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Kevin M. Rice

Marshall University, rice9@marshall.edu

Sarath Meduru

Marshall University

Sunil K. Kakarla

Marshall University, kakarla@marshall.edu

Anjaiah Katta

Marshall University, katta@marshall.edu

Sriram P. Mupparaju

Marshall University

*See next page for additional authors*

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**Authors**

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# Chronic Paracetamol Treatment Influences Indices of Reactive Oxygen Species Accumulation in the Aging Fischer 344 X Brown Norway Rat Aorta

Kevin M. Rice<sup>1</sup>, Sarath Meduru<sup>1</sup>, Sunil K Kakarla<sup>2</sup>, Anjaiah Katta<sup>2</sup>, Sriram P. Mupparaju<sup>1</sup>, Brent Kidd<sup>1</sup>, Lynne J. Goebel<sup>3</sup>, and Eric R. Blough<sup>1,4</sup>

<sup>1</sup> Center for Diagnostic Nanosystems, Marshall University, Huntington WV; <sup>2</sup> Department of Pharmacology, Physiology and Toxicology, Joan C. Edwards School of Medicine, Marshall University, Huntington WV; <sup>3</sup> Department of Internal Medicine and Geriatrics, Joan C. Edwards School of Medicine, Marshall University, Huntington WV; <sup>4</sup> Department of Pharmaceutical Science and Research, School of Pharmacy, Marshall University, Huntington, WV

**Abstract.** Previous reports have demonstrated that increased levels of reactive oxygen species (ROS) and alterations in cell signaling characterize aging in the Fischer 344 X Brown Norway (FBN) rat aorta. Other work has suggested that increases in ROS may be related to vascular wall thickening and the development of hypertension. Paracetamol (acetaminophen) is a potent antioxidant that has been found to diminish free radicals in ischemia-reperfusion studies. However, it remains unclear whether chronic paracetamol administration influences signaling or ROS accumulation in the aging aorta. FBN rats (27 months old; n=8) were subjected to 6 months of treatment with a therapeutic dose of paracetamol (30 mg/kg/day) and compared to age-matched untreated FBN rat controls (n=8). Compared to measurements in the aortae of 6-month old animals, tunica media thickness, tissue superoxide levels, and protein oxidation levels were  $38 \pm 7\%$ ,  $92 \pm 31\%$ , and  $7 \pm 2\%$  higher in the aortae of 33-month control animals ( $p \leq 0.05$ ). Chronic paracetamol treatment decreased tunica media thickness and the amount of oxidized protein by  $13 \pm 4\%$  and  $30 \pm 1\%$ , respectively ( $p \leq 0.05$ ). This finding of diminished aortic thickening was associated with increased phosphorylation (activation) of the mitogen activated protein kinases and diminished levels of the anti-apoptotic protein Bcl-2. Taken together, these data suggest that chronic paracetamol treatment may decrease the deleterious effects of aging in the FBN rat aorta

**Key words:** aging; aorta; paracetamol; ROS; cell signaling

## Introduction

It is estimated that by the year 2035, nearly one in four individuals will be 65 years of age or older [1]. Since cardiovascular diseases – such as coronary artery disease, peripheral vascular disease, hypertension, and chronic heart failure – are more prevalent among older persons, understanding why aging may be associated with increased incidence of cardiovascular disease will perforce take on greater significance [1]. An emerging hypothesis, described as the free radical theory of aging, suggests that

aging occurs through the gradual accumulation of free radical damage to biomolecules [2]. Inherent to this theory is that oxidative stress (often measured by alterations in levels of serum protein C, TNF-alpha and other cytokines) increases with advancing age. It is thought that oxidative stress both promotes and is induced by vascular disease and risk factors that lead to vascular disease.

Paracetamol (acetaminophen) is one of the most widely used of all drugs, with a wealth of experience clearly establishing it as the standard antipyretic and analgesic for mild to moderate pain. Recent ischemia-reperfusion work has suggested that paracetamol exhibits antioxidant properties that may be cardio-protective and anti-arrhythmic under conditions of increased oxidative stress [3].

Address correspondence to Eric Blough; Center for Diagnostic Nanosystems; Room 241N, Robert C. Byrd Biotechnology Science Center Building; Department of Pharmaceutical Science and Research; 1700 3rd Ave. Marshall University; Huntington, WV 25755-1090; e-mail: blough@marshall.edu

Whether paracetamol might offer protection against the effects of age-associated increases in vascular oxidative stress is currently unclear.

Previous work by our laboratory has reported that aging in the Fischer 344 X Brown Norway (FBN) rat aorta is characterized by increased levels of reactive oxygen species (ROS) and alterations in the regulation of the stress-activated signaling molecule adenosine monophosphate-activated protein kinase (AMPK), the apoptotic regulators Bax and Bcl-2, and the mitogen-activated protein kinases (MAPK) [4]. These data are important given that these molecules and pathways may be involved in regulating vascular smooth muscle growth, which can give rise to increases in vessel thickness and blood pressure if allowed to proceed unregulated. Additional studies have demonstrated that the function and activity of specific MAPKs such as p38 kinase, extracellular-signal-regulated kinase 1/2 (ERK 1/2-p44/p42), as well as the c-Jun N-terminal kinase (JNK), have been found to be regulated by ROS in vascular tissues [5-7]. While other research has demonstrated that paracetamol is involved in the regulation of the MAPKs and apoptosis in hepatocytes [8-10], how paracetamol may regulate these molecules in the aging aorta has, to our knowledge, not yet been investigated. Therefore, the purpose of this study was to determine how aging and paracetamol treatment alter the production of ROS and markers of oxidative stress in the aging FBN rat aorta. We hypothesized that chronic treatment with therapeutic doses of paracetamol would result in a diminished age-associated increase in aortic ROS and activation of stress-activated signaling.

## Materials and Methods

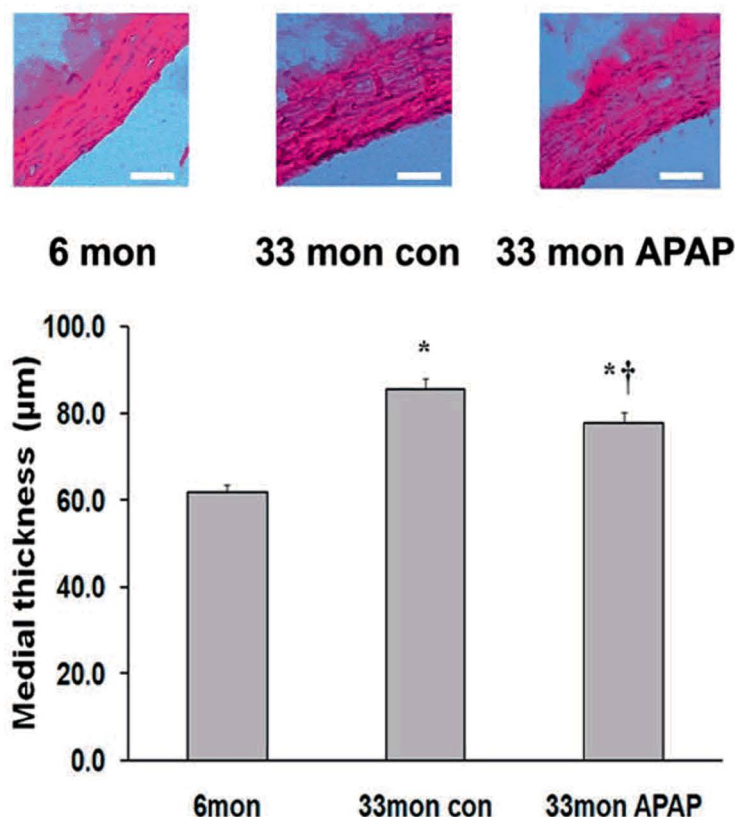
**Animals.** All procedures were performed in accordance with the Marshall University Institutional Animal Care and Use Committee (IACUC) guidelines, using the criteria outlined by the American Association of Laboratory Animal Care (AALAC) as set forth in the Animal Welfare Act (PL89-544, PL91-979, and PL94-279). Fischer 344/NNiaHSD x Brown Norway/BiNia (FBN) rats aged 6 and 27 months, were purchased from the National Institute on Aging colony at Harlan. Animals were housed two per cage in an AALAC

approved vivarium with a 12 hour light-dark cycle and a temperature maintained at  $22 \pm 2^\circ\text{C}$ , and fed *ad libitum*. All animals were allowed to acclimatize for two weeks before initiation of any treatment or procedures. All animals were examined for precipitous weight loss, failure to thrive, or unexpected gait alterations, and animals with apparent abnormalities or tumors were removed from the study. Periodic weight measurements were taken throughout the duration of the study.

**Materials.** Acetaminophen tablets or pure compound used in the study was provided by McNeil Pharmaceuticals (Fort Washington, PA). Antibodies against Bax, Bcl-2, and mouse IgG, goat IgG, and rabbit IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against AMPK- $\alpha$ , p44/42(ERK1/2-MAPK), p38-MAPK, SAPK/JNK - MAPK, p-AMPK- $\alpha$  (Thr 172), p-p44/42 (Thr 202/Tyr 204), p-p38 (Thr 180/Tyr 182), p-SAPK/JNK (Thr 183/Tyr 185), biotinylated protein ladder, and mouse and rabbit IgG antibodies were purchased from Cell Signaling Technology (Beverly, MA). Precast 10% SDS-PAGE gels were procured from Cambrex Biosciences (Baltimore, MD). Enhanced chemiluminescence (ECL) western blot detection reagent was acquired from Amersham Biosciences (Piscataway, NJ). Restore western blot stripping buffer was obtained from Pierce (Rockford, IL), and 3T3 cell lysates were from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were purchased from Sigma (St Louis, MO).

**Chronic paracetamol treatment.** 27-month old FBN rats (n=8) were subjected to 6 months – thus, up to the age of 33 months – of treatment with 30 mg/kg/day of acetaminophen administered through drinking water. Age-matched FBN control animals (n=8) were maintained under the same environmental conditions without any drug treatment.

**Aortic specimen collection.** Rats were anesthetized with a ketamine-xylazine (4:1) cocktail (50 mg/kg IP) and supplemented as necessary for reflexive response. In a sterile environment, the ventral surface of the thorax was shaved and the superficial musculature was exposed by means of a transverse incision through the skin distal to the



**Figure 1. Aortic medial thickness is altered with aging and paracetamol treatment in F344/N X BN rat.** The medial thickness of aortae from 6-month and 33-month old control and 33-month paracetamol-treated rats. Data are presented as means  $\pm$  SE. Insets: representative H & E stained aortic sections. (\*) indicates significant difference from adult (6-month old) value ( $P < 0.05$ ). (†) indicates significant difference from the 33-month old control value ( $P < 0.05$ ).  $n = 8$  for all groups. Scale Bar = 50  $\mu\text{m}$ .

thoracic cavity. After midline laparotomy and perforation of the heart, the aorta was isolated and removed from its origin at the left ventricle to the renal arch and placed in Krebs-Ringer bicarbonate buffer (KRB) containing 118mM NaCl, 4.7mM KCl, 2.5mM  $\text{CaCl}_2$ , 1.2mM  $\text{KH}_2\text{PO}_4$ , 1.2mM  $\text{MgSO}_4$ , 24.2mM  $\text{NaHCO}_3$  and 10mM  $\alpha$ -D-glucose, (pH 7.4) equilibrated with 5%  $\text{CO}_2$  / 95%  $\text{O}_2$  and maintained at 37°C. Isolated aortae were cleaned of connective tissue, weighed and immediately snap frozen in liquid nitrogen.

#### **Histology and oxidative fluorescent microscopy.**

Aortic specimens were serially sectioned (8  $\mu\text{m}$ ) using an IEC Microtome cryostat and collected on poly-lysine coated slides. After fixing in acetone (22°C for 2 min), sections were stained with

hematoxylin and eosin, mounted, and cover-slipped. Morphometric evaluation was performed with the use of a computerized imaging analysis system (Image J). Medial thickness in micrometers was calculated from the average of eight different points of cross section. Hydroethidine (HE), an oxidative fluorescent dye, was used to visualize superoxide production in situ [11, 12]. HE is permeable to cells and in the presence of superoxide ( $\text{O}_2^{\cdot -}$ ) is oxidized to Ethidium bromide, where it intercalates unable DNA [13]. Because Ethidium is unable to cross the cell membranes, extracellular  $\text{O}_2^{\cdot -}$  would not be expected to significantly contribute to the observed cellular fluorescence [14]. Since neither hydroxyl radical,  $\cdot\text{NO}$ , peroxyxynitrite,  $\text{H}_2\text{O}_2$ , hypochlorite, nor singlet  $\text{O}_2$  significantly oxidizes HE, an increase in Et fluorescence is thought to specifically indicate  $\text{O}_2^{\cdot -}$  generation within the fluorescing cell. Briefly, aortic sections were incubated for 30 min at 37°C with 5mM HE. After extensive washing with PBS and mounting (permount), tissue was visualized under fluorescence using an Olympus fluorescence microscope

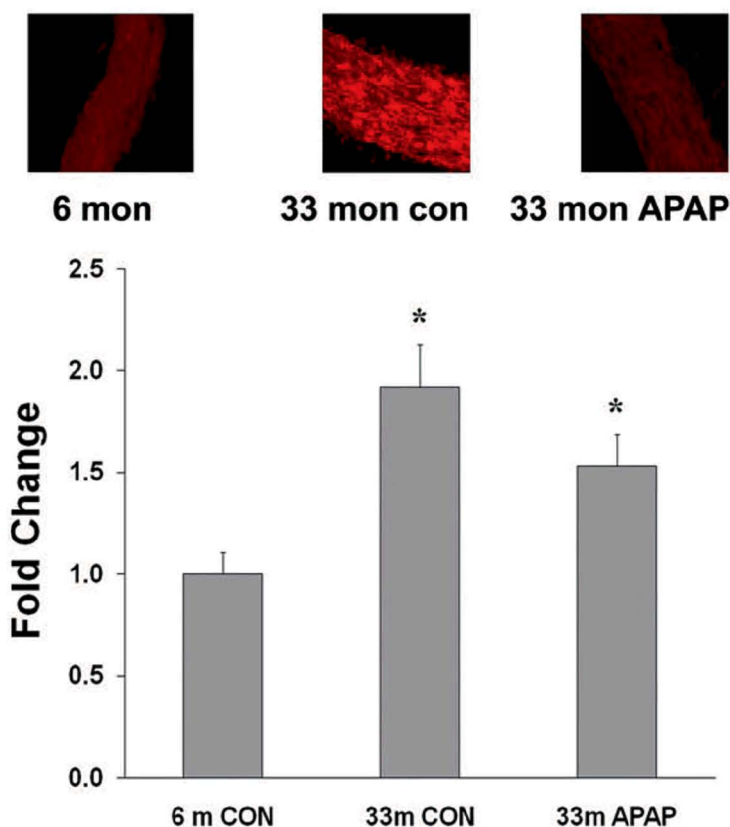
(Melville, NY) and analyzed using imaging software (AlphaEaseFC). The intensity of fluorescent Et-stained nuclei was calculated by digitizing images and then determining the average pixel intensity of six randomly positioned regions (1000  $\text{mm}^2$ ) per arterial cross section. Six images per vessel were analyzed with  $\geq 500$  nuclei per vessel examined.

**Immunoblot analysis.** Tissues were pulverized in liquid nitrogen using a mortar and pestle until a fine powder was obtained. After washing with ice-cold PBS, pellets were lysed on ice for 15 minutes in T-PER (2mL/1g tissue weight) (Pierce, Rockford, IL) and centrifuged for 10 minutes at 2000 X g to pellet particulate matter. Protein concentrations of homogenates were determined in triplicate via the Bradford method (Pierce), using bovine serum



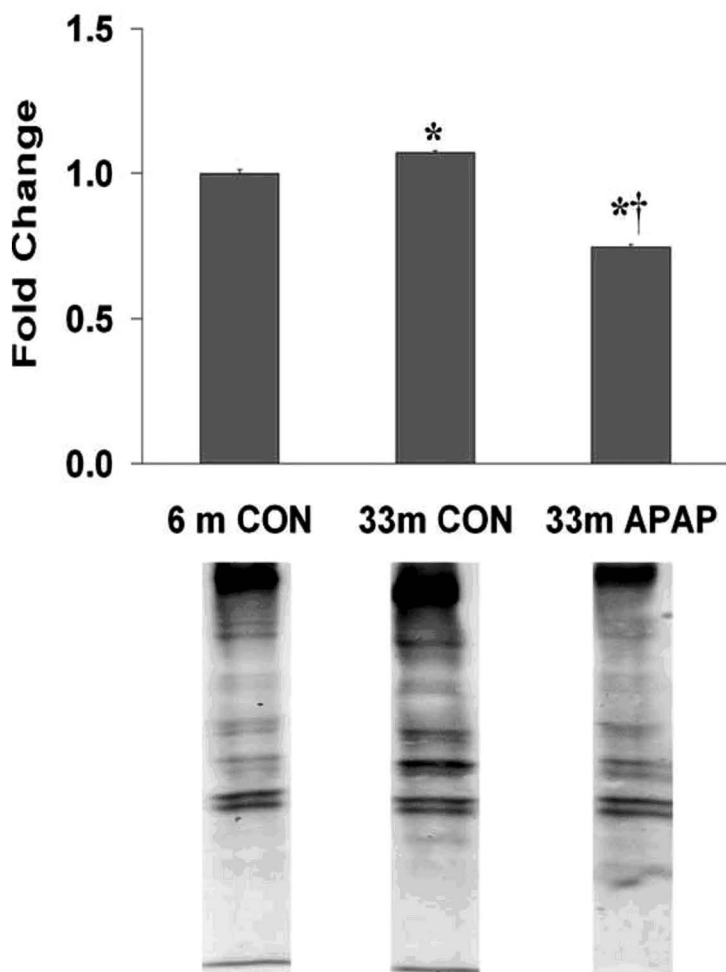
albumin as a standard. Samples were diluted to a concentration of 2.0 µg/µl in SDS loading buffer and boiled for 5 min. 40 µg of total protein per sample was separated on a 10% SDS-PAGE gel. Transfer of protein onto nitrocellulose membranes was performed using standard conditions [15]. To verify transfer of proteins and equal loading of lanes, the membranes were stained with Ponceau S.

For immunodetection, membranes were blocked in 5% Milk TBST for 1 h at room temperature and then incubated with the appropriate primary antibody overnight. After washing in TBST, the membranes were exposed to horseradish peroxidase-labeled IgG (HRP) secondary antibody for 1 h. Protein bands were visualized with ECL (Amersham Biosciences). Exposure time was adjusted to keep the integrated optical densities (IODs) within a linear and non-saturated range. Band signal intensity was quantified by densitometry using a flatbed scanner (Epson Perfection 3200 PHOTO) and imaging software (AlphaEaseFC). Molecular weight markers (cell signaling) were used as molecular mass standards, and NIH 3T3 cell lysates were included as positive controls. A total of three SDS-PAGE gels were run for each experimental set to evaluate changes in dependent variable tissue content and basal phosphorylation where applicable. Immunoblots were stripped with restore western blot stripping buffer as described by the manufacturer to obtain direct comparisons between expression and phosphorylation levels of different signaling molecules. After verifying the absence of residual HRP activity by treating the membrane with the ECL reagent, membranes were washed and re-probed with HRP labeled antibody. To minimize potential experimental error associated with membrane stripping, the order of antibody incubation was randomized between experiments.



**Figure 2. Alterations in ROS with aging and paracetamol treatment in F344/N X BN rat aortae.** Detection of vascular superoxide by dihydroethidium (hydroethidine) with aging in the aortae of 6-month and 33-month old control and 33-month old paracetamol-treated F344/N X BN rats. The increase in superoxide involves all layers within the vessel wall. Scale Bar = 50 µm. Quantification of aortic ROS as determined by intensity of fluorescent Et-stained nuclei. Results are expressed as percent of the 6-month integrated optical density (IOD) value. An asterisk (\*) indicates significant difference from the 6-month old control value ( $P < 0.05$ ), (†) indicates significant difference from the 33-month old control value ( $P < 0.05$ ).  $n = 8$  for all groups.

**OxyBlot™ Analysis.** OxyBlot analysis was performed, according to manufacturer's specifications (Millipore, Billerica, MA), to identify carbonyl groups that are introduced into the amino acid side chain after oxidative modification of proteins. The level of protein oxidation was determined by an Oxidized Protein Detection Kit (OxyBlot, Chemicon Cat# S7150-Kit). The OxyBlot kit derivatizes carbonyl groups to a 2, 4-dinitrophenylhydrazine (DNP) moiety. The DNP moiety can then be detected using anti-DNP antibodies and is a method to assay for one form of oxidative damage to a protein. The proteins were derivatized as per the protocol given in the kit. These proteins were separated on 10% SDS-PAGE gels and transferred



**Figure 3. Alterations in Protein oxidation with aging and paracetamol treatment in F344/N X BN rat aortae.** Protein isolates from the aortae excised from 6-month old and 33-month old control and 33-month old paracetamol-treated F344/N X BN rats were analyzed by immunoblotting for changes in protein oxidation. Data are presented as means  $\pm$  SE. Insets: representative blots for protein oxidation. An asterisk (\*) indicates significant difference from the 6-month old control value,  $p < 0.05$ , (†) indicates significant difference from the 33-month old control value ( $P < 0.05$ ).  $n = 8$  for all groups.

onto nitrocellulose membranes. After the transfer, membranes were blocked with 2.5% BSA (in Tris Buffered Saline [TBS] with 0.2% Tween-20) for 1 h at room temperature. The nitrocellulose membrane was exposed to a primary rabbit anti-DNPH protein antibody from Chemicon Oxyblot (1:200 working dilution) for 1 h, and then to a secondary antibody [Goat Anti-Rabbit IgG (HRP-conjugated)] diluted in the blocking solution 1:500 for 1 h at room temperature. After every step,

membranes were washed in washing buffer (TBS with 0.2% Tween-20). Protein bands were visualized with ECL (Amersham Biosciences). Band signal intensity was quantified by densitometry using a flatbed scanner (Epson Perfection 3200 PHOTO) and imaging software (AlphaEaseFC).

**Statistical Methods.** Results are presented as mean  $\pm$  SEM. Statistical analyses were performed using Sigma Plot 11.2 statistical software. Comparisons between morphologic indices and protein expression were evaluated by One Way Analysis of Variance (ANOVA) followed by a Student-Newman-Keuls post-hoc analysis. The level of significance accepted *a priori* was  $\leq 0.05$ .

## Results

### ***Aging is associated with increased aortic wall thickness and evidence of increased ROS.***

The tunica media thickness in aortae from the 33-month old control animals was  $38 \pm 7\%$  higher than that of the 6-month old control animals ( $p \leq 0.05$ ) (Figure 1). Ethidium (Et) fluorescence was seen throughout the aortic cross section with prominent signal in both the endothelial and medial portions (Figure 2). Compared to levels in the 6-month old control animals, superoxide levels, as determined by the oxidation of HE to Et and the quantification of Et fluorescence, were  $91 \pm 31\%$  higher in the aortae of

33-month control animals ( $p \leq 0.05$ ) (Figure 2). Similarly, protein oxidation as determined by the OxyBlot assay was  $7 \pm 2\%$  higher in the 33-month old control animals ( $p \leq 0.05$ ) (Figure 3).

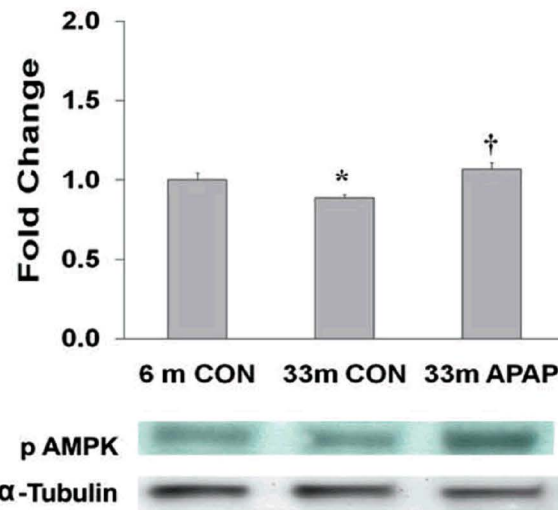
To determine whether aging influenced the total amount of AMPK- $\alpha$  present in the aorta, gel electrophoresis and immunoblot analysis using antibodies that recognize both the unphosphorylated and phosphorylated forms of AMPK- $\alpha$  were performed. There were no differences between 6- and

33-month old control aortae in the total content of AMPK- $\alpha$  (data not shown). Compared to levels in the 6-month old control animals, the basal phosphorylation levels of AMPK- $\alpha$  were  $11 \pm 6\%$  lower in the 33-month old control animals ( $p \leq 0.05$ ) (Figure 4).

**Aging is associated with changes in MAPK signaling and diminished expression of the apoptotic regulator Bax.** The MAPK proteins play an important role in propagating external stimuli into the cytoplasm and nucleus. The p38-MAPK content was  $19 \pm 7\%$  less in the 33-month old control aortae than in the aortae from the 6-month old animals ( $p < 0.05$ ) (Figure 5). No change in the total level of the p44 (ERK 1) - MAPK was observed with aging. However, the amount of p42 (ERK 2) - MAPK was  $11 \pm 3\%$  lower in the 33-month old control aortae than in the 6-month old control aortae ( $p \leq 0.05$ ) (Figure 5). The JNK-1 MAPK levels in the 33-month old control aortae were  $19 \pm 9\%$  less than those found in the 6-month old control aortae ( $p \leq 0.05$ ) (Figure 5), while JNK-2 MAPK levels were  $29 \pm 5\%$  lower in the 33-month old control aortae compared to the 6-month old control aortae. The JNK-3 MAPK levels in the 33-month old control aortae were not significantly different from levels in the 6-month old control aortae (Figure 5).

The phosphorylated p38-MAPK content in the 33-month old control aortae was  $43 \pm 12\%$  less than that observed in the 6-month old control aortae ( $p \leq 0.05$ ) (Figure 6). The activated levels of p44 (ERK 1) - MAPK and p42 (ERK 2) - MAPK in the 33-month old control aortae were  $21 \pm 3\%$  and  $26 \pm 2\%$  lower than those in the 6-month old control aortae, respectively ( $p \leq 0.05$ ) (Figure 6). The phosphorylated levels of JNK-1 and JNK-3 MAPKs were  $25 \pm 5\%$  and  $22 \pm 3\%$  less than that found in 6-month old control animals ( $p \leq 0.05$ ) (Figure 6).

With aging, the level of Bax expression decreased by  $28 \pm 9\%$  in the 33-month old control aortae compared to in the 6-month old control aortae ( $p \leq 0.05$ ) (Figure 7). Bcl-2 levels were not altered with aging.

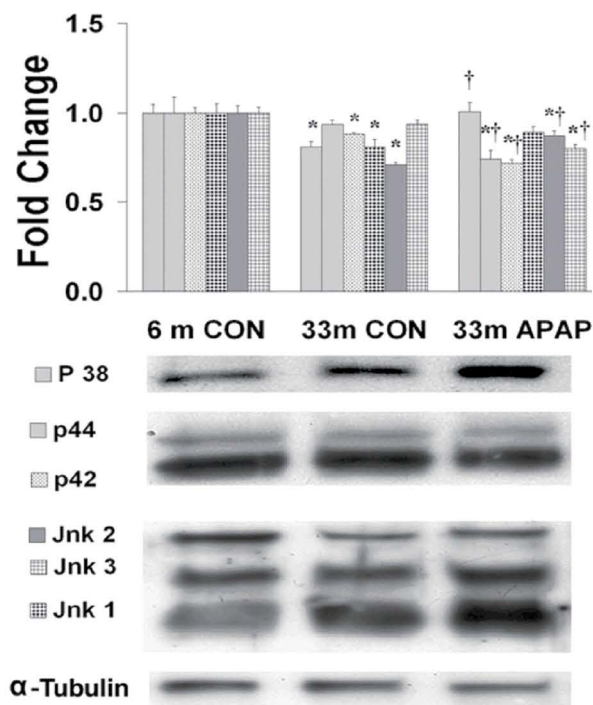


**Figure 4. Aging and paracetamol treatment alters phosphorylation status of signaling protein AMPK  $\alpha$  in F344/N X BN rat aortae.** Protein isolates from the aortae excised from 6-month old and 33-month old control and 33-month old paracetamol-treated F344/N X BN rats were analyzed by immunoblotting for changes in phosphorylated AMPK  $\alpha$ . Data are presented as means  $\pm$  SE. Insets: representative blots for phosphorylated AMPK  $\alpha$ . An asterisk (\*) indicates significant difference from the 6-month old control value,  $p < 0.05$ , (†) indicates significant difference from the 33-month old control value ( $P < 0.05$ ).  $n = 8$  for all groups.

**Paracetamol treatment decreases age-associated aortic thickening and protein oxidation.** The tunica media thickness in the paracetamol-treated aortae was  $12 \pm 4\%$  less than that of the aortae from the 33-month old control animals ( $p \leq 0.05$ ) (Figure 1). No significant differences in the levels of  $O_2^{\cdot -}$  were found between the 33-month old control aortae and the paracetamol-treated aortae (Figure 2). The levels of oxidized proteins as determined by OxyBlot assay were  $30 \pm 1\%$  less in the 33-month paracetamol aortae ( $p \leq 0.05$ ) (Figure 3).

**Paracetamol treatment alters AMPK- $\alpha$ , p44-p42 (ERK 1/2)-, p38-, and JNK-MAPK signaling.** There were no differences in the total content of AMPK- $\alpha$  between the 33-month old control and the 33-month old paracetamol-treated aortae (data not shown). Compared to the levels of the 33-month old control aortae, the basal phosphorylation levels of AMPK- $\alpha$  were  $20 \pm 6\%$  more in the 33-month old paracetamol-treated aortae ( $p \leq 0.05$ )





**Figure 5. Aging and paracetamol treatment differentially affects the concentration of proteins involved in aortic signaling.** Protein isolates from the aortae excised from 6-month old and 33-month old control and 33-month old paracetamol-treated F344/N X BN rats were analyzed by immunoblotting for changes in total p44/p42 (ERK 1/2), p38 and Jnk protein expression. Data are presented as means  $\pm$  SE. Insets: representative blots for total p44/p42 (ERK 1/2), p38 and Jnk. An asterisk (\*) indicates significant difference from the 6-month old control value,  $p < 0.05$ . (†) indicates significant difference from the 33-month old control value ( $P < 0.05$ ).  $n = 8$  for all groups.

(Figure 4). The p38- MAPK content in the 33-month old paracetamol-treated aortae was  $24 \pm 8\%$  more than that seen in the aortae from the 33-month old control animals ( $p \leq 0.05$ ) (Figure 5). The p44 and p42- MAPK levels are  $20 \pm 7\%$  and  $18 \pm 2\%$  lower in the 33-month old paracetamol-treated aortae than in the 33-month old control aortae, respectively ( $p \leq 0.05$ ) (Figure 5). There was no significant difference in the expression of total JNK-1 MAPK levels between the 33-month old control and the 33-month old paracetamol-treated aortae (Figure 5). The JNK-2 MAPK levels in paracetamol treated aortae were  $22 \pm 4\%$  higher than in the 33-month old control aortae ( $p \leq 0.05$ ). The JNK-3 MAPK levels in paracetamol-treated

aortae were  $14 \pm 4\%$  higher than in the 33-month old control aortae ( $p \leq 0.05$ ) (Figure 5).

The phosphorylated p38- MAPK content in the 33-month old paracetamol-treated aortae was not significantly different than that in the 33-month old control aortae (Figure 6). The activated levels of p44 (ERK 1) - MAPK and p42 (ERK 2) - MAPK were  $26 \pm 2\%$  and  $15 \pm 2\%$  higher with treatment, respectively ( $p \leq 0.05$ ) (Figure 6). Compared to the levels in the 33-month old control animals, the phosphorylated levels of JNK- 1 and JNK- 3 MAPKs were  $16 \pm 6\%$  and  $41 \pm 3\%$  higher in the paracetamol-treated aortae ( $p \leq 0.05$ ) (Figure 6).

#### **Paracetamol treatment alters the regulation of Bcl-2 but not Bax.**

The level of Bcl-2 protein was decreased by  $13 \pm 4\%$  in the 33-month old paracetamol-treated animals compared to the 33-month old controls ( $p \leq 0.05$ ) (Figure 7). Bax levels were not altered with paracetamol treatment.

#### **Discussion**

Previous work from our lab has suggested that aging is associated with increased levels of oxidative stress [4]. Here we investigate if chronic paracetamol treatment affects the regulation of previously identified indices of oxidative stress in the aging FBN rat aorta. Our findings suggest that chronic treatment with a therapeutic dose of paracetamol alters aortic morphology and the level of aortic oxidative stress.

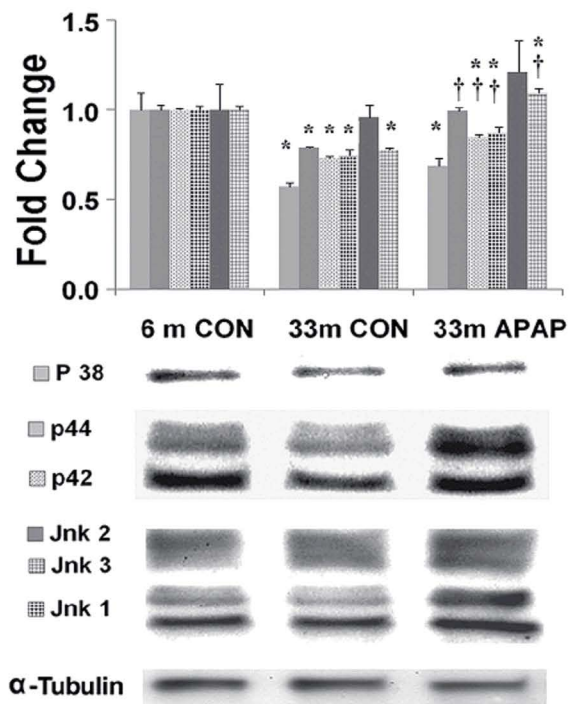
#### **Alterations in aortic tunica media thickness and ROS.**

Our results concur with previous studies that showed an increase in tunica media thickness associated with advancing age [16, 17]. It has been generally accepted that increases in vascular ROS are linked to vascular smooth muscle cellular (VSMC) proliferation and hypertrophy [18-23], and therefore may act as a hypertrophic/hyperplastic effector to thicken the arterial wall. This possibility is important as increases in wall thickness are often associated with increased vessel stiffness, a primary risk factor for the development of hypertension [24, 25]. Herein, we observed that paracetamol treatment appears to significantly decrease age-related increases in aortic wall thickness (Figure 1). Whether this decrease in aortic wall

thickening with paracetamol is due to diminished VSMC hypertrophy or replication is currently unclear.

In the present study we used dihydroethidium (HE) staining of the aortic cross sections to examine the efficacy of paracetamol treatment in attenuating aortic ROS levels [26]. Our data suggest that aging in FBN rat aortae is characterized by a marked elevation in the level of superoxide anion ( $O_2^{\cdot -}$ ) and that the amount of superoxide anion elevation is unaltered with paracetamol treatment (**Figure 2**). In addition to superoxide levels, other ROS indices include the level of protein oxidation. Because of its highly diffusible nature across phospholipid membranes [27], peroxynitrite is known to initiate oxidative modification of proteins. In our present study, protein oxidation in the rat aortae (as determined by OxyBlot™ analysis) was found to be elevated with age, a finding consistent with previously reported data [28]. The elevation in the protein oxidation was decreased with paracetamol treatment (**Figure 3**). Although we suspect that decreases in protein oxidation with paracetamol treatment can be attributed to the antioxidant properties of the paracetamol molecule, why paracetamol decreases protein oxidation levels but not the amount of superoxide remains unclear.

**Alterations in AMPK activity with age and paracetamol treatment.** AMP-activated protein kinase (AMPK) is a stress-activated protein kinase that functions as a metabolic sensor of cellular ATP levels. Several investigators have shown that AMPK is also a redox-sensitive enzyme [29, 30]. AMPK is thought to be activated by phosphorylation when the ratio of ATP: ADP falls. Once activated, AMPK switches on catabolic pathways that generate ATP while switching off ATP-consuming processes such as protein synthesis [31]. Our data suggest that aging is associated with a decrease in AMPK- $\alpha$  (Thr 172) phosphorylation. The decrease in AMPK- $\alpha$  (Thr 172) phosphorylation we show with aging is similar to the change in AMPK phosphorylation one would expect to see in cells undergoing hypertrophy [32]. With paracetamol treatment, the level of AMPK- $\alpha$  (Thr 172) phosphorylation appears to increase (**Figure 4**). This increase in the phosphorylation of AMPK- $\alpha$  (Thr 172) would be expected to decrease protein

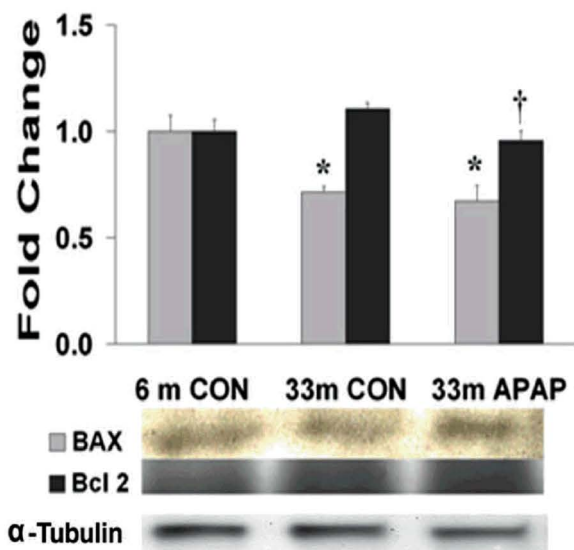


**Figure 6. Aging and paracetamol treatment alters phosphorylation status of signaling proteins p38, p44/p42 (ERK 1/2)-MAPK, and Jnk-MAPK in F344/N X BN rat aortae.** Protein isolates from the aortae excised from 6-month old and 33-month old control and 33-month old paracetamol-treated F344/N X BN rats were analyzed by immunoblotting for changes in phosphorylated p38, p44/p42 (ERK 1/2), and Jnk-MAPK protein expression. Data are presented as means  $\pm$  SE. Insets: representative blots for phosphorylated p38. An asterisk (\*) indicates significant difference from the 6-month old control value,  $p < 0.05$ , (†) indicates significant difference from the 33-month old control value ( $P < 0.05$ ).  $n = 8$  for all groups.

synthesis and may have contributed, at least in part, to the diminished aortic wall thickening we see with paracetamol treatment.

**Alterations in the regulation of MAPK and the Bax / Bcl-2 ratio with acetaminophen treatment.** The mitogen-activated protein kinase (MAPK) family has been shown to be activated by exogenous  $H_2O_2$  and by endogenously-generated ROS in VSMCs stimulated with growth factors [23]. The extracellular-signal-regulated kinase (ERK)-MAPK proteins play a major role in cell proliferation and differentiation, as well as survival [33]. The ROS sensitivity of MAPK proteins has been subject to controversy, since some groups have





**Figure 7. Aging and paracetamol treatment alters basal levels of apoptotic regulators BAX and Bcl-2 in F344/N X BN rat aortae.** Protein isolates from the aortae excised from 6-month old and 33-month old control and 33-month old paracetamol-treated F344/N X BN rats were analyzed by immunoblotting for changes in BAX and Bcl-2 protein expression. Data are presented as means  $\pm$  SE. *Insets*: representative blots for BAX and Bcl-2. An asterisk (\*) indicates significant difference from the 6-month old control value,  $p < 0.05$ , (†) indicates significant difference from the 33-month old control value ( $P < 0.05$ ).  $n = 8$  in all groups.

found these proteins to be sensitive [34, 35] while others have found them to be insensitive [36, 37]. In our present study, the amount of phosphorylated ERK1/2- and c-Jun N-terminal kinase (JNK) – MAPK, but not p38, were lowered with age but increased with paracetamol treatment (**Figure 6**). The reason(s) for which paracetamol may increase the phosphorylation of MAPK proteins in the aging FBN aorta is currently not clear. Given that increases in MAPK phosphorylation have been implicated in the initiation of cellular apoptosis, it is possible that the treatment-induced decreases in aortic wall thickness we observed may be related to increased levels of age-related VSMC apoptosis.

The over-proliferation of VSMCs has been posited to play a role in the development of several cardiovascular disorders. Reduced MAPK activity can lead to the down-regulation of the cyclin-dependent kinase (cdk) inhibitor p21 which, in turn, can cause VSMC proliferation and hypertrophy [38]. Our data indicate that paracetamol may regulate

cell proliferation by elevating ERK1/2 MAPK activity. This up-regulation of ERK1/2 MAPK activity could potentially lead to an up-regulation of p21 and the inhibition of VSMC proliferation, thereby preventing vascular wall thickening. Future experiments designed to directly test this assertion will no doubt be useful in determining whether this mechanism actually exists.

It is well established that increased aortic wall thickening can cause an increase in vessel stiffness, a primary risk factor for the development of hypertension [24, 25]. To investigate the possibility that paracetamol diminishes age-associated aortic wall thickening by causing increased levels of apoptosis, we examined how paracetamol treatment affected the ratio of Bcl-2 to Bax. Bcl-2 is an evolutionary conserved protein that blocks apoptosis [39]. Conversely, Bax is a pro-apoptotic signaling protein of the Bcl-2 family that has been proposed to promote cell death by dimerizing anti-apoptotic proteins. It is thought that the susceptibility of a cell to apoptotic signals is regulated by the ratio of anti- to pro-apoptotic proteins [40, 41]. In the present study, we observed that paracetamol treatment decreased the amount of Bcl-2, a finding which is consistent with an increase in VSMC apoptosis. Whether these changes are directly related to the decrease in aortic thickness seen with paracetamol treatment is currently unclear.

In summary, our data indicate that paracetamol treatment decreases age-associated increases in aortic wall thickening and protein oxidation. These changes were associated with alterations in AMPK and MAPK signaling and are consistent with the anti-oxidant properties of paracetamol. Further experiments will be necessary to determine the exact mechanisms through which paracetamol may act to improve vascular health during aging.

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