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Divergent effects of aging and sex on vasoconstriction to endothelin in coronary arterioles

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Abstract

The risk for cardiovascular disease increases with advancing age; however, the chronological development of heart disease differs in males and females. The purpose of this study was to determine whether age-induced alterations in responses of coronary arterioles to the endogenous vasoconstrictor, endothelin, are sex-specific. Coronary arterioles were isolated from young and old male and female rats to assess vasoconstrictor responses to endothelin (ET), and ETa and ETb receptor inhibitors were used to assess receptor-specific signaling. In intact arterioles from males, ET-induced vasoconstriction was reduced with age, whereas age increased vasoconstrictor responses to ET in intact arterioles from female rats. In intact arterioles from both sexes, blockade of either ETa or ETb eliminated age-related differences in responses to ET; however, denudation of arterioles from both sexes revealed age-related differences in ETa-mediated vasoconstriction. In arterioles from male rats, ETa receptor protein decreased, whereas ETb receptor protein increased with age. In coronary arterioles from females, neither ETa nor ETb receptor protein changed with age, suggesting age-related changes in ET signaling occur downstream of ET receptors. Thus, aging-induced alterations in responsiveness of the coronary resistance vasculature to endothelin are sex-specific, possibly contributing to sexual dimorphism in the risk of cardiovascular disease with advancing age.

Keywords

rat; vasodilation; BQ123; BQ788

INTRODUCTION

Considerable evidence demonstrates that sex plays an important role in the development of cardiovascular disease with advancing age. Specifically, endothelial dysfunction occurs

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more than a decade later in women compared to men (5). Recent data (19, 20) indicate that endothelium-dependent dilation declines with age in coronary arterioles from both male and female rats; however, the underlying mechanisms that contribute to the decline in endothelial function are sex-specific. In contrast, little is known with regard to sex-specific adaptations of vasoconstrictor responses that occur in the coronary vasculature with advancing age. Previous work indicates that endothelial modulation of vasoconstrictor responses increases with age in coronary arterioles from male rats (31); however, because estrogen exerts a potent vasodilatory influence in the vasculature, it is plausible that a decline in circulating estrogen could lead to an increase in vasoconstrictor responses of coronary arterioles of aged female rats.

Advancing age causes a decrease in cardiac function (1) and reduces maximal and submaximal coronary blood flow in aged rats (14) and humans (9). Endothelin (ET) is a 21-amino acid vasoconstrictor peptide that is released from the coronary vasculature in response to a stimulus from cardiac myocytes (25), causing a potent and long-lasting coronary vasoconstriction (40). Responsiveness to ET declines with age in the aorta isolated from female rats (2) but are enhanced in large coronary arteries from male rats (17) with advancing age. A decrease in ET-induced vasoconstriction in coronary resistance arterioles occurred with advancing age in male rats (32); however, it remains to be determined whether age alters ET-mediated responses of smaller caliber vessels from females as it does in males.

ET has been shown to be involved in determining basal coronary arteriolar tone, and a reduction of ET contributes to the elevation of coronary blood flow during periods of increased metabolism (26). The long-lasting vasoconstriction caused by ET can redirect coronary blood flow in order to promote subendocardial perfusion, and this vasoconstriction has been proposed to prevent excessive back flow from the coronary circulation (25). Thus, age-related alterations in responsiveness of coronary arterioles to ET may be important to regulation of blood flow in the aged heart. ET has the potential to stimulate both dilation, through ETb receptors on the endothelium, and constriction, through ETa and ETb receptors on the vascular smooth muscle cell (32). Thus, the net effect of ET depends on the relative distribution and density of each specific subtype of receptor. Although we have previously shown that advancing age decreases vasoconstriction to ET in coronary arterioles from males (32), the effects of advancing age on ET signaling through specific ET receptor subtypes have not been investigated in the coronary circulation of males or females. Therefore, the goals of this study were to 1) determine whether age-induced alterations in vasoconstrictor responses of coronary arterioles are sex-specific, and 2) determine the effects of advancing age on signaling through ETa and ETb receptors in coronary arterioles from male and female rats.

MATERIALS AND METHODS

Animals

Young (6 mo; n = 54) and old (24 mo; n = 48) male and young (n = 35) and old (n = 34) female Fischer-344 rats were obtained from Harlan (Indianapolis, IN). All procedures were approved by the Institutional Animal Care and Use Committees at the Institutional Animal Care and Use Committees at West Virginia University and University of Florida and conformed to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (National Research Council, Washington D.C., Revised 1996). Rats were separated by sex and housed two per cage and maintained on a 12:12-h light-dark cycle at 23° C. All rats were fed standard rat chow and water *ad libitum*.

Microvessel Preparation

Rats were anesthetized (isoflurane 3%/O_2 balance) and euthanized by removal of the heart. The heart was rinsed and placed in cold (4°C) physiological saline solution (PSS) containing 145.0 mM NaCl, 4.7 mM KCl, 2.0 mM $CaCl_2$, 1.17 mM $MgSO_4$, 1.2 mM NaH_2PO_4, 5.0 mM glucose, 2.0 mM pyruvate, 0.02 mM EDTA, 3.0 mM MOPS buffer, and 1 g/100 ml BSA, pH 7.4. In order to study vessels that contribute significantly to coronary vascular resistance (7) , only arterioles <150 μm in diameter were dissected from the left ventricular free wall with the aid of a dissection microscope (Olympus SVH10). The arterioles were transferred to a Lucite chamber containing PSS equilibrated with room air. The ends of the arteriole were cannulated with a micropipette and secured with nylon suture. The chamber containing the cannulated arteriole was then placed on an inverted microscope (Olympus IX70) equipped with a video camera and micrometer (Panasonic BP310; Texas A&M Cardiovascular Research Institute) to measure intraluminal diameter. The coronary arterioles were then pressurized at 45 mmHg with two hydrostatic columns. Arterioles unable to hold pressure due to leaks or branches were discarded. Arterioles without leaks were warmed to 37°C and allowed to equilibrate for 40 minutes before beginning assessment of vasoconstrictor responses.

Responses to Endothelin

To determine whether aging alters sensitivity and/or maximal responses to ET, a concentration-response curve to ET was generated. Changes in diameter were measured in response to cumulative additions of ET (1×10^{-11} M – 3×10^{-8} M; 5-minute stages) to the vessel bath.

ET Receptor Blockade

To determine the contribution of ET receptor subtypes in the age-related alteration of ET-mediated constriction, the ET concentration-response was evaluated in the presence of either a specific ETa receptor (BQ123, 1×10^{-6} M) or ETb receptor (BQ788, 3×10^{-8} M) antagonist (27).

Removal of the Endothelium

To determine the role of the endothelium in modulating ET-induced vasoconstriction, the endothelium was removed, and the above experiments were repeated. The endothelium was denuded by passing approximately 10 ml of air through the vessel lumen. Complete lack of vasodilation to 3×10^{-5} M ACh, indicated by no change or a decrease in diameter, confirmed removal of the endothelium.

Passive Pressure Responses

In order to determine maximal diameter and passive responses to increasing pressure, the solution in the bath and pressure lines was replaced with calcium-free PSS containing 2.0 mM EDTA. Arterioles were washed every 15 minutes and allowed to completely relax at 45 mmHg for 45 minutes. Maximal diameter at 45 mmHg was recorded, and then the passive pressure response was determined by lowering the pressure reservoirs to 0 mmHg, and recording diameters as pressure was increased incrementally by ~10 mmHg to 100 mmHg. This procedure was performed in arterioles from females only, as a similar experiment has already been performed in arterioles from males (32).

Determination of ETa and ETb receptor protein

Coronary arterioles (n = 4/rat) were immediately snap frozen and stored at –80°C until ready for use. After addition of 15 ul lysis buffer (1x Laemmli buffer + 5% β-mercaptoethanol), arterioles were solubilized by repeating a series of vortexing, spinning, and boiling (3x),

sonication (1x), and a final series of vortexing, spinning and boiling. Protein content was assessed by NanoOrange assay (Molecular Probes). Equal amounts of protein (5 μg) were electrophoresed on 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes. Following blocking (6% nonfat dry milk), membranes were incubated with primary antibodies overnight at 4 °C as described previously (ETa 1:1000, Sigma E9780, ETb 1:1000, Sigma E9905). Positive controls for ETa and ETb antibodies were previously performed using rat testis (17, 36). After washing, membranes were incubated with the appropriate horseradish peroxidase-conjugated species-specific anti-IgG (1 h). Peroxidase activity was detected by enhanced chemiluminescence (Super Signal West Femto, Pierce). Densitometric analysis of immunoblot films was performed using NIH ImageJ 1.38x Analysis Software (National Institutes of Health, Bethesda, MD). Equal loading was confirmed by visual inspection of Sypro Ruby staining.

Immunohistochemical analysis of ETa and ETb receptor protein

Coronary arterioles were cannulated, pressurized, and fixed in Bouin's solution. Fixed arterioles were placed in optimal cutting temperature (OCT) compound and stored at -80°C, until cutting of 4 micron sections. Sections were incubated in blocking solution (PBS containing 5% goat serum and 3% Triton) for 1 hr, followed by overnight incubation in anti-ETa (1:400, Sigma E9780) or anti-ETb (1:400, Sigma E9905) primary antibodies at 4°C. After washing, sections were incubated in FITC-conjugated secondary antibody (1:400) for fluorescence development. Smooth muscle α-actin (1:250), with Texas Red-conjugated secondary antibody (1:200), was applied as marker of vascular smooth muscle cells. FITC staining located on the lumen side that did not overlap with red staining of smooth muscle α-actin was designated as endothelial fluorescence. The level of fluorescence in a single arteriolar section was determined by corrected fluorescence density according to the following equation:

$$\text{Corrected fluorescence density} = \text{Integrated density} - (\text{Area of vessel section} \times \text{mean background fluorescence})$$

In a subset of samples, sections were incubated with ETa or ETb in the presence of 3×10^{8} M endothelin. The presence of this saturating concentration of endothelin reduced ETa fluorescence by 76±3% and ETb fluorescence by 79±3%. Similarly, staining with secondary fluorescent antibodies in the absence of the respective primary antibody (ETa or ETb) eliminated fluorescence.

Solutions and Drugs

Albumin was purchased from USB Chemicals (Cleveland, OH). All other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Data Analysis

Data are expressed as means ± standard error.

$$\text{Spontaneous Tone} \quad (\%) = [(D_M - D_T)/D_M) \times 100$$

where D_M is the maximal diameter recorded at 45 mmHg and D_T is the steady-state baseline diameter recorded at the same pressure. Constriction to ET was expressed by the following equation:

$$\text{Constriction} \quad (\%) = [(D_b - D_s)/D_b] \times 100$$

where D_b is the baseline diameter immediately prior to addition of the first dose of vasoconstrictor agonist, and D_s is the steady state diameter measured after addition of each dose. Passive myogenic response curve was generated using normalized diameter for every pressure point according to the formula as following:

$$\text{Normalized Diameter} = D_s / D_{max}$$

where D_{max} is the maximal inner diameter recorded at a pressure of 45 mmHg under Ca^{2+}-free conditions and D_s is the steady diameter after each pressure change. The concentration that produced 50% of the maximal vasoconstriction to the agonist was designated as the EC_{50}.

Concentration-diameter curves were evaluated by three-way repeated measures ANOVA in order to detect differences within (concentration) and between (age, sex, or drug treatment) factors. Pairwise comparisons were made by post-hoc analysis (Bonferroni) when a significant main effect was found. Two-way ANOVA was used for comparisons of animal and vessel characteristics. In all statistical analyses, *n* indicates the number of animals in each group. Significance was set at $P \leq 0.05$.

RESULTS

Animal and Vessel Characteristics

Animal characteristics are presented in Table 1. Old male and female rats had a higher body weight and heart weight than young male and female rats. Heart weight to body weight (HW/BW) ratio was increased with age in males, but decreased in females. In intact arterioles, spontaneous tone was similar between all groups (Table 2). Denudation significantly increased spontaneous tone in arterioles from old males and females, but did not alter tone in arterioles from young males and females. Denuded arterioles from old females exhibited greater spontaneous tone than those from young females. BQ123 increased the level of tone in young and old females as compared to intact vessels from the same age group. Arterioles from old males exhibited less tone after BQ123 treatment as compared to those from young males, and this was significantly less than the tone developed by arterioles from old females. Similar to denuding, treatment with BQ788 increased tone in intact arterioles from old males and females, but did not alter tone in intact arterioles from young males and females (Table 2).

Response to ET

Old age altered the vasoconstriction to ET in coronary arterioles from both male and female rats (Fig 1). As shown previously, overall ET-induced vasoconstriction was impaired in arterioles from old males compared to those from young males (Fig 1A). In contrast, age increased overall vasoconstriction to ET in arterioles from female rats (Fig 1B). Comparing between sexes, overall ET-induced vasoconstriction was greater in arterioles from old female rats compared to arterioles from old male rats (Table 3, Fig 1A vs. Fig. 1B, significant $\geq 3e^{-11}$ [M]), but there were no significant differences in overall ET response curves, EC_{50} or maximal constriction between young males and females (Figure 1, Table 3). Following denudation, age-related differences in overall ET-induced curves were abolished in coronary arterioles from male rats (Fig 2A), but remained in coronary arterioles from female rats (Fig 2B, significant $3e^{-11} – 1e^{-8}$ [M]). Additionally, vasoconstriction responses to ET were greater in denuded arterioles from young and old male rats than those in arterioles from young and old female rats (Fig 2A vs. 2B, $P < 0.0001$ and $P < 0.001$, respectively). Removal of the endothelium impaired vasoconstriction to ET in arterioles from young ($P = 0.007$) and old ($P = 0.03$) female rats. In contrast, denuded arterioles from

young (P = 0.003) and old (P = 0.0001) male rats exhibited greater overall vasoconstriction to ET than intact arterioles from young and old male rats, respectively.

ETa receptor signaling

There were no age-related differences in ET-induced vasoconstriction in intact arterioles after pretreatment with BQ788, an ETb receptor inhibitor, in either males or females (Fig 3A and 3B). Blockade of ETb signaling with BQ788 increased overall vasoconstriction to ET (Fig 3A vs. 1A) in intact arterioles from old male rats, but decreased overall vasoconstriction to ET (Fig 3B vs. 1B) in intact arterioles from old female rats. In contrast, treatment with BQ788 did not alter responses of intact arterioles from young male and young female rats. In the presence of BQ788, intact arterioles from young female rats showed greater overall vasoconstriction to ET as compared to intact arterioles from young male rats (Figure 3A vs. 3B, significant $3e^{-11}$ – $3e^{-9}$ [M]). Following denudation, overall ET-induced vasoconstriction in the presence of BQ788 remained greater in arterioles from young males compared to those from old males (Fig 4 A, significant ≥ $3e^{-9}$ [M]). In the presence of BQ788, overall vasoconstriction to ET in denuded coronary arterioles from old females was greater than in denuded arterioles from young females (Fig 4 B). In denuded arterioles from young male and young female rats, and old female rats, treatment with BQ788 did not alter ET-induced vasoconstrictor responses; however, in denuded arterioles from old male rats, BQ788 treatment reduced overall ET-induced vasoconstriction (Fig 4A vs 2A).

ETb receptor signaling

The ETa receptor blocker, BQ123, reduced ET-induced vasoconstriction in intact arterioles from all groups of rats. BQ123 eliminated age-related differences in ET-induced vasoconstriction in intact arterioles from both males and females (Fig 3). Similarly, in denuded arterioles from both males and females, age-related differences to ET-induced vasoconstriction were abolished by pretreatment with BQ123 (Fig 4). In the presence of BQ123, there were significant differences in EC_{50} between young males and females (significant ≥ $3e^{-9}$ [M]), and maximal constriction between young males and females and old males and females (Table 3, Fig 3A and 3B). In the presence of BQ123, denuded arterioles from both young and old male rats exhibited greater overall ET-induced vasoconstriction and greater maximal constriction as compared to denuded arterioles young and old female rats, respectively (Table 3, Fig.4A vs Fig. 4B, $P < 0.05$). In the presence of BQ123, denuded arterioles from males exhibited greater ET-induced vasoconstriction as compared to intact arterioles (Fig. 3A vs. 4A, $P < 0.05$). Overall responses to ET were similar in denuded and intact arterioles from females treated with BQ123 (Fig. 3B vs. 4B,); however, but EC_{50} was shifted to the left after denudation in young females (intact w/ BQ123: $3.2e^{-8}$ vs. denuded w/ BQ123: $8.2e^{-9}$ [M], Table 3) and maximal constriction was significantly greater in denuded arterioles from both young and old females (Table 3).

Arteriolar Distensibility

To determine whether structural changes contributed to age-induced changes in ET-mediated vasoconstriction, incremental distensibility curves were determined in coronary arterioles from young and old female rats. There were no differences in distensibility between arterioles from male and female rats, (Fig 5A vs Fig 5B). Age did not alter distensibility in arterioles from female or male rats (32).

ETa and ETb receptor proteins

ETa receptor protein was decreased in arterioles from old males compared to young males (Fig 6 A). Conversely, ETb receptor protein was increased in coronary arterioles from old

males compared to young males (Fig 6 B). There were no age-related differences in ETa or ETb receptor protein in coronary arterioles from females (Fig 6 C,D).

Similarly, immunofluorescence analysis indicated that ETa receptor expression was reduced in arterioles isolated from old males compared to young males (Fig 7A) and ETb receptor expression was increased with aging in males (Fig 7B). In addition, relative ETb protein expression in the endothelium was increased in coronary arterioles from old males as compared to young males (Fig 7C). There were no age-related differences in total ETa, total ETb, or endothelial ETb receptor protein levels observed in coronary arterioles from females (Fig 7 D, E, F).

DISCUSSION

The primary findings of the present study are as follows: 1) ET-induced vasoconstriction is differentially altered with age in coronary resistance arterioles from male and female rats; 2) in contrast to other investigations on conduit arteries from rats and humans, vasoconstriction in response to ET decreased in coronary resistance arterioles from aged males, whereas constrictor responses to ET increased with age in arterioles from females (Fig 1); 3) in arterioles from males, the age-related decrement in ET-induced vasoconstriction is accompanied by a decrease in the ETa receptor protein and an increase in ETb receptor protein (Fig 6, 7). In contrast, coronary arterioles from aged females exhibited increased responsiveness to ET independent of changes in either the ETa or ETb receptor protein.

Arterial Vessel Heterogeneity in ET-mediated Constriction

Coronary blood flow is regulated by the release of a combination of relaxing and constricting factors released by myocytes in response to changes in metabolism. In particular, coronary blood flow is regulated mainly by vasoactive responses of coronary arterioles with diameter less than 150 μm (22). ET functions as a modulator of basal vascular tone in the heart and regulates coronary blood flow during periods of basal metabolism (25). Long-lasting vasoconstriction caused by ET can redirect coronary blood flow in order to promote subendocardial perfusion and has been proposed to prevent excessive back flow from the coronary circulation (25). Considerable heterogeneity exists in age-related adaptations of vascular responsiveness to ET, and depends on which specific vascular bed is being investigated. For example, a decrease (10), no change (18), or increase (11) in the vasoconstriction to ET with advancing age has been shown in mesenteric arteries, aorta, and gastrocnemius arterioles in rats, respectively. Age-induced enhancement of ET-mediated vasoconstriction has been reported in the human male forearm (42). The current findings confirm our previous report of decreased vasoconstriction to ET in coronary arterioles from aged male rats (32). In contrast, in large coronary arteries from aged male rats, ET-mediated vasoconstriction is increased compared to arteries from young rats (15, 37). ET administration, in the presence of inhibition of endothelial NO synthase and prostacyclin release, resulted in greater reduction of coronary flow in hearts from old male rats (13), suggesting that age-related impairment of ET-mediated constriction is specific to the resistance vasculature in male hearts. Thus, despite evidence for global reductions in responsiveness of the coronary circulation to ET with age, the currently reported decrement in resistance vasculature responsiveness to ET with age may contribute to altered blood flow distribution in the hearts of senescent male rats (14, 39).

In contrast to the male literature, there are extremely few studies addressing the age-related response to ET in any vascular bed in females. To our knowledge, this is the first study to investigate the effects of age on ET-mediated vasoconstriction in the coronary resistance arterioles of female rats. Age-related increases in vasoconstrictor responses to 5-HT in mesenteric arteries (35) and KCl and norepinephrine (NE) in aortas (2) from females have

been reported, along with augmented plasma ET-1 levels in senescent females (2, 4, 43). These data suggest that females exhibit an enhanced vasoconstrictor profile along with an increase in ET levels as age progresses, potentially contributing to the heightened ET-induced vasoconstriction in coronary arterioles shown in the present study. In contrast, Stauffer et al. (34) reported greater ET_A-mediated vasoconstrictor tone in the forearm of older men as compared to age-matched women. In large epicardial arteries, maximal constriction to ET decreases in senescent females (2). These divergent results are likely due to differential functional responses of conduit and resistant arteries, especially in the coronary circulation.

ETa Contribution in Males

In our previous (32) and present study, passive pressure responses from males and females were unaltered with advancing age, suggesting that age-dependent responses to ET were receptor-dependent. ET stimulates both dilation and constriction of arterioles (32) and mediates its effects via two distinct G-coupled protein receptor subtypes. ETa receptors are the major receptor subtype involved in the vasoconstrictor response to ET and are localized on the vascular smooth muscle cell (23). ETb receptors located on the endothelial cell mediate vasodilation through the release of relaxing factors, but can also exert vasoconstriction through ETb receptors located on the vascular smooth muscle (23). Thus, the net effect of ET depends on the relative distribution and density of each specific subtype of receptor. Reports in the literature suggest that age-related differences in the ET system of the human coronary circulation occur predominantly as a result of changes in ETa receptor (34, 42). Our results in coronary arterioles from male rats are consistent with these reports: 1) age-related differences in the response to ET remained after denudation and after BQ788 pretreatment (Fig 4 A), 2) loss of ETa-mediated vasoconstriction was accompanied by a reduction of ETa receptor protein (Fig 6 A and 7A), and 3) treatment with BQ123 eliminated age-related differences in response to ET in denuded arterioles from male rats. Thus, our data indicate that a decrease in ETa receptor protein in vascular smooth muscle contributes to the age-related decrement in ET-mediated constriction in male rats.

ETb Contribution in Males

The ETb receptor on the endothelium is distinctive from the ET receptors on the smooth muscle due to the signaled release of relaxing factors, in particular NO (38). The endothelial ETb receptor has also been shown to modulate the vasoconstrictor effects of ET bound to ETa or ETb receptors on the VSM (38). In arterioles from male rats, ETb protein content increased with old age (Fig. 6 B and 7B). The increased ETb protein was not localized to vascular smooth muscle (Fig 7H) in arterioles from old males. Additionally, ETb blockade reduced responsiveness to ET only in intact arterioles from old males, suggesting that the age-related decrease in the vasoconstrictor response to ET is related to increased expression of endothelial ETb receptors. Seo and Luscher (30) found that stimulation of ETb receptors on the endothelium of renal arteries release more NO with advancing age in male rats. The loss of age-related differences in intact coronary arterioles of male rats after treatment with BQ788 also suggests that the endothelial ETb receptor exerts a greater vasodilatory stimulus in coronary arterioles from aged males compared to those from young males (Fig 1 A and Fig 3 A). Additionally, our finding that ET-mediated constriction was similar in denuded arterioles from young and old rats treated with BQ123 suggests that alterations in ETb receptor expression occurs predominantly in the endothelium.

Endothelium-Independent Signaling in Females

ETa and ETb receptor protein levels were unchanged in coronary arterioles from females with advancing age, suggesting that the age-related increase in ET-mediated constriction occurs as a result of alterations in post-receptor signaling mechanisms of the vascular

smooth muscle. Since age-related differences in ET-mediated vasoconstriction remained after denudation and pretreatment of denuded vessels with BQ788 in females (Fig 4 B), endothelium-independent mechanisms downstream of ETa, such as Ca^{2+} handling likely contribute to age-related differences. Large arteries from female rats exhibit lower b-myosin and higher levels of sarcoplasmic reticulum Ca^{2+}-ATPase expression compared to those from male rats (41). Lopes et al. (21) found that aged female rats exhibited an increase in colon smooth muscle contraction and suggested this might be due to increases in Ca^{2+} stores. In mesenteric resistance arteries from aged male rats, impaired Ca^{2+}-induced Ca^{2+}-release resulted in greater stored Ca^{2+} and heightened contractile responses to phenylephrine (29); however, the effects of age on intracellular Ca^{2+} handling has not been investigated in arteries from females. In aggregate, these studies suggest that alterations in Ca^{2+} handling that occur with age could lead to heightened vasoconstriction to ET as seen in coronary arterioles from aged female rats.

In addition to Ca^{2+} alterations, NO feedback on ET signaling and hormone levels could also explain the divergent response to ET observed in aged males and females. In 1990, Boulanger and Luscher (3) first demonstrated that NO inhibits formation of ET in the aorta and suggested that an impaired release of NO from the vasculature may lead to an exaggerated ET production. In addition, NO has been shown to actively displace ET from its receptor binding site on VSM (12) and can directly bind to thiol groups on the ET receptor causing reduction of the thiol groups and the production of active s-nitrosothiols, a stable NO metabolite that can contribute to vasodilation (8, 33). Our laboratory has shown a decrement in NO-mediated vasodilation in coronary arterioles from aged female rats, related to decreasing circulating estrogen levels (16), whereas coronary arterioles from male rats exhibit an increase in eNOS mRNA with advancing age (32). These sex-specific NO regulatory mechanisms could contribute to the directionally opposite changes in ET-mediated constriction of coronary arterioles from aged male and female rats.

Endothelium-Dependent Signaling in Females

In addition to the potential VSM effects, modulation of ET-induced constriction by endothelial factors clearly differs in arterioles from males compared to arterioles from females. A significant endothelial-derived constrictor influence appears to contribute to the ET-mediated vasoconstriction in coronary arterioles from both young and old females, as exhibited by a decrease in ET-mediated constriction induced by denudation (Fig. 1B vs. Fig. 2B). In intact arterioles from old females, blockade of ETb with BQ788 significantly reduced ET-induced constriction (Fig 3 B vs. 1 B, $P < 0.05$), suggesting that the endothelium-dependent constriction is mediated by the ETb receptor. This finding is consistent with our previous report of an age-induced increase in endothelium-dependent constriction in coronary arterioles from female rats (19). Denudation of arterioles from young female rats also decreased constriction to ET; however, if ET induces constriction through release of an endothelium-derived constricting factor in arterioles from young rats, this constriction does not appear to be mediated through ETb, since BQ788 did not alter constrictor responses to ET in these vessels. Further studies are needed to elucidate the endothelial signaling pathway that contributes to ET-induced constriction in arterioles from females. Age may alter vasoconstriction to ET in coronary arterioles from females through combined changes in endothelial constrictor and dilator influences (19).

Conclusion

ET has been shown to be increased in chronic diseases such as congestive heart failure (24), myocardial infarction (28), and hypertension (6). Because the risk for these diseases increases with advancing age, and because these diseases are accompanied by coronary vascular dysfunction, it is important to determine how advancing age alters vasoreactive

responses to ET of the coronary resistance vasculature. This study provides insight into sex-specific changes in mechanisms by which age alters reactivity to ET in coronary arterioles. These differences may provide therapeutic targets for management of cardiovascular disease in the elderly.

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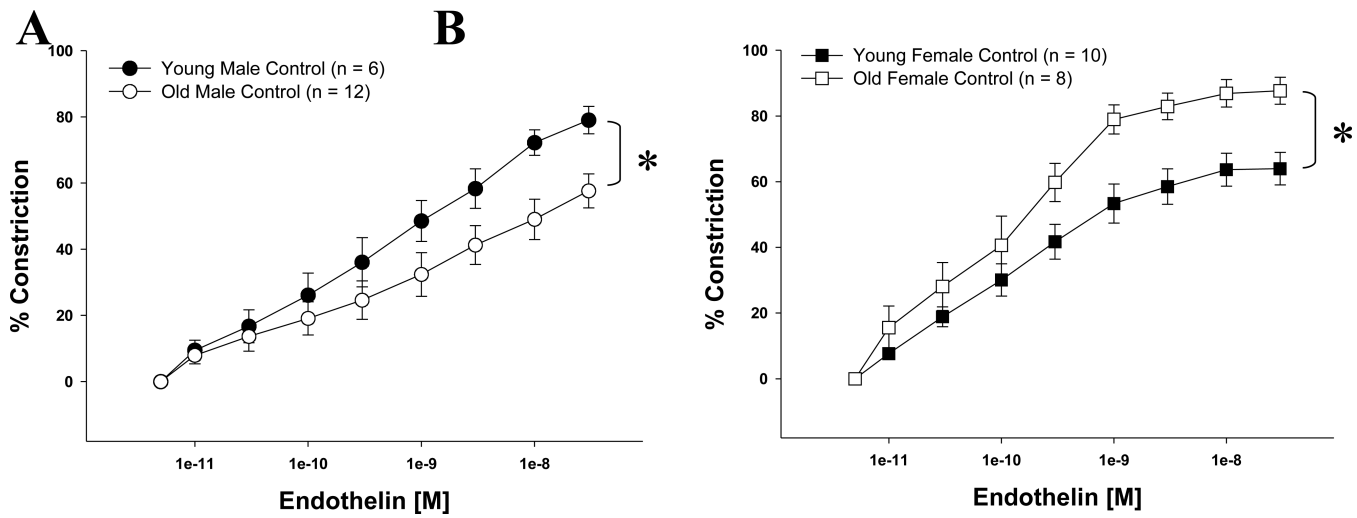

Figure 1.

Vasoconstriction to ET in coronary arterioles from young and old male (A) and female (B) rats. ET-induced vasoconstriction was decreased with advancing age in males (A), but increased in aged females (B). Values are means ± SE. * Indicates significant age-related difference vs. young control, ($P \leq 0.05$).

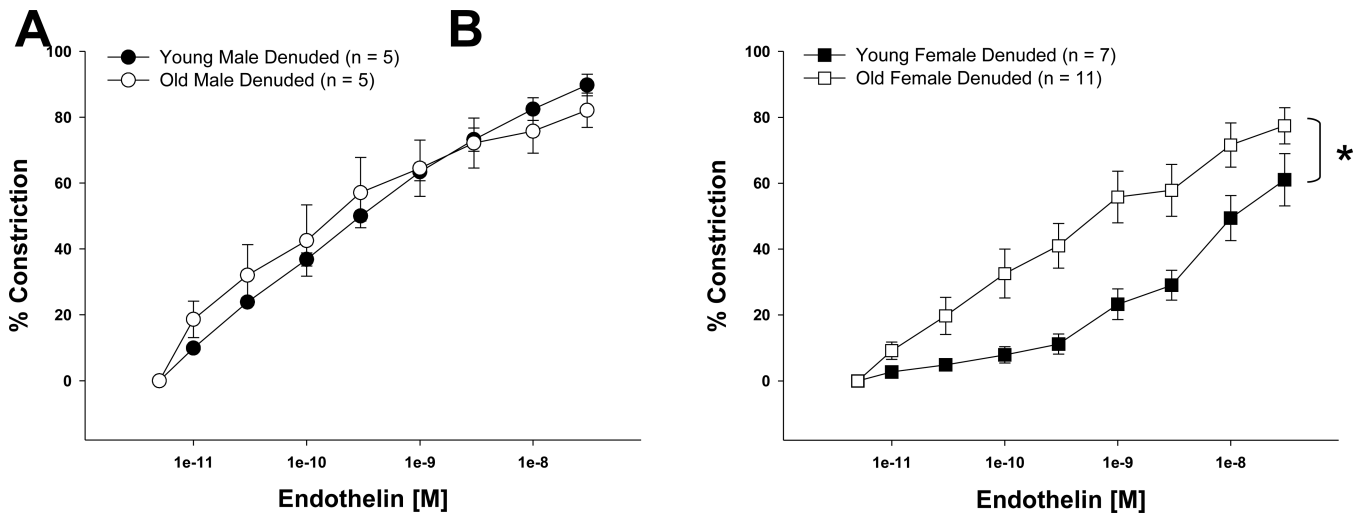

Figure 2.

ET-induced vasoconstriction in denuded coronary arterioles from young and old males (A) and females (B). Age-related differences in vasoconstriction to ET was abolished after denudation in males (A), but remained in females (B). Values are means ± SE. * Indicates significant age-related difference vs. young control, ($P \leq 0.05$).

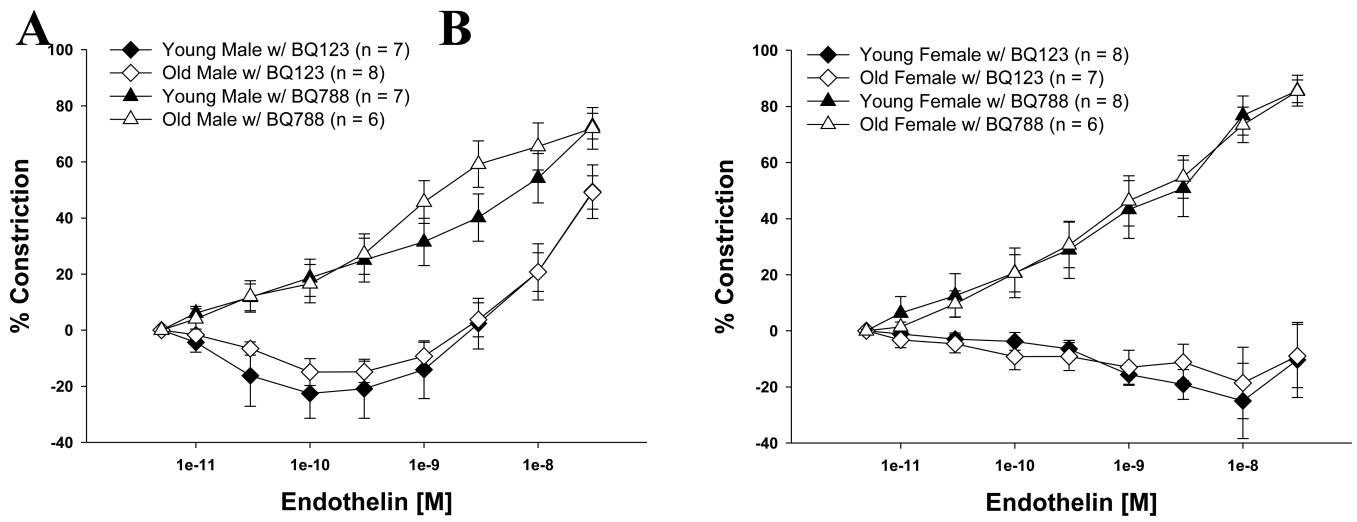

Figure 3.

Vasoconstriction to ET after pretreatment with BQ123, an ETa receptor inhibitor, or BQ788, an ETb receptor inhibitor, in coronary arterioles from young and old males (A) and females (B). No age-related differences were found in ET-induced vasoconstriction in coronary arterioles after inhibition of ETa or ETb receptors in either sex. Values are means ± SE.

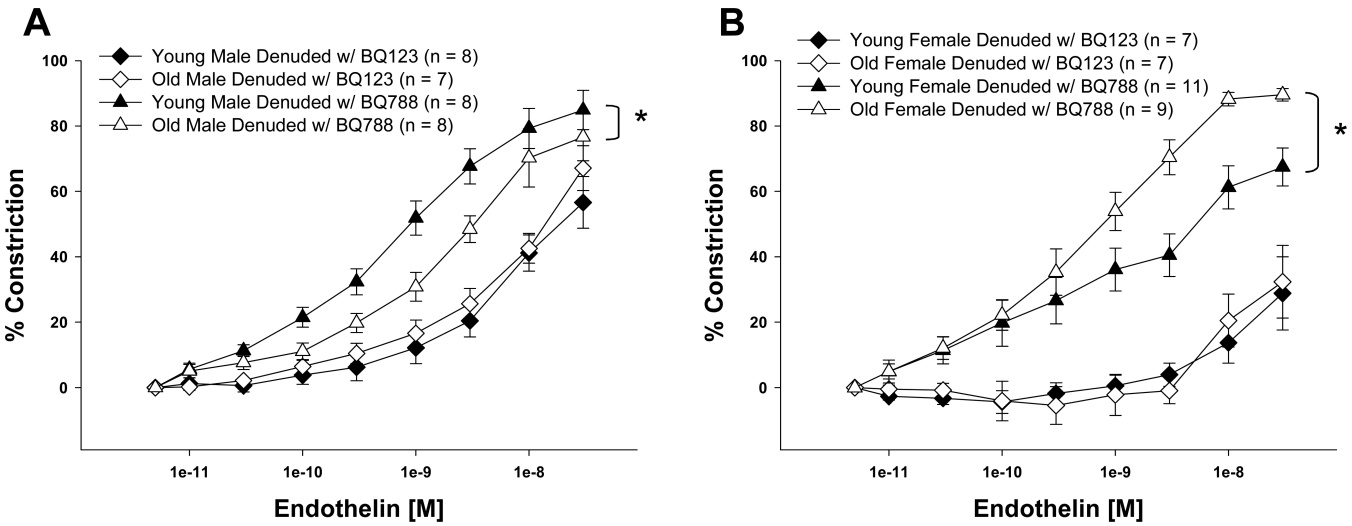

Figure 4.

ET-induced vasoconstriction in denuded coronary arterioles after inhibition of ETa (BQ123) or ETb (BQ788) receptors in young and old males (A) and females (B). No age-related differences were found in ETb-mediated vasoconstriction in denuded coronary arterioles from either sex. Age-related differences in the vasoconstriction to ET persisted in the presence of ETb blockade in arterioles from males (A) and females (B). Values are means ± SE. * Indicates significant age-related difference vs. young control, ($P \leq 0.05$).

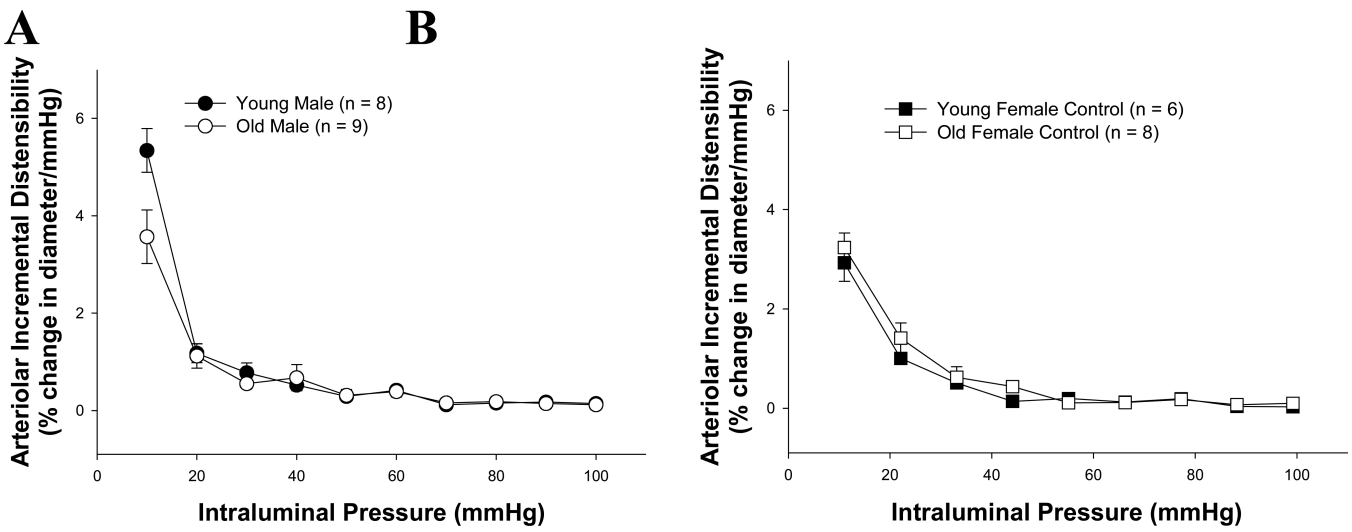

Figure 5.

Incremental distensibility in coronary arterioles from young and old, male and female rats. No age-associated differences were detected. Values are means ± SE.

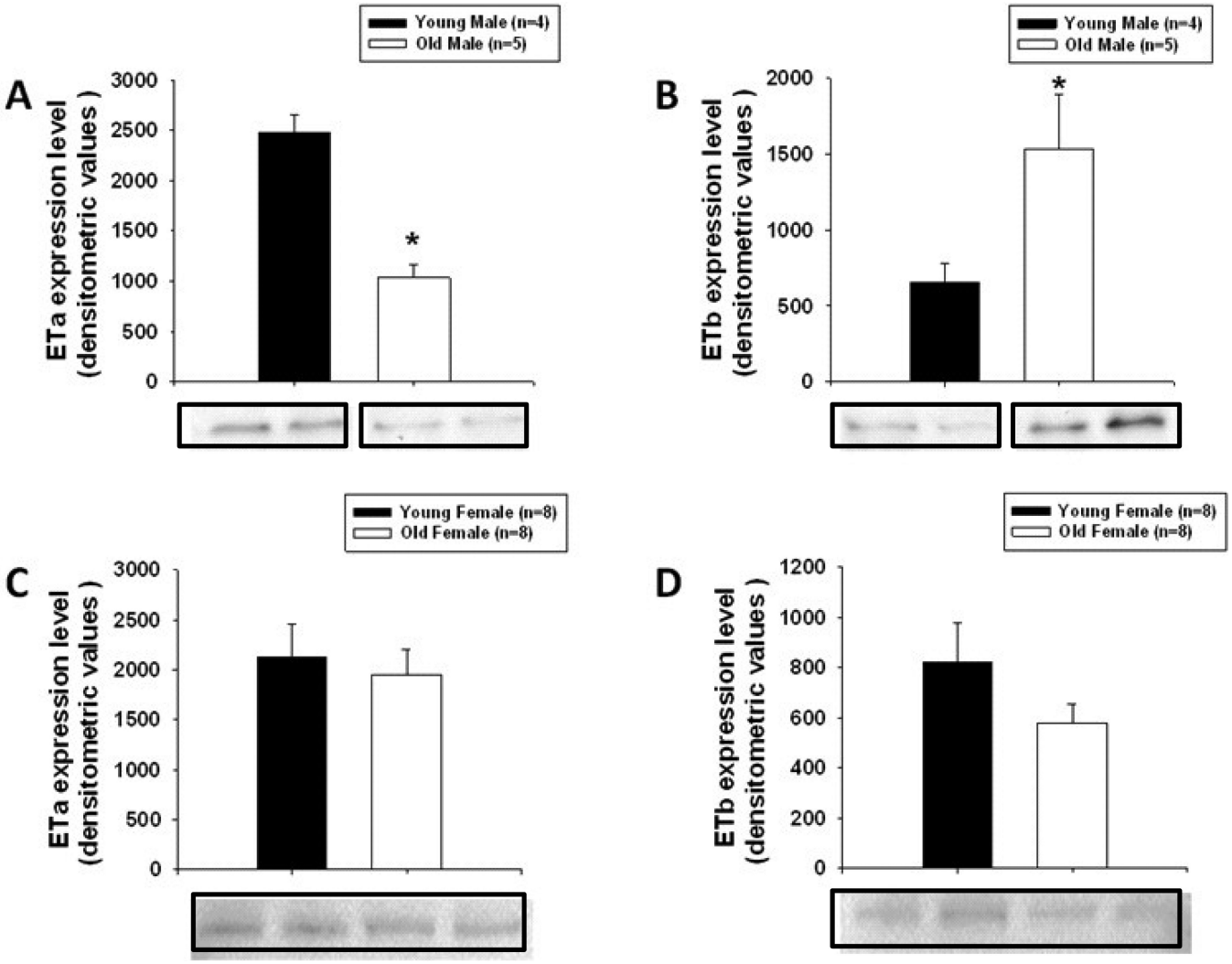

Figure 6.

Advancing age in males caused a decrease in ETa protein expression (A), but an increase in ETb protein expression (B) in coronary arterioles (Young male, n = 4; Old male, n = 5). No age-related differences were found in ETa or ETb protein expression in coronary arterioles from females (C,D) (n = 8 per group). Representative blots of either ETa or ETb receptor protein (~45 kd) are shown below graphs. Equal loading was confirmed by Sypro Ruby staining for total protein. Values are means ± SE. * Indicates significant age-related difference vs. young control, ($P \leq 0.05$).

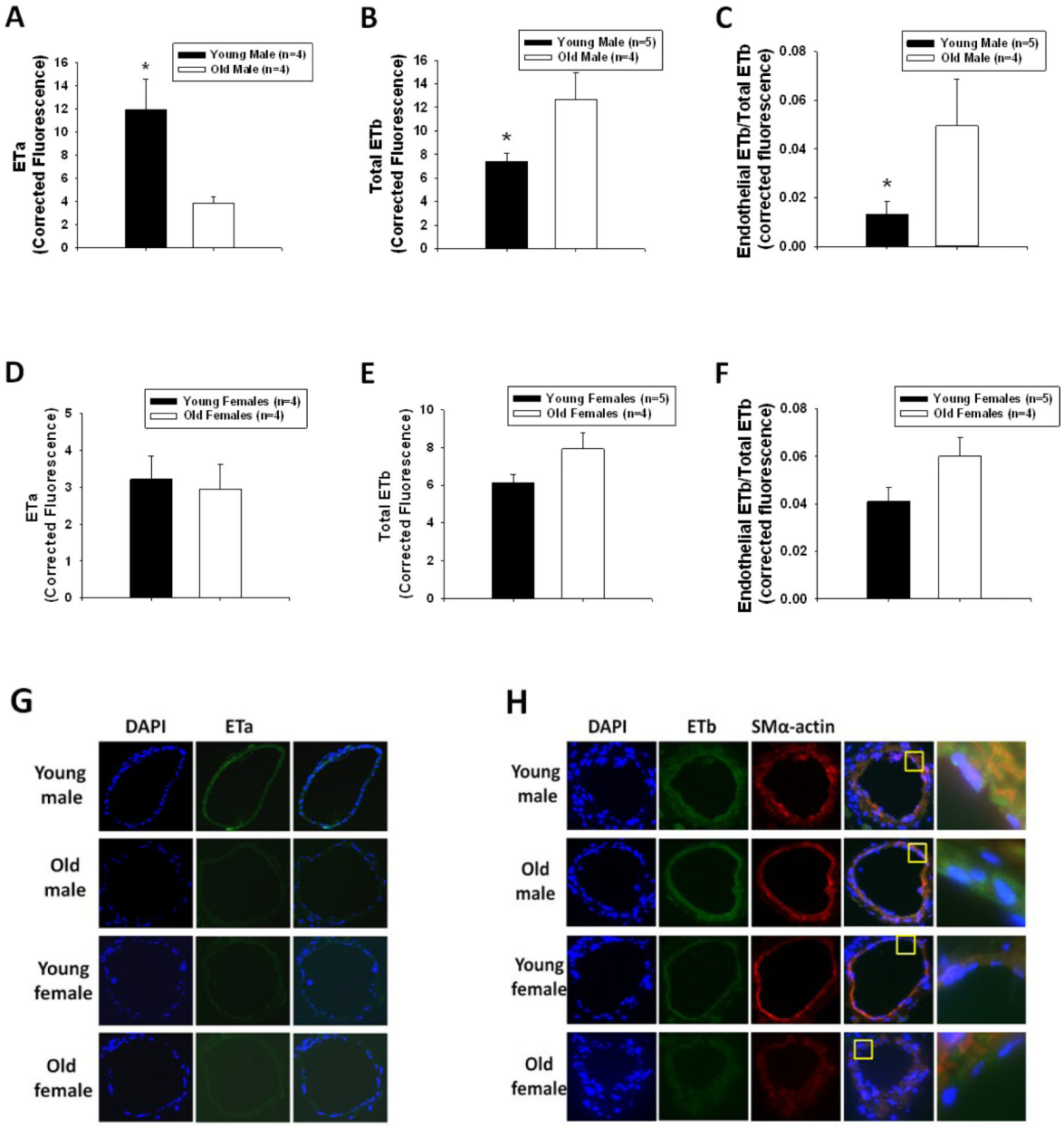

Figure 7.

Immunohistochemistry analysis for ETa and ETb expressions in coronary arterioles from young and old males (A, B, C) and females (D, E, F). ETa expression level was reduced with advancing age in males (A), but not in females (D). Total ETb expression was increased by aging in males (B), but not in females (E). Age differences in relative expression of ETb in endothelial cells versus total ETb expression were found in males (C), but not in females (F). Representative fluorescence staining of ETa (G) and ETb (H) are shown. Values are means ± SE. * Indicates significant age-related difference vs. old control, ($P \leq 0.05$).

Table 1

Animal characteristics of young and old male and female rats.

	Young Male	Old Male	Young Female	Old Female
Body Weight (g)	330±6 (54)	394±5 * (48)	205±2 † (35)	298±3 *† (34)
Heart Weight (mg)	1007±18	1385±23 *	582±9 †	774±12 *†
HW/BW (mg/g)	3.06±0.04	3.54±0.08 *	2.83±0.03 †	2.62±0.05 *†

Values are means ± SE.

* Indicates significant age-effect

† Indicates significant age-matched sex difference, ($P \leq 0.05$).

Table 2

Tone development in coronary arterioles of young and old male and female rats.

	Young Male	Old Male	Young Female	Old Female
Maximal Diameter (μm) (n)	128±4 (44)	133±4 (50)	124±4 (51)	127±4 (51)
Spontaneous Tone (%)				
Endothelium Intact	18±7 (6)	10±5 (12)	19±5 (10)	9±6 (8)
Denuded	24±7 (5)	29±7‡ (5)	24±6 (7)	40±5*‡ (11)
Post BQ123	36±4 (15)	24±4* (16)	36±4†‡ (15)	36±4†‡ (14)
Post BQ788	26±4 (18)	28±4‡ (17)	26±4 (19)	31±4‡ (18)

Values are means ± SE.

*Indicates significant age-effect

†Indicates significant age-matched sex difference

‡Indicates significant treatment effect compared to spontaneous tone in endothelium intact vessels ($P \leq 0.05$).

Table 3

EC_{50} and maximal constriction to ET in coronary arterioles of young and old male and female rats.

	Young Male		Old Male		Young Female		Old Female	
	EC_{50} ([M])	**Maximal Constriction (%)**	**EC_{50} ([M])**	**Maximal Constriction (%)**	**EC_{50} ([M])**	**Maximal Constriction (%)**	**EC_{50} ([M])**	**Maximal Constriction (%)**
Endothelium Intact	1.1±0.6(e^{-9})	79.0±4.1	2.5±1.2(e^{-9})	57.6±5.1*	1.7±0.3(e^{-10})	64.2±4.9	1.6±0.5(e^{-10})	88.4±4.1*†
with BQ123	1.2±0.2(e^{-8})	49.4±9.6	1.1±0.2(e^{-8})	49.1±6.0	3.2±1.5(e^{-8})†‡	-10.4±13.4†‡	1.9±1.1(e^{-8}) *‡	-12.6±13.9†‡
with BQ788	4.1±2.3(e^{-9})	72.2±4.5	7.4±2.3(e^{-10})	71.9±7.4	2.2±0.9(e^{-9})	85.6±5.5	1.7±0.7(e^{-9})	85.3±4.0
Denuded	2.2±0.3(e^{-10})	89.8±3.3	2.0±1.1(e^{-10})	82.1±5.2	3.7±1.4(e^{-9})	61.0±7.9	1.7±1.0(e^{-9})	77.4±5.5
with BQ123	4.9±1.1(e^{-9})	56.6±7.9†	6.2±1.5(e^{-9})	67.1±6.9	8.2±2.4(e^{-9})$^{\#}$	27.8±11.7$^{\dagger\#}$	6.2±1.1(e^{-9})	31.3±11.8$^{\dagger\S\#}$
with BQ788	8.2±2.0(e^{-10})	84.9±6.0	1.9±0.3(e^{-9})	76.6±7.3	2.1±1.0(e^{-9})	67.5±5.5	8.3±2.4(e^{-10})	89.5±1.9*

Values are means ± SE.

*Indicates significant age-effect

†Indicates significant age-matched sex difference

‡Indicates significant treatment effect compared to age- and sex-matched endothelium-intact

§Indicates significant treatment effect compared to age- and sex-matched denuded

$^{\#}$Indicates significant denudation effect compared to age-, sex- and treatment-matched non-denuded ($P \leq 0.05$).