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Increased Endothelial Exocytosis and Generation of Endothelin-1 Contributes to Constriction of Aged Arteries

Aditya Goel, Baogen Su, Sheila Flavahan, Charles J. Lowenstein,
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Rationale: Circulating levels of endothelin (ET)-1 and endogenous ET_A-mediated constriction are increased in human aging. The mechanisms responsible are not known.

Objective: Investigate the storage, release, and activity of ET-1 system in arteries from young and aged Fischer-344 rats.

Methods and Results: After NO synthase inhibition (L-NAME), thrombin contracted aged arteries, which was inhibited by endothelial denudation, ET_A receptor antagonism (BQ123), and ECE inhibition (phosphoramidon, SM19712) or by inhibiting exocytosis (TAT-NSF, N-ethylmaleimide-sensitive factor inhibitor). Thrombin did not cause endothelium-dependent contraction of young arteries. In aged but not young arteries, thrombin rapidly increased ET-1 release, which was abolished by endothelium denudation or TAT-NSF. L-NAME did not affect ET-1 release. ET-1 immunofluorescent staining was punctate and distinct from von Willebrand factor (VWF). VWF and ET-1 immunofluorescent intensity was similar in young and aged quiescent arteries. Thrombin rapidly increased ET-1 staining and decreased VWF staining in aged but had no effect in young aortas. After L-NAME, thrombin decreased VWF staining in young aortas. NO donor DEA-NONOate (1 to 100 nmol/L) reversed thrombin-induced exocytosis in young (VWF) but not aged L-NAME-treated aortas (VWF, ET-1). Expression of preproET-1 mRNA and ECE-1 mRNA were increased in aged compared to young endothelium. BigET-1 levels and contraction to exogenous BigET-1 (but not ET-1) were also increased in aged compared to young arteries.

Conclusions: The stimulated exocytotic release of ET-1 is dramatically increased in aged endothelium. This reflects increased reactivity of exocytosis, increased expression and storage of ET-1 precursor peptides, and increased expression of ECE-1. Altered endothelial exocytosis of ET-1 and other mediators may contribute to cardiovascular pathology in aging. (*Circ Res.* 2010;107:242-251.)

Key Words: aorta ■ mesenteric arteries ■ Weibel-Palade bodies ■ thrombin ■ von Willebrand factor

Human aging is associated with an increase in baseline systemic vasoconstriction and increased circulating levels of endothelin (ET)-1.¹⁻⁴ Indeed, inhibition of ET_A receptors causes powerful vasodilation in aged but not young individuals^{5,6} and normalizes the reduced blood flow and increased vascular resistance in elderly subjects.⁵ Constriction to exogenous ET-1 appears diminished in the elderly suggesting that the increased activity of ET-1 reflects the increased levels of the mediator.⁶ Increased ET-1 production can cause oxidant stress, endothelial dysfunction, vascular inflammation and vascular remodeling, and likely contributes to age-related cardiovascular dysfunction.⁷⁻⁹ Indeed, ET-1 contributes to stiffening and calcification of central arteries,¹⁰⁻¹⁴ which is a prominent characteristic of the aging vascular system and is associated with increased risk for stroke, myocardial infarction, heart failure and mortality in the elderly.¹⁵

ET-1 is the major vascular ET isoform and is produced predominantly by the endothelium.^{9,16} In cultured endothelial cells, ET-1 is released continuously through a constitutive pathway that is regulated principally through gene transcription and translation.^{9,16} However, stimulation of cultured endothelial cells also causes a rapid increase in ET-1, which suggests storage of ET-1 or its precursors and regulated exocytosis.¹⁷ Indeed, ET-1 has been localized to Weibel-Palade bodies (WPBs), the most recognizable endothelial storage granule, although the primary localization in cultured and native endothelium may be in distinct storage granules.¹⁷⁻²² This pattern of partial localization in WPBs is similar to other endothelial mediators, including tissue plasminogen activator.²³⁻²⁶ ET-1 is formed from precursor peptides by proteolytic processing. PreproET-1 mRNA is translated, stripped of its signal sequence and further cleaved by a furin-like peptidase to generate BigET-1.^{9,16} Further process-

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From the Department of Anesthesiology (A.G., B.S., S.F., D.E.B., N.A.F.), Johns Hopkins University, Baltimore, Md; and Department of Medicine (C.J.L.), University of Rochester, Rochester, NY 14642.

Correspondence to Nick Flavahan, Department of Anesthesiology, Johns Hopkins University, Ross-R1159, 720 Rutland Ave, Baltimore, MD 21205. E-mail nflavah1@jhmi.edu

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ing to biologically active ETs is achieved predominantly by endothelin-converting enzyme (ECE).^{9,16} Despite the evidence for increased activity of ET-1 in the aging vascular system, no previous studies have directly analyzed the exocytotic release of ET-1 from aging endothelial cells. Therefore, the present experiments were performed to investigate the ET-1 signaling system in aging native arterial endothelium.

Methods

Young (3 to 4 months) and aged (18 to 20 months) Fischer-344 rats were used in the study, which was approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University and complied with the NIH Guide for the Care and Use of Laboratory Animals. Thoracic aorta and mesenteric arteries were rapidly and carefully removed from anesthetized rats into cold physiological buffer solution. Tissues were processed for measurement of vasomotor activity or endothelial mediator expression and release by ELISA, laser scanning microscopy and real-time PCR.

An expanded Methods section is available in the Online Data Supplement at <http://circres.ahajournals.org>.

Results

Endothelium, ET-1, and Thrombin-Induced Contractions

Aortas

In aged aortas, thrombin (1 U/mL) caused a transient dilation that peaked at $37.7 \pm 5.9\%$ ($n=6$) of the contraction to phenylephrine (Figure 1). After NO synthase (NOS) inhibition (L-NAME, 100 $\mu\text{mol/L}$), the relaxation to thrombin (1 U/mL) was abolished and converted to rapid contraction (Figure 1). In endothelium-denuded arteries (with or without L-NAME), thrombin (1 U/mL) caused only a small contractile response, suggesting that the transient dilation and the major component of the contraction to thrombin were mediated by the endothelium (Figure 1 and Online Figure I). Although the selective ET_A receptor antagonist BQ123 (1 $\mu\text{mol/L}$) did not affect the transient dilation to thrombin, it markedly reduced the contraction to thrombin in L-NAME-treated aged aortas (Figure 1). The inhibitory effect of BQ123 in L-NAME-treated aged aortas was similar to the inhibitory effect of endothelial denudation (Figure 1). The ET_B receptor antagonist BQ788 (1 $\mu\text{mol/L}$, alone or in presence of BQ123) did not affect contractions to thrombin in aged L-NAME-treated aortas (Online Figure II).

In young aortas, thrombin (1 U/mL) caused relaxation that was greater in magnitude and was more sustained than in aged blood vessels ($69.9 \pm 6.0\%$ of the contraction to phenylephrine, $n=5$, $P < 0.01$ compared to aged arteries) (Figure 1). As in aged aortas, NOS inhibition (L-NAME, 100 $\mu\text{mol/L}$) abolished the relaxant response to thrombin and converted it to contraction (Figure 1). However, the contractile response to thrombin in young aortas was not affected by ET receptor antagonism (BQ123 [1 $\mu\text{mol/L}$] and/or BQ788 [1 $\mu\text{mol/L}$]) or by endothelial denudation (Figure 1 and Online Figure II).

Exogenous ET-1 (in the presence of L-NAME, 100 $\mu\text{mol/L}$), caused similar concentration-dependent contractions of young and aged aortas that were virtually abolished by the ET_A receptor antagonist BQ123 (1 $\mu\text{mol/L}$) (Online Figure III).

Non-standard Abbreviations and Acronyms

BQ123	cyclo(D-Asp-Pro-D-Val-Leu-D-Trp)
BQ788	<i>N</i> -[(cis-2,6-dimethyl-1-piperidiny)carbonyl]-4-methyl-L-leucyl-1-(methoxycarbonyl)-D-tryptophyl-D-norleucine sodium salt
DEA-NONOate	diethylamine NONOate
ECE	endothelin-converting enzyme
ET	endothelin
L-NAME	<i>N</i> ^o -nitro-L-arginine methyl ester hydrochloride
LSM	laser scanning microscope
NOS	nitric oxide synthase
SM19712	4-chloro- <i>N</i> -[[[4-cyano-3-methyl-1-phenyl-1 <i>H</i> -pyrazol-5-yl]amino]carbonyl]benzenesulfonamide sodium
TAT-NSF	human immunodeficiency virus transactivator of transcription (TAT) protein transduction domain fused to a <i>N</i> -ethylmaleimide-sensitive factor (NSF) homohexamization domain
TAT-CON	human immunodeficiency virus transactivator of transcription (TAT) protein transduction domain fused to scrambled amino acid sequence of <i>N</i> -ethylmaleimide-sensitive factor (NSF) homohexamization domain
ULVWF	ultralarge von Willebrand factor
VWF	von Willebrand factor
WPB	Weibel-Palade body

Mesenteric Arteries

In control quiescent arteries, thrombin (1 U/mL) did not affect the baseline diameter of young or aged mesenteric arteries. However, in the presence of L-NAME (100 $\mu\text{mol/L}$), thrombin (1 U/mL) caused constriction that was significantly increased in aged compared to young arteries (Online Figure IV).

In aged L-NAME-treated arteries, thrombin-induced constriction was markedly reduced by endothelial denudation or by antagonism of ET_A and ET_B receptors (BQ123 [1 $\mu\text{mol/L}$], BQ788 [1 $\mu\text{mol/L}$]) (Online Figure IV). Constriction to phenylephrine was not significantly affected by these interventions (Online Figure IV).

The small constriction to thrombin in young L-NAME-treated arteries was not affected by endothelial denudation or by antagonism of ET_A and ET_B receptors (BQ123 [1 $\mu\text{mol/L}$], BQ788 [1 $\mu\text{mol/L}$]) (Online Figure IV).

Exogenous ET-1 caused similar concentration-dependent constrictions of young and aged L-NAME-treated mesenteric arteries that were virtually abolished by antagonism of ET_A and ET_B receptors (BQ123 [1 $\mu\text{mol/L}$], BQ788 [1 $\mu\text{mol/L}$]) (Online Figure V).

Endothelial Exocytosis and Thrombin-Induced Contractions

TAT-NSF is a cell-permeable inhibitor of endothelial exocytosis that acts by blocking the activity of NSF (*N*-ethylmaleimide-sensitive factor), a critical component of the exocytotic machinery.^{27,28} In aged rat aortas treated with L-NAME (100 $\mu\text{mol/L}$), the endothelium-dependent contrac-

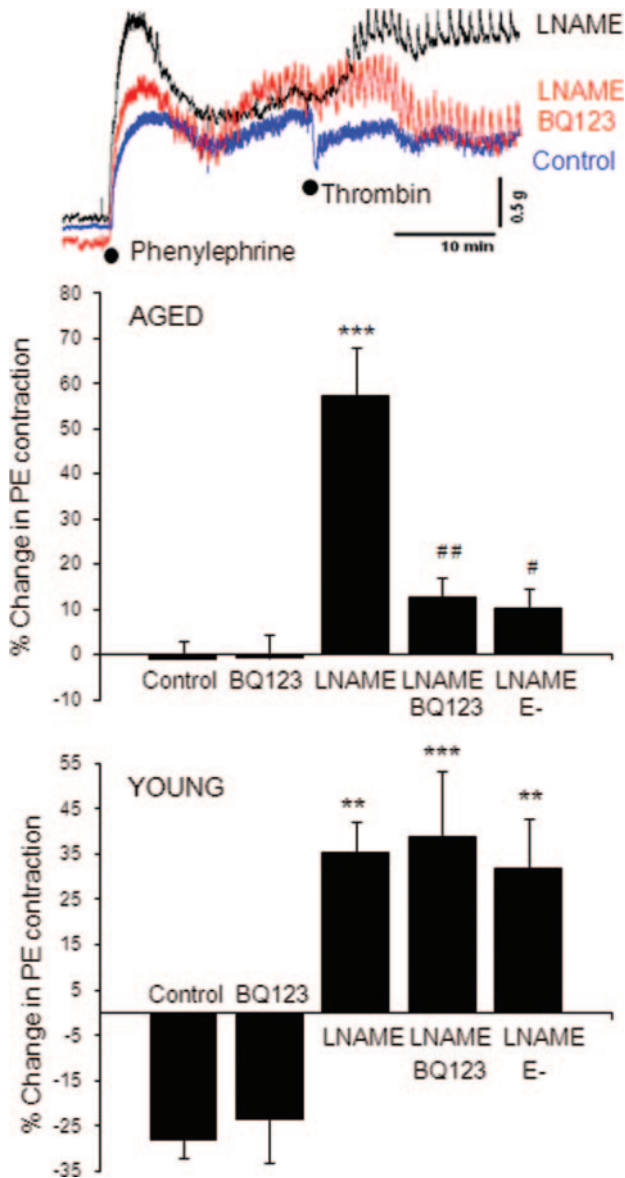


Figure 1. Thrombin-induced vasomotor responses in aged and young rat aortas. Responses to thrombin (1 U/mL) were assessed in aortic rings contracted with phenylephrine in the absence or presence of the ET_A antagonist BQ123 (1 μmol/L) and/or the NOS inhibitor L-NAME (100 μmol/L) and in endothelium-denuded rings (E-). **Upper trace** is a representative experiment in aged aortas, whereas the **lower graphs** present combined data for aged (**top**) or young aortas (**bottom**). Responses to thrombin were determined after 5 minutes, expressed as a percentage change in the contraction to phenylephrine and presented as means±SEM for n=3 to 6. *Significant difference from control (**P<0.01; ***P<0.001); #significant difference from L-NAME-treated aortas (#P<0.05; ##P<0.01).

tion to thrombin (1 U/mL) was significantly reduced by TAT-NSF (1 μmol/L) but not by a control scrambled peptide (TAT-CON, 1 μmol/L) (Figure 2).

ET-1 Release and Aging Endothelium

In aged aortas, thrombin (1 U/mL, 5 minutes) caused a rapid 3.7-fold increase in ET-1 release that was prevented by endothelial denudation or by the exocytosis inhibitor TAT-NSF (1 μmol/L), but not affected by the control peptide

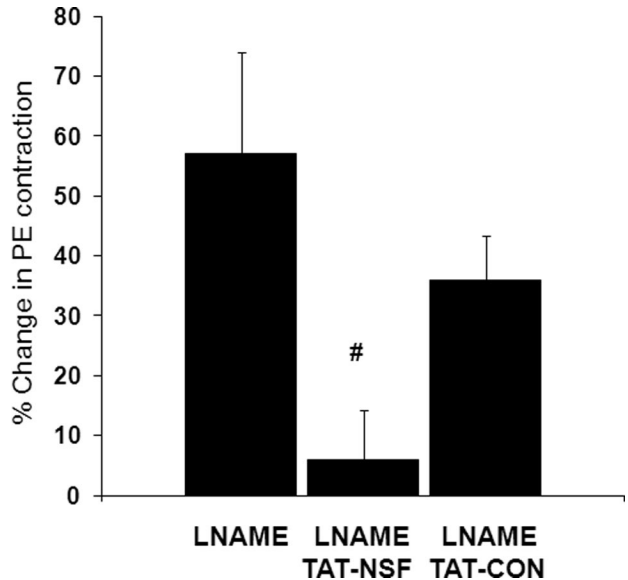


Figure 2. Effect of TAT-NSF, a cell-permeable inhibitor of endothelial exocytosis on endothelium-dependent contractions to thrombin (1 U/mL) in aged rat aorta. Thrombin-induced contraction was assessed in aortic rings, which were treated with the NOS inhibitor L-NAME (100 μmol/L) and contracted with phenylephrine, in the absence or presence of TAT-NSF or a control scrambled peptide TAT-CON (each at 1 μmol/L). Responses to thrombin were determined after 5 minutes, expressed as a percentage change in the contraction to phenylephrine and presented as means±SEM for n=5. #Significant difference from L-NAME-treated aortas (P<0.05).

(TAT-CON) (Figure 3). Inhibition of NOS (L-NAME, 100 μmol/L) did not significantly affect ET-1 release under basal conditions or in response to thrombin in aged aortas (Figure 3).

In young aortas, thrombin did not affect ET-1 release in the presence or absence of L-NAME (Figure 3). The basal release

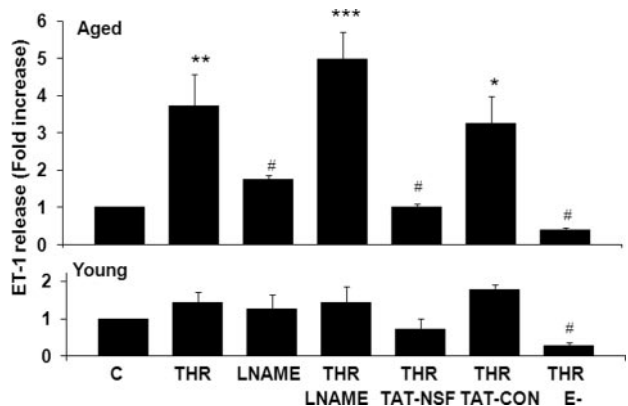


Figure 3. Analysis of the release of ET-1 from aged and young rat aortas. Release of ET-1 from aortic segments (**upper**: aged; **lower**: young) was determined under basal conditions and following stimulation with thrombin (1 U/mL, 5 minutes) in the absence or presence of the NOS inhibitor L-NAME (100 μmol/L), the inhibitor of exocytosis (TAT-NSF), or a control scrambled peptide (TAT-CON) (each at 1 μmol/L) and in endothelium-denuded rings (E-). ET-1 levels were determined by ELISA, expressed relative to basal unstimulated conditions, and presented as means±SEM for n=4 to 5. *Significant difference from control (C) (*P<0.05, **P<0.01, ***P<0.001); #significant difference from thrombin (THR)-treated aortas, P<0.05.

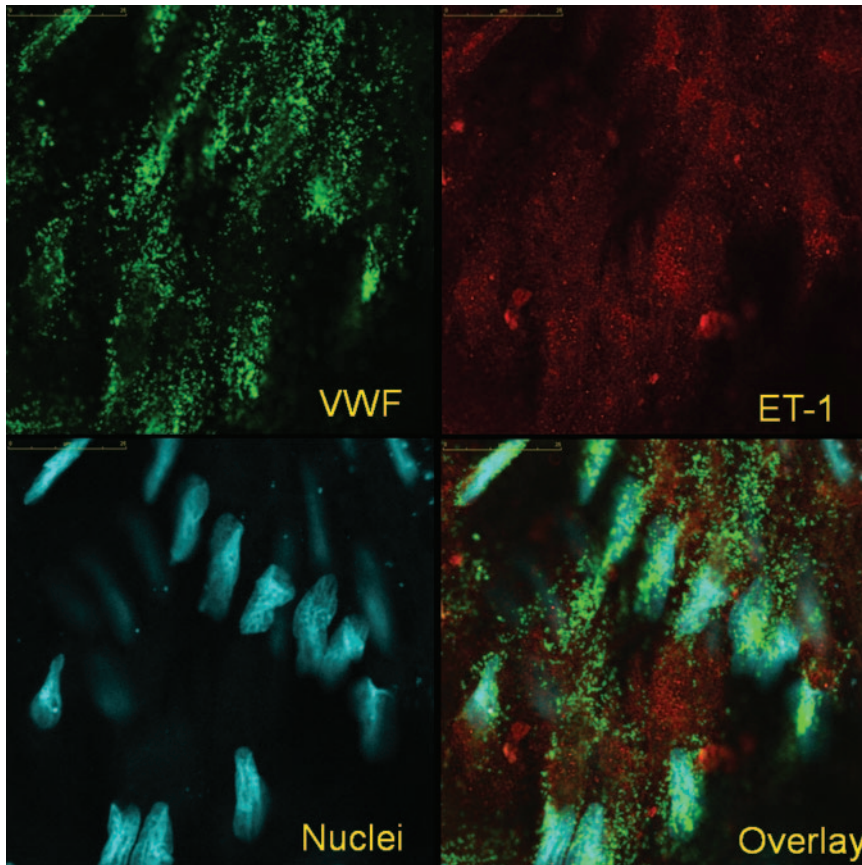


Figure 4. Localization of ET-1 and VWF in endothelial cells of aged rat aorta. Representative LSM images of endothelial cells lining the lumen of aged rat aorta, which was stained for VWF (green), ET-1 (red), and nuclei (blue). Individual images and the overlay are presented. **Bar:** 25 μm . To enhance visualization of ET-1, this image was acquired using image accumulation (2 images).

of ET-1 was not significantly different between young and aged aortas (111.4 ± 23.7 and 87.1 ± 20.1 pg/g dry tissue, respectively, $n=5$, $P=NS$).

Imaging ET-1 and WPBs in Native Endothelium

VWF immunofluorescent staining identified WPBs in the native endothelium of young and aged aortas (Figures 4 and 5; Online Figure VI). ET-1 immunofluorescence revealed punctate staining in the endothelium that was distinct from WPBs (Figure 4). Under control unstimulated conditions, there was no significant difference in the intensity of VWF or of ET-1 staining between young and aged endothelium (Figure 5). Thrombin (1 U/mL, 5 minutes) did not significantly affect ET-1 or VWF staining in young control aortas (Figure 5). However, thrombin (1 U/mL, 5 minutes) significantly increased ET-1 immunofluorescence and decreased VWF immunofluorescence associated with the endothelium of aged control aortas (Figure 5). Acute inhibition of ECE (SM19712, 200 $\mu\text{mol/L}$ 30 minutes before thrombin) prevented the thrombin-induced increase in ET-1 immunofluorescence but not the thrombin-induced decrease in VWF immunofluorescence in aged aortas (Figure 6).

Although thrombin had no significant effect on WPBs in young control aortas, in the presence of L-NAME (100 $\mu\text{mol/L}$) thrombin caused a dramatic mobilization of these endothelial storage granules, evidenced by the presence of VWF immunofluorescent aggregates and strands (Online Figure VII) and by a significant decrease in intensity of VWF fluorescence (Figure 7). In aged arteries, the effect of throm-

bin was not influenced by the presence of L-NAME (Online Figure VII; Figures 5 and 7).

The effects of the NO donor DEA-NONOate were analyzed in L-NAME-treated aortas. DEA-NONOate (0.1 to 100 nmol/L) caused similar relaxation of aged and young aortas contracted with phenylephrine (Figure 7A). However, in aged arteries, DEA-NONOate (1 to 100 nmol/L) did not significantly affect the stimulation of endothelial exocytosis by thrombin (1 U/mL), which was characterized by increased fluorescent staining for ET-1 (Figure 7B) and decreased fluorescent staining for VWF (Figure 7C). In contrast, DEA-NONOate (1 to 100 nmol/L) significantly reversed the thrombin-induced decrease in VWF staining in young arteries (Figure 7C). Thrombin (1 U/mL) did not affect ET-1 immunofluorescence in young L-NAME-treated aortas (Figure 7B).

Processing of ET-1 in Young and Aging Arteries

In aged L-NAME-treated aortas, the rapid contraction to thrombin (1 U/mL) was inhibited by acute treatment with ECE inhibitors SM19712 (200 $\mu\text{mol/L}$) or phosphoramidon (30 $\mu\text{mol/L}$) (each 30 minutes before thrombin), which caused similar inhibition as the ET_A receptor antagonist BQ123 (Online Figure VIII). The ECE inhibitors did not affect the contractile response to thrombin in young aortas (Online Figure VIII).

Analysis of aortic lysates revealed a 3.2-fold increase in the ET-1 precursor Big ET-1 in aged compared to young blood vessels (Figure 8A), which was associated with increased expression of preproET-1 mRNA in aged aortas (Figure 8C).

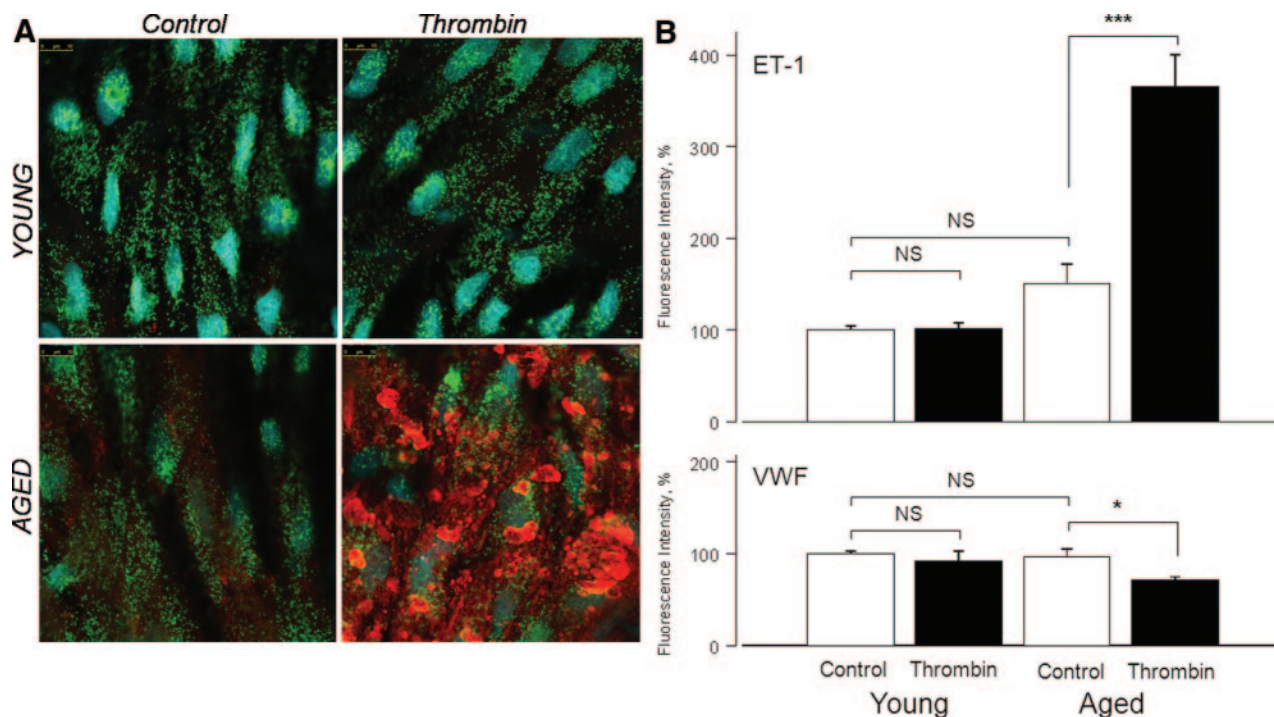


Figure 5. Effect of thrombin (1 U/mL, 5 minutes) on immunofluorescent staining for VWF and ET-1 in endothelial cells lining young and aged rat aortas. **A**, Representative LSM images of native endothelial cells, which were stained for VWF (green), ET-1 (red), and nuclei (blue). Aortas from young (upper) and aged rats (lower) were processed under control conditions (left) and following stimulation with thrombin (1 U/mL, 5 minutes) (right). Bar: 10 μ m. **B**, Quantification of immunofluorescent images. Fluorescence intensity is expressed as a percentage of the signal in young control aortas and is presented as means \pm SEM (N=11 to 19 scanned images for individual groups; n=3 animals). *Statistically significant difference ($P < 0.05$; $***P < 0.001$). NS indicates not significant.

Endothelial denudation reduced the aortic expression of preproET-1 mRNA and abolished the difference in expression between young and aged blood vessels.

Expression of ECE-1 mRNA was also increased in aged compared to young aorta (Figure 8C). Endothelial denudation reduced the expression of ECE-1 mRNA in aged aortas and abolished the difference in expression between young and aged blood vessels (Figure 8C). ECE-2 expression was similar in aged and young aortas (Figure 8C). Consistent with increased ECE-1 expression, the ET-1 precursor, BigET-1 (30 nmol/L) caused contraction that was significantly greater in aged compared to young aortas (Figure 8B). The ECE inhibitor phosphoramidon (30 μ mol/L) decreased the contraction to BigET-1 in young and aged arteries, and after phosphoramidon, there was no longer any significant difference between young and aged aortas (Figure 8B).

Discussion

The results of the present study demonstrate that ET-1 is generated during stimulated endothelial exocytosis in aged but not in young arteries and that this ET-1 contributes to constriction of the aged vascular system. ET-1 was formed predominantly and rapidly during the exocytotic process. An aging-associated specific increase in the stimulated generation of ET-1 reflects increased expression and storage of ET-1 precursors and increased expression of ECE-1 in aged endothelium. Stimulated exocytosis from aged endothelium was also less sensitive to inhibition by NO compared to young arteries, and so an important restraint on endothelial

exocytosis is diminished. The increased ability of aged endothelium to generate ET-1, combined with an increased excitability of the exocytotic process may contribute to the cardiovascular pathology of aging.

Endothelial secretagogues such as thrombin can cause rapid release of mediators from distinct storage granules in cultured endothelial cells, as well as de novo synthesis of endothelial vasodilators including NO.^{24,29,30} Indeed, thrombin caused NOS and endothelium-dependent relaxation, which was decreased in aged compared to young aortas. This is consistent with the known effect of aging to decrease endothelium-dependent relaxation and NO activity.^{31–33} After NOS inhibition, the thrombin response converted to rapid contraction, which was increased in aged compared to young arteries. The aging-dependent increase in thrombin-induced contraction was entirely dependent on the endothelium (absent in endothelium-denuded arteries) and was prevented by blocking ET receptors. Studies on cultured endothelial cells have suggested that ET-1 release can occur through two processes: a constitutive pathway regulated principally through gene transcription and synthesis of ET-1 precursors, and a stimulated pathway that results from rapid exocytosis of stored peptides.^{9,16,17} Thrombin rapidly increased the release of ET-1 from aged but not young aortas, consistent with the thrombin-induced contractile responses. The stimulated release of ET-1 from aged aortas was abolished by endothelial denudation or by TAT-NSF, a cell-permeable inhibitor of NSF and endothelial exocytosis.^{27,28,34,35} TAT-NSF also inhibited the endothelium-dependent contraction to thrombin in

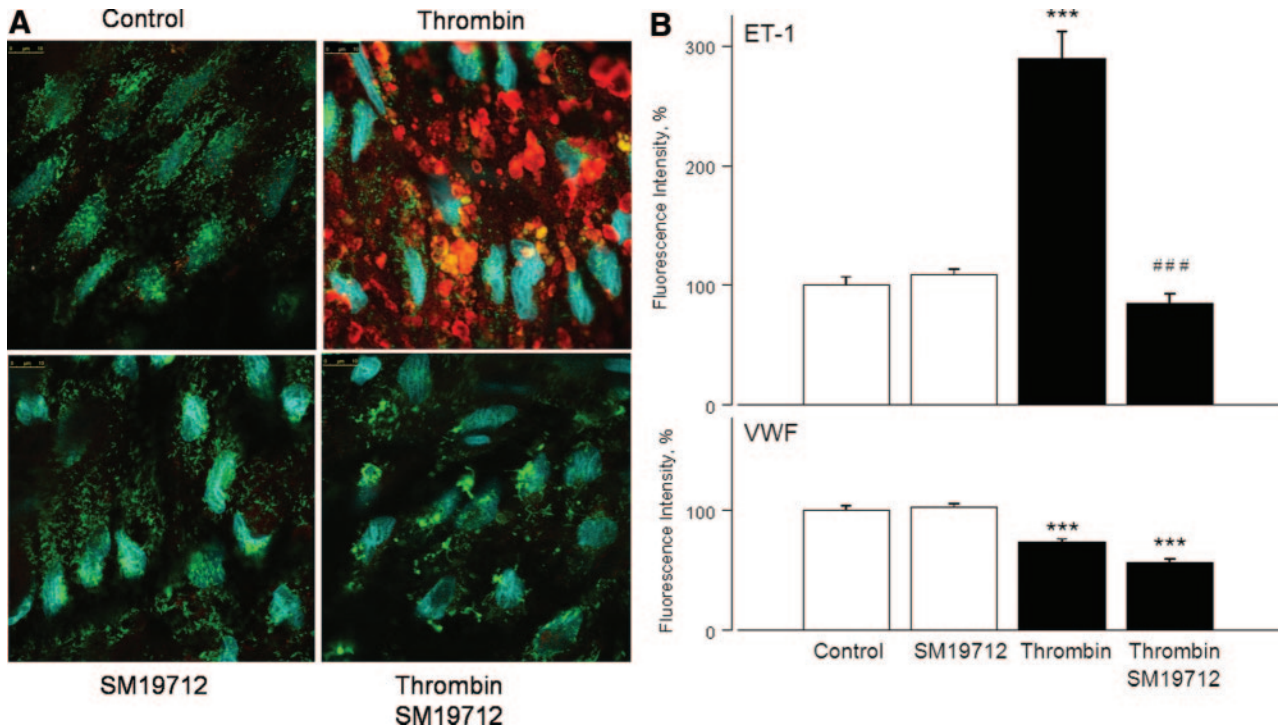


Figure 6. Effect of ECE inhibitor SM19712 (200 $\mu\text{mol/L}$, 30 minutes) on thrombin-induced (1 U/mL, 5 minutes) changes in immunofluorescent staining for VWF and ET-1 in endothelial cells lining aged rat aortas. **A**, Representative LSM images of native endothelial cells, which were stained for VWF (green), ET-1 (red), and nuclei (blue). Aortic segments from aged rats were incubated in the presence and absence of SM19712 followed by incubation in the presence and absence of thrombin (1 U/mL, 5 minutes). **Bar**: 10 μm . **B**, Quantification of immunofluorescent images. Fluorescence intensity is expressed as a percentage of the signal in control aortas and presented as means \pm SEM (N=16 to 23 scanned images for individual groups; n=3 animals). *Significant difference from control (** $P < 0.001$); #significant difference from thrombin treated aortas (### $P < 0.001$).

aged aorta. The scrambled control peptide TAT-CON^{27,28} did not significantly affect the thrombin-induced release of ET-1 or the resulting endothelium-dependent contraction occurring in aged aortas. In contrast to the stimulated release of ET-1, the basal release of the peptide, which may reflect the constitutive pathway, was not significantly different between young and aged aortas. Therefore, aged arterial endothelial cells have an increased ability to rapidly generate ET-1 through regulated exocytosis, which subsequently causes vasoconstriction of the aging vascular system.

Endothelium-derived NO is an important and powerful endogenous inhibitor of endothelial exocytosis.^{34–37} However, in aged arteries, NOS inhibition did not significantly affect the release of ET-1 occurring under basal conditions or following stimulation with thrombin. An altered role of endogenous NO activity in regulating endothelial exocytosis of young and aged aortas was evident when imaging exocytosis of WPBs. VWF is the major component of WPBs and is stored as large multimers reaching an ultimate size of 20 000 kDa (ultralarge or ULVWF).^{38,39} Stimulated release of ULVWF does not occur from individual WPBs: multiple granules fuse before release and exocytosis is associated with formation of craters on the endothelial surface.^{38,40} After exocytosis, ULVWF can be unfurled by flow revealing a pearls-on-a-string appearance with adherent platelets.^{41,42} In control aged arteries, thrombin caused a significant decrease in intensity of VWF staining concomitant with the appearance of ULVWF strands and aggregates, all consistent with

stimulated exocytosis of the protein. In contrast, thrombin did not alter the intensity or pattern of VWF staining in young control arteries. However, after NOS inhibition, thrombin caused the same pattern of ULVWF aggregation and release, and a decrease in VWF staining in young and aged arteries. These results suggest that endogenous NO normally restrains endothelial exocytosis in young arteries, but this restraint is diminished in aged arteries. This could reflect a generalized aging-induced decrease in endothelial NO activity.^{31–33} However, although NOS inhibition did not influence thrombin-induced endothelial exocytosis in aged arteries, it was an absolute requirement to observe the resulting thrombin-induced ET-1-mediated constriction of these blood vessels. Although the NOS-dependent endothelium-dependent relaxation to thrombin was partly reduced in aging arteries, NOS activity was still associated with marked inhibition of contractions to phenylephrine or thrombin in aging blood vessels. This suggests that aging arteries may retain sufficient endothelial NOS activity to relax smooth muscle but not to inhibit exocytosis. Aging can cause uncoupling of NOS resulting in increased generation of reactive oxygen species (ROS) and decreased production of NO by the enzyme.⁴³ Altered generation of NOS-derived mediators could then contribute to differential regulation of endothelial and smooth muscle function. However, ROS such as H_2O_2 not only mediate dilation to uncoupled NOS,⁴⁴ but also inhibit endothelial exocytosis.⁴⁵ Furthermore, catalase (1200 U/mL), which degrades H_2O_2 , did not act like L-NAME and had no effect on constriction to

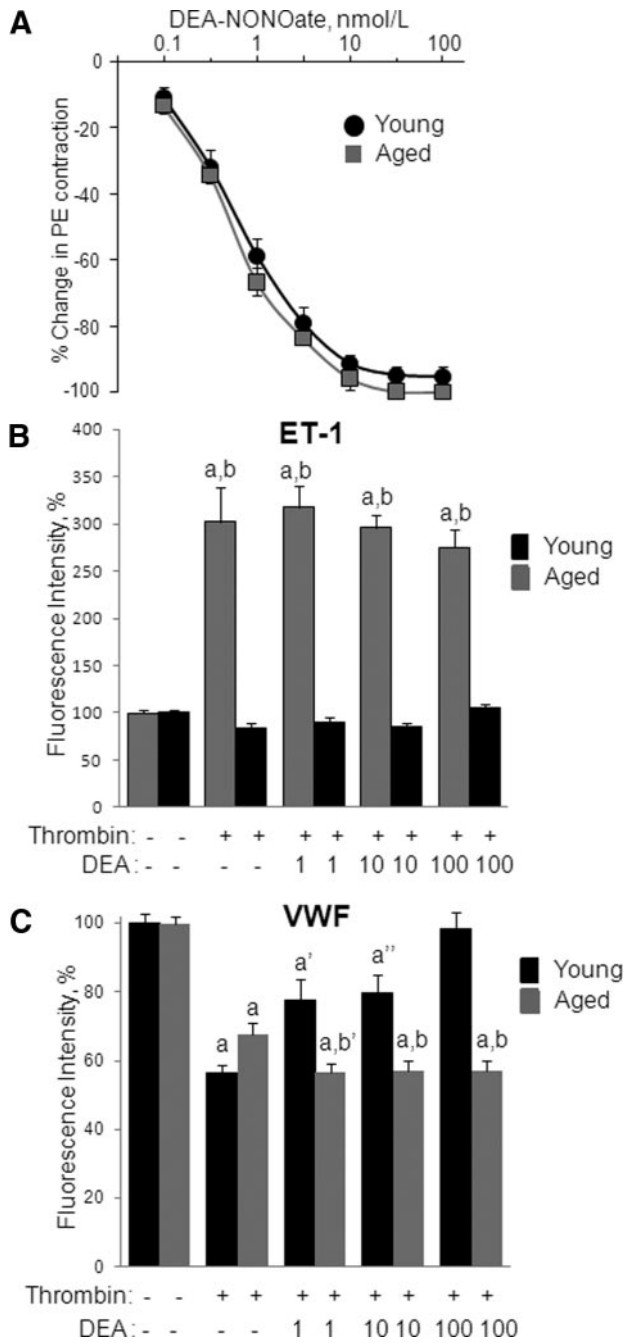


Figure 7. Effects of the NO donor DEA-NONOate on smooth muscle and endothelium of young and aged aortas treated with the NOS inhibitor L-NAME (100 μ mol/L). **A**, Effect of DEA-NONOate (0.1 to 100 nmol/L) to cause dilation of phenylephrine-contracted young and aged aortas. Responses are expressed as a percentage change in the contraction to phenylephrine and presented as means \pm SEM for $n=6$. **B and C**, Effect of DEA-NONOate (1, 10, 100 nmol/L; 10 minutes) on thrombin-induced exocytosis of ET-1 (**B**) or VWF (**C**) from native endothelium of aged and young aortas, determined by quantification of immunofluorescent staining. Fluorescence intensity was expressed as a percentage of the signal in control aortas and presented as means \pm SEM ($N=23$ to 54 scanned images for individual groups; $n=5$ [**B**] or 3 [**C**] animals). **a**, significantly different from corresponding control arteries (**a**, $P<0.001$; **a'**, $P<0.01$; **a''**, $P<0.05$); **b**, significantly different from corresponding young aortas (**b**, $P<0.001$; **b'**, $P<0.01$).

phenylephrine or dilation to thrombin in aged aortas (data not shown). Therefore, the most likely explanation is that exocytosis in aging endothelial cells has a reduced sensitivity to endogenous NO. Indeed, exogenous NO (DEA-NONOate) had a markedly decreased ability to inhibit endothelial exocytosis in aged compared to young arteries. Interestingly, there was no difference in the ability of exogenous NO to cause smooth muscle relaxation in young and aged arteries. These results therefore highlight a novel form of aging-induced endothelial dysfunction, namely a selective insensitivity of endothelial exocytosis to the inhibitory effects of NO. This may reflect an altered pattern of S-nitrosylation or reduced cyclic GMP signaling, which can mediate the modulatory effects of NO on endothelial exocytosis.^{27,34,35,46,47}

Enhanced activity of endothelial exocytosis cannot solely explain the increased ET-1 activity in aging arteries. Although NO synthase inhibition enabled exuberant thrombin-induced exocytosis in young aortas, it did not uncover thrombin-induced release of ET-1 in these arteries. This indicates that in addition to enhanced exocytosis, aging also caused specific changes in endothelial ET-1 processing.

ET-1 immunofluorescence revealed diffuse punctate staining distinct from VWF suggesting that the primary storage site in native aortic endothelium is in small granules and not WPBs, which is consistent with previous studies in cultured and native endothelium.^{17–22} Under quiescent conditions, there was no significant difference in ET-1 staining between the endothelium of young and aged aortas. However, thrombin caused a dramatic and rapid increase in ET-1 staining associated with the endothelium of aged but not young arteries. These results suggest that the increased activity of ET-1 in aging arteries does not reflect increased storage of the active peptide, and that the dramatic thrombin-induced generation of ET-1 in aged arteries reflects rapid formation of ET-1. Indeed, the thrombin-induced ET-1-mediated contraction and increase in ET-1 immunofluorescent staining were markedly reduced by acute treatment of the aortas with ECE inhibitors, which would not affect ET-1 that had already been formed and stored in endothelial granules. ECE inhibition did not affect the ability of thrombin to cause exocytosis of WPBs and release of VWF. These results suggest that ET-1 is stored as a precursor and that it is formed rapidly during the exocytotic process; a process that is dramatically increased in aged compared to young endothelium. Indeed, there was increased expression of preproET-1 mRNA and also of ECE-1 mRNA in aged compared to young aortas. In each case, the increased expression was abolished by endothelium denudation indicating that it reflected a selective increase in endothelial expression. The increase in preproET-1 mRNA expression was paralleled by increased concentrations of BigET-1 in aortic lysates of aged compared to young aortas. Likewise, the functional significance of the increase in ECE-1 mRNA was demonstrated by the marked increase in phosphoramidon-sensitive contractions to exogenous BigET-1 in aged compared to young arteries. In contrast to BigET-1, exogenous ET-1 caused contraction that was similar in young and aged arteries. Therefore, the increased generation of ET-1 in aged arteries results from increased expression of endothelial precursors and converting enzyme and an increased

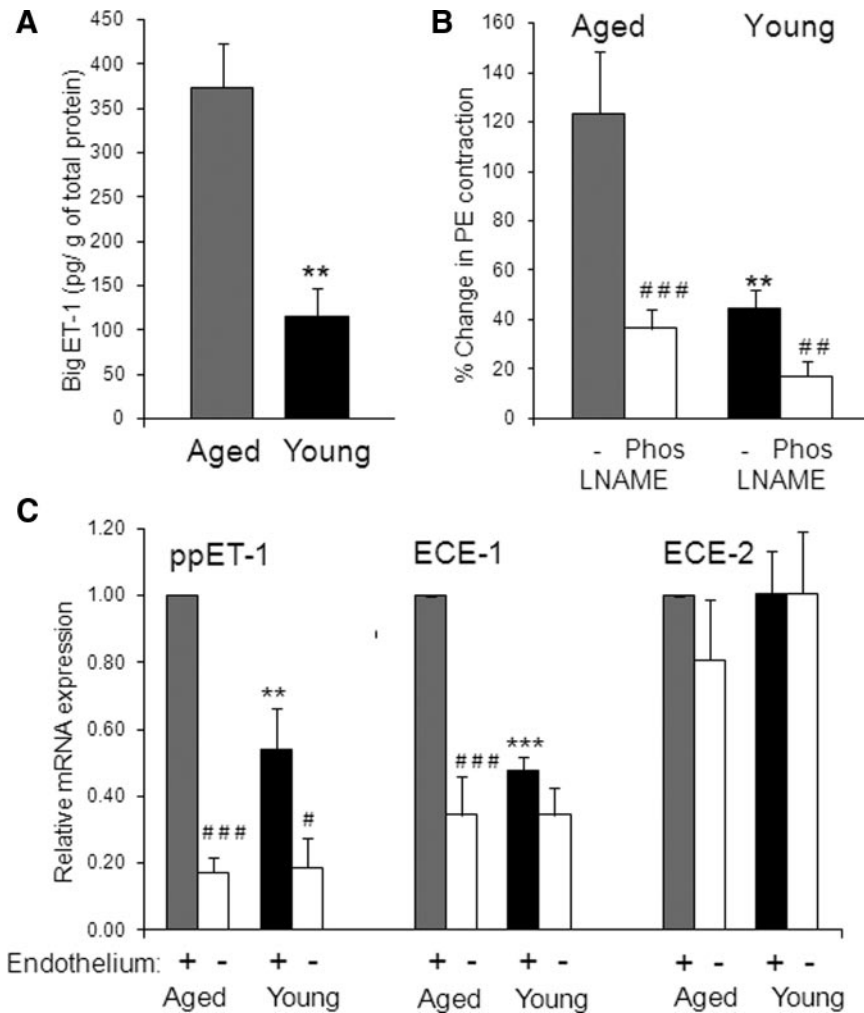


Figure 8. Processing of ET-1 in aortas of young and aged rats. **A**, Quantification of BigET-1 content of aged and young aortas determined by ELISA of aortic lysates. Data are presented as picograms per gram of aortic protein and is presented as means±SEM, n=4. **Significant difference from aged aortas (P<0.01). **B**, Contractions to BigET-1 (30 nmol/L) in aged (left) and young (right) aortas in the absence and presence of the ECE inhibitor phosphoramidon (Phos) (30 μmol/L). Aortic rings were treated with the NOS inhibitor L-NAME (100 μmol/L) and contracted with phenylephrine. Responses to BigET-1 were assessed after 15 minutes, expressed as a percentage change in contraction to phenylephrine and presented as means±SEM for n=6 to 7. **Significant difference from corresponding group in aged aortas (P<0.01); #significant difference from aortas not treated with phosphoramidon (##P<0.01; ###P<0.001). **C**, Expression of mRNA for preproET-1 (ppET-1), ECE-1, and ECE-2 in endothelium-containing (+) and endothelium-denuded (-) aortas from young and aged rats. mRNA was determined using real-time PCR, expressed relative to levels in aged endothelium-containing aortas, and presented as means±SEM for n=5 or 7. *Significant difference from corresponding aged aortas (endothelium+ or -) (**P<0.01; ***P<0.001); #significant difference from endothelium+ (#P<0.05; ###P<0.001).

sensitivity of the exocytotic process, compared to young arteries. Increased expression of preproET-1 and ECE-1 may result from (or be amplified by) chronic exposure to altered hemodynamics, including arterial stiffness, or altered mediator activity within the aging blood vessel wall, including diminished NO activity, increased oxidant stress and inflammatory activation of the endothelium. Altered activity of the ET-1 signaling pathway may be an important early component of the aging endothelial phenotype. Although endothelial senescence in cultured systems is associated with a loss of WPBs and ET-1 immunofluorescence, presenescent aging of cultured endothelial cells increases ET-1 expression.^{48,49} Likewise, ECE-1 expression and circulating levels of ET-1 are increased in third generation telomerase-deficient mice.⁵⁰

Human aging is associated with increased circulating levels of ET-1 and VWF.^{1-4,51} Increased ET-1 production can cause oxidant stress, endothelial dysfunction, vascular inflammation, and vascular remodeling^{7,8} and likely contributes to age-related cardiovascular dysfunction including arterial stiffening and atherosclerosis.⁹⁻¹⁵ Likewise, VWF is an important thrombotic mediator and increased VWF levels are associated with myocardial infarction and mortality in individuals with vascular disease.⁵² Increased expression of ET-1 precursors and converting enzyme and increased excitability of the exocytotic process

likely contributes to increased circulating levels of these mediators and to the cardiovascular pathology of aging.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- Studies in cultured endothelial cells have demonstrated that endothelin (ET)-1 is released both continuously via a constitutive pathway and rapidly in response to stimulated exocytosis, suggesting storage of ET-1 or its precursors in endothelial storage granules such as Weibel-Palade Bodies.
- ET-1, which can cause oxidant stress, endothelial dysfunction, vascular inflammation, vascular constriction and vascular remodeling, has increased activity in aging individuals and likely contributes to age-related cardiovascular dysfunction.
- No previous studies have directly assessed the stimulated endothelial exocytosis of ET-1 or other mediators from native arteries or its potential modulation in the aging vasculature.

What New Information Does This Article Contribute?

- Stimulation of endothelium caused rapid exocytotic release of ET-1 from aged but not young arteries, and the released ET-1 contributed to constriction of aged arteries.
- The generation of ET-1 in aging arteries occurred during the exocytotic process and reflected rapid conversion of its precursor Big ET-1 by endothelin-converting enzyme (ECE)-1, both of which had increased expression in aged endothelium.
- Aging also increased the excitability of the exocytotic process because of a decreased sensitivity of aging endothelium to the

inhibitory effects of nitric oxide, resulting in increased stimulated exocytosis of stored mediators from aging endothelium.

Despite evidence for increased ET-1 activity in the aging vascular system, no previous studies have directly analyzed the endothelial exocytotic release of ET-1 from aging arteries. We report that stimulation of native arteries with a secretagogue (thrombin) can cause a rapid exocytotic release of ET-1 from the endothelium of aged but not young arteries, resulting in constriction of aged arteries. Interestingly, there was no difference in storage of ET-1 between young and aged endothelial cells, and the increased activity of ET-1 in aged arteries became evident only during the stimulated exocytotic process. Compared to young endothelial cells, aging cells stored increased levels of the ET-1 precursor, Big ET-1, and had increased expression of preproET-1 and ECE-1, which cleaves BigET-1. Indeed, acute inhibition of ECE, which would not inhibit stored ET-1, abolished ET-1 release and the associated constriction in aged arteries. Aging arteries also displayed a novel type of endothelial dysfunction, namely a diminished ability of endothelium-derived NO to inhibit the stimulated exocytotic release of endothelial mediators, including ET-1 and von Willebrand factor. The increased ability of aged endothelium to generate ET-1, combined with an increased excitability of the exocytotic process may contribute to the cardiovascular pathology of aging.

SUPPLEMENTAL MATERIAL

METHODS

Experimental animals: Young (3-4 months) and aged (18-20 months) Fischer-344 rats were used in the study (National Institute of Aging, Bethesda, MD). Use of animals was approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University and complied with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health. Rats were anesthetized using isoflurane gas, then heparin (3,000 IU) was administered in the left ventricle to prevent clotting. The thoracic aorta and small mesenteric arteries were rapidly and carefully removed in cold Krebs-Ringer-bicarbonate solution (118.3 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 25.0 mM NaHCO₃, 11.1 mM glucose) (control solution) and cleaned of adherent tissue.

Aortic Contraction Studies: For isometric tension recording, aortic rings (~ 2.5 mm long) were suspended between two stainless steel triangular wires in organ baths containing 20 ml of cold control solution, gassed with 95% O₂/5% CO₂ (Radnoti Glass Technology, Monrovia, CA). The tension of the aortic rings was continually monitored using a data acquisition system (Acqknowledge, BIOPAC Systems Inc, CA). Rings were stretched incrementally by 0.1 gm every 2 min; when passive tension reached 1.5 gm buffer temperature was warmed to 37°C and maintained at that temperature for the duration of the experiment. Preliminary experiments demonstrated that contractions to KCl (60 mM) were maximal at this level of passive tension for both age groups indicating that the rings were at their optimal passive tension. After a period of equilibration (45 min), the rings were stimulated with KCl (60 mmol/L) every 15-20 min until contractile responses were stable (3 to 4 times). Rings were then incubated in the absence and presence of L-NAME (N^G-nitro-L-arginine methyl ester, 100 µmol/L) a nitric oxide synthase (NOS) inhibitor and/or BQ123 (1 µmol/L) an ET_A receptor inhibitor, BQ788 (1 µmol/L) an ET_B receptor inhibitor, phosphoramidon (30 µmol/L) or SM19712 (200 µmol/L) endothelin converting enzyme inhibitors, TAT-NSF (1 µmol/L), a cell permeable inhibitor of exocytosis (comprising the human immunodeficiency virus transactivator of transcription protein transduction domain, TAT, fused to the homohexamization domain of N-ethylmaleimide-sensitive factor, NSF), or a scrambled control peptide TAT-CON (1 µmol/L)^{1,2}. After 30 min, aortic rings were contracted with phenylephrine to approximately 50% of the response to KCl (60 mmol/L) (0.1 µmol/L for L-NAME treated or endothelium-denuded rings, 1 µmol/L for other rings). Once the phenylephrine contraction was stable, responses to thrombin (1U/ml), ET-1 (0.1 to 10 nmol/L), Big-ET1 (30 nmol/L), or DEA-NONOate (0.1 to 1 nmol/L) were evaluated. Apart from L-NAME, none of the inhibitors had any significant effects on the contractile response to phenylephrine. Endothelial denudation was achieved by rubbing the luminal surface with a cotton-tipped applicator and was confirmed by lack of dilation to acetylcholine (1 µmol/L) during contraction with phenylephrine (0.1 µmol/L). Thrombin (1U/ml) did not cause contraction under basal quiescent conditions (i.e in the absence of phenylephrine).

Mesenteric Artery Constriction Studies: Small mesenteric arteries were isolated from the same anatomical location, cannulated at both ends with glass micropipettes, secured using 12-0 nylon monofilament suture, and placed in a microvascular chamber (Living Systems, Burlington, VT)³. Blood vessels were studied in the absence of flow and maintained at a constant transmural pressure (P_{TM}) of 60 mmHg. The chamber was placed on the stage of an inverted microscope

(Nikon TMS-F; Nikon, Tokyo, Japan) connected to a video camera and superfused with control solution (maintained at 37°C, gassed with 16% O₂, 5% CO₂, balance N₂; pH 7.4). The blood vessel image was projected onto a video monitor and internal diameters continuously determined and recorded by a video dimension analyzer (Living Systems) and BIOPAC data acquisition system (Santa Barbara, CA). At a P_{TM} of 60 mmHg, the internal diameter of mesenteric arteries were 292.4 ± 11.8 μm (n = 7) and 267.0 ± 9.9 μm (n = 5) for aged and young animals, respectively. Mesenteric arteries were allowed to equilibrate for 45 min before commencing experiments. In some experiments, endothelial cells were removed by gently passing a wire (70 μm in diameter) through the vessel lumen. This procedure abolished endothelium-dependent relaxation to acetylcholine (1 μmol/L), assessed during constriction to phenylephrine (by ~ 30% of baseline diameter). Vasoconstrictor responses to thrombin (1 U/ml) or to exogenous ET-1 (0.3 and 3 nmol/L) were determined under basal unstimulated conditions (i.e. in the absence of other agonists) in the presence of L-NAME (100 μmol/L), and in the absence and presence of combined inhibition of ET_A and ET_B receptors (BQ123, 1 μmol/L and BQ788, 1 μmol/L).

ET-1 Generation: Endothelium-containing and endothelium-denuded segments of aortas were equilibrated in 120 μL of control solution for 45 min at 37°C (5% CO₂, balance air). The segments were then incubated in the absence and presence of L-NAME (100 μmol/L), TAT-NSF (1 μmol/L), or TAT-CON (1 μmol/L) for 30 min before being stimulated by thrombin (1U/ml, 5 mins). On completion of the incubations, the supernatant was removed and assayed for ET-1 using a commercial ELISA kit according to the manufacturer's instructions (R&D Systems, Minneapolis, MN).

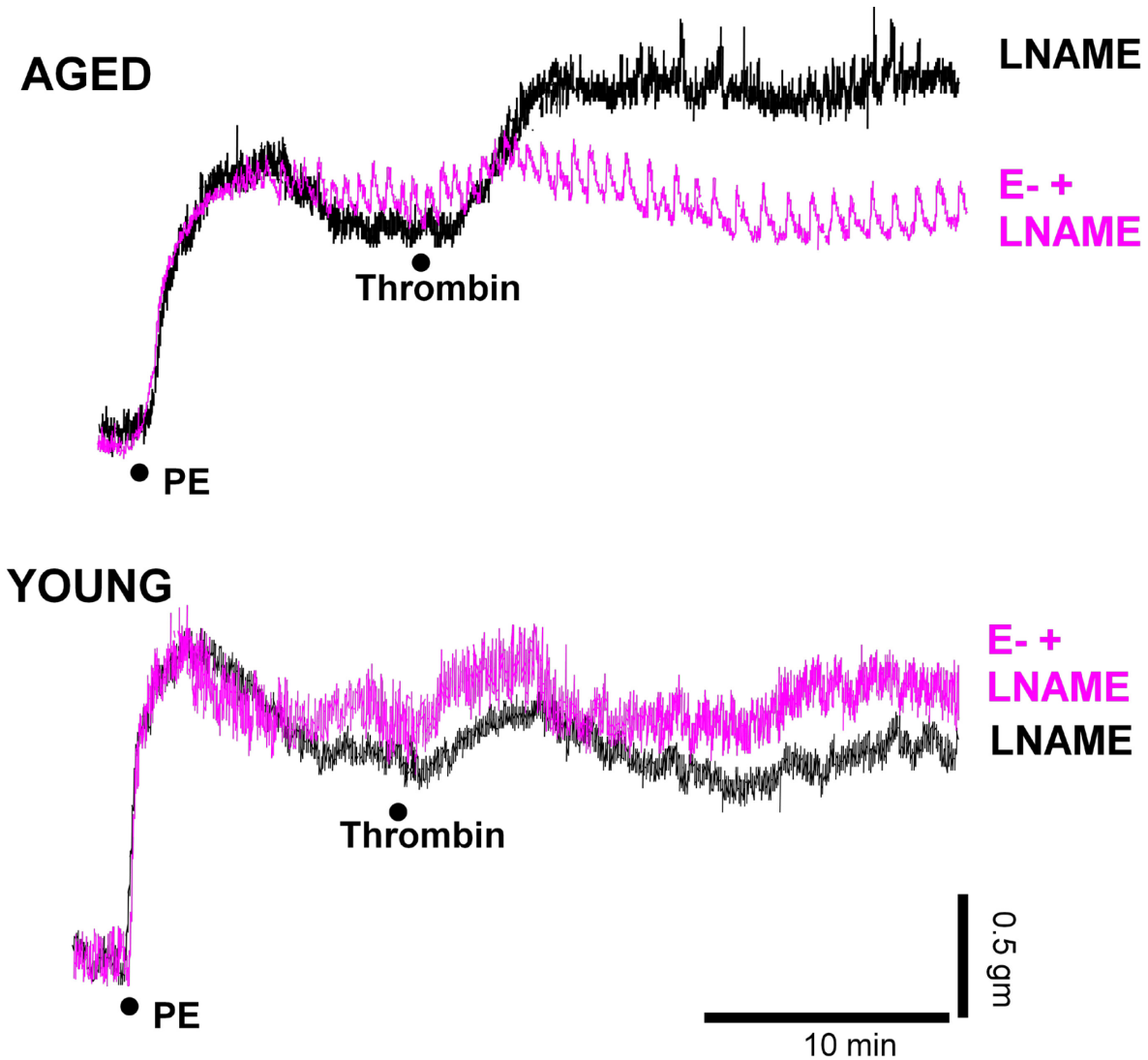
Big Endothelin Levels: Aortic segments from young and aged animals were equilibrated in 120 μL control solution for 45 min at 37 ° C (5% CO₂, balance air). Segments were then lysed in 100 μL PBS containing 0.5 % Triton -X, 30 μmol/L phosphoramidon, 1 μL of HaltTM protease/phosphatase inhibitor cocktail (Thermo Scientific, IL) for 30 min. Big ET-1 levels were analyzed by ELISA (Assay Designs, MI) according to the manufacturers protocol.

Endothelial Imaging: Aortic segments were equilibrated in 120 μL of control solution for 75 mins at 37°C (5% CO₂, balance air) before being incubated in the absence and presence of thrombin (1U/ml) for 5 mins. In some experiments, the aortic segments were incubated with L-NAME (100 μmol/L) or with the ECE inhibitor SM19712 (200 μmol/L) for 30 mins prior to the addition of thrombin, or with DEA-NONOate (1 to 100 nmol/L) for 10 mins prior to thrombin. On completion of the incubations, aortic segments were fixed in 3% paraformaldehyde in PBS (4°C, 30 min) followed by 3 rinses in PBS before being cut open and pinned on a silicone-coated dish (lumen exposed). Aortas were permeabilized (Triton-X, 0.5%, 15 min), rinsed (PBS, 3 times) and incubated in goat serum (1.5 %, 15 min) to reduce nonspecific binding. To visualize WPBs, aortas were incubated with a primary polyclonal rabbit antibody to rat von Willebrand factor (VWF, 1:100 dilution, Santa Cruz Biotechnology, CA) for 90 min, rinsed (PBS, 3 times) then incubated with an AlexaFluor 488 goat anti rabbit antibody (1:200 dilution, Invitrogen, Carlsbad, CA) for 90 min. To visualize ET-1, aortas were incubated with a primary mouse monoclonal antibody to rat ET-1 (1:1000 dilution, Calbiochem, CA) for 90 min, rinsed (PBS, 3 times) then incubated with an AlexaFluor 568 goat anti mouse antibody (1:200, Invitrogen) for 90 min. After rinsing (PBS, 3 times) aortas were incubated with Draq5 (5 μmol/L, 30min) (Biostatus Ltd, Leicestershire, United Kingdom) to label nuclei.

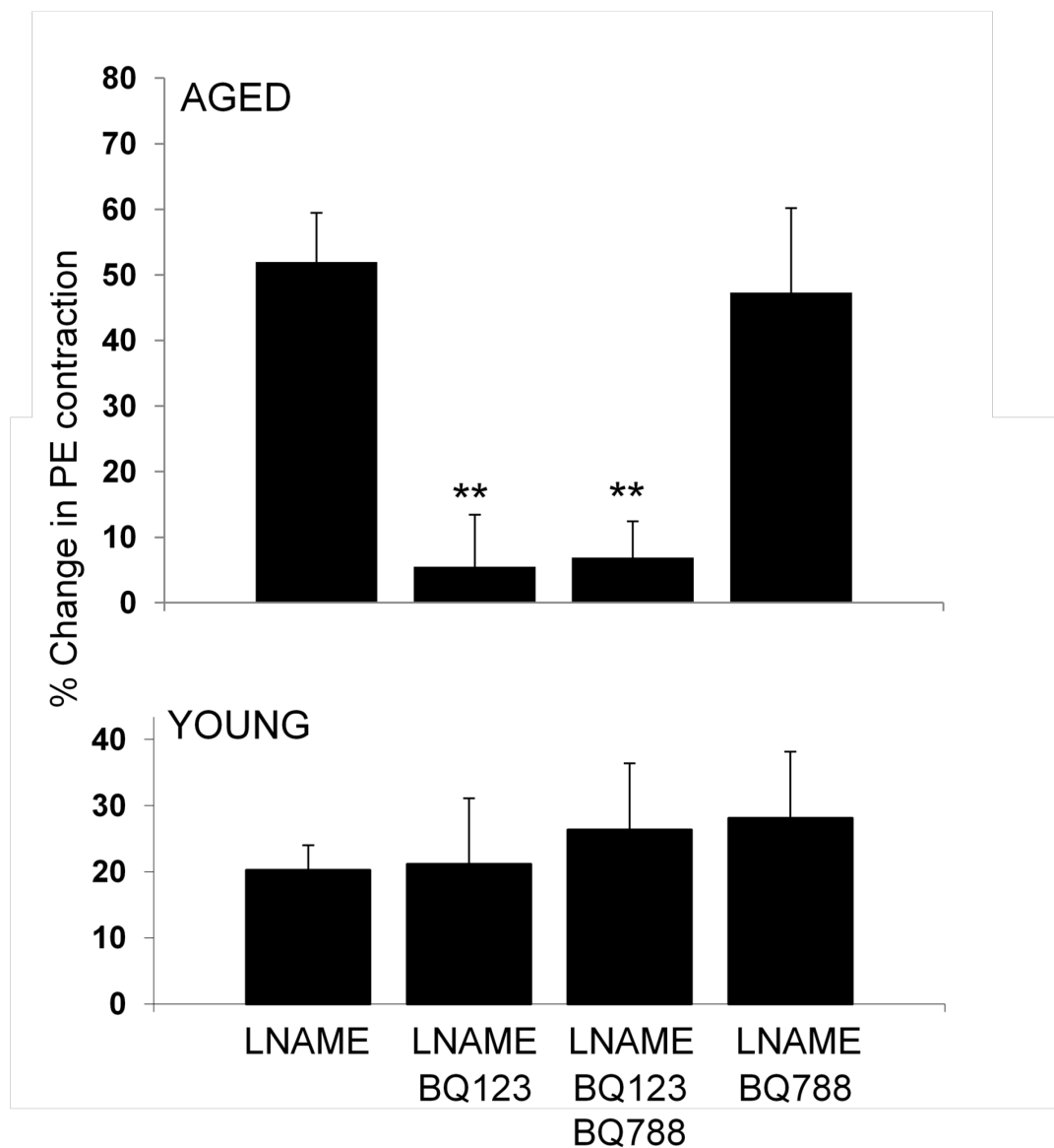
Samples were viewed using a Leica AOBs-equipped SP5 laser scanning microscope. Images (1024x1024 pixels) were obtained using sequential acquisition, a pinhole of 1 Airy unit, scan speed of 400 Hz, and 8 line averaging. Unless specifically stated, image accumulation was not used. For VWF (AlexaFluor488), excitation was at 488 nm and emission was captured from 495 to 542 nm, for ET-1 (AlexaFluor 568) excitation was at 568 nm and emission was captured from 584 to 630 nm, and for DRAQ excitation was at 633 nm and emission was from 656 to 744 nm. Images are presented in their original unprocessed condition. Paired aortas from young and aged animals were processed at the same time using the same instrument settings. For quantification, the average intensity of each fluorescent signal was determined for the entire endothelial area that was in focus within each image. For each pair of aortas, the mean of the average signal intensities in young arteries was set as 100% and all images were expressed relative to that value. When analyzing the effect of DEA-NONOate or SM19712 in aged arteries, a similar approach was used. Control and treated arteries were processed at the same time, and the mean of the average signal intensities in control arteries was set as 100% and all images were expressed relative to that value.

Real-time PCR: Aortic rings were homogenized and total RNA extracted using Trizol (Invitrogen), according to the manufacturer's instructions. First-strand cDNA was synthesized by reverse transcription of 2µg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc, CA), in a total volume of 20 µl, according to the manufacturer's suggestions. The gene fragments were specifically amplified using TaqMan® Universal PCR Master Mix (Applied Biosystems Inc, CA) and 1 µl of first-strand cDNA with real-time RT-PCR. Internal variations were normalized using 18S rRNA. Taqman probes and primers for internal control (18s rRNA) were purchased from Perkin-Elmer. The following primers and probes were designed using Primer 3 (Steave Rozen and Jelen J. Skaletsky, MIT center for genome Research, MA): Preproendothlin, forward: 5'-CGTCCCGTATGGACTAGGAA-3', reverse: 5'-GGCTCGGAGTTCTTTGTCTG-3' and probe: 5'-/56-FAM/AGGGAACAGATGCCAGTGTGC/36-TAMSP/-3'. ECE-1, forward: 5'-GCAACCAGCTCTTCTTCCTG-3', reverse: 5'-AGTTGGAGAGTGAGCCGATG-3' and probe: 5'-56-FAM/CGGTCCGCACAGAGAGCT/36-TAMSP/-3'. ECE-2: forward; 5'-GTTCTTCGTGGGATTTGCTC-3', reverse; 5'-CTCGGGAGTTGGAGAGAGTG-3', probe; 5'-56-FAM/CGGTCCGCACAGAGAGCT/36-TAMSP/-3'. Reactions were carried out in a 7500 Fast Real-Time PCR System (Applied Biosystems Inc, CA) at: 50°C for 2 min, 95°C for 10 min followed by 40 cycles of incubation at 95°C for 15 sec, 60°C for 1 min.

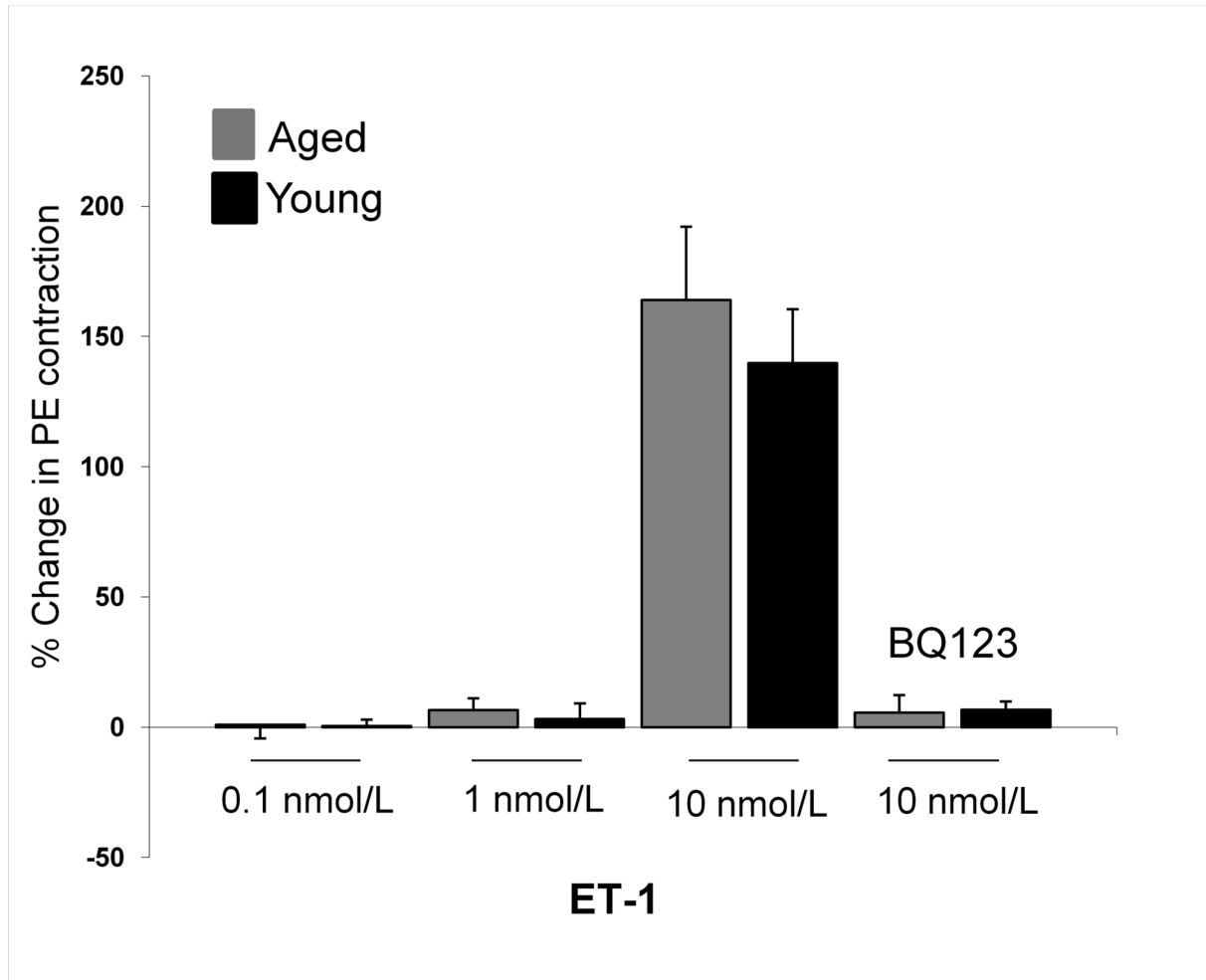
Statistical Analysis: Statistical evaluation of the data was performed by Student's t test for paired or unpaired observations. When more than two means were compared, analysis of variance was used. If a significant F value was found, Tukey's test for multiple comparisons was employed to identify differences among groups. Data are expressed as means ± S.E.M. for n number of experiments, where n equals the number of animals from which blood vessels were studied. Values were considered to be statistically different when $P < 0.05$.



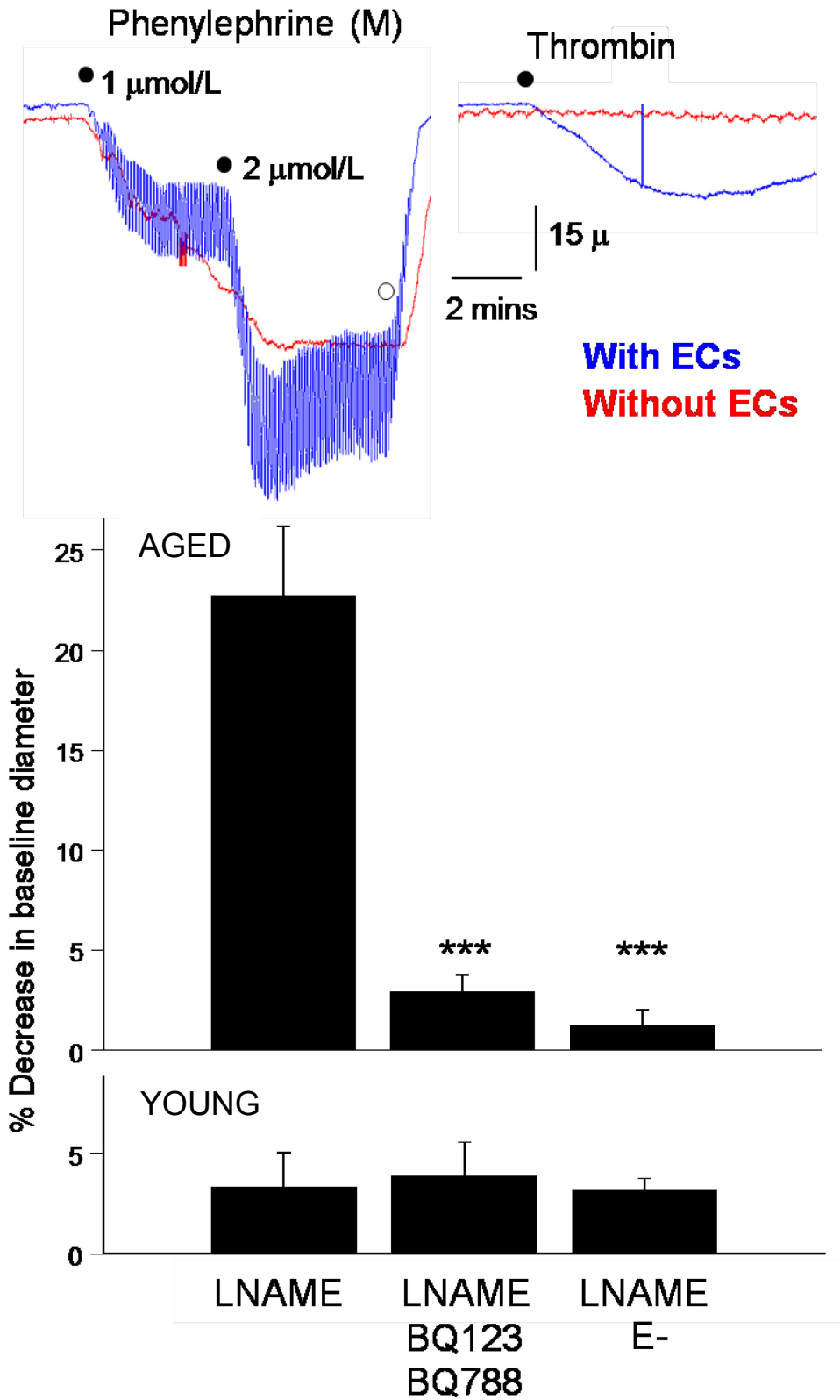
Supplemental Figure I: Representative tracing demonstrating the effect of endothelial denudation on the contractile response to thrombin (1 U/ml) in young and aged aortas. Responses to thrombin were assessed in isolated endothelium-containing (black) and endothelium-denuded (pink) aortic rings treated with L-NAME (100 $\mu\text{mol/L}$) and contracted with phenylephrine (PE).



Supplemental Figure II: Effect of ET receptor antagonists on the contractile response to thrombin (1U/ml) in aortas from aged (upper panel) and young rats (lower panel). Responses to thrombin (1U/ml) were assessed in isolated aortic rings treated with L-NAME (100 $\mu\text{mol/L}$) and contracted with phenylephrine in the absence or presence of the ET_B receptor antagonist BQ788 (1 $\mu\text{mol/L}$) and/or the ET_A receptor antagonist BQ123 (1 $\mu\text{mol/L}$). Contractile effects were assessed after 5 mins, expressed as a percentage change in the stable contraction to phenylephrine, and are presented as means \pm SEM for $n = 5$. *, denotes significant difference from L-NAME-treated arteries (**, $P < 0.01$).

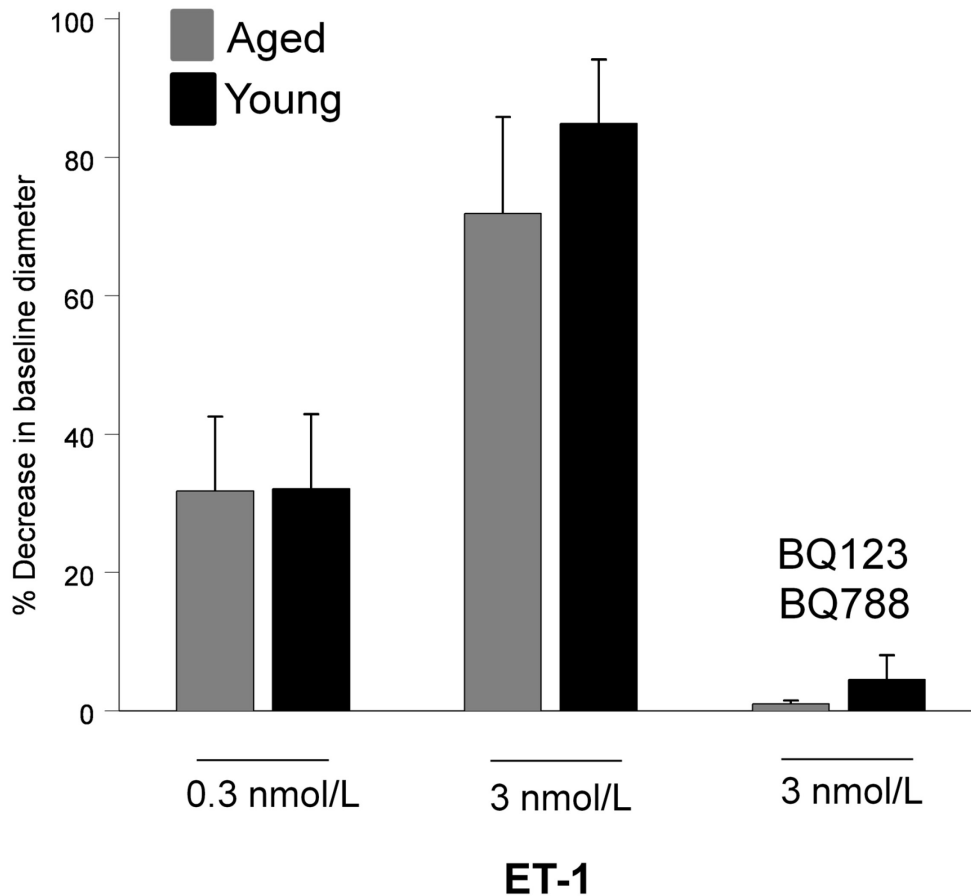


Supplemental Figure III: Contractile responses to ET-1 (0.1 to 10 nmol/L) in aortas from aged and young rats. Responses to ET-1 were assessed in aortic rings treated with the NO synthase inhibitor L-NAME (100 μ mol/L) and contracted with phenylephrine. Contractile effects were assessed after 5 mins, expressed as a percentage change in the stable contraction to phenylephrine, and are presented as means \pm SEM for n = 5. In some rings, the inhibitory effects of the ET_A receptor antagonist BQ123 (1 μ mol/L) was confirmed using ET-1 (10 nmol/L) in young and aged aortas (n = 3 or 5).

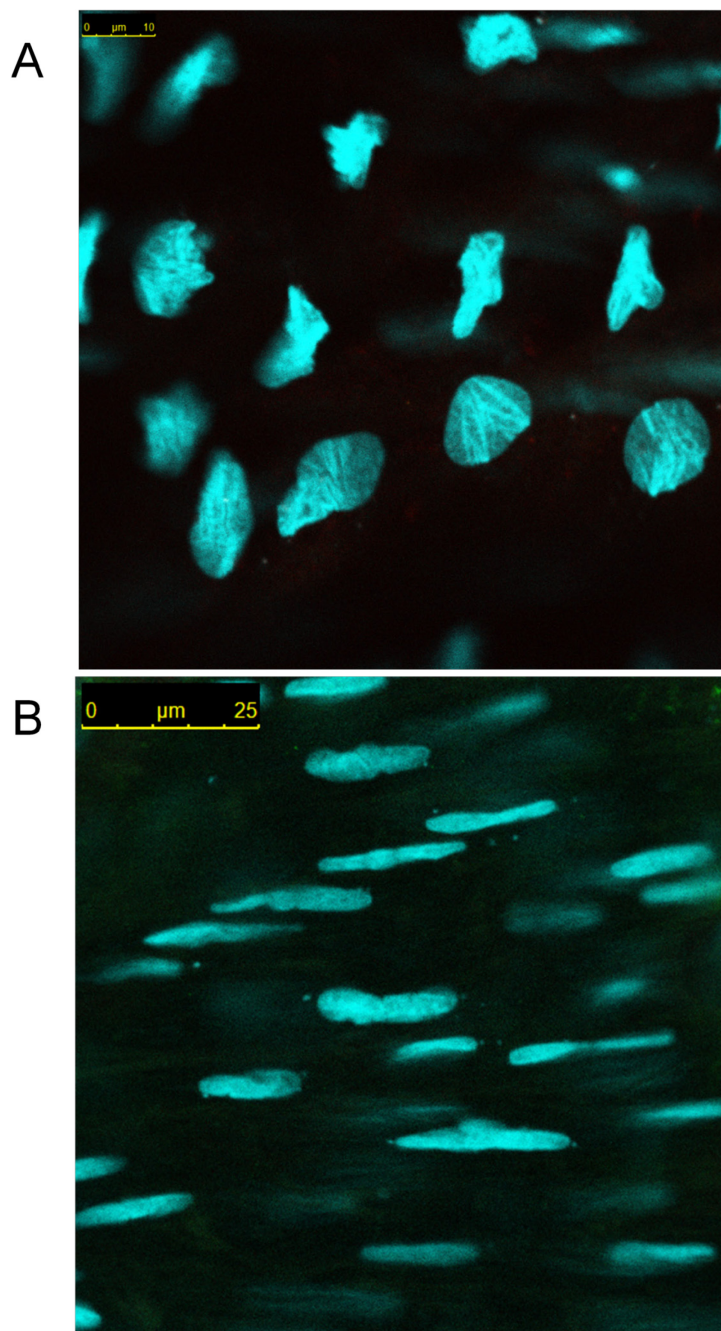


Supplemental Figure IV: Analysis of thrombin-induced constrictor responses in mesenteric arteries from aged and young rat aortas. Responses to thrombin (1U/ml) were assessed under

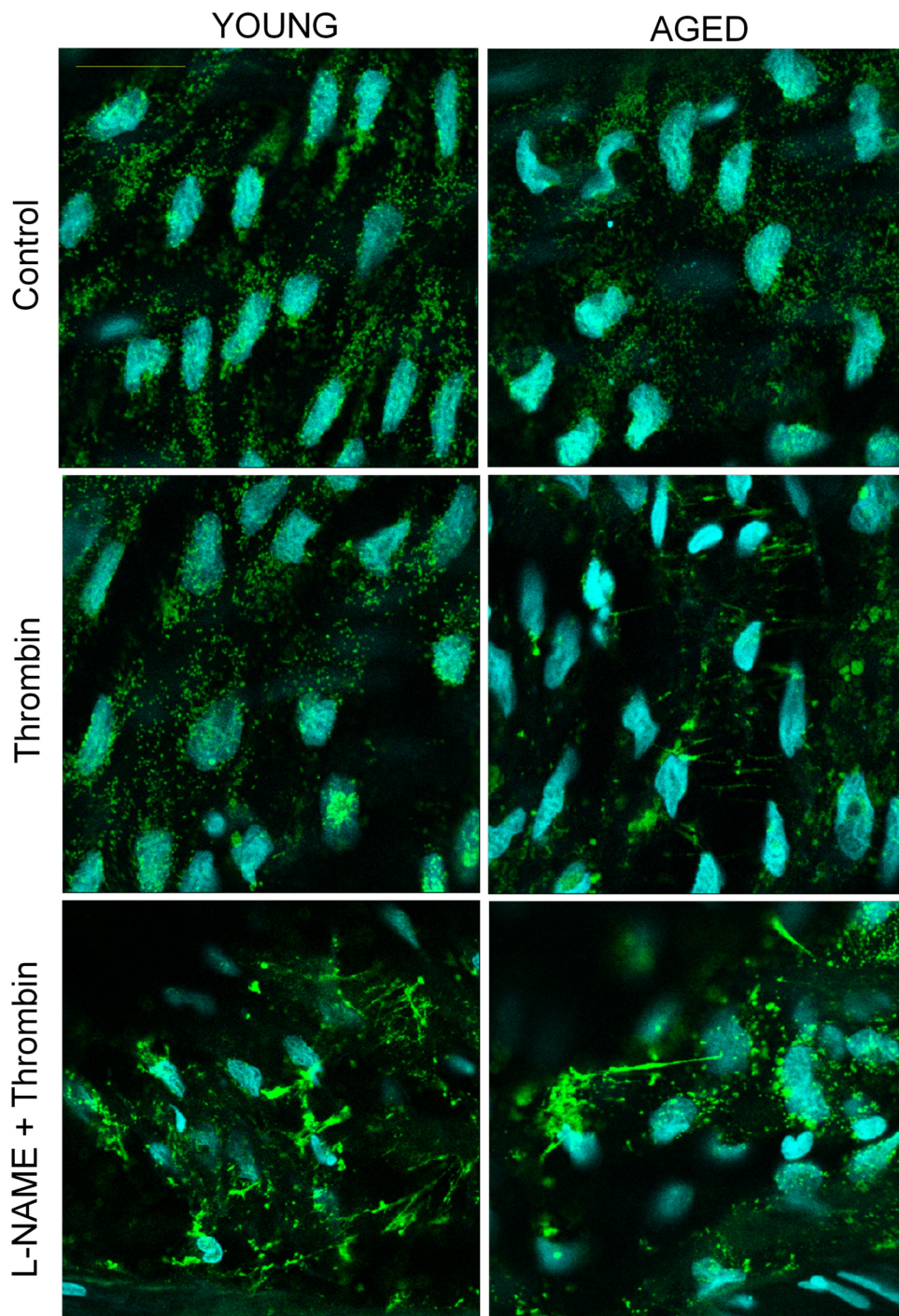
quiescent conditions in the presence of the NO synthase inhibitor L-NAME (100 $\mu\text{mol/L}$) and in the absence or presence of combined ET_A and ET_B receptor antagonism (BQ123, 1 $\mu\text{mol/L}$; BQ788 1 $\mu\text{mol/L}$) or in endothelium-denuded rings (E-). The upper trace is a representative experiment in aged mesenteric arteries, whereas the lower graphs present combined data for aged (upper graph) and young aortas (lower graph). Responses to thrombin were determined at the peak of the response (~ 5 mins), expressed as a percentage decrease in the stable baseline internal diameter, and are presented as means \pm SEM for $n = 3$ to 6. *, denotes significant difference from L-NAME-treated arteries (***, $P < 0.001$).



Supplemental Figure V: Contractile responses to ET-1 (0.3 and 3 nmol/L) in mesenteric arteries from aged and young rats. Responses to ET-1 were assessed in quiescent arteries treated with the NO synthase inhibitor L-NAME (100 μ mol/L). Contractile effects were assessed at the peak of the response, expressed as a percentage decrease in the baseline internal diameter, and are presented as means \pm SEM for n = 4 or 5. The inhibitory effect of combined ET_A and ET_B receptor antagonism (BQ123, 1 μ mol/L; BQ788, 1 μ mol/L) was assessed using ET-1 (3 nmol/L) in young and aged aortas (n = 4 or 5).

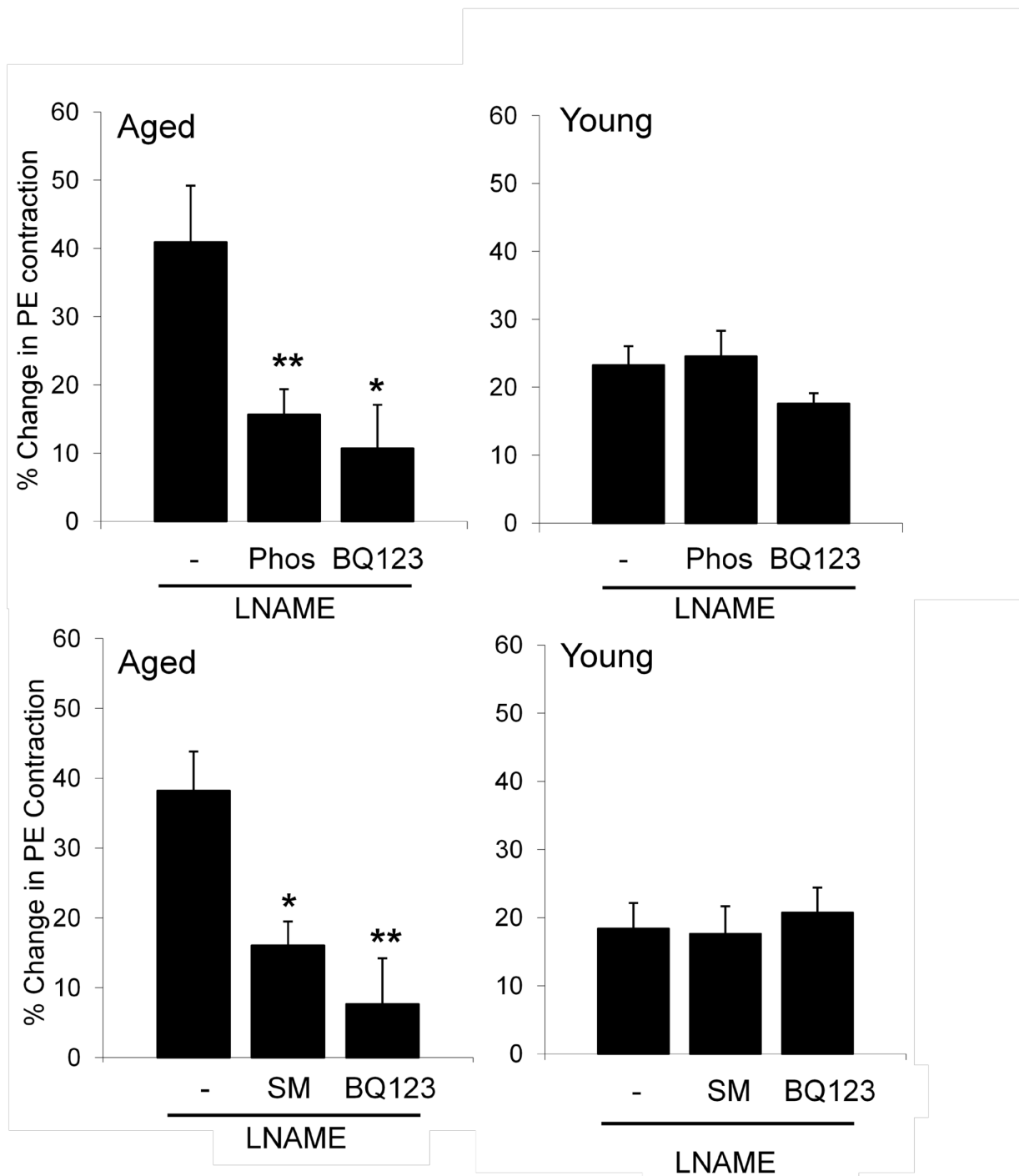


Supplemental Figure VI: Negative controls for immunofluorescent staining of aged aortic endothelium (A) and smooth muscle cells (B). Aortic segments from aged animals were processed for immunofluorescent staining of VWF (green) and ET-1 (red) as in other experiments with the exception that in: (A) the primary antibodies were excluded, whereas in (B) all antibodies were included but the image was captured from the underlying smooth muscle cells.



Supplemental Figure VII: Representative LSM images demonstrating the effect of thrombin (1U/ml, 5 mins) on immunofluorescent staining for VWF in endothelial cells lining young (left) and aged (right panels) rat aortas. The effect of thrombin was evaluated in untreated aortas and arteries treated with an NOS inhibitor (L-NAME 100 μ mol/L). Aortas were subsequently

stained for VWF (green) and nuclei (blue). Similar results were obtained in blood vessels from three additional pairs of animals. Bar: 25 μ m



Supplemental Figure VIII: Effect of ECE inhibitors phosphoramidon (30 $\mu\text{mol/L}$) (*A*) or SM19712 (SM, 200 $\mu\text{mol/L}$) (*B*) on the contractile response to thrombin (1 U/ml) in aged (left panels) and young (right panels) rat aortas. Endothelium-dependent contractions to thrombin were assessed in aortic rings that were treated with the NO synthase inhibitor L-NAME (100 $\mu\text{mol/L}$) and contracted with phenylephrine. For comparison, the effect of the ET_A receptor antagonist BQ123 (1 $\mu\text{mol/L}$) was assessed in paired aortic rings. Responses to thrombin were determined after 5 mins, expressed as a percentage change in the contraction to phenylephrine,

and are presented as means \pm SEM for n = 5. *, denotes significant difference from L-NAME-treated aortas (*, P < 0.05; **, P < 0.01).

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