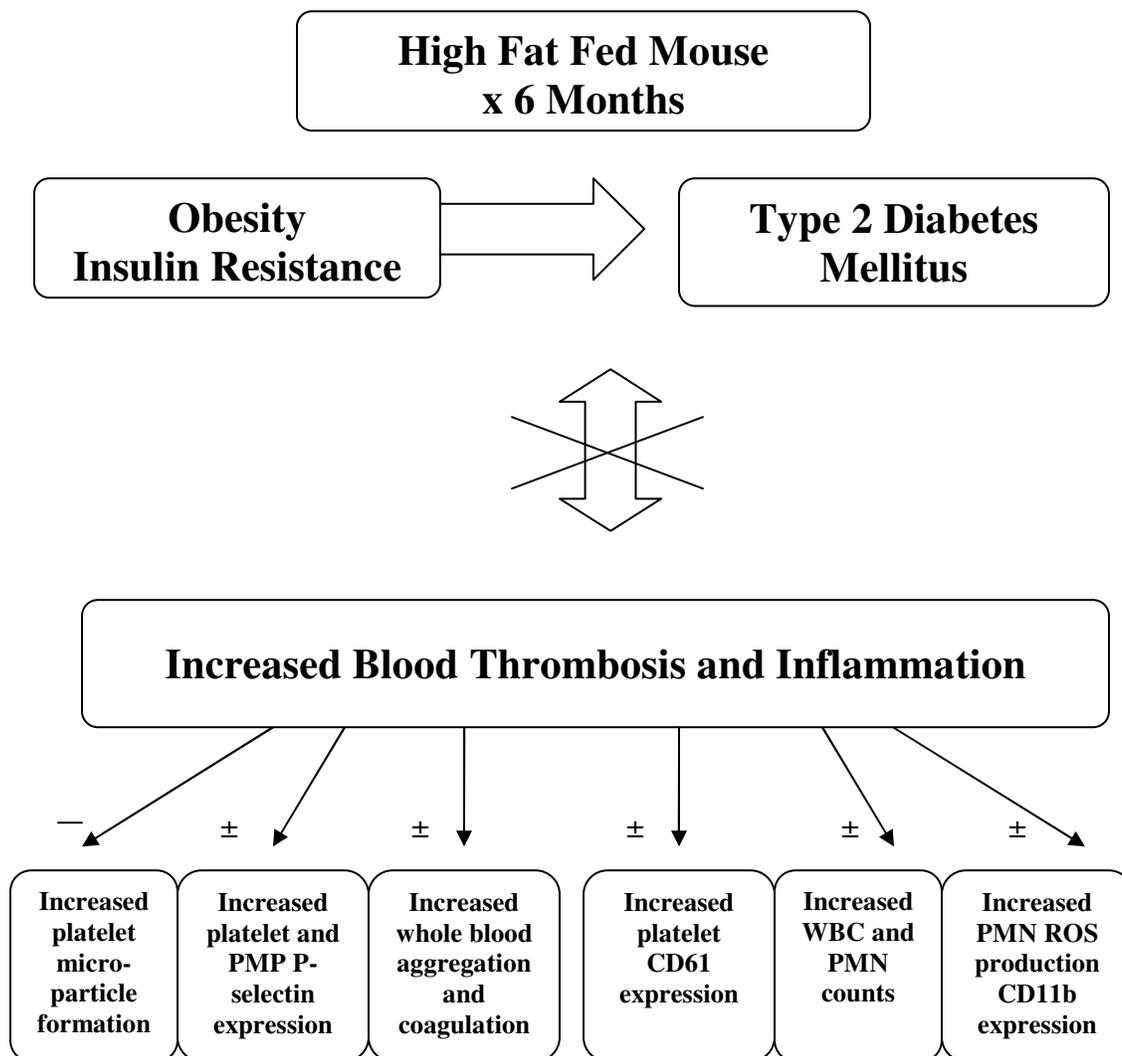


Figure 7.4 The effects of obesity and type 2 diabetes in high fat fed mice for 6 months on thrombotic and inflammatory markers of activation. '+' signifies an increase, '±' no difference, and '-' a decrease in the measurement from non-obese, non-diabetic, control mice.



After 4 months on the high fat diet, obese, diabetic mice demonstrated a significant elevation in WBC count, but after 6 months on the diet the WBC decreased and was no longer different from chow fed, control mice. In contrast to studies in humans, the PMN fraction of the WBC was lower at both time points. PMN function was also altered in the obese diabetic mice, and after 4 months of the diet, PMNs were unable to fully increase expression of CD11b after LPS stimulation compared to chow fed mice.

This result was significantly correlated with increasing blood sugar and HOMA scores, and unlike the coagulation parameters, the CD11b response appears to be related to both blood sugar and insulin resistance. The PMNs of the obese and diabetic mice did not demonstrate any difference in ROS production until after 6 months of the diet, in which ROS production in response to PMA was significantly increased.

In summary, it appears that PMN function is reduced in mice fed a high fat diet (decreased PMN counts, LPS stimulated CD11b expression), although total WBC count was increased after 4 months of the diet. These results suggest that both the thrombotic and inflammatory responses to diet induced obesity and diabetes in mice do not demonstrate thrombotic, platelet, or PMN hyperactivity as seen in humans with these diseases. As such, it appears that these mice would not be suitable mouse models to study the pathogenesis of inflammatory blood changes related to obesity and type 2 diabetes.

#### *High Fat Fed Mouse Injected with Low Dose Streptozotocin*

Neither the *db/db* and *ob/ob* mouse models nor the high fat fed (4 or 6 months) mouse model of obesity and type 2 diabetes demonstrated prothrombotic or inflammatory

changes similar to that seen in human obese and type 2 diabetic patients. The high fat fed mouse model of obesity and diabetes, however, do not demonstrate a severe form of type 2 diabetes. Therefore, we hypothesized that with more severe hyperglycemia, obese/diabetic mice would demonstrate a thrombotic and inflammatory state similar to humans. To test this, we used a mouse model of later stage type 2 diabetes, in which mice are fed a high fat diet for one month, then injected with streptozotocin (STZ), and fed the diet another month. STZ increases nitric oxide species that damage the pancreas (Haluzik, & Nedvidkova, 2000), but the dose delivered to these mice was small and does not damage the pancreas enough to produce type 1 diabetes.

Similar to the previous mouse models, the high fat fed mice injected with STZ demonstrated metabolic changes similar to humans with diabetes, exhibiting significant obesity, hyperglycemia, and hypercholesterolemia. The degree of hyperglycemia and hypercholesterolemia was greater in this model compared with the mice fed a high fat diet alone. Additionally, the high fat +STZ mice demonstrated hyperinsulinemia and elevated HOMA-IR scores that were consistent with insulin resistance, but these results were not statistically significant because of the small number of experiments in these groups.

Similar to that seen previously in the other mouse models of obesity and type 2 diabetes, these mice demonstrated lower platelet counts than the control mice. However, these mice demonstrated significantly more platelet clumping after venipuncture. There was no difference in whole blood aggregation in the high fat fed mice injected with STZ compared to control mice, but because whole blood aggregation is effected by platelet

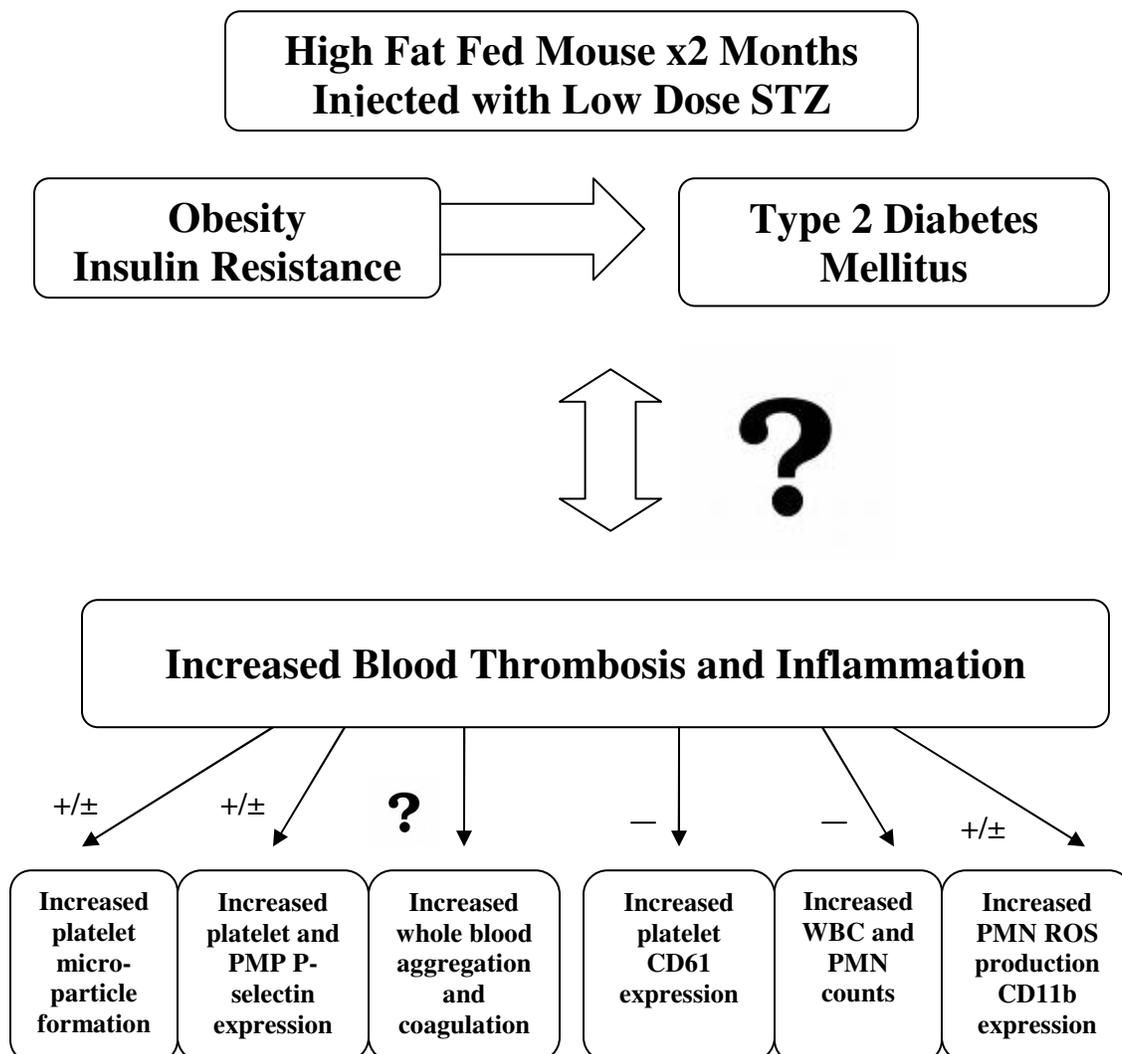
count, the aggregation response may actually have been higher compared to control mice without the clumping of platelets. The measurement of higher levels of platelet clumping may signify that these platelets are hyperaggregable, but that this is not measurable during the whole blood aggregation procedure because platelet aggregates are heavier than single platelets and fall out of solution (WB diluted with saline), and thus are unable to adhere to the measuring probe. There was a non-statistical increase in platelet markers of activation (PMPs, PMP/platelet P-selectin expression) in the high fat fed+ STZ obese, diabetic mice. We postulate that with more experiments (only *n* of 4 in these groups) or with a more prolonged diabetic state (greater than one month diabetes in this study), these mice may have demonstrated a significant increase in these platelet markers compared to chow fed mice.

The PMN response to obesity and a more severe diabetic state in the high fat fed + STZ mice demonstrated a chronic level of inflammation not observed in the other mouse models of obesity and diabetes, although not entirely similar to that seen in humans. The PMN fraction of the WBC count decreased with increasing hyperglycemia, similar to that seen in the high fat fed mice for 4 and 6 months. The explanation for this result is not clear, but may be species related (Mestas, & Hughes, 2004). The decreased production of PMNs in response to obesity and diabetes is contrary to that observed in diabetic humans, in whom the PMN count is elevated (van Oostrom, et al., 2004; Shurtz-Swirski, et al., 2001). The HF+STZ mice demonstrated a significant increase in baseline CD11b expression, although similar to the high fat fed mice, LPS stimulation of CD11b was attenuated in these mice. Both of these changes were significantly correlated with

rising hyperglycemia. Thus, it appears that with rising blood sugar, the PMN numbers are reduced, and the remaining PMNs, although chronically activated, are not able to respond to a stimulus as robustly as PMNs from lean, non-diabetic mice. Again, the reason for this response is not clear and may represent an altered function of PMNs in response to obesity and diabetes similar to humans (McManus, et al., 2001).

From our study it is difficult to determine if the high fat fed +STZ mouse model demonstrates similar thrombotic and inflammatory changes due to obesity and type 2 diabetes as humans (Figure 7.5). The results from the platelet indices were not significant, but may have demonstrated difference with more experiments or with prolonged obesity and diabetes. The PMN count decreased significantly in these mice, and demonstrated chronic low level activation at baseline, but were unable to respond to stimuli as robustly as PMNs from lean, chow fed mice. In conclusion, the high fat fed mouse injected with STZ represents the human alterations of thrombosis and inflammation in obesity and diabetes more closely than the other mouse models tested, but further analysis is needed to fully appreciate these changes.

Figure 7.5 The effects of obesity and type 2 diabetes in high fat fed mice for 2 months injected with low dose STZ on thrombotic and inflammatory markers of activation. '+' signifies an increase, '±' no difference, and '-' a decrease in the measurement from non-obese, non-diabetic, control mice.



## Effects of Obesity and Type 2 Diabetes on Ischemic Stroke and Reperfusion

The final two aims of this project were to examine the 1) infarct size and neurologic function, and 2) changes in platelet and leukocyte function after stroke and reperfusion in obese, type 2 diabetic mice compared to lean, nondiabetic mice. To our knowledge, this is the first study that attempted to examine these effects in obese/diabetic mice.

The MCAO procedure in the mouse is known to have significant rates of mortality (Duckworth, Butler, Mesquita, Collier, Collier, & Pennypacker, 2005) and high interexperimental variability (Connolly, Winfree, Stern, Soloman, & Pinsky, 1996). The MCAO experiments in this project were not immune from these complications; all five of the obese diabetic mice died either intra- or post-operatively. Additionally, two of the five lean, non-diabetic mice died postoperatively and only two of those that lived had measurable infarcts.

While the small number of experiments limits the conclusions that can be made from these results, we found that mice demonstrated circulating PMN and platelet activation in response to ischemic stroke and reperfusion. Although the total white blood count was decreased, mice developed significant neutrophilia after ischemic stroke and 24 hours of reperfusion. The PMNs demonstrated a non-significant increase in oxidative burst and the number of PMNs positive for CD11b was significantly elevated. Additionally, platelet microparticle (PMP) P-selectin expression was significantly elevated after MCAO, consistent with previous research (McCabe, et al., 2004). The numbers of PMPs non-significantly decreased after MCAO and reperfusion due to

sequestration in the microcirculation or the binding to leukocytes. Conversely, because the infarct sizes in the two mice that underwent MCAO and reperfusion were relatively small compared to studies from other laboratories (Hata, et al., 1997), the activation of platelets and PMNs may have been lower than if the mice had developed larger infarcts.

In conclusion, mice appear to demonstrate similar platelet and PMN changes that humans experience after ischemic stroke and reperfusion, and are good models for studying this process. However, because of the technical demands of the procedure, there is high mortality and variability associated with MCAO in mice. Obese, diabetic mice introduce even more technical difficulties because of their larger size, variable response to anesthesia, and worse outcome after surgery. In light of our findings, it may be practical to use a different model of MCAO and reperfusion that demonstrates higher success rates (i. e. rat models), particularly in the setting of obesity and diabetes.

### Conclusions and Future Studies

This dissertation project covered several aims. Initially, we developed a reliable flow cytometry method to measure both platelet and PMN markers of inflammation in mice. The use of flow cytometry and several other whole blood methodologies enabled us to measure indices of thrombosis and inflammation in four mouse models of obesity and type 2 diabetes. We felt that these models did not represent diabetic human alterations in platelets and PMNs, but this knowledge is essential for our laboratory and others to understand the biologic differences in thrombosis and inflammation in obese, diabetic mice. Additionally, we were able to measure these indices after ischemic stroke

and 24 hours of reperfusion, and demonstrated that peripheral blood alterations occur after this event in mice, similar to humans.

This dissertation project encountered some technical difficulties. Because of their size, mice have very small blood volumes that made it difficult to perform several whole blood methods without adaptation to this volume (flow cytometry). Additionally, we found that mice, in general, do not demonstrate thrombotic and inflammatory changes with obesity and diabetes. There are possibilities of altering mice, so that they demonstrate platelet and PMN similarities to humans (i.e. genetic manipulation, bone marrow transplant) that then could be used in future studies of experimental stroke and reperfusion. Finally, mice are known to have high mortality with MCAO, and the obese, diabetic mice were prone to even higher mortality rates. These observations may indicate that to proceed with examining thrombotic and inflammatory changes in obesity and diabetes and their impact on stroke and reperfusion, these experiments may need to be conducted in a different type of animal model or in a human population of diabetics that have a cerebral ischemic event.

The mouse model that demonstrated platelet and PMN changes most closely resembling humans was the high fat fed mouse injected with STZ. This mouse model demonstrated a non-significant increase in expression of platelet and platelet microparticle P-selectin and no difference in whole blood aggregation. Increasing the number of experiments and performing platelet aggregation studies in samples without platelet clumping (addition of prostaglandin to the sample, or *in vivo* measurement of aggregation) may clarify if these mice demonstrate markers of platelet activation and

hyperaggregation. Additionally, with a more prolonged diabetic state (these mice were diabetic for 1 month), the expression of these activation markers may increase and they may demonstrate hyperaggregation with whole blood aggregometry or other platelet aggregation studies.

This study was important to fully understand the blood cell changes in obese, diabetic mice in order to determine their applicability to studying vascular complications in obesity and type 2 diabetes. The results of this project have future implications for development of blood cell biomarkers to predict the progression of obesity and type 2 diabetes to complications of vascular disease. Additionally, this work contributes to the knowledge of platelet and PMN activation after ischemic stroke and reperfusion in mice and may lead to further development of therapeutic treatments for patients with diabetes that develop ischemic stroke.

## APPENDIX A

NEUROLOGIC EVALUATION FOR MICE AFTER CEREBRAL ISCHEMIA AND 24  
HOUR REPERFUSIONComposite Neurological Score  
(Hattori, et al., 2000, and Hata, 1998)

Observe mouse on open bench and lift by tail to measure forelimb strength:

0= no deficit

1= forelimb weakness and torso turning to ipsilateral side when held by its tail

2= circling to affected side, but normal posture at rest

3= unable to bear weight on affected side, leaning to contralateral side at rest

4= no spontaneous locomotor activity or barrel rolling

5= coma

General Neurological Deficit Tests  
(Adapted from Clark, et al., 1997)

The animal is observed on the open bench top without interference or stimulation.

## 1. Hair:

0= Normal- animals have a sleek, clean, and well-groomed coat

1= Animals with piloerection and/or dirt that is confined to one or two areas (usually the nose and the eyes)

2= Animals with piloerection and/or dirt in more than two locations

## 2. Ears:

0= Mouse ears are normally stretched latero-posteriorly, briskly responding in an upright position to noise

1= Lope-eared animals have relaxed ears uni- or bi-laterally without being stretched posteriorly, but respond to noise

2= Lope-eared animals have relaxed ears uni- or bi-laterally without being stretched posteriorly, and do not respond to noise

## 3. Eyes:

0= Eyes that are open, clean, and able to track movements swiftly

1= Open eyes that show a watery, mucous discharge or slowly follow movement

2= Open eyes that display a dark discharge

3= When an eye cleft is not circular but ellipsoid and is accompanied by discharge

4= Closed eyes

4. Posture: the animal is observed on the open bench top, and then placed in the palm of the observer's hand and rocked gently to observed stability.
  - 0= Animal stands upright on four limbs, it's back remains parallel with the ground, and quickly uses its limbs to stabilize during a gentle rocking motion
  - 1= Animal's back is hunched at rest and while walking, and lowers its body during rocking instead of using its limbs to stabilize
  - 2= Animal's head or part of the trunk rests on the ground or it shows evident difficulty of stabilization
  - 3= Animal reclines to one side, may be able to turn to an upright position with strain
  - 4= Animal lies as placed and does not turn to an upright, prone position
  
5. Spontaneous activity: the animal is allowed to move on the open bench top, in an area at least 4 ft<sup>2</sup>.
  - 0= An alert animal that vigorously explores
  - 1= An animal that appears alert but is calm and quiet, and starts and stops exploring repeatedly and slowly
  - 2= A listless animal that moves sluggishly in place, but does not explore
  - 3= An animal that is lethargic or stuporous barely moves in place
  - 4= An animal that does not move spontaneous except for occasional response upon handling
  
6. Epileptic behavior: the animal is observed in the cage and on the open bench top. The observation of the epileptic behavior at any time during the experiment is counted, not just during the direct scoring period for that animal. Scores for epileptic behavior are higher than other scores to account for masking of other signs that may be caused by epileptic behavior.
  - 0= An animal without epileptic manifestations
  - 3= An animal that is timid and dramatically bolts from handling, and may exhibit hyperactivity such as repeated climbing of the cage wall or aimless rocking motions
  - 6= An animal that appears aggressive and tense with a glazed look and hyperexcitability
  - 9= Extreme hyperexcitability demonstrated by gallop-like running following stimulation or Jackson-type (focal) seizures
  - 12= Grand mal seizures with change in breathing or consciousness

Focal Neurological Deficit Tests  
(Adapted from Clark, et al., 1997)

7. Body symmetry: the animal is observed at rest on the open bench top without handling.
  - 0= Normal animals have both sides of the body off the ground, with all limbs symmetrically placed under the body, and the tail extends straight back behind the animal
  - 1= Slight asymmetry with the body leaning to the paretic side (or animal rests on its back legs), and the tail is consistently away from the midline. Limb asymmetry is not evident
  - 2= Moderate asymmetry with the body leaning to the paretic side (or animal rests on its back legs), paretic limbs are extended outward, and the tail is extended away from the midline
  - 3= Prominent asymmetry with the body curving and the paretic side leaning on the ground
  - 4= Extreme asymmetry with body and tail tightly curved with paretic side constantly on the ground
  
8. Gait: the animal is observed in motion in the open bench top.
  - 0= A normal gait that is flexible, symmetrical, and fast
  - 1= A mechanical gait, with a stiff, inflexible walk, hunch-backed posture while moving, or slower speed
  - 2= Slight limping, with asymmetry in grasping or during motion
  - 3= Heavy limping, drifting, or falling, with obvious deficiency in gait
  - 4= Does not walk spontaneously, and motion upon stimulation is confined to three steps
  
9. Climbing: the animal is placed in the middle of a gripping surface held at 45° angle from the bench top. For a gripping surface we use 40 X 20 cm foam.
  - 0= An animal that quickly climbs to the top or bottom edge of the surface
  - 1= An animal that slowly climbs with a visible strain
  - 2= An animal that can hold its place on the slope, but does not advance upward
  - 3= An animal that gradually slips downward
  - 4= An animal that immediately falls downward with no attempt to prevent fall
  
10. Circling behavior: the animal is observed in motion in the open bench top.
  - 0= A normal animal that that turns to both the right and left equally
  - 1= Predominantly turns to one side
  - 2= Circles to one side, although not constantly
  - 3= Circles steadily to one side only
  - 4= Pivoting or swaying, sluggish circling in place, or no movement

11. Front limb symmetry: the animal is suspended over the bench top by its tail, and the position and motion of the front limbs are observed.
- 0= Both limbs are extended to the bench top and vigorously moving
  - 1= Light asymmetry between limbs, with paretic limb not fully extended or flexing rapidly
  - 2= Moderate asymmetry with paretic limb slightly adducted to chest, accompanied by slight twisting of the body towards the paretic sides
  - 3= Prominent asymmetry with paretic limb steadily flexed and adducted to the chest
  - 4= Limbs move slightly or do not move, asymmetry may be present but is light
12. Compulsory circling: the animal is lifted by the tail, with only the front limbs on the bench top with a hard smooth surface. In this handstand position, limb weakness is displayed by a circling behavior caused when the animal extends its forelimbs and attempts forward motion.
- 0= An animal that extends both forelimbs equally
  - 1= An animal that extends both forelimbs, but starts to circle predominantly to one side
  - 2= An animal that circles only to one side, may show slower motion than healthier animals
  - 3= An animal that pivots sluggishly to one side, does not rotate a full circle
  - 4= An animal that does not advance, upper body may rest on the bench top, and movements are brief and slow
13. Sensory response: on the open bench top, the observer uses a pen or similar instrument to test the animal's response. Approaching from behind the animal with a pen, the base of the whiskers and the tip of the ears are touched lightly.
- 0= Symmetrical response, including turning of the head toward the stimulation and moving away from the stimulation
  - 1= Delayed or muted response from the paretic side, with normal response from the opposite side
  - 2= Absent response from the paretic side, normal response from the opposite side
  - 3= Absent response from the paretic side, diminished response from the opposite side
  - 4= Proprioceptive response absent bilateral

APPENDIX B

VERIFICATION OF PARTICIPATION

Institutional Animal Care  
and Use Committee



P.O. Box 210101  
Tucson, AZ 85721-0101

**Verification of Participation  
For Dissertation Files**

By The Institutional Animal Care and Use Committee (IACUC)  
PHS Assurance No. A-3248-01 -- USDA No. 86-3

Name of Participant/Certification Number:

**Melissa Maes, BS - #5685**

IACUC Assigned Protocols - PI/Department:

**#02-165 - "Inflammatory and Thrombotic Blood Cell Interactions in Stroke"  
Leslie S. Ritter, PhD - Neurology/College of Nursing**

Participation Status was Confirmed: **August 25, 2005**

Linda S. Musgrave, IACUC Program Coordinator

Institutional Official: Leslie P. Tolbert, Ph.D.  
Vice President for Research

DATE: August 26, 2005

DATE: August 26, 2005

## APPENDIX C

## VERIFICATION OF REVIEW 2002-2005

Institutional Animal Care  
and Use Committee



P.O. Box 210101  
Tucson, AZ 85721-0101

Verification of Review  
By The Institutional Animal Care and Use Committee (IACUC)  
PHS Assurance No. A-3248-01 -- USDA No. 86-3

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use. The following listed proposal has been granted *Final Approval* according to the review policies of the IACUC:

PROTOCOL CONTROL NUMBER/TITLE:

**#02-165 - "Inflammatory and Thrombotic Blood Cell Interactions in Stroke"**

PRINCIPAL INVESTIGATOR/DEPARTMENT:

**Leslie S. Ritter, PhD - Neurology/College of Nursing**

GRANTING AGENCY:

**NIH**

SUBMISSION DATE: **September 13, 2002**

APPROVAL DATE: **November 21, 2002**      APPROVAL VALID THROUGH\*: **November 20, 2005**

\*When projects or grant periods extend past the above noted expiration date, the Principal Investigator will submit a new protocol proposal for full review. Following IACUC review, a new Protocol Control Number and Expiration Date will be assigned.

REVIEW STATUS FOR THIS PROJECT WAS CONFIRMED ON: **November 22, 2002**

REVISIONS (if any):

MINORITY OPINIONS (if any):

*Richard C. Powell*

Richard C. Powell, PhD, MS  
Vice President for Research

DATE: November 22, 2002

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.

## APPENDIX D

## VERIFICATION OF REVIEW 2005-2006

Institutional Animal Care  
and Use Committee



P.O. Box 210101  
Tucson, AZ 85721-0101

**Verification of Review**  
**By The Institutional Animal Care and Use Committee (IACUC)**  
**PHS Assurance No. A-3248-01 -- USDA No. 86-3**

The University of Arizona IACUC reviews all sections of proposals relating to animal care and use.  
The following listed proposal has been granted *Final Approval* according to the review policies of the IACUC:

PRINCIPAL INVESTIGATOR/DEPARTMENT: **Leslie Ritter, PhD - Nursing/Neurology**

PROTOCOL CONTROL NUMBER/TITLE:

**#05-171 - "Inflammatory and Thrombotic Blood Cell Interactions in Stroke"**

ACTIVE AUTHORIZATION PERIOD\*:

INITIATION DATE: **December 19, 2005**

EXPIRATION DATE: **December 18, 2008**

\* Projects scheduled to continue longer than the originally approved 3-year authorization period, will require the submission of a new protocol proposal to undergo full review. Following IACUC review, a new Protocol Control Number and Authorization Period will be assigned to commence immediately following the original expiration date.

GRANTING AGENCY: **NIH**

REVIEW INFORMATION:

SUBMISSION DATE: **November 16, 2005**

FINAL REVIEW DATE: **December 15, 2005**

RESULTS OF GRANT TO PROTOCOL REVIEW:

No Significant Discrepancies noted    Discrepancies noted below    Not Applicable:

REVISIONS/MINORITY OPINIONS (if any):

*Leslie P. Tolbert*

Institutional Official: Leslie P. Tolbert, PhD  
Vice President for Research

AUTHORIZATION STATUS FOR THIS PROJECT WAS CONFIRMED ON: **December 20, 2005**

This approval authorizes only information as submitted on the Animal Protocol Review Form, Amendments, and any supplemental information contained in the file noted as reviewed and approved by the IACUC.

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