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Aging influences multiple incidices of oxidative stress in the aortic media of the Fischer 344/NNia × Brown Norway/BiNia rat

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Abstract

Here, we determine the influence of aging on multiple markers of oxidative stress in the aorta of adult (6-month), aged (30-month) and very aged (36-month) Fischer 344/NNiaHSd × Brown Norway/BiNia (F344/N × BN) rats. Compared to adults, increases in $O_2^{\cdot-}$ as determined by oxidation of hydroethidine (HE) to ethidium (Et) were increased 79.7 ± 7.0% in 36-month aortae and this finding was highly correlated with increases in medal thickness ($r = 0.773$, $p < 0.01$) and total protein nitration ($r = 0.706$, $p < 0.01$) but not Ki67, a marker for cell proliferation. Regression analysis showed that increases in aortic superoxide anion ($O_2^{\cdot-}$) with aging were significantly correlated with changes in the expression and/or regulation of proteins involved in metabolic (AMPK-α), signaling (mitogen activated protein kinases (MAPKs) along with c-Src), apoptotic (Bax, Bcl-2, Traf-2) and transcriptional (NF-κB) activities. These results suggest that the aging F344/N × BN aorta may be highly suited for unraveling the molecular events that lead to age-associated alterations in aortic oxidative stress.

Keywords: *Aging, aorta, reactive oxygen species, cardiovascular disease*

Introduction

Recent epidemiological studies have demonstrated that aging, by itself, confers a greater risk for cardiovascular diseases than do the other major risk factors such as plasma lipid levels, smoking, diabetes or sedentary life style [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 increases with advancing age. How aging may affect vascular levels of oxidative stress has not been well studied. Indeed, a review of the literature suggests that the majority of data collected on this subject has been obtained through the investigation of cultured cells [3–5]. These data are important given the pathophysiological consequences of enhanced oxidative stress in the intact vessel include the attenuation of endothelium-dependent dilation, the induction of cellular damage and inflammation and vascular remodeling [6,7]. Moreover, oxidative stress both promotes and is induced by vascular disease and risk factors that lead to vascular disease.

The measurement of oxidative stress is difficult due to the presence of endogenous control systems designed to quickly detect and correct alterations in oxidant levels. Complicating matters, oxidative stress can result from diminished antioxidant protection as well as increased free radical production. DNA, lipid and protein oxidation products provide an extensive and growing array of potential biomarkers, although our understanding of the relation between their status

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in cells and tissues remains to be elucidated [8,9]. In an attempt to gain greater physiological insight into effects of oxidative stress, recent studies have examined how oxidative stress may alter the content and phosphorylation status of various cellular proteins. Increased levels of oxidative stress *in vitro*, following vascular injury, or during pathological remodeling have been shown to be associated with vascular smooth muscle cell (VSMC) proliferation. The Ki67 is a prototypic cell cycle related nuclear protein, expressed by proliferating cells in all phases of the active cell cycle (G1, S, G2 and M phase) but absent in resting (G0) cells [10,11]. How aging and oxidative stress may affect Ki67 protein expression is unknown. Similarly, the function and activity of specific mitogen activated protein kinases (MAPKs), such as p38 kinase, extracellular signal-regulated kinase 1/2 (ERK 1/2-p42/p44), as well as the c-Jun *N*-terminal kinase (JNK) have been found to be regulated by oxidative stress in vascular tissues [12–14]. Several tyrosine kinases are activated by oxidative stress in particularly the Src family of kinases [15]. Investigations have also identified other markers of oxidative stress, namely: heat shock proteins (HSP), AMP-activated kinase (AMPK), protein nitro-tyrosine levels, along with apoptotic related factors Bcl-2 and Bcl2 associated protein × (BAX) [16–19]. Little information is available on whether the tissue content of these molecules is altered in the aging vasculature. The purpose of this study was to determine how aging affects multiple, previously identified markers of oxidative stress in the rat aorta. We hypothesized that aging would be associated with increases in the aortic content or basal phosphorylation of oxidative stress markers. To this end, analyses of oxidative markers was performed in aortas from young (6 month), aged (30 month) and very aged (36 month) Fischer 344/NNiaHSd × Brown Norway/BiNia (F344/N × BN) rats. Probability of survival curves generated by the National Institute of Aging (NIA) for the F344/N × BN suggested that rats of these ages corresponded roughly to humans in their third, sixth and eighth decade of life respectively [20]. This latter time point was selected due to the fact cardiovascular dysfunction in humans accelerates during the eighth decade of life, and because this age also represents one of the fastest growing segments of the aging population in the United States (21). We demonstrate that aging in the F344/N × BN increases oxidative stress, and this alteration is strongly correlated with alterations in indices of oxidative stress.

Results

Aortic wall thickness and $O_2^{\cdot-}$ are increased with aging

Compared to 6-month animals, body mass increased ~24 (421 ± 17 vs 549 ± 22 g) and ~12% (421 ± 17 vs 482 ± 28 g) in 30- and 36-month animals ($p < 0.05$). Systolic blood pressure was not significantly altered at 30- (143 ± 3 vs 131 ± 8 mm Hg) or 36-months (143 ± 3 vs 135 ± 4 mm Hg) of age. No evidence of age associated pathology (e.g. intimal fibrosis, fibrolipid plaques, or ulceration) was observed in any of the cross sections (Figure 7). Compared to the 6-month animals, aging increased the tunica media thickness of the aorta 11.29 and 21.1% at 30- and 36-month, respectively ($p<0.01$). Ethidium (Et) fluorescence was seen throughout the aortic cross section with prominent signal in both the endothelial and medial portions (Figure 1(A)). Levels of superoxide anion $O_2^{\cdot-}$, as determined by oxidation of HE to Et and the quantification of Et fluorescence were increased 55.5 ± 9.7% in 30- and 79.7 ± 7.0% in 30- and 36-month aortae compared to the levels determined in the 6-month aortas (Figure 1(B)). The amount of Ki67 (Figure 7(B)) immunoreactive signal of 6- and 36-month aortic cross sections was minimal and not altered with aging. Immunoreactivity was not detected in negative control sections incubated in the absence of primary antibody (data not shown).

Tyrosine nitration is increased with aging in the F344/N × BN rat aortae

Reactive oxygen species (ROS) may interact to form agents that modify cellular proteins. To investigate the possibility of $O_2^{\cdot-}$ associated changes in protein structure, we examined aortae for evidence of nitration. Superoxide has been shown to react with nitric oxide to form peroxynitrite which in turn is able to modify proteins by interacting with tyrosine residues to form 3-nitro-tyrosine [22]. To determine whether aging influenced the total amount of nitrated tyrosine residues present in the aorta, gel electrophoresis and immunoblot analysis using an antibody that recognize proteins and peptides containing nitrated tyrosine residues were performed. Compared to aortae obtained from 6-month animals, tyrosine nitration increased 65.9% in the 30-month aortae 65.9 and 78.4% in the 36-month aortae ($p < 0.05$) (Figure 2, Table I). The spatial location of immunoreactive nitro-tyrosine proteins in the aortic wall was examined by immunohistochemistry. With aging, we observe an increase in the amount of immunoreactive nitro-tyrosine signal throughout the medial and endothelial portions of the aorta (Figure 7(F)). These data are consistent with our previous findings of an increase in $O_2^{\cdot-}$ with aging in the F344/N × BN aorta and suggest that increased ROS may also participate in age-related aortic protein modification.

Phosphorylation of the metabolic sensor protein AMPK-α is altered with aging

AMPK is a metabolite-sensing protein that senses nutritional and environmental stresses and acts as

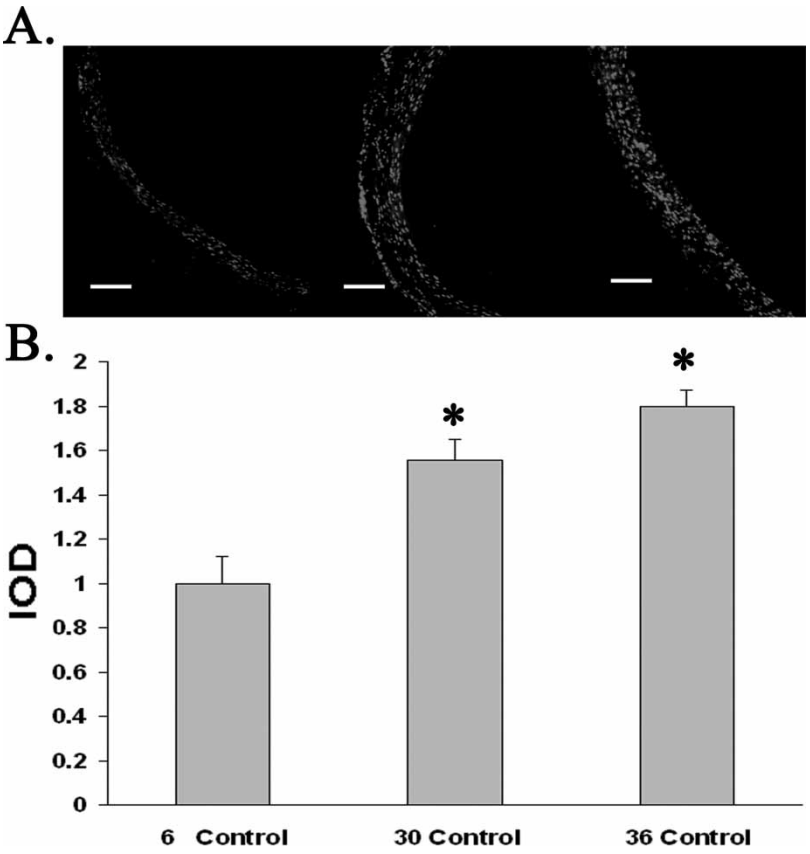

Figure 1. Aging increases aortic ROS. (A) Dectection of vascular superoxide by dihydroethidium (hydroethidine) with aging in the aortae of 6-, 30- and 36-month F433/N × BN aorta. The increase in superoxide involves all layers within the vessel wall. Bar indicates 20 μm. (B) Quantification of aortic ROS 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 young adult (6 month) value, $p < 0.05$, $n = 4$ vessels per age group.

a metabolic master switch [23]. To determine whether aging influenced the total amount of AMPK-α present in the aorta, gel electrophoresis and immunoblot analysis using antibodies that recognize both the unphosphorylated and phosphorylated forms of AMPK-α was performed. Compared to aortae obtained from 6- or 36-month animals, AMPK-α content was increased 55.1% in the 30-month aortae 55.1% ($p < 0.05$) (Figure 3, Table I). Because AMPK-α is activated by phosphorylation it was important to establish if aging in the aorta was characterized by changes in the basal phosphorylation level of the threonine 172 residue, a necessary and sufficient step for AMPK-α activation [24] and thus an established marker for AMPK-α activity [25]. Compared to 6-month aortae, the basal phosphorylation of AMPK-α (Thr 172) decreased 18.2% in the 30-month aortae and 43.7% in the 36-month aortae, ($p < 0.05$) (Figure 3, Table I). To determine whether the vascular smooth muscle was the specific cellular site for alterations in protein expression we also examined the localization of p-AMPK-α (Thr 172) by immunohistochemical analysis. Consistent with our immunoblotting results, p-AMPK-α (Thr 172) showed a substantial decrease with age in the 36-month aorta compared to the 6-month (Figure 7(C)). Immunoreactivity was not detected in negative control sections incubated in the absence of primary antibody (data not shown).

The aortic content and basal phosphorylation of the signaling molecules Src, p44/p42 (ERK 1/2)-, p38- and JNK-MAPK are altered with aging

The Src and MAPK proteins play a key role in propagating external stimuli into the cytoplasm and nucleus. As we have previously described for the MAPK proteins [26], compared to aortae obtained from 6-month animals, p38 and JNK-MAPK content increased in the 30-month aortae 83.4 and 81.4%, respectively, with JNK-MAPK remaining elevated by 72.3% in the 36-month aortae, respectively ($p < 0.05$) (Figure 3). Compared to 6-month aortae, the basal phosphorylation of p44/p42 (Thr 202/Tyr 204)-MAPK decreased 31.3% in the 30-month aorta and 26.2% in the 36-month aorta ($p < 0.05$) (Figure 4). Conversely, with aging the aortic content of Src was increased 89.8 and 92.7% in the 30- and 36-month aortae ($p < 0.05$) (Figure 3). The phosphorylation of Src (Tyr 527), Src (Tyr 416), p38 (Thr 180/Tyr 182)-MAPK and JNK (Thr 183/Tyr 185)-MAPK increased 88.0%, 49.5%,

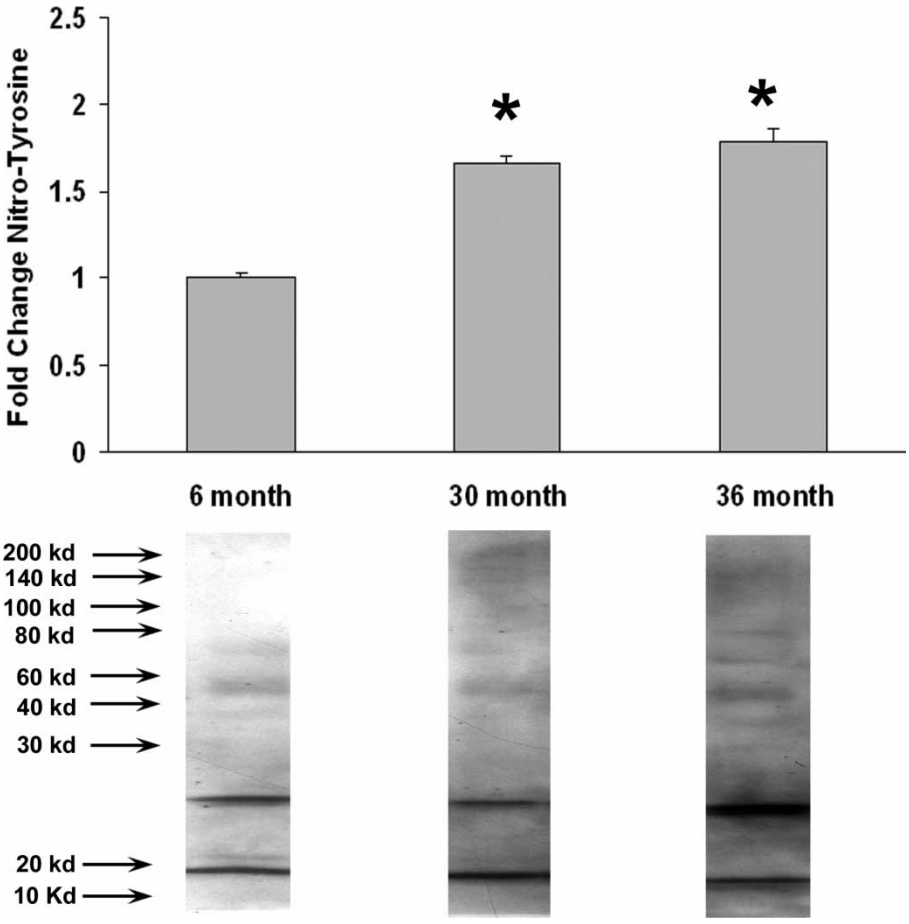

Figure 2. Increasing age in the aorta is associated with increases the nitration of tyrosine residues. Proteins isolated from the aortae of young adult (6 month), aged (30 month) and very aged (36 month) rats were analyzed by immunoblotting for age-related changes in the extent of protein tyrosine nitration. Results are expressed as percent of the 6 month value. An asterisk (*) indicates significant difference from the young adult (6 month) value, $p < 0.05$ or less, $n = 4$ observations per group.

128.7% and 23.9% in the 30-month aortae ($p < 0.05$) (Figure 4). However, in the 36-month aortae only the phosphorylation of Src (Tyr 527) and p38 (Thr 180/Tyr 182)-MAPK was above that of the 6-month control, demonstrating increases of 77.7% and 97.0%, respectively, ($p < 0.05$) (Figure 4, Table I). Src (Tyr 416) and JNK (Thr 183/Tyr 185)-MAPK phosphorylation levels in the 36-month aorta were not different from those in the 6-month aorta (Figure 4, Table I). To determine whether the vascular smooth muscle was the specific cellular site for alterations in protein expression, we also examined the localization of Src, p-Src (Tyr 527), p42/p44 (ERK 1/2)-MAPK using immunohistochemistry (Figure 7). Only minimal Src (Figure 7(D)), p-Src (Tyr 527) (Figure 7(E)) and p42/p44 (ERK 1/2)-MAPK (Figure 7(F)) immunoreactive signal was detected in the intima and adventitia portions of the vascular wall of 6- and 36-month aortae with no immunoreactivity detected in the absence of primary antibody (data not shown). Consistent with our immunoblotting results, Src and p-Src (Tyr 527) showed marked increases in immunofluorescent activity in the 36- compared to the 6-month aortae. Conversely, the basal levels of p42/p44 (ERK 1/2)-MAPK showed no change with age.

Aging increases the aortic content of the apoptotic regulators Bax, Bcl-2 and Traf-2

Bax, Bcl-2 and Traf-2 are key regulators of apoptosis. With aging, Bax and Bcl-2 content was increased in the 30-month age group by 56.6% and 78.9%, respectively. These changes in Bax and Bcl-2 were maintained with advancing age with the aortic content of Bax and Bcl-2 elevated 145.3 and 172.0%, respectively ($p < 0.05$) (Figure 5, Table I) in 36-month compared to 6-month aortae. Traf-2 exhibited increases of 56.0% and 60.2% in the 30- and 36-month aged aortae, respectively, ($p < 0.05$) (Figure 5, Table I). These data suggest that aortic content of apoptotic regulators is increased with aging.

Aging increases aortic HSP27 but not HSP70

Exposure to cellular stress is thought to increase the cellular content of the HSPs. Compared to aortae

Table I. Tissue concentration and basal levels of protein phosphorylation in aortae excised from young adult (6 month), aged (30 month) and very aged (36 month) F344/N × BN F1 hybrid rats.

	6 Month	30 Month	36 Month
Metabolic			
AMPK-α	100.0 ± 6.4	+155.1 ± 3.2*	+16.1 ± 6.0
p-AMPK-α (Thr 172)	100.0 ± 1.0	−18.2 ± 3.1*	−43.7 ± 4.5*
Signaling			
JNK	100.0 ± 23.2	+181.4 ± 9.5*	+172.3 ± 3.8*
p-JNK (Thr 183/Tyr 185)	100.0 ± 2.9	+123.9 ± 4.1*	+114.5 ± 1.6
p38	100.0 ± 7.9	+183.4 ± 17.6*	+110.1 ± 9.5
p-p38 (Thr 180/Tyr 182)	100.0 ± 3.2	+228.7 ± 2.6*	+197.0 ± 13.6*
p44/p42	100.0 ± 6.3	+16.7 ± 5.0	+13.2 ± 0.5
p-p44/p42 (Thr 202/Tyr 204)	100.0 ± 3.2	−31.3 ± 2.4*	−26.2 ± 0.4*
Src	100.0 ± 2.7	+189.8 ± 1.3*	+192.7 ± 2.0*
p-Src (Tyr 416)	100.0 ± 5.5	+149.5 ± 6.3*	+109.6 ± 4.3
p-Src (Tyr 527)	100.0 ± 7.6	+188.0 ± 3.2*	+177.7 ± 6.0*
Apoptotic regulators			
Bax	100.0 ± 5.7	+156.6 ± 7.5*	+245.3 ± 3.6*
Bcl-2	100.0 ± 3.5	+178.9 ± 8.6*	+272.0 ± 9.9*
Traf-2	100.0 ± 10.7	+156.0 ± 2.6*	+160.2 ± 7.5*
HSP			
HSP 27	100.0 ± 3.4	+129.2 ± 2.7*	+169.5 ± 4.8*
HSP 70	100.0 ± 6.3	+110.0 ± 5.3	+100.3 ± 4.1
Nitration			
Nitro-tyrosine	100.0 ± 3.3	+165.9 ± 4.5*	+178.4 ± 7.7*
Transcription factors			
NF-κB	100.0 ± 5.2	+175.4 ± 13.1*	+121.5 ± 2.0

Data was obtained from immunoblotting analysis using antibodies that recognize the unphosphorylated or phosphorylated forms of the proteins. Data are presented as percentages of the young adult value ± SE. The results reflect the analysis of combined protein comparisons of four individual aortas from each age group. An asterisk (*) designates significant differences from 6-month age group ($p < 0.05$).

obtained from 6-month animals, HSP27 content increased in the 30-month aortae 29.2% and 69.5% in the 36-month aortae ($p < 0.05$) (Figure 6, Table I). The aortic content of HSP70 did not change with aging.

Aging alters the expression of the transcription protein NF-κB

Nuclear factor of kappa light polypeptide gene enhancer in B-cells (NF-κB) is a transcription factor that has been implicated in the control of inflammatory responses, cellular growth and apoptosis [27]. To determine whether aging influenced the total amount of NF-κB present in the aorta, gel electrophoresis and immunoblot analysis using antibodies that recognize the unphosphorylated form of this protein were performed. Compared to aortae obtained from 6-month animals, NF-κB content increased in the 30-month aortae 75.4% ($p < 0.05$) (Figure 6, Table I) before returning to baseline levels in the 36-month animals.

Discussion

To our knowledge it has not been determined how aging affects multiple, previously identified markers of oxidative stress in the rat aorta. We demonstrate that aging in the F344/N × BN aorta is strongly correlated with, and characterized by elevations in aortic superoxide anion ($O_2^{\cdot-}$) and protein nitration. The increase in these markers of ROS are in turn significantly correlated with age-associated increases in medial thickness along with changes in the expression and/or regulation of proteins involved in metabolic (AMPK-α), signaling (MAPKs along with c-Src), apoptotic (Bax, Bcl-2, Traf-2) and transcriptional (NF-κB) activities. These data suggest that age-associated changes in oxidative stress are related to changes in aortic morphology and multiple signaling pathways which influence cell function.

The use of lucigenin to detect $O_2^{\cdot-}$ in tissue has recently become a subject of controversy, due to the fact lucigenin may itself enhance $O_2^{\cdot-}$ formation [28,29]. Chemiluminescence is capable of detecting $O_2^{\cdot-}$ within tissues, however, it is not a sensitive measurement of O_2 throughout the vessel wall due to the tissues ability to quench emitted photons [30]. Because of these limitations, we utilized an *in situ* assay employing the oxidative fluorescent dye HE to detect ROS with aging in the aortae. HE easily diffuses across cell membranes, where it is directly oxidized by $O_2^{\cdot-}$ to Et, intercalates with DNA and fluoresces red. We observed an increase in Et fluorescence with aging in the F344/N × BN aorta. An unexpected finding was the marked elevation of Et fluorescence within the media of aged and very aged F344/N × BN aortae,

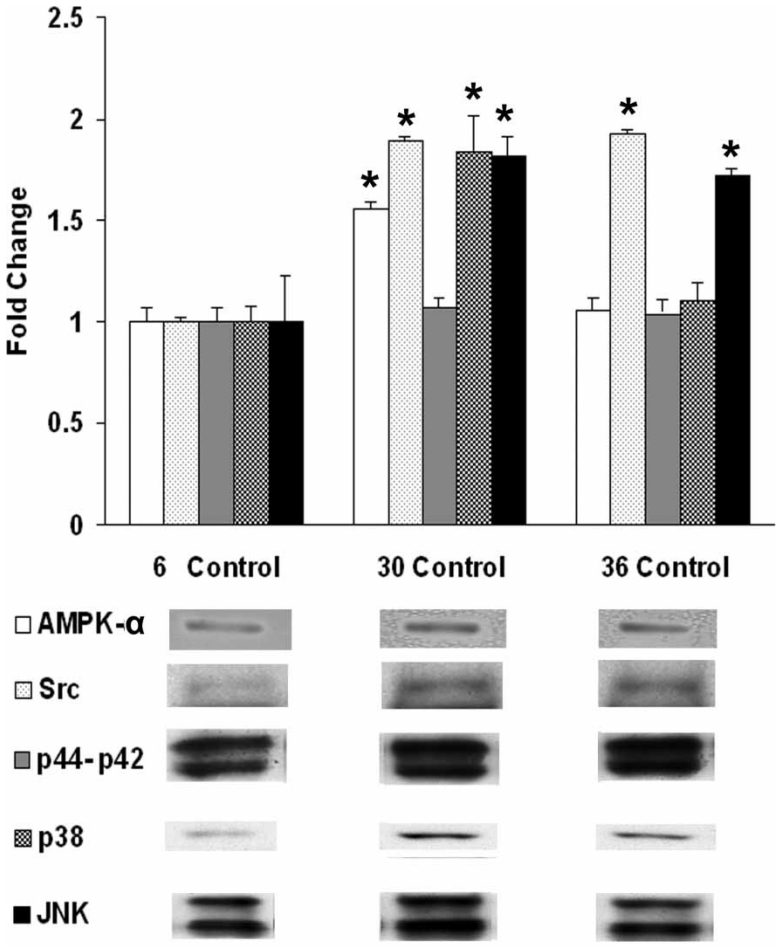

Figure 3. Aging differentially affects the concentration of proteins involved in aortic signaling. Aortic segments from young adult (6 month), aged (30 month) and very aged (36 month) rats were analyzed by immunoblotting for age-related changes in total AMPK-α, Src, p44/p42 (ERK 1/2), p38 and JNK protein expression. Results are expressed as percent of the 6 month value. An asterisk (*) indicates significant difference from the young adult (6 month) value, $p < 0.05$ or less, $n = 4$ observations per group.

suggesting increased $O_2^{\cdot-}$ production by SMCs. We believe we are amongst the first to describe these findings.

The source of the increased $O_2^{\cdot-}$ we observe with aging is unknown. The mitochondrial oxidation–reduction system and cytochrome P450 monooxygenase are well known generators of $O_2^{\cdot-}$ radicals, however, there are several other potential candidates for ROS generation, including xanthine oxidase, lipoxygenase, mitochondrial oxidase, NO synthase (NOS) and NADH/NADH oxidase [31–36]. Although, many of these sources could potentially produce ROS, it is thought that the NADH/NAD(P)H oxidase is the main cause of ROS generation in the VSMC [37–40]. Further, it has been postulated that NAD(P)H oxidase is also a major contributor to age-related increases in vascular ROS. For example, Hamilton et al. [41] showed that increased $O_2^{\cdot-}$ generation in the aged rat arota was associated with increases in the aortic content of p22phox, an integral subunit of the NAD(P)H oxidase. Csiszar and co-workers using an isolated coronary preparation showed that inhibitors to NAD(P)H oxidase substantially inhibited age-related increases in $O_2^{\cdot-}$ generation while inhibitors of NOS or xanthine oxidase did not [42]. We speculate that age-related increases in aortic NAD(P)H oxidase may be a reasonable explanation for our findings of increased $O_2^{\cdot-}$ generation in the aged F344/N × BN aorta.

ROS and especially $O_2^{\cdot-}$ have been linked to formation of the powerful oxidant peroxynitrite [22]. Peroxynitrite because of its highly diffusible across phospholipid membranes [43] is known to initiate oxidative modification of proteins, including the nitration of tyrosine. Tyrosine nitration of specific proteins attenuates their tyrosine phosphorylation [44] and thus is able to inactivate proteins whose activity depends on phosphorylation of tyrosine residues. For example, agonist-induced calcium signaling in endothelial cells is regulated by tyrosine phosphorylation and peroxynitrite is able to inhibit this response [45]. Similarly, nitration of prostacyclin synthetase and Mn-superoxide dismutase induced by overproduction of peroxynitrite have been shown to

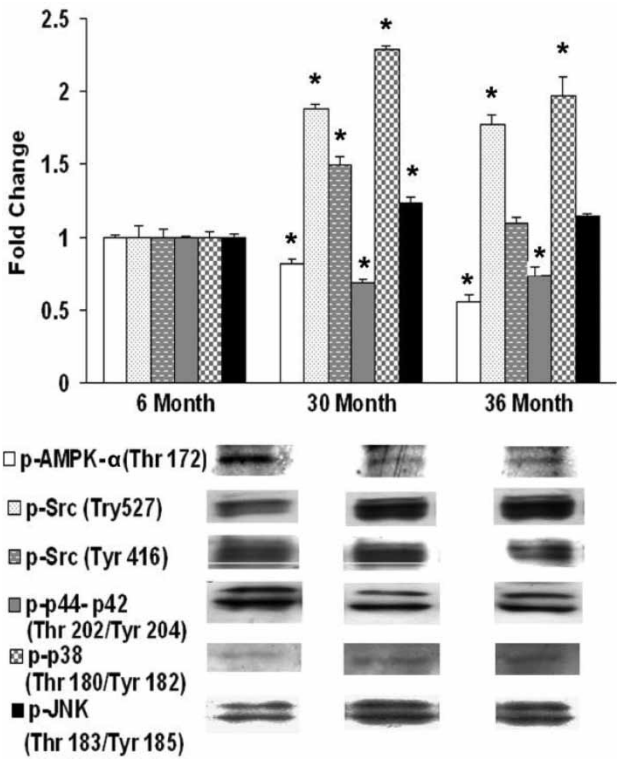

Figure 4. Aging differentially affects the phosphorylation status of proteins involved in aortic signaling. Protein extracts obtained from the aortae of young adult (6 month), aged (30 month) and very aged (36 month) rats were analyzed by immunoblotting for age-related changes in phosphorylated AMPK-α (Thr 172), Src (Tyr 527), Src (Tyr 416), p44/p42 (ERK 1/2) (Thr 202/ Tyr 204), p38 (Thr 180/Tyr 182) and JNK (Thr 183/Tyr 185) protein expression. Results are expressed as percent of the 6 month value. An asterisk (*) indicates significant difference from the young adult (6 month) value, $p < 0.05$ or less, $n = 4$ observations per group.

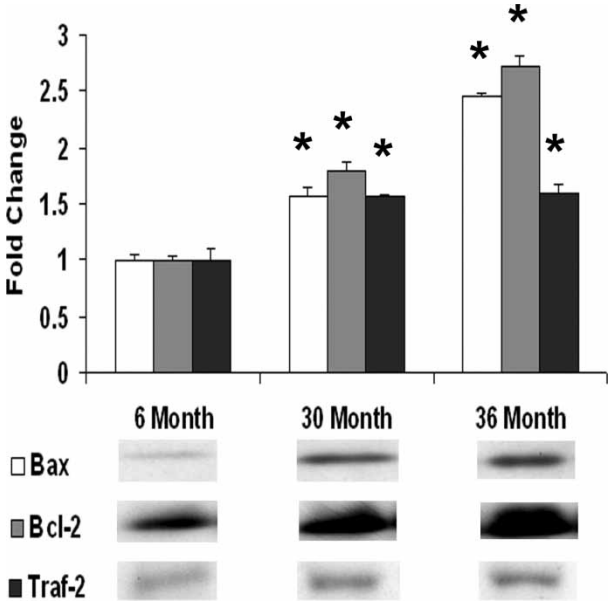

Figure 5. Aging increases the basal levels of apoptotic regulators. Protein isolates from aortae excised from young adult (6 month), aged (30 month) and very aged (36 month) rats were analyzed by immunoblotting for age-related changes in Bax, Bcl-2 and Traf-2 protein expression. Results are expressed as percent of the 6 month value. An asterisk (*) indicates significant difference from the young adult (6 month) value, $p < 0.05$ or greater, $n = 4$ observations per group.

contribute to the development of endothelial dysfunction in a number of disease states including atherosclerosis and aging [46,47]. With aging we demonstrate evidence for the *in vivo* formation of peroxynitrite, as determined by immunoblotting and quantification of proteins exhibiting nitro-tyrosine residues. Our findings of an increased immunoreactive signal following immunohistochemistry are consistent with these findings. These data suggest a link between peroxynitrite and the age-related vascular dysfunction seen by others in the aging F344/N × BN rat model [48,49].

It has been generally accepted that increases in vascular ROS are linked to VSMC proliferation and hypertrophy [50,51]; and therefore, may act as a hypertrophic/hyperplastic effector to thicken the arterial wall. Similar to previous reports (26, 52), tunica media thickness was found to significantly increase with age. This age-associated increase in aortic medial thickness was found to be highly correlated with an elevation in vessel $O_2^{\cdot-}$ levels as determined by HE staining (Figure 1). These data suggest that the increased $O_2^{\cdot-}$ in the arterial wall of aged F344/N × BN rats may contribute to age-associated arterial wall thickening. Conversely, aging did not appear to be associated with any changes in the amount of aortic Ki67 immunoreactivity (Figure 7(B)). As Ki67 is a sensitive marker of cell proliferation this finding suggests that age-related increases in F344/N × BN aortic media thickness may

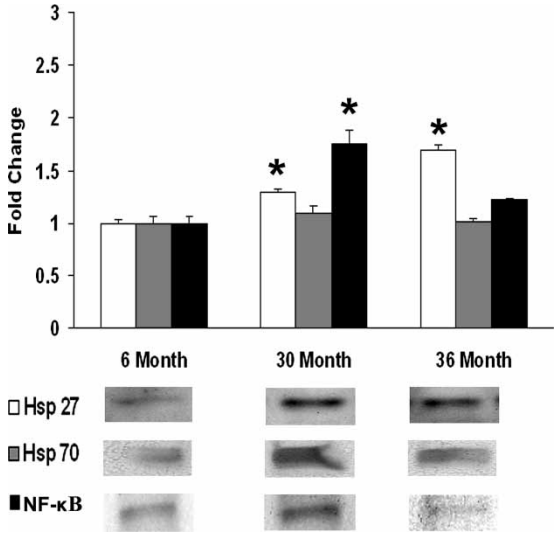

Figure 6. Aging alters aortic HSP27 levels but not HSP70. Aortic segments from young adult (6 month), aged (30 month) and very aged (36 month) rats were analyzed by immunoblotting for age-related changes in HSP27, HSP70 and NF-κβ protein expression. Results are expressed as percent of the 6 month value. An asterisk (*) indicates significant difference from the young adult (6 month) value, $p < 0.05$ or greater, $n = 4$ observations per group.

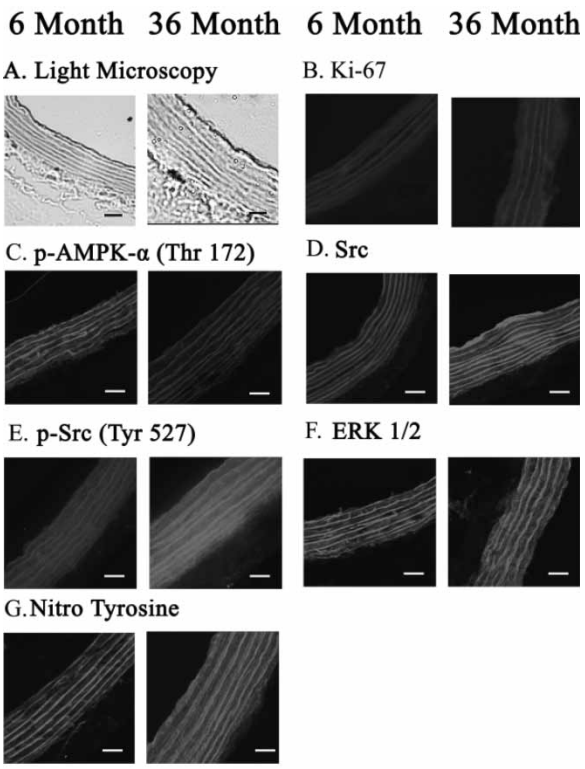

Figure 7. Aging in the F344/N × BN aorta is characterized by alterations in several signal transduction pathways. (A) Photomicrographs of light microscopy images obtained from the analysis of aortic segments from young adult (6 month) and very aged (36 month) rats. Fluorescent micrographs showing antibody labeled Ki67 (B), phosphorylated AMPK-α (Thr 172) (C), total Src (D), phosphorylated Src (Tyr 527) (E) total p44/p42 (ERK1/2) (F) and nitro-tyrosine (G) of aortic segments derived from young adult (6 month) and very aged (36 month) rats. Bar indicates 10 μm.

be due to cellular hypertrophy rather than hyperplasia. Further studies designed to directly address this question will no doubt be useful in determining which of these two processes is responsible for altering aortic wall thickness. A previous report has indicated systolic blood pressure is increased in 30- vs 6-month old F344/N × BN rats [52]. However, our results show no age-associated elevation of blood pressure in these animals suggesting the described changes in vessel morphology are not a result of hypertensive stimuli. We demonstrate that when normalized based on total protein, the aortic content of JNK-MAPK, c-Src, HSP-27, Bax, Bcl-2, Traf-2 and nitro-tyrosine-reactive proteins are significantly increased with aging (Table I). Furthermore, the basal levels of p38-MAPK (Thr 180/Tyr182) and Src (Tyr 527) phosphorylation are increased while the phosphorylation levels of AMPK-α (Thr 172) and p44/p42 (ERK 1/2-MAPK) (Thr 202/Tyr 204)-MAPK are decreased. These alterations appear to occur coincident with, and are significantly correlated with age-associated increases in $O_2^{\cdot-}$ levels and medial thickening (Table II). The physiological impact of these changes in vascular smooth muscle and aging await further clarification.

Several lines of evidence have indicated that the MAPK proteins along with c-Src are involved in vascular remodeling. For example, it has recently been reported that MAPK proteins were sensitive to oxidative stress while others have found that MAPK family members are required for cardiac hypertrophy (53, 54). Similarly, the nonreceptor tyrosine kinase Src is thought to play a key role in signaling events associated with VSMC contraction, growth and migration [55–57]. Studies have demonstrated that c-Src is an important upstream regulator of MAPK activation by ROS [58,59]. These findings are consistent with the high correlations we observe with alterations in the aortic content or basal phosphorylation of these proteins and increases in medial thickening (Table II). Additional evidence employing cell culture has suggested that c-Src may be involved in $O_2^{\cdot-}$ generation by stimulating p47phox activation and chronically by increasing the protein content of gp91phox, p22phox and p47phox [15,60]. Emerging evidence indicates the VSMC NAD(P)H oxidase is a major source of vascular ROS [32,38]. Whether age-related alterations in Src levels or phosphorylation play a similar role in altering the activation or expression of the vascular NAD(P)H oxidase is currently under investigation in our laboratory. AMPK is a metabolite-sensing protein that functions in a protective role under metabolic stress conditions such as hypoxia, ischemia and ROS [23]. Whenever the cellular ATP:ADP ratio falls, AMPK is thought to be activated by phosphorylation. Once activated, AMPK switches on catabolic pathways that generate ATP while switching off ATP-consuming processes. Our data suggest that aging is associated with a decrease in AMPK-α (Thr 172) phosphorylation (Figures 4 and 7). Although, we did not directly measure AMPK activity in this investigation, it is known that Thr172 phosphorylation is a necessary and sufficient step for AMPK activation [24] and is thus an established marker for AMPK activity [25]. AMPK activation has been shown to inhibit protein synthesis [61]. The decrease in AMPK-α (Thr 172) phosphorylation we show with aging is similar to the change in AMPK phosphorylation one would expect to see in cells undergoing hypertrophy [62]. Our data demonstrate a strong negative correlation between an age-associated decrease in AMPK-α (Thr 172) phosphorylation and an increase in medial thickening with no age associated increase in Ki67. This is consistent with previous findings examining muscle hypertrophy (63). The mechanism(s) by which AMPK acts to regulate cell growth are unclear. One possible mechanism by which AMPK may influence cell growth is through its interaction with the Akt/mammalian target of rapamycin (mTOR) pathway [64]. These data are intriguing since the Akt/mTOR pathway has been demonstrated as a key regulator of cell growth that also contains multiple potential sites for regulatory integration with AMPK [65]. HSP27 is a member of the family

Table II. Regression analysis of the relationship between expression levels of specific proteins and HE staining intensity, age and the thickness of the tunica media of aortae obtained young adult (6 month), aged (30 month) and very aged (36 month) F344/N × BN F1 hybrid rats.

	HE	Age	Medial Thickness
Independent variable			
HE	N.T.	0.807‡	0.773‡
Age	0.807‡	N.T.	0.896‡
Medial Thickness	0.773‡	0.896‡	N.T.
Metabolic			
AMPK-α	0.168	0.506†	0.176
p-AMPK-α (Thr 172)	0.736‡	0.804‡	0.860‡
Signaling			
JNK	0.546†	0.678†	0.575†
p-JNK (Thr 183/Tyr 185)	0.437⋆	0.555†	0.393⋆
p38	0.269	0.331⋆	0.045
p-p38 (Thr 180/Tyr 182)	0.627†	0.853‡	0.605†
p44/p42	0.120	0.170	0.077
p-p44/p42 (Thr 202/Tyr 204)	0.653†	0.908¶	0.712‡
Src	0.746‡	0.979¶	0.823‡
p-Src (Tyr 416)	0.292	0.376⋆	0.044
p-Src (Tyr 527)	0.688†	0.852‡	0.643†
Apoptotic regulators			
Bax	0.796‡	0.843‡	0.915¶
Bcl-2	0.737‡	0.907¶	0.910¶
Traf-2	0.663†	0.826‡	0.733‡
HSP			
HSP 27	0.625†	0.832‡	0.878‡
HSP 70	0.090	0.246	0.058
Nitration			
Nitro-tyrosine	0.706‡	0.941¶	0.829‡
Transcription factors			
NF-κB	0.171	0.459⋆	0.165

The results reflect the analysis of combined proteins and thickness comparisons of four individual aortas from each age group. An asterisk (⋆) indicates low correlation between parameters ($p < 0.05$), (†) indicates moderate correlation ($p < 0.05$), (‡) indicates high correlation ($p < 0.05$), (¶) indicates very high correlation ($p < 0.05$). N.T. (not tested).

of small HSPs that has been involved in the protection against environmental or physiological stress. In vitro studies have demonstrated that Hsp27 acts as a molecular chaperone, as an actin capping-decapping protein stabilizing the cytoskeleton and as a defense mechanism against elevated ROS [66]. We speculate that age-associated increases in Hsp27 may be a compensatory mechanism to circumvent the augmented ROS we show with aging in the F344/N × BN aorta.

We demonstrate that the aortic content of Bcl-2 and Bax are highly correlated with ROS levels, aging and medial thickening (Table II). Bcl-2 is thought to act as negative regulator of apoptosis and has been thought to protect cells from ROS, although the mechanism by which this latter event occurs remains unclear. Conversely, Bax has been implicated to promote or accelerate cell death with recent data showing that BAX may be able to induce apoptosis by both caspase-dependent and -independent mechanisms [67]. It has been proposed that cell viability may depend on the ratio of the level of Bcl-2 to that of Bax [68]. Our data suggests that Bcl-2 and Bax are upregulated with aging to a similar degree. Given the role that Bcl-2 may play in protecting the cell against elevated ROS [69], it is conceivable that age-related increases in Bcl-2 may be a mechanism employed with aging to protect the cell against elevated $O_2^{\cdot -}$. As the ratio of Bcl-2 to Bax is thought to regulate cell viability, our findings of Bax regulation paralleling that of Bcl-2 suggest that this strategy may act as a means to ensure constancy of the Bcl-2 to Bax ratio. Such a mechanism, if present, may act to minimize unnecessary cell death.

In summary, we have found that aging in the F344/N × BN aorta is characterized by increased levels of ROS and alterations in several signal transduction pathways. Further information on the individual or combined effects of these changes may reveal their role in aging aortic morphology and function. Due to the similarities between the aging F344/N × BN and humans [70–73], our data suggest that the aging F344/N × BN aorta may be highly suited for unraveling the molecular events that lead to age-associated alterations in aortic ROS.

Experimental procedures

Animals

All procedures were performed as outlined in the Guide for the Care and Use of Laboratory Animals as approved by the Council of the American Physiological Society and the Animal Use Review Board of Marshall University. Adult (6 month), aged (30 month) and very aged (36 month) male F344/N × BN rats were obtained from the NIA. Probability of survival curves generated by the NIA has indicated that rats of these ages generally represent humans in their third, sixth and eighth decade of life, respectively.

Rats were barrier housed individually in an AAALAC approved vivarium. Housing conditions consisted of a 12:12 h dark–light cycle and temperature was maintained at 22 ± 2°C. Animals were provided food and water *ad libitum*. Rats were allowed to recover from shipment for at least 2 weeks before experimentation began. During this time the animals were carefully observed and weighed weekly to ensure none exhibited signs of failure to thrive, such as precipitous weight loss, disinterest in the environment or unexpected gait alterations. Systolic blood pressure was determined with the animal unanesthetized using a programmed electrosphygmomanometer with pneumatic tail cuff (Narco-Biosystems, Houston, TX). Animals were acclimated to the procedure for a minimum of 3 days prior to obtaining blood pressure.

Materials

Antibodies against Bax, Bcl-2, HSP27, HSP70, and Traf-2, mouse IgG, goat IgG and rabbit IgG antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Antibodies against AMPK-α, p44/42(ERK1/2-MAPK), p38-MAPK, SAPK/JNK-MAPK, NF-κB, Src, p-AMPK-α (Thr 172), p-p44/42 (Thr 202/Try 204), p-p38 (Thr 180/Try 182), p-SAPK/JNK (Thr 183/Tyr 185), p-Src (Tyr 416), p-Src (Tyr527), nitro-tyrosine, biotinylated protein ladder, mouse and rabbit IgG antibodies were purchased from cell signaling technology (Beverly, MA). The Ki67 antibody was obtained from Novocastra Laboratories (Norwell, MA). Precast 10% SDS-PAGE gels were procured from Cambrex Biosciences (Baltimore, MD) and 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).

Vessel 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 aseptic 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 thoracic cavity. After midline laparotomy and perforation of the heart, the aorta was isolated and removed from the left ventricle to the renal arch and placed in Krebs-Ringer bicarbonate buffer (KRB) containing; 118 mM NaCl, 4.7 mM KC1, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24.2 mM $NaHCO_3$ and 10 mM α-D-glucose, (pH 7.4) equilibrated with 5% CO_2/95% 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 μm) using an IEC Minotome cryostat and collected on poly-lysine coated slides. After fixing in acetone, (−20°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 (Olympus MicroSuite™ Basic). Medial thickness in micrometer 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* [74,75]. HE is freely permeable to cells and in the presence of $O_2^{\cdot-}$ is oxidized to Et bromide, where it is trapped by intercalating with the DNA [76]. Because Et is impermeable to cell membranes, extracellular $O_2^{\cdot-}$ would not be expected to significantly contribute to the observed cellular fluorescence [77]. Neither hydroxyl radical, $^{\cdot}NO$, peroxynitrite, H_2O_2, hypochlorite, nor singlet O_2 significantly oxidizes HE, as such, an increase in Et fluorescence is thought to specifically indicate $O_2^{\cdot-}$ generation within the fluorescing cell. Briefly, aortic sections were incubated for 30 min at 37 °C with 5 μM 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 (Olympus MicroSuite™ Basic, Olympus America, Melville, NY). 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 μm^2) per arterial cross section. Eight images per vessel were analyzed with ≥500 nuclei per vessel examined.

Immunohistochemistry

Immunostaining for Ki67, p-AMPK-α (Thr 172), Src, p-Src (Tyr 527), p42/p44 (ERK 1/2) and nitrotyrosine was visualized by immunofluorescence as

outlined by the antibody manufacturer. Briefly, sections were washed three times with phosphate buffered saline (PBS) containing 0.5% Tween 20 (PBS-T), pH 7.5. After incubation for 30 min in a blocking solution (5% BSA) sections were incubated with specific antisera diluted in PSB-T (antibody dilution of 1:100) for 1 h at 24°C in a humidified chamber. After washing three times in PBS, sections were incubated with Texas RED anti-rabbit mouse IgG (1:200) for 30 min at 24°C in a humidified chamber. DAPI was also included in the secondary antibody solution at a concentration of 1.5 μg/ml in order to visualize cell nuclei. After a final PBS wash and mounting, specimens were visualized by epifluorescence using an Olympus fluorescence microscope (Melville, NY) fitted with a 40 × objective. Images were recorded digitally using a CCD camera and analyzed using Olympus MicroSuite™ Basic from Olympus America (Melville, NY).

Immunoblot analysis

Tissues were pulverized in liquid nitrogen using a mortar and pestle until a fine powder was obtained and washed three times with ice cold PBS as described previously [26]. Samples were then suspended on ice in 100 μl of RIPA buffer (50 mM Tris, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM EDTA, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepsatin, 1 mM PMSF, 1 mM Na_3VO_4 and 1 mM NaF) to obtain cytoplasmic proteins and centrifuged for 10 min at 10,000 rpm.

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 1.5 mg/ml in SDS-loading buffer and boiled for 5 min. Thirty μg of total protein for each sample was separated on a 10% SDS-PAGE gel. Transfer of protein onto nitrocellulose membranes was performed using standard conditions [78]. 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 secondary antibody for 1 h and 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 Pefection 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 reacting the membrane with the ECL reagent, membranes were washed and reprobed. To minimize potential experimental error associated with membrane stripping, the order of antibody incubation was randomized between experiments.

Data analysis

Results are presented as mean ± SEM. Data were analyzed by using the SigmaStat 3.0 statistical program. A one-way analysis of variance on ranks was performed for overall comparisons with the Student-Newman-Keuls *post hoc* test used to determine differences between groups. Regression analysis of the dependent variables was performed across age groups using values from four individual aortae from each group. Significance of correlation was analyzed by one-way ANOVA. The level of significance accepted *a priori* was ≤ 0.05.

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