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Age-Associated Alterations of Morphology and Protein Signaling in the Female F344xBN Rat Aorta

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Abstract

The Fischer 344 x Brown Norway F1 (F344xBN) male rat has been shown to undergo many of the same age-associated vascular changes seen in humans [1]. However, limited research has been done to determine if the female F344xBN rat is a good aging female rodent model to study age-associated changes in the vasculature. Previously we reported cardiac increases in oxidative-nitrosative stress and mitochondrial-mediated apoptosis in this model [2]. Aortae from 6-, 26-, and 30-month female F344xBN rats were stained with hematoxylin and eosin, and a trichrome stain to determine intima-medial thickness and fibrosis, respectively. Age-associated changes in expression and phosphorylation of proteins were measured by immunoblotting. Aging in the female F344xBN rat was associated with an increase in aortic intima-medial thickness, activation of p44/42 MAPK, and Hsp27 expression and decreased activation of NF-κβ p50. Hsp90 expression decreased with age in the female F344xBN aorta. There were no age-associated changes in activation of eNOS or Akt or expression of the apoptotic regulators Bax and Bcl-2. Taken together, these data are consistent with the possibility that the female F344xBN rat may be an appropriate animal model to study age-associated changes in the cardiovascular system.

Keywords: Aging; Female; Vasculature

Introduction

Cardiovascular disease remains the leading cause of death despite new discoveries in medical technology and increased awareness/education [3-5]. Aging in the human vasculature is associated with increased dilation of the lumen, thickening of the media and intima, increased stiffness, and endothelial dysfunction [5]. Sex may also play a role in age-associated alterations in vascular structure. Lower incidences of CVD-associated morbidity and mortality in premenopausal women compared to age-matched men have been reported [6]. Currently, the NIA recommends the F344xBN rat as an aging model for various age associated pathologies including CVD. This model has been shown to be excellent to study age-associated changes in the vasculature of male rats; however, no investigations have been performed to determine if age-associated changes are present in the female F344xBN rat [7-9] .

In addition to structural and functional changes, there is also evidence that aging may also affect the regulation of several signaling pathways including the mitogen-activated protein kinases (MAPK), nuclear factor-κβ (NF-κβ), endothelial nitric oxide synthase (eNOS), heat shock proteins (Hsp), and apoptotic signaling in the male rat aorta [1,10-13]. The MAPKs are serine/threonine protein kinases that play a role in the regulation of cellular proliferation, differentiation, development, cell cycle, and cell death [14]. The major MAPK signaling pathways include the extracellular signal-regulated protein kinase cascade (p44/42 cascade), c-Jun amino-terminal kinase/stress-activated protein kinase cascade (JNK/SAPK), and the p38-MAPK cascade. Signaling through the MAPK pathways is activated by growth factors, cytokines, physical, and chemical stress which causes the activation of the upstream activator of MAPK kinase kinase [15] and the MAPK kinase (MKK) [14]. The MKK then phosphorylates the downstream MAPK on serine and threonine residues leading to MAPK activation [14]. In the aorta, the MAPKs have been shown to participate in several different processes including vascular smooth muscle proliferation, contraction, migration, differentiation, and cell survival following activation by oxidative-nitrosativestress [16-20]. Whether aging may affect the regulation of these pathways in the aging female rat aorta is currently unclear.

The production of nitric oxide (NO) is stimulated by growth factors, mechanical forces, estrogen, hydrogen peroxide, and angiotensin II [21-24]. The endothelial nitric oxide synthase (eNOS), like most enzymes, is regulated by phosphorylation as it is thought that eNOS phosphorylation by Akt and AMP kinase (AMPK) is associated with increased activity. In addition to phosphorylation, eNOS is also indirectly regulated by calmodulin and Hsp90 which function to stabilize eNOS levels [25,26]. It is thought that aging is associated with increased endothelial dysfunction that is characterized by decreased NO levels [26-29]. Whether the decreases in NO with aging are due to decreased NOS levels, NO production or increased NO scavenging is not entirely understood [30-32]. The purpose, therefore, of this study

was to examine if age-associated changes in structure and protein signaling are present in the aging female F344xBN aorta. We hypothesized that aging female F344xBN, similar to that seen in humans, would be associated with increased intima-medial thickness and alterations in the regulation of intracellular signaling pathways.

Materials and Methods

Animals

All procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals as approved by the Council of the American Physiological Society, the Animal Use Review Board of Marshall University, as well as the Public Health Service Animal Welfare Policy. Adult (6-month), aged (26-month), and very aged (30-month) female F344xBN rats were obtained from the NIA and housed two per cage in an Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) approved vivarium. Animals were housed under the following conditions: 12 h-12 h light-dark cycle and temperature of 22 ± 2˚C; food and water were provided ad libitum. Rats were allowed to recover from shipment for at least two weeks before experimentation, during which time the animals were carefully observed and weighed weekly. Rats were removed from the study if they had signs of failure to thrive such as precipitous weight loss, disinterest in environment, or unexpected gait alterations.

Materials

Antibodies against p38 [#9212], p-p38 MAPK (T180/Y182) [#4631], p44/42 MAPK [#9102], p-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) [#4377], SAPK/JNK [#9252], p-JNK [#9251], AMPKα [#2532], p-AMPKα (Thr172) [#2535], eNOS [#9572], p-eNOS (Ser1177) [#9571], NF-κβ p65 [#3987], p-NF-κβ p65 (Ser536) (93H1) [#3033], Hsp27 (rodent preferred) [#2442], Bcl-2 (50E3) [#2870], Akt [#9272], phospho-Akt(Ser473) [#9271], phospho-Akt(Thr308) [#9275], HSP90 (C45G5) Rabbit mAB [#4877], GAPDH (14C10) Rabbit mAB [#2118], 3T3 Control Cell Extracts [#9203], biotinylated protein ladder [#7727], mouse and rabbit IgG antibodies [#7076, #7074] were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against NF-κβ p50 [#sc-8414], p-NF-κβ p50 [#sc-33022], HSP70 (K-20) [#sc-1060], HeLa Whole Cell Lysate [sc-2200], L6 +IGF Cell Lysate [sc-24127], and Bax (N-20) [#sc-493] were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The following materials were acquired and used for immunoblotting procedures: Precision Plus Protein Dual Color Standards (Bio-Rad, Hercules, CA, [#161-0374]); precast 10% and 15% PAGE r Gold Precast Gels (Lonza, Rockland, ME); AmershamHybond-enhanced chemiluminescence (ECL) membranes (Amersham Biosciences, Piscataway, NJ, [RPN2020D]); ECL western blot detection reagent (Amersham Biosciences, Piscataway, NJ); Restore western blot stripping buffer (Pierce, (Rockford, IL); and albumin from bovine serum (minimum 98% electrophoresis) (Sigma, St. Louis, MO). All other chemicals were purchased from Sigma (St. Louis, MO).

Aorta collection

Anesthetization of female F344xBN rats was achieved with an intraperitoneal injection of ketamine (40 mg/kg) and xylazine (10 mg/kg), supplemented as necessary for reflexive responses. A midline laparotomy was performed in order to remove the aorta from the left ventricle to the branching of the renal arteries. The aortae were stored in a Krebs-Ringer bicarbonate buffer (118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 24.2 mM $NaHCO_3$, 10 mM α-D-glucose; pH 7.4) equilibrated with 5% CO_2/95% O_2 andmaintained at 37°C as previously described by Rice and colleagues [12] for the removal of blood and connective tissue. Aortae were weighed before they were snap frozen in liquid nitrogen.

Histological analysis

Frozen aortae (n=4) were sectioned (8 μm) onto poly-lysine coated slides using an IECMinotome Cryostat. Aortic sections were stained using hematoxylin and eosin stain to determine morphology.

Immunoblot analysis

Aortic tissues were pulverized in liquid nitrogen and washed in ice cold PBS as previously published [12]. The samples were centrifuged at 4000 x g at 4°C for 20 minutes. The pellet was resuspended in TPER buffer supplemented with 0.5 M EDTA, 0.1 M EGTA, 1.0 M MgCl2, 0.1 M NaVO3, 0.5 M PMSF, phosphatase inhibitor cocktail 3 (P0044, Sigma), and proteinase inhibitor cocktail (P8340, Sigma). Samples were incubated on ice for 30 minutes and vortexed every five minutes during the incubation. The samples were centrifuged at 4000 x g at 4°C for 20 minutes, after which the supernatants were then transferred into new tubes and stored at -80°C. Protein concentration was measured using the Pierce 660 nm Protein Assay (Rockford, IL), following manufacturer's instructions. Briefly, concentrations of triplicates of each sample and bovine serum albumin as a standard were measured using a SpectraMaxPlus 384 kinetic microplate reader (Molecular Devices, Sunnyvale, CA). Each sample was diluted to 5 μg/μl using SDS-loading buffer and boiled for 5 minutes at 95°C. Proteins were separated on 10% and15 % SDS-PAGE gels and transferred to nitrocellulose membrane in order to probe with primary and secondary antibodies as described previously [12]. Chemoluminescent images were captured using the FlourChemE system, and band intensity was determined using Alphaview software (Cell Biosciences, CA). GAPDH band intensity was used to normalize the band intensity of the signaling protein.

Statistical methods

Results are given as mean ± SEM. The statistical software Sigma Stat 11.0 was used to perform statistical analyses. Age comparisons between morphologic indices and protein expression were evaluated by One Way ANOVA, or Kruskal-Wallis One Way Analysis of Variance on Ranks with the Student-Newman-Keuls, or Dunn's methods as the post hoc test, respectively. Regression analysis was performed with dependent variables against the independent variables age and intima-medial thickness. The level of significance accepted a priori was ≤ 0.05.

Results

Aortic intima-medial thickness increases with age in the female F344xBN

As reported previously, body weight was increased at 26- (274.0 ± 4.9 g) and 30-months (321.3 ± 7.2 g) compared to that observed in the 6-month female F344xBN rats [2]. Aortic intima-medial thickness was higher at 26- (97.3 + 4.0 μm) and at 30-months (140.2 + 2.0 μm) compared to that observed in the 6-month old animals (86.2 + 6.3 μm)

(Figure 1). No apparent changes in structure were observed with trichrome staining.

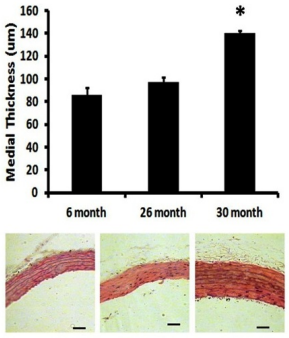

Figure 1: Aging increases intima-medial thickness in the female f344xbn aorta. Hematoxylin and eosin staining of 6-, 26-, and 30-month female f344xbn aortae. Bar indicates 100 µm. n=4 aortae per age group.

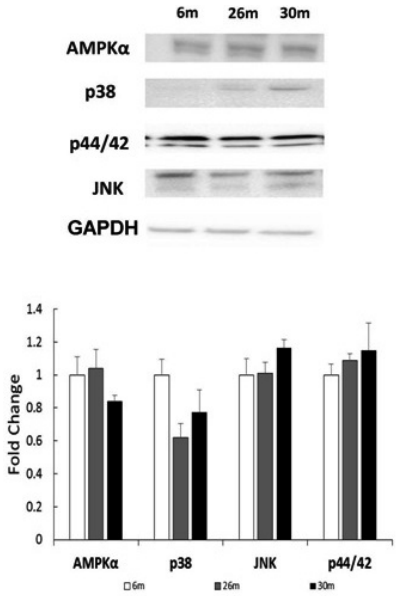

Figure 2: aging does not alter the expressions of mapks and ampk-αlpha in the f344xbn aorta: total levels of aortic ampkα as well as p38, p44/42, and jnk mapks were determined by immunoblotting in 6-, 26-, and 30-month female rats. Results were normalized to gapdh expression and expressed as fold change of the 6-month value. n=5 aortae per group.

Phosphorylation of p44/42 MAPK is altered with aging but not AMPKα, p38 MAPK, or JNK MAPK

No age-associated changes in expression or phosphorylation of AMPKα or total MAPK protein levels were observed with aging (Table 1 and Figure 2). Compared to that found in the 6-month aortae, the phosphorylation (activation) of p44/42 MAPK at Thr202 and Tyr204 was increased 125% at 26-months and 187% at 30-months (Table 1 and Figure 3). The phosphorylation of p38 and JNK MAPK activation did not change with age.

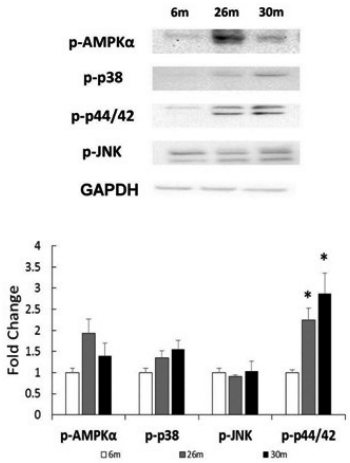

Figure 3: phosphorylation status of p44/42 mapk is increased with age in the female rat aorta but not p38 mapk, jnk mapk, or ampk-alpha. Age-related changes in phosphorylated ampkα in addition to p38, p44/42, and jnk mapk expression were analyzed by immunoblotting in 6-, 26-, and 30-month female rat aortae. Results were normalized to gapdh expression and expressed as fold change of the 6-month value. An asterisk (*) indicates significance difference from the 6-month value, $p<0.05$ or less, n=5 aortae per group.

No change in eNOS, Akt, or apoptosis in the aging female F344xBN aorta

Immunoblotting was used in order to determine age-associated alterations in expression and/or activity of eNOS. Aging was not associated with a significant change in the regulation of eNOS and Akt (Figures 4A and 4B and Table 1). No significant difference was found in the expression of Bax, Bcl-2, or the Bax/Bcl-2 ratio with aging (Table 1 and Figure 5).

Differential regulation of heat shock proteins in the aging female aorta

Hsp27 expression increased 32% at 30-months when compared to that observed in 6-month aortae (Figure 6 and Table 1). Conversely, Hsp90 protein levels were decreased 59% and 52% at 26- and 30-months (Figure 6 and Table 1). The expression of Hsp70 was unaltered with aging (Figure 6 and Table 1).

Activation of NF-κβ p50 is decreased with age

The protein levels of NF-κβ p50 and NF- κβ p65 did not change with age. The ratio of total to phosphorylated NF-κβ p50 was decreased 50% and 55% at 26- and 30-months, respectively, compared to that found in the 6-month old animals (Figure 7 and Table 1).

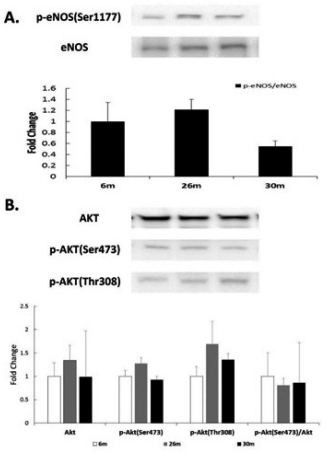

Figure 4: enos and akt activation are not altered with aging in the female f344xbn aorta. Immunoblotting was used to detect (a) the ratio p-enos(ser1177)/total enos expression. (b) akt, p-akt (ser473), and p-akt (thr308) in 6-, 26-, and 30-month female rat aortae. Results were normalized to gapdh expression and expressed as fold change of the 6-month value, n=5 aortae per group.

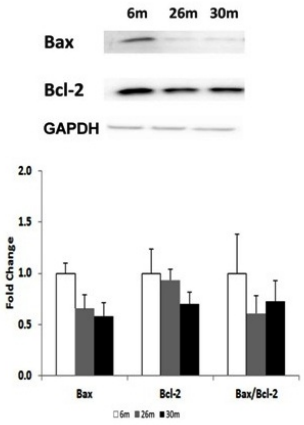

Figure 5: No age-associated increase in apoptosis with age in the female rat aorta. Protein expression of bax and bcl-2 were detected by immunoblotting in 6-, 26-, and 30-month female rat aortae. Results were normalized to gapdh expression and expressed as fold change of the 6-month value. (an asterisk (*) indicates significant difference from the 6-monthvalue ($p<0.05$)), n=5 aortae per group. There are no *

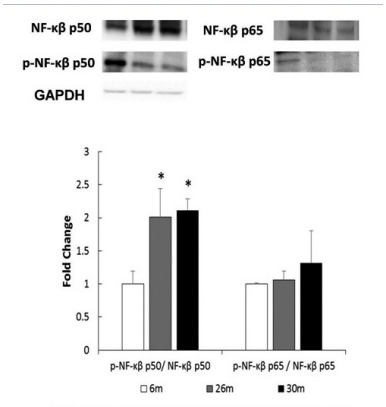

Figure 6: Differential regulation of hsps in the aging female rat aorta. Age-related changes in hsp27, hsp70, and hsp90 expression were analyzed by immunoblotting in 6-, 26- and 30-month female rat aortae. Results were normalized to gapdh expression and expressed as fold change of the 6-month value. An asterisk (*) indicates significant difference from the 6-month value ($p<0.05$) or less, what does † indicate?, n=5 aortae per group.

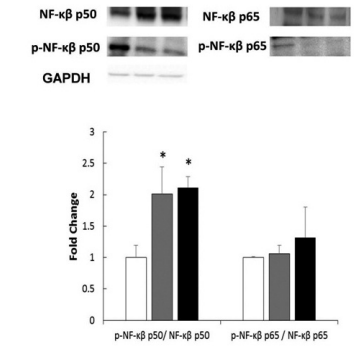

Figure 7: activation of nf-κβ p50 is decreased with age. Transcriptional regulation in the 6-, 26-, and 30-month female f344xbn aorta was determined by analyzing the expression of nf-κβ p50, p-nf-κβ p50, nf-κβ p65, and p-nf-κβ p65. Results were normalized to gapdh expression and expressed as percent of the 6-month value. An asterisk (*) indicates significance difference from the 6-month value ($p<0.05$) or less, n=5 aortae per group.

Discussion

An increase in aortic intima-medial thickness has been shown to be correlated with the development of CVD [33]. Consistent with previous work from our laboratory using the male F344xBN model [1], we found that aging in the female F344xBN aorta is also characterized by increases in intima-media thickness. To investigate the potential mechanism(s) responsible for this finding, we next examined the regulation of MAPK signaling. The MAPK proteins play a role in several different signaling pathways and are involved in the control of cell growth, proliferation, survival, motility, and differentiation [34]. It is thought that the MAPKs participate in the

pathogenesis of aortic dysfunction in several diseases [35]. In the male F344xBN aorta, the phosphorylation (activation) of p44/42, p38, and JNK MAPKs was reduced, increased, or slightly increased with age [13].

	6-month	26-month	30-month
Metabolic			
AMPKα	100 ± 6.4	+ 4.1 ± 11.2	-16.1 ± 3.5
p-AMPKα	100 ± 10.8	+93.3 ± 33.1	+39.0 ± 30.8
Signaling			
JNK	100 ± 13.3	+1.0 ± 6.6	+16.3 ± 5.1
p-JNK	100 ± 9.9	-9.8 ± 4.2	+2.8 ± 24.4
p38	100 ± 22.1	-38.2 ± 8.8	-22.7 ± 13.7
p-p38	100 ± 9.7	+34.5 ± 17.0	+54.6 ± 22.0
p44/42	100 ± 14.7	+8.9 ± 4.0	+14.9 ± 16.5
p-p44/42	100 ± 6.4	+124.5 ± 28.4*	+187.3 ± 47.6*
eNOS	100 ± 7.3	+76.5 ± 15.9	+110.5 ± 39.9*
p-eNOS (Ser1177)	100 ± 30.2	+125.3 ± 52.7	+23.8 ± 43.0
Akt	100 ± 29.1	+34.4 ± 31.9	-1.5 ± 35.7
p-Akt (Ser473)	100 ± 12.8	+27.5 ± 12.1	-7.5 ± 7.7
p-Akt (Thr308)	100 ± 21.2	+69.2 ± 48.3	+35.6 ± 12.4
Apoptotic Regulators			
Bax	100.0 ± 11.8	-34.7 ± 16.7	-41.9 ± 16.1
Bcl-2	100.0 ± 29.1	-6.8 ± 13.3	-30.1 ± 14.3
Heat shock proteins			
Hsp27	100 ± 6.8	+2.2 ± 3.5*	+31.8 ± 6.2*
Hsp70	100 ± 8.1	+55.5 ± 23.6	+19.7 ± 11.4
Hsp90	100 ± 20.3	-59.4 ± 5.2*	-52.0 ± 9.3*
Transcription Factors			
NF-κβ p50	100 ± 13.1	+65.8 ± 40.9	+49.3 ± 17.7
p NF-κβ p50	100 ± 8.6	-20.3 ± 5.6	-31.0 ± 4.4*
NF-κβ p65	100 ± 10.3	+3.0 ± 19.9	-11.9 ± 28.8
p NF-κβ p65	100 ± 11.9	+5.1 ± 9.4	-2.9 ± 7.8

Table 1: aortic tissue expression for total and phosphorylated proteins in aorta [from 6- month, 26-month, and 30-month female f344xbn rats. Data are presented as percentages changes? Of 6-month adult value ± SE. Values for proteins were obtained from n=5 aortae per age group. An asterisk (*) indicates significant difference from 6-month age group ($p<0.05$).]

Conversely, in the female F334xBN aorta, the activation of p44/42 MAPK was significantly increased with age. Previous studies have found that this age-associated increase of p44/42 MAPK activation was associated with increases in VSMC proliferation [34] and migration [36-38]. Consistent with these findings, we also noted that elevations in p44/p42 MAPK phosphorylation appeared to be highly correlated with increases in intima-medial thickness (Figure 1 and Table 2). Whether this increase in p44/p42 MAPK phosphorylation is solely responsible for the aortic remodeling we observed in the current study is currently unclear and will require further investigation.

	Age	Intima-medial Thickness
Independent variable		
Age	N.T.	0.741 (0.022) *
Intima-medial thickness	0.741 (0.022) *	N.T.
Metabolic		
AMPKα	0.326 (0.391)	0.690 (0.04) *
p-AMPKα	0.555 (0.121)	0.067 (0.864)
Signaling		
JNK	0.389 (0.301)	0.498 (0.172)
p-JNK	0.044 (0.91)	0.024 (0.952)
p38	0.547 (0.128)	0.082 (0.834)
p-p38	0.740 (0.023) *	0.692 (0.039) *
p44/42	0.364 (0.336)	0.204 (0.589)
p-p44/42	0.888 (0.001) *	0.732 (0.025) *
eNOS	0.837 (0.005) *	0.709 (0.032) *
p-eNOS (Ser1177)	0.384 (0.307)	0.189 (0.627)
Akt	0.132 (0.736)	0.214 (0.581)
p-Akt(Ser473)	0.123 (0.753)	0.354 (0.35)
p-Akt(Thr308)	0.486 (0.185)	0.075 (0.849)
Apoptotic Regulators		
Bax	0.725 (0.027) *	0.483 (0.188)
Bcl-2	0.377 (0.317)	0.579 (0.102)
Heat Shock Proteins		
Hsp27	0.615 (0.078)	0.903 (<0.001) *
Hsp70	0.519 (0.152)	0.004 (0.993)
Hsp90	0.834 (0.005) *	0.388 (0.302)
Transcription Factors		
NF-κβ p50	0.851 (0.004)	0.701 (0.035)
p-NF-κβ p50	0.617 (0.077)	0.329 (0.387)
NF-κβ p65	0.120 (0.758)	0.159 (0.682)
p-NF-κβ p65	0.001 (0.999)	0.032 (0.934)

Table 2: regression analysis of the relationship between signaling proteins to age and intima-medial thickness in the aortae of 6-, 26-, and 30-month old female f344xbn rats. Values for proteins and

thickness were obtained from n=5 aortae for Each age group. Reported as r2 and p value, * indicated $p<0.05$. N.t. (not tested).

Age-associated endothelial dysfunction has been linked to changes in expression and activity of eNOS [39]. It is currently unclear how aging may affect eNOS expression and activity. In the current study, we found no significant change in total eNOS expression or phosphorylation (activation) with increasing age. Consistent with this finding, we also found that expression and phosphorylation of Akt, which functions as an upstream regulator of eNOS, is also unchanged with aging. Like Akt, Hsp90 also plays an important role in the regulation of eNOS and NO production. It is thought that association of Hsp90 with eNOS increases NO generation [40]. We found a decrease in Hsp90 expression with increasing age (Figure 6). This decreased expression may be associated with a reduction in NO production. Whether this finding is associated with diminished aortic relaxation or alterations in animal blood pressure with aging will require additional study.

Heat shock proteins are induced by cell stress and function to stabilize protein structure or to protect the cell from injury [41,42]. Supporting this notion, with aging we found that Hsp27 expression was increased in the female F344xBN aorta (Figure 6). Similar to our findings for p44/p42 MAPK, this increase in Hsp27 was also found to be highly correlated to increases in intima-medial thickness (Table 2). Previous data has suggested that decreased levels of Hsp27 are associated with the pathogenesis of atherosclerosis [43]. Whether this increase in Hsp27 levels is a compensatory response to aging or diminished estrogen levels to maintain aortic function with aging is currently unclear.

NF-κβ is an important proinflammatory transcription factor that induces transcription of chemokines, cytokines, adhesion molecules, secondary inflammatory enzymes, and anti-apoptotic factors [44,45]. Previous work in our laboratory demonstrated that aging was not associated with alterations in NF-κβ expression in the male F344xBN [46]. Conversely, in the present study we found that the phosphorylation (activation) of NF-κβ p50 was increased with age. Why aging might increase NF-κβ p50 activity levels is not yet clear, however, previous studies have demonstrated that increases in NF-κβ expression are regulated, at least in part, by ROS levels [17-19,47] and that it may function in the control of vascular smooth muscle cell proliferation [48]. Whether NF-κβ p50 might functions in a similar manner in the aging F344xBN aorta will require further investigation.

Conclusion

In conclusion, our data suggest that aging in the female F344xBN aorta is characterizedby increases in intima-medial thickness, increased p44/42 MAPK activation, decreased Hsp90, increased Hsp27 protein levels, and increased activation of NF-κβ p50. This combination of age-associated signaling alterations may be a compensatory response by the aorta to mitigate the age-related loss in circulating estrogen. Whether similar findings are also seen in aging women is currently unclear. Additional work is needed to more fully understand the underlying mechanisms of the age-associated changes in the aging female F344xBN aorta.

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