ROLE OF NADPH OXIDASE IN OXIDATIVE STRESS AND AGING

By

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Abstract

As people age, their preferential adipose tissue stores tend to shift from subcutaneous to visceral, which could lead to diabetes, since visceral adipose tissue is insulin resistant. One of the proposed mechanisms for this shift is due to elevated levels of oxidative stress in adipose tissue cells due to aging, as a result of increased levels of NADPH oxidase (NOX). The goal of these experiments is to determine the existence and significance of this potential correlation. Visceral, subcutaneous, and stromal vascular adipose tissue samples from 18 week and 20 month old mice were probed for p47phox levels, a crucial component of NOX, and the levels of oxidative stress were also determined through a dinitrophenolhydrazine reaction in the OxyBlot analysis. In order to achieve these goals, electrophoresis gels were run, the gels were transferred through standard Western blot procedures, then probed with primary and secondary antibodies, and finally scanned and analyzed to determine differences in signal. As of yet, there have not been any statistically significant differences in oxidative stress levels determined in young and old mice, but further experiments will be conducted in order to validate these findings.

Introduction

Obesity and the closely associated adult-onset or type 2 diabetes are both epidemics that are increasing in prevalence in these modern times[11]. In an obese individual, adipocytes hypertrophy as they grow to accommodate excess energy intake and storage in the form of triglyceride, resulting in expansion of adipose tissue. As a result, the existing vascular structures within adipose tissue may not be sufficient in delivering vital oxygen to the cells. As aging occurs, there tends to be a shift in adipose tissue deposition from subcutaneous to the visceral depot, leading to visceral obesity, which has been shown to correlate with insulin resistance and

consequently, diabetes[6]. Indeed, obesity and aging are the two most important risks factors for developing type 2 diabetes[11].

One abnormality seen in adipose tissue of obese animals is hypoxia[7]. If oxygen supplies cannot reach cells due to insufficient vascular structures, a state of hypoxia becomes induced[10]. Hypoxia in adipose tissue is associated with induction of unfolded protein response in the endoplasmic reticulum (ER stress), increase in the presence of inflammatory cytokines and macrophages, and also oxidative stress[5]. One potential source of oxidative stress in adipose tissue is NADPH oxidase, or NOX, which serves to convert molecular oxygen to superoxide anions. These reactive oxygen species partake in signaling that leads to the generation of new vasculature (angiogenesis)[1]. Specifically, the p47phox subunit of this enzyme has been identified as a key component to this process[2]. NOX activates vascular endothelial growth factor (VEGF), which promotes angiogenesis in order to cope with hypoxia and ER stress. In turn, VEGF has also been linked to upregulation of NOX[4]. It is a possibility that the activity of NOX increases with aging and demand for angiogenesis, leading to an exaggerated state of oxidative stress due to overproduction of superoxide anions. The goal of this project is to determine if not only correlation, but causation, exists among increased NOX activity during aging, oxidative stress, shift in adipose tissue deposition, and development of type 2 diabetes.

One of the main reasons that p47phox is determined to be a crucial subunit of NOX is not only that it is one of four regulatory subunits, but also because of its NOX activity-enhancing role within the angiotensin II system that increases blood pressure, yet another side effect of obesity[9]. p47phox is especially essential in upregulation of NOX activity within neutrophils, leading to the release of cytokines, which then, in turn, can further stimulate p47phox in vascular smooth muscle, as well as in endothelium. This relationship to muscle in vasculature may then

directly connect with the previously mentioned issue of hypoxia and consequent angiogenesis as a result of adipose tissue hypertrophy. These conclusions and correlations were drawn after a series of p47phox gene knockout experiments[10]. Mice without the p47phox gene, when stimulated with angiotensin II, did not produce an oxidative stress response in their vasculature.

The major hypothesis to be tested in this study is that p47phox and thus NOX is a major contributor to oxidative stress in aging adipocytes, specifically in visceral adipose tissue, as opposed to subcutaneous adipose tissue or stromal vascular fraction of visceral adipose tissue. This may directly lead to increased visceral adipose tissue deposition and decreased subcutaneous adipose tissue deposition, and ultimately, diabetes. Along the way, it is anticipated that induction of VEGF and angiogenesis will be a consequence of increased NOX activity.

Methods and Materials

Gel electrophoresis

11% SDS denaturing gels were made and run with samples at 120 Volts for 1 ½ hours each in SDS Tris-Glycine running buffer. Samples were loaded with SDS loading dye, DTT to prevent oxidation, vortexed to mix the solutions, heated and weighted down to prevent sample evaporation, for 15 minutes at 70ºC, then centrifuged for 1 minute prior to loading. PageRuler Plus prestained protein ladder was used to determine the molecular weights of the various proteins and components run in the gel.

Western blot transfer

Gels run through electrophoresis were then transferred in pairs onto 8 cm x 13 cm nitrocellulose membranes at 162 milliAmps, for 2 hours and 20 minutes. Membranes were

stained and shaken with Ponceau-S for 30 seconds, the stain was washed off with deionized water, and then the membrane was scanned for a loading control. Membranes were then placed in a blocking buffer consisting of 10% milk in TBST, pH adjusted to 7.4, overnight in a 4ºC refrigerator. Membranes were probed with an ABBiotech p47phox rabbit primary antibody, 1:625 in the blocking buffer, along with 1:50 sodium azide, overnight in a 4ºC refrigerator. Membranes were washed 6 times, 1 hour minutes each, with TBST, and then probed with a 1:2500 goat anti-rabbit secondary 800 nm infrared-conjugated antibody, in 5% BSA in TBST, 1:50 sodium azide, overnight in a 4ºC refrigerator. Membranes were then washed 5 times, 20 minutes each. The membranes were scanned with the Licor Odyssey scanner at 800 nm, and the exposure was analyzed with ImageJ. The data was further analyzed through Microsoft Excel in regards to relative integrated signal intensity to determine differences in enzyme expression.

In the subcutaneous adipose tissue experiment, Odyssey signals from a prior run of the samples was used to adjust the relative loading volumes of sample in order to normalize the protein content loaded. The rest of the sample volume was made up with lysis buffer. Following an initial scan of newly run samples, the membrane was washed 4 times, 10 minutes each, and then incubated with calf serum in order to bind albumin and remove it from the membrane, as it was overshadowing the p47phox signal. The membrane was washed in the same manner afterwards, following an additional scan with the Odyssey apparatus.

As a loading control, the membrane was then incubated with a β-actin primary antibody, followed by a 680 nm IR-conjugated goat anti-rabbit secondary antibody, 1:5000 in 5% BSA in TBST, with 1:100 sodium azide added as well. The washing procedure in between the two incubations was the same as previously stated.

BCA assay (Thermo Scientific)

A BCA assay was run on the newest subcutaneous fat samples from young and old mice, after running an initial gel, in order to normalize the amount of protein loaded in each sample and to avoid overloading. Standard BSA solutions with deionized water were made with concentrations of 2000 µg/mL, 1500 µg/mL, 1000 µg/mL, 750 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 25 µg/mL, and 0 µg/mL through serial dilutions. 25 µL of each unknown sample and standard were pipetted into a 96-well plate with 2 replicates each, and 200 µL of the working reagent solution (50:1 ratio of working reagents A:B, as specified by the kit) and incubated at 37ºC for 30 minutes. The samples were analyzed for absorbance with a plate reader, the results were recorded into excel, averaged, and using Beer's Law, the standard linear curve was plotted and protein concentration was found based on absorbance with this calculated relationship.

OxyBlot Analysis (Millipore)

The OxyBlot analysis was used in order to detect oxidative stress levels in fat samples from young and old mice through a reaction involving DNPH. 2% β-mercaptoethanol was added to all samples to reduce the samples and thus prevent oxidation outside of the reaction. 5 µL volumes of samples were used, and using the results of a prior BCA assay, actual sample volume was calculated so that each sample contained the required 20 µg of protein for optimal efficiency in the assay. The remaining volume was made up with lysis buffer (see adipose tissue homogenization). Two replicates of each sample were aliquoted, and 6% SDS was added and incubated for 15 minutes at room temperature. 1X DNPH solution, provided in the kit, was added to one sample in each set, while 1X derivatization-control solution, also provided within the kit, was added to the other sample in each set. The samples were then incubated for 30

minutes at room temperature, and neutralization solution was added to all samples to end the reaction. The samples were left for 2 days in the 4ºC refrigerator. Samples were run through gel electrophoresis, without vortexing or centrifuging, and with 0.02% bromophenol blue as the loading dye. A standard protein, provided within the kit, was used as a positive control in the gel. All other parameters were as previously outlined in regular gel electrophoresis, and the Western transfer procedure, Ponceau-S stain, and incubation procedure were followed as before as well. The blocking buffer was 1% BSA in PBST, and the washes consisted of 2 rinses with PBST, 1 15 minute wash, and 2 5 minute washes. The primary antibody was provided through the kit, and the secondary antibody was the same goat anti-rabbit 800 nm IR conjugated as with other Western blot transfers. Data analysis was performed the same way in order to determine differences in oxidative stress levels in young and old mice, as demonstrated by the degree of presence of the DNPH.

Adipose Tissue Sample Homogenization

Subcutaneous adipose tissue samples were thawed on ice, weighed and then lysis buffer was added to the sample in an epitube in a ratio of 1 µL to 1 mg. Lysis buffer consisted of 1 M HEPES, 1M sodium chloride, 200 mM sodium orthovanadate, 1M sodium fluoride, 1:100 Sigma 1 phosphatase inhibitor cocktail, 1:100 Sigma 2 phosphatase inhibitor cocktail, 1:2000 10 mg/mL aprotinin, 1:2000 10 mg/mL pepstatin, 1:2000 10 mg/mL leupeptin, and 10 mM PMSF. Samples were homogenized for 10 seconds each, and then centrifuged at 1000 rpm for 10 minutes. The supernatant was removed and placed into separate epitubes, which made up the samples to be run.

For stromal vascular samples, 100 µL of the aforementioned lysis buffer was added to the epitubes, and sonicated for 15 seconds on high. Samples were then centrifuged for 15 minutes at 2500 rpm, 4ºC, and the supernatant was removed.

For adipocytes, a new, more concentrated (10X) lysis buffer was made, as the existing samples were already suspended, so as to add less fluid and thus not dilute them further, and also to avoid the use of standard phosphatase inhibitors. The lysis buffer consisted of 1 M HEPES, 5 M sodium chloride, 200 mM sodium orthovanadate, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 1:2000 10 mg/mL aprotinin, 1:2000 10 mg/mL pepstatin, 1:2000 10 mg/mL leupeptin, and 10 mM PMSF. Samples were thawed on ice prior to the addition of lysis buffer, and then they underwent 3 cycles of freeze-thaw in order to fully lyse the cells. Samples were then centrifuged for 15 minutes at 2500 rpm and 4ºC, and the resulting pellet and fluid below the lipid layer were removed for p47phox probing.

Results

Visceral and subcutaneous adipose tissue samples were analyzed primarily due to their direct relevance to the shift in adipose tissue deposition between these two locales. Stromal vascular samples were analyzed because of the prevalence of lymphocytes and other immune cells within this particular tissue type, especially neutrophils. Neutrophils are stimulated by p47phox, which then leads to the release of inflammatory cytokines and consequently, inflammation, which is a common affliction within obese individuals. Within this project, visceral, subcutaneous, as well as stromal vascular samples from various 18 week and 20 month old mice were processed through homogenization, sonication, cell lysis, and centrifugation in order to separate out the key components, particularly within the resulting pellet. The samples

were then run through gel electrophoresis (denaturing SDS-PAGE) and subsequently transferred through Western blots, probed with the p47phox antibody to determine levels of the NADPH Oxidase enzyme, and then scanned with the Licor Odyssey at 800 nm. The data was further analyzed through ImageJ and the levels of expression across the various mice were compared. Oxidative stress was also measured directly in a different approach included within this project. Dinitrophenylhydrazine (DNPH) oxidative stress level analysis (Millipore) was used on a separate set of samples, all visceral, from 14 separate mice samples: 7 were 18 weeks old, and 7 were 20 months old. The reaction is based upon the premise that under conditions of oxidative stress, protein side chains are derivatized to become carbonyl functional groups. This assay tags these carbonyl groups where present in a derivatization reaction, parallel to a non-treated negative control, as well as a pre-treated positive control. The samples were run through an SDS-PAGE gel, followed by western blot transfer, specific antibody probing, as provided by the assay kit, and analysis of the entire signal from each sample to determine overall oxidative stress levels through carbonyl expression.

With the OxyBlot analysis, although there appeared to be a difference in DNPH signal between young and old mice, suggesting differences in oxidative stress levels in the two cohorts, the results did not reach statistical significance ($p = 0.16$). Further experiments will be conducted with newer visceral, subcutaneous, and stromal vascular samples in order to see if consistent results are produced, or if there is, in fact, a vast difference in oxidative stress, as well as p47phox signaling.

In terms of the p47phox experiments, so far, the newest subcutaneous samples (harvested in November 2011) have resulted in a signal with p47phox, albeit faint. A single visceral sample from a previous experiment from a 20 month old mouse was run alongside the old mouse

subcutaneous samples, as visceral adipose tissue was hypothesized to be the tissue with the greatest amount of p47phox expression, and thus it was determined to be a sufficient comparison on eyeball. The signal was attempted to be intensified through incubation with calf serum in order to bind excess albumin, a band on the membrane that overshadowed the signal of p47phox. As a result, there was a slight increase in signal intensity that will be analyzed in the near future. In order to normalize these results through data analysis, the membrane was incubated in β-actin primary antibody, followed by a 680 nm IR-conjugated secondary antibody so as to scan at a different channel and wavelength than the original antibody so that signals did not interfere. Half of the young samples revealed a strong β-actin band, while the other ones did not; most of the old samples had a weak band, which, upon analysis and normalization, is projected to show a significant difference in p47phox signaling in young and old mice, in that the old mice are, on eyeball, anticipated to show a greater amount of p47phox. Actin is an element of a cell's cytoskeleton, but due to the lysis and sample aliquots, there is not a guarantee that that component of the cell will be present in the sample. As a result, this is not an entirely accurate normalization control, and in order to provide for more statistical strength and significance in future analyses, another normalization control will be run through GAPDH so as to bring out signals in a greater number of samples for a greater sample size.

A preliminary gel has been run of the newest stromal vascular and adipocyte fractions (also harvested in November 2011), which has shown to be severely overloaded for the most part. The subsequent gel has recently been run, and is in the process of being transferred as a Western in order to probe for p47phox. The previous gel has been used to estimate the factor by which each sample size is to be reduced by, and it is anticipated that a more moderately loaded membrane will result. Since the previous gel did in fact produce a signal for p47phox when

scanned with the Odyssey, albeit too strong, there is hope that there will be more modest results to follow shortly.

Discussion

The problem being investigated consists of whether there is a correlation between aging and levels of NADPH oxidase and consequent oxidative stress levels in adipocytes in young and old mice. Currently, there are not any significant statistical differences in these levels across the two age groups in terms of oxidative stress, as demonstrated by a high p-value with regards to the OxyBlot experiment, but as for the p47phox subcutaneous experiments, there is promise of a significant increase in p47phox expression in old mice versus young, which is to be confirmed through ImageJ analysis following β-actin and GAPDH probing.

Several times, one of the major obstacles in data analysis was a high presence of background when scanning the membrane with the Licor Odyssey, which skewed the results. There were also several incidences where there was not any signal when analyzed with the Odyssey, and prior to that, there were not any signals with chemiluminescent exposure or film exposure either. The solution to the latter problem became to use IR conjugated secondary antibody in order to use Licor Odyssey software to scan the membranes. In the former problem, new antibodies were made as the old ones were stripped off. In some cases there was overloading, so the sample loading size was reduced during gel electrophoresis, based on BCA assay results, or through determination of relative concentrations of samples through ImageJ and Excel analysis. With the BCA assay, the original samples had absorbances outside of the standard range, leading to inaccurate results with trivial differences in absorbance. There were also problems determining protein concentration in adipose tissue samples due to a component in

the adipose tissue blocking the assay from performing to its full and accurate potential. Less samples were used the second time, but even though the absorbances were within the standard range, there were not any major differences in absorbance, due to adipose tissue masking the BCA results. As a result, a smaller volume of samples was loaded in the second gel in order to control for overloading. The final problem involved standardizing a procedure for analyzing the Odyssey scans with ImageJ so as to not incorporate signals into the analysis that were part of the background and nonspecific binding, as opposed to the specific signal being probed for. Other aforementioned problems include determining a valid normalization control to induce signal across the majority of samples to bolster the significance of statistical analysis. In terms of sample loading during gel electrophoresis, many samples were extremely delicate and burst prior to loading, becoming viscous as their DNA leaked out of the cell, limiting the quantity able to be loaded, also skewing results due to uneven loading.

In consulting more literature, one of the potential reasons that NOX levels may not be elevated in aging, while oxidative stress could be, is that NOX is not the only means by which a cell can accumulate reactive oxygen species. Reactive oxygen species are also generated through the electron transport chain during aerobic metabolism, and thus contribute to oxidative stress levels without directly altering NOX levels[3]. Current literature consistently reiterates the strong correlation between aging, elevated NOX levels, and consequent elevated oxidative stress levels, especially due to reactive oxygen species, alluding to us that we must continue repeating these experiments in order to produce results consistent with these myriads of concurrent research.

To continue further in this project, the newest subcutaneous samples will be probed with GAPDH and then analyzed with ImageJ and Excel for statistical significance. The stromal vascular and adipocyte suspension samples will be probed for p47phox and any further

normalization controls deemed necessary for statistical analysis. Furthermore, the OxyBlot analysis will be used on this set of samples to analyze the oxidative stress across the various tissue types, and either dispel previous negative results from the OxyBlot, or demonstrate support for our hypothesis. Afterwards, there are several assays, such as the ELISA assay, which have potential to be used in this project in order to measure VEGF levels, for instance, or to determine the degree of hypoxia in the cells.

Figures

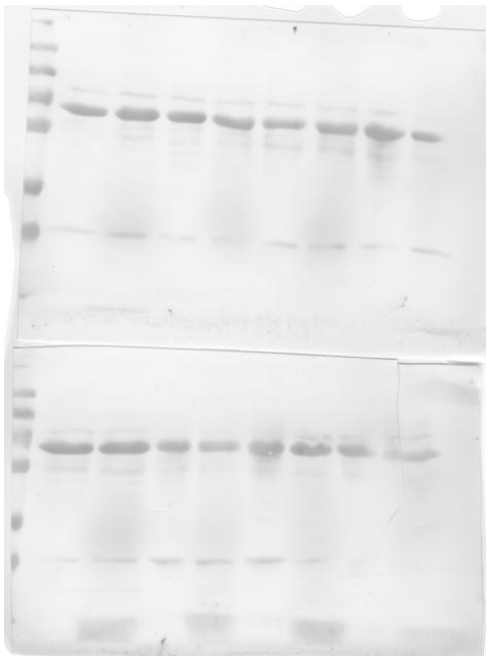

Figure 1

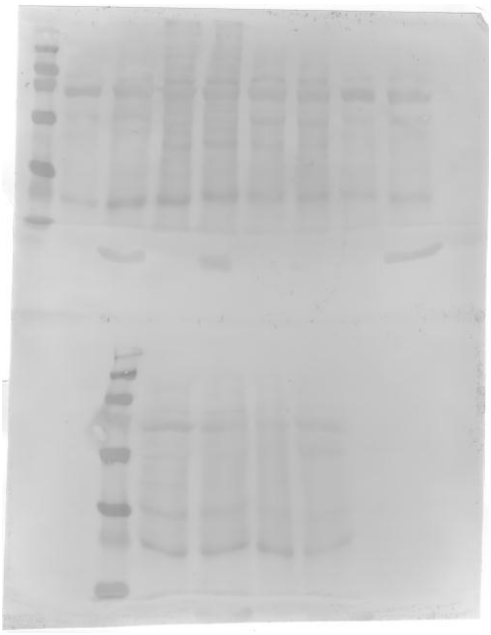

Figure 2

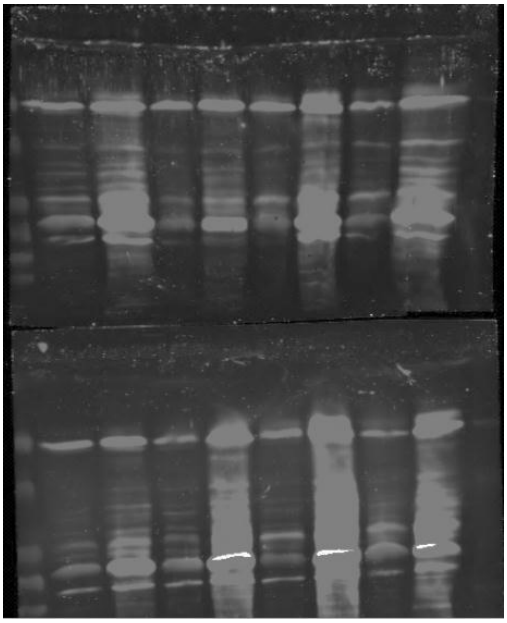

Figure 3

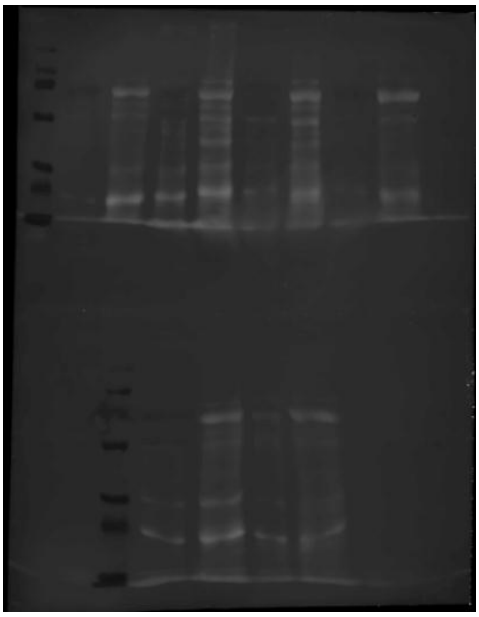

Figure 4

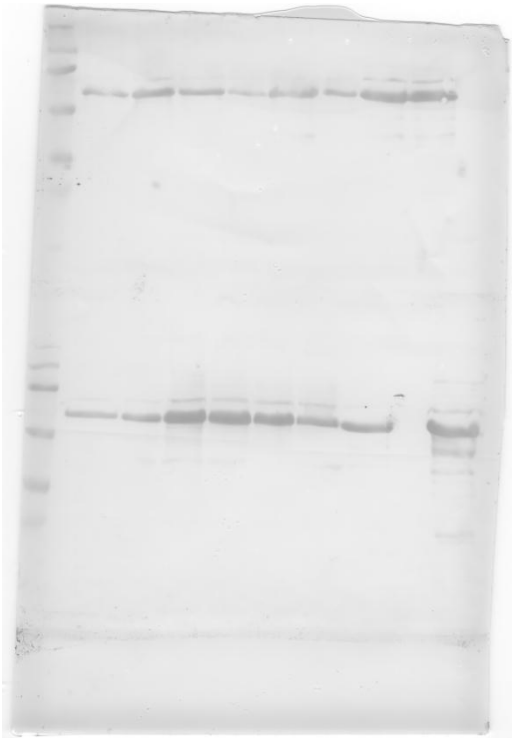

Figure 5

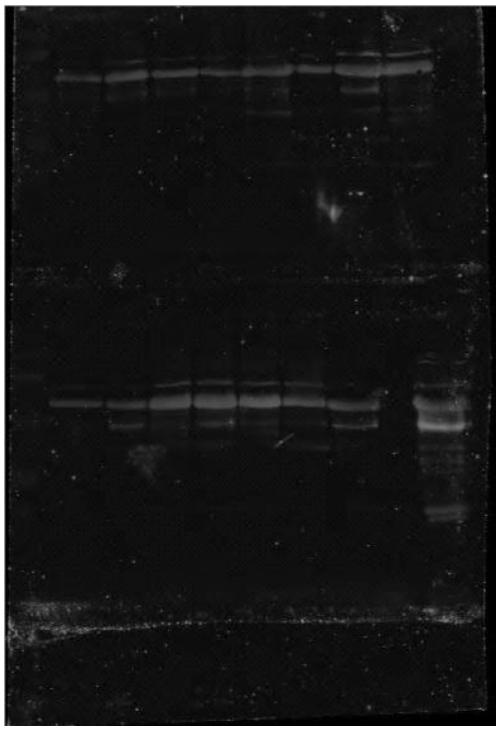

Figure 6

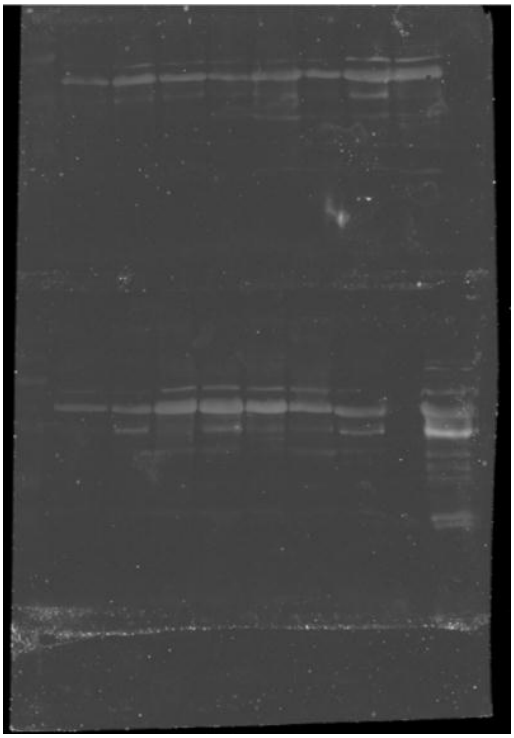

Figure 7

Figure Legend

Figure 1: Ponceau stain of OxyBlot samples, young and old mice, showing even protein loading. Loading order of top gel (left to right) marker, 20 month visceral 1 – DNPH, 20 month visceral 1 + DNPH, 20 month visceral 2 – DNPH, 20 month visceral 2 + DNPH, 18 week visceral 1 – DNPH, 18 week visceral 1 + DNPH, 18 week visceral 2 – DNPH, 18 week visceral 2 + DNPH, OxyBlot positive control. Bottom gel loading order (left to right): marker, 20 month visceral 3 – DNPH, 20 month visceral 3 + DNPH, 20 month visceral 4 – DNPH, 20 month visceral 4 + DNPH, 18 week visceral 3 – DNPH, 18 week visceral 3 + DNPH, 18 week visceral 4 – DNPH, 18 week visceral 4 + DNPH, OxyBlot positive control.

Figure 2: Ponceau stain of OxyBlot samples, young and old mice, showing even protein loading. Top gel loading order (left to right): marker, 20 month visceral 5 – DNPH, 20 month visceral 5 + DNPH, 20 month visceral 6 – DNPH, 20 month visceral 6 + DNPH, 18 week visceral 5 – DNPH, 18 week visceral 5 + DNPH, 18 week visceral 6 – DNPH, 18 week visceral 6 + DNPH, OxyBlot positive control. Bottom gel loading order (left to right): marker, 20 month visceral 7 – DNPH, 20 month visceral 7 + DNPH, 18 week visceral 7 – DNPH, 18 week visceral 7 + DNPH.

Figure 3: Odyssey scan of OxyBlot samples (see Figure 1 legend for loading order).

Figure 4: Odyssey scan of OxyBlot samples (see Figure 2 legend for loading order).

Figure 5: Ponceau stain of subcutaneous young and old mice samples, after incubation with calf serum. Top gel loading order (left to right): marker, 18 week subcutaneous 1, 18 week subcutaneous 2, 18 week subcutaneous 3, 18 week subcutaneous 4, 18 week subcutaneous 5, 18 week subcutaneous 6, 18 week subcutaneous 7, 18 week subcutaneous 8. Bottom gel loading order (left to right): Marker, 20 month subcutaneous 1, 20 month subcutaneous 3, 20 month subcutaneous 4, 20 month subcutaneous 5, 20 month subcutaneous 6, 20 month subcutaneous 7, 20 month subcutaneous 8, blank, 18 week visceral 1 (from older sample set)

Figure 6: Odyssey scan of subcutaneous young and old mice samples, after incubation with calf serum (see Figure 5 legend for loading order).

Figure 7: β-actin Odyssey scan of subcutaneous young and old mice samples as an additional loading control (see Figure 5 legend for loading order)

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