3-1-2009

Acetaminophen prevents aging-associated hyperglycemia in aged rats: effect of aging-associated hyperactivation of p38-MAPK and ERK1/2

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Recommended Citation

Wu, M., Desai, D. H., Kakarla, S. K., Katta, A., Paturi, S., Gutta, A. K., Rice, K. M., Walker, E. M. and Blough, E. R. (2009), Acetaminophen prevents aging-associated hyperglycemia in aged rats: effect of aging-associated hyperactivation of p38-MAPK and ERK1/2. Diabetes Metab. Res. Rev., 25: 279–286. doi: 10.1002/dmrr.932

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Acetaminophen prevents aging-associated hyperglycemia in aged rats: effect of aging-associated hyperactivation of p38-MAPK and ERK1/2

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Abstract

Background: Aging-related hyperglycemia is associated with increased oxidative stress and diminished muscle glucose transporter-4 (Glut4) that may be regulated, at least in part, by the mitogen-activated protein kinases (MAPK).

Methods: To test the possibility that aging-related hyperglycemia can be prevented by pharmacological manipulation of MAPK hyperactivation, aged (27-month) Fischer 344/NNiaHSD × Brown Norway/BiNia F1 (F344BN) rats were administered acetaminophen (30 mg / kg body weight / day) for 6 months in drinking water.

Results: Hepatic histopathology, serum aspartate aminotransferase and alanine aminotransferase analysis suggested that chronic acetaminophen did not cause hepatotoxicity. Compared to adult (6-month) and aged (27-month) rats, very aged rats (33-month) had higher levels of blood glucose, phosphorylation of soleus p38-MAPK and extracellular regulated kinase 1/2 (ERK1/2), superoxide and oxidatively modified proteins ($P < 0.05$), and these changes were associated with decreased soleus Glut4 protein abundance ($P < 0.05$). Chronic acetaminophen treatment attenuated age-associated increases in blood glucose by 61.3% ($P < 0.05$), and increased soleus Glut4 protein by 157.2% ($P < 0.05$). These changes were accompanied by diminished superoxide levels, decreases in oxidatively modified proteins (− 60.8 %; $P < 0.05$) and reduced p38-MAPK and ERK1/2 hyperactivation (− 50.4 % and − 35.4% respectively; $P < 0.05$).

Conclusions: These results suggest that acetaminophen may be useful for the treatment of age-associated hyperglycemia.

Keywords: acetaminophen; aging; hyperglycemia; glucose transporter-4; mitogen-activated protein kinase; reactive oxygen species

Introduction

Aging in humans and rats is typically characterized by a diminished capacity for glucose disposal and metabolism, increases in blood glucose levels and chronic hyperglycemia that if allowed to proceed unchecked can lead to increased morbidity and mortality [1, 2]. Blood glucose homeostasis and glucose uptake into muscle is positively correlated with the percentage of oxidative muscle fibers [3, 4], and is regulated, in large part, by glucose transporter-4 (Glut4) [2, 5]. The expression of muscle Glut4 has been shown to be decreased with aging in both humans and the Fischer 344/NNiaHSD × Brown Norway/BiNia F1 (F344BN) rat strain [2, 6]. Why aging is associated with decreased Glut4 protein levels is not well understood. The stress-responsive kinase, p38 mitogen-activated protein kinase (p38-MAPK), has been found to be involved in the regulation of Glut4

expression [7-9], and under conditions of stress or disease, such as that seen in type 2 diabetes, elevated and prolonged p38-MAPK activation is associated with decreased Glut4 expression [8, 9]. In addition to p38-MAPK, regulation of glucose uptake has also been found to be influenced by the activation (phosphorylation) of the extracellular regulated kinase 1/2 (ERK1/2)-MAPK as the phosphorylation of this molecule may be involved in regulating insulin receptor signaling and Glut4 translocation [10-12]. The factors which regulate the phosphorylation of MAPK are not entirely understood, however MAPK hyperactivation has been found to be associated with elevated reactive oxygen species (ROS) [13, 14]. Whether a link exists between aging-related increases in the elevated blood glucose levels, muscle ROS, MAPK phosphorylation, and alteration of muscle Glut4 levels is not known.

Here we hypothesize that aging-related increases in blood glucose and muscle ROS levels are associated with the hyperactivation of MAPK that results in the decreased Glut4 level and hyperglycemia. Further, we hypothesize that these changes in MAPK hyperactivation and hyperglycemia, if present, can be reversed by pharmacological intervention. To test these possibilities, we examined the relationship between blood glucose levels, Glut4 level, MAPK phosphorylation and ROS in the oxidative soleus muscle of aged (33-month) F344BN rats that had been daily treated with acetaminophen (N-acetyl-para-aminophenol (APAP), 30 mg / kg body weight / day) for 6 months. Acetaminophen was chosen for investigation since it is easily tolerated and on the basis of previous data demonstrating that this compound exhibits potent anti-oxidant activity [15, 16]. Our data suggest that acetaminophen may be effective in preventing hyperglycemia during aging and that this alteration is associated with the diminished ageing-associated MAPK hyperactivation and increases in muscle Glut4 abundance.

Materials and Methods

Animals

The F344BN rats (National Institute on Aging, Bethesda, MD), aged 6- and 27-month, were housed in an AAALAC approved vivarium. Housing conditions consisted of a 12:12 h light / dark cycle and temperature was maintained at 22 ± 2°C. Water and food (LabDiet 5001, PMI Nutrition International, LLC, Brentwood, MO) were provided *ad libitum*. All procedures were approved by the Marshall University Institutional Animal Care and Use Committee.

Acetaminophen treatment and tissue collection

The F344BN rats (27-month) were given acetaminophen (30 mg / kg body weight / day, Sigma-Aldrich, Inc., St. Louis, MO) for 6 months in drinking water. Age-matched F344BN rats were maintained as controls. Rats were anesthetized with a ketamine-xylazine (4:1) cocktail (50 mg/kg, I/P) and supplemented as necessary for reflexive response prior to tissue collections. Soleus muscles and liver were removed, weighed, frozen in liquid nitrogen, and stored at − 80 °C.

Biochemical analysis of blood sample

Blood glucose was measured using a TrueTrack Smart System blood glucose monitor (Home Diagnostics, Fort Lauderdale, FL). Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using a Dimension RxL Clinical Chemistry Analyzer (Dade Behring, Newark, DE).

Western blot analysis

Portions of individual soleus muscles were pooled and homogenized in a TPER (Pierce, Rockford, IL) extraction buffer (2 mL / g tissue) that contained protease inhibitors (P8340, Sigma-Aldrich, Inc., St. Louis, MO) and phosphatase inhibitors (P5726, Sigma-Aldrich, Inc., St. Louis, MO). After incubation on ice for 30 min, the homogenate was collected by centrifuging at 12,000 g for 5 min at 4 °C. Protein concentration of homogenates was determined via the Bradford method (Fisher Scientific, Rockford, IL). Homogenate samples were boiled in a Laemmli 2 × sample buffer (Sigma-Aldrich, Inc., St. Louis, MO) for 5 min. Thirty microgram of total protein each sample was separated on a 10 % PAGEr Gold Precast gel (Lonza, Rockland, ME), and then transferred to nitrocellulose membranes. The gel was stained with a RAPIDstain™ protein stain reagent (G-Biosciences, St. Louis, MO) to monitor equal protein loading and transference efficiency. Membranes were blocked with 5 % milk in TBS containing 0.5% Tween-20 (TBST) for 1 h and then incubated with primary antibody overnight at 4 °C. After washing with TBST, the membranes were incubated with the corresponding secondary antibodies conjugating with horseradish peroxidase (HRP) (anti-rabbit (#7074) or anti-mouse (#7076), Cell Signaling Technology, Danvers, MA) for 1 h at room temperature. Protein bands were visualized following reaction with ECL reagent (Amersham ECL™ Western Blotting reagent RPN 2106, GE Healthcare Bio-Sciences Corp., Piscataway, NJ). Target protein levels were quantified by an AlphaEaseFC image analysis software (Alpha Innotech, San Leandro, CA) and normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primary antibodies against Glut4 (#2299), p38-MAPK (#9212), phospho-p38-MAPK (Thr180/Tyr182) (p-p38-MAPK, #9216), extracellular regulated kinase 1/2 (ERK1/2, #9102), phospho-ERK1/2 (Thr202/Tyr204) (p-ERK1/2, #4377), c-Jun N-terminal Kinase 1/2 (JNK1/2, #9252), phospho-JNK1/2 (Thr183/Tyr185) (p-JNK1/2, #9251) and GAPDH (#2118) were purchased from Cell Signaling Technology (Beverly, MA).

Dihydroethidium staining

Soleus muscles were serially sectioned (8 μm) using an IEC Microtome cryostat and the sections were collected on poly-lysine coated slides. The fluorescent superoxide indicator dihydroethidium (Hydroethidine, HE) was used to evaluate superoxide levels as outlined elsewhere [17]. Upon oxidation, dihydroethidium is intercalated within DNA exhibiting a bright fluorescent red. Briefly, frozen tissue sections were washed with phosphate buffered saline (PBS) for 5 min, and then incubated with 10 μM of dihydroethidium (Molecular Probes, Eugene, OR) for 1 h at room temperature. After washing with PBS (3 × 5 min) fluorescence was visualized using an Olympus BX51 microscope (Olympus America, Melville, NY) equipped with Olympus WH 10 × widefield eyepieces and Olympus UPlanF1 40 × / 0.75 objective lens.

Analysis of oxidatively modified proteins

An OxyBlot™ protein oxidation detection kit (Millipore, Billerica, MA) was employed for the quantitative analysis of oxidative modification of proteins by oxygen free radicals and other reactive species according to the manufacturer's instructions. Briefly, 7 μg total protein of each soleus homogenate was derivatized by reaction with 2, 4-dinitrophenylhydrazine (DNPH) at room temperature for 15 min before separation using 10 % PAGEr Gold Precast gels. Oxidized proteins were detected by immunoblotting using the anti-DNP antibody provided in the kit.

Hematoxylin and eosin staining (H&E)

Portions of liver were serially sectioned (8 μm) using an IEC Microtome cryostat and stained with H&E, dehydrated and mounted (Perimount, Fisher Scientific, Fairlawn, NJ) according to standard methods. The stained sections were visualized under an Olympus BX51 microscope equipped with Olympus WH 10 × widefield eyepieces and Olympus UPlanF1 10 × / 0.30 objective lens.

Data analysis

Results are presented as means ± SEM. The effects of age and acetaminophen treatment were analyzed using the GLM procedure (SAS 9.1 for Windows, SAS Institute Inc., Cary, NC). Means were calculated by the LSMEANS procedure and multiple comparisons were performed using the Tukey-Kramer test to determine differences between groups. Values of $p < 0.05$ were considered to be statistically significant.

Results

Chronic acetaminophen treatment did not cause hepatotoxicity in aged F344BN rats

In humans, it is possible that chronic acetaminophen treatment may cause hepatotoxicity. To address this possibility, we examined hepatic histopathology along with serum ALT and AST. Routine H&E staining failed to demonstrate any morphological differences between 33-month age-matched and acetaminophen-treated liver sections (Figure 1A). Similarly, we observed no differences in serum AST ($P = 0.60$) or ALT ($P = 0.053$) between two control and treatment groups (Figure 1B). Consistent with other acute and chronic studies with similar or higher acetaminophen dosages [18, 19], our data suggest that chronic acetaminophen (30 mg / kg body weight / day) ingestion for up to 6 months does not cause hepatotoxicity in aged rats.

Acetaminophen diminished aging-associated increase of blood glucose levels

Blood glucose levels in the 33-month old rats were 85.2 % and 37.4 % higher than that observed in 6- and 27-month rats, respectively ($P < 0.05$; Figure 2). Compared to age-matched control animals, six months of acetaminophen treatment decreased blood glucose levels by 61.3 % ($P < 0.05$).

Acetaminophen increased Glut4 protein abundance in the soleus of aged rats

Compared to that in adult animals (6-month), Glut4 protein levels were lower in the soleus muscles of 27- and 33-month control rats, respectively (−50.5 % and −44.8 %; $P < 0.05$; Figure 3). After 6 months of acetaminophen treatment Glut4 protein abundance was

increased by 157.2% when compared to the age-matched control animals ($P < 0.05$; Figure 3).

Acetaminophen decreased the aging-associated hyperactivation of MAPK

The p38-MAPK and ERK1/2 total protein levels were not altered with aging or acetaminophen treatment (Figure 4 and 5). Compared to that in adult rats (6-month), the phosphorylation of p38-MAPK (Thr180 / Tyr182) and ERK1/2 (Thr 202/Tyr 204) were 154.8 % and 42.7 % higher ($P < 0.05$; Figure 4 and 5) in the soleus muscles of 33-month control rats, respectively. Acetaminophen treatment restored both p-p38 MAPK and p-ERK1/2 levels to that observed in the 6- and 27-month rats ($P > 0.05$; Figure 4 and 5).

Acetaminophen decreased aging-associated increases of superoxide and the amount of oxidatively modified proteins in the aged soleus

Compared to that observed in the 6-month control and 33-month treated animals, dihydroethidium reactivity appeared to be visibly increased in the 27- and 33-month control animals (Figure 6). Similarly, the abundance of oxidatively modified proteins was 73.0 % higher in 33-month control animals compared to that observed in 6-month animals ($P < 0.05$; Figure 7). Compared to 33-month control animals, chronic acetaminophen treatment decreased the abundance of oxidatively modified proteins by 60.8 % ($P < 0.05$; Figure 7).

Discussion

Aging-related hyperglycemia and insulin resistance in humans has been well documented [2]. Using F344BN rats we demonstrate similar findings, and more importantly, show that age-associated increases in blood glucose can be corrected by chronic acetaminophen treatment. Why aging may be associated with alterations in blood glucose levels is not fully understood, however it is thought that decreases in muscle Glut4 protein content and increases in oxidative stress may be involved. We observed a similar relationship in aging rats and further, demonstrate that acetaminophen administration may function to lower blood glucose levels in aging animals by increasing the abundance of skeletal muscle Glut4 protein levels.

Similar to previous research using humans and other rodent models, we found that aging appeared to decrease the expression of skeletal muscle Glut4 levels [2]. It has been reported that activation of p38-MAPK is involved in down-regulating Glut4 expression, while the inhibition of p38-MAPK appears to increase both basal and insulin-stimulated glucose uptake during the development of insulin resistance [8, 9, 20]. In agreement with previous reports [21, 22], we demonstrate that aging dramatically increases the activation (phosphorylation) of p38-MAPK in the F344BN soleus. Further, and consistent with studies using non-aged subjects examining the relationship between p38-MAPK activation and hyperglycemia [8, 20], our results also demonstrate that aging-related increases p38-MAPK phosphorylation are inversely proportional to Glut4 protein levels. Taken together, these findings suggest that the age-associated hyperactivation of p38-MAPK may contribute to the decreases in muscle Glut4 expression and further increases in blood glucose levels.

Compared to adult (6-month) and aged (27-month) animals, very aged (33-month) control rats exhibited 85.2% and 37.4% increases in blood glucose, respectively, and these

changes were associated with increases in muscle ROS and the amount of oxidatively modified proteins (Figure 6 and 7). Coinciding with these changes we observed a marked increase in p38-MAPK activation in the soleus muscles of the very aged F344BN animals (Figure 4). The cause(s) of this age-associated increase in the amount of phosphorylated p38-MAPK at 33-months is not known. Previous reports have suggested that p38-MAPK activation is elevated with increasing ROS and exposure to high level of glucose [8, 10, 13, 14]. Whether these factors alone or in combination with other influences are the cause of the elevated p38-MAPK phosphorylation we observe here will require further study.

To test the assertion that age-associated increases in ROS may play a role in hyperactivation of p38-MAPK, we examined the antioxidant potential of acetaminophen administration to influence muscle ROS and hence reduce p38-MAPK activation. For these experiments, animals were given acetaminophen (30 mg / kg body weight / day) chronically over a period of 6 months. Similar to studies that have examined the effect of acetaminophen on liver and heart ROS levels [15, 16, 23], we observed that acetaminophen treatment decreased indices of skeletal muscle ROS in the aging F344BN soleus (Figure 6). Matching the reduced ROS, chronic acetaminophen treatment drastically decreased the content of proteins oxidatively modified by oxygen free radicals and other reactive species (Figure 7), and importantly, we observed that these alterations in ROS and oxidatively modified proteins were accompanied by a 50.4% decrease in basal p38-MAPK phosphorylation (activation) levels (Figure 4).

The signaling mechanisms that regulate blood glucose homeostasis and the expression of Glut4 protein in skeletal muscle are complicated and have not been fully elucidated. In addition to p38-MAPK, it is also thought that other MAPKs may be involved in regulating glucose uptake. In agreement with that observed in aging human [21], the phosphorylation (activation) of ERK1/2 (Thr 202/Tyr 204) was higher in the soleus muscles obtained from the 33-month control rats than that in the 6-month rats (Figure 5). Conversely, the phosphorylation of JNK1/2 was unchanged with either aging or acetaminophen treatment (data not shown). Why ERK1/2 phosphorylation is altered with aging is not known, but it is possible that this increased ERK1/2 activation may be a compensatory mechanism elicited by the muscle to try and increase glucose uptake in the face of increased blood glucose levels, as the phosphorylation of ERK1/2 has been shown to enhance Glut4 translocation to the cellular membrane and increase glucose uptake [10]. Alternatively, others have suggested that the activation of ERK1/2 is, under some conditions, associated with an increase in the serine phosphorylation of IRS-1 and an inhibition of insulin receptor signaling [11, 12]. Whether the increase in ERK1/2 phosphorylation with aging is in compensation to the effects of hyperglycemia or contributes to the development of hyperglycemia is not clear, nonetheless, we demonstrate here that chronic acetaminophen treatment is capable of restore p-ERK1/2 levels to that observed in 6- and 27-month rats ($P > 0.05$) (Figure 7). Future studies using other time points in the aging spectrum to examine these possibilities will no doubt be helpful in increasing our understanding of the role that ERK1/2 may play in regulating aging-associated hyperglycemia.

In summary, the data of the present study are consistent with the notion that aging-associated increases in ROS may act to increase MAPK hypreactivation and that these alteration are associated with the decreased Glut4 abundance and hyperglycemia. Chronic

acetaminophen treatment at a therapeutic dosage appears to decrease intracellular ROS and this finding is associated with diminished aging-associated MAPK hyperphosphorylation, increased Glut4 abundance and an improvement in blood glucose levels (Figure 8). Whether these findings are mechanistically linked to each other will require further experimentation. Nonetheless, these data suggest that acetaminophen may be useful for the prevention of age-associated hyperglycemia. Future studies using other animal models, different antioxidants or other type of analysis may be warranted to determine the efficacy of using acetaminophen or other ROS inhibitors for the treatment of aging-associated hyperglycemia.

Figure legends:

Figure 1. Chronic acetaminophen treatment at the dosage of 30 mg / kg body weight / day for 6 months does not cause hepatotoxicity in the aged rats. A. Hepatic histopathology: hematoxylin and eosin staining (H&E) was used to evaluate hepatic morphological difference between 33-month control (33C) and acetaminophen-treated (33T) rats. Liver sections were viewed under an Olympus BX51 microscope at a magnification of 100 × as described under *Materials and Methods*. B. Serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using Dimension RxL Clinical Chemistry Analyzer. There were no difference in AST (P = 0.60) and ALT (P = 0.053) levels between age-matched control (33C) and treated (33T) animals. U / L: unit / liter.

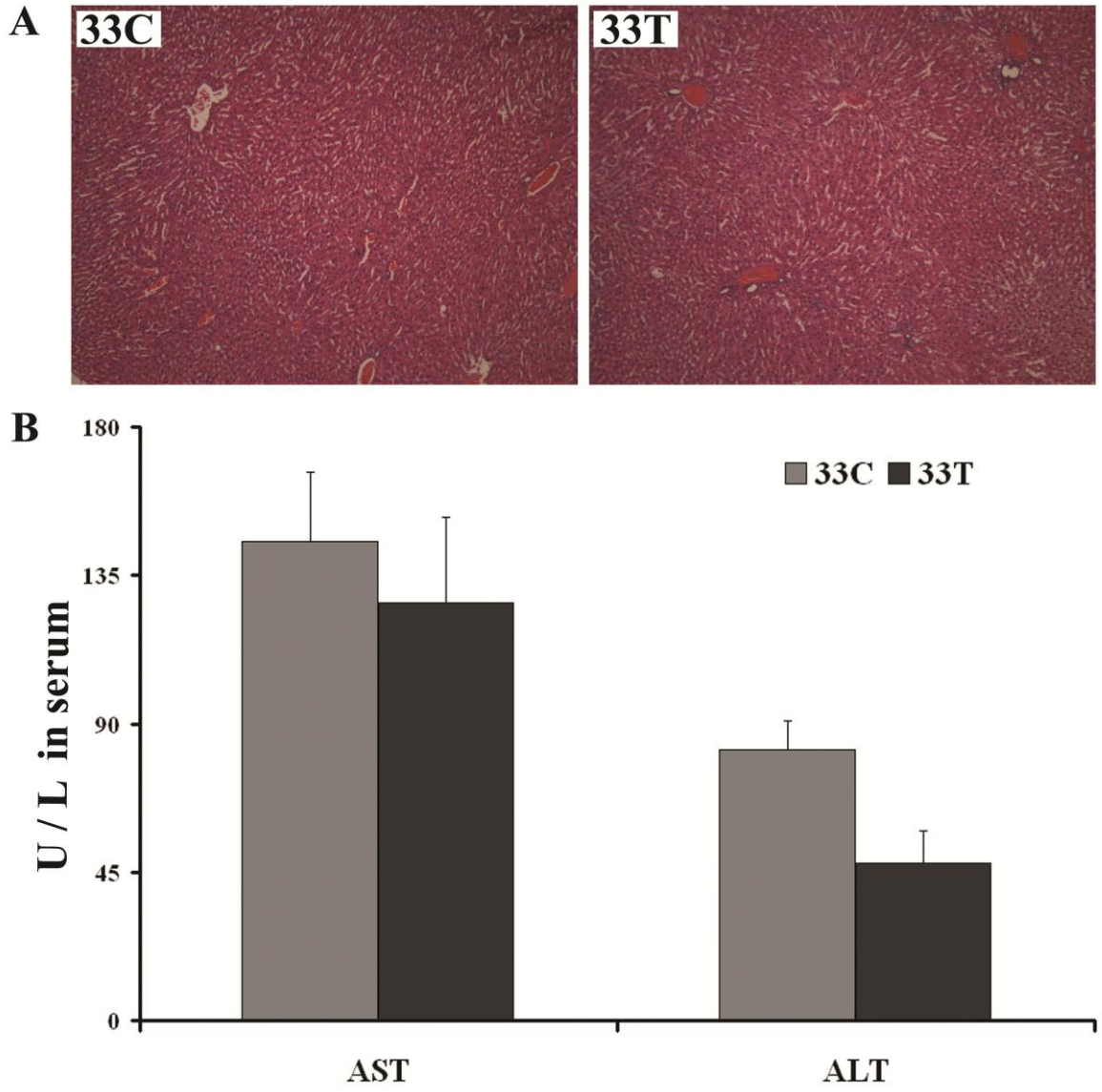


Figure 2. Acetaminophen (APAP) decreased aging-associated increase of blood glucose levels. Blood glucose levels of control (at the age of 6-, 27- and 33-month) and acetaminophen-treated (at the age of 29-, 31- and 33-month) rats were measured using a TrueTrack Smart System blood glucose monitor. abc: groups without the same letter were significantly different ($P < 0.05$).

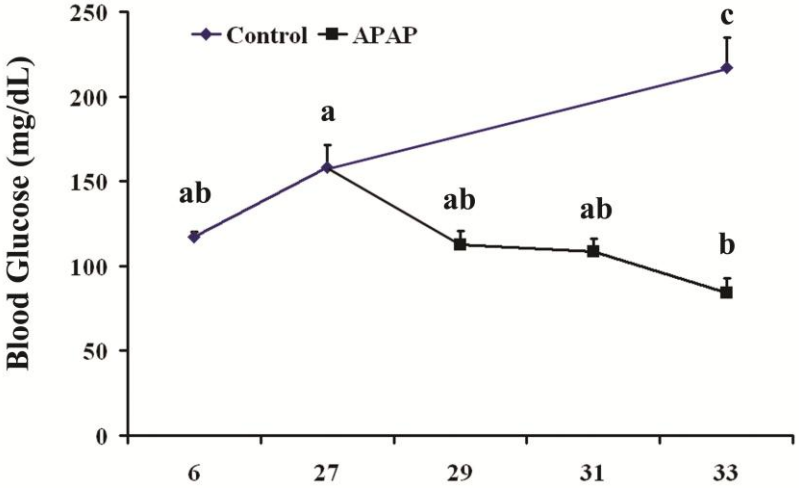


Figure 3. Acetaminophen increased glucose transporter-4 (Glut4) protein abundance in the soleus of aged rats. The Glut4 protein in the soleus of 6-, 27-, 33-month control (33C) and acetaminophen-treated (33T) rats were determined by Western blot. abc: groups without the same letter were significantly different ($P < 0.05$).

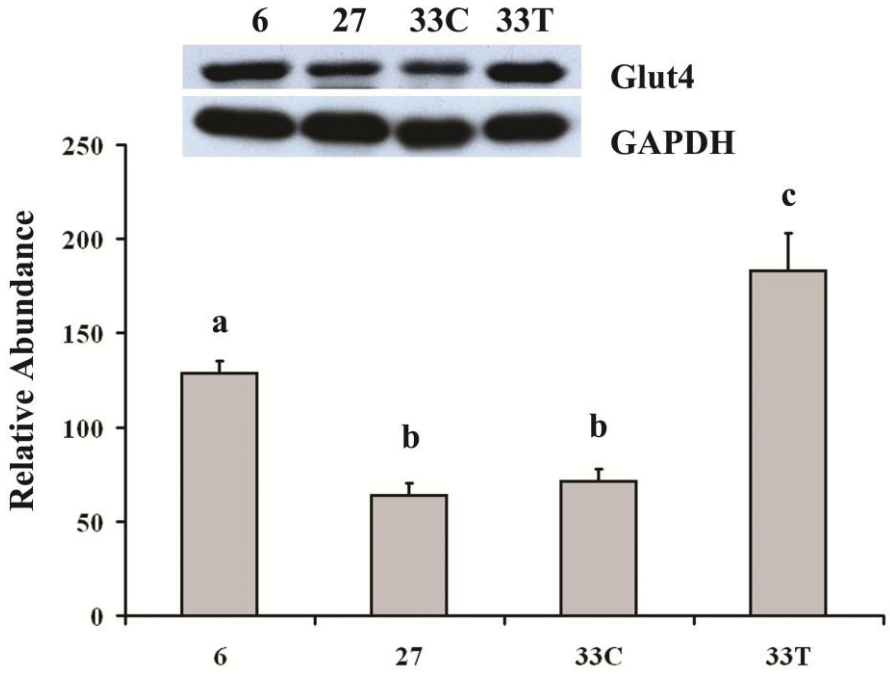


Figure 4. Acetaminophen decreased aging-associated hyperactivation of p38 mitogen-activated protein kinase (p38-MAPK) in the aged soleus. The p38-MAPK total protein and phosphorylation of p38-MAPK (Thr180 / Tyr182) (p-p38 MAPK) at the age of 6-, 27-, 33-month control (33C) and acetaminophen-treated (33T) rats were determined by Western blot. There was no difference of p38-MAPK total protein between groups (P = 0.20). ab: groups without the same letter were significantly different (P < 0.05).

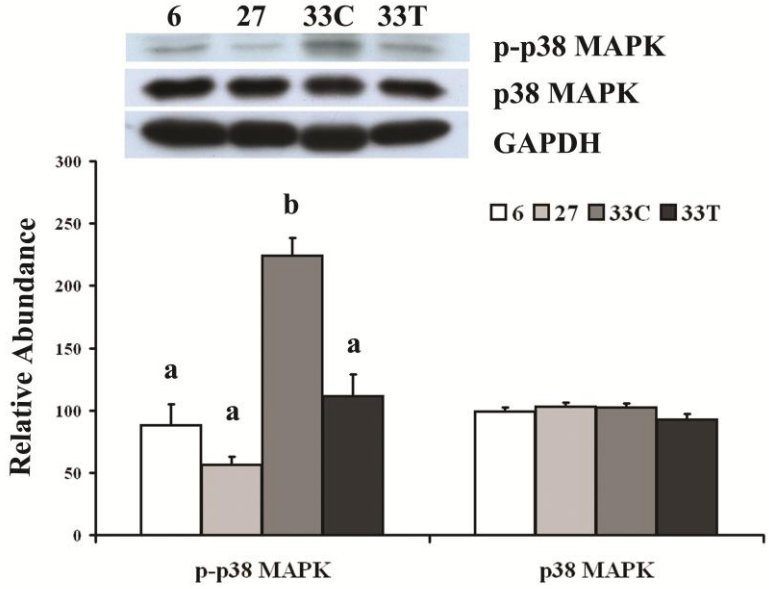


Figure 5. Acetaminophen decreased aging-associated hyperactivation of extracellular regulated kinase 1/2 (ERK1/2) in the aged soleus. The ERK1/2 total protein and phosphorylation of ERK1/2 (Thr202/Tyr204) (p-ERK1/2) at the age of 6-, 27-, 33-month control (33C) and acetaminophen-treated (33T) rats were determined by Western blot. There was no difference of ERK1/2 total protein between groups (P = 0.50). ab: groups without the same letter were significantly different (P < 0.05).

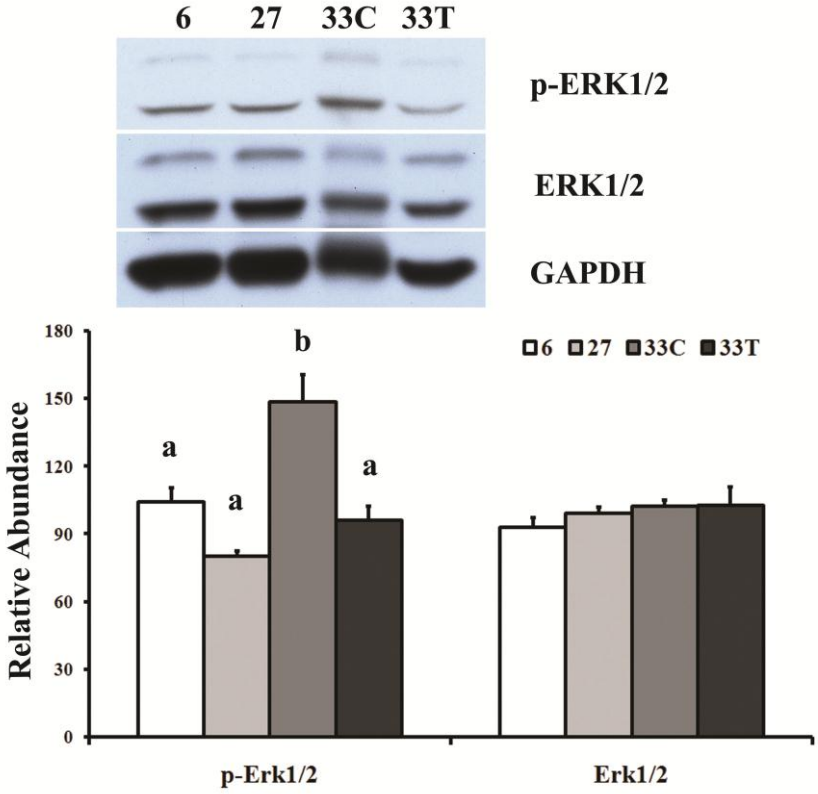


Figure 6. Acetaminophen decreased aging-associated increase of superoxide in the soleus. The abundance of superoxide at the age 6-, 27-, 33-month control (33C) and acetaminophen-treated (33T) rats were determined by the superoxide indicator dihydroethidium. When oxidized, dihydroethidium was intercalated within DNA, therefore exhibiting a bright fluorescent red. The stained sections were viewed under Olympus BX51 microscope at a magnification of 400 × as described under *Materials and Methods*.

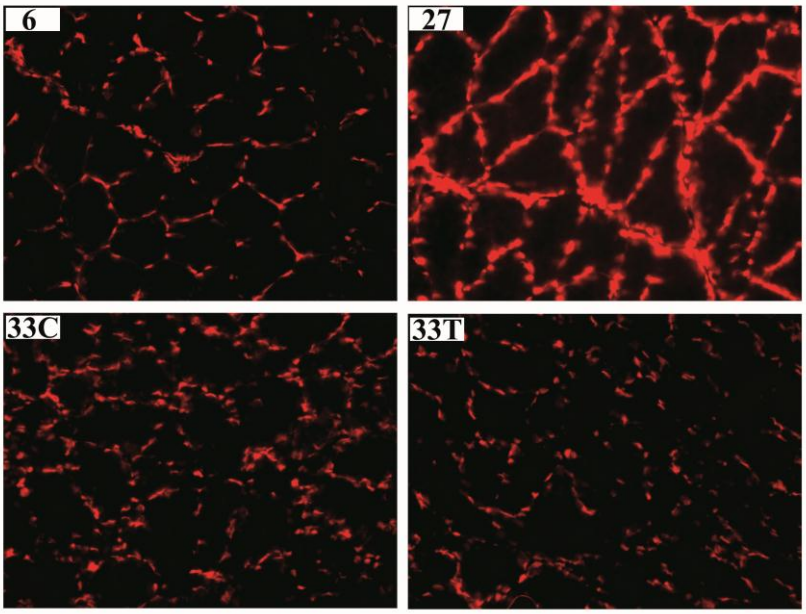


Figure 7. Acetaminophen decreased aging-associated increase of oxidatively modified proteins in the soleus. A. OxyBlot: Oxidatively modified proteins in each homogenate were derivatized by reaction with 2, 4-dinitrophenylhydrazine (DNPH), and then detected by immunoblotting. Non-derivatized homogenate was used as negative control (NC). ST: molecular weight protein standards; BPL: biotinylated protein ladder from Cell Signaling Technology (Beverly, MA). B. Relative abundance of oxidatively modified proteins in the soleus of 6-, 27-, 33-month control (33C) and acetaminophen-treated (33T) rats. abc: groups without the same letter were significantly different ($P < 0.05$).

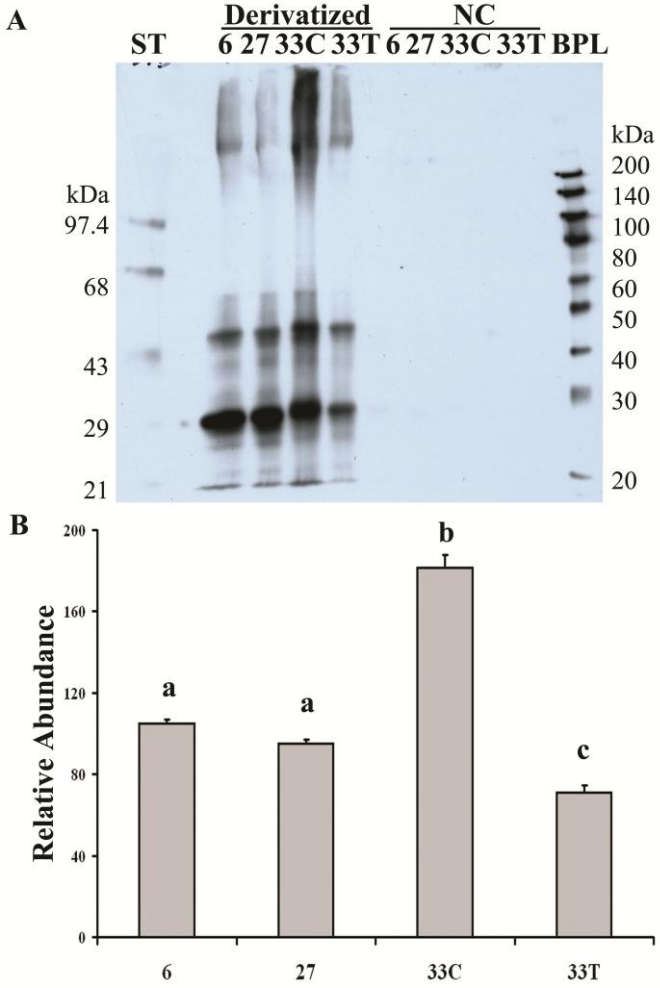
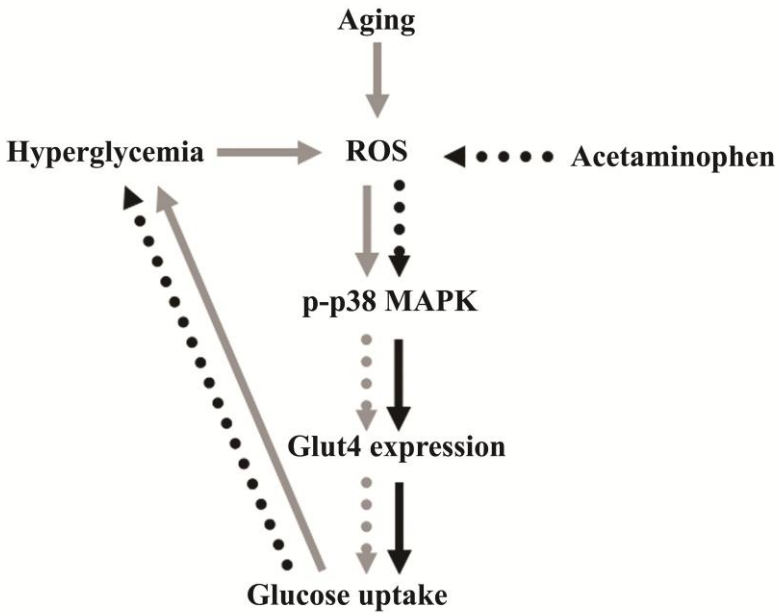


Figure 8. Proposed scheme for the mechanism underlying acetaminophen regulation of blood glucose in the aged rats.

Aging is associated with increases in reactive oxygen species (ROS), increased phosphorylation of p38 mitogen-activated protein kinase (p38-MAPK) and decreased glucose transporter-4 (Glut4) expression that result in diminished glucose uptake. Elevated blood glucose (hyperglycemia) may in turn act to further increase ROS, over-activate p38-MAPK and decrease Glut4 expression, which may lead to further dysregulation of glucose homeostasis. Chronic acetaminophen treatment during the aging process acts to prevent hyperglycemia possibly by alteration in muscle ROS levels, p38-MAPK phosphorylation and changes in Glut4 expression. Solid lines represent increase or activation, while dotted lines represent decrease or inhibition.



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