Potential Role of NADPH Oxidase Subunit Neutrophil Cytosolic Factor 1 (NCF1) in Regulation of Adipocyte Hormone Adiponectin and Aging-Associated Lipodystrophy

By

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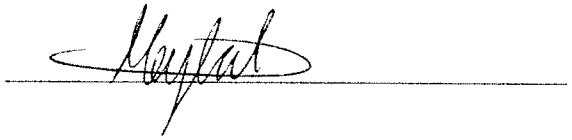
Signed: 

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Abbreviations Used

BCA – Bicinchoninic Acid; BSA – Bovine Serum Albumin; DTT – Dithiothreitol; EDTA – Ethylenediaminetetraacetic acid; HMW – Higher Molecular Weight; NADPH – Nicotinamide Adenine Dinucleotide Phosphate; PBS – Phosphate buffered saline; SDS – Sodium dodecyl sulfate; TBS – Tris Buffered Saline .

Abstract

Adipose tissue is important in regulating whole body insulin action. Inflammation in adipose tissue results in impaired response to insulin, but the causes behind onset of inflammation are not fully understood. Oxidative stress is an established cause of inflammation and obesity is associated with increased levels of reactive oxygen species and increased expression of NADPH oxidase subunits in adipose tissue. To examine if NADPH oxidase is a mediator of inflammation in adipose tissue, the circulating and adipose tissue levels of the anti-inflammatory adipokine adiponectin in homozygous *Ncf1*^{m1j} mice lacking the critical NADPH oxidase subunit NCF1 were analyzed. *Ncf1*^{m1j} mice displayed an 80% increase in the higher molecular weight (HMW) isoform of adiponectin in serum. This was associated with a 70% increase in the ratios of HMW to hexameric adiponectin in the visceral adipose tissue depot. Additionally, adiponectin levels of mice from two age groups were analyzed. When comparing between the two age groups, 20 months and 18 weeks, the younger age group displayed a 47% increase in levels of total adiponectin in the adipose tissue depot when compared with the older age group. This was accompanied by a 4.4 fold decrease in the levels of NADPH oxidase. These results indicate that *Ncf1*^{m1j} mice as well as mice from the younger age group have a metabolic profile consistent with being more insulin sensitive. The potential underlying mechanism is decreased inflammation due to increased production of HMW adiponectin. The suggested pathway involves NADPH oxidase.

Introduction

Diabetes is a major cause of morbidity and mortality in the United States. According to the American Diabetes Association, 23.6 million children and adults in the U.S. (7.8% of the population) have diabetes as of 2007 (1). Unfortunately, this number is expected to increase over the coming years as diabetes becomes a leading pandemic disease. The Center for Disease Control (CDC) estimates that one in three children born in U.S. after 2000 will develop diabetes during their life time (4).

Type 2 diabetes, the most common form of diabetes, is characterized by decreased responsiveness to insulin (1). Normally, insulin is necessary for maintaining whole body glucose homeostasis. In diabetes, the buildup of glucose in the blood can lead to complications such as hypertension, heart attack, stroke, visual impairment, and kidney disease (1). At the present, the two main factors that are related to the increased incidence of type 2 diabetes are obesity and the larger number of aging population.

Adipose tissue plays a major role in the regulation of insulin action (9). A special characteristic of the adipose tissue is its ability to expand or shrink according to the body's needs. When adipose tissue expands it allows for more energy to be stored, while shrinking of the tissue occurs when triglycerides are oxidized to release energy to cope with different forms of stress.

Long-term adipose tissue expansion in obesity is characterized by hypoxia, in which the lack of oxygen results in adipocyte death as well as inflammation of the adipose tissue (3). Adipose tissue inflammation is accompanied by infiltration of macrophages and lymphocytes and contributes to local and systemic insulin resistance (3).

Aging is associated with loss of subcutaneous adipose tissue and increased deposition of fat in visceral adipose tissue as well as in liver and skeletal muscle (5). Visceral adipose tissue

has been shown to correlate with the development of these diseases (8). On the other hand, subcutaneous adipose tissue is thought to protect against the development of cardiovascular diseases as well as type 2 diabetes (11). Aging is characterized by a form of lipodystrophy in which a loss of subcutaneous adipose depot was accompanied by expansion of visceral adipose depot.

The main goal of this study is to examine the changes in both subcutaneous and visceral adipose tissue associated with aging and obesity. Because of the close similarities between obesity and the process of aging, we hypothesize that the development of insulin resistance associated with these conditions share common causes. Specifically, oxidative stress in subcutaneous tissue contributes to insulin resistance and decreased production of the adipocyte hormone adiponectin in both obesity and aging.

Adiponectin, a protein hormone produced and secreted by the adipose tissue, regulates many metabolic processes (7). As its concentration in serum is negatively correlated with insulin resistance (7), it has been used as a marker for insulin responsiveness. In order to determine the degree of insulin resistance associated with aging, adiponectin levels in serum and in adipose tissue extracted from mouse of various ages will be analyzed.

Obesity is associated with increased levels of reactive oxygen species as well as increased expression of the radical-generating NADPH oxidase in the adipose tissue (6). The NADPH oxidase complex is comprised of membrane associated flavocytochrome protein (6). This protein consists of multiple subunits and cytosolic components. When the incorporation of the Neutrophil Cytosolic Factor 1 (NCF1), also known as p47phox subunit of the enzyme, into the complex is impeded, NADPH oxidase activity is inhibited (10).

Overall there are five types of NADPH oxidase, NOX1 through NOX5. Each enzyme is characterized by a distinct catalytic subunit while some of them share a few of the additional subunits (Fig. 1). Despite the similar structure and function of the different enzymes, they differ in their activation mechanism. NOX2 requires p22phox, p47phox, p67phox and Rac (2). In order to activate NOX2, p47phox phosphorylation is required. Based on prior work, p47phox has been designated as the organizer subunit of NOX2. This came from an observation done in phagocytes where p67phox, p40phox and Rac all failed to translocate to the membrane in neutrophils from patients lacking p47phox (2).

This study aims to understand the role of oxidative and inflammatory stress in adipose tissue in inducing insulin resistance. Results from these projects might shed light on the pathway by which obesity and aging can lead to the development of type 2 diabetes.

Material and Methods

Animals

Ncf1^{m1j}/*Ncf1*^{m1j} and age/sex-matched control mice were purchased from Jackson Labs (Bar Harbor, ME) and housed in a barrier facility at Arizona Health Sciences Center under 12-hr dark and light cycles. All procedures involving animals were performed by personnel under, and in accordance with, Dr. Craig Stump's protocol approved by University of Arizona Institutional Animal Care and Use Committee.

Adipose tissue sample preparation

Epidydimal Adipose tissue samples were dissected out of the mice using scissors and tweezers. The fat was then weighted and wrapped in foil. The wrapped samples were then submerged in liquid nitrogen before transferred to -80°C freezer.

Processing of the samples was done by homogenization of the fat. The samples were thawed, placed individually in 2mL centrifuge tubes and mixed with lysis buffer containing: 1X PBS (137mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 2mM KH_2PO_4, pH 7.4), 1:2000 aprotinin, 1:2000 pepstatin, 1:2000 leupeptin, 0.17mg/mL PMSF and 1mM EDTA. For each mg of fat, 1 uL of buffer was used. Power Gen 125 by Fisher Scientific was used in order to homogenize the samples. The instrument was set to level 5 and the samples were mixed till the fat and the buffer were incorporated. The homogenized samples were then centrifuged using Eppendorf Centrifuge 5417R at 5000 RPM and in 7°C for 5 minutes. Using a gel loading pipet tip, the middle layer containing the soluble fat proteins was removed and placed in a new tube. The tip of the gel loading pipet tip was wiped with a paper towel in between insertion to the same sample and was

changed in between insertions to different samples. Finally, the samples were placed back in -80°C freezer for storage.

Prior to loading the samples on an electrophoresis gel, a protein assay of the total protein concentration of each sample was preformed by using BCA Protein Assay Kit by Thermo Scientific. BSA standards were prepared in concentrations ranging from 2000mg/mL to 25mg/mL. The standards samples and the adipose tissue samples were mixed with the working reagent prepared according to the protocol in the assay kit. 25uL of both BSA standards and fat samples were then placed in a 96 well plate and 200uL of the prepared working reagent were added to each well. The plate was cover and placed for incubation at 37°C for 30 minutes. At the end of the incubation time the plate was cooled to room temperature and the absorbance of each sample was measured at 562nm using VARIOSKAN Flash plate reader by Thermo Fisher Scientific. The standards' absorption values were used to generate a standard curve and the line equation was used to determine the fat samples' concentration.

Protein analysis– Western blots (Native gels)

Gel Preparation

Gels were prepared using a 1.5mm gel plate by BIO-RAD. Gel's percentages were 5% stacking and 12% resolving. The stacking gel was made using: 30% Polyacrylamide, 1M Tris-Acetate pH=6.8 (30.29g Tris Base per 250mL, Glacial Acetic Acid was used to titrate to pH 6.8), 10% Ammonium persulfate, TEMED and H_2O. The resolving gel was made using: 30% Polyacrylamide, 1.5M Tris-Acetate pH=8.8 (90.86g Tris Base per 500mL, Glacial Acetic Acid was used to titrate to pH 8.8), 10% Ammonium persulfate, TEMED and H_2O.

Sample Loading

For adipose tissue samples 50ug of protein was loaded. For serum samples 2uL of sample was loaded. Prior to loading of the samples 4X loading buffer (for 10mL of loading buffer: 1.25mL 1M Tris pH 6.8, 5mL Glycerol, 0.02g Orange G Dye Solution and H_2O) and H_2O were added.

Electrophoresis

The gels were placed in a BIO-RAD electrophoresis gel box. Upon loading of the samples onto the gel, 1X Tris-Glycine running buffer was added (25mM Tris-Base, 20mM Glycine, 0.1% SDS). The gel was ran at 150V until the dye band fell off.

Transfer

Wet transfer was used for native gels. Blot paper, nitrocellulose membrane and the native gel were placed inside a transfer cassette. The cassette was placed in a BIO-RAD transfer box and transfer buffer was added (1X Tris-Glycine, 20% v/v Methanol, 0.015% w/v SDS and H_2O). The transfer was done overnight at 25V.

Protein analysis– Western blots (Denaturing gels)

Gel Preparation

Gels were prepared using a 1.5mm gel plate by BIO-RAD. Gel's percentages were 5% stacking and 10% resolving. The stacking gel was made using: 30% Polyacrylamide, 1M Tris-HCl pH 6.8 (0.052M Tris Base, 0.948M Tris-HCl, NaOH and HCl were used to titrate to pH 6.8), 10% Ammonium persulfate, 10% SDS, TEMED and H_2O. The resolving gel was made using:

30% Polyacrylamide, 1.5M Tris-HCl pH 8.8 (1.27M Tris Base, 0.23M Tris-HCl, NaOH and HCl were used to titrate to pH 8.8), 10% Ammonium persulfate, 10% SDS, TEMED and H_2O.

Loading

For adipose tissue samples 50ug of protein was loaded. For serum samples 2uL of sample was loaded. Prior to loading of the samples 5X loading buffer (106mM Tris-HCl, 141mM Tris Base, 2% SDS, 10% EDTA, 0.51mM Glycerol, 0.22mM SERVA Blue G250 1% solution, 0.175mM Phenol Red 1% solution and H_2O), 10X DTT and H_2O were added. Prior to loading, the samples were heated in a water bath at 95°C for 15 minutes.

Electrophoresis

The gels were placed in a BIO-RAD electrophoresis gel box. Upon loading of the samples onto the gel, 1X Tris-Glycine running buffer was added (25mM Tris-Base, 20mM Glycine, 0.1% SDS). The gel was ran at 150V until the dye band fell off.

Transfer

Semi-dry transfer was used for denaturing gels. Blot paper, nitrocellulose membrane and the denaturing gel were placed inside the TE77X Semi-Dry transfer unit by Hoefer. A small amount of Transfer buffer was added and the device was set to constant AMP setting. The AMP level was determined according to: (area of the blot paper used in cm)x(0.8). The transfer was preformed for 1.5 hours.

Ponceau Staining

Upon successful transfer of either native gels or denaturing gels the blots were removed from the transfer apparatus and were washed with 1X PBS for 5 minutes. In order to assess the protein loading and transfer quality, the blots were placed in a Ponceau Membrane Staining solution for 3 minutes or until protein bands were visible. Excess ponceau solution was washed using H_2O and the blots were scanned. The blots were then placed in 1X PBS for 5 minutes to wash any ponceau solution residues before being placed overnight in blocking buffer comprised of 5% dry milk powder in TBST (4.5mM Tris Base, 20.5mM Tris-HCl, 150mM NaCl, 3mM KCl, H_2O and 0.1% v/v Tween-20 pH 7.4).

Adiponectin Probing

Upon successful transfer and overnight incubation with blocking buffer the blot was incubated with primary antibody for 12 hours at 4°C. For Native gels the primary antibody used was a rabbit anti-serum raised against recombinant globular adiponectin (Genset) at a concentration of 1:5000 in blocking buffer. For denaturing gels the primary antibody used was directed at the N terminus of mouse adiponectin at a concentration of 1:5000 in blocking buffer.

At the end of the incubation time, for both native and denaturing gels, the antibody was removed and the blot was washed 3 times for 10 minutes with TBST followed by additional 30 minute blocking with blocking buffer at room temperature. Upon removal of the blocking buffer the blot was incubated with secondary antibody, 1:2000 Horseradish peroxidase – conjugated anti-rabbit antibody by Jackson ImmunoResearch Laboratories, for 2 hours at room temperature.

At the end of the incubation time the secondary antibody was removed and the blot was washed 3 times for 10 minutes with TBST and then twice for 5 minutes with 1X PBS. The

membrane was then treated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and exposed using Chemidoc Photometrics 1394 Camera by BIO-RAD, to view the protein bands.

NADPH Oxidase Probing

Only denaturing gels were used for this purpose. The gels and the samples were loaded and prepared in the same way described above. Upon successful transfer and overnight incubation with blocking buffer the blot was incubated with primary antibody for 12 hours at 4°C. The primary antibody used was $p47^{phox}$ aimed at the NCF1 subunit of NADPH oxidase by Abbiotec at a concentration of 1:1000 in blocking buffer.

At the end of the incubation time, the antibody was removed and the blot was washed 3 times for 10 minutes with TBST followed by additional 30 minute blocking with blocking buffer at room temperature. Upon removal of the blocking buffer the blot was incubated with secondary antibody, 1:2000 Horseradish peroxidase – conjugated anti-rabbit antibody by Jackson ImmunoResearch Laboratories, for 2 hours at room temperature.

At the end of the incubation time the secondary antibody was removed and the blot was washed 3 times for 10 minutes with TBST and then twice for 5 minutes with 1X PBS. The membrane was then treated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and exposed using Chemidoc Photometrics 1394 Camera by BIO-RAD, to view the protein bands.

Data Analysis

Images obtained with the Chemidoc Photometrics 1394 Camera by BIO-RAD were analyzed with ImageJ. The integrated intensity of each protein band was measured and the background intensity was subtracted from to assure consistency and accuracy. Finally, the total protein loading was determined by analysis of the Ponceau staining image of the blot. The data obtained for the intensity of the specific protein bands was then normalized to the intensity of the corresponding protein loading.

Results

NADPH oxidase is a major source of reactive oxygen species (ROS). To assess if NADPH oxdiase plays a role in the inflammatory status of adipose tissue, we examined the levels of different oligomeric forms of adiponectin, an anti-inflammatory adipocyte hormone, in mice lacking the critical NCF1 subunit of NADPH oxidase. As shown in Fig. 2, homozygous *Ncf1*^{m1j} mice displayed an 80% increase in the higher molecular weight (HMW) isoform of adiponectin in serum without significant differences in hexamers or trimers. This was associated with a 70% increase in the ratio of HMW to hexameric adiponectin in the visceral adipose tissue depot (Fig. 3).

Angiotnesin II was previously shown to increase NADPH oxidase activity (2). Treatment with angiotensin II caused a decrease in hexameric isoform of adiponectin in serum (Fig. 2). However, there was no significant difference between wild type and *Ncf1*^{m1j} mice (Fig. 2), indicating the effect of angiotensin II on serum adiponectin levels was independent of NADPH oxidase activity. Similarly, no significant difference was observed in the ratio of HMW to hexameric adiponectin in the visceral adipose tissue depot (Fig. 3).

To assess the functional fitness of adipose tissue in aging, serum levels of adiponectin oligomers in 20-month- and 18-week-old mice were assessed using native immunoblot analysis. As shown in Fig. 4, 20-month-old mice displayed a tendency for having a decreased ratio of HMW to hexameric adiponectin in the visceral adipose tissue depot. However, no significant changes in total adiponectin levels were observed between the 20-month- and the 18-week-old mice in the visceral adipose depot (Fig. 5). In contrast to visceral adipose tissue, subcutaneous adipose depot from 20-month-old mice had higher levels of HMW and hexameric adiponectin but a lower ratio of HMW to hexameric adiponectin (Fig. 6).

Given the lack of dramatic differences in the levels of HMW or hexameric adiponectin in visceral or subcutaneous adipose tissue between 18-week and 20-month old mice, it was surprising that 20-month old mice had an approximately 50% decrease in serum HMW adiponectin (Fig. 7). This may be due to impaired secretion from adipocytes or decreased stability of adiponectin in circulation from aged mice.

Results described above suggest that NCF1 and aging are both factors that could contribute to decreased levels of HMW adiponectin in circulation. This led us to examine if the protein levels of the critical NADPH oxidase subunit NCF1 are increased in visceral adipose tissue from 20-month-old mice when compared with that from 18-week-old mice. As shown in Fig. 8, the cohort of younger age group analyzed displayed a 47% increase in levels of total adiponectin in the adipose tissue depot when compared with the older age group (Fig. 8). This was accompanied by a 4.4 fold decrease in the levels of NADPH oxidase subunit NCF1 when compared with aged mice (Fig. 8). The increase in visceral adipose tissue NCF1 in aged mice was not associated with changes in the amount of epididymal fat pad mass (Fig. 9).

Future Plans

Currently there are still some tissues and sera left from a high-fat diet study of NCF1 -/- mice that remain to be processed. Additional data from this study will provide new insight into the role of NADPH oxidase in obese mice versus aged mice. In terms of developing a module of a larger U-type proposal, the project with the highest potential is to examine infiltration of leukocytes including macrophages, neutrophils, and lymphocytes in adipose tissue from aging mice. A proposal listing this project as a subaim is being developed.

Discussion

In recent decades, type 2 diabetes has become a leading pandemic and with a growing number of individuals being diagnosed with it each year. Type 2 diabetes is characterized by decreased responsiveness to insulin (1). Therefore, it is important to analyze the causes and the mechanisms by which insulin resistance develop. At the present, two major risk factors are known to contribute to the increasing number of type 2 diabetes incidents: obesity and aging.

In obese individuals, the expansion of adipose tissue leads to local as well as systemic inflammation, which in turn leads to the development of insulin resistance (3). Additionally, obesity is associated with increased levels of reactive oxygen species as well as the expression of the radical-generating NADPH oxidase in the adipose tissue (6).

The process of aging includes the loss of the subcutaneous adipose tissue (5), which is thought to confer protection from the development of type 2 diabetes and cardiovascular diseases. In addition, loss of subcutaneous adipose tissue in aging is accompanied by gain of visceral adipose tissue (5), which correlates with the development of these diseases (11). Fig. 9 shows that the corresponding weights of visceral and subcutaneous adipose tissue collected from mice in the two age groups followed a similar trend.

Due to the close similarities between the process of aging and obesity we hypothesized that insulin resistance in both cases shares common causes. In particular, we tested if oxidative stress is a major contributing factor leading to insulin resistance and decreased production of the adipocyte hormone adiponectin.

Using mice lacking the critical subunit NCF1 allowed us to assess the role of NADPH oxidase in circulating adiponectin oligomer levels. Homozygous *Ncf1*^{m1j} mice displayed an 80% increase in the higher molecular weight (HMW) isoform of adiponectin in serum (Fig. 2) and a

70% increase in the ratio of HMW to hexameric adiponectin in the visceral adipose tissue depot (Fig. 3). These results indicate that *Ncf1*^{m1j} mice have a metabolic profile consistent with being more insulin sensitive. The potential underlying mechanism is decreased inflammation due to increased production of HMW adiponectin.

Angiotensin II treatment, which was used in order to increase NADPH oxidase activity, caused an overall decrease in the hexameric isoform of adiponectin in serum but no difference was observed between wild type and *Ncf1*^{m1j} mice (Fig. 2). This indicates that the effect of angiotensin II serum adiponectin was independent of changes in NADPH oxidase activity.

In order to understand the relationship between obesity, aging and insulin resistance, serum and adipose tissue levels of the protein adiponectin were analyzed in mice of two age groups, 20 months and 18 weeks. Examination of adiponectin levels in the visceral adipose tissue revealed a decreased ratio of HMW to hexameric adiponectin among 20 months old mice (Fig. 4) with no significant change in the total adiponectin levels when comparing the two age groups (Fig. 5). This is consistent with 20-month-old mice being less insulin sensitive.

In contrast to visceral adipose tissue, the subcutaneous adipose tissue of 20-month-old mice was characterized by higher levels of HMW and hexameric isoform of adiponectin (Fig. 6). This was accompanied by a decrease in the ratio of HMW to hexameric adiponectin (Fig. 6). It is important to note that the increase of the two adiponectin isoforms among the 20 month-old group was observed despite of a decrease in amount of subcutaneous adipose tissue (Fig. 9), implying that the increase observed was independent of the amount of tissue collected.

Surprisingly, even though we observed an increase in the levels of HMW adiponectin in the subcutaneous tissue of 20 month-old mice, analysis of serum samples taken from the two age groups revealed about 50% decrease in HMW adiponectin among the 20-month-old mice (Fig. 7).

This may reflect decreased secretion of adiponectin from adipose tissue or increased clearance from circulation in 20-month-old mice. If there are changes in post-translational modifications of adiponectin associated with aging, it may account for decreased circulating HMW adiponectin. Adiponectin stability in serum is known to be affected by its sialic acid content.

To asses the role of NADPH oxidase in the development of insulin resistance we determined the levels of the critical NADPH oxidase subunit, NCF1, alongside with the levels of protein hormone adiponectin in visceral adipose tissue from 18-week and 20-month old mice. This revealed a 47% increase in the levels of total adiponectin among the younger group (Fig. 8). This increase in adiponectin levels was accompanied by a 4.4-fold decrease in the levels of NCF1 subunit (Fig. 8). This shows the correlation between the levels of adiponectin and NADPH oxidase.

It has been proposed that adiponectin can be used as a marker for insulin responsiveness since its concentration in serum has been shown to be negatively correlated with insulin resistance (7). The decrease in total adiponectin levels among 20-month-old mice (Fig. 5 and Fig. 8) indicates that they were less insulin-sensitive. These results along with the dramatic increase in the levels of NADPH oxidase among the older age group reveal a correlation between insulin resistance and the expression of the enzyme.

In order to directly correlate NADPH oxidase levels with oxidative stress and inflammation, further experiments will include activity assay of NADPH oxidase in adipose tissue from both age groups. Additionally, levels of protein modification as well as inflammatory factors that might be present due to oxidative stress would be measured.

Results from this study show that obesity and aging share similarities in the effects they have on the adipose tissue. Changes in the composition of adiponectin in the adipose tissue as

well as in circulation could indicate a profile of reduced insulin sensitivity among both homozygous *Ncf1*^{m1j} and older mice. While the reasons for these changes are still unclear, the study suggests a correlation between adiponectin levels and levels of NADPH oxidase. This helps establish a potential mechanism by which inflammation due to increase oxidative stress can be a major cause for type 2 diabetes.

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Figure Legends

Figure 1. Basic structure of NADPH Oxidase NOX2 (badrad). NOX2 activation requires p22phox, p47phox, p67phox and Rac. Additionally, in order to activate NOX2 p47phox phosphorylation is required (Badrad).

Figure 2. Native immunoblot analysis of adiponectin oligomers from plasma of wild type and NCF1-/- mice treated with saline or angiotensin II (AngII). Each sample contained 2uL of serum and was loaded and probed according to the procedure described in the Material and Methods section. Data analysis was done using ImageJ and the amount of signal produced was normalized to the total protein content. n=8 to 10 for each group.

Figure 3. Native immunoblot analysis of adiponectin oligomers from visceral (epidydimal) adipose tissue depot of wild type and NCF1-/- mice treated with saline or angiotensin II (AngII). Each sample contained 50ug of protein and was loaded and probed according to the procedure described in the Material and Methods section. Data analysis was done using ImageJ and the amount of signal produced was normalized to the total protein content. n=8 to 10 for each group.

Figure 4. Native immunoblot analysis of adiponectin oligomers from visceral (epidydimal) adipose tissue depot of 20 months and 18 weeks old mice. Each sample contained 50ug of protein and was loaded and probed according to the procedure described in the Material and Methods section. Data analysis was done using ImageJ and the amount of signal produced was normalized to the total protein content. n=7 per group.

Figure 5. Denaturing immunoblot analysis of total adiponectin from the visceral (epidydimal) adipose tissue depot of 20 months and 18 weeks old mice. Each sample contained 50ug of protein and was loaded and probed according to the procedure described in the Material and Methods section. Data analysis was done using ImageJ and the amount of signal produced was normalized to the total protein content. n=7 per group.

Figure 6. Native immunoblot analysis of adiponectin oligomers from subcutaneous (inguinal) adipose tissue depot of 20 months and 18 weeks old mice. Each sample contained 50ug of protein and was loaded and probed according to the procedure described in the Material and Methods section. Data analysis was done using ImageJ and the amount of signal produced was normalized to the total protein content. n=7 per group.

Figure 7. Native immunoblot analysis of adiponectin oligomers from plasma of 18-week and 20-month old mice. Each sample contained 1uL of serum and was loaded and probed according to the procedure described in the Material and Methods section. Data analysis was done using ImageJ and the amount of signal produced was normalized to the total protein content. n=7 for each group.

Figure 8. Denaturing immunoblot analysis of total adiponectin from the visceral (epidydimal) adipose tissue depot of 20 months and 18 weeks old mice with their corresponding Neutrophil Cytosolic Factor 1 levels. Each sample contained 50ug of protein and was loaded and probed according to the procedure described in the Material and Methods section. Data analysis was

done using ImageJ and the amount of signal produced was normalized to the total protein content n=5 for 20 months group and n=3 for 18 weeks group.

Figure 9. Visceral (epidydimal) and subcutaneous (inguinal) fat pad weight (mg) in 20-month and 18-week old male mice. n=7 for each group.

Figure 1 – general structure of NADPH Oxidase, NOX2 (2)

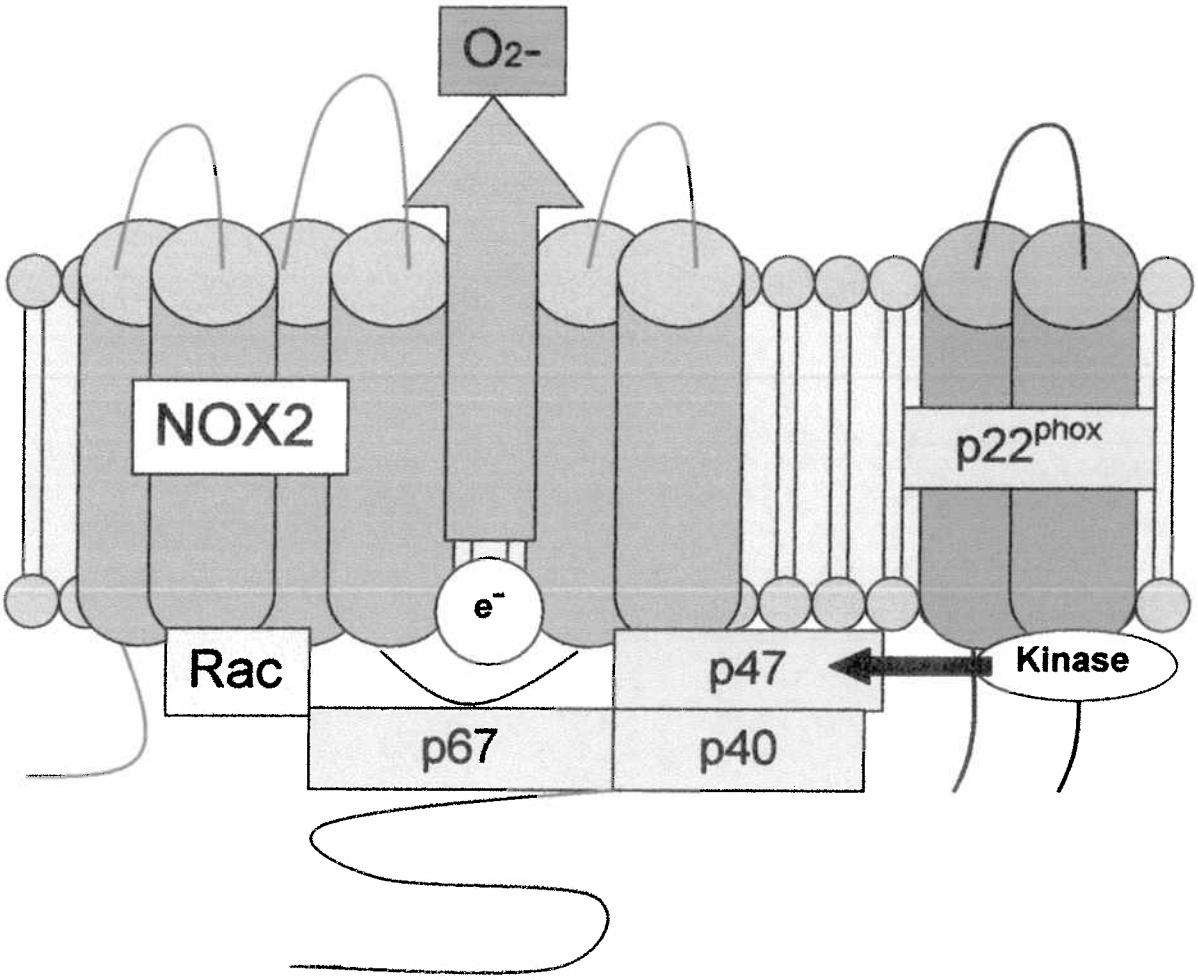
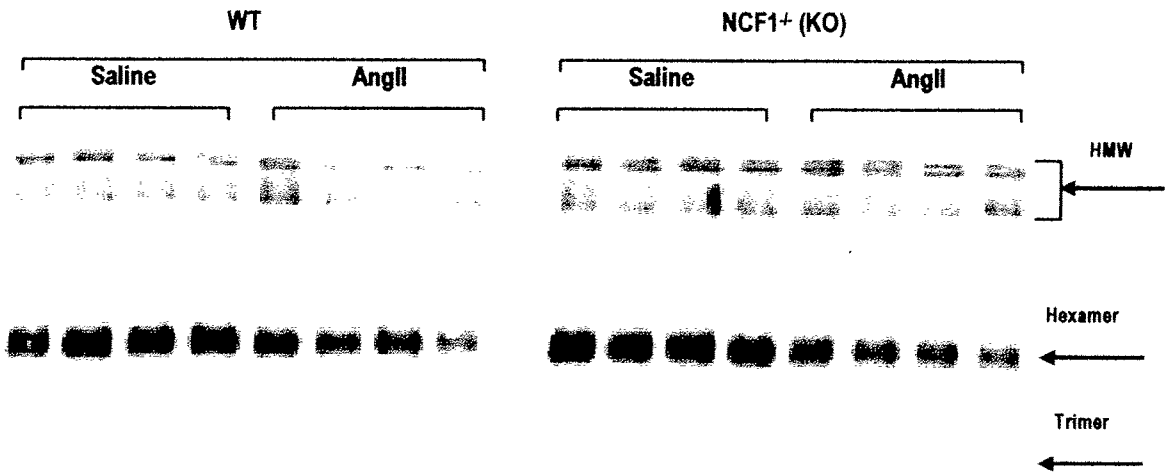


Figure 2 - Increased Higher Molecular Weight (HMW) Isoform of Adiponectin in Serum of NCF1 -/- mice



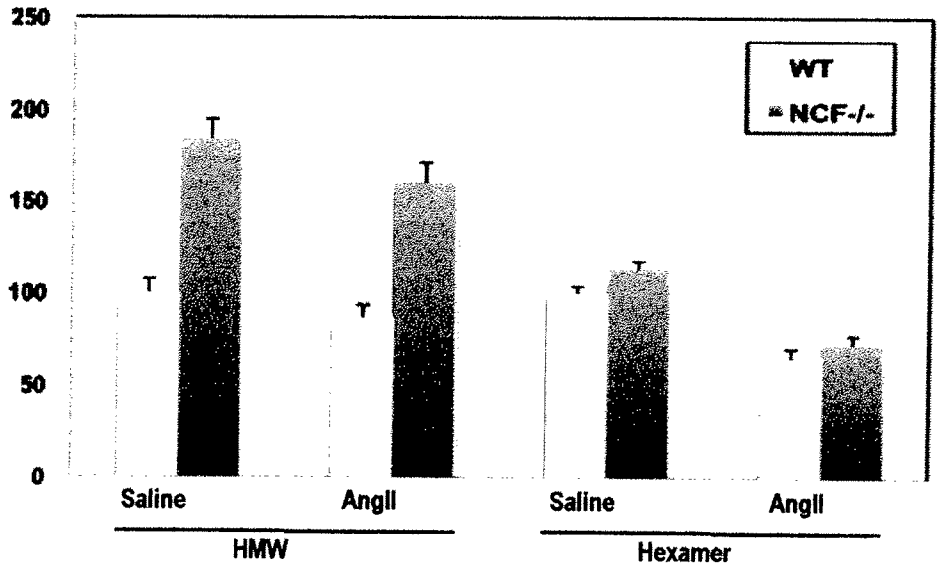
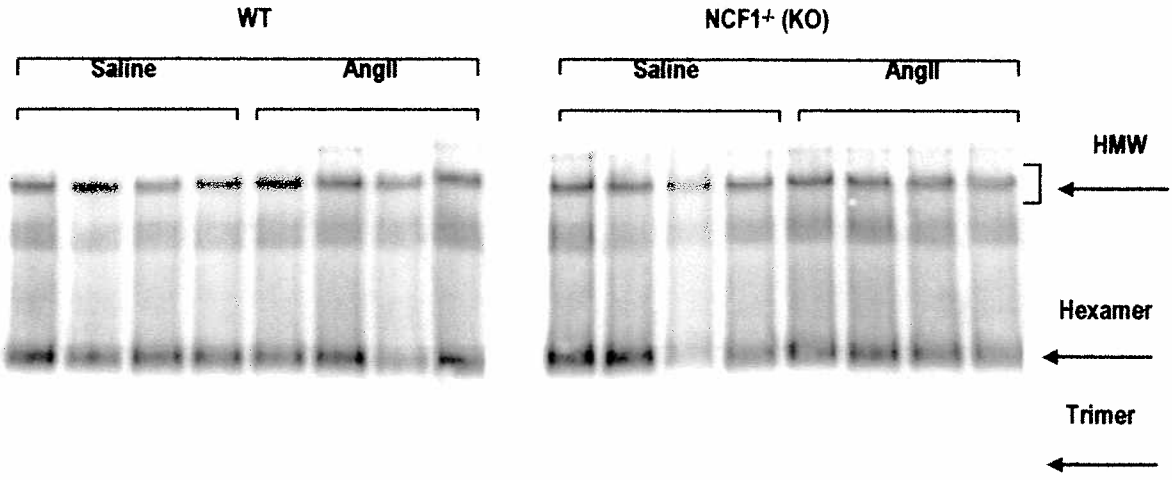


Figure 3 – Increased ratio of HMW to Hexameric Adiponectin in Visceral Adipose Tissue Depot of NCF1 -/- mice



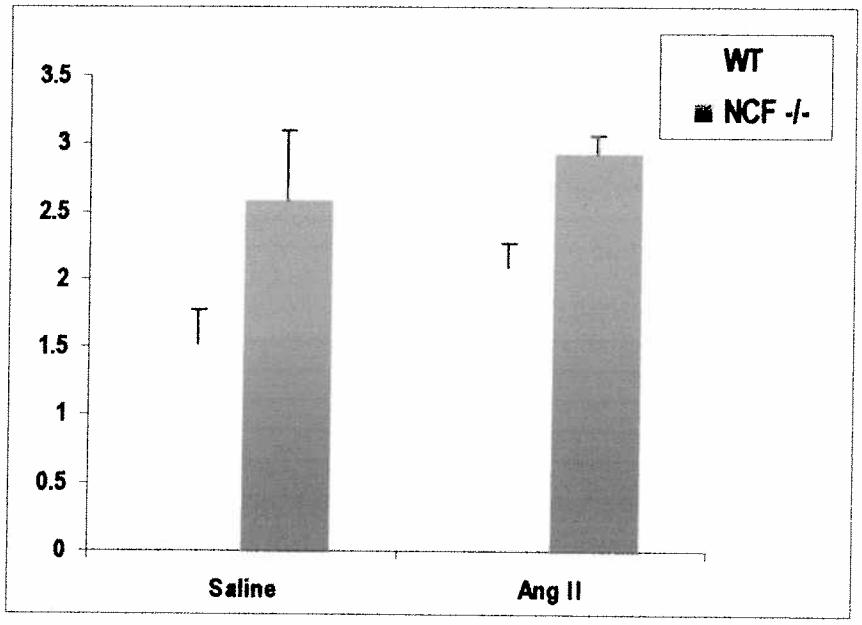
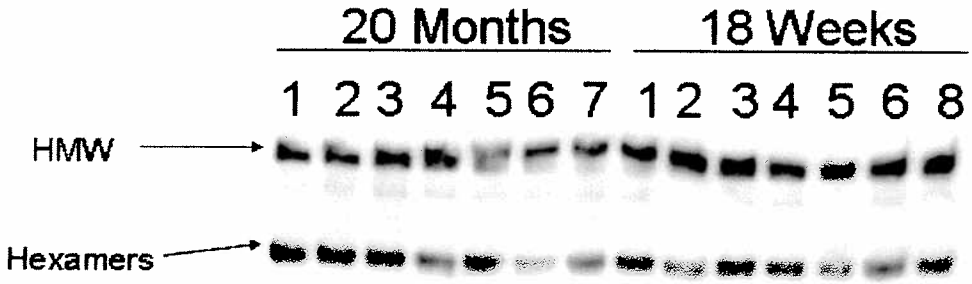


Figure 4 - Increased ratio of HMW to Hexameric Adiponectin in Visceral Adipose Tissue Depot of 18 Weeks Old Mice



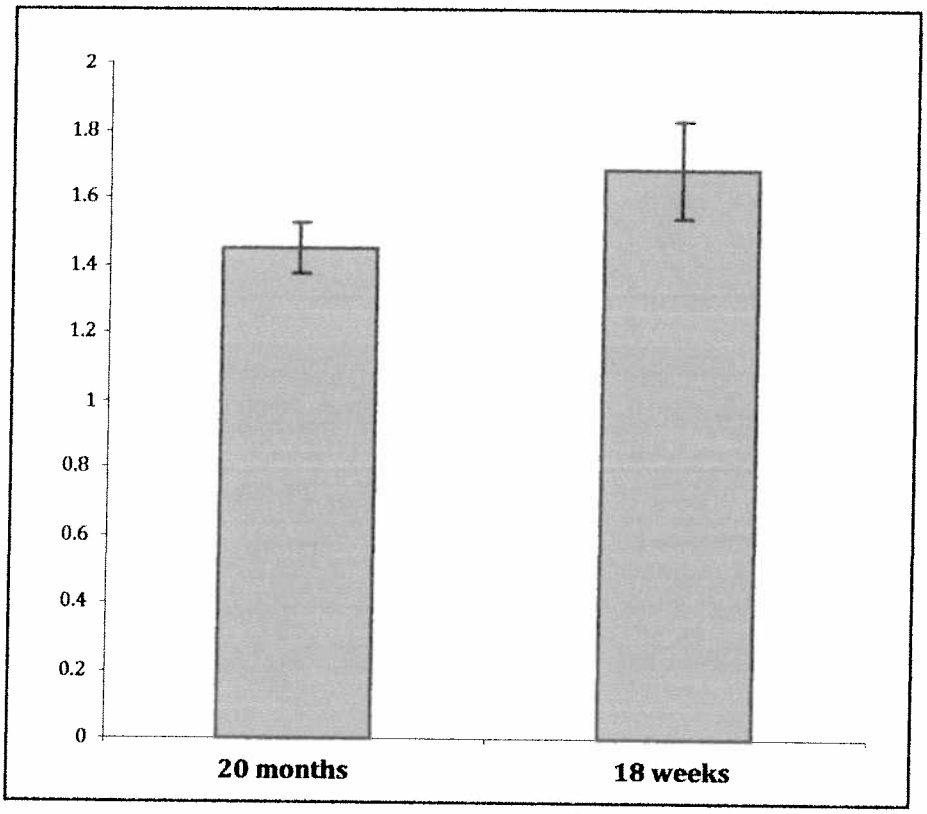


Figure 5 - Total Adiponectin Levels in Visceral Adipose Tissue Depot of 18 Weeks Old Mice

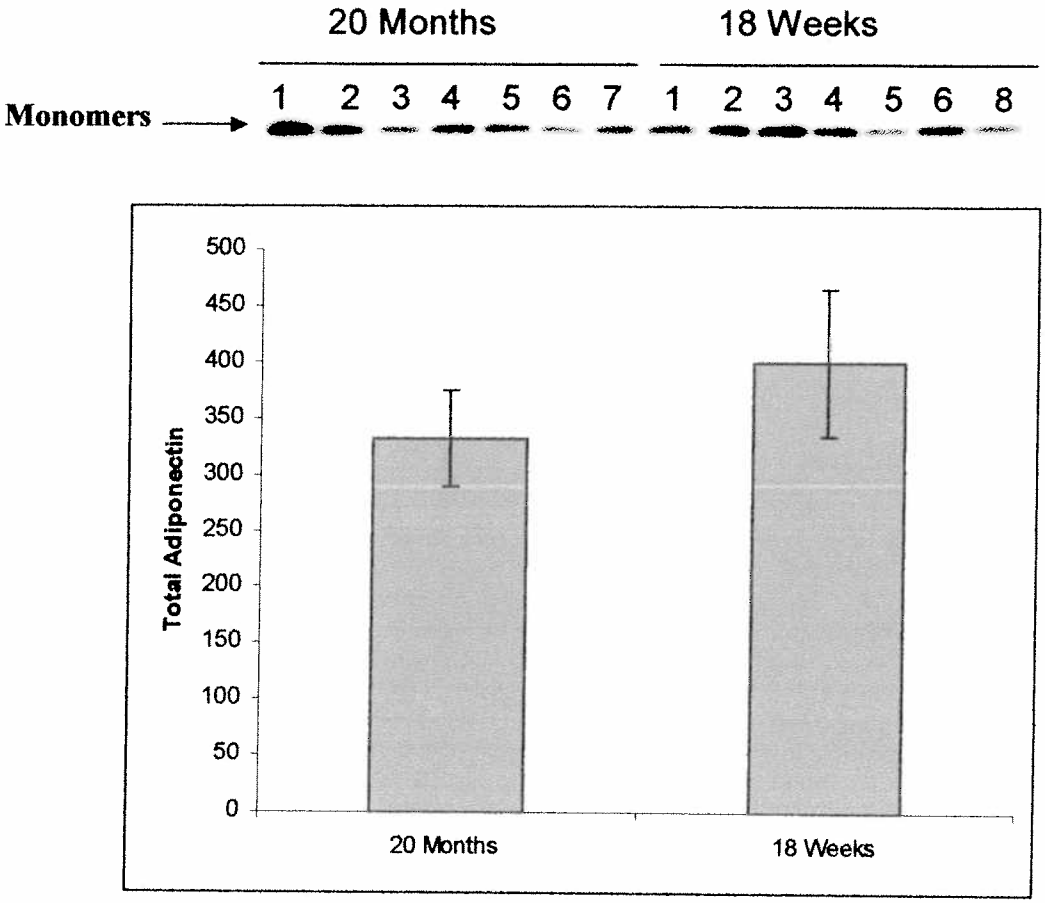
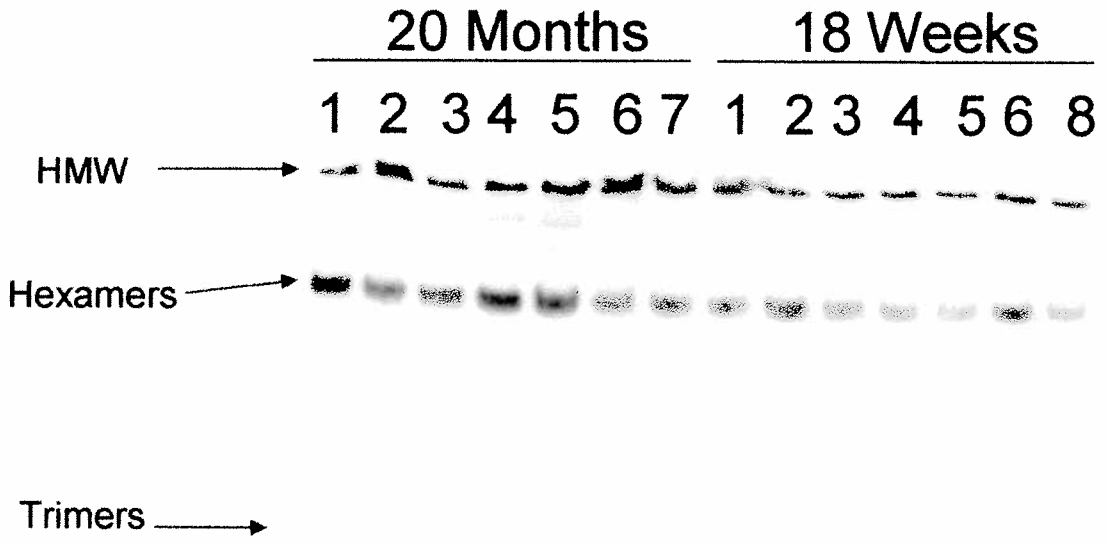


Figure 6 - Decreased Ratio of HMW to Hexameric Adiponectin but Increased Levels of Both HMW and Hexameric Adiponectin in Subcutaneous Adipose Tissue Depot of 20 Month Old Mice



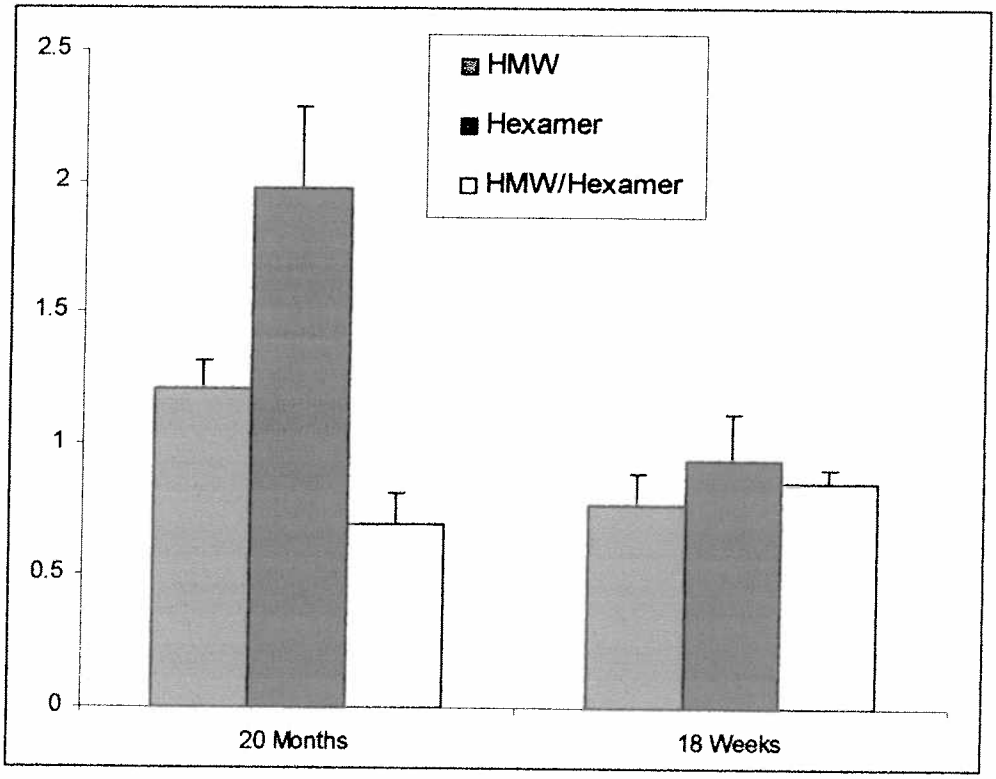


Figure 7 – Decreased Serum HMW Adiponectin in 20-Month Old Mice

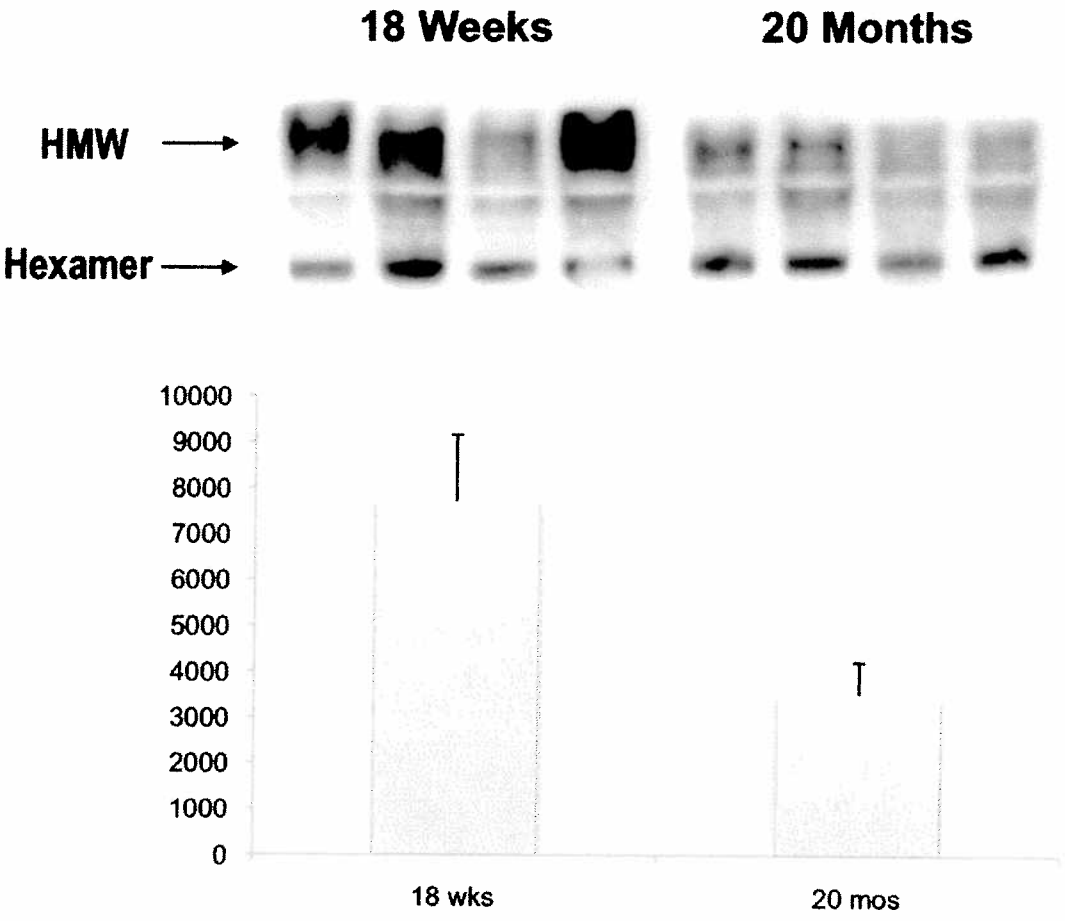
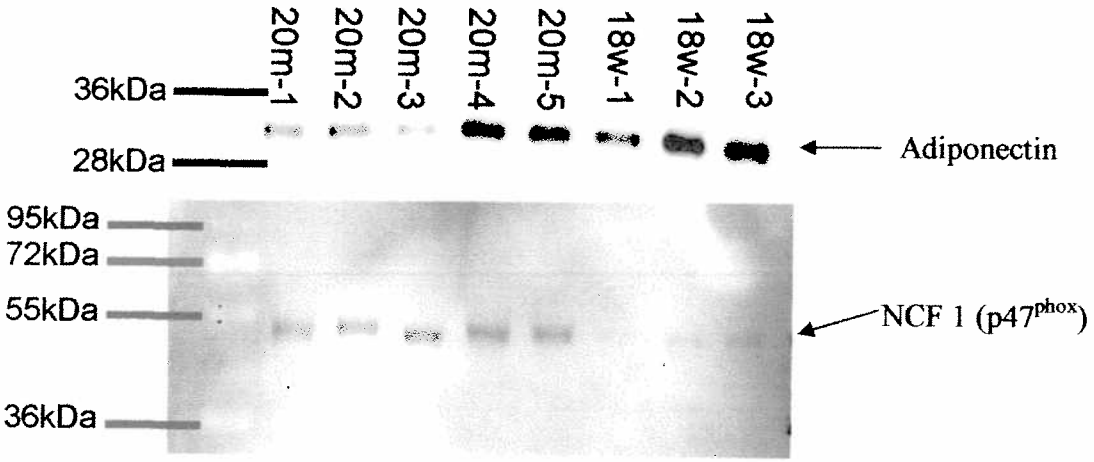


Figure 8 – Increased Total Adiponectin and Decreased Neutrophil Cytosolic Factor 1 in Visceral Adipose Tissue Depot of 18 Weeks Old Mice



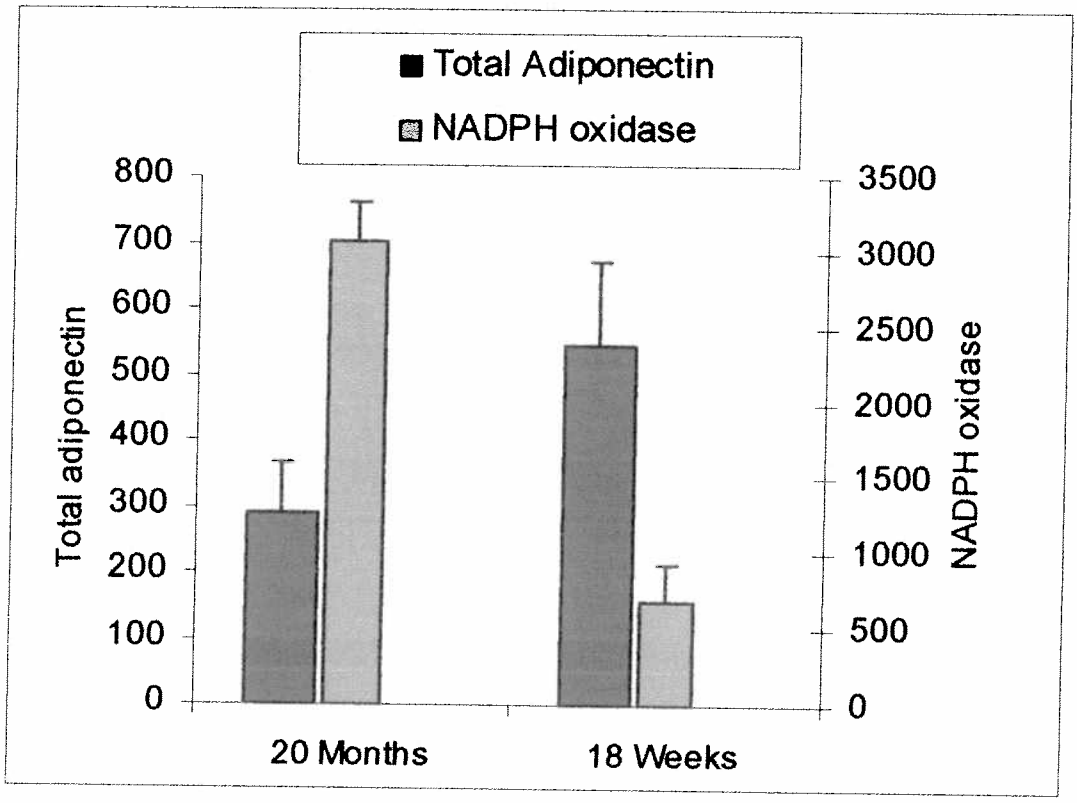


Figure 9 - Visceral and subcutaneous fat pad weight (mg) in 20-month and 18-week old male mice