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Functional heterogeneity of NADPH oxidase-mediated contractions to endothelin with vascular aging

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ABSTRACT

Aims: Aging, a physiological process and main risk factor for cardiovascular and renal diseases, is associated with endothelial cell dysfunction partly resulting from NADPH oxidase-dependent oxidative stress. Because increased formation of endothelium-derived endothelin-1 (ET-1) may contribute to vascular aging, we studied the role of NADPH oxidase function in age-dependent contractions to ET-1.
Main methods: Renal arteries and abdominal aortas from young and old C57BL6 mice (4 and 24 months of age) were prepared for isometric force measurements. Contractions to ET-1 (0.1–100 nmol/L) were determined in the presence and absence of the NADPH oxidase-selective inhibitor gp91ds-tat (3 μmol/L). To exclude age-dependent differential effects of NO bioactivity between vascular beds, all experiments were conducted in the presence of the NO synthase inhibitor L-NAME (300 μmol/L).
Key findings: In young animals, ET-1-induced contractions were 6-fold stronger in the renal artery than in the aorta ($p < 0.001$); inhibition of NADPH oxidase by gp91ds-tat reduced the responses to ET-1 by 50% and 72% in the renal artery and aorta, respectively ($p < 0.05$). Aging had no effect on NADPH oxidase-dependent and -independent contractions to ET-1 in the renal artery. In contrast, contractions to ET-1 were markedly reduced in the aged aorta (5-fold, $p < 0.01$ vs. young) and no longer sensitive to gp91ds-tat.
Significance: The results suggest an age-dependent heterogeneity of NADPH oxidase-mediated vascular contractions to ET-1, demonstrating an inherent resistance to functional changes in the renal artery but not in the aorta with aging. Thus, local activity of NADPH oxidase differentially modulates responses to ET-1 with aging in distinct vascular beds.

Introduction

Endothelin-1 (ET-1) is the predominant isoform of three distinct isopeptides constitutively secreted by endothelial and other vascular cells, and the most potent endogenous vasoconstrictor known (Yanagisawa et al., 1988; Kohan et al., 2011). The renal artery is particularly sensitive to ET-1 (Clozel & Clozel, 1989; Pernow et al., 1989; Widmer et al., 2006), and an increase in renal artery tone may lead to reduced kidney perfusion and subsequent activation of the renin–angiotensin system, which contributes to the ET-1-dependent regulation of basal vasomotor tone and blood pressure (Kohan et al., 2011; Haynes & Webb, 1994; Barton & Shaw, 1997). However, ET-1 also induces vascular oxidative stress, inflammation and remodeling (Amiri et al., 2004, 2008). Indeed, ET-1 contributes to vascular stiffening and calcification with aging, which are all major independent cardiovascular risk factors and associated with cardiovascular complications such as myocardial infarction, stroke, and renal injury (Zieman et al., 2005).

ET-1 activates two G protein-coupled receptors, ET_A and ET_B (Kohan et al., 2011). In the vascular wall, smooth muscle cells predominantly express ET_A receptors to mediate vasoconstriction, although contractions in response to ET_B receptor activation have also been reported for some vascular beds (Kohan et al., 2011). However, ET_B receptors are predominantly found on endothelial cells, where their activation results in the release of the vasodilators nitric oxide (NO) and prostacyclin; moreover, ET_B receptors are important for ET-1 clearance (Kohan et al., 2011). ET-1 acts in concert with other endothelium-derived contracting factors to balance the activity of endothelium-derived relaxing factors (Feletou & Vanhoutte, 2006). However, vascular aging impairs endothelial cell function favoring the production of contracting factors, including ET-1 (Barton, 2010; Seals et al., 2011). We have previously shown that aging increases circulating ET-1 levels, functional

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endothelin converting enzyme activity in the aorta, as well as ET-1 expression in conduit and renal arteries of otherwise healthy, normotensive animals (Barton et al., 1997; Goettsch et al., 2001). Accordingly, aging augments endothelial ET-1 expression (Donato et al., 2009) and ET-1-dependent vascular tone in human arteries (Van Guilder et al., 2007; Thijssen et al., 2007; Westby et al., 2011). These findings point towards an increase in ET-1 bioactivity with vascular aging, as also evidenced from the increased exocytotic ET-1 release in aged endothelial cells (Goel et al., 2010).

Many of the detrimental effects of vascular aging have been attributed to the increased generation of oxygen-derived free radicals, particularly superoxide (Barton, 2010; Seals et al., 2011; Oudot et al., 2006; Donato et al., 2007). Although reactive oxygen species can stimulate ET-1 production, ET-1 on the other hand may also induce superoxide generation by activating NADPH oxidase (Pollock & Pollock, 2005). In young rats, ET-1 enhances NADPH oxidase activity in carotid arteries (Li et al., 2003), and induces contractions of renal arteries and aorta that are partly mediated by NADPH oxidase-derived superoxide (Loomis et al., 2005; Just et al., 2008). Moreover, activation of vascular NADPH oxidase is likely involved in impaired endothelium-dependent vasodilation and vascular remodeling due to ET-1 overproduction in transgenic mice (Amiri et al., 2004). Likewise, NADPH oxidase has been identified as an important source of ET-1 stimulated superoxide production in mammary arteries and saphenous veins of patients with coronary artery disease (Cerrato et al., 2012). These findings suggest that generation of NADPH oxidase-derived superoxide may contribute to the ET-1-dependent regulation of vascular homeostasis in physiology and disease.

It is however not known whether vascular aging affects contractile responses to ET-1 mediated by NADPH oxidase. Given the physiological importance and high sensitivity of the renal vasculature to ET-1 (Kohan et al., 2011; Clozel & Clozel, 1989; Pernow et al., 1989; Widmer et al., 2006), the present study was therefore designed to determine whether age affects ET-1-induced contractions, particularly through NADPH oxidase, in the renal artery. Parallel experiments were conducted in the aorta, which has previously been shown to be sensitive to ET-1-related vascular aging (Barton et al., 1997; Goettsch et al., 2001).

Materials and methods

Materials

ET-1 was from American Peptide (Sunnyvale, CA, USA), the NADPH oxidase-selective inhibitor gp91ds-tat (Rey et al., 2001) from Anaspec (Fremont, CA, USA), and the NO synthase inhibitor L-N^G-nitroarginine methyl ester (L-NAME) from Cayman Chemical (Ann Arbor, MI, USA). All other drugs were from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions were prepared according to the manufacturer's instructions, and diluted in physiological saline solution (PSS, composition in mmol/L: 129.8 NaCl, 5.4 KCl, 0.83 $MgSO_4$, 0.43 NaH_2PO_4, 19 $NaHCO_3$, 1.8 $CaCl_2$, and 5.5 glucose; pH 7.4) to the required concentrations before use.

Animals

Young and old male C57BL6 mice (4 and 24 months of age, mean body weight 29 ± 1 g and 31 ± 1 g, respectively, Harlan Laboratories, Indianapolis, IN) were bred and housed at the animal research facility of the University of New Mexico Health Sciences Center. Animals had free access to standard rodent chow and water, with a 12 hour light–dark cycle. All procedures were approved by the University of New Mexico Institutional Animal Care and Use Committee and carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Isolated vessel preparation

After mice were euthanized by intraperitoneal injection of sodium pentobarbital (2.2 mg/g body weight), renal arteries and the abdominal aorta were immediately excised and transferred into cold (4 °C) PSS. Vessels were carefully dissected free from adherent connective tissue and fat, cut into rings 2 mm in length, and transferred to organ chambers of a Mulvany–Halpern myograph (620M Multi-Channel Myograph System, Danish Myo Technology, Aarhus, Denmark) containing PSS. Renal artery rings were mounted using two 25 μm tungsten wires threaded through the vessel lumen and secured to mounting jaws, whereas abdominal aorta rings were transferred onto 200 μm stainless steel pins. The jaws or pins were connected either to a micropositioner or to a force transducer for the recording of isometric tension.

Vascular pharmacology studies

After equilibrating for 30 min in PSS (37 °C, pH 7.4, bubbled with 21% O_2, 5% CO_2 and balanced N_2), vascular rings were stretched stepwise until the optimal passive tension for generating force during isometric contraction was reached. Vessels were equilibrated for an additional period of 30 min (renal artery) or 45 min (abdominal aorta), and repeatedly exposed to K^+ (PSS with equimolar substitution of 60 mmol/L potassium for sodium) to confirm vascular smooth muscle integrity and to determine maximal contractile responses. The role of NADPH oxidase was studied by randomly treating the left or right renal artery as well as one of two neighboring rings of the abdominal aorta with the Nox-selective inhibitor gp91ds-tat (3 μmol/L for 30 min) (Rey et al., 2001; Park et al., 2004; Miller et al., 2005). Gp91ds-tat consists of a 9-amino acid peptide of the Nox1/Nox2 catalytic subunits of NADPH oxidase (at the interface with $p47^{phox}$, which is essential for activity) linked to the 11-amino acid HIV-tat peptide, which facilitates cellular entry (Rey et al., 2001; Brandes et al., 2010). After the incubation period, vessels were exposed to cumulative concentrations of ET-1 (0.1–100 nmol/L) or to the predominantly α_1-adrenergic agonist phenylephrine (1 μmol/L). All experiments were performed following inhibition of NO synthase by L-NAME (300 μmol/L for 30 min) to unmask contractions in the aorta (Widmer et al., 2006), and to exclude ET_B receptor-stimulated NO release (Kohan et al., 2011) as well as potential differences in NO bioavailability between vascular beds and age groups (Barton, 2010; Seals et al., 2011).

Data calculation and statistical analyses

Data are expressed as mean ± SEM; *n* equals the number of animals used. Contractions to ET-1 are given relative to K^+ (60 mmol/L)-induced responses. Fitting of dose–response curves to calculate area under the curve (AUC), EC_{50} values (as negative logarithm, pD_2) and maximal responses was performed as described by DeLean et al. (1978). Data was analyzed using two-way analysis of variance (ANOVA) followed by Bonferroni's post-hoc test (Prism version 5.0 for Macintosh, GraphPad Software, San Diego, CA, USA). A $p < 0.05$ value was considered significant.

Results

The renal artery is resistant to ET-1-related functional aging

To study the functional effects of aging on ET-1-dependent vascular tone, we first determined contractile responses in young and old mice (4 and 24 months of age). ET-1 induced potent contractions in the renal artery of young animals that were 6-fold stronger compared to the abdominal aorta (102 ± 4% vs. 18 ± 4%, $n = 4–8$, $p < 0.001$, Fig. 1A). In the aorta, aging reduced contractions to ET-1 by 78% (from 18 ± 4% to 4 ± 1%, $n = 5–8$, $p < 0.01$, Fig. 1A), whereas there was no change in the renal artery (102 ± 4% vs. 92 ± 8%, $n = 4–5$, $p =$ n.s.

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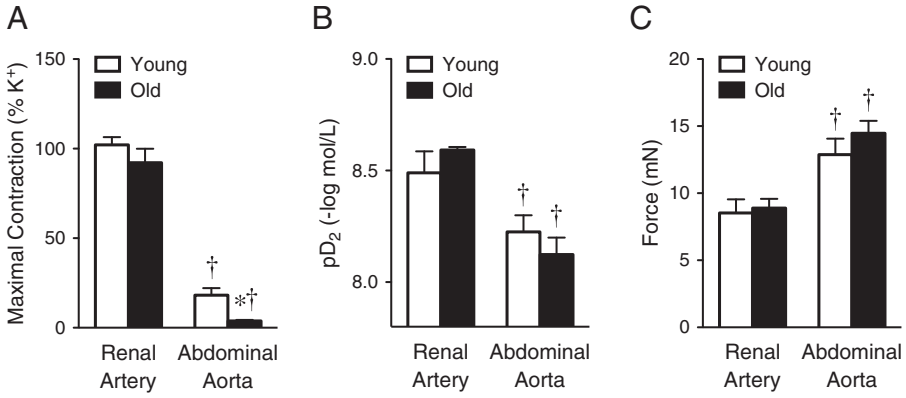

Fig. 1. Effect of aging on contractions to endothelin-1 and K^+ in the renal artery and abdominal aorta. Maximal effects (A) and the sensitivity (pD_2 values, B) of endothelin-1-induced contractions in young (4 months) and old (24 months) mice were calculated by fitting of dose–response curves (0.1–100 nmol/L) (DeLean et al., 1978). K^+ (60 mmol/L) was utilized to determine maximal smooth muscle contractile capacity for generating force (C). *$p < 0.01$ vs. young animals; †$p < 0.05$ vs. renal artery ($n = 4–12$).

vs. young, Fig. 1A). Consistent with these findings, the sensitivity to ET-1 was slightly but significantly greater in the renal artery compared to the abdominal aorta of young and old mice ($n = 4–8$, $p < 0.05$, Fig. 1B). Age-dependent differential effects on responses to ET-1 were likely not due to an altered contractile function of the smooth muscle, since the force response to K^+ (60 mmol/L) in either vascular bed was unaffected by aging (Fig. 1C). Taken together, these findings indicate that responses to ET-1 in the renal artery are highly potent and resistant to vascular aging.

Local activity of NADPH oxidase regulates ET-1-induced contractions

We next studied whether contractions to ET-1 depend on functional NADPH oxidase with vascular aging, a condition characterized by increased oxidative stress (Barton, 2010; Seals et al., 2011; Oudot et al., 2006; Donato et al., 2007). In renal arteries of both young and old animals, the NADPH oxidase-selective inhibitor gp91ds-tat (Rey et al., 2001) potently and equally reduced ET-1-induced contractions (50% reduction, $n = 4–6$, $p < 0.001$, Fig. 2), consistent with the preserved response to ET-1 with aging in this vessel. The sensitivity to ET-1 (pD_2 values) remained unaffected by gp91ds-tat (not shown). Similarly, contractile responses to the predominantly α_1-adrenergic agonist phenylephrine (1 μmol/L) did not differ between young and old animals (106 ± 5% vs. 102 ± 3%, $n = 5$, $p =$ n.s.) and were comparably reduced by gp91ds-tat, independent of age (25% and 22% reduction, $n = 5$, $p < 0.01$). These findings further corroborate the observation that NADPH oxidase-dependent and -independent responses in the renal artery are resistant to functional aging.

In the abdominal aorta, however, inhibition of NADPH oxidase activity in young animals reduced responses to ET-1 to the level seen in old animals (4-fold, from 18 ± 4% to 5 ± 1%, $n = 4–8$, $p < 0.05$, Fig. 3). In contrast, the blunted response to ET-1 in aged abdominal aortas was unaffected by inhibition of NADPH oxidase (4 ± 1% vs. 4 ± 2%, $n = 5$, $p =$ n.s., Fig. 3), indicating that aging reduces contractions to ET-1 in the abdominal aorta by abolishing the contribution of NADPH oxidase.

Discussion

The present study investigated how NADPH oxidase and the physiological aging process affect ET-1-dependent contractions in the renal artery and abdominal aorta of healthy mice. We show that ET-1 induces highly potent NADPH oxidase-dependent and -independent responses in the renal artery that are resistant to vascular aging. In contrast, ET-1-induced contractions in the abdominal aorta are weak and further reduced by aging due to loss of NADPH oxidase activity. These findings are the first demonstration of an age-dependent, localized role of NADPH oxidase in specific vascular beds that determines ET-1-dependent arterial tone and suggest that the renal artery is resistant to NADPH oxidase-related functional aging.

An augmented release of ET-1 and other contracting factors by endothelial cells plays a significant role in the pathophysiology of vascular aging (Barton, 2010; Seals et al., 2011). In addition to enhanced vasomotor tone (Van Guilder et al., 2007; Thijssen et al., 2007; Westby et al., 2011), age-dependent increases in the bioactivity of ET-1 have been implicated in vascular oxidative stress, inflammation and

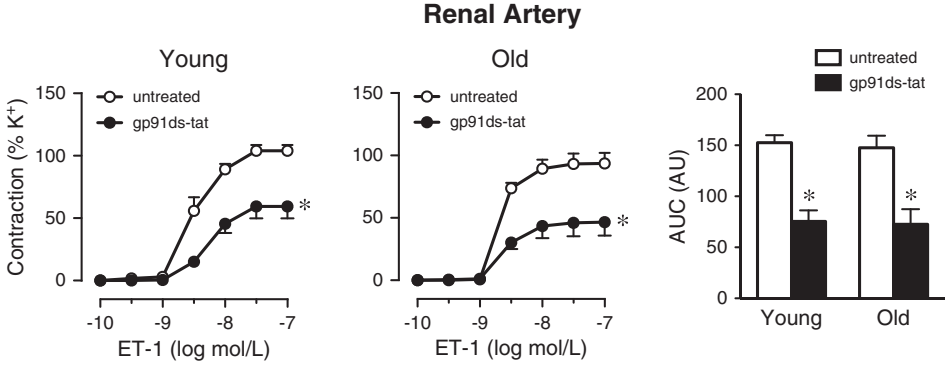

Fig. 2. NADPH oxidase-dependent contractions to endothelin-1 (ET-1) in the renal artery during vascular aging. Concentration-dependent responses to ET-1 were determined in young (4 months) and old (24 months) mice in the presence and absence of the NADPH oxidase-selective inhibitor gp91ds-tat (3 μmol/L). Area under the curve (AUC) of ET-1-induced contractions is expressed as arbitrary units (AU). *$p < 0.001$ vs. untreated ($n = 4–6$).

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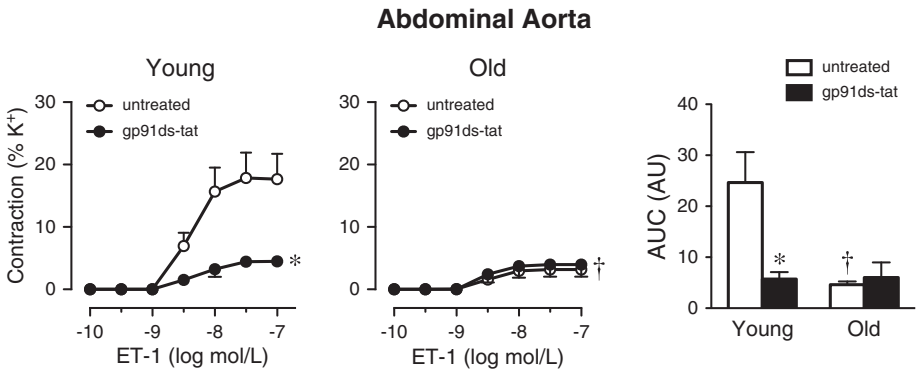

Fig. 3. Effect of aging on NADPH oxidase-dependent contractions to endothelin-1 (ET-1) in the abdominal aorta. Concentration-dependent responses to ET-1 were determined in young (4 months) and old (24 months) mice in the presence and absence of the NADPH oxidase-selective inhibitor gp91ds-tat (3 μmol/L). Area under the curve (AUC) of ET-1-induced contractions is expressed as arbitrary units (AU). $^*p < 0.05$ vs. untreated; $\dagger p < 0.05$ vs. young animals ($n = 4$–8).

remodeling (Amiri et al., 2004, 2008), which in turn promote arterial stiffening and calcification (Zieman et al., 2005). Although ET-1-induced contractions display a marked heterogeneity between vascular beds and are generally less potent in mice compared to other species (Widmer et al., 2006; Wiley & Davenport, 2004), the renal vascular bed and particularly the main renal artery are highly sensitive to ET-1 as shown in the present and previous studies (Kohan et al., 2011; Clozel & Clozel, 1989; Pernow et al., 1989; Widmer et al., 2006). We now demonstrate that the potent responses to ET-1 are preserved in old mice, suggesting that the responsiveness to ET-1 in the renal vasculature, unlike the aorta, remains intact with aging. It is, however, important to note that acute, exogenous application of ET-1 might not necessarily reflect its chronic autocrine and paracrine actions within the vascular microenvironment (Kohan et al., 2011). In fact, aging has been found to increase endogenous vascular and renal ET-1 bioavailability (Goettsch et al., 2001; Donato et al., 2009; Lattmann et al., 2005), which in turn can down-regulate ET_A receptor expression (Lattmann et al., 2005; Clozel et al., 1993; Kuc & Davenport, 2000), potentially leading to reduced responsiveness to ET-1 as observed in other vascular beds (Barton et al., 1997; Ishihata et al., 1991; Shipley & Muller-Delp, 2005; Modrick et al., 2012). However, the preserved, potent contractions to ET-1 in the aged renal artery argue against such a compensatory change in vascular ET_A receptor function. Despite the fact that the kidney already displays the greatest ET-1 concentration of all tissues under physiological conditions (Kitamura et al., 1989) that increases further with aging (Lattmann et al., 2005), other mechanisms known to potentiate ET-1-induced responses such as cross-talk with the renin-angiotensin or the adrenergic systems (Kohan et al., 2011) might also contribute to the maintained high responsiveness to ET-1 in the aged renal artery.

The finding that ET-1-induced contractions remain unaffected with vascular aging in the renal artery but not in the abdominal aorta is strengthened by the fact that responses are independent of NO bioavailability, which may be affected by both aging and superoxide production (Barton, 2010; Seals et al., 2011). Thus, since all experiments were performed in the presence of the NO synthase inhibitor L-NAME, the observed age-dependent differences in ET-1-dependent contractility between vascular beds are unrelated to basal or endothelin ET_B receptor-stimulated NO release as previously found in rat coronary arterioles (Shipley & Muller-Delp, 2005). The use of the NO synthase inhibitor also excludes potential confounding effects on vascular reactivity resulting from altered expression levels of endothelial and inducible NO synthase with aging (Goettsch et al., 2001). Moreover, it is unlikely that the blunted response to ET-1 in the abdominal aorta of aged animals was due to a non-specific decline in smooth muscle contractile function, since the responsiveness to K^+ did not change with age. Furthermore, K^+-induced contractions in abdominal aortas and renal arteries varied by only 1.5-fold and thus cannot account for the observed marked differences in ET-1-dependent contractility between those vascular beds.

Increased production of oxygen-derived free radicals by NADPH oxidase, uncoupled endothelial NO synthase, and xanthine oxidase has been implicated in the physiology of vascular aging (Barton, 2010; Seals et al., 2011; Oudot et al., 2006; Donato et al., 2007). Since vascular responses to ET-1 partly depend on its ability to stimulate superoxide production by NADPH oxidase (Amiri et al., 2004; Pollock & Pollock, 2005; Li et al., 2003; Loomis et al., 2005; Just et al., 2008), we hypothesized that NADPH oxidase function might, at least in part, determine contractile responses to ET-1 with aging. In arteries of young mice, we found that the NADPH oxidase-selective inhibitor gp91ds-tat (Rey et al., 2001) largely reduces contractions to ET-1. Gp91ds-tat was originally designed to be a selective inhibitor for the Nox2 catalytic subunit (Rey et al., 2001), but likely also inhibits the assembly of Nox1 due to its high sequence homology to the Nox2 isoform (Brandes et al., 2010; Williams & Griendling, 2007). Thus, the data from the present study suggest that ET-1-dependent contractions depend on the activity of the inducible, superoxide-generating Nox1 or Nox2 isoform (Brandes et al., 2010). In line with our observations, a previous study in rats demonstrated that apocynin attenuates ET-1-dependent reductions in renal blood flow by 35% (Just et al., 2008), although apocynin might not be considered a specific NADPH oxidase-specific inhibitor, since it may also exert potent antioxidant and other effects (Brandes et al., 2010). In the present study we now demonstrate that contractions to ET-1 are indeed NADPH oxidase-dependent, and that the NADPH-oxidase dependent contribution to the ET-1 response remains unaffected by aging in the renal artery. In contrast, NADPH oxidase-dependent contractions to ET-1 are abolished in the abdominal aorta of otherwise healthy aged animals, indicating an age-dependent, localized role of NADPH oxidase in the regulation of ET-1-dependent responses in the arterial vascular tree. Of note, these findings are not inconsistent with previous reports showing increased vascular ROS activity with aging (Barton, 2010; Seals et al., 2011; Oudot et al., 2006; Donato et al., 2007), since agonists other than ET-1 may differentially regulate functional NADPH oxidase activity. Furthermore, alternative vascular sources of ROS might become activated in the murine renal artery and abdominal aorta with aging (Barton, 2010; Seals et al., 2011).

To the best of our knowledge, the present study is the first demonstration of a specific role of NADPH oxidase activity for the regulation of vasomotor tone in different vascular beds. However, the underlying mechanisms remain unclear. Different levels of expression or activity of components of the NADPH oxidase multienzyme complex that are sensitive to vascular aging, such as Nox2 and p47phox (Oudot et al., 2006; Donato et al., 2007; Takenouchi et al., 2009), might contribute to the observed heterogeneous responsiveness to ET-1 in different vascular beds. Furthermore, activity of NADPH oxidase might be locally regulated by factors known to contribute to oxidative stress-driven

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vascular aging, such as the aging-associated genes klotho (Wang et al., 2012) and silent information regulator 1 (SIRT1) (Zarzuelo et al., 2013), or the transcription factor JunD (Paneni et al., 2013).

Conclusions

The present study demonstrates that contractions to ET-1 in the aorta and renal artery of healthy young mice to a substantial degree depends on NADPH oxidase activity, one of the major vascular sources of reactive oxygen species. With aging, localized regulation of NADPH oxidase activity appears to determine the functional response to ET-1 in different vascular beds. Of note, the preserved, highly potent and partly NADPH oxidase-dependent reactivity to ET-1 in the aged renal artery might have clinical implications. Indeed, both oxidative stress and increased ET-1 bioactivity have been implicated in age-dependent impaired endothelial cell function (Barton, 2010; Seals et al., 2011), which is associated with arterial stiffening and sclerosis (Zieman et al., 2005), and consecutive renal injury (Zhou et al., 2008). Antagonizing ET-1-dependent effects have previously been found to improve endothelial function in individuals with early coronary artery disease (Reriani et al., 2010), and even to reverse renal aging and glomerular vascular injury (Ortmann et al., 2004). Moreover, treatment with an ET_A receptor antagonist reduces arterial stiffness in patients with chronic kidney disease (Dhaun et al., 2009). Thus, similar treatment strategies might be suitable to protect from age-induced changes in the renal vascular bed, such as impaired renal hemodynamics, renal arterial sclerosis and subsequent renal ischemia that are critically involved in progressive loss of kidney function with aging (Zhou et al., 2008).

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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