CHRONIC NOREPINEPHRINE SUPPRESSION INDUCES A COMPENSATORY B-CELL ADAPTATION THAT ENHANCES INSULIN SECRETION AFTER ALLEVIATION OF THE CATECHOLAMINE INHIBITION IN FETAL SHEEP

By

Xiaochuan Chen

_____

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF ANIMAL SCIENCES

In Partial Fulfillment of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2012

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Xiaochuan Chen

Entitled CHRONIC NOREPINEPHRINE SUPPRESSION INDUCES A COMPENSATORY B-CELL ADAPTATION THAT ENHANCES INSULIN SECRETION AFTER ALLEVIATION OF THE CATECHOLAMINE INHIBITION IN FETAL SHEEP

And recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

_____ Date: 4/13/2012
Dr. Ronald E. Allen

_____ Date: 4/13/2012
Dr. Robert J. Collier

_____ Date: 4/13/2012
Dr. Kurt J. Griffin

_____ Date: 4/13/2012
Dr. Sean W. Limesand

_____ Date: 4/13/2012
Dr. Ronald M. Lynch

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

_____ Date: 4/20/2012
Dissertation Director: Dr. Sean W. Limesand

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in The University Library to be made available to borrowers under rules of the Library.

Brief quotations from this thesis are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Xiaochuan Chen

ACKNOWLEDGEMENTS

This dissertation is dedicated foremost to my parents for their constant support and encouragement for all my endeavors in the pursuit of this degree. I thank my parents, Yu Chen and Xiaomei Fei, for instilling the importance of higher education and giving me the motivation to achieve my goals. I would like to thank all the professors, who have helped me throughout my graduate studies at The University of Arizona. I deeply appreciate my advisor, Dr. Sean W. Limesand, for his support, guidance, and friendship. He is warm-hearted, knowledgeable, and a patient mentor that has been a major influence throughout my master and doctoral training. I would like to thank my fellow laboratory members, Mrs. Miranda J. Anderson, Mrs. Mandie M. Dunham, Dr. Alice S. Green, Mr. Octavio B. Mendivil, Mr. Antoni R. Macko, Dr. Kyle L. Flann, Ms. Amy C. Kelly, Dr. Laura E. Wright, Dr. Dustin T. Yates, and Dr. Catherine M. Herway, who aided me in my research and raised my spirit every day. Finally, I am deeply grateful to all those who collaborated in bringing this project to fruition.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Placental insufficiency-induced intrauterine growth restriction (IUGR) increases risk of mortality and morbidity in newborn infants and domestic animals. IUGR fetuses are typically exposed to prolonged hypoxemia, hypoglycemia, and hypercatecholaminemia, which results in perinatal pancreatic β-cell dysfunction. Recent evidence indicates that chronic exposure to norepinephrine *in utero* suppresses insulin secretion through α2-adrenergic receptors (ARs), but if the adrenergic actions are blocked compensatory hyper insulin secretion response is observed in the IUGR sheep fetus. In the current studies, we demonstrate that chronic NE exposure alone can produce the compensatory enhancement of β-cell responsiveness following termination of a chronic NE infusion. In the fetus NE was continuously infused at 1-4 μg/min for seven days starting at 131 days of gestational age (term = 145 days). During treatment, NE infused fetuses had higher ($P < 0.05$) plasma NE concentrations and lower ($P < 0.01$) insulin concentrations than vehicle infused control fetuses. Glucose stimulated insulin secretion (GSIS), which measures β-cell function, prior to NE treatment was not different between treatments. However, insulin concentrations during hyperglycemic steady state period of GSIS studies and area under the curve of glucose-potentiated arginine-induced insulin secretion were higher ($P < 0.01$) than control values and this augmentation was confirmed at 3 hours, 24 hours, and five days in NE-infused fetuses after discontinuing the infusion. Pancreatic islets isolated within 10 hours post NE infusion had lower ($P < 0.05$) mRNA

expression of α1D (58%), α2A (43%), α2C (42%), β1 (67%) adrenergic receptors (ARs), and uncoupling protein 2 (40%) compared to islets from controls. Isolated islets from NE-infused fetuses 5 days after NE treatment had lower ($P < 0.05$) inhibitory responsiveness from NE and a greater ($P < 0.05$) maximal insulin release with glucose simulation in static incubations compared to controls. These findings show that following chronic NE exposure insulin secretion responsiveness was augmented and was coupled with desensitized adrenergic signaling. Moreover, this compensatory β-cell enhancement persists for days indicating chronic NE exposure permanently alters β-cell responsiveness.

CHAPTER I

INTRODUCTION

Intrauterine growth restriction (IUGR) is associated with increased perinatal morbidity and mortality, which limits profits in domestic livestock production and increases public health care costs in human populations (Hales and Barker, 2001; Wu et al., 2006). To survive insufficient nutrient supply *in utero*, a common etiology for IUGR, the fetus responds by sparing nutrients for critical organs. This results in asymmetric growth in fetuses because neuronal tissues are preserved at the expense of visceral organs, thus identifying a phenotype for fetuses with IUGR. In growth restricted fetuses, the physiological function of their organs also adapts and these adaptations can lead to a pathophysiology in metabolism in adulthood that manifests as human metabolic diseases or remains unforeseen to slow postnatal performance traits in livestock production systems (Barker, 1995; Chen et al., 2010; Desai et al., 1995; Langley and Jackson, 1994; Limesand et al., 2005; Swenne et al., 1987).

Type 2 diabetes is one of the adult onset metabolic disorders that can manifest from IUGR. Human fetuses complicated with IUGR are hypoxemia, hypoglycemia, and have glucose intolerance (Nicolini et al., 1990). These findings indicate that β-cell dysfunction is an antecedent for the metabolic disorders in adulthood (Hales et al., 1991). Several models of IUGR exist for fetal sheep to characterize the *in utero* adaptation and to define mechanisms that formulate the persistent β-cell dysfunction. One model of IUGR that shares all of

the symptoms described in human pregnancy is hyperthermia-induced placental insufficiency and IUGR sheep fetus (Bell et al., 1987). It was shown that sheep fetuses with placental insufficiency have hypoxemia, hypoglycemia, hypoinsulinemia, reduced pancreatic β-cell mass (Limesand et al., 2005), and impaired β-cell function (Limesand et al., 2006). Another key factor found in this model that correlates with human IUGR fetuses is elevated plasma norepinephrine concentrations, due to fetal hypoxemia and hypoglycemia (Greenough et al., 1990; Limesand et al., 2006; Paulick et al., 1985). Elevated fetal norepinephrine has been shown to slow fetal growth rates by suppressing insulin secretion, a major anabolic hormone in the fetus (Fowden, 1989; Fowden and Hay, 1988; Fowden et al., 1986). Chronic norepinephrine exposure has also been associated with β-cell dysfunction, which in this case appears to enhanced β-cell glucose responsiveness if the adrenergic response is blocked with receptor antagonists (Leos et al., 2010).

The aim of this dissertation is to investigate the effect of chronic norepinephrine exposure as a fetal programming mechanism for pancreatic β-cell dysfunction. We determine whether chronic norepinephrine suppression contributes the dysregulation of insulin secretion during the chronic exposure and whether this leads to a compensatory augmention in insulin secretion responsiveness after chronic exposure in normal uncompromised sheep fetuses. It is also unknown if this causes the permanent impairment in β-cell responsiveness, leading to metabolic complications in the later life. Understanding the mechanisms

underlying the abnormalities of β-cell function associated with elevated norepinephrine will be necessary to identify potential therapeutic targets to alleviate the complications of IUGR fetus in both the livestock industry and human health.

CHAPTER II

LITERATURE REVIEW

Intrauterine Growth Restriction in Human

Events during early development have a profound impact on one's risk for developing adult metabolic diseases. Fetuses developing in poor nutrient condition undergo global metabolic reprogramming to promote fetal survival. This altered fetal program is essential to preserve normal neuron development at the expense of systemic growth of the fetus (Barker et al., 2009; Roseboom et al., 2001). Barker and Osmond (Barker and Osmond, 1986) were the first to postulate that altered development *in utero* contributes to permanent metabolic, physiologic, and structural alterations in postnatal life, resulting in the manifestations of adult diseases.

According to the World Health Organization (WHO, 2002), birth weight is one of the most sensitive and important indications of future health outcomes in children. Low birth weight (LBW) in humans has been defined as a weight at birth less than 2500 grams. LBW can be a consequence of preterm birth, defined as birth before 37 completed weeks of gestation, or due to small size for gestational age, SGA, defined as weight for gestation <10th percentile, or both. An infant suffering from IUGR is also defined as being below the 10% percentile of the recommended gender-specific birth weight for gestational age reference curves (Williams et al., 1982). In contrast to SGA, IUGR refers to a reduction in expected fetal growth trajectory. Multiple adverse conditions inhibit the fetuses' ability to

reach their genetic growth potential. IUGR is usually responsible for SGA, but not all IUGR infants are SGA.

IUGR is a major public health problem in developing countries. The incidence of LBW infants can be as high as 30% in Latin America. In those LBW infants, prematurity accounts for 5-7%, the rest is a result of IUGR caused by chronic fetal malnutrition (Villar and Belizan, 1982). There are two different types of IUGR, symmetric and asymmetric growth restriction. Growth restriction beginning in early gestation is symmetric IUGR and characterized by all internal organs being reduced in size proportionally. Symmetric IUGR accounts for 20% to 25% of all cases of IUGR. On the other hand, asymmetric IUGR, caused by interference with growth later in gestation, is characterized by the head being normal in size, but the abdomen is spared (Lockwood and Weiner, 1986).

In humans, LBW and disproportion in head circumference, length, and weight, are important phenotypic markers of fetal compensation to malnutrition. Epidemiological studies indicate that low birth weight, caused by poor nutrition *in utero*, is closely associated with increased mortality in childhood and an increased incidence of metabolic diseases in later life. The pathogenesis is not based on phenotype but on altered genetic expression as a consequence of an adaptation to environmental changes during fetal development (Breier et al., 2001). Clinical and experimental studies have shown that maternal nutrient status was a key factor that affects prenatal and postnatal health outcomes (Barker and Clark, 1997; Godfrey and Barker, 2000).

The Dutch famine (1944-1945) is a tragic event in history that makes a compelling and clear illustration of the adverse outcomes that arise in nutrient deprived fetuses (Ravelli et al., 1976). During a discrete period during World War II, the daily nutritional intake of pregnant women was approximately 400 to 800 calorie, not sufficient to sustain their energy requirements (Ravelli et al., 1998). Fetuses exposed to nutrient restriction during pregnancy were compared with babies born before or after the famine. Because of the discrete period of famine, early, mid, and late exposure to caloric restriction could be examined. Infants born after famine exposed during mid or late gestation had lower birth weights, lengths, and placental weights compared to those exposed during early gestation or unexposed. Interestingly, glucose intolerance was a consequence of Dutch famine progeny. Fetus exposed to the famine had higher fasting proinsulin concentration, and higher 120 min plasma glucose and insulin concentration than those unexposed (Ravelli et al., 1998).

Glucose intolerance could become one of the critical factors associated with the permanent β-cells dysfunction in the later life (Hales et al., 1991; Ravelli et al., 1998). IUGR human fetuses exhibit a slower return to euglycemic level and have a blunted insulin response following a glucose challenge (Nicolini et al., 1990). Human IUGR fetuses have marked reductions in endocrine pancreas area and lower plasma insulin concentrations at term (Economides et al., 1991; van Assche and Aerts, 1979; Van Assche et al., 1977). Postnatally, infants with

severe IUGR have a higher incidence of β-cell dysfunction, glucose intolerance, and type 2 diabetes in adult life (Cook et al., 1993; Hales et al., 1991).

Intrauterine Growth Restriction in Livestock

IUGR is also a major problem in domestic animal production. A variety of practical production situations, including multifetal pregnancies due to improved reproductive management, undernutrition because of limited forage, illness, and exposure to warm or cold environment, cause impairment of fetal growth. The significance of IUGR in livestock production can be divided into four areas: 1) prenatal and perinatal death losses, 2) postnatal growth rates, 3) reproductive fecundity, and 4) carcass characteristics at slaughter (Wu et al., 2006). These areas may not be recognized until adulthood, or remain as an unforeseen expense absorbed by livestock producers.

In sheep, adverse effects of IUGR are related to higher death losses at birth, reduced rate of growth, and poor wool and meat production in the later life (De Blasio et al., 2007a; Shelton, 1964). Foals with lower birth weights have stunted athletic performance (Platt, 1978), whereas, the larger piglets tend to grow faster, compared to smaller littermates (Campbell and Dunkin, 1982; Dwyer et al., 1993). Thus, fetal growth restriction during pregnancy is associated with poor quality, quantity, and performance of animals for food production.

Skeletal muscle development is impaired in small piglets (Campbell and Dunkin, 1982; Dwyer et al., 1993). Primary muscle fibers formed are the same in both

large and small littermates, however, secondary muscle fibers are fewer in the smaller piglets with 17% fewer total fibers number formed at birth (Wigmore and Stickland, 1983). As a result, they generally have less muscle fibers in prenatal life and impaired weight gain during the postnatal period (Handel and Stickland, 1987; Wigmore and Stickland, 1983). The body fat is higher in low birth weight pigs than in the high birth weight group at one year old age (Poore and Fowden, 2004). A fatter carcass and higher muscle lipid content at market, indicating a poorer meat quality, were reported in those pigs with low birth weight (Gondret et al., 2005; Powell and Aberle, 1980). Relative lower lean to fat mass also indicates to a risk factor of health, including insulin insensitivity and glucose intolerance (Kahn et al., 2001).

Ewes fed 50% of their regular nutrient exhibit fetal growth restriction at mid gestation (Vonnahme et al., 2003). These IUGR fetuses have fewer nephrons in the kidney, indicating a higher incidence of cardiovascular disease (Gopalakrishnan et al., 2005). They also have slower growth rates and less carcass quality with a higher fat to lean mass ratio (Ford et al., 2007).

Chronic environmental heat stress during pregnancy induces IUGR in sheep (Bell et al., 1987), causing higher mortality at birth (Shelton, 1964), dysregulation of insulin-glucose metabolism (Limesand et al., 2006), and higher risk of adipose deposit in later life (Chen et al., 2010). High ambient temperatures are unavoidable in many geographical regions, and contribute to fetal nutritional deprivations and higher production costs due to higher perinatal morbidity and

mortality. Those neonates have slower postnatal growth rates, impaired functions of organs, and lower efficiency of feed utilization in domestic animals (Alexander and Williams, 1971; Bell et al., 1987).

Adaptation of Complications in IUGR

In order to promote their survival, IUGR fetuses develop a thrift phenotype to initiate a sparing response mechanism to adapt to a poor nutrition environment (Hales and Barker, 2001). The function of insulin is to promote both prenatal and postnatal growth performance by activating glucose uptake in the peripheral tissues, including liver, adipose, and skeletal muscle. In addition, insulin is one of the key factors to exert systemic glucose sparing effect in poor nutrient environment. Lower plasma insulin helps redirect limited glucose from insulin sensitive tissues to insulin insensitive brain and nervous tissue, which are critical for fetal survival (Hales and Barker, 2001).

Therefore, insulin plays an important role to mediate growth path during intrauterine development. However, how fetal adaptation in poor nutrient environment associated with impairment of β-cell function is not fully answered. Understanding fetal programming mechanisms in pancreatic β-cell dysfunction, which impair glucose homeostasis, will allow us to improve the health of small offspring in both domestic livestock and human.

Insulin

The β-cell of the pancreatic islet is the only cell type that synthesizes and secrets insulin in response to nutrient secretagogues (Nussey and Whitehead, 2001). Insulin is released in pulses every 10 min or so and has a half-life in the systemic circulation of approximately 3 min in human (Nussey and Whitehead, 2001) and 23 min in fetal sheep (Colwill et al., 1970). In order to maintain blood glucose concentrations, insulin acts on the liver, adipose tissue, and muscle to stimulate uptake, utilization, and storage of glucose (Nussey and Whitehead, 2001). Insulin is also a major growth-promoting hormone in fetus (Fowden et al., 1989; Hill, 1982). Insulin deficiency induced by pancreatectomy significantly lowers growth of skeleton and soft tissues and lowers metabolism in the sheep fetus (Fowden et al., 1986). Exogenously infusing insulin in those fetuses could restore the growth rate back to normal values (Fowden et al., 1989). Therefore, the appropriate production and secretion of insulin ensure a normal fetal growth *in utero*.

Glucose stimulated insulin secretion

Insulin secretion increases in response to elevated blood glucose concentrations (Figure 2.1). Glucose is transported into the β-cell by facilitated diffusion through the glucose transporter (GLUT) 2, which is a high capacity and low affinity glucose transporter on the plasma membrane (Efrat, 1997; Guillam et al., 1997). Once glucose enters the β-cell, metabolic pathways, such as glycolysis, Krebs cycle, and respiratory chain, metabolize glucose to form the high-energy

compound ATP. The rise in cellular ATP/ADP ratio initiates an insulin-releasing signal, by closing an ATP-regulated K^+ (K_{ATP}) channel, depolarizing the β-cell and opening voltage-dependent Ca^{2+} channels. The influx of Ca^{2+} in the β-cell triggers the exocytosis of insulin stored in secretory granules (Lang, 1999).

This K_{ATP}-dependent process initiates the first phase of insulin secretion. It is very rapid and secretion of insulin occurs within one minute of glucose exposure (Straub and Sharp, 2002). A few minutes later after docked insulin granules have been depleted, there is a more gradual and sustained increase in insulin release that can last for several hours in normal individuals and is dependent on *de novo* synthesis of insulin (Straub and Sharp, 2004). Newly synthesized insulin plays an integral part in refilling insulin storage (Curry et al., 1968). K_{ATP}-independent actions help to sustain insulin secretion for several hours if elevated hyperglycemia persists (Curry et al., 1968; Henquin et al., 2006; Wang and Thurmond, 2009). However, the rate of production of sustained insulin secretion might be influenced by the first phase GSIS (Henquin et al., 2006). Therefore, β-cell function is comprehensively coordinated by hyperglycemia to ensure the entire insulin release.

A common feature of insulin release requires an influx of Ca^{2+} from outside of the cell into the cytosol (Ammala et al., 1993; Ashcroft and Gribble, 1999; Jitrapakdee et al., 2010; Matschinsky et al., 1998; Straub and Sharp, 2004) . The L-type Ca^{2+} channel is responsible for the entry of Ca^{2+} across the plasma membrane and determines exocytosis in β-cells (Gopel et al., 2004). Opening

and closing of these L-type Ca^{2+} channels are mediated by the β-cell membrane potential, which is in turn regulated by the activity of the K_{ATP} channel. In pancreatic β-cells, the K_{ATP} channel is formed from two types of subunits: a pore-forming subunit, Kir6.2, and a regulatory subunit, the sulphonylurea receptor 1 (SUR1) (Inagaki et al., 1997). Pancreatic β-cells isolated from homozygous Kir6.2 knockout mice lack K_{ATP} channel activity, leading to the defect in GSIS (Miki et al., 1998). Mutations of SUR1 commonly abolish the ability of the K_{ATP} channel to response to increased ATP/ADP, resulting in impairing β-cell function and causing diabetes (Nestorowicz et al., 1996; Otonkoski et al., 1999; Shyng et al., 1998; Thomas et al., 1995a). Thus, the K_{ATP} channel is important and require for normal insulin release.

The endoplasmic reticulum (ER) is also a major site for Ca^{2+} storage and supplement cytosolic increase of Ca^{2+} in β-cells (Borge et al., 2002; Luciani et al., 2009; Shepherd et al., 2000). This is different from K_{ATP} channel stimulated Ca^{2+} influx from extracellular sources (Miki et al., 1998). Sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) is a protein responsible for Ca^{2+} uptake into the endoplasmic reticulum (ER) lumen. Ca^{2+} release from the ER is mediated by inositol 1,4,5-trisphosphate (IP3) via IP3 receptors (IP3Rs) and cyclic adenosine diphosphate ribose via the ryanodine receptors (RyRs) (Putney, 1999; Rossi and Sorrentino, 2010). Abnormalities in ER Ca^{2+} regulation cause the development of type 2 diabetes in several rodent models and human (Levy et al., 1994; Roe et al., 1994). In the diabetic mice (Roe et al., 1994) and rats (Varadi et al., 1996), a

loss of SERCA activity is associated with defects in the patterns of glucose-stimulated changes in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and insulin secretion. By mediating with Ca^{2+} mobilization, the expressions of IP3Rs and RyRs are also associated with GSIS from β-cells (Lee et al., 1999; Luciani et al., 2009).

Regulation of insulin secretion

Various regulators associated with insulin secretion signaling pathway ensure normal β-cell function. Glucose is the key secretagogue for normal insulin secretion (Figure 2.1). Other nutrients, including non-esterified fatty acids (NEFAs) and certain amino acids are also insulin secretagogues. Meanwhile, the maintenance of this β-cell responsiveness requires hormones, such as glucagon-like peptide 1 and catecholamines, to positively or negatively regulate insulin secretion.

Nutrients

As discussed above, the fuel hypothesis is the primary stimulant for insulin secretion by increase ATP production (Shepherd et al., 2000). An anaplerotic enzyme, pyruvate carboxylase (PC) catalyzes the formation oxaloacetate from pyruvate, increases the metabolism of glucose, indicating a positive correlation with the rate of insulin secretion in β-cell (MacDonald, 1993a, b). Reduction of PC expression in INS-1 and rat islets decreases GSIS responsiveness during incubation (Hasan et al., 2008; Xu et al., 2008). The activity and expression of PC are also decreased in pancreatic islets of human patients with type 2

diabetes (MacDonald et al., 2009). As the rate-limiting enzyme during glucose metabolism, the activity of PC plays important role to influence insulin secretion from β-cell.

Uncoupling protein 2 (UCP2) is another modulator of β-cell metabolism to affect insulin secretion by regulating proton leak in the mitochondria, (Fleury et al., 1997). By creating a mitochondrial proton leak, UCP2 separates oxidative phosphorylation from ATP synthesis with energy dissipated as heat. Thus, overexpression of UCP2 lowers the net increase of ATP generation and inhibits GSIS in rat islets (Chan et al., 1999). UCP2 knockout mice exhibit improved GSIS and lower glucose concentrations during an intraperitoneal glucose tolerance test (Dalgaard, 2011; Zhang et al., 2001). Therefore, the activity of those enzymes associated with glucose metabolism influence insulin secretion from β-cell.

NEFAs also potentiate insulin release in response to glucose and non-glucose secretagogues (Dobbins et al., 1998b; Prentki et al., 2002). The concentration of NEFA in plasma directly modifies effects on insulin secretion and clearance (Dobbins et al., 1998b; Stein et al., 1996). Studies with isolated pancreatic islets or insulin secreting cell lines from rodents as well as humans, and in perfused pancreas models both *ex vivo* as well as *in vivo*, have clearly defined a stimulatory role for fatty acids in both basal and glucose-stimulated insulin secretion (Balent et al., 2002; Boden et al., 1998; Crespin et al., 1973; Dobbins et al., 1998a; Dobbins et al., 1998b; Stein et al., 1996). The underling mechanism is

that glucose-induced increase of citrate levels increases the concentrations of malonyl-CoA in the β-cell, which inhibits fatty acid oxidation by decreasing carnitine palmitoyl transferase-1 activity. As a result, an increased *de novo* lipid synthesis elevates levels of long-chain acyl CoA, and stimulates diacylglycerol through protein kinase C (PKC) signaling (Brun et al., 1993; Corkey et al., 1989), resulting in the sustained insulin secretion in β-cell. NEFA also involves with binding to the G-protein-coupled receptor GPR40 on the β-cell membrane, resulting in elevating intracellular Ca^{2+} to stimulate exocytosis (Briscoe et al., 2003; Itoh et al., 2003). Fatty acyl-CoA generation is also responsible for exocytosis of insulin by acting PKC signaling in the β-cell (Prentki et al., 2002).

Amino acids can also directly or indirectly stimulate insulin secretion. Arginine is a potentiator of insulin release and stimulates insulin secretion in the presence of glucose (Hermans et al., 1987). As a positively charged amino acid, L-arginine directly activates insulin granule exocytosis, by depolarizing the plasma membrane of β-cell and stimulating Ca^{2+} influx in the β-cell (Smith et al., 1997). L-glutamate participates in nutrient induced insulin secretion, as a potentiating factor to stimulate GSIS (Maechler and Wollheim, 1999). It has allosteric effects on insulin secretory granules to promote Ca^{2+}-dependent exocytosis in the β-cell (Hoy et al., 2002). Overexpression of L-glutamate decarboxylase causes a reduced glutamate concentration with a lower GSIS in INS-1 cells (Rubi et al., 2001). Leucine and metabolic intermediates also play a significant to control insulin secretion in β-cell. It is believed that leucine or its transaminated product

α-ketoisocaproate could directly regulate K_{ATP} channel, resulting in increase of $[Ca^{2+}]_i$ (Branstrom et al., 1998; Gao et al., 2003). Leucine also could indirectly control insulin secretion by enhancing glutaminolysis through allosterically activating glutamate dehydrogenase, a key enzyme controlling the oxidation of glutamate (Smith and Stanley, 2008). Thus, any defect in those regulators of intracellular glucose metabolism impairs insulin secretion from β-cells.

Adrenergic signaling

Acute exposure to catecholamines, including epinephrine and norepinephrine, inhibits insulin secretion from pancreatic β-cell (Figure 2.1). The inhibitory action of norepinephrine is specific to α2-ARs, since neither α1- nor β-adrenergic agonists support insulin secretion (Laychock and Bilgin, 1987). Norepinephrine binds to α2-ARs and inhibits insulin secretion through multiple pathways by 1) activating ATP-sensitive K^+ channels and repolarizing of the β-cells; 2) inhibiting L-type Ca^{2+} channels; 3) decreasing activity of adenylate cyclase; and 4) inhibiting of exocytosis of insulin vesicle from pancreatic β-cells (Sharp, 1996; Zhao et al., 2010). Exogenous elevation of cAMP and Ca^{2+} into adrenergically suppressed β-cell only partially restores insulin secretion during norepinephrine inhibition (Nakaki et al., 1983; Nilsson et al., 1988), therefore, norepinephrine must also act via inhibition of glucose utilization in β-cell to lower K_{ATP}-independent pathways in pancreatic islets (Laychock and Bilgin, 1987).

Chronic exposure to high plasma catecholamines concentrations results in a loss of total receptor-binding sites with an accompanying loss in downstream effector

stimulation (Benovic et al., 1990; Chen et al., 2010; Collins et al., 1991). Overexpression of α2A-AR in pancreatic β-cells suppresses insulin secretion and exhibits altered regulation of glucose homeostasis, resulting in glucose intolerance in mice (Devedjian et al., 2000). Blocking α2A adrenergic signaling in those cells by antagonist yohimbine recovers insulin release back to normal (Rosengren et al., 2010). On the other hand, α2A-AR deficient mice exhibit a lack of inhibitory function to catecholamines in pancreatic β-cell, resulting in hyperinsulinemia and reduced blood glucose (Fagerholm et al., 2004; Savontaus et al., 2008). Hence, nutrients and hormones modify insulin secretion from pancreatic β-cells, by either stimulating or inhibiting the regulators in glucose stimulated insulin secretion signaling pathway.

Catecholamines

Catecholamines are synthesized from the amino acid tyrosine and are secreted from the adrenal chromaffin cells as well as sympathetic neurons (Sherwood, 2007). A cascade of enzymes catalyzes the whole process of catecholamine synthesis. Tyrosine hydroxylase is the rate-limiting step in the conversion of tyrosine to dihydroxyphenylalanine. The final step in the generation of epinephrine from norepinephrine is catalyzed by phenylethanolamine N-methyltransferase (PNMT), which is induced by cortisol at late gestational age (Wong, 2006). Catecholamines are stored within secretory granules with chromogranin and released in response to activate coupled Ca^{2+} influxes, such as stimulation from hypoxemia and stress (Nussey and Whitehead, 2001; Wong,

2006). The half-life of catecholamines is very short, such as a couple of minutes (Eisenhofer et al., 2004; Padbury et al., 1986). Plasma catecholamine concentrations are regulated by synthesis, secretion, and degradation, but their varied biological actions are regulated by multiple adrenergic receptors (ARs). Monoamine oxidase and catechol-O-methyltranferase are enzymes that degrade catecholamines in the liver and kidneys (Nussey and Whitehead, 2001).

Epinephrine and norepinephrine act through ARs, which belong to G-protein coupled receptors, to maintain metabolic homeostasis of the body (Altan et al., 2007; Schaak et al., 2007). There are three main types of ARs, α1-ARs, α2-ARs and β-ARs, and each has multiple subtypes, including α1A-, α1B- and α1D-AR, α2A-, α2B- and α2C-AR, and β1-, β2- and β3-AR. The α1-ARs are Gq-associated receptors that stimulate IP3 and increase intracellular Ca^{2+}; α2-ARs are G_i-associated receptors that suppress cAMP and lower intracellular Ca^{2+}; and β-AR are G_s-associated receptors that stimulate adenylate cyclase to increase intercellular cAMP concentrations. By binding to different subtype of ARs, catecholamines activate or inhibit different signal transduction pathways to regulate physiological function of tissues and organs (Nussey and Whitehead, 2001).

During fetal development catecholamine synthesis begins at mid-gestation. Due to lower PNMT concentrations in the fetal adrenal medulla, norepinephrine, instead of epinephrine, is the predominant catecholamine secreted into the fetal circulation (Simonetta et al., 1997). Catecholamines do not cross the placenta

from either maternal or fetal circulation (Bzoskie et al., 1995; Saarikoski, 1974). The major role is to maintain and promote the maturation of cardiovascular and respiration system fetus (Butler et al., 1995; Thomas et al., 1995b).

In human fetuses, norepinephrine concentrations increase during hypoxemia and are higher in IUGR fetuses (Divers et al., 1981; Greenough et al., 1990; Paulick et al., 1985). Not only do catecholamines inhibit fetal insulin secretion from β-cell, but they also augment blood pressure, activate gluconeogenesis and glycogenolysis in the liver, and promote lipolysis of fat (Sherwood, 2007), resulting in impairment of insulin-glucose homeostasis IUGR fetuses. Chronic norepinephrine exposure has the potential effect on insulin secretion by manipulating adrenergic signaling during fetal development. Permanent disruption of ARs in β-cell may contribute fetal adaptation associated with impaired β-cell function (Leos et al., 2010), leading to permanent metabolic complications in the later life.

Impairment of β-cell Function in IUGR Animal Models

An appropriate animal model is critical and necessary to understand the pathologic and physiologic mechanisms that impair β-cell function during IUGR. Animal models have the advantage to study several kinds of treatment effects associated with IUGR, including overnutrition, undernutrition, environment factors, disease, and toxins. Animal model systems also make it possible to investigate acute, long-term, and chronic effects by avoiding ethical issue in human (Holemans et al., 2003). Maternal hyperthermia (Bell et al., 1987; Galan

et al., 1999; Regnault et al., 2002b), underfeeding (Dumortier et al., 2007; Vonnahme et al., 2003; Young and Widdowson, 1975) and overfeeding adolescent (Wallace et al., 1996; Wallace et al., 2004), uterine carunclectomy (De Blasio et al., 2007a; Gatford et al., 2008; Owens et al., 1989; Robinson et al., 1979), and umbilical artery ligation (UAL) (Emmanouilides et al., 1968; Oh et al., 1975) are popular methods to induce IUGR in the animal models and disrupt maternal nutrient supply to the fetus. Important features are similar to human IUGR, including hypoxemia, hypoglycemia, hypoinsulinemia, elevated norepinephrine concentration, lower birth weight, and asymmetrical growth (Morrison, 2008; Wallace et al., 2005).

Maternal undernutrition model

Maternal nutrition restriction decreases fetal growth and placental weight in guinea pig (Young and Widdowson, 1975), rat (Boujendar et al., 2002; Dumortier et al., 2007; Garofano et al., 1997), and sheep (Ford et al., 2007; Luther et al., 2007; Vonnahme et al., 2003). Among those animals, insufficient maternal nutrition is associated with impaired fetal β-cell function and metabolic complications in their postnatal life.

Maternal undernutrition with 30% of food restriction in guinea pigs reduces birth weight in male offspring. In addition, pancreatic β-cell function is reprogrammed with higher fasting plasma insulin, higher insulin-glucose ratio, and higher plasma glucose area under the curve during glucose tolerance tests (Kind et al., 2003).

Maternal 50% caloric restriction in pregnant Wistar rats from day 15 of gestation leads to an 18% reduction in birth weight of the offspring (Garofano et al., 1997, 1998). Pancreas weight, insulin content, islet density, and β-cell mass are all reduced at 1 day old. Insulin content and β-cell mass remain decreased at 21 days old, from impairment of β-cell differentiation (Garofano et al., 1997). Another similar study also shows low energy diet with 50% restriction of the daily calories intake lowers 12% fetal growth at 21 days of gestational age (dGA) with significantly decreased pancreas and liver weight of rats (Dumortier et al., 2007). This low energy diet raises the fetal corticosterone concentration, which in turn lowers the expression of pancreatic transcription factors, including neurogenin 3 and PDX-1, resulting in inhibited β-cell differentiation and lower β-cell mass (Dumortier et al., 2007).

Not only caloric restriction, but also low protein diets, 8% protein versus 20% in control dams during the last week of gestation, lowers fetal weight, pancreas weight, β-cell mass, and islet size in Wistar rat (Boujendar et al., 2002; Dumortier et al., 2007). In contrast to the decreased differentiation of gene expression induced by caloric restriction, low protein diet leads to lower proliferation and increased apoptosis of β-cells with normal differentiation rates (Boujendar et al., 2002; Dumortier et al., 2007). Lower expression of vascular endothelial growth factor (VEGF), VEGF receptor 2, and insulin-like growth factor (IGF) 2 in islets is associated with this lower vascularization and proliferation rates that decrease β-cell mass (Boujendar et al., 2003; Dumortier et al., 2007; Petrik et al., 1998).

However, because of immature development of rat β-cells (Hole et al., 1988; Rorsman et al., 1989), neither control nor nutrient restricted fetal islets respond to a glucose challenge during insulin responsiveness test (Cherif et al., 1996).

In adult sheep, severe maternal undernutrition arising in any part of pregnancy decreases fetal growth rate (Ford et al., 2007; Vonnahme et al., 2003). Growth restriction is exacerbated, if the undernutrition occurs later in gestation. Restriction of 50% energy intake in pregnant ewes causes 32% reduction of fetal weight at 80 dGA (Vonnahme et al., 2003). In another underfeeding study of pregnant sheep, restriction of feed intake to 70% of recommended levels lead to lower angiogenic growth factors in the whole placentome, compromised placental vascular development (Luther et al., 2007), and reduced uterine blood flow (Chandler et al., 1985). The smaller fetuses have lower plasma glucose concentrations and a greater brain to liver weight ratio at 130 dGA (Luther et al., 2007). As the result, insulin and glucose concentrations are significantly lower in undernutrition induced IUGR fetuses (Osgerby et al., 2002). Fetal glucose utilization is not changed, because enhanced fetal gluconeogenesis from liver supplements to the lack of placental glucose supply in undernourished ewes (Leury et al., 1990). In postnatal life, maternal undernutrition at early to mid-gestation impairs β-cell function and results in glucose intolerance at 63 days of age, and a lower insulin response at 280 days of age (Ford et al., 2007).

In a similar study on pregnant ewes, a strong relationship between maternal undernutrition and impaired insulin-glucose homeostasis was demonstrated,

because of glucose intolerance and insulin resistance in their offspring (Gardner et al., 2005). Lambs from nutrient restricted ewes exhibit greater fat-to-lean ratio at 280 days of age (Ford et al., 2007) and 1 year of age (Gardner et al., 2005), indicating higher adiposity in adulthood. Similar as the aforementioned characters of human IUGR, maternal undernutrition in various animal models significantly disrupts placental and fetal growth *in utero*, permanently impairs physiological function of organs, and metabolic complications in the adult life.

Umbilical artery ligation model

Ligaturing around the umbilical arteries, UAL, minimizes blood circulation in the uterus. Decreased blood supply causes severe IUGR in pigs (Lafeber et al., 1984), rats (Emmanouilides et al., 1968), and sheep (Emmanouilides et al., 1968; Hobel et al., 1970). In guinea pig, UAL reduces maternal placental blood flow and impairs the growth and development of the fetuses, leading to 40% lower body weight in one third of fetuses (Lafeber et al., 1984). Small fetuses exhibit significantly lower plasma glucose, insulin (Jones et al., 1984; Jones et al., 1990), resulting in asymmetric fetal growth (Lafeber et al., 1984).

In sheep model, single UAL reduces fetoplacental blood flow (Supramaniam et al., 2006), produces hypoxemia (Emmanouilides et al., 1968; Hobel et al., 1970), and increases fetal brain-to-liver weight ratio (Newnham et al., 1986). Chronic elevation of norepinephrine and epinephrine concentration is observed after UAL (Newnham et al., 1986) and causes blunt pulmonary function associated with disrupted adrenergic signaling (Oyama et al., 1992).

Uterine artery ligation in rats at 19.5 dGA restricts fetal and placental growth as well as lowers fetal insulin concentration (De Prins et al., 1983; De Prins and Van Assche, 1982). As an important trans-activating factor of maturation of β-cells, PDX-1 mRNA expression is lower in UAL fetuses and adults (Park et al., 2008). Postnatally, β-cell mass and proliferation rate are significantly reduced in rats born following UAL (De Prins and Van Assche, 1982; Simmons et al., 2001). They have impaired glucose tolerance, insulin sensitivity, and insulin secretion in the first week of age (Simmons et al., 2001) indicating higher risk of metabolic complications in their later life.

Uterine carunclectomy model

The uterine carunclectomy of placental restriction (UC-PR) is another well-established IUGR sheep model (McMillen et al., 2001). Unlike humans and rodents, sheep have a cotyledonary placenta, which compose about 100 placentomes, as the functional units for nutrient exchange between mother and fetus. In this model most of endometrial caruncles, the maternal portion of the placentomes, are surgically removed from the uterus of the non-pregnant ewe prior to mating. This lowers the number of placentation sites and restricts placental growth, causing fetal growth restriction (Robinson et al., 1979).

In UC-PR ewes, placental and fetal weights are 64% and 30% reduced, respectively, compared with controls at 0.96 of gestation (Simonetta et al., 1997). Decreased fetal and placental weights are associated with poor placental function, especially leading to chronic fetal hypoxemia and hypoglycemia (Owens

et al., 1989; Phillips et al., 1996). UC-PR impairs of β-cell function, lowers glucose- and arginine-stimulated insulin secretion, and leads to glucose intolerance (Owens et al., 2007), without affecting insulin sensitivity of peripheral tissues (Owens et al., 2007) and β-cell area or mass (Gatford et al., 2008) in the near term fetal sheep.

The weight of the adrenal gland is increased and plasma cortisol concentration is elevated (Morrison, 2008). Even though the expression of PNMT in chromaffin cells is low during fetal stage, enhanced cortisol could stimulate PNMT by regulating PNMT mRNA to generate epinephrine by converting norepinephrine in adrenal medulla of UC-PR fetuses (Wong, 2006). As the result, fetal plasma concentrations of both epinephrine and norepinephrine are approximately two times higher in the UC-PR than in the control group throughout late gestation (Simonetta et al., 1997).

Postnatally, UC-PR neonates at 35 days of age exhibit impaired glucose tolerance due to β-cell insufficiencies (De Blasio et al., 2007a). Even though fasting plasma insulin concentration is not different, glucose tolerance is impaired, revealing a depressed insulin secretion for the first 15 min (De Blasio et al., 2007a). UC-PR lambs also exhibit lower maximum plasma glucose concentration and decreased rate of glucose clearance from 30–50 min during glucose tolerance test. Body mass is not different in young UC-PR sheep, but visceral fat, including perirenal, retroperitoneal, and omental fat, in absolute and relative terms, are increased, reflecting the decrease in muscle mass (De Blasio

et al., 2007a). Young UC-PR sheep at 18 months postnatal age fails to maintain adequate insulin secretion despite compensatory enhanced β-cell mass in male (Gatford et al., 2008). Overall, UC-PR impairs growth performance and induces a defect in pancreatic β-cell function, consistent with the human IUGR during prenatal and postnatal life (Hales et al., 1991; Hales and Ozanne, 2003).

Environmental heat stress model

Increased mortality rates and lower birth weights in sheep lambs was observed in the summer months in northern Australia (Shelton and Huston, 1968). Experimental conditions, designed to mimic this environmental heat stress showed sheep fetuses are growth restricted (Bell et al., 1987; Limesand et al., 2006; Thureen et al., 1992). Subsequent reports on heat stress in pregnant sheep show that abnormalities in placental structures and functions impair the uteroplacental transportation of nutrients and oxygen, resulting in placental insufficiency (Galan et al., 2001; Limesand et al., 2004; Regnault et al., 2003; Regnault et al., 2002a). As a result, fetal growth restriction induced by heat stress exhibits increased brain to liver weight ratio, restricted peripheral tissues growth, and reprogramed function of organs *in utero* (de Vrijer et al., 2004; Regnault et al., 1999).

In those IUGR fetal sheep placental growth is significantly impaired, resulting in lower placental weight, decreased number, diameter, and surface area of villi, and reduce arterial numbers in placenta (Regnault et al., 2003; Regnault et al., 2002b). Consequently, these abnormalities in placental structure lower nutrient

and oxygen transportation capacity and restrict fetal growth. IUGR fetal sheep at 0.85 of gestation, after 57 days in hyperthermic treatment, exhibit 47% lower glucose concentration and 69% decreased insulin secretion than normal fetuses (Limesand et al., 2006). They also have 38% lower pO_2 with relative 4 times higher norepinephrine concentration compared to controls (Limesand et al., 2006). The plasma norepinephrine concentrations of placental insufficiency induced IUGR (PI-IUGR) fetuses are negatively associated with the total arterial blood oxygen content (Limesand et al., 2006). In addition to this lower umbilical nutrients uptake, the unchanged glucose utilization in PI-IUGR fetuses also appears to require fetal glucose production, indicating synthesis the *de novo* glucose (Hay et al., 1984; Limesand et al., 2007). Enhanced norepinephrine will stimulate hepatic glucose production via gluconeogenesis in PI-IUGR, by activating hepatic gluconeogenic enzymes, such as phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, and cAMP response element binding protein. By regulating insulin secretion, elevated plasma catecholamine concentrations may also redirect the glucose to the vital organs, such as the brain, to supply adequate nutrients for normal neurological development (Rudolph, 1984). As a result, IUGR fetuses have to produce 40% of the glucose to meet energy demands and increase insulin sensitivity in peripheral tissues to adapt to this malnutrition *in utero* (Limesand et al., 2007).

The pancreas is significantly compromised in heat stress induced IUGR sheep fetuses (Van Assche et al., 1977). They have 59% lower pancreas weight, 76%

less β-cell mass, and slower rates of β-cell replication at 0.9 of gestation (Limesand et al., 2005). Less pancreatic progenitor epithelium at the beginning of the third trimester (unpublished data) and decreased growth factors, including IGF and FGF, have been shown to partially account for the lower of β-cell mass (Chen et al., 2012).

Consequently, they exhibit lower plasma insulin concentration and responsiveness to the glucose and other non-glucose secretagogues (Limesand et al., 2006). However, administration of AR antagonists blocks both α- and β-ARs and provides a full recovery of GSIS responsiveness in PI-IUGR fetal sheep (Leos et al., 2010), indicating an enhanced compensatory β-cell responsiveness with relative lower β-cell mass. Postnatally, since there is no stress responsive to stimulate catecholamine, newborn IUGR lambs show greater GSIS than normal lambs at 8-day age (unpublished data). Therefore, chronic hyperthermia causes growth restriction associated with placental insufficiency. Impairment of β-cell function and dysregulation of glucose homeostasis are present in IUGR prenatal life.

Summary of β-cell Dysfunction in IUGR Animal Models

Understanding the mechanisms underlying impaired regulation of insulin secretion could provide significant information to allow us to better manage IUGR animals and design therapeutic strategies to improve the β-cell function in IUGR infants. As mentioned above, hypoxemia and hypoglycemia are common features among IUGR fetuses. Cells in the adrenal medulla synthesize and

secrete norepinephrine in response to a series of stimuli, such as acute hypoxemia (Padbury et al., 1987a). The oxygen-sensitive K^+ channels depolarize the chromaffin cells, activating the voltage-gated L-type Ca^{2+} channels to increase $[Ca^{2+}]_i$ concentrations and trigger the release of catecholamine (Douglas and Rubin, 1963, 1961; Vogt, 1952). Clinic studies have shown that IUGR human fetuses suffer chronic higher norepinephrine (Divers et al., 1981; Fowden and Forhead, 2004) with related lower insulin concentration (Nicolini et al., 1990) compare to uncompromised fetuses during pregnancy.

IUGR sheep fetuses adapt to placental insufficient environment associated with hypoxemia, hypoinsulinemia, hypoglycemia, elevated norepinephrine (Limesand et al., 2006). Chronic enhancement of norepinephrine *in utero* is closely associated with impairment of GSIS (Limesand et al., 2006), damage pancreatic β-cell mass in IUGR fetuses (Limesand et al., 2005), compensatory enhanced β-cell responsiveness after removing adrenergic inhibitory function (Leos et al., 2010). Whether only the chronic norepinephrine suppression contributes the dysregulation of insulin secretion through adrenergic signaling during fetal programming is not determined. It is also unknown if this causes the permanent impairment of insulin-glucose metabolism, leading to metabolic complications in the later life. Investigating the mechanism underlying the abnormalities of β-cell function associated with chronic elevated norepinephrine will be necessary to identify potential therapeutic targets to alleviate the complications of IUGR in both livestock industry and human public health.

Advantage of Ovine Model

Pregnant sheep provide advantages as an animal model to study the development and physiological function of pancreatic β-cell from IUGR (Green et al., 2010; Regnault et al., 2002a). Compared to rodent model, the sheep pancreas development shares more similarities, in both morphological and temporal aspects, to humans (Cole et al., 2009; Green et al., 2010). Pancreas development is a highly coordinated process with structural and cytological characteristics. Pancreas originates from the dorsal and ventral region of the foregut endoderm at 0.50 of gestation in the mouse and rat, which is much later than 0.16 of gestation in the sheep, and 0.09 in the human (Cole et al., 2009; Green et al., 2010). In addition, onset of mature islets cell replication and isletogenesis also exhibits temporal differences among species. The formation of islet-like structures happens earlier in humans and sheep than in rodents, who do not form until 0.78 of gestation (Jensen et al., 2000; Slack, 1995). In humans and sheep, however, aggregates begin forming shortly after the onset of differentiation, resulting in primitive islets by 0.27 of gestation in humans (Bocian-Sobkowska et al., 1999; Nieto, 2002; Piper et al., 2004) and 0.23 in sheep (Cole et al., 2009).

On the insulin secretion aspect, human fetal β-cells exhibit integrated insulin response following the glucose challenge during late gestational age (Nicolini et al., 1990). Rat β-cells only show a poor insulin responsive to nutrients during fetal life compared to adulthood (Bliss and Sharp, 1994). Because of an immature

oxidative metabolism pathway in fetal β-cells, glucose fails to generate the proper signal for the initiation of the insulin secretion (Hole et al., 1988; Rorsman et al., 1989). Also, K_{ATP}-channels are insensitive to increased ATP/ADP levels (Rorsman et al., 1989) and fail to depolarize the β-cell membrane (Hole et al., 1988), even though L-type Ca^{2+} channels are present in the islet at embryonic day (E) 19.5 (Hole et al., 1988). The ability of glucose to stimulate insulin secretion could not be detected at E 15, 17, or 18 (Rhoten, 1980). GSIS responsiveness of fetal rats during late gestational age is limited, usually monophasic and insensitive to Ca^{2+} channel blockers (Hole et al., 1988), which is different from the matured β-cell in adult rat with biphasic insulin release (Curry et al., 1968).

In sheep, the insulin response to glucose and arginine are detectable early in the mid-gestation. The β-cell responsiveness continues to increase over the second half of gestation and reach a maximum at 130 dGA (Aldoretta et al., 1998). Similar as the rat during perinatal stage (Mendonca et al., 1998), newborn lambs have much higher GSIS responsiveness than the fetuses before parturition (Philipps et al., 1978). The enhancement of β-cell responsiveness in sheep is consistent with insulin-glucose relationship, induced by the altering of internal environment (Philipps et al., 1978), but different from the further maturation of insulin signaling pathway in the rat β-cells (Hole et al., 1988).

It is practical to maintain indwelling catheters in the sheep fetus for long periods during gestation to study chronic outcome after administrating norepinephrine

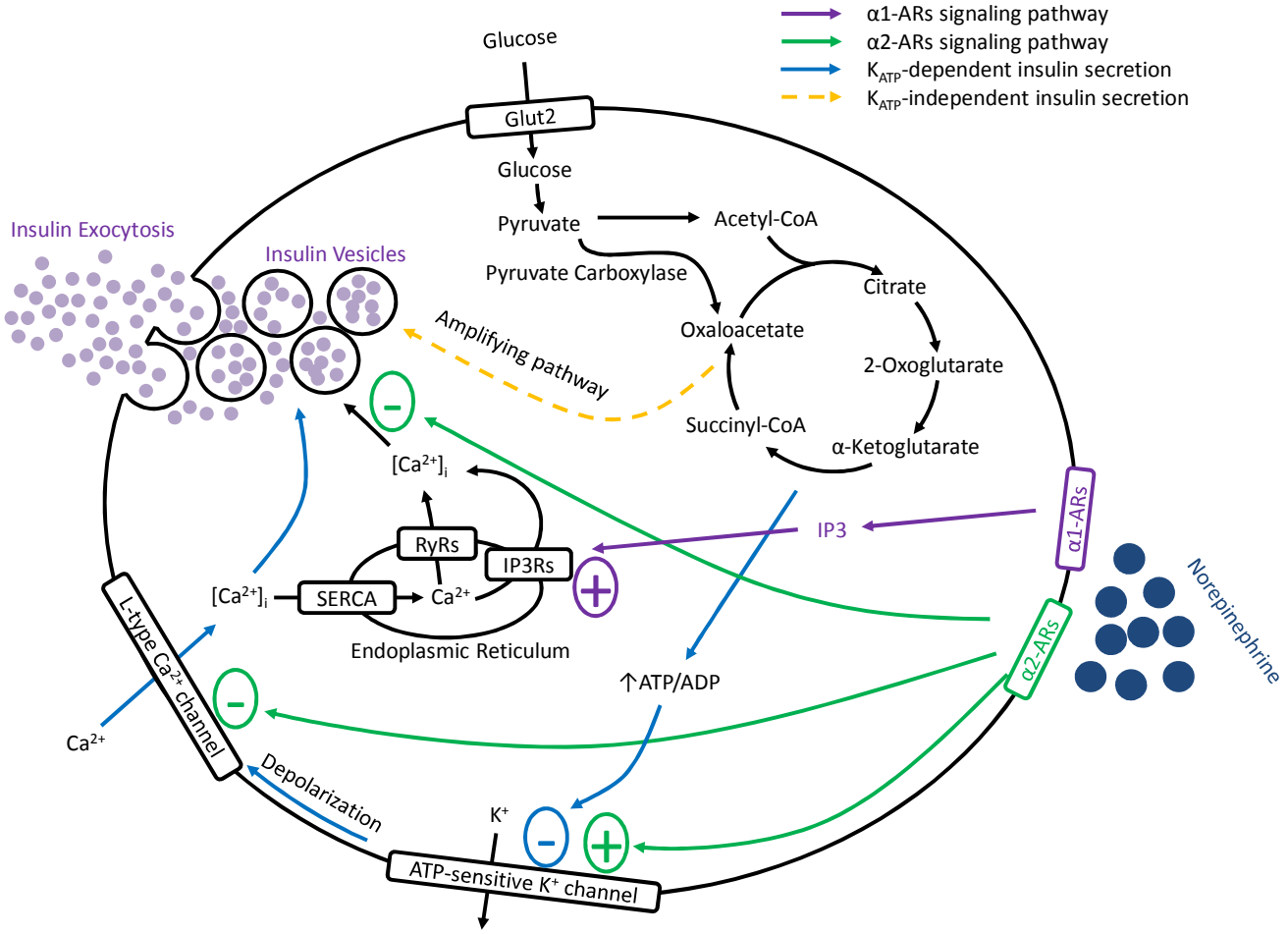
(Padbury et al., 1987b), glucose (Rozance et al., 2009b), amino acids (Rozance et al., 2009a), IGF-1 (Eremia et al., 2007), or insulin (Limesand et al., 2009). Investigating β-cell function is also feasible in fetal sheep and in isolated pancreatic islets *ex vivo* (Limesand et al., 2006). Previous studies showed that chronic norepinephrine infusion attenuates plasma insulin concentration, inhibits fetal weight gain, and leads to asymmetrical growth restriction during in sheep fetuses (Bassett and Hanson, 1998, 2000). However, whether chronic norepinephrine exposure impairs β-cell responsiveness associated with disrupted adrenergic signaling is unknown. In order to test the impact of chronic norepinephrine suppression associated with the dysregulation of insulin secretion through adrenergic signaling during fetal programming, therefore, applying a chronic infusion of norepinephrine to sheep fetuses is critical and necessary to investigate β-cell function associated with adrenergic regulation during fetal development.

Conclusion

IUGR is a significant problem in domestic animals and public health, resulting in morbidity and mortality in fetal life and higher risk of developing metabolic diseases in adulthood. Human IUGR fetus and animal IUGR models exhibit fetal condition of hypoxemia, hypoglycemia, hypoinsulinemia, elevated norepinephrine during pregnancy (Cetin et al., 2001; Limesand et al., 2006; Newnham et al., 1986; Nicolini et al., 1990; Pardi et al., 2002; Wong, 2006). In order to survival, IUGR fetuses adapt to the nutrient deprived environment and insulin-glucose

metabolism is impaired consequently. Pancreatic β-cell development and function are significantly disrupted in IUGR fetuses (Nicolini et al., 1990). In IUGR sheep fetuses, impaired GSIS is associated with elevated norepinephrine concentration and altered adrenergic signaling in pancreatic islets (Leos et al., 2010; Limesand et al., 2006). It is unknown whether chronic norepinephrine exposure alone programs the pancreatic β-cells and contributes the enhanced compensatory β-cell responsiveness. Therefore, we postulate that chronic norepinephrine suppression impairs insulin signaling and desensitizes adrenergic regulation in pancreatic islets to contribute permanent β-cell adaptation, resulting in persistently enhanced GSIS after removing norepinephrine suppression. To test this hypothesis, we plan to investigate the pancreatic β-cell function associated with ARs in the fetal sheep after chronic NE infusion.

Figure 2.1. Glucose stimulated and norepinephrine regulated insulin secretion. ARs, adrenergic receptors; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentrations; Glut2, glucose transporter 2; SERCA, Sarco(endo)plasmic reticulum Ca^{2+}-ATPase; IP3, inositol 1,4,5-trisphosphate; IP3Rs, inositol 1,4,5-trisphosphate receptors; RyRs, ryanodine receptors.



CHAPTER III

ENHANCED GLUCOSE STIMULATED INSULIN SECRETION RESPONSIVENESS IN THE FETAL SHEEP AFTER CHRONIC NOREPINEPHRINE INFUSION SUPPRESSION

Abstract

Intrauterine growth restricted fetuses are typically exposed to prolonged hypoxemia and hypoglycemia, resulting in perinatal pancreatic β-cell dysfunction. Recent evidence indicates that chronic exposure to norepinephrine (NE), which suppresses insulin secretion, will elicit a β-cell compensatory response that augments insulin secretion during an adrenergic blockade in IUGR fetuses. We demonstrate that chronic NE exposure alone can produce the β-cell compensatory responsiveness once removing NE suppression. NE was continuously infused into fetal sheep at 1-4 μg/min through 131-137 days of gestational age. During the 7-day treatment, NE infused fetuses had higher ($P < 0.05$) plasma NE concentrations than vehicle infused controls. Plasma glucose concentrations were increased 1.6-fold ($P < 0.01$), and insulin concentrations were 48% lower ($P < 0.01$) in NE fetuses versus controls. Glucose stimulated insulin secretion (GSIS) prior to NE treatment was not different between treatments; however, insulin secretion responsiveness was suppressed during NE infusion and exhibited 4-fold higher during hyperglycemic state ($P < 0.01$) in NE fetuses after discontinuing the infusion compared to controls. Pancreatic islets from the NE fetuses had lower ($P < 0.05$) mRNA concentrations for α1D

(58%), α2A (43%), α2C (42%), β1 (67%) adrenergic receptors (ARs), and uncoupling protein 2 (40%), but insulin, pancreatic duodenal homeobox 1, glucose transporter 2, ATP-regulated potassium channel, and L-type voltage-dependent calcium channel α1D subunit mRNA concentrations were not different. Islet insulin content was also not different. These findings indicate that chronic exposure to elevated NE persistently suppresses insulin secretion. However, when the NE infusion was terminated, compensatory GSIS hyper-responsiveness was observed and associated with dysregulation of ARs in pancreatic islets.

Introduction

Intrauterine growth restriction (IUGR) fetuses with placental insufficiency suffer from hypoglycemia and hypoxemia, resulting in chronically elevated catecholamine concentrations, including epinephrine (Epi) and norepinephrine (NE), compare to uncompromised fetuses (Divers et al., 1981; Fowden and Forhead, 2004). In pancreatic β-cell, NE primarily binds to α2-adrenergic receptors (ARs), but not α1- or β-ARs (Laychock and Bilgin, 1987), to inhibit insulin secretion from pancreatic β-cells (Sharp, 1996; Zhao et al., 2010). Abnormal expression of ARs in pancreatic β-cells impairs insulin secretion and increases the incidence of type 2 diabetes (Doronin et al., 2002; Rosengren et al., 2010; Urano et al., 2004).

In pancreatic β-cells, glucose metabolism is a potent stimulation for insulin secretion. Glucose enters the pancreatic β-cells through the glucose transporter

2 (Glut2) for glycolysis in the cytoplasm and oxidative phosphorylation in the mitochondria. PDX-1, primarily expressed in β-cells, regulates the insulin gene and Glut2, which are required for proper β-cell function (Ahlgren et al., 1998; Ohlsson et al., 1993). The uncoupling proteins (UCPs) are involved in regulating cellular ATP production on the inner mitochondrial membrane (Krauss et al., 2005). Among the UCP family, only uncoupling protein 2 (UCP2) is expressed in pancreatic β-cells and plays a role in regulating GSIS (Chan et al., 1999). Due to glucose metabolism, the elevated cellular ATP/ADP ratio closes K_{ATP} channels (Kir6.2) and depolarizes the β-cell (Ashcroft, 1988). Depolarization activates L-type voltage-dependent calcium channels, which are responsible for the majority of all Ca^{2+} channels, causing increased Ca^{2+} entry in the β-cell (Gopel et al., 2004). Finally, increased intracellular Ca^{2+} concentrations stimulate insulin exocytosis from β-cells (Herchuelz et al., 1980; Straub and Sharp, 2002).

A fetal sheep model with hyperthermia-induced placental insufficiency and IUGR shares many similarity with human IUGR, such as asymmetric growth restriction, chronic hypoinsulinemia and hypoglycemia, and hypercatecholaminemia (De Blasio et al., 2007b; Green et al., 2010; Regnault et al., 2002a). Endocrine pancreas development and function was impaired in these IUGR fetuses. Specifically, glucose stimulated insulin secretion (GSIS) was blunted (Limesand et al., 2006) and β-cell mass was reduced (Limesand et al., 2005) in near-term fetuses. Suppression of adrenergic signaling with antagonists revealed an enhancement in GSIS responsiveness in IUGR fetuses, making their maximal

insulin concentrations equivalent to uncompromised controls, even though β-cell mass was 56% less in IUGR fetuses (Leos et al., 2010).

Chronic exposure to high catecholamine concentrations has been shown to down-regulate ARs by reducing mRNA synthesis and increasing protein degradation among various organs and tissue (Benovic et al., 1990; Chen et al., 2010; Collins et al., 1991). Thus, we postulate that during long term elevated NE exposure contributes to enhanced compensatory GSIS responsiveness associated with abnormal adrenergic regulation of insulin secretion. In this study, we determined if a chronic infusion of NE would continuously suppress insulin concentrations in the fetal sheep and whether GSIS responsiveness, AR expression, and regulators of insulin secretion would be disrupted after discontinuing the NE infusion.

Method and Materials

Animal preparation

Pregnant Columbia-Rambouillet crossbred ewes carrying single pregnancies were purchased from Nebekar Ranch, Lancaster, CA, USA, and managed in compliance with the Institutional Animal Care and Use Committee at The University of Arizona. All animal experiments were conducted at the Agricultural Research Complex, Tucson, AZ, USA, which is accredited by the National Institutes of Health, the United States Department of Agriculture, and the American Association for Accreditation of Laboratory Animal Care.

Sheep fetuses were randomly assigned to the control group (n=6) or the chronic NE infusion group (n=6). All the ewes were fed Standard-Bread Alfalfa Pellets (Sacate Pellet Mills, AZ) and water ad libitum. At 126 ± 1 days of gestational age (dGA), indwelling polyvinyl catheters were surgically placed in fetuses and ewes as described previously (Limesand and Hay, 2003). Fetal catheters for blood sampling were placed in the abdominal aorta via hind limb pedal arteries, and infusion catheters were placed in the femoral veins via the saphenous veins. Maternal catheters were placed in the femoral artery and vein for arterial sampling and venous infusions, respectively. All catheters were tunneled subcutaneously to the ewe's flank, exteriorized through a skin incision, and kept in a plastic mesh pouch sutured to the ewe's skin. Ewes were allowed to recover for approximately 5 days before the first GSIS study was conducted.

Treatment protocol

In the chronic NE treatment group, norepinephrine biturtrate (Bedford Laboratories, OH) diluted in NE buffer (0.3% ascorbic acid in 0.9% sterile saline) was continuously infused in the fetal vein. During the 7-day infusion period, the dose of NE concentration started at 1 µg/min for the first 24 hours, increased to 2 µg/min for days 2-4, and finally to 4 µg/min for days 5-7 as previously described by (Bassett and Hanson, 1998). The chronic NE infusion was initiated at 131 dGA, which is 0.9 of gestation for sheep. Control fetuses received an infusion of NE buffer. After the final GSIS study, both ewes and fetuses were euthanized with an overdose of sodium pentobarbital (86 mg/kg; Euthasol, Virbac Animal

Health, Fort Worth, TX). Perirenal adipose, liver, spleen, heart, lung, and kidneys were dissected and weighed from both groups.

GSIS and glucose-potentiated arginine-induced insulin secretion (GPAIS) studies

At 131 dGA, a GSIS study was performed in all sheep fetuses to establish their initial β-cell function before the treatment. After completion of the 7-day treatment period, the infusion was stopped and a GSIS study was performed to investigate the impact of the treatment on β-cell function. In fetal sheep the rate of NE clearance was shown to be 178 ± 28 ml/kg·min (Padbury et al., 1986). Thus, the final GSIS studies were initiated 3 h after terminating the infusion to prevent NE influence on GSIS. Each NE fetus also received a GSIS study at 135 dGA (mid NE treatment) to evaluate the NE suppression of insulin secretion during the treatment.

Insulin secretion in response to glucose was measured with a square-wave hyperglycemic clamp as previously reported (Green et al., 2011). Briefly, a continuous transfusion of maternal blood into the fetus (10 ml/h) was started 30 min before baseline sampling to compensate for blood collection throughout the entire study. Three baseline blood samples were collected 5 min apart before beginning the hyperglycemic clamp. Following the last baseline sample, at time 0 min, a 2.5 ml 33% dextrose bolus (16.67 mM glucose) was given to the fetuses and followed immediately by a continuous infusion of 33% dextrose. The infusion rate was varied to maintain fetal glucose concentration around 2.4 mM, which is

the recommended value to reveal 90% of maximum insulin secretion responsiveness of single sheep fetuses (Green et al., 2011). During the hyperglycemic clamp, three fetal arterial plasma samples were collected every 5 min starting from 35 min. Following the hyperglycemic clamp, 5 ml of arginine solution (261 mg arginine, 1 ml of 2 M sodium acetate, and 4 ml saline) was constantly infused into a fetal vein for 4 min, and four plasma samples were collected at 5, 10, 20, and 30 min after the beginning of the infusion.

All the fetal blood was collected in syringes lined with EDTA (Sigma-Aldrich, St. Louis, MO) and centrifuged (16,000 g) for 2 min at 4 ℃ in EDTA-treated 1.5 ml Eppendorf tubes. Plasma was collected form the red blood cell pellet for glucose and insulin concentration measurements. Blood samples (2 mL) for plasma NE concentration measurements were spiked with 20 µl (1:100) of 0.5 mM EDTA and 0.33 mM reduced glutathione mixture and then plasma was separated and stored at -80 C. During the baseline and steady-state hyperglycemic clamp periods, fetal blood samples were also collected in syringes lined with dried heparin (Elkins-Sinn, Inc., Cherry Hill, NJ, USA) for blood gas and oxygen saturation determinations.

Biochemical analysis

Blood pH, oxygen saturation, and hemoglobin concentrations were measured with ABL 725 (Radiometer, Copenhagen, Denmark). The average temperature of a sheep is 39.1 ℃, which was used for the temperature co rrection in pH and pO_2 calculations. Plasma glucose concentrations were measured immediately on a

YSI model 2700 SELECT Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH). Plasma insulin concentrations were measured by an ovine insulin ELISA (ALPCO Diagnostics, Windham, NH). Plasma NE concentrations were analyzed by Noradrenaline ELISA (Labor Diagnostika Nord GmbH & Co.KG, Germany).

Pancreatic islet isolation

As described previously (Limesand et al., 2006), islets were obtained from the fetal pancreas with a retrograde perfusion Liberase BlendZyme III (0.175 mg/ml; Roche, Indianapolis, IN) into the pancreatic ducts. The digested tissue was diluted and purified in Krebs Ringer Buffer containing 0.5% BSA. After isolation, all the islets were cultured overnight at 37 ℃ in 95 % O2/5% CO2 in RPMI 1640 media containing 2.8 mM glucose (Sigma-Aldrich) supplemented with 2% fetal bovine serum and 1× penicillin-streptomycin-neomycin (50 U, 50 µg, and 100 µg, respectively, Sigma-Aldrich). On the next day, islets were handpicked into a 1.5 ml Eppendorf tube and centrifuged at 800 g for 5 min. After removing the supernatant, the pellets were stored at -80 ℃ until R NA and protein were extracted. Isolated islet insulin content was measured from the insulin concentration within pancreatic islets and normalized by total protein concentration, determined with BCA protein assay (Thermo Fisher Scientific Inc, Rockford, IL).

RNA extraction

Total RNA and protein was extracted from isolated pancreatic islets by following the manufacture instruction of the AllPrep DNA/RNA/Protein Mini Kit (QIAGEN, Valencia, CA). The quality and concentration of the RNA was determined by measuring absorbance at 260 and 280 nm with the NanoDrop ND-1000 Spectrophotometer (NanoDrop; Wilmington, DE). RNA integrity was evaluated with an Experion Automated Electrophoresis System (BioRad Laboratories, Hercules, CA). The PCR amplification was performed on RNA (~1 µg) that was reverse transcribed into cDNA with Superscript III reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions.

Primers design and complement cDNA cloning

Synthetic oligonucleotide primers were designed against sequences for ovine α1(A, B, D)-ARs, α2(A, B, C)-ARs, β(1, 2, 3)-ARs (Chen et al., 2010), insulin (GenBank accession number: U00659), pancreatic duodenal homeobox 1 (PDX-1, JF728303), Glut2 (AJ318925), UCP2 (forward, 5'-TCC CCT GTC GAC GTG GTC AAG A-3'; reverse, 5'-TGC AGC TGG AGC CAA TGC TGA TCA G-3'), Kir6.2 (forward, 5'-ACA GGA CGT GTT CAC CAC GCT-3'; reverse, 5'-CGA AAA GGA GTG GAT GCT GGT AAC G-3'), and L-type voltage-dependent calcium channel α1D subunit (CACNA1D, forward, 5'-GCC GGG AAG ACC CGG AGA TAC A-3'; reverse, 5'-AGG CCG CTC AAA GTC CAA GCT G-3'). Complement cDNA was cloned as previously described by (Chen et al., 2010).

Briefly, synthetic oligolnucliotide primers for cloning were designed to keep each amplicon between 250-500 bp in length. PCR products were inserted into the TOPO TA cloning expression vector pCRII (Invitrogen Life Technologies). After transformed into competent cells to culture overnight and purified by QIAprep Spin Miniprep Kit (QIAGEN), inserted DNA was sequenced (The University of Arizona Sequencing Service) and prepared for following studies. All nucleotide sequence results were submitted to GenBank (www.ncbi.nlm.nih.gov/genbank/).

Quantitative real time-PCR (qPCR)

The relative expression levels of all mRNA transcripts were determined by quantitative PCR using SYBR Green (QIAGEN) in an iQ5 Real-Time PCR Detection System (BioRad). After the initial denaturation for 15 min, all reactions went through 40 cycles of 96 ℃ (30 sec), annealing temp erature (30 sec), and 72 ℃ (10 sec) at which point the fluorescent measureme nt was collected. Optimal annealing temperatures for different primer sets were determined using a temperature gradient (54-62 ℃) and examined on a n agarose gel for specificity. Melt curve analysis, starting at 60 ℃ with a n increase of 0.2 ℃ every 6 seconds to 96 ℃, was performed at the end of the am plification to confirm product homogeneity.

PCR efficiency was determined with gene-specific plasmid DNA, for which threshold cycles (C_t) were linear over eight orders of magnitude. Samples were run on triplicate RT-PCR reactions. The results were normalized to the reference gene ribosomal protein S15 for each RT reaction by qPCR and the average ΔC_t

was analyzed by the comparative ΔC_t method (C_t gene of interest – C_t reference gene), and fold change was determined by the Pfaffl method (Pfaffl, 2001).

Statistical analysis

The weights (body and organs), qPCR (ΔC_t), and western immunoblot were analyzed by paired t-test to determine differences between control and NE infusion treatment group. GSIS responsiveness was evaluated by comparing the average insulin concentrations between treatment groups at baseline and hyperglycemia steady state. GPAIS responsiveness was examined by area under the curve against from mean basal plasma insulin concentrations for each sheep fetus (Prism 5.01, GraphPad Software, La Jolla, CA, USA). Biochemical, hematological, and hormone measurements during both daily and GPAIS studies were subjected to an ANOVA that accounts for the fetal sheep as random effects (proc mixed, SAS 9.2, SAS Institute Inc., Cary, NC). GSIS studies during chronic NE infusion was compared to GSIS studies of NE fetuses prior to treatment by applying nonparametric Kruskal-Wallis test (proc npar1way, SAS 9.2, SAS Institute Inc.). All values are expressed as the mean ± SEM. P-values ≤ 0.05 were considered significant.

Results

Bodyweight, feed intake, and organ weights

Before treatment, maternal body weight was not different between control (56.0 ± 4.5 kg) and NE ewes (49.8 ± 3.3 kg). During the treatment period, feed and water

intake were not different between control (1.76 ± 0.13 kg, 6.85 ± 0.71 L) and NE ewes (1.50 ± 0.06 kg, 5.48 ± 0.26 L). At necropsy, fetal weights were not different between control fetuses (3.8 ± 0.4 kg) and NE infused fetuses (3.3 ± 0.2 kg). The weight of the spleen was lower in NE treated fetuses (5.4 ± 0.3 g, $P < 0.05$) compared to control fetuses (7.7 ± 0.8 g). No other treatment effect was found for fetal organ weights (Table 3.1).

Daily fetal plasma values

Prior to treatment, fetal plasma NE, glucose, and insulin concentrations were not different between the treatment groups (Figure 3.1). During the 7-day infusion, plasma NE concentrations were on average 9-fold higher in NE fetuses (9343 ± 1047 pg/mL, $P < 0.01$) than control groups (1059 ± 142 pg/mL; Figure 3.1A). Mean insulin concentrations were lower (0.12 ± 0.01 µg/L) in NE infused fetuses than control fetuses (0.31 ± 0.03 µg/L, $P < 0.01$, Figure 3.1C). NE-infused fetuses had higher plasma glucose concentrations (1.54 ± 0.04 mM versus 0.99 ± 0.03 mM, $P < 0.01$, Figure 3.1B) and partial pressure of oxygen (pO_2, 31.29 ± 0.65 mmHg versus 22.98 ± 0.56 mmHg, $P < 0.01$, Figure 3.1D) than controls. Hematocrit and blood pH were not different between the two groups throughout 7-day infusion.

Fetal GSIS and GPAIS

During all GSIS studies, the mean glucose infusion rate for maintaining the hyperglycemic clamp was not different between the two treatment groups (Table

3.2). Hematocrit, pO_2, and pH at baseline and during the hyperglycemic clamp were not different between NE fetuses and controls before the chronic infusion (Table 3.2). However, after the infusion treatment, NE fetuses had 12% lower ($P < 0.01$) hematocrit at both baseline and hyperglycemic states, 1.24-fold higher pO_2 ($P < 0.01$) and 5.34-fold higher insulin ($P < 0.01$) at baseline, and 1.34-fold higher pO_2 ($P < 0.01$, Table 3.2) during the hyperglycemic steady-state than the control group.

Before treatment the plasma insulin concentration at baseline and hyperglycemic steady-state during GSIS (Figure 3.3) and GPAIS studies (Table 3.2) were not different between controls and NE fetuses. The GSIS studies performed during the chronic NE infusion only in NE fetuses showed decreased (Figure 3.2) plasma insulin concentrations at baseline and hyperglycemic steady state conditions (0.09 ± 0.01 µg/L, basal; and 0.40 ± 0.16 µg/L, hyperglycemic; $P < 0.01$) compared to the pretreatment GSIS study (0.44 ± 0.05 µg/L, basal; and 0.99 ± 0.10 µg/L, hyperglycemic). Three hours after terminating the 7-day NE infusion, NE fetuses had 5-fold higher insulin concentrations at baseline and 4-fold higher insulin concentration during the hyperglycemic clamp of the GSIS studies compared to controls and their pretreatment study (Figure 3.3, $P < 0.01$). The plasma NE concentrations were also elevated in NE infused fetuses (3189 ± 456 pg/mL, $P < 0.01$) versus controls (904 ± 140 pg/mL, Table 3.2). In the control group, there was no difference in glucose responsiveness or baseline and hyperglycemic steady state plasma insulin concentrations between the

pretreatment and post treatment GSIS study (Figure 3.3). Insulin concentrations following the arginine bolus reached maximum values after 5 minutes in all fetuses. The GPAIS area under the curve (Figure 3.4.A) was also higher in NE fetuses (199.13 ± 39.39 μg·min/L, $P < 0.01$, Figure 3.4.B) compared to controls (56.58 ± 5.62 μg·min/L) after removing the chronic NE infusion.

Islet characteristics

In order to interpret compensatory enhancement of GSIS in NE fetuses, we evaluated factors regulating aspects of insulin synthesis and secretion. After the 7-day infusion, NE fetuses had lower ($P < 0.05$, Figure 3.5) mRNA concentrations for α1D (58%), α2A (43%), α2C (42%), β1- (67%) ARs, and UCP2 (40), but insulin, PDX-1, Glut2, Kir6.2, CACNA1D, and other ARs mRNA expressions were not different in pancreatic islets. Islet insulin content, normalized by total protein concentration, was not different between NE fetuses (113.59 ± 48.64 μg/L/mg/L) and control animals (35.61 ± 10.65 μg/L/mg/L).

Discussion

In the current study, we show that a chronic NE infusion inhibited insulin secretion throughout a 7-day treatment coupled with greater plasma glucose in the fetal sheep. NE treated fetal sheep had compensatory augmentation in GSIS after terminating the NE infusion, even though plasma NE concentrations remained elevated compared to controls. The mRNA concentrations for α1D, α2A, α2C, β1-ARs, and UCP2 were lower in pancreatic islets from the NE-

infused fetuses compared to control fetuses. These findings show that chronic elevated NE contributes to a compensatory increase in insulin secretion and impairs adrenergic signaling β-cell responsiveness. Moreover, the suppression of UCP2 mRNA would improve islet glucose metabolism, which might link adrenergic signaling to mitochondrial function in sheep fetal pancreatic islets.

As expected, there was no difference in GSIS responsiveness between the two groups before the treatment (Figure 3.3), and insulin secretion in control fetuses was not different following the 7-day infusion treatment (Figure 3.3). Comparisons between the two GSIS studies in control fetuses demonstrate that there is no developmental increase in β-cell responsiveness at this gestational stage. During the NE infusion, insulin concentrations for NE fetuses at basal and hyperglycemic steady states were suppressed by elevated NE compared to the study prior to treatment. After removing the chronic NE infusion, basal and hyperglycemic insulin concentrations were ~5-fold higher than control fetuses or pretreatment measurement in NE fetuses (Figure 3.3). These finding support the hypothesis that NE induces a compensatory hyper-insulin secretion state in IUGR fetuses, as observed after administering adrenergic antagonists (Leos et al., 2010).

To our surprise, the enhanced insulin secretion responsiveness was observed despite higher than normal plasma NE concentrations. In addition this occurred after chronic hyperglycemia from the NE infusion. According to previous studies, fetal sheep whole body clearance rate of NE, which is not associated with

plasma catecholamine concentration, is 178 ± 28 ml/kg·min in near term sheep fetuses, which were similar in gestation age to our fetuses (Padbury et al., 1986). Since the average blood volume in fetal sheep is 120 ml/kg at 131 dGA (Brace, 1983), the half-life of circulating plasma NE is extremely short, not longer than a couple of minutes. However, in NE fetuses during the final GSIS study, three hours after removing the NE infusion, plasma NE was still 3-fold higher than age-matched control fetuses (Table 3.2), indicating that chronic NE exposure significantly decreases NE clearance as previously described (Bzoskie et al., 1997). Despite NE's inhibitory effect on insulin release, NE fetuses with relative higher NE concentrations still had stronger GSIS responsiveness than control fetuses.

During the 7-day NE treatment, chronic hyperglycemia and hyperoxemia were found in NE fetuses. This represents another confounding factor that can influence β-cell function, and is most likely due to reduced glucose and oxygen utilization in peripheral tissues (Milley et al., 1986). Previous studies show that 7 to 10 days of chronic hyperglycemia impairs β-cell function in fetal sheep (Carver et al., 1995). Two weeks of pulsatile hyperglycemia treatment also impairs GSIS and causes 63% greater reactive oxygen species (ROS) accumulation in pancreatic islets at 11.1 mM glucose incubation compared to controls (Green et al., 2012). Therefore, chronic hyperglycemic should cause islet failure because of oxidative stress. NE, however, overcomes the hyperglycemic impairment and this

is most likely due to decrease islet oxidative metabolism (Laychock and Bilgin, 1987) and reduced ROS production.

Isolated pancreatic islets from NE fetuses have lower expression of α1D, α2A, α2C, β1-ARs, and UCP2 mRNA transcripts (Figure 3.5) compared to control fetuses. No augmentation was found for any β-cell regulatory factors of the insulin-signaling pathway. Chronic exposure to high catecholamine concentrations has been shown to persistently reduce both mRNA and protein expression of ARs (Benovic et al., 1990; Chen et al., 2010; Collins et al., 1991). NE inhibits insulin secretion principally through α2-adrenergic signaling, but not α1- or β-ARs (Laychock and Bilgin, 1987). Thus, this lower expression of α2A- and α2C-ARs might lead to decreased α2-adrenergic inhibitory function in regulating insulin secretion (Sharp, 1996), resulting in higher basal insulin as well as higher insulin responsiveness during GSIS studies after removing 7-day NE infusion.

On the other hand, as the exclusive proton leak regulator in the mitochondria of β-cells, UCP2 negatively affects β-cell responsiveness by decreasing ATP production (Dalgaard, 2011; Zhang et al., 2001). Chronic hyperglycemia induces higher UCP2 mRNA and protein expression in rat and human islet cultures (Brown et al., 2002; Patane et al., 2002). In glucokinase knock-out mice, either incubated with or without chronic hyperglycemia, decreased glucose utilization lowers UCP2 expression and activity in pancreatic islets (Dalgaard, 2011). Thus, expression and function of UCP2 are associated with the rate of glucose

utilization in pancreatic islets. Laychock and Bilgin showed that α2-ARs inhibit islets glucose utilization rates (Laychock and Bilgin, 1987). Thus, NE might work as the switch to turn down/off glucose utilization in β-cells similar to the glucokinase mutated mice. Consequently, NE infusion decreased islet glucose utilization rates and caused UCP2 expression to decrease. Decreased UCP2 expression was also found in isolated pancreatic islets from IUGR sheep fetuses, which have NE concentrations 5 fold greater than control fetuses and in mouse insulinoma cell line (MIN6), cultured for both 72 or 96 hours with epinephrine (unpublished data). Consistent with this hypothesis, lower UCP2 expression in pancreatic islets increases GSIS responsiveness after removing the 7-day NE infusion. Moreover, due to the regulation of α2-adrenergic signaling on glucose utilization in β-cells (Laychock and Bilgin, 1987), the decreased α2-ARs caused by chronic NE exposure could lead to lower inhibitory regulation in glucose utilization. Therefore, the desensitization of α2-ARs induced by chronic NE exposure, which not only directly lowers the α2-adrenergic regulation of insulin secretion, but also indirectly down regulates UCP2 expression, resulting in higher GSIS after removing NE suppression.

Our current 7-day NE infusion scheme is physiologically relevant and provides insight into the compensatory insulin secretion mechanism in the IUGR ovine model (Leos et al., 2010). During the NE infusion, fetal sheep had 9-fold higher NE with 60% lower insulin secretion in NE fetuses relative to the control group (Figure 3.1.A). These results resemble findings for placental insufficiency-

induced IUGR sheep fetuses, which have 69% suppression in their mean plasma insulin concentration (Limesand et al., 2006). However, the control group had slightly higher plasma NE concentration during GSIS studies before the chronic infusion treatment, and it persisted to last GSIS studies after removing 7-day infusion (Table 3.2). According to previous studies on thresholds for physiological effects of plasma catecholamines in fetal sheep, higher NE concentration in control fetuses would not exceed the physiological threshold for glucose *de novo* synthesis or fatty acid mobilization (Padbury et al., 1987b).

The chronic NE exposure appears to re-program β-cell responsiveness to glucose, which may persist and impair normal growth patterns in the neonate. For example, carunclectomy IUGR fetal sheep not only suffer from chronic elevated NE (Jones and Robinson, 1983), but also have impaired β-cell responsiveness (Owens et al., 2007). They have smaller body size at birth, increased GSIS and insulin sensitivity as young lambs, and catch-up growth during adult life (Gatford et al., 2008). Consequently, by inhibiting insulin secretion, NE plays an important role in helping to ensure fetal survival despite undernutrition. However, if this adaptive mechanism persists, compensatory GSIS responsiveness might not always be beneficial, resulting in IUGR offspring being at greater risk for obesity and dyslipidemia in postnatal life.

To summarize, a 7-day infusion of NE into fetal sheep continually suppresses insulin concentrations as well as contributes to desensitized α2-adrenergic signaling and down-regulated UCP2 in pancreatic islets. Termination of the

chronic NE infusion after 1 week revealed a hyper-responsiveness of GSIS, indicating that chronic suppression by NE resulted in enhanced compensation of β-cell function. These data begin to explain how endocrine factors, like catecholamines, may facilitate prenatal adaptations to for a thrifty phenotype *in utero*, and contribute to catch-up growth after birth by developing compensatory increased insulin secretion, resulting in early onset obesity and type 2 diabetes during postnatal life.

Figure 3.1. Daily norepinephrine (**A**), glucose (**B**), insulin (**C**), and pO_2 (**D**) throughout 7-day norepinephrine infusion treatment. NE, norepinephrine; pO_2, partial pressure of oxygen.

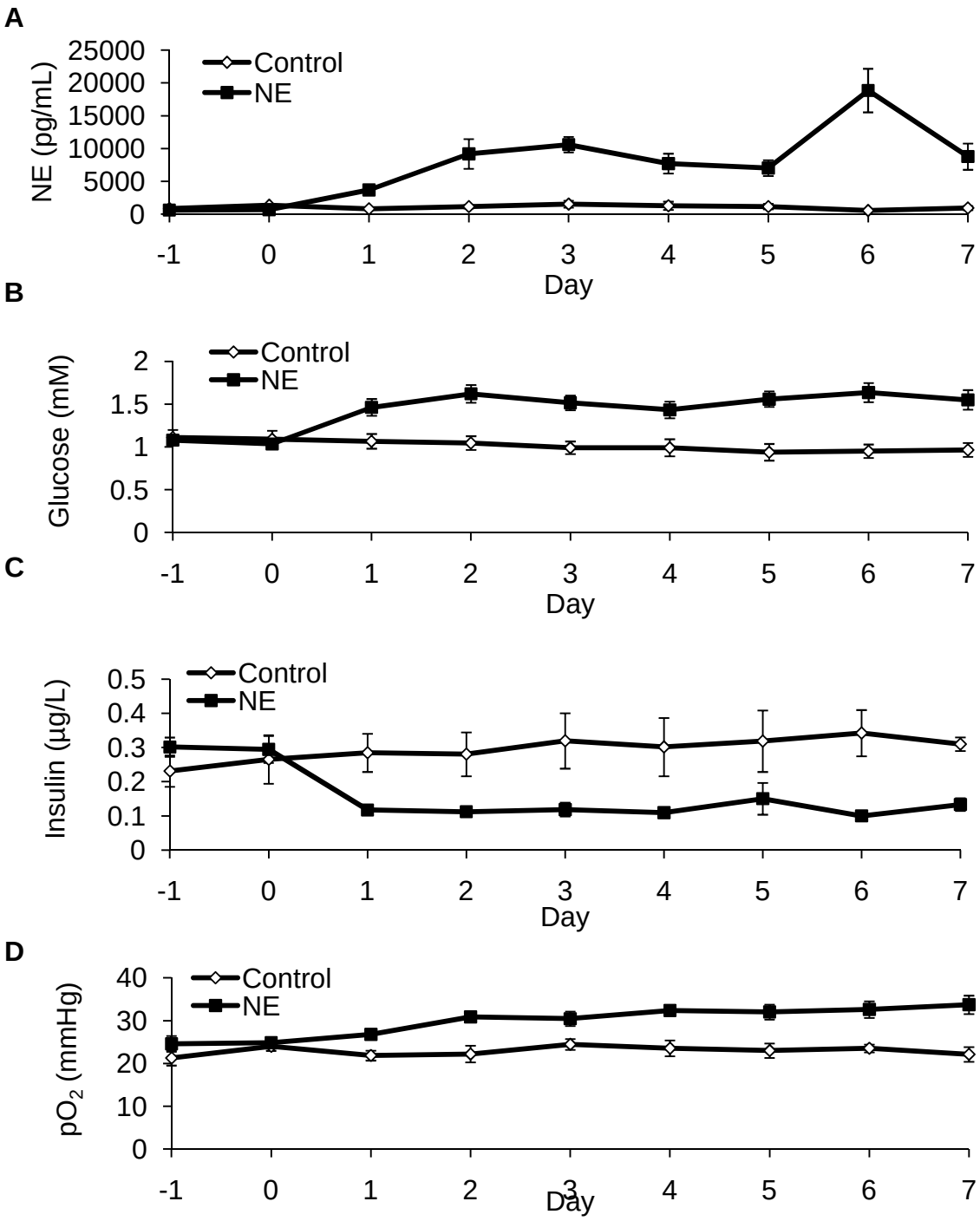


Figure 3.2. Insulin concentration of norepinephrine-infused fetuses during glucose stimulated insulin secretion studies prior to and during chronic norepinephrine infusion in fetal sheep. Significance is indicated by **, P<0.01.

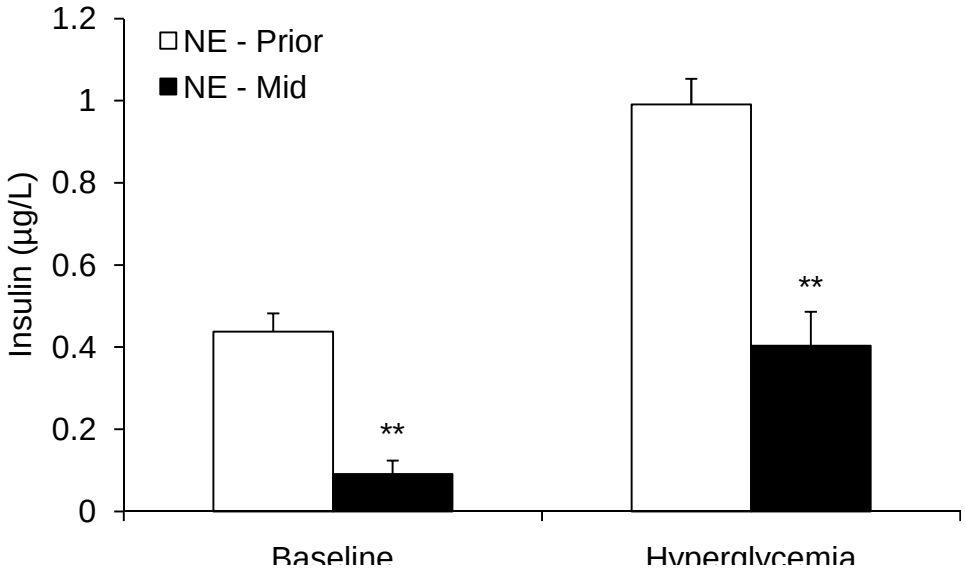


Figure 3.3. Insulin concentration of control and norepinephrine treated fetuses during glucose stimulated insulin secretion studies prior to and post of chronic norepinephrine infusion. Significance is indicated by **, P<0.01.

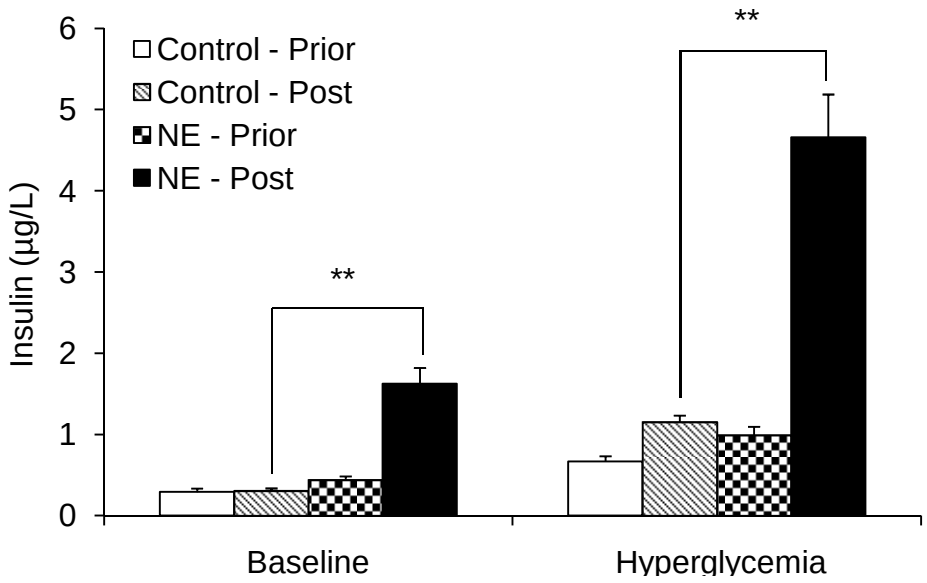


Figure 3.4. Insulin concentration of control and norepinephrine treated fetuses during glucose potentiated arginine induced insulin secretion studies (**A**) after removing chronic norepinephrine infusion. Glucose potentiated arginine induced insulin secretion responsiveness was analyzed by area under the curve (**B**). Significance is indicated by **, P<0.01.

A

B

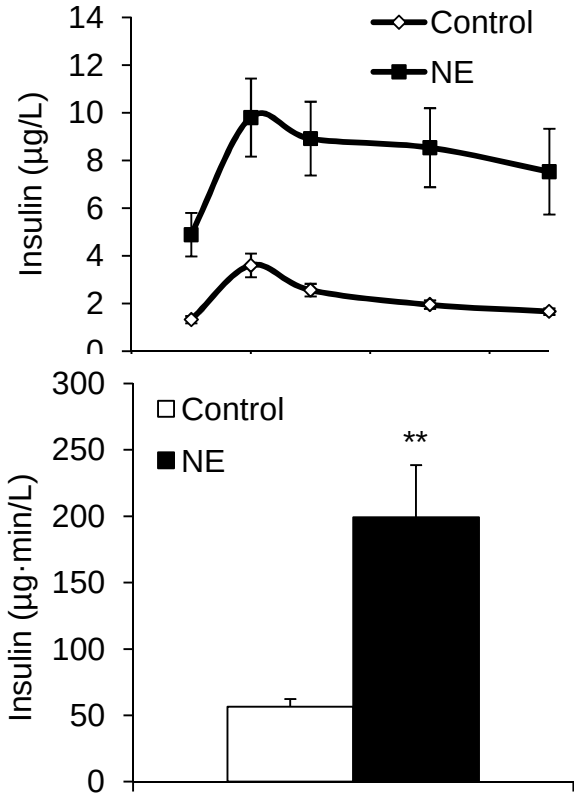


Figure 3.5. mRNA expression in isolated pancreatic islets from chronic norepinephrine-infused fetal sheep. Significance is indicated by *, P<0.05, or **, P<0.01. AR, adrenergic receptor; UCP2, uncoupling protein 2.

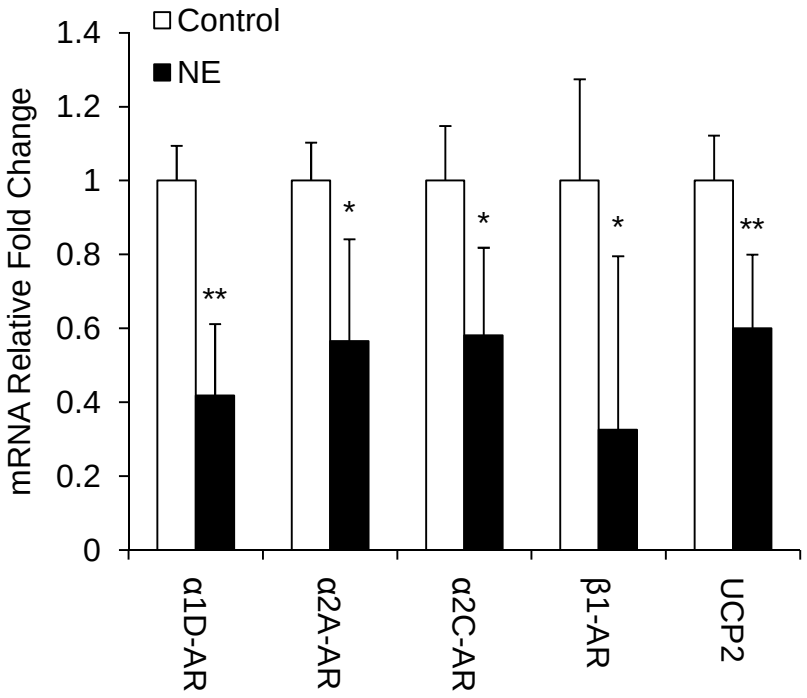


Table 3.1. Organ and tissue weights in control and norepinephrine-infused fetal sheep. NE, norepinephrine. Significance is indicated by *, P<0.05.

	Tissue Weight, g	
	Control (n = 6)	NE (n = 6)
Brain	52.5 ± 1.7	50.9 ± 1.5
Heart	25.0 ± 2.2	24.4 ± 1.1
Kidneys	19.9 ± 1.5	18.4 ± 1.2
Liver	98.8 ± 11.6	88.3 ± 5.1
Lungs	123.8 ± 8.9	109.8 ± 5.9
Perirenal Fat	13.8 ± 1.5	15.2 ± 0.8
Spleen	7.7 ± 0.8	5.4 ± 0.3*

Table 3.2. Sheep fetal plasma glucose, insulin, hematocrit, partial pressure of oxygen (pO_2), and norepinephrine (NE) levels during glucose stimulated insulin secretion and glucose potentiated arginine induced insulin secretion studies prior to as well as after terminating chronic NE infusion treatment. N/S, not significant.

GSIS Study	Parameter	Condition	Control	NE	P-value
Prior to Norepinephrine Infusion					
	Glucose (mmol/hr)	Infusion	10.75 ± 1.44	10.69 ± 1.12	N/S
	Glucose (mM)	Baseline	1.19 ± 0.07	1.14 ± 0.03	N/S
		Hyperglycemia	2.48 ± 0.03	2.24 ± 0.03	< 0.05
	Insulin (µg/L)	Baseline	0.29 ± 0.04	0.44 ± 0.05	N/S
		Hyperglycemia	0.67 ± 0.06	0.99 ± 0.10	N/S
	Insulin (µg·min/L)	Arginine	42.12 ± 4.97	63.38 ± 9.76	N/S
	Hematocrit (%)	Baseline	34.78 ± 0.82	34.00 ± 1.00	N/S
		Hyperglycemia	34.73 ± 0.80	34.22 ± 0.96	N/S
	pO_2 (mmHg)	Baseline	24.48 ± 0.96	24.29 ± 0.83	N/S
		Hyperglycemia	23.66 ± 0.99	23.75 ± 0.70	N/S
	NE (pg/mL)	Baseline	1262.33 ± 178.76	588.89 ± 65.86	N/S
		Hyperglycemia	1434.80 ± 98.11	693.23 ± 95.80	< 0.05
Post Norepinephrine Infusion					
	Glucose (mmol/hr)	Infusion	14.79 ± 1.90	10.09 ± 1.59	N/S
	Glucose (mM)	Baseline	0.97 ± 0.05	1.15 ± 0.04	< 0.01
		Hyperglycemia	2.38 ± 0.03	2.37 ± 0.03	N/S
	Insulin (µg/L)	Baseline	0.30 ± 0.03	1.62 ± 0.20	< 0.01
		Hyperglycemia	1.15 ± 0.08	4.66 ± 0.53	< 0.01
	Insulin (µg·min/L)	Arginine	56.58 ± 5.62	199.13 ± 39.39	< 0.01
	Hematocrit (%)	Baseline	34.74 ± 0.91	30.53 ± 0.87	< 0.01
		Hyperglycemia	35.00 ± 0.84	30.68 ± 0.85	< 0.01
	pO_2 (mmHg)	Baseline	24.57 ± 0.77	30.52 ± 0.80	< 0.01
		Hyperglycemia	24.08 ± 0.88	32.19 ± 1.06	< 0.01
	NE (pg/mL)	Baseline	903.80 ± 140.37	3189.40 ± 469.70	< 0.01
		Hyperglycemia	1329.35 ± 181.32	3415.22 ± 392.04	< 0.01

CHAPTER IV

CHRONIC NOREPINEPHRINE DESENSITIZES A2-ADRENERGIC RECEPTORS TO ENHANCE INSULIN SECRETION IN FETAL SHEEP

Abstract

Placental insufficiency lowers fetal oxygen and glucose concentrations, which chronically elevate fetal plasma norepinephrine (NE) concentrations. Previous studies in uncompromised sheep fetus designed to isolate NE actions show that chronic exposure to NE suppresses insulin secretion through α2-adrenergic receptors (ARs) and increases plasma glucose concentrations. Removing fetuses from the chronic NE exposure results in acute β-cell hyper-responsiveness to glucose due in part to lower mRNA expression of α2-ARs in pancreatic islets. The aim of this study is to determine if the compensatory mechanism of NE is dependent on hyperglycemia and persists in fetal sheep after chronic NE exposure. In addition, we evaluated whether the desensitization of α2-ARs is associated with compensatory insulin secretion. We investigated β-cell responsiveness both in fetal sheep *in vivo* and in isolated pancreatic islet *ex vivo*. NE was continuously infused into fetal sheep at 1-4 μg/min through 130-136 days of gestational age. Insulin was infused into the ewe to maintain fetal euglycemia during the NE treatment period. Fetal glucose stimulated insulin secretion (GSIS) was tested with a square-wave hyperglycemic clamp prior to chronic treatments, 1 day, and 5 days after terminating the NE infusion. After the last GSIS study, pancreatic islets were isolated and incubated with

hyperglycemic condition with various NE concentrations (0.0001, 0.001, 0.01, 0.1, 1, and 10 μM) to determine insulin secretion. During the 7 day treatment period, NE-infused fetuses had 11 times higher ($P < 0.05$; n=8) NE plasma concentration than vehicle infused controls (n = 7). Plasma insulin concentrations were lower (0.15 ± 0.02 versus 0.42 ± 0.02 μg/L, $P < 0.01$) than controls. Plasma glucose concentrations were not different between treatment groups. GSIS responsiveness before treatment was similar between groups. However, both 1 day and 5 days after discontinuing NE infusion, NE fetuses had 2-fold greater ($P < 0.05$) GSIS than control groups at hyperglycemic status. Isolated islets from NE infused fetuses had lower ($P < 0.05$) responsiveness to NE in static incubations than control with IC50 of 5.40 ± 1.10 nM and 2.17 ± 0.90 nM, respectively. NE islets also showed greater maximal ($P < 0.05$) insulin secretion than control (2.12 ± 0.2 versus 1.38 ± 0.15 μg/L/islets, $P < 0.05$). These findings show that chronic NE exposure, not hyperglycemia, leads to the post treatment hyper insulin secretion responsiveness coupled with desensitized α2-ARs signaling. Moreover, this compensatory enhancement in fetal β-cell persists for 5 days after removing NE infusion indicates chronic NE exposure continues to effect β-cell responsiveness.

Introduction

Placental insufficiency-induced intrauterine growth restriction (PI-IUGR) increases risk of mortality and morbidity in newborn infants (Garite et al., 2004). IUGR human fetuses are exposed to hypoxemia, hypoglycemia, and glucose

intolerance (Nicolini et al., 1990). Fetal hypoxemia stimulates oxygen-sensitive K^+ channels in the adrenal medulla (Douglas and Rubin, 1963, 1961; Vogt, 1952) to elevate plasma norepinephrine (NE) concentrations (Divers et al., 1981). Elevated plasma NE binds to α2-adrenergic receptors (ARs) on pancreatic β-cell to inhibit insulin secretion (Sharp, 1996). Chronic NE exposure as observed in IUGR fetuses alters the fetal β-cell function. Overexpression of α2A-AR, induced by a single-nucleotide polymorphism, reduces insulin secretion, impairs glucose-stimulated insulin secretion (GSIS), and increases the risk for type 2 diabetes in humans (Rosengren et al., 2010).

Similarly, PI-IUGR sheep fetuses have attenuated GSIS (Limesand et al., 2006) and less pancreatic β-cell mass (Limesand et al., 2005). Administrating of AR antagonists, which block both α- and β-adrenergic receptors, results in a full recovery in GSIS responsiveness in PI-IUGR fetal sheep (Leos et al., 2010). In normal sheep fetuses, chronic infusion of elevated NE inhibits insulin secretion and reduces fetal growth rate at 0.9 of gestation (Bassett and Hanson, 1998). Our previous work provide evidence that a 7-day NE infusion caused enhanced compensatory GSIS in the fetal sheep and reduced mRNA expression of α2A- and α2C-AR in pancreatic islets after terminating the chronic NE infusion (Chapter III).

Chronic NE infusion also produces hyperglycemia (Bassett and Hanson, 1998, 2000), which alone has been shown impairment of glucose tolerance and insulin secretion in sheep fetuses (Carver et al., 1995; Dabelea et al., 2000; Green et

al., 2012; Sobngwi et al., 2003). Thus, in order to understand the influence of chronic NE exposure on β-cell function *in utero*, it is necessary to maintain euglycemia during the chronic NE infusion. It is also unknown whether desensitization of ARs in fetal islets induced by chronic NE exposure contributes permanent enhancement of GSIS in fetus *in vivo* and specific β-cell adaptation of increased insulin secretion *ex vivo*. In this study, we determined whether the enhanced β-cell responsiveness persists after chronic NE infusion in fetal sheep without hyperglycemic influence. We also identified whether isolated pancreatic islets develop compensatory GSIS resulting from adrenergic desensitization after chronic NE infusion in sheep fetuses. Overall, understanding the mechanisms underlying the adverse programming effects of chronic NE will be necessary to identify potential therapeutic targets to alleviate compensatory enhance insulin secretion for later life.

Method and Materials

Animal preparation

Pregnant Columbia-Rambouillet crossbred ewes carrying single pregnancies were purchased from Nebekar Ranch, Lancaster, CA, USA, and managed in compliance with the Institutional Animal Care and Use Committee at The University of Arizona. All animal experiments were conducted at the Agricultural Research Complex, Tucson, AZ, USA, which is accredited by the National Institutes of Health, the United States Department of Agriculture, and the American Association for Accreditation of Laboratory Animal Care.

All the ewes were fed Standard-Bread Alfalfa Pellets (Sacate Pellet Mills, AZ) and water ad libitum. At 125 days of gestational age (dGA), indwelling polyvinyl catheters were surgically placed in fetus and ewe as described previously (Limesand and Hay, 2003). Fetal catheters for blood sampling were placed in the abdominal aorta via hind limb pedal arteries, and infusion catheters were placed in the femoral veins via the saphenous veins. Maternal catheters were placed in the femoral artery and vein for arterial sampling and venous infusions, respectively. All catheters were tunneled subcutaneously to the ewe's flank, exteriorized through a skin incision, and kept in a plastic mesh pouch sutured to the ewe's skin. Ewes were allowed to recover for approximate 5 days before the first GSIS study was conducted.

Chronic NE infusion and euglycemic adjustment

Sheep fetuses were randomly assigned to the control group (n=7) or the chronic NE infusion group (n=8). At 130 dGA, NE (Norepinephrine Bitartrate, Bedford Laboratories, OH, USA) was infused into the treatment group continually for 7 days by following preset rate, 1, 2, 2, 2, 4, 4, 4 μg/min, on each day (Figure 4.1). Because fetal glucose concentration depends on ewe's glucose concentration, a chronic intravenous insulin infusion (30-60 pmol/min/kg, Humulin R; Eli Lilly, Indianapolis, IN, USA) was infused into the ewe's femoral vein to maintain fetal glucose concentration at normal fetal glucose level during 7-day NE treatment. Control fetuses were received an infusion of NE buffer (0.9% saline and 0.3%

ascorbic acid) and ewes were administrated infusion of insulin buffer (0.9% saline and 0.5% bovine serum albumin).

GSIS and glucose-potentiated arginine-induced insulin secretion (GPAIS) studies

Three GSIS studies were performed before (GSIS#1), 1-day after (GSIS#2), and 5-day after (GSIS#3) NE infusion on each fetal sheep *in vivo* (Green et al., 2011; Limesand et al., 2006). Briefly, a continuous transfusion of maternal arterial blood into the fetus (10 mL/h) was started 30 min prior to baseline sampling and maintained for the duration of the study to compensate for blood collection. All sample times are presented relative to the start of the fetal glucose bolus and continuous glucose infusion at time 0. Baseline plasma glucose and insulin concentrations were determined at -15, -10, and -5 min. The hyperglycemic clamp was initiated with a dextrose bolus of 1.19 ± 0.04 mmol/kg estimated fetal weight to the fetus followed by a constant infusion of 33% dextrose in saline to increase and maintain fetal arterial plasma glucose concentration at 2.4 mmol/L, which produces a near-maximal GSIS response in singleton sheep fetuses (18, 33). At the onset of the glucose infusion, fetal arterial samples were collected every 5-10 min for the initial 30 min to establish the hyperglycemic steady state, after which fetal samples were collected at 45, 50, and 55 min (hyperglycemic period).

In order to test the maximum insulin responsiveness, GPAIS, a following arginine bolus (0.5 mmol/kg) was administrated right after GSIS study. GSIS

responsiveness was evaluated by comparing the average insulin concentrations between treatment groups at baseline and hyperglycemia steady state. GPAIS responsiveness was analyzed by area under the curve against mean insulin concentration during baseline for each sheep fetus (Prism 5.01, GraphPad Software, USA).

Biochemical analysis

Fetal arterial blood samples of both daily and *in vivo* β-cell responsiveness studies were collected in syringes lined with EDTA (Sigma-Aldrich, St. Louis, MO, USA). Plasma was separated in EDTA treated 1.5 mL Eppendorf tubes by centrifuging (16,000 g) for 2 min at 4 ℃. Plasma glucose and lactate concentrations were measured immediately by using an YSI model 2700 SELECT Biochemistry Analyzer (Yellow Springs Instruments, Yellow Springs, OH, USA). The remaining plasma was stored at -80 ℃ both in 2 mL Eppendorf tubes for insulin measurement and in the tubes with 20 µl of 0.5 mM EDTA and 0.33 mM reduced glutathione mixture for NE concentration measurements (Limesand et al., 2006). During the basal and steady state hyperglycemic clamp periods, fetal blood samples were also collected in syringes lined with dried heparin (Elkins-Sinn, Inc., Cherry Hill, NJ, USA) for blood gas and oxygen saturation measurement.

Blood pH, oxygen saturation and hemoglobin concentrations were measured with an ABL 725 (Radiometer, Copenhagen, Denmark). Average core body temperature of sheep, 39.1 ℃, was applied for the temperature correction.

Plasma insulin concentrations were measured by an ovine insulin ELISA (ALPCO Diagnostics, Windham, NH, USA), with the 9.1% inter-assay and 8.5% intra-assay coefficients value. Plasma NE concentrations were analyzed by Noradrenaline ELISA (Labor Diagnostika Nord GmbH & Co.KG, Nordhorn, Germany), with the 22.5% inter-assay and 19.6% intra-assay coefficients value.

Necropsy and islet isolation

Within 5-hour after the final GSIS studies, ewes and fetuses were killed with an overdose of sodium pentobarbital (86 mg/kg) and phenytoin sodium (11 mg/kg, Euthasol; Virbac Animal Health, Fort Worth, TX, USA). Fetal organs were dissected, weighed, and collected. The hepatic portion of pancreas was collected for islet isolation (Limesand et al., 2006). The splenic portion of pancreas was fixed in 4% paraformaldehyde overnight and prepared for morphological study (Leos et al., 2010).

Islet insulin secretion

Isolated islets, 6 control and 6 NE treated fetuses, were cultured overnight at 37 ℃ with 95% O_2/5% CO_2 in RPMI-1640 medium (Sigma-Aldrich) supplemented with 2% fetal bovine serum, 2.8 mM glucose, and penicillin–streptomycin (50 U and 50 mg; Sigma-Aldrich) (Limesand et al., 2006). For static islets incubation studies, fetal islets were washed twice in KRB with 0.5% BSA and a third time in KRB/BSA supplemented with 10 μM forskolin (Sigma-Aldrich). All media was equilibrated to 37 ℃ and 95% O_2/5% CO_2. Ten islets were handpicked (n = 3-4

replicates/condition) and incubated at 37 ℃ in 1 mL KRB/BSA/forskolin media with 11 different incubation conditions listed in Table 4.1. After the 1-hour 37 ℃ incubation, islets were pelleted by centrifugation (800 g) at 4 ℃ for 3 min. The media was removed for insulin releasing measurement, and then insulin was extracted from pellets within acid–ethanol (1 M HCl/70% ethanol). Both media and islet extract were stored in -20 ℃ for at least ov ernight before performing the insulin assay.

Islets insulin secretion associated with adrenergic regulation was summarized in NE concentration dose response curve with a logarithmic scale. The effectiveness of ARs in inhibiting insulin secretion is calculated into half-maximal inhibitory concentration (IC50). The fractional insulin secretion value, which is released insulin concentration normalized by total insulin concentration, was analyzed in log (inhibitor) versus response nonlinear curve (Prism 5.01) to generate IC50 value for each animal.

Histology of fetal pancreatic endocrine cells

Twenty tissue sections of 6 µm were cut from frozen OCT-embedded control (n = 7) and NE treated fetal pancreases (n = 6) for histological and morphometric evaluation. Pancreatic sections were dried in incubator for 30 min at 37 ℃ and rehydrated in water for twice for 5 min (Leos et al., 2010). Sections were microwaved twice for 5 min at 60% power in 10 mM citric acid buffer, pH 6, cooled for 20 min, and washed three times in PBS for 10 min. Sections were blocked with 0.5% NEN blocking reagent (Perkin Elmer, Waltham, MA, USA) for

1 hour. Mature pancreatic endocrine hormones were identified in the fetal sheep pancreas with guinea pig anti-porcine insulin (1:500; Dako, Carpinteria CA, USA), mouse anti-porcine glucagon (1:500; Sigma-Aldrich), rabbit anti-human somatostatin (1:500; Dako), and rabbit anti-human pancreatic polypeptide (1:500; Dako) (Leos et al., 2010; Limesand et al., 2005). Primary antisera were diluted in 1% BSA in PBS and incubated at 4℃ overnight; negati ve controls were included for which the primary antiserum was omitted. After this incubation, the pancreatic sections were washed three times for 10 min with PBS; immunocomplexes were detected with affinity-purified secondary antiserum conjugated to Cy2 (rabbit), Texas Red (mouse), or 7-amino-4-methylcoumarin-3-acetic acid (AMCA, guinea pig; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) diluted 1:500 in 1% BSA in PBS for 60 min at room temperature. The pancreatic sections were washed three times for 10 min each with PBS and mounted in 50% glycerol and 10 mM Tris-HCl, pH 8.

Fluorescent images were visualized on a Leica DM5500 microscope system and digitally captured with a Spot Pursuit 4 Megapixel CCD camera (Diagnostic Instruments, Sterling Heights, MI, USA). Morphometric analysis was performed with ImagePro 6.0 software (Media Cybernetics, Silver Spring, MD, USA). Positive areas were determined for 25 fields of view (FOV = 0.39 mm2) on two pancreas sections per animal separated by ≥ 120 µm intervals (total area = 19.5 mm^2; coefficient of variation between sections, 10.7%).

Statistical analysis

Statistical analysis for fetal and organs weights, qPCR (ΔCt), IC50, and immunohistology were evaluated by paired t-test to determine differences between control and treatment group. Biochemical, hematological, and hormone measurements from both daily, GSIS studies, GPAIS studies, and islets incubation were subjected to an ANOVA that account for the fetal sheep as random effects in SAS Proc GLM (SAS version 9.2, SAS Institute Inc., Cary, NC, USA). All values are expressed as the mean ± SEM. P-values ≤ 0.05 were considered significant.

Results

Bodyweight, feed intake, and organ characters

Maternal body weight was not different between control (56.8 ± 3.9 kg) and NE ewes (52.8 ± 3.3 kg). During the treatment period, the average daily feed and water intake were not different between control (1.8 ± 0.1 kg and 7.5 ± 0.9 L) and NE ewes (1.9 ± 0.1 kg and 7.2 ± 0.6 L).

Average age at necropsy of control fetuses (139.9 ± 0.5 dGA, 3 male and 4 female) was not different from NE treated fetuses (140.8 ± 0.8 dGA, 7 male and 1 female). No treatment effect was found for total bodyweight, carcass weight, and organ weights, including kidneys, lung, liver, brain, heart, perirenal adipose, and spleen, in sheep fetuses (Table 4.2).

Daily plasma profile in fetal sheep

Prior to treatment, fetal plasma glucose, insulin, NE, pressure of oxygen (pO_2), and hematocrit concentrations were not different between treatment groups (Figure 4.2). Plasma lactate concentration was higher in NE fetuses (2.53 ± 0.11 mM) than control (2.02 ± 0.17 mM, $P < 0.05$) before the chronic NE infusion.

During 7-day infusion period, the average plasma NE concentration was 11-fold higher in NE fetuses (8969 ± 1287 pg/mL, $P < 0.01$) than control group (840 ± 99 pg/mL; Figure 4.2.A), indicating that NE infusion exceed the threshold for physiological responsiveness in fetal sheep (Padbury et al., 1987b). Mean insulin concentrations of NE fetuses were lower (0.15 ± 0.02 µg/L) than control fetuses (0.42 ± 0.02 µg/L, $P < 0.01$, Figure 4.2.C) during 7-day infusion period. NE-infused fetuses had a higher pO_2 (26.54 ± 0.54 mmHg, $P < 0.01$, Figure 4.2.D) than controls (20.96 ± 0.32 mmHg). One day after terminating NE infusion, NE treated fetal sheep had higher plasma insulin concentrations and blood pO_2 ($P < 0.01$, Table 4.3). However, both parameters returned to normal at 5-day after terminating the NE infusion. Glucose, lactate, pH, or hematocrit was not different between two groups throughout the entire treatment.

***In vivo* insulin secretion responsiveness in fetal sheep**

The mean glucose infusion rate for maintaining the hyperglycemic clamp was not different from two groups (Figure 4.3.A). NE and pO_2 were not different between NE fetuses and control before chronic infusion treatment (Table 4.3). However,

one day after removing 7-day infusion, NE fetuses had a 1.3-fold higher pO_2 ($P < 0.01$, Table 4.3) and 5.3-fold higher NE concentration ($P < 0.01$) compared to control fetus at baseline of the GSIS study. Five days after stopping the 7-day NE infusion, pO_2 was normal, but plasma NE concentrations remained elevated 2.5-fold ($P < 0.05$, Table 4.3) in NE fetuses compared to controls. Plasma hematocrit or pH was not different between the two groups in all the GSIS studies.

Before chronic infusion, GSIS responsiveness was not different between treatments groups (Figure 4.4). At 1-day and 5-day after removing chronic NE infusion treatment, insulin secretion of NE-infused fetuses at hyperglycemic steady-state was 2 times higher than control as well as themselves prior to the treatment (Table 4.3 and Figure 4.4). No difference in insulin concentration at baseline or hyperglycemic steady-state for was found between GSIS studies in control fetuses.

Insulin concentrations following the arginine bolus reached maximum values after 5 minutes in all treatments. Analyzed by area under the curve (Figure 4.3.B), GPAIS responsiveness of NE treated fetuses was higher ($P < 0.05$, Figure 4.4) both 1-day and 5-day after infusion, compared to control.

Ex vivo insulin secretion and morphological studies in pancreatic islets

Among the islets incubation with no glucose, 1.1 mM glucose, 11.1 mM glucose plus 30 mM KCl, and 11.1 mM glucose on ice, there was a study effect ($P < 0.05$)

on insulin stimulated incubation versus negative control and a treatment effect ($P < 0.05$) on NE-infused fetuses, resulting in higher insulin secretion from islets.

Incubating isolated islets with 11.1 mM glucose plus a range of NE concentrations (0, 0.0001, 0.001, 0.01, 0.1, 1, and 10 µM) showed that NE fetal islets were less ($P < 0.05$) responsive to NE inhibition than control islets. The NE islet IC50 has 5.40 ± 1.10 versus 2.17 ± 0.90 nM in contents (Figure 4.5). Islets from NE fetuses also had greater maximal ($P < 0.05$) insulin secretion versus control islets (2.12 ± 0.20 versus 1.38 ± 0.15 ng/L/islet, $P < 0.05$, Figure 4.6). Insulin contents from isolated pancreatic islets were not different between NE (20.9 ± 4.1 ng/islet) and controls (17.1 ± 3.3 ng/islet).

The pancreas morphology was not different between treatments. No difference was found insulin (β-cells), glucagon (α-cells), or the combination of somatostatin (δ-cells) and pancreatic polypeptide cells area (Table 4.4).

Discussion

In the present study, we evaluated the islets physiological response to chronic adrenergic suppression in insulin secretion of β-cell after a 7-day NE infusion in the fetal sheep. Chronic exposure to elevated NE with euglycemia contributes to sustaining enhancement of β-cell responsiveness after terminating the NE infusion *in vivo*. Pancreatic islets of NE fetuses show permanently decreased inhibition of β-cell responsiveness, caused by adrenergic desensitization, and greater maximal insulin secretion during islets incubation with NE concentration

gradient *ex vivo*. The enhanced compensatory β-cell responsiveness from impaired adrenergic regulation in pancreatic islets may improve insulin-glucose metabolism in the postnatal life.

Chronic NE exposure is detrimental to placental NE transporter function (Bzoskie et al., 1997), leading to a lower NE clearance rate in fetal sheep (Bzoskie et al., 1995). In our present study, plasma NE concentrations of NE fetuses remained 4.9-fold and 2.5-fold higher than control 1-day and 5-day after terminating NE infusion, respectively (Figure 4.2.A). Despite increased NE inhibits insulin secretion through α2-ARs, NE fetuses still had significantly higher GSIS and GPAIS responsiveness than control animals after removing chronic infusion (Figure 4.4). Meanwhile, maintaining euglycemia in NE fetuses during NE infusion provided evidence that chronic NE exposure, not hyperglycemia, leads to this persistent adaptations of β-cell function, resulting in enhanced compensatory GSIS in fetal sheep.

Catecholamines via α2-ARs are regulators of insulin secretion from β-cells. In the previous study, a 7-day NE exposure lowers mRNA expression of α2A- and α2C-AR for 43% and 42%, respectively, in the pancreatic islets (Chapter III). Our current results support less activity of adrenergic receptors in β-cell, because both the IC50 and maximal insulin secretion are higher in isolated pancreatic islets after 5-day NE infusion in fetal sheep.

In mice, overexpression of α2A-AR in pancreatic β-cells influences glucose homeostasis by suppressing insulin secretion, resulting in glucose intolerance in

mice (Devedjian et al., 2000). Blocking α2A-adrenergic signaling with an antagonist, yohimbine, recovers insulin release in α2A-AR mutated mice (Rosengren et al., 2010). Conversely, α2A-AR deficient mice lack inhibitory action from α2A-AR in pancreatic β-cell, and are hyperinsulinemic and hypoglycemic (Fagerholm et al., 2004; Savontaus et al., 2008). These findings support that lower α2-ARs expression in isolated pancreatic islets of fetal sheep could significantly raise maximal insulin secretion after terminating chronic NE infusion (Figure 4.6). Moreover, islets showed higher insulin releasing during *ex vivo* incubation with stimulatory glucose (Figure 4.6). However, chronic NE infusion does not change total insulin content of islets or islet architecture in fetal sheep. Therefore, desensitization of ARs induced by chronic exposure of elevated NE directly contributes lower adrenergic inhibition of insulin secretion in β-cell, resulting in a persistent enhancement of compensatory β-cell responsiveness to glucose.

In conclusion, chronic elevated NE exposure, not hyperglycemia, contributes persistent enhancement of β-cell responsiveness after removing NE infusion in fetal sheep. Lower inhibitory action of α2-ARs caused by desensitization in pancreatic islets contributes to the manifestation of persistent β-cell adaptation. Permanent impairment of β-cell responsiveness with enhanced compensatory GSIS may improve glucose utilization and increase fat deposition when exposed to energy-enriched diets in postnatal life (Modan et al., 1985). Dyslipidemia in

return produces insulin resistance and increases the risk of glucose intolerance and type 2 diabetes in adulthood.

Figure 4.1. Treatment design of chronic norepinephrine infusion with euglycemic adjustment in fetal sheep.

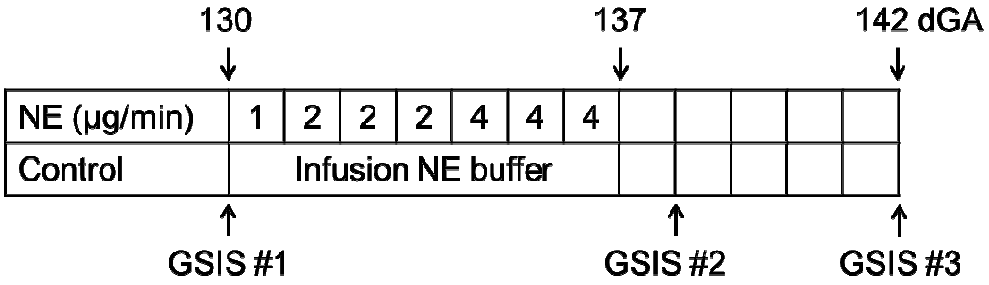


Figure 4.2. Daily plasma norepinephrine (**A**), glucose (**B**), insulin concentration (**C**), and partial pressure of oxygen (**D**) of fetal sheep throughout 7-day norepinephrine infusion and 5-day post treatment.

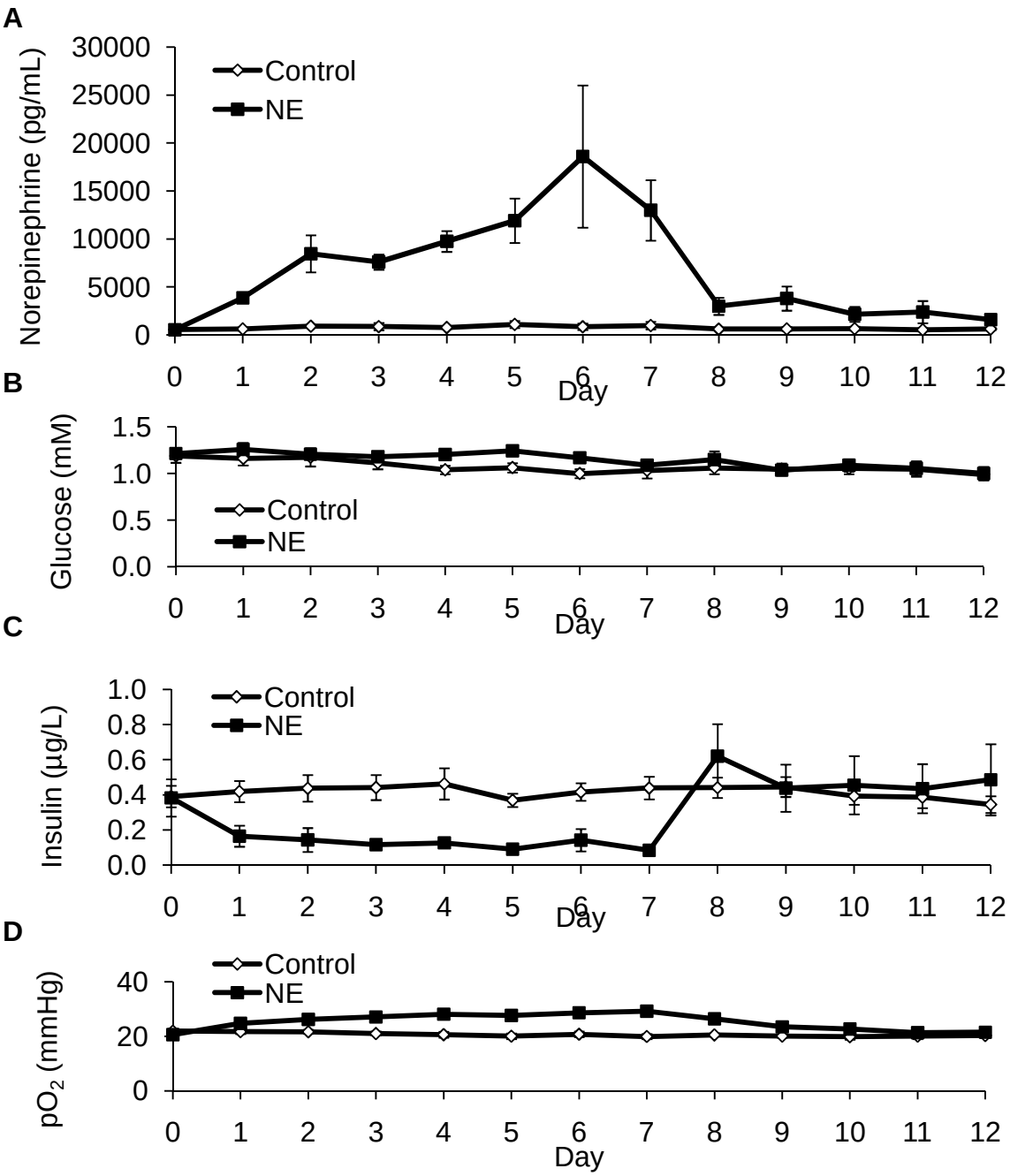
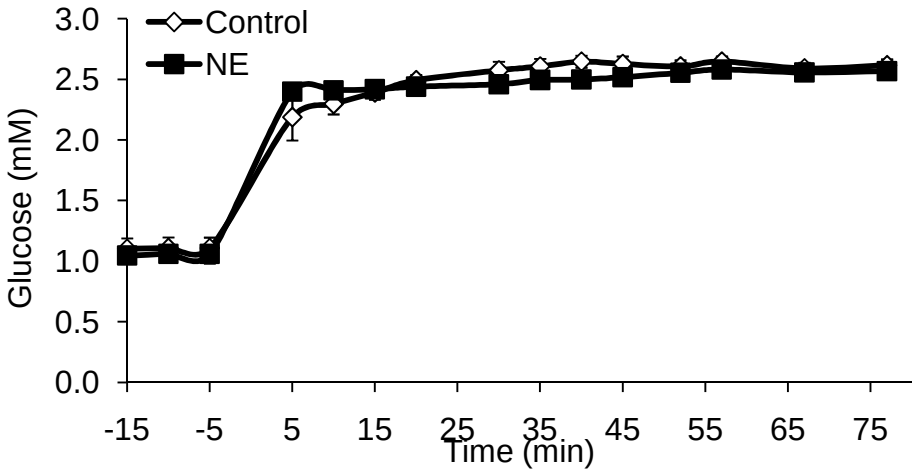


Figure 4.3. Sheep fetal glucose (**A**) and insulin (**B**) concentration during glucose stimulated insulin secretion and glucose-potentiated arginine-induced insulin secretion studies 5-day after terminating chronic norepinephrine infusion.

A



B

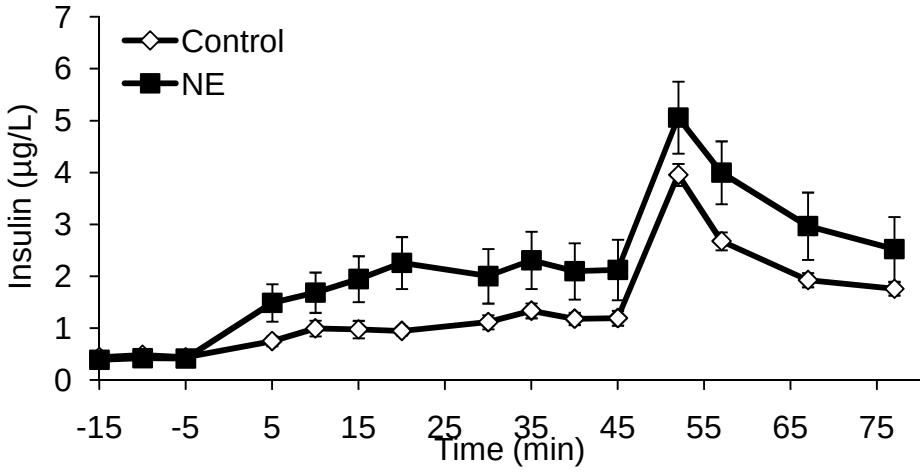


Figure 4.4. Sheep fetal insulin secretion during baseline and hyperglycemic state in glucose stimulated insulin secretion studies as well as insulin concentration analyzed by area under the curve during glucose-potentiated arginine-induced insulin secretion studies. $P < 0.05$.

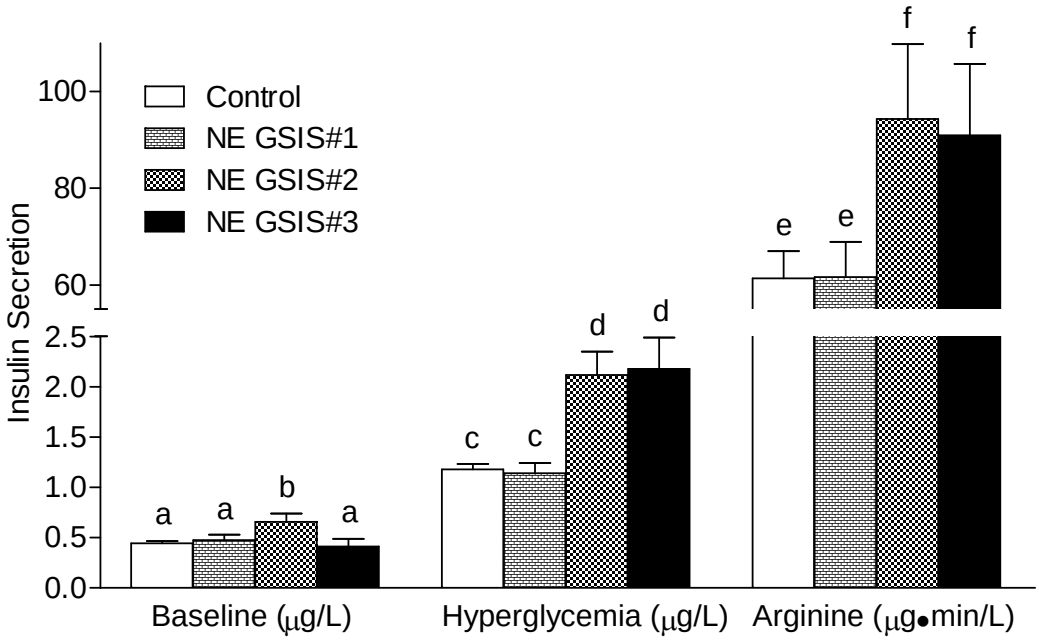


Figure 4.5. Half-maximal inhibitory concentration (IC50) of norepinephrine inhibition during 11.1 mM glucose with NE concentration gradient incubation in isolated pancreatic islets from sheep fetuses after chronic infusion. Significance is indicated by *, $P < 0.05$.

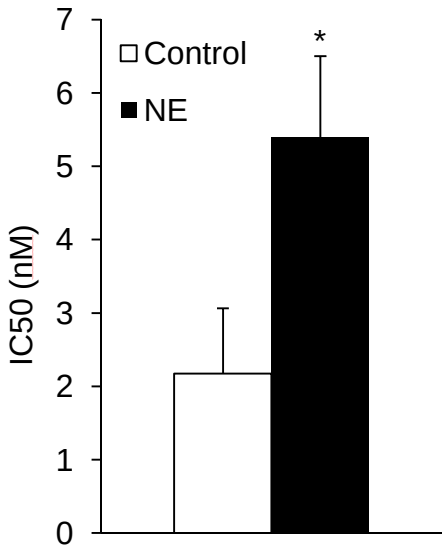


Figure 4.6. Insulin secretion during 11.1 mM glucose with norepinephrine concentration gradient incubation in isolated pancreatic islets from sheep fetuses after chronic infusion.

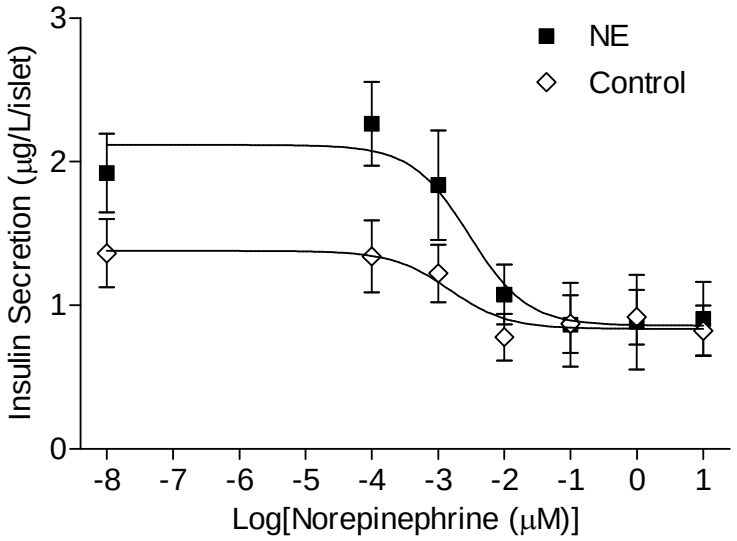


Table 4.1. Isolated pancreatic islets static incubation conditions.

Number	Incubation Conditions
1	0 mM gluocse
2	1.1 mM glucose
3	11.1 mM glucose
4	11.1 mM glucose + ice
5	1.1 mM glucose + 30 mM KCl
6	10 µM NE + 11.1 mM glucose
7	1 µM NE + 11.1 mM glucose
8	0.1 µM NE + 11.1 mM glucose
9	0.01 µM NE + 11.1 mM glucose
10	0.001 µM NE + 11.1 mM glucose
11	0.0001 µM NE + 11.1 mM glucose

Table 4.2. Organ and tissue weights in control and norepinephrine-infused fetal sheep. NE, norepinephrine.

	Tissue Weight, g	
	Control (n = 7)	NE (n = 8)
Brain	55.3 ± 2.3	53.4 ± 2.2
Heart	29.5 ± 1.8	27.7 ± 1.6
Kidneys	26.0 ± 1.8	24.9 ±1.4
Liver	106.5 ± 9.6	106.7 ± 8.1
Lungs	134.3 ± 10.2	133.5 ± 9.6
Perirenal Fat	14.0 ± 1.7	13.9 ± 0.8
Spleen	10.1 ± 1.1	8.6 ± 0.9

Table 4.3. Sheep fetal plasma glucose, insulin, partial pressure of oxygen (pO_2), and norepinephrine (NE) levels during glucose stimulated insulin secretion and glucose potentiated arginine induced insulin secretion studies before, 1-day, and 5-day after terminating chronic NE infusion treatment. N/S, not significant.

GSIS Study	Parameter	Condition	Control	NE	P-value
GSIS#1	Glucose (mmol/hr)	Infusion	11.00 ± 1.20	12.53 ± 0.67	N/S
	Glucose (mM)	Baseline	1.28 ± 0.06	1.14 ± 0.04	<0.05
		Hyperglycemia	2.53 ± 0.06	2.45 ± 0.02	N/S
	Insulin (µg/L)	Baseline	0.45 ± 0.04	0.47 ± 0.05	N/S
		Hyperglycemia	1.21 ± 0.08	1.13 ± 0.10	N/S
	Insulin (µg·min/L)	Arginine	60.66 ± 11.77	61.59 ± 7.28	N/S
	pO_2 (mmHg)	Baseline	21.28 ± 0.30	20.76 ± 0.58	N/S
		Hyperglycemia	20.25 ± 0.32	20.88 ± 0.48	N/S
	NE (pg/mL)	Baseline	503.96 ± 78.13	528.82 ± 84.72	N/S
		Hyperglycemia	605.48 ± 104.06	722.39 ± 130.47	N/S
GSIS#2	Glucose (mmol/hr)	Infusion	14.18 ± 0.90	11.75 ± 1.12	N/S
	Glucose (mM)	Baseline	1.07 ± 0.03	1.15 ± 0.05	N/S
		Hyperglycemia	2.48 ± 0.03	2.58 ± 0.04	N/S
	Insulin (µg/L)	Baseline	0.42 ± 0.04	0.66 ± 0.08	<0.01
		Hyperglycemia	1.21 ± 0.08	2.12 ± 0.23	<0.01
	Insulin (µg·min/L)	Arginine	64.09 ± 11.74	94.30 ± 15.57	0.08
	pO_2 (mmHg)	Baseline	20.05 ± 0.53	26.03 ± 0.59	<0.01
		Hyperglycemia	19.93 ± 0.47	24.71 ± 0.76	<0.01
	NE (pg/mL)	Baseline	590.70 ± 61.04	3113.49 ± 559.93	<0.01
		Hyperglycemia	620.65 ± 67.24	3546.22 ± 645.93	<0.01
GSIS#3	Glucose (mmol/hr)	Infusion	14.28 ± 1.12	13.66 ± 0.77	N/S
	Glucose (mM)	Baseline	1.11 ± 0.05	1.06 ± 0.04	N/S
		Hyperglycemia	2.63 ± 0.03	2.50 ± 0.03	<0.05
	Insulin (µg/L)	Baseline	0.46 ± 0.04	0.41 ± 0.08	N/S
		Hyperglycemia	1.24 ± 0.08	2.18 ± 0.31	<0.01
	Insulin (µg·min/L)	Arginine	58.90 ± 3.81	90.96 ± 14.76	0.08
	pO_2 (mmHg)	Baseline	20.48 ± 0.51	20.71 ± 0.41	N/S
		Hyperglycemia	19.27 ± 0.54	20.32 ± 0.47	N/S
	NE (pg/mL)	Baseline	563.53 ± 108.17	1590.15 ± 171.63	<0.01
		Hyperglycemia	672.16 ± 91.82	1498.38 ± 233.70	0.05

Table 4.4. Endocrine pancreas morphometry of α-, β-, δ-, and pancreatic polypeptide-cells in fetal sheep after chronic infusion.

	Control (n = 7)	NE (n = 6)
α-cells area, %	33.4 ± 1.5	35.5 ± 2.4
β-cells area, %	43.6 ± 1.9	44.0 ± 3.9
δ- and PP-cells area, %	23.0 ± 2.3	20.5 ± 1.6

CHAPTER V

SUMMARY OF MAJOR FINDINGS AND OPPORTUNITIES FOR FURTHER RESEARCH

The objective of this dissertation was to evaluate the effects of chronic norepinephrine exposure on pancreatic β-cell function in fetal sheep to determine their role in fetal programming. Discussion of the major findings and limitations are presented with implications for future research.

Elevated norepinephrine (NE) undoubtedly contributes to complications in the IUGR fetus, specifically in suppression of insulin secretion. Previous studies showed that NE binds α2-adrenergic receptors (ARs) to inhibit insulin secretion from pancreatic β-cells acutely through a bevy of cellular mechanisms (Sharp, 1996). IUGR fetal sheep have chronically elevated NE concentrations, hypoinsulinemia, diminished glucose stimulated insulin secretion (GSIS) (Limesand et al., 2006), and lower pancreatic β-cell mass (Limesand et al., 2005). They were also found to develop a compensatory insulin secretion response, which was shown by an enhancement of GSIS during acute AR blockade relative to β-cell mass (Leos et al., 2010), and this hyper-secretion could predispose IUGR infants to metabolic complications in later life.

In order to investigate the chronic effects of NE exposure on fetal pancreatic β-cells, we infused NE into uncompromised sheep fetuses for a week. This treatment significantly lowers insulin concentrations and increases glucose. After removing the norepinephrine inhibition, a compensatory enhancement in GSIS is

observed. We also show that expression of α2A- and α2C-ARs are lower in pancreatic islets from chronic NE treated sheep fetuses. As a result, chronic infusion of NE into fetal sheep continually suppresses insulin concentrations, but also desensitizes the β-cells to NE. Termination of the chronic norepinephrine infusion after 1 week revealed a persistent hyper-responsiveness in GSIS, indicating chronic suppression by norepinephrine results in β-cell compensation in fetal sheep.

This first study indicates that chronic NE suppresses GSIS but after chronic inhibition is alleviated hyper-secretion is observed. It is unknown whether this enhancement of insulin responsiveness is specifically associated with pancreatic β-cells and functional adaptations in adrenergic signaling. It is also important to determine whether enhanced GSIS after termination of NE infusion persists providing evidence for islet programming. Finally, chronic hyperglycemia was observed in the first study (Chapter III), which can augments insulin secretion and β-cell expansion in adults. Chronic hyperglycemia has been shown to impair insulin secretion in fetal pancreatic β-cell (Carver et al., 1995; Green et al., 2012). Therefore, eliminating hyperglycemic influence and better isolate adrenergic signaling within the pancreatic β-cell will accurately interpret the chronic NE suppression induced compensatory enhanced insulin secretion.

During the 7-day norepinephrine treatment with euglycemic adjustment, basal insulin concentrations from norepinephrine treated fetuses were significantly lower, but recovered to normal after removing norepinephrine suppression. Both

1-day and 5-day after discontinuing the infusion, norepinephrine treated fetuses had greater insulin concentrations than controls at hyperglycemic steady state conditions, indicating persistence of enhanced compensatory GSIS secretion responsiveness after chronic norepinephrine exposure. Glucose stimulated arginine potentiated insulin secretion also was higher in norepinephrine treated fetal sheep, both 1-day and 5-day after infusion. Moreover islet specific effects were shown to persist in static incubations. Islets from norepinephrine treated fetuses were less responsive to norepinephrine inhibition than control islets. Norepinephrine treated fetuses also had greater maximal insulin secretion than controls with stimulatory glucose concentrations. No difference was found in pancreatic islet insulin content or β-cell mass. Therefore, chronic exposure to elevated NE programmed β-cells GSIS enhancement in euglycemic fetuses. The hypersensitivity persists for 5 days after termination of NE exposure in the fetus, and fetal pancreatic islets show that the persistent compensatory enhancement is specific to β-cells. Desensitization of α2-adrenergic signaling may partially explain the persistent β-cell adaptation.

Future Directions for Research

Regulation of glucose in liver

In the nutrient deprived environment, IUGR fetuses appear to have normal glucose utilization rates with hypoinsulinemia and have enhanced fetal glucose production from the liver, indicating whole body insulin-glucose metabolic complications (Desai and Hales, 1997; Hay et al., 1984; Limesand et al., 2007).

Key hepatic enzymes and transcription factors that regulate hepatic gluconeogenesis, including glucose 6 phophatase (Glc-6-Pase), phosphoenolpyruvate carboxykinase (PEPCK), and phosphorylated cAMP response element-binding protein. Interestingly, expressions of these factors are increased in IUGR fetal liver, which confirms their capacity for glucose production.

Several studies showed that glucose production in the liver, including both stimulation of glycogenolysis and gluconeogenesis, is facilitated by α1- and β-adrenergic signaling (Erraji-Benchekroun et al., 2005; Exton and Park, 1968) and inhibited by insulin (Sherwood, 2007). This indicates a pivotal role for NE in the IUGR fetus's ability to maintain glucose, and elucidates a need to better understand the potential consequences of chronic norepinephrine exposure on the liver. In previous 7-day NE treated fetal sheep, mRNA expressions of all the ARs are not different, but rate-limiting enzymes of gluconeogenesis, including PEPCK, peroxisome proliferator-activated receptor gamma coactivator 1-alpha, and Glc-6-Pase, are decreased in livers from chronic norepinephrine treated fetal sheep. Despite the minor role of gluconeogenesis in normal fetal physiology during normal gestation, lower expression of key gluconeogenic enzymes might impair glucose regulation in the liver postnatally. The down-regulation of gluconeogenesis at the liver could also be exacerbated by compensatory enhanced insulin responsiveness. Thus, gluconeogenesis could be further inhibited in those fetuses after chronic norepinephrine exposure. Therefore, in

order to understand whole-body glucose regulation after chronic norepinephrine exposure in fetal sheep, it is necessary to study glucose produce, uptake, utilization, and storage as well as insulin sensitivity in liver.

Regulation of free fatty acids in perirenal fat

Not only signaling pathways of gluconeogenesis are disrupted in liver, but also mRNA expression of β2-AR (Figure 5.2), the most potent stimulator of fat mobilization, is significantly lower in perirenal fat coupled with 2-day enhancement of non-esterified fatty acid (NEFA, Figure 5.3) from chronic norepinephrine treated fetal sheep. IUGR fetal sheep, which also have chronic elevation of norepinephrine, appear to have similar β2-AR desensitization with lower lipolysis in perirenal fat during perinatal stage (Chen et al., 2010). Impaired fat metabolism potentially increases the risk of dyslipidemia, lipid immobilization, and obesity in IUGR offspring. However, whether this same β2-AR desensitization in perirenal adipose tissue contributes to impairment of lipolysis is unknown in chronic norepinephrine treated fetal sheep. Norepinephrine challenge test with relative NEFA measurement will provide evidence to explore the fat metabolism after chronic norepinephrine exposure in fetal sheep.

Intrinsic regulation of insulin secretion

Besides adrenergic and Ca^{2+} signaling in the of control insulin secretion, other G protein coupled receptors (GPCRs), including free fatty acid receptor (GPR40), glucagon-like peptide-1 (GLP-1), and gastric inhibitory polypeptide (GIP) (Winzell

and Ahren, 2007), help modulate β-cell insulin secretion. As norepinephrine is also a GPCR ligand, it is important to understand the physiological function of those various insulin regulators of GPCRs after chronic norepinephrine exposure.

On the other hand, the endoplasmic reticulum (ER, Figure 2.1) is also a major site to regulate intracellular Ca^{2+} ($[Ca^{2+}]_i$) (Borge et al., 2002; Luciani et al., 2009; Shepherd et al., 2000). Sarco(endo)plasmic reticulum Ca^{2+}-ATPase (SERCA) is a protein responsible for Ca^{2+} uptake into the ER lumen. Ca^{2+} release from the ER is mediated by inositol 1,4,5-trisphosphate (IP3) via IP3 receptors (IP3Rs) and cyclic adenosine diphosphate ribose via the ryanodine receptors (RyRs) Abnormalities in Ca^{2+} regulation is related to the development of type 2 diabetes in several rodent models and in humans (Levy et al., 1994; Roe et al., 1994). Whether the decreased α2-ARs (Chapter III, Figure 3.5), G_i-associated receptors, are associated with glucose metabolism in islets is unanswered. On the other hand, GSIS in pancreatic β-cells is principally regulated by $[Ca^{2+}]_i$. Therefore, understanding the ER Ca^{2+} regulation, regulated by SERCA, IP3Rs, and RyRs, will provide fully assessment to interpret the enhanced GSIS with lower ARs after chronic norepinephrine suppression in fetal sheep.

In conclusion, chronic exposure of elevated norepinephrine contributes to a compensatory enhancement of GSIS after removing norepinephrine suppression in fetal sheep. Adrenergic signaling is significantly impaired and specifically leads to hyper-responsiveness of insulin secretion in pancreatic β-cell, but this is independent of insulin content or increase β-cell mass. However,

comprehensively investigating insulin-glucose regulation in liver, adrenergic impact in adipose tissue, and intrinsic regulation of insulin secretion will provide a complete evaluation of the organisms physiology after chronic norepinephrine exposure to define its role in fetal programming.

Figure 5.1. mRNA expression in liver from 7-day norepinephrine-infused fetal sheep. Glc-6-Pase, glucose 6-phosphatase; NE, norepinephrine treated sheep fetus; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha. Significance is indicated by *, $P < 0.05$, or **, $P < 0.01$.

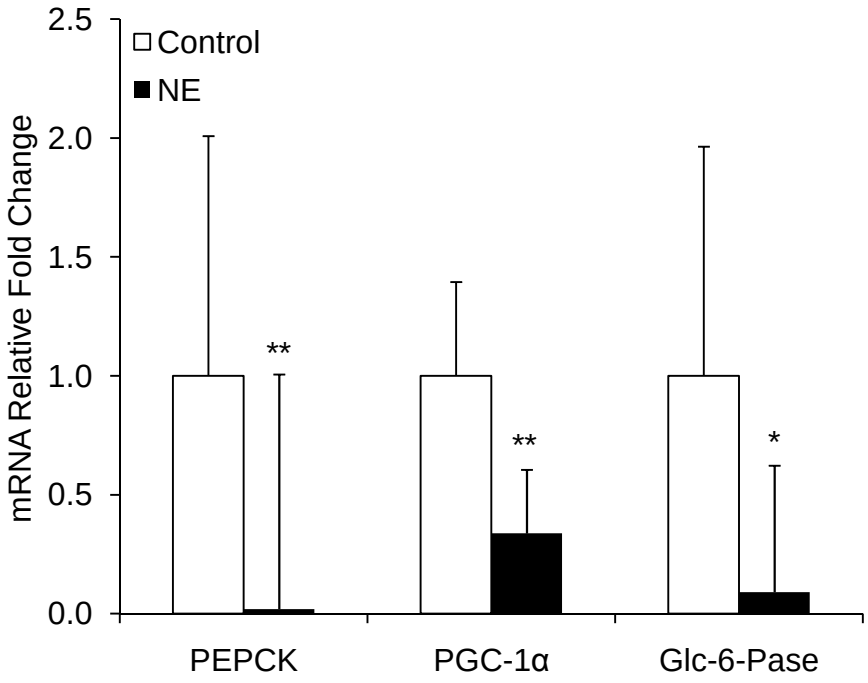


Figure 5.2. mRNA expression in perirenal fat from 7-day norepinephrine-infused fetal sheep. AR, adrenergic receptor; NE, norepinephrine treated sheep fetus. Significance is indicated by *, $P < 0.05$.

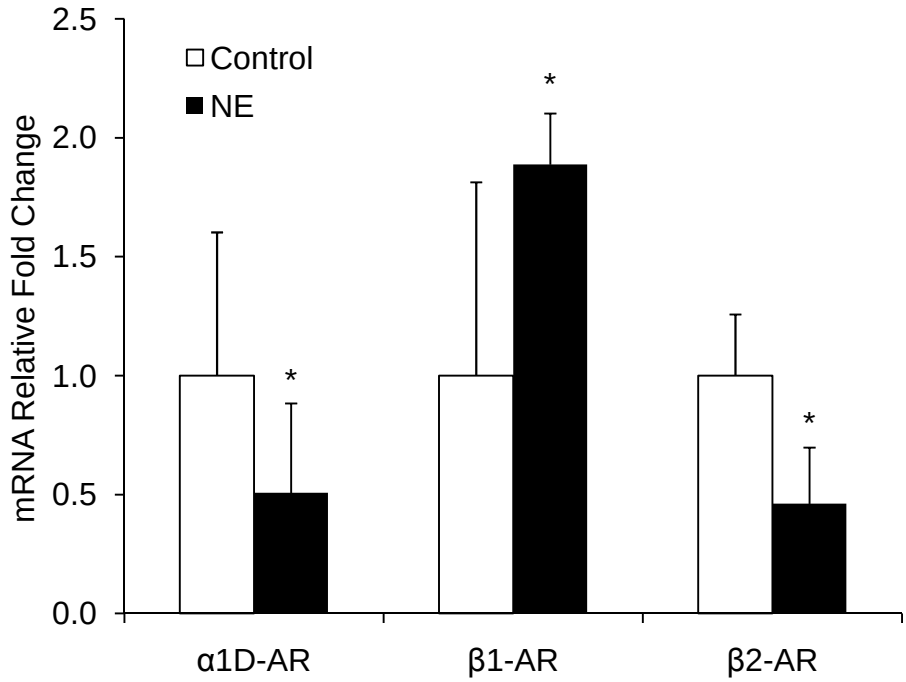
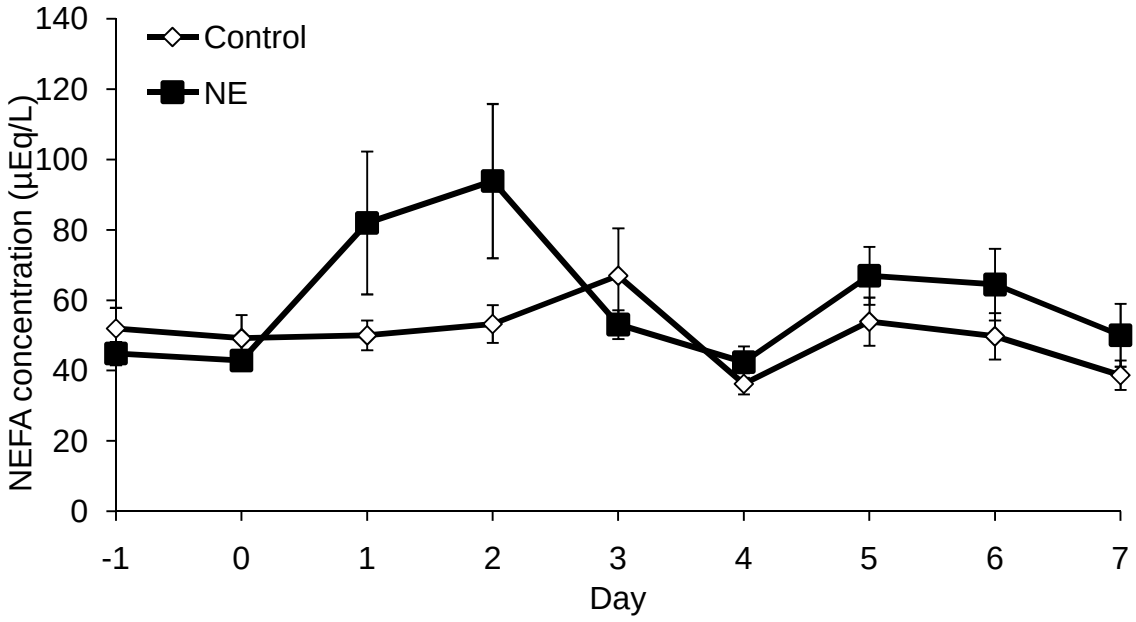


Figure 5.3. Daily non-esterified fatty acid throughout 7-day norepinephrine infusion treatment in fetal sheep. NE, norepinephrine treated sheep fetus; NEFA, non-esterified fatty acid.



REFERENCES

Ahlgren, U., J. Jonsson, L. Jonsson, K. Simu, and H. Edlund. 1998. beta-cell-specific inactivation of the mouse Ipf1/Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. Genes Dev 12: 1763-1768.

Aldoretta, P. W., T. D. Carver, and W. W. Hay, Jr. 1998. Maturation of glucose-stimulated insulin secretion in fetal sheep. Biol Neonate 73: 375-386.

Alexander, G., and D. Williams. 1971. Heat stress and development of the conceptus in domestic sheep. The Journal of Agricultural Science 76: 53-72.

Altan, V. M., E. Arioglu, S. Guner, and A. T. Ozcelikay. 2007. The influence of diabetes on cardiac beta-adrenoceptor subtypes. Heart Fail Rev 12: 58-65.

Ammala, C. et al. 1993. Exocytosis elicited by action potentials and voltage-clamp calcium currents in individual mouse pancreatic B-cells. J Physiol 472: 665-688.

Ashcroft, F. M. 1988. Adenosine 5'-triphosphate-sensitive potassium channels. Annu Rev Neurosci 11: 97-118.

Ashcroft, F. M., and F. M. Gribble. 1999. ATP-sensitive K+ channels and insulin secretion: their role in health and disease. Diabetologia 42: 903-919.

Balent, B. et al. 2002. Acute elevation of NEFA causes hyperinsulinemia without effect on insulin secretion rate in healthy human subjects. Annals of the New York Academy of Sciences 967: 535-543.

Barker, D. J. 1995. Fetal origins of coronary heart disease. BMJ 311: 171-174.

Barker, D. J., and P. M. Clark. 1997. Fetal undernutrition and disease in later life. Rev Reprod 2: 105-112.

Barker, D. J., and C. Osmond. 1986. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. Lancet 1: 1077-1081.

Barker, D. J., C. Osmond, E. Kajantie, and J. G. Eriksson. 2009. Growth and chronic disease: findings in the Helsinki Birth Cohort. Ann Hum Biol 36: 445-458.

Bassett, J. M., and C. Hanson. 1998. Catecholamines inhibit growth in fetal sheep in the absence of hypoxemia. Am J Physiol 274: R1536-1545.

Bassett, J. M., and C. Hanson. 2000. Prevention of hypoinsulinemia modifies catecholamine effects in fetal sheep. Am J Physiol Regul Integr Comp Physiol 278: R1171-1181.

Bell, A. W., R. B. Wilkening, and G. Meschia. 1987. Some aspects of placental function in chronically heat-stressed ewes. J Dev Physiol 9: 17-29.

Benovic, J. L., J. J. Onorato, M. G. Caron, and R. J. Lefkowitz. 1990. Regulation of G protein-coupled receptors by agonist-dependent phosphorylation. Soc Gen Physiol Ser 45: 87-103.

Bliss, C. R., and G. W. Sharp. 1994. A critical period in the development of the insulin secretory response to glucose in fetal rat pancreas. Life Sci 55: 423-427.

Bocian-Sobkowska, J., M. Zabel, W. Wozniak, and J. Surdyk-Zasada. 1999. Polyhormonal aspect of the endocrine cells of the human fetal pancreas. Histochemistry and cell biology 112: 147-153.

Boden, G., X. Chen, and N. Iqbal. 1998. Acute lowering of plasma fatty acids lowers basal insulin secretion in diabetic and nondiabetic subjects. Diabetes 47: 1609-1612.

Borge, P. D. et al. 2002. Insulin receptor signaling and sarco/endoplasmic reticulum calcium ATPase in beta-cells. Diabetes 51 Suppl 3: S427-433.

Boujendar, S., E. Arany, D. Hill, C. Remacle, and B. Reusens. 2003. Taurine supplementation of a low protein diet fed to rat dams normalizes the vascularization of the fetal endocrine pancreas. J Nutr 133: 2820-2825.

Boujendar, S. et al. 2002. Taurine supplementation to a low protein diet during foetal and early postnatal life restores a normal proliferation and apoptosis of rat pancreatic islets. Diabetologia 45: 856-866.

Brace, R. A. 1983. Blood volume and its measurement in the chronically catheterized sheep fetus. Am J Physiol 244: H487-494.

Branstrom, R., S. Efendic, P. O. Berggren, and O. Larsson. 1998. Direct inhibition of the pancreatic beta-cell ATP-regulated potassium channel by alpha-ketoisocaproate. J Biol Chem 273: 14113-14118.

Breier, B. H., M. H. Vickers, B. A. Ikenasio, K. Y. Chan, and W. P. Wong. 2001. Fetal programming of appetite and obesity. Mol Cell Endocrinol 185: 73-79.

Briscoe, C. P. et al. 2003. The orphan G protein-coupled receptor GPR40 is activated by medium and long chain fatty acids. J Biol Chem 278: 11303-11311.

Brown, J. E., S. Thomas, J. E. Digby, and S. J. Dunmore. 2002. Glucose induces and leptin decreases expression of uncoupling protein-2 mRNA in human islets. FEBS Lett 513: 189-192.

Brun, T., E. Roche, K. H. Kim, and M. Prentki. 1993. Glucose regulates acetyl-CoA carboxylase gene expression in a pancreatic beta-cell line (INS-1). J Biol Chem 268: 18905-18911.

Butler, T. G., G. Simonetta, M. L. Roberts, and I. C. McMillen. 1995. Impact of gestational age on the catecholamine responses of the fetal sheep adrenal to cholinergic stimulation in vitro. Experimental physiology 80: 767-777.

Bzoskie, L., L. Blount, K. Kashiwai, J. Humme, and J. F. Padbury. 1997. The contribution of transporter-dependent uptake to fetal catecholamine clearance. Biol Neonate 71: 102-110.

Bzoskie, L. et al. 1995. Placental norepinephrine clearance: in vivo measurement and physiological role. Am J Physiol 269: E145-149.

Campbell, R. G., and A. C. Dunkin. 1982. The effect of birth weight on the estimated milk intake, growth and body composition of sow-reared piglets. Animal Science 35: 193-197.

Carver, T. D., S. M. Anderson, P. A. Aldoretta, A. L. Esler, and W. W. Hay, Jr. 1995. Glucose suppression of insulin secretion in chronically hyperglycemic fetal sheep. Pediatr Res 38: 754-762.

Cetin, I. et al. 2001. The endocrine and metabolic profile of the growth-retarded fetus. J Pediatr Endocrinol Metab 14 Suppl 6: 1497-1505.

Chan, C. B. et al. 1999. Overexpression of uncoupling protein 2 inhibits glucose-stimulated insulin secretion from rat islets. Diabetes 48: 1482-1486.

Chandler, K. D., B. J. Leury, A. R. Bird, and A. W. Bell. 1985. Effects of undernutrition and exercise during late pregnancy on uterine, fetal and uteroplacental metabolism in the ewe. Br J Nutr 53: 625-635.

Chen, X. et al. 2010. beta2-Adrenergic receptor desensitization in perirenal adipose tissue in fetuses and lambs with placental insufficiency-induced intrauterine growth restriction. J Physiol 588: 3539-3549.

Cherif, H., B. Reusens, S. Dahri, C. Remacle, and J. J. Hoet. 1996. Stimulatory effects of taurine on insulin secretion by fetal rat islets cultured in vitro. J Endocrinol 151: 501-506.

Cole, L., M. Anderson, P. B. Antin, and S. W. Limesand. 2009. One process for pancreatic beta-cell coalescence into islets involves an epithelial-mesenchymal transition. J Endocrinol 203: 19-31.

Collins, S., M. G. Caron, and R. J. Lefkowitz. 1991. Regulation of adrenergic receptor responsiveness through modulation of receptor gene expression. Annu Rev Physiol 53: 497-508.

Colwill, J. R. et al. 1970. Insulin-induced hypoglycemia in the ovine fetus in utero. Endocrinology 87: 710-715.

Cook, J. T. et al. 1993. Association of low birth weight with beta cell function in the adult first degree relatives of non-insulin dependent diabetic subjects. BMJ 306: 302-306.

Corkey, B. E. et al. 1989. A role for malonyl-CoA in glucose-stimulated insulin secretion from clonal pancreatic beta-cells. J Biol Chem 264: 21608-21612.

Crespin, S. R., W. B. Greenough, 3rd, and D. Steinberg. 1973. Stimulation of insulin secretion by long-chain free fatty acids. A direct pancreatic effect. J Clin Invest 52: 1979-1984.

Curry, D. L., L. L. Bennett, and G. M. Grodsky. 1968. Dynamics of insulin secretion by the perfused rat pancreas. Endocrinology 83: 572-584.

Dabelea, D. et al. 2000. Intrauterine exposure to diabetes conveys risks for type 2 diabetes and obesity: a study of discordant sibships. Diabetes 49: 2208-2211.

Dalgaard, L. T. 2011. UCP2 mRNA expression is dependent on glucose metabolism in pancreatic islets. Biochem Biophys Res Commun.

De Blasio, M. J., K. L. Gatford, I. C. McMillen, J. S. Robinson, and J. A. Owens. 2007a. Placental restriction of fetal growth increases insulin action, growth, and adiposity in the young lamb. Endocrinology 148: 1350-1358.

De Blasio, M. J., K. L. Gatford, J. S. Robinson, and J. A. Owens. 2007b. Placental restriction of fetal growth reduces size at birth and alters postnatal growth, feeding activity, and adiposity in the young lamb. Am J Physiol Regul Integr Comp Physiol 292: R875-886.

De Prins, F., A. Van Assche, and R. D. Milner. 1983. C-peptide levels in amniotic fluid in experimental fetal growth retardation. Biol Neonate 43: 181-185.

De Prins, F. A., and F. A. Van Assche. 1982. Intrauterine growth retardation and development of endocrine pancreas in the experimental rat. Biol Neonate 41: 16-21.

de Vrijer, B., T. R. Regnault, R. B. Wilkening, G. Meschia, and F. C. Battaglia. 2004. Placental uptake and transport of ACP, a neutral nonmetabolizable amino acid, in an ovine model of fetal growth restriction. Am J Physiol Endocrinol Metab 287: E1114-1124.

Desai, M., N. J. Crowther, S. E. Ozanne, A. Lucas, and C. N. Hales. 1995. Adult glucose and lipid metabolism may be programmed during fetal life. Biochem Soc Trans 23: 331-335.

Desai, M., and C. N. Hales. 1997. Role of fetal and infant growth in programming metabolism in later life. Biological reviews of the Cambridge Philosophical Society 72: 329-348.

Devedjian, J. C. et al. 2000. Transgenic mice overexpressing alpha2A-adrenoceptors in pancreatic beta-cells show altered regulation of glucose homeostasis. Diabetologia 43: 899-906.

Divers, W. A. et al. 1981. Amniotic fluid catecholamines and metabolites in intrauterine growth retardation. Am J Obstet Gynecol 141: 608-610.

Dobbins, R. L., M. W. Chester, M. B. Daniels, J. D. McGarry, and D. T. Stein. 1998a. Circulating fatty acids are essential for efficient glucose-stimulated insulin secretion after prolonged fasting in humans. Diabetes 47: 1613-1618.

Dobbins, R. L. et al. 1998b. A fatty acid- dependent step is critically important for both glucose- and non-glucose-stimulated insulin secretion. J Clin Invest 101: 2370-2376.

Doronin, S., E. Shumay, H. Y. Wang, and C. C. Malbon. 2002. Akt mediates sequestration of the beta(2)-adrenergic receptor in response to insulin. J Biol Chem 277: 15124-15131.

Douglas, W. W., and R. P. Rubin. 1961. The role of calcium in the secretory response of the adrenal medulla to acetylcholine. J Physiol 159: 40-57.

Douglas, W. W., and R. P. Rubin. 1963. The mechanism of catecholamine release from the adrenal medulla and the role of calcium in stimulus-secretion coupling. J Physiol 167: 288-310.

Dumortier, O. et al. 2007. Different mechanisms operating during different critical time-windows reduce rat fetal beta cell mass due to a maternal low-protein or low-energy diet. Diabetologia 50: 2495-2503.

Dwyer, C. M., J. M. Fletcher, and N. C. Stickland. 1993. Muscle cellularity and postnatal growth in the pig. J Anim Sci 71: 3339-3343.

Economides, D. L., K. H. Nicolaides, and S. Campbell. 1991. Metabolic and endocrine findings in appropriate and small for gestational age fetuses. J Perinat Med 19: 97-105.

Efrat, S. 1997. Making sense of glucose sensing. Nat Genet 17: 249-250.

Eisenhofer, G., I. J. Kopin, and D. S. Goldstein. 2004. Catecholamine metabolism: a contemporary view with implications for physiology and medicine. Pharmacological reviews 56: 331-349.

Emmanouilides, G. C., D. E. Townsend, and R. A. Bauer. 1968. Effects of single umbilical artery ligation in the lamb fetus. Pediatrics 42: 919-927.

Eremia, S. C., H. A. de Boo, F. H. Bloomfield, M. H. Oliver, and J. E. Harding. 2007. Fetal and amniotic insulin-like growth factor-I supplements improve growth rate in intrauterine growth restriction fetal sheep. Endocrinology 148: 2963-2972.

Erraji-Benchekroun, L. et al. 2005. Overexpression of beta2-adrenergic receptors in mouse liver alters the expression of gluconeogenic and glycolytic enzymes. Am J Physiol Endocrinol Metab 288: E715-722.

Exton, J. H., and C. R. Park. 1968. Control of gluconeogenesis in liver. II. Effects of glucagon, catecholamines, and adenosine 3',5'-monophosphate on gluconeogenesis in the perfused rat liver. J Biol Chem 243: 4189-4196.

Fagerholm, V. et al. 2004. Altered glucose homeostasis in alpha2A-adrenoceptor knockout mice. European journal of pharmacology 505: 243-252.

Fleury, C. et al. 1997. Uncoupling protein-2: a novel gene linked to obesity and hyperinsulinemia. Nat Genet 15: 269-272.

Ford, S. P. et al. 2007. Maternal undernutrition during early to mid-gestation in the ewe results in altered growth, adiposity, and glucose tolerance in male offspring. J Anim Sci 85: 1285-1294.

Fowden, A. L. 1989. The role of insulin in prenatal growth. J Dev Physiol 12: 173-182.

Fowden, A. L., and A. J. Forhead. 2004. Endocrine mechanisms of intrauterine programming. Reproduction 127: 515-526.

Fowden, A. L., and W. W. Hay, Jr. 1988. The effects of pancreatectomy on the rates of glucose utilization, oxidation and production in the sheep fetus. Q J Exp Physiol 73: 973-984.

Fowden, A. L., P. Hughes, and R. S. Comline. 1989. The effects of insulin on the growth rate of the sheep fetus during late gestation. Q J Exp Physiol 74: 703-714.

Fowden, A. L., X. Z. Mao, and R. S. Comline. 1986. Effects of pancreatectomy on the growth and metabolite concentrations of the sheep fetus. J Endocrinol 110: 225-231.

Galan, H. L. et al. 1999. Relationship of fetal growth to duration of heat stress in an ovine model of placental insufficiency. Am J Obstet Gynecol 180: 1278-1282.

Galan, H. L. et al. 2001. Cotyledon and binucleate cell nitric oxide synthase expression in an ovine model of fetal growth restriction. J Appl Physiol 90: 2420-2426.

Gao, Z. et al. 2003. Distinguishing features of leucine and alpha-ketoisocaproate sensing in pancreatic beta-cells. Endocrinology 144: 1949-1957.

Gardner, D. S. et al. 2005. Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. Am J Physiol Regul Integr Comp Physiol 289: R947-954.

Garite, T. J., R. Clark, and J. A. Thorp. 2004. Intrauterine growth restriction increases morbidity and mortality among premature neonates. Am J Obstet Gynecol 191: 481-487.

Garofano, A., P. Czernichow, and B. Breant. 1997. In utero undernutrition impairs rat beta-cell development. Diabetologia 40: 1231-1234.

Garofano, A., P. Czernichow, and B. Breant. 1998. Postnatal somatic growth and insulin contents in moderate or severe intrauterine growth retardation in the rat. Biol Neonate 73: 89-98.

Gatford, K. L. et al. 2008. Impaired beta-cell function and inadequate compensatory increases in beta-cell mass after intrauterine growth restriction in sheep. Endocrinology 149: 5118-5127.

Godfrey, K. M., and D. J. Barker. 2000. Fetal nutrition and adult disease. Am J Clin Nutr 71: 1344S-1352S.

Gondret, F. et al. 2005. Influence of piglet birth weight on postnatal growth performance, tissue lipogenic capacity and muscle histological traits at market weight. 93: 137-146.

Gopalakrishnan, G. S. et al. 2005. Influence of maternal pre-pregnancy body composition and diet during early-mid pregnancy on cardiovascular function and nephron number in juvenile sheep. Br J Nutr 94: 938-947.

Gopel, S. et al. 2004. Capacitance measurements of exocytosis in mouse pancreatic alpha-, beta- and delta-cells within intact islets of Langerhans. J Physiol 556: 711-726.

Green, A. S. et al. 2012. Chronic pulsatile hyperglycemia reduces insulin secretion and increases accumulation of reactive oxygen species in fetal sheep islets. J Endocrinol 212: 327-342.

Green, A. S. et al. 2011. Characterization of glucose-insulin responsiveness and impact of fetal number and sex difference on insulin response in the sheep fetus. Am J Physiol Endocrinol Metab 300: E817-823.

Green, A. S., P. J. Rozance, and S. W. Limesand. 2010. Consequences of a compromised intrauterine environment on islet function. J Endocrinol 205: 211-224.

Greenough, A., K. H. Nicolaides, and H. Lagercrantz. 1990. Human fetal sympathoadrenal responsiveness. Early human development 23: 9-13.

Guillam, M. T. et al. 1997. Early diabetes and abnormal postnatal pancreatic islet development in mice lacking Glut-2. Nat Genet 17: 327-330.

Hales, C. N., and D. J. Barker. 2001. The thrifty phenotype hypothesis. Br Med Bull 60: 5-20.

Hales, C. N. et al. 1991. Fetal and infant growth and impaired glucose tolerance at age 64. BMJ 303: 1019-1022.

Hales, C. N., and S. E. Ozanne. 2003. The dangerous road of catch-up growth. J Physiol 547: 5-10.

Handel, S. E., and N. C. Stickland. 1987. The growth and differentiation of porcine skeletal muscle fibre types and the influence of birthweight. J Anat 152: 107-119.

Hasan, N. M. et al. 2008. Impaired anaplerosis and insulin secretion in insulinoma cells caused by small interfering RNA-mediated suppression of pyruvate carboxylase. J Biol Chem 283: 28048-28059.

Hay, W. W., Jr., J. W. Sparks, R. B. Wilkening, F. C. Battaglia, and G. Meschia. 1984. Fetal glucose uptake and utilization as functions of maternal glucose concentration. Am J Physiol 246: E237-242.

Henquin, J. C., M. Nenquin, P. Stiernet, and B. Ahren. 2006. In vivo and in vitro glucose-induced biphasic insulin secretion in the mouse: pattern and role of cytoplasmic Ca2+ and amplification signals in beta-cells. Diabetes 55: 441-451.

Herchuelz, A., N. Thonnart, A. Sener, and W. J. Malaisse. 1980. Regulation of calcium fluxes in pancreatic islets: the role of membrane depolarization. Endocrinology 107: 491-497.

Hermans, M. P., W. Schmeer, and J. C. Henquin. 1987. The permissive effect of glucose, tolbutamide and high K+ on arginine stimulation of insulin release in isolated mouse islets. Diabetologia 30: 659-665.

Hill, D. E. 1982. Fetal effects of insulin. Obstet Gynecol Annu 11: 133-149.

Hobel, C. J., G. C. Emmanouilides, D. E. Townsend, and K. Yashiro. 1970. Ligation of one umbilical artery in the fetal lamb; experimental production of fetal malnutrition. Obstet Gynecol 36: 582-588.

Hole, R. L., M. C. Pian-Smith, and G. W. Sharp. 1988. Development of the biphasic response to glucose in fetal and neonatal rat pancreas. Am J Physiol 254: E167-174.

Holemans, K., L. Aerts, and F. A. Van Assche. 2003. Fetal growth restriction and consequences for the offspring in animal models. Journal of the Society for Gynecologic Investigation 10: 392-399.

Hoy, M. et al. 2002. Increase in cellular glutamate levels stimulates exocytosis in pancreatic beta-cells. FEBS Lett 531: 199-203.

Inagaki, N., T. Gonoi, and S. Seino. 1997. Subunit stoichiometry of the pancreatic beta-cell ATP-sensitive K+ channel. FEBS Lett 409: 232-236.

Itoh, Y. et al. 2003. Free fatty acids regulate insulin secretion from pancreatic beta cells through GPR40. Nature 422: 173-176.

Jensen, J. et al. 2000. Independent development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: a role for the notch pathway in repression of premature differentiation. Diabetes 49: 163-176.

Jitrapakdee, S., A. Wutthisathapornchai, J. C. Wallace, and M. J. MacDonald. 2010. Regulation of insulin secretion: role of mitochondrial signalling. Diabetologia 53: 1019-1032.

Jones, C. T., H. N. Lafeber, and M. M. Roebuck. 1984. Studies on the growth of the fetal guinea pig. Changes in plasma hormone concentration during normal and abnormal growth. J Dev Physiol 6: 461-472.

Jones, C. T., H. N. Lafeber, T. P. Rolph, and J. T. Parer. 1990. Studies on the growth of the fetal guinea pig. The effects of nutritional manipulation on prenatal growth and plasma somatomedin activity and insulin-like growth factor concentrations. J Dev Physiol 13: 189-197.

Jones, C. T., and J. S. Robinson. 1983. Studies on experimental growth retardation in sheep. Plasma catecholamines in fetuses with small placenta. J Dev Physiol 5: 77-87.

Kahn, S. E. et al. 2001. Obesity, body fat distribution, insulin sensitivity and Islet beta-cell function as explanations for metabolic diversity. J Nutr 131: 354S-360S.

Kind, K. L. et al. 2003. Effect of maternal feed restriction during pregnancy on glucose tolerance in the adult guinea pig. Am J Physiol Regul Integr Comp Physiol 284: R140-152.

Krauss, S., C. Y. Zhang, and B. B. Lowell. 2005. The mitochondrial uncoupling-protein homologues. Nat Rev Mol Cell Biol 6: 248-261.

Lafeber, H. N., T. P. Rolph, and C. T. Jones. 1984. Studies on the growth of the fetal guinea pig. The effects of ligation of the uterine artery on organ growth and development. J Dev Physiol 6: 441-459.

Lang, J. 1999. Molecular mechanisms and regulation of insulin exocytosis as a paradigm of endocrine secretion. Eur J Biochem 259: 3-17.

Langley, S. C., and A. A. Jackson. 1994. Increased systolic blood pressure in adult rats induced by fetal exposure to maternal low protein diets. Clin Sci (Lond) 86: 217-222; discussion 121.

Laychock, S. G., and S. Bilgin. 1987. Alpha 2-adrenergic inhibition of pancreatic islet glucose utilization is mediated by an inhibitory guanine nucleotide regulatory protein. FEBS Lett 218: 7-10.

Lee, B., J. C. Jonas, G. C. Weir, and S. G. Laychock. 1999. Glucose regulates expression of inositol 1,4,5-trisphosphate receptor isoforms in isolated rat pancreatic islets. Endocrinology 140: 2173-2182.

Leos, R. A. et al. 2010. Chronic exposure to elevated norepinephrine suppresses insulin secretion in fetal sheep with placental insufficiency and intrauterine growth restriction. Am J Physiol Endocrinol Metab 298: E770-778.

Leury, B. J., K. D. Chandler, A. R. Bird, and A. W. Bell. 1990. Effects of maternal undernutrition and exercise on glucose kinetics in fetal sheep. Br J Nutr 64: 463-472.

Levy, J., J. R. Gavin, 3rd, and J. R. Sowers. 1994. Diabetes mellitus: a disease of abnormal cellular calcium metabolism? Am J Med 96: 260-273.

Limesand, S. W., and W. W. Hay, Jr. 2003. Adaptation of ovine fetal pancreatic insulin secretion to chronic hypoglycaemia and euglycaemic correction. J Physiol 547: 95-105.

Limesand, S. W., J. Jensen, J. C. Hutton, and W. W. Hay, Jr. 2005. Diminished beta-cell replication contributes to reduced beta-cell mass in fetal sheep with intrauterine growth restriction. Am J Physiol Regul Integr Comp Physiol 288: R1297-1305.

Limesand, S. W., T. R. Regnault, and W. W. Hay, Jr. 2004. Characterization of glucose transporter 8 (GLUT8) in the ovine placenta of normal and growth restricted fetuses. Placenta 25: 70-77.

Limesand, S. W., P. J. Rozance, L. D. Brown, and W. W. Hay, Jr. 2009. Effects of chronic hypoglycemia and euglycemic correction on lysine metabolism in fetal sheep. Am J Physiol Endocrinol Metab 296: E879-887.

Limesand, S. W., P. J. Rozance, D. Smith, and W. W. Hay, Jr. 2007. Increased insulin sensitivity and maintenance of glucose utilization rates in fetal sheep with placental insufficiency and intrauterine growth restriction. Am J Physiol Endocrinol Metab 293: E1716-1725.

Limesand, S. W., P. J. Rozance, G. O. Zerbe, J. C. Hutton, and W. W. Hay, Jr. 2006. Attenuated insulin release and storage in fetal sheep pancreatic islets with intrauterine growth restriction. Endocrinology 147: 1488-1497.

Lockwood, C. J., and S. Weiner. 1986. Assessment of fetal growth. Clinics in perinatology 13: 3-35.

Luciani, D. S. et al. 2009. Roles of IP3R and RyR Ca2+ channels in endoplasmic reticulum stress and beta-cell death. Diabetes 58: 422-432.

Luther, J. et al. 2007. Placental growth, angiogenic gene expression, and vascular development in undernourished adolescent sheep. Biol Reprod 77: 351-357.

MacDonald, M. J. 1993a. Estimates of glycolysis, pyruvate (de)carboxylation, pentose phosphate pathway, and methyl succinate metabolism in incapacitated pancreatic islets. Arch Biochem Biophys 305: 205-214.

MacDonald, M. J. 1993b. Metabolism of the insulin secretagogue methyl succinate by pancreatic islets. Arch Biochem Biophys 300: 201-205.

MacDonald, M. J. et al. 2009. Decreased levels of metabolic enzymes in pancreatic islets of patients with type 2 diabetes. Diabetologia 52: 1087-1091.

Maechler, P., and C. B. Wollheim. 1999. Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. Nature 402: 685-689.

Matschinsky, F. M., B. Glaser, and M. A. Magnuson. 1998. Pancreatic beta-cell glucokinase: closing the gap between theoretical concepts and experimental realities. Diabetes 47: 307-315.

McMillen, I. C. et al. 2001. Fetal growth restriction: adaptations and consequences. Reproduction 122: 195-204.

Mendonca, A. C., E. M. Carneiro, J. R. Bosqueiro, S. C. Crepaldi-Alves, and A. C. Boschero. 1998. Development of the insulin secretion mechanism in fetal and neonatal rat pancreatic B-cells: response to glucose, K+, theophylline, and carbamylcholine. Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas / Sociedade Brasileira de Biofisica ... [et al.] 31: 841-846.

Miki, T. et al. 1998. Defective insulin secretion and enhanced insulin action in KATP channel-deficient mice. Proc Natl Acad Sci U S A 95: 10402-10406.

Milley, J. R., J. S. Papacostas, and B. K. Tabata. 1986. Effect of insulin on uptake of metabolic substrates by the sheep fetus. Am J Physiol 251: E349-356.

Modan, M. et al. 1985. Hyperinsulinemia. A link between hypertension obesity and glucose intolerance. J Clin Invest 75: 809-817.

Morrison, J. L. 2008. Sheep models of intrauterine growth restriction: fetal adaptations and consequences. Clin Exp Pharmacol Physiol 35: 730-743.

Nakaki, T., T. Nakadate, S. Yamamoto, and R. Kato. 1983. Inhibition of dibutyryl cyclic AMP-induced insulin release by alpha-2 adrenergic stimulation. Life Sci 32: 191-195.

Nestorowicz, A. et al. 1996. Mutations in the sulonylurea receptor gene are associated with familial hyperinsulinism in Ashkenazi Jews. Hum Mol Genet 5: 1813-1822.

Newnham, J. P. et al. 1986. Differential response of ovine placental lactogen levels in maternal and fetal circulations following single umbilical artery ligation in fetal sheep. Placenta 7: 51-64.

Nicolini, U., C. Hubinont, J. Santolaya, N. M. Fisk, and C. H. Rodeck. 1990. Effects of fetal intravenous glucose challenge in normal and growth retarded fetuses. Horm Metab Res 22: 426-430.

Nieto, M. A. 2002. The snail superfamily of zinc-finger transcription factors. Nat Rev Mol Cell Biol 3: 155-166.

Nilsson, T., P. Arkhammar, P. Rorsman, and P. O. Berggren. 1988. Inhibition of glucose-stimulated insulin release by alpha 2-adrenoceptor activation is parallelled by both a repolarization and a reduction in cytoplasmic free Ca2+ concentration. J Biol Chem 263: 1855-1860.

Nussey, S., and S. A. Whitehead. 2001. Endocrinology : an integrated approach. Bios, Oxford.

Oh, W., K. Omori, C. J. Hobel, A. Erenberg, and G. C. Emmanouilides. 1975. Umbilical blood flow and glucose uptake in lamb fetus following single umbilical artery ligation. Biol Neonate 26: 291-299.

Ohlsson, H., K. Karlsson, and T. Edlund. 1993. IPF1, a homeodomain-containing transactivator of the insulin gene. EMBO J 12: 4251-4259.

Osgerby, J. C., D. C. Wathes, D. Howard, and T. S. Gadd. 2002. The effect of maternal undernutrition on ovine fetal growth. J Endocrinol 173: 131-141.

Otonkoski, T. et al. 1999. A point mutation inactivating the sulfonylurea receptor causes the severe form of persistent hyperinsulinemic hypoglycemia of infancy in Finland. Diabetes 48: 408-415.

Owens, J. A., J. Falconer, and J. S. Robinson. 1989. Glucose metabolism in pregnant sheep when placental growth is restricted. Am J Physiol 257: R350-357.

Owens, J. A. et al. 2007. Restriction of placental growth in sheep impairs insulin secretion but not sensitivity before birth. J Physiol 584: 935-949.

Oyama, K. et al. 1992. Single umbilical artery ligation-induced fetal growth retardation: effect on postnatal adaptation. Am J Physiol 263: E575-583.

Padbury, J. et al. 1987a. Effect of fetal adrenalectomy on catecholamine release and physiologic adaptation at birth in sheep. J Clin Invest 80: 1096-1103.

Padbury, J. F., J. K. Ludlow, M. G. Ervin, H. C. Jacobs, and J. A. Humme. 1987b. Thresholds for physiological effects of plasma catecholamines in fetal sheep. Am J Physiol 252: E530-537.

Padbury, J. F., J. K. Ludlow, J. A. Humme, and Y. Agata. 1986. Metabolic clearance and plasma appearance rates of catecholamines in preterm and term fetal sheep. Pediatr Res 20: 992-995.

Pardi, G., A. M. Marconi, and I. Cetin. 2002. Placental-fetal interrelationship in IUGR fetuses--a review. Placenta 23 Suppl A: S136-141.

Park, J. H., D. A. Stoffers, R. D. Nicholls, and R. A. Simmons. 2008. Development of type 2 diabetes following intrauterine growth retardation in rats is associated with progressive epigenetic silencing of Pdx1. J Clin Invest 118: 2316-2324.

Patane, G. et al. 2002. Role of ATP production and uncoupling protein-2 in the insulin secretory defect induced by chronic exposure to high glucose or free fatty acids and effects of peroxisome proliferator-activated receptor-gamma inhibition. Diabetes 51: 2749-2756.

Paulick, R., E. Kastendieck, and H. Wernze. 1985. Catecholamines in arterial and venous umbilical blood: placental extraction, correlation with fetal hypoxia, and transcutaneous partial oxygen tension. J Perinat Med 13: 31-42.

Petrik, J., E. Arany, T. J. McDonald, and D. J. Hill. 1998. Apoptosis in the pancreatic islet cells of the neonatal rat is associated with a reduced expression of insulin-like growth factor II that may act as a survival factor. Endocrinology 139: 2994-3004.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 29: e45.

Philipps, A. F., B. S. Carson, G. Meschia, and F. C. Battaglia. 1978. Insulin secretion in fetal and newborn sheep. Am J Physiol 235: E467-474.

Phillips, I. D. et al. 1996. Placental restriction alters the functional development of the pituitary-adrenal axis in the sheep fetus during late gestation. Pediatr Res 40: 861-866.

Piper, K. et al. 2004. Beta cell differentiation during early human pancreas development. J Endocrinol 181: 11-23.

Platt, H. 1978. Growth and maturity in the equine fetus. Journal of the Royal Society of Medicine 71: 658-661.

Poore, K. R., and A. L. Fowden. 2004. The effects of birth weight and postnatal growth patterns on fat depth and plasma leptin concentrations in juvenile and adult pigs. J Physiol 558: 295-304.

Powell, S. E., and E. D. Aberle. 1980. Effects of birth weight on growth and carcass composition of swine. J Anim Sci 50: 860-868.

Prentki, M., E. Joly, W. El-Assaad, and R. Roduit. 2002. Malonyl-CoA signaling, lipid partitioning, and glucolipotoxicity: role in beta-cell adaptation and failure in the etiology of diabetes. Diabetes 51 Suppl 3: S405-413.

Putney, J. W., Jr. 1999. TRP, inositol 1,4,5-trisphosphate receptors, and capacitative calcium entry. Proc Natl Acad Sci U S A 96: 14669-14671.

Ravelli, A. C. et al. 1998. Glucose tolerance in adults after prenatal exposure to famine. Lancet 351: 173-177.

Ravelli, G. P., Z. A. Stein, and M. W. Susser. 1976. Obesity in young men after famine exposure in utero and early infancy. N Engl J Med 295: 349-353.

Regnault, T. R. et al. 2003. The relationship between transplacental O2 diffusion and placental expression of PlGF, VEGF and their receptors in a placental insufficiency model of fetal growth restriction. J Physiol 550: 641-656.

Regnault, T. R., H. L. Galan, T. A. Parker, and R. V. Anthony. 2002a. Placental development in normal and compromised pregnancies-- a review. Placenta 23 Suppl A: S119-129.

Regnault, T. R., R. J. Orbus, F. C. Battaglia, R. B. Wilkening, and R. V. Anthony. 1999. Altered arterial concentrations of placental hormones during maximal placental growth in a model of placental insufficiency. J Endocrinol 162: 433-442.

Regnault, T. R. et al. 2002b. Placental expression of VEGF, PlGF and their receptors in a model of placental insufficiency-intrauterine growth restriction (PI-IUGR). Placenta 23: 132-144.

Rhoten, W. B. 1980. Insulin secretory dynamics during development of rat pancreas. Am J Physiol 239: E57-63.

Robinson, J. S., E. J. Kingston, C. T. Jones, and G. D. Thorburn. 1979. Studies on experimental growth retardation in sheep. The effect of removal of a endometrial caruncles on fetal size and metabolism. J Dev Physiol 1: 379-398.

Roe, M. W. et al. 1994. Defective glucose-dependent endoplasmic reticulum Ca2+ sequestration in diabetic mouse islets of Langerhans. J Biol Chem 269: 18279-18282.

Rorsman, P. et al. 1989. Failure of glucose to elicit a normal secretory response in fetal pancreatic beta cells results from glucose insensitivity of the ATP-regulated K+ channels. Proc Natl Acad Sci U S A 86: 4505-4509.

Roseboom, T. J. et al. 2001. Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. Mol Cell Endocrinol 185: 93-98.

Rosengren, A. H. et al. 2010. Overexpression of alpha2A-adrenergic receptors contributes to type 2 diabetes. Science 327: 217-220.

Rossi, D., and V. Sorrentino. 2010. The multiple alternatives of intracellular calcium signaling: a functionally distinct RyR splicing variant in pancreatic islets. Islets 2: 383-385.

Rozance, P. J. et al. 2009a. Prolonged maternal amino acid infusion in late-gestation pregnant sheep increases fetal amino acid oxidation. Am J Physiol Endocrinol Metab 297: E638-646.

Rozance, P. J., S. W. Limesand, J. S. Barry, L. D. Brown, and W. W. Hay, Jr. 2009b. Glucose replacement to euglycemia causes hypoxia, acidosis, and decreased insulin secretion in fetal sheep with intrauterine growth restriction. Pediatr Res 65: 72-78.

Rubi, B., H. Ishihara, F. G. Hegardt, C. B. Wollheim, and P. Maechler. 2001. GAD65-mediated glutamate decarboxylation reduces glucose-stimulated insulin secretion in pancreatic beta cells. J Biol Chem 276: 36391-36396.

Rudolph, A. M. 1984. Oxygenation in the fetus and neonate--a perspective. Semin Perinatol 8: 158-167.

Saarikoski, S. 1974. Fate of noradrenaline in the human foetoplacental unit. Acta physiologica Scandinavica. Supplementum 421: 1-82.

Savontaus, E., V. Fagerholm, O. Rahkonen, and M. Scheinin. 2008. Reduced blood glucose levels, increased insulin levels and improved glucose tolerance in alpha2A-adrenoceptor knockout mice. European journal of pharmacology 578: 359-364.

Schaak, S., J. Mialet-Perez, C. Flordellis, and H. Paris. 2007. Genetic variation of human adrenergic receptors: from molecular and functional properties to clinical and pharmacogenetic implications. Curr Top Med Chem 7: 217-231.

Sharp, G. W. 1996. Mechanisms of inhibition of insulin release. Am J Physiol 271: C1781-1799.

Shelton, M. 1964. Relation of Birth Weight to Death Losses and to Certain Productive Characters of Fall-Born Lambs. Journal of Animal Science 23: 355-359.

Shelton, M., and J. E. Huston. 1968. Effects of high temperature stress during gestation on certain aspects of reproduction in the ewe. J Anim Sci 27: 153-158.

Shepherd, R. M. et al. 2000. Hyperinsulinism of infancy: towards an understanding of unregulated insulin release. European Network for Research into Hyperinsulinism in Infancy. Arch Dis Child Fetal Neonatal Ed 82: F87-97.

Sherwood, L. 2007. Human physiology : from cells to systems. 6th ed. Thomson/Brooks/Cole, Australia ; Belmont, CA.

Shyng, S. L. et al. 1998. Functional analyses of novel mutations in the sulfonylurea receptor 1 associated with persistent hyperinsulinemic hypoglycemia of infancy. Diabetes 47: 1145-1151.

Simmons, R. A., L. J. Templeton, and S. J. Gertz. 2001. Intrauterine growth retardation leads to the development of type 2 diabetes in the rat. Diabetes 50: 2279-2286.

Simonetta, G., A. K. Rourke, J. A. Owens, J. S. Robinson, and I. C. McMillen. 1997. Impact of placental restriction on the development of the sympathoadrenal system. Pediatr Res 42: 805-811.

Slack, J. M. 1995. Developmental biology of the pancreas. Development 121: 1569-1580.

Smith, P. A. et al. 1997. Electrogenic arginine transport mediates stimulus-secretion coupling in mouse pancreatic beta-cells. J Physiol 499 (Pt 3): 625-635.

Smith, T. J., and C. A. Stanley. 2008. Untangling the glutamate dehydrogenase allosteric nightmare. Trends Biochem Sci 33: 557-564.

Sobngwi, E. et al. 2003. Effect of a diabetic environment in utero on predisposition to type 2 diabetes. Lancet 361: 1861-1865.

Stein, D. T. et al. 1996. Essentiality of circulating fatty acids for glucose-stimulated insulin secretion in the fasted rat. J Clin Invest 97: 2728-2735.

Straub, S. G., and G. W. Sharp. 2002. Glucose-stimulated signaling pathways in biphasic insulin secretion. Diabetes Metab Res Rev 18: 451-463.

Straub, S. G., and G. W. Sharp. 2004. Hypothesis: one rate-limiting step controls the magnitude of both phases of glucose-stimulated insulin secretion. Am J Physiol Cell Physiol 287: C565-571.

Supramaniam, V. G., G. Jenkin, J. Loose, E. M. Wallace, and S. L. Miller. 2006. Chronic fetal hypoxia increases activin A concentrations in the late-pregnant sheep. BJOG 113: 102-109.

Swenne, I., C. J. Crace, and R. D. Milner. 1987. Persistent impairment of insulin secretory response to glucose in adult rats after limited period of protein-calorie malnutrition early in life. Diabetes 36: 454-458.

Thomas, P. M. et al. 1995a. Mutations in the sulfonylurea receptor gene in familial persistent hyperinsulinemic hypoglycemia of infancy. Science 268: 426-429.

Thomas, S. A., A. M. Matsumoto, and R. D. Palmiter. 1995b. Noradrenaline is essential for mouse fetal development. Nature 374: 643-646.

Thureen, P. J., K. A. Trembler, G. Meschia, E. L. Makowski, and R. B. Wilkening. 1992. Placental glucose transport in heat-induced fetal growth retardation. Am J Physiol 263: R578-585.

Urano, Y. et al. 2004. Desensitization of the inhibitory effect of norepinephrine on insulin secretion from pancreatic islets of exercise-trained rats. Metabolism 53: 1424-1432.

van Assche, F. A., and L. Aerts. 1979. The fetal endocrine pancreas. Contributions to gynecology and obstetrics 5: 44-57.

Van Assche, F. A., F. De Prins, L. Aerts, and M. Verjans. 1977. The endocrine pancreas in small-for-dates infants. Br J Obstet Gynaecol 84: 751-753.

Varadi, A., E. Molnar, C. G. Ostenson, and S. J. Ashcroft. 1996. Isoforms of endoplasmic reticulum Ca(2+)-ATPase are differentially expressed in normal and diabetic islets of Langerhans. Biochem J 319 (Pt 2): 521-527.

Villar, J., and J. M. Belizan. 1982. The relative contribution of prematurity and fetal growth retardation to low birth weight in developing and developed societies. Am J Obstet Gynecol 143: 793-798.

Vogt, M. 1952. The secretion of the denervated adrenal medulla of the cat. British journal of pharmacology and chemotherapy 7: 325-330.

Vonnahme, K. A. et al. 2003. Maternal undernutrition from early- to mid-gestation leads to growth retardation, cardiac ventricular hypertrophy, and increased liver weight in the fetal sheep. Biol Reprod 69: 133-140.

Wallace, J. M., R. P. Aitken, and M. A. Cheyne. 1996. Nutrient partitioning and fetal growth in rapidly growing adolescent ewes. J Reprod Fertil 107: 183-190.

Wallace, J. M., R. P. Aitken, J. S. Milne, and W. W. Hay, Jr. 2004. Nutritionally mediated placental growth restriction in the growing adolescent: consequences for the fetus. Biol Reprod 71: 1055-1062.

Wallace, J. M., T. R. Regnault, S. W. Limesand, W. W. Hay, Jr., and R. V. Anthony. 2005. Investigating the causes of low birth weight in contrasting ovine paradigms. J Physiol 565: 19-26.

Wang, Z., and D. C. Thurmond. 2009. Mechanisms of biphasic insulin-granule exocytosis - roles of the cytoskeleton, small GTPases and SNARE proteins. J Cell Sci 122: 893-903.

WHO. 2002. Meeting of advisory group on maternal nutrition and low birthweight.

Wigmore, P. M., and N. C. Stickland. 1983. Muscle development in large and small pig fetuses. J Anat 137 (Pt 2): 235-245.

Williams, R. L. et al. 1982. Fetal growth and perinatal viability in California. Obstet Gynecol 59: 624-632.

Winzell, M. S., and B. Ahren. 2007. G-protein-coupled receptors and islet function-implications for treatment of type 2 diabetes. Pharmacology & therapeutics 116: 437-448.

Wong, D. L. 2006. Epinephrine biosynthesis: hormonal and neural control during stress. Cell Mol Neurobiol 26: 891-900.

Wu, G., F. W. Bazer, J. M. Wallace, and T. E. Spencer. 2006. Board-invited review: intrauterine growth retardation: implications for the animal sciences. J Anim Sci 84: 2316-2337.

Xu, J., J. Han, Y. S. Long, P. N. Epstein, and Y. Q. Liu. 2008. The role of pyruvate carboxylase in insulin secretion and proliferation in rat pancreatic beta cells. Diabetologia 51: 2022-2030.

Young, M., and E. M. Widdowson. 1975. The influence of diets deficient in energy, or in protein, on conceptus weight, and the placental transfer of a non-metabolisable amino acid in the guinea pig. Biol Neonate 27: 184-191.

Zhang, C. Y. et al. 2001. Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. Cell 105: 745-755.

Zhao, Y., Q. Fang, S. G. Straub, M. Lindau, and G. W. Sharp. 2010. Noradrenaline inhibits exocytosis via the G protein betagamma subunit and refilling of the readily releasable granule pool via the alpha(i1/2) subunit. J Physiol 588: 3485-3498.