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ORIGINAL CONTRIBUTION

Sex-difference in expression and function of beta-adrenoceptors in macrovessels: role of the endothelium

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Abstract Estrogen modulates adrenergic reactivity of macrovessels, resulting in weaker α-adrenergic vasoconstriction in females than males. However, the mechanisms governing this important sex-specific difference are not well understood. We hypothesized that vessels of females express more dilatory β-adrenoceptors, which counteract constrictive effects of α-adrenoceptors. This hypothesis was tested using aortas of normotensive (WKY) and hypertensive rats (SHR), along with human mammary artery. Selective blockade of β_1 (CGP20712) or β_3 (SR59230A), but not β_2 (ICI118,551) adrenoceptors, greatly increased α-adrenergic constriction (norepinephrine) of aorta in female SHRs, but not in male SHRs at 12 weeks of age. Consistently, the selective β_1/β_2 (isoproterenol) and β_3-adrenergic (BRL37344) relaxation was stronger in female SHRs than in males. Removal of endothelium and use of L-NMMA abolished sex-difference in α-adrenergic constriction and β-adrenergic relaxation. Immunostainings revealed endothelial localization of β_1- and β_3-adrenoceptors. mRNA levels of aortic β_1- and β_3-, but not β_2-adrenoceptors were markedly higher in female than in male SHRs. The sex-specific differences in α-adrenergic constriction and β-adrenoceptor mRNA levels were age-dependent, predominantly present up to 29 weeks and disappeared at 36 weeks of age. The sex-specific difference was not strain-dependent and was similarly present in normotensive WKY rats. Human mammary artery of women showed a weaker α-adrenergic constriction than arteries of men. This sex-specific difference was prominent at 45–65 years and disappeared with aging. Our results convincingly demonstrate that female macrovessels express more dilatory β_1- and β_3-adrenoreceptors than male vessels with a predominant endothelial localization. This sex-specific difference is functionally relevant in young adults and is attenuated with aging.

Keywords Beta-adrenoceptors · Vascular endothelium · Vascular tone regulation · Sex-difference · Human mammary artery

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Introduction

The sympathetic nervous system (SNS) is the major regulator of vascular tone [20, 45]. The major transmitter of sympathetic activation in vasculature norepinephrine targets two major classes of receptors—alpha (α) and beta (β), collectively termed adrenoceptors [20, 45]. While α-adrenoceptors mediate vasoconstriction, β-adrenoceptors mediate vasodilatation [42, 45]. So far three types of β-adrenoceptors were identified—β_1, β_2 and β_3 [38, 43]. Although in some organs these receptors have a relatively specific distribution [43], all three forms are present in the vasculature, and strongly involved in regulation of vascular tone [6, 38]. Although adrenoceptor-dependent regulation

of vessel tone is highly prominent at the level of small resistance vessels [18], also in large arteries both α- and β-adrenoceptors are expressed [6, 20, 38, 43]. The balance of α- and β-adrenoceptor-mediated effects in epicardial coronary arteries has been shown to be shifted to α-vasoconstriction during atherosclerosis [3, 31] which may facilitate acute ischemia precipitation. This demonstrates that the balance between α-vasoconstrictive and β-vasodilatory adrenoceptors can strongly modulate vascular reactivity to adrenergic stimulation. More chronically, adrenergic receptors of macrovessels, specifically α-adrenoceptors, are implicated in mediation of adverse remodeling during hypertension [11, 13, 24, 32]. This includes direct effects of norepinephrine on aortic hyperplasia and fibrosis [11], which may result in enhanced aortic stiffness [4, 7, 40]. Consecutively, a stiffer aorta may increase systolic pressure and hence augment left ventricular afterload, which in the long term may result in ventricular dysfunction [40]. Furthermore, over-activation of SNS may lead to endothelial dysfunction [1]—another factor potentially contributing to adverse structural alterations in aorta [2, 33]. These adverse effects of SNS seem to be mediated by norepinephrine primarily via α-adrenoceptors [15]. In contrast, β-adrenoceptors are considered to have more beneficial effects with respect to large vessel remodeling [14, 24, 34, 41]. This may be largely due to the effect of β-adrenoceptor activation on the bioavailability of nitric oxide (NO), which is known to have protective effects in vessels remodeling [14, 24, 34, 41]. Because bioavailability of NO requires a normal endothelial function [15, 37], impaired β-adrenoceptor signaling and reduced bioavailability of NO during endothelial dysfunction may be related to each other.

An overwhelming amount of data demonstrates that reactivity of resistance vessels to adrenergic stimulation is sex-specific [12, 16, 19, 21, 22, 28]. While more studies report sex-differences with selective β-adrenergic stimulation [16, 19, 21], demonstrating that females react stronger than males, few studies demonstrate that vessels of females react with less vasoconstriction to norepinephrine than males [22, 28]. Sex-specific responses to adrenergic stimulation are supported by the evidence that estrogen receptors are expressed on endothelial (ECs) and vascular smooth muscle (SMCs) cells [29] and under the influence of estrogens these receptors may change vascular reactivity to adrenergic stimulation [5, 27, 44]. The estrogen-dependent modulation of resistance vessels may represent one mechanism that contributes to the difference in systolic blood pressure between females and males [39]. On the level of macrovessels the sex-specific modulation of adrenergic stimulation may at least partially explain the lower incidence of vascular disease in premenopausal women compared to men [36]. It is imperative to note that while many studies point to the existence of sex-specific reactivity to adrenergic stimulation, the evidence also suggests that males have a preponderance toward impaired NO production due to endothelial dysfunction compared to age-matched females [28]. Because bioavailability of NO may strongly determine the sensitivity to adrenergic stimulation [22], it may be expected that males with endothelial dysfunction respond with stronger vasoconstriction toward norepinephrine and less vasodilation toward isoprenaline than females. Because there are no studies to date, which comprehensively address sex-differences in expression and balance of vascular β-adrenoceptors, it is still unclear whether a sex-difference in reactivity to adrenergic stimulation is a result of impaired NO bioavailability due to endothelial dysfunction in males or whether there is primarily a sex-specific difference in expression of β-adrenoceptors. In our work, we tested the hypothesis that there is a sex-specific difference in the expression of β-adrenoceptors in aorta between female and male SHRs, which is of functional relevance. In addition, we addressed the importance of the endothelium as a prevailing site of sex-specific adrenoceptor expression with functional importance. Because endothelial function is known to change in response to age [8], we included SHRs with ages ranging from 5 to 36 weeks. In addition, we tested whether differences found in the SHR model are of more general importance by performing similar experiments in WKY. Finally, to obtain more insights into the translational relevance of our results, we investigated the sex-specific reactivity to norepinephrine using isolated human mammary artery from patients undergoing bypass surgery.

Methods

Animals

Handling of animals was approved by the institutional ethics committee and the local authorities (permission 24-9168.24-1/2012-16 and 24-9168.11-1/2009-46). Spontaneously hypertensive rats (SHR) were used as an animal model of hypertension in comparison with their normotensive genetic controls—Wistar Kyotos (WKY). 5-, 12-, 29- and 36-week-old SHRs and WKYs were used for the study. The major experiments were performed on SHRs at 12 weeks of age, because neither male nor female SHRs at this age had endothelial dysfunction, but they were hypertensive. Older SHRs were used to test the hypothesis under the conditions of aging, chronic hypertension and endothelial dysfunction. Young SHRs at 5 weeks of age were used as normotensive controls to SHRs at 12 weeks of age. Six rats per group (sex, strain and age) were used. Female and male rats were purchased from Charles River

Table 1 Body weight (g)

Age (weeks)	Female		Male	
	WKY	SHR	WKY	SHR
5	85 ± 6	89 ± 4	111 ± 7	115 ± 6
12	201 ± 9	209 ± 3	310 ± 9	318 ± 5
29	265 ± 9	271 ± 8	569 ± 9	571 ± 8
36	280 ± 5	289 ± 11	601 ± 7	603 ± 9

Laboratories (Germany) at least 2 weeks younger than the study age group. Aortas of β_1/β_2 double knockout mice for testing the specificities of anti-β_1- and β_2-adrenoceptor antibodies were used. Isolation of aorta was performed after euthanasia with intraperitoneal injection of ethyl carbamate (1.3 g/kg body weight). Euthanasia was proved with disappearance of pain and corneal reflexes. Body weights of animals are presented in Table 1.

Materials

Selective blockers of β_1-(CGP 20712), β_2-(ICI 118.551) and β_3-(SR 59230A) adrenoceptors, as well as β_3-agonist (BRL 37344) and isoprenaline (cat. No. 1747) were purchased from Tocris. Norepinephrine (cat. No. A7257) and phenylephrine (cat. No. P6126) were purchased from Sigma, whereas L-NMMA (cat. No. 106-C01-G001) from Alexis. Anti-β_1 (sc568), -β_2 (sc569) and -β_3 (sc50436) primary antibodies were obtained from Santa Cruz.

Assessment of aortic reactivity

A Mulvany myograph was used to assess development of aortic wall tension and relaxation in response to norepinephrine and isoprenaline, respectively. Assessment was performed as previously described [17, 46]. After isolation, thoracic aortas were prepared by removing adherent adipose tissue and cut into 2 mm rings. The rings were then mounted on a wire transducer of the myograph (Power Lab/400, AD-Instruments, Spechbach, Germany) located in an organ chamber filled with physiological salt solution (equilibrated with 5/95% CO_2/O_2, pH 7.4, 37 °C). Vessel rings were stretched with a resting tension equivalent to an intraluminal pressure of 100 mm Hg. Contraction was induced with potassium-enriched solution, containing mmol/L concentrations of 123.7 KCl, 1.17 $MgSO_4$, 1.18 KH_2PO_4, 25 $NaHCO_3$, 5.5 glucose, 0.027 EDTA and 2.5 $CaCl_2$. After this, various concentrations of norepinephrine and isoprenaline were applied to test development of tension and vasorelaxation, respectively. A selective β_3-adrenoceptor agonist (BRL 37344) was also used. These experiments were performed with and without selective blockers of β-adrenoceptor subtypes. In some experiments, norepinephrine and isoprenaline were used on aortic segments with and without endothelium. Removal of endothelium was performed using a cotton wire as previously described [23, 35]. In addition, the vessels were tested for endothelium-dependent vasorelaxation using acetylcholine in various concentrations.

Human mammary arteries

Data of myograph measurements on human mammary artery ($n = 133$) were used to address the question whether macrovessels of women and men respond differently in response to a standardized maximal norepinephrine (10 µmol/L) stimulus. Part of this work has been published [17]. However, sex-specific difference in responses to norepinephrine had not been analyzed. In this work, norepinephrine was used as a standard procedure to precontract vessels to assess ACh-dependent vasorelaxation. We now analyzed data from 103 vessels from men and 30 vessels from women patients permitting to search retrospectively for sex-specific differences of the vasoconstrictive effect of norepinephrine. The use of these vessels had been approved by the Ethics Committee of the Medical Faculty, TU Dresden (EK 307-12-2007) conforming to NIH Guidelines. The experimental procedures have been described in detail in Garbe at. al. [17].

Measurement of systolic blood pressure

A tail-cuff method employing electro-sphygmomanometer (FMI, Seeheim, Germany) was used to measure systolic blood pressure (SBP) on conscious animals as previously described [30]. Measurements were performed in the time frame of 8 to 11 am, twice for each respective age group. Average values from two measurements of SBP are presented.

RNA isolation and reverse transcription qPCR (RT-qPCR)

Isolation of total mRNA and RT-qPCR was performed as previously described [35]. Briefly, TRIzol Reagent (Life Technologies) was used for isolation of total mRNA from aorta according to the user manual. Tissue sections were homogenized using a power homogenizer (Polytron). 1 µg of total mRNA was reverse transcribed using high-capacity cDNA reverse transcription kit (Invitrogen) followed by cDNA amplification using SensiMix SYBR (Invitrogen) and respective primers (Table 2). The kits for reverse transcription and cDNA amplification were used as instructed by the manufacturer.

Table 2 List of primers

β1	5′-CAGGTGAACTGTAACTGACT-3′ (sense)
	5′-CTCACCTCACAGGTCAGGAG-3′ (antisense)
β2	5′-ACGGTTTCCTAAAGCGATTC-3′ (sense)
	5′-TTGGTAACCTGGTCGTCTG-3′ (antisense)
β3	5′-TCCTATTCCCGCTCATCT-3′ (sense)
	5′-CGAGCTTCCGGATACGA-3′ (antisense)

Immunofluorescent staining

Aorta was stained for β-adrenoceptors as previously described [35]. Briefly, 5-µm-thick paraffin tissue sections were dewaxed with xylene (3 × 5 min) followed by hydration with ethanol (2 min in 100, 96 70, 40%) and distilled water. Then, slides were microwave irradiated for 10 min at 1000 W in 0.01 M sodium citrate buffer (pH 6.0) followed by cooling down for 30 min at RT. Aortic sections were blocked in 1% bovine serum albumin for 20 min at RT. Then, sections were incubated in primary antibodies diluted (1:100) in antibody diluent at 4 °C overnight. Unbound primary antibodies were removed by washing three times 5 min with PBS containing 0.1% Tween followed by application of secondary antibodies (1:200) for 1 h at RT. Sections were mounted with mounting media and imaged using fluorescence microscopy (ApoTome, Zeiss, Germany).

Statistical analysis

Mean values from six independent experiments were analyzed using two-way ANOVA. Bonferroni post hoc test was used to correct multiple comparisons. Analysis was performed in GraphPad Prism version 6 for windows (GraphPad Software, San Diego, CA, USA). Significance was accepted at a P value smaller than 0.05. All values are expressed as mean ± SEM.

Results

Aorta of young female SHRs show weaker α-adrenergic wall tension and stronger β-adrenergic relaxation than males

Because α-adrenoceptors are responsible for vasoconstriction, whereas β-adrenoceptors mediate vasodilation [20, 42, 45], the quantitative balance of these receptors in aorta may affect aortic reactivity to the adrenergic stimulus. Norepinephrine increased aortic wall tension both in female and male SHRs in a concentration-dependent manner (Fig. 1a). However, aorta of female SHRs showed significantly ($P < 0.01$) lower tension to norepinephrine than males. To test whether this sex-specific reactivity to norepinephrine is not limited to SHRs we used their genetic controls—female and male WKYs of the same age and assessed reactivity of aorta to norepinephrine. Similar to SHRs, female WKYs developed significantly ($P < 0.05$) lower tension than males (Fig. 1b). Notably, this difference was not as pronounced as that seen in SHRs. Aortic relaxation to the β_1/ β_2-agonist isoprenaline was significantly ($P < 0.01$) stronger in female SHRs than males (Fig. 1c). In WKYs, the difference was less pronounced, but still significant ($P < 0.05$, Fig. 1d). Aorta of female SHRs and WKYs reacted with a ~20% relaxation to the β_3 agonist (BRL37344), whereas aorta of males did not relax at all (Fig. 1e, f). This sex-specific difference in β_3 agonist-induced aortic relaxation was significant ($P < 0.01$). Unlike with norepinephrine, neither WKYs nor SHRs showed a sex-related difference in KCl-induced vasoconstriction (Table 3). Because bioavailability of NO can affect the reactivity of vessels to adrenergic stimulation, we assessed endothelium-dependent aortic relaxation in response to ACh. Endothelium-dependent aortic relaxation in response to ACh was not different between female and male SHRs (Table 4). Moreover, aortic relaxations in SHRs were not different from WKYs. These findings indicate that at an age of 12 weeks SHRs did not have altered endothelial function and that the stronger reactivity of male SHRs to norepinephrine was unlikely a result of impaired availability of NO in males.

Selective blockade of β_1- and β_3-adrenoceptors equalizes sex-difference in α-adrenergic wall tension and β-adrenergic relaxation of aorta

To identify the role of β-adrenoceptor subtypes in sex-differences of aortic reactivity to norepinephrine and isoprenaline, aortas were pre-incubated with selective blockers of β-adrenoceptors for 30 min before application of norepinephrine. The β_1-blocker significantly ($P < 0.01$) increased aortic tension in response to norepinephrine in female SHRs, but not in males (Fig. 2a, b). The β_2-blocker significantly increased aortic tension in response to norepinephrine both in female ($P < 0.05$) and male ($P < 0.05$) SHRs (Fig. 2c, d). Similar to the blockade of β_1-adrenoceptors, β_3-blockade significantly increased aortic tension to norepinephrine in female but not in male SHRs ($P < 0.05$, Fig. 2e, f). Pretreatment of aortas with a selective β_1-blocker significantly decreased aortic relaxation to isoprenaline ($P < 0.01$) in female SHRs, but not in males (Fig. 3a, b), whereas the β_2-blocker significantly decreased aortic relaxation towards isoprenaline both in female ($P < 0.05$) and male ($P < 0.01$) SHRs (Fig. 3c, d). Use of a β_3-adrenoceptor blocker abolished the β_3-agonist-induced aortic relaxation in female SHRs, but did not affect aortas of males (Fig. 3e, f). These results demonstrate that, while blockade of β_2-adrenoceptors does not affect sex-

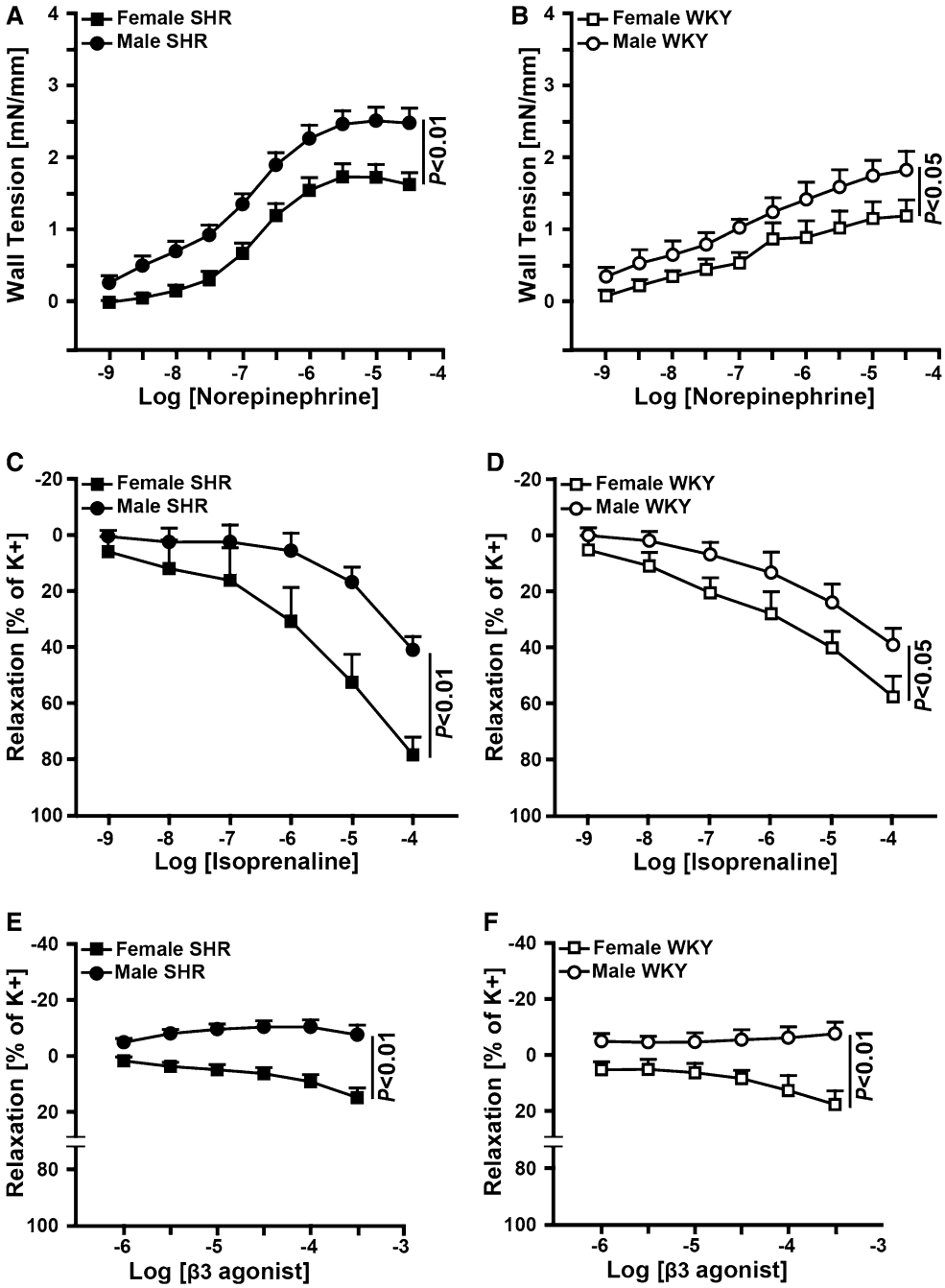

Fig. 1 Development of α-adrenergic wall tension and β-adrenergic relaxation in aorta of 12-week-old female and male SHRs and WKYs. **a** Development of aortic wall tension in response to norepinephrine in female and male SHRs and **b** WKYs. **c** Relaxation in response to isoprenaline in female and male SHRs and **d** WKYs. **e** Relaxation in response to β_3-agonist (CGP20712) in female and male SHR and **f** WKYs. Aorta of female SHRs and WKYs generally developed less tension in response to norepinephrine, but relaxed stronger to isoprenaline and β_3-adrenoceptor agonist than males. $n = 6$

Table 3 Maximal wall tension of aorta to KCl (mN/mm)

Age (weeks)	Female		Male	
	WKY	SHR	WKY	SHR
5	3.2 ± 0.14	2.7 ± 0.08	2.9 ± 0.06	2.1 ± 0.09
14	2.3 ± 0.19	2.94 ± 0.2	2.5 ± 0.35	2.8 ± 0.06
29	2.5 ± 0.15	2.7 ± 0.25	2.4 ± 0.2	2.7 ± 0.08
36	3.6 ± 0.51	3.3 ± 0.41	3.4 ± 0.15	3.2 ± 0.28

difference in adrenergic constriction and relaxation of aorta in SHRs, selective blockade of β_1- and β_3-adrenoceptors diminishes this difference.

Aorta of young female SHRs has higher mRNA levels of β_1- and β_3-adrenoceptors than males

In support of the functional assessments, mRNA levels of β-adrenoceptors were assessed. mRNA level of β_1-

Table 4 Maximal relaxation of aorta to ACh and SNP (% of norepinephrine)

Age (weeks)		Female		Male	
		SHR	WKY	SHR	WKY
5	ACh	102.1 ± 4.9	102.6 ± 1.5	100.7 ± 1.4	103.1 ± 1.3
	SNP	103.7 ± 2.6	103.4 ± 1.9	102.4 ± 0.8	100.6 ± 1.8
14	ACh	92.5 ± 5.2	104.9 ± 22.6	95.6 ± 5.6	98.2 ± 5.9
	SNP	100.9 ± 28.1	114.6 ± 7.8	116.4 ± 18.8	104.6 ± 12.1
29	ACh	94.4 ± 9.5	98.5 ± 11.5	53.5 ± 7.7** ##	93.9 ± 7.9
	SNP	107.5 ± 5.9	91.8 ± 14.8	104.7 ± 15.3	101.7 ± 6.4
36	ACh	18.1 ± 6.5##	101.4 ± 13.3	6.1 ± 9##	83.9 ± 2.1
	SNP	66.5 ± 11.3#	119.1 ± 11.7	41.2 ± 12.1##	95.6 ± 6

$n = 6$

ACH acetylcholine, *SNP* sodium nitroprusside

** $P < 0.01$ versus age-matched female SHR

# $P < 0.05$, ## $P < 0.01$ versus age- and sex-matched WKY

adrenoceptors in aorta of male SHRs was significantly ($P < 0.01$) lower than that in female aorta (Fig. 4a). This sex-specific difference in β_1-adrenoceptor expression was less pronounced but still significant ($P < 0.05$) in aorta of WKYs (Fig. 4b). There was no sex-specific difference in mRNA level of β_2-adrenoceptors either in SHRs or in WKYs (Fig. 4c, d). Similar to β_1, mRNA of β_3-adrenoceptor was also significantly ($P < 0.01$) lower in male than in female aortas from SHRs and WKYs (Fig. 4e, f).

Endothelial β-adrenoceptors and NO govern sex-specific difference in constriction and relaxation of aorta

Because activation of vascular β-adrenoceptors was proposed to be linked to production of NO [14], we further tested whether endothelium contributes to the sex-specific difference in aortic relaxation and constriction in SHRs. While isoprenaline caused pronounced relaxation in intact aorta of female SHRs, endothelium-denuded aortas did not relax in response to isoprenaline (Fig. 5a). A similar effect was observed in aortas from male SHRs (Fig. 5b). Compared to intact aorta, the β_3-agonist failed to cause relaxation in endothelium-denuded aorta of female SHRs (Fig. 5c). There was no relaxation in aorta of male SHRs in response to the β_3-agonist, either in presence or absence of endothelium (Fig. 5d). Finally, compared to intact aorta, norepinephrine-induced aortic tension was significantly ($P < 0.01$) increased in endothelium-denuded aorta from female SHRs (Fig. 5e). In males, the difference was less pronounced but still significant (Fig. 5f). Because removal of endothelium does not provide direct evidence of involvement of NO in sex-specific differences in constriction and relaxation of aorta, we performed additional experiments using L-NMMA. Results show that the sex-difference in norepinephrine-induced aortic tension was markedly reduced with L-NMMA (Fig. 6a). Consistently, the sex-difference in isoprenaline-induced aortic relaxation was largely reduced with L-NMMA (Fig. 6b). In addition to norepinephrine, we used phenylephrine (selective α_1-adrenoceptor agonist) to provide better evidence for a specific involvement of β-adrenoceptors in the sex-specific difference in constriction and relaxation of aorta. Unlike norepinephrine, phenylephrine-induced aortic tension was not different between sexes (Fig. 6c). The small difference observed at the highest concentration was most likely caused by co-activation of β-adrenoceptors due to decreased specificity of phenylephrine at this concentration. Notably, this difference was abolished with L-NMMA. Immunostaining was used to provide supportive evidence on the localization of β-adrenoceptors in aorta of female and male SHRs. Results show that strong fluorescent signals of β_1-, β_2- and β_3-adrenoceptors are localized on the endothelium of female aorta (Fig. 6d). Of note, while fluorescent signal of β_3-adrenoceptor is prominent in aorta of female SHRs, no fluorescent signal was detected for β_3-adrenoceptor in aorta of male SHRs (Fig. 6d). To demonstrate the specificity of antibodies and rule out the fluorescence of elastin fibers in aorta, we stained aortas of wild type and β1/β2 double knockout mice. There was neither a positive staining for β-adrenoceptors nor background fluorescence of elastic fibers in aorta of knockout mice (Fig. 6e). Collectively these results show that functionally relevant β-adrenoceptors are located on endothelium and therefore β-adrenergic relaxation of aorta strongly depends on the endothelium and released NO both in female and male SHRs. Removal of endothelium or blockade of NO production augments α-adrenergic wall tension and inhibits β-adrenoceptor-induced relaxation more profoundly in female SHRs than males.

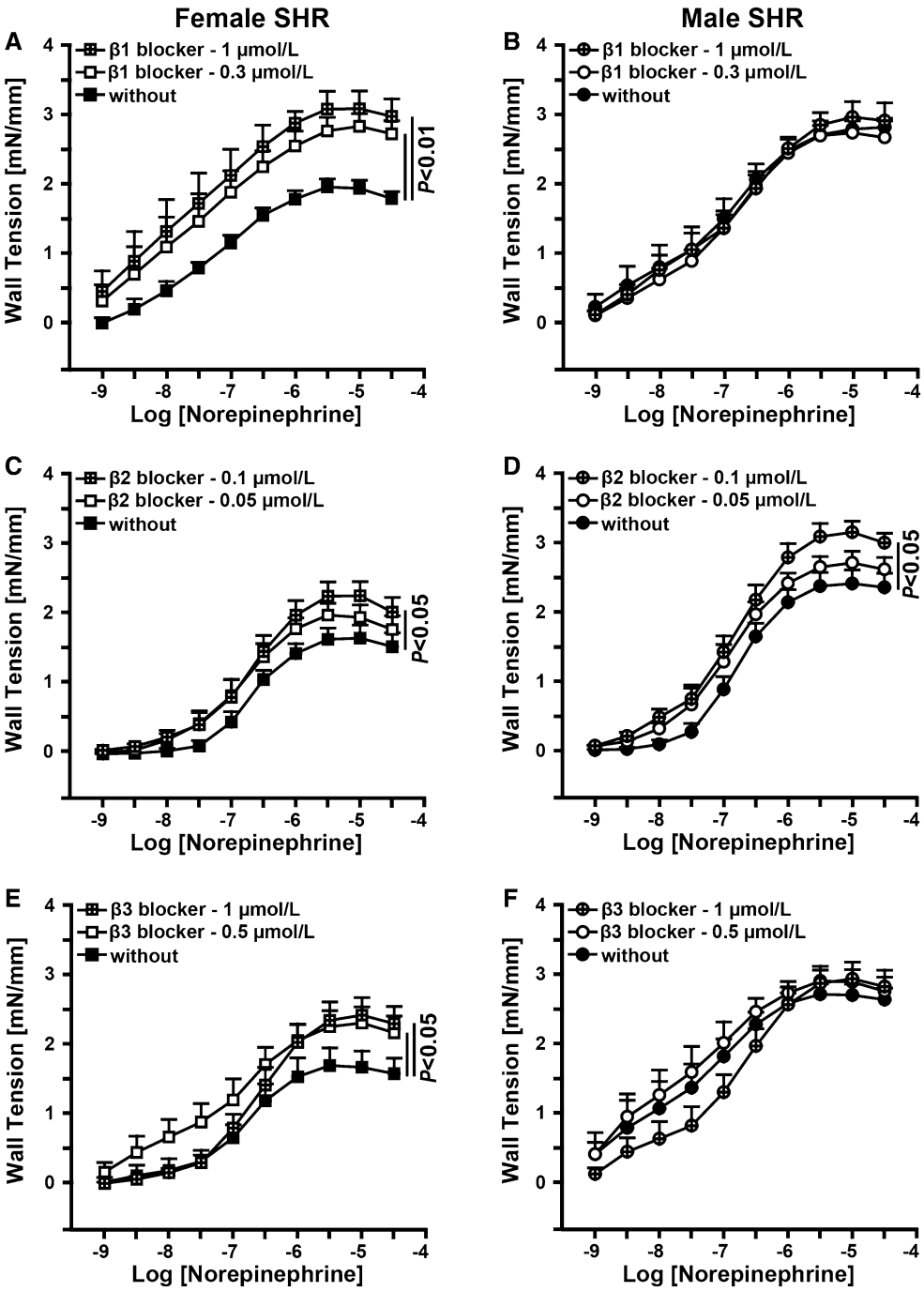

Fig. 2 Effects of selective β-adrenoceptor blockade on α-adrenergic wall tension in aorta of 12-week-old female and male SHRs. **a** Selective β_1-blockade in norepinephrine-dependent aortic wall tension in female and **b** male SHRs. **c** Selective β_2-blockade in norepinephrine-dependent aortic wall tension in female and **d** male SHRs. **e** Selective β_3-blockade in norepinephrine-dependent aortic wall tension in female and **f** male SHRs. Selective β_1- and β_3-blockade increased aortic tension in female, but not in male SHRs. Selective β_2-blockade increased aortic tension both in female and male SHRs. $n = 6$

Sex-specific differences in aortic reactivity to norepinephrine is attenuated with aging and hypertension in SHRs

We further investigated the potential influence of age and hypertension on sex-specific reactivity of aorta to norepinephrine in SHRs and WKYs. For this, we additionally tested SHRs and WKYs at ages of 5, 29 and 36 weeks. Norepinephrine-induced aortic tension was significantly ($P < 0.05$) lower in 5-week-old female SHRs than males (Fig. 7a). Similarly, 29-week-old SHRs also showed significantly ($P < 0.01$) lower aortic tension than males (Fig. 7b). In fact, the sex-specific difference in this age group was even more pronounced compared to the other age groups. However, at the age of 36 weeks there was no significant difference in development of aortic tension in response to norepinephrine between female and male SHRs (Fig. 7c). Aortas of 5-, 29- and 36-week-old female WKYs

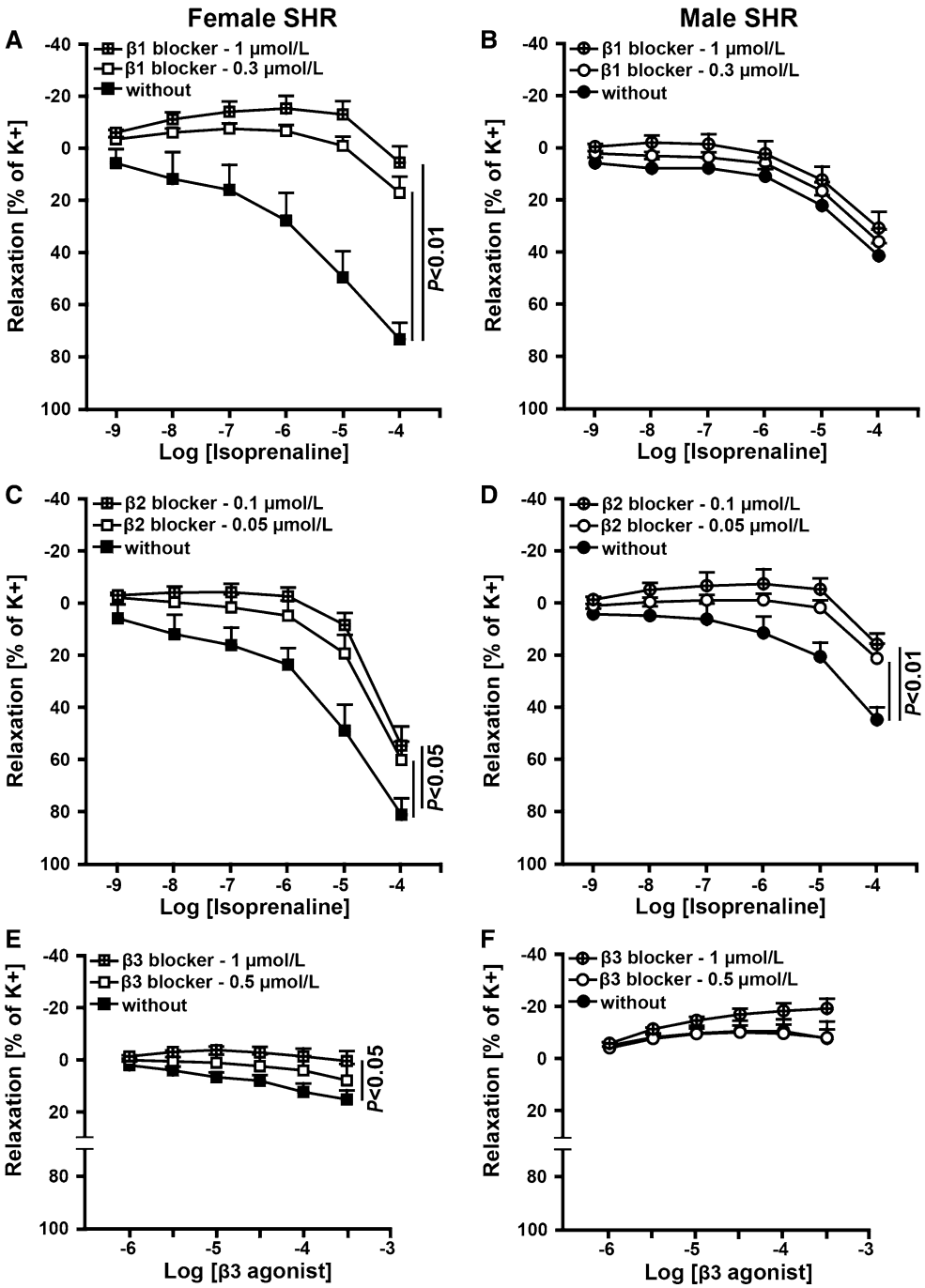

Fig. 3 Effects of selective β-blockade on β-adrenergic relaxation in aorta of 12-week-old female and male SHRs. **a** Selective β_1-blockade in isoprenaline-dependent aortic relaxation in female and **b** male SHRs. **c** Selective β_2-blockade in isoprenaline-dependent aortic relaxation in female and **d** male SHRs. **e** Selective β_3-blockade in β_3-agonist (CGP20712)-dependent aortic relaxation in female and **f** male SHRs. Selective β_1- and β_3-blockade abolished the aortic relaxation in female, but not in male SHRs. Selective β_2-blockade diminished the aortic relaxation both in female and male SHRs. $n = 6$

showed moderate, but significantly ($P < 0.05$) lower tension development in response to norepinephrine than males (Fig. 7d–f). Notably, the age group of 29-week-old male SHRs showed significantly ($P < 0.01$) impaired endothelium-dependent relaxation compared to their WKY counterparts (Table 4). However, at 29 weeks endothelium-dependent relaxation in female SHRs was not different from that of WKYs (Table 4). At the age of 36 weeks, endothelium-dependent relaxation was significantly ($P < 0.01$) impaired both in female and male SHRs when compared with age-matched WKYs (Table 4).

Quantification of β-adrenoceptor mRNA levels showed that 5-week-old male SHRs had significantly lower aortic mRNA levels of β_1—($P < 0.05$) and β_3—($P < 0.01$) adrenoceptors than females (Fig. 8a). Pronounced differences were also observed at the age of 29 weeks (β_1—$P < 0.01$, β_3-adrenoceptors $P < 0.01$, Fig. 8b). However, 36-week-old SHRs did not show a significant difference in

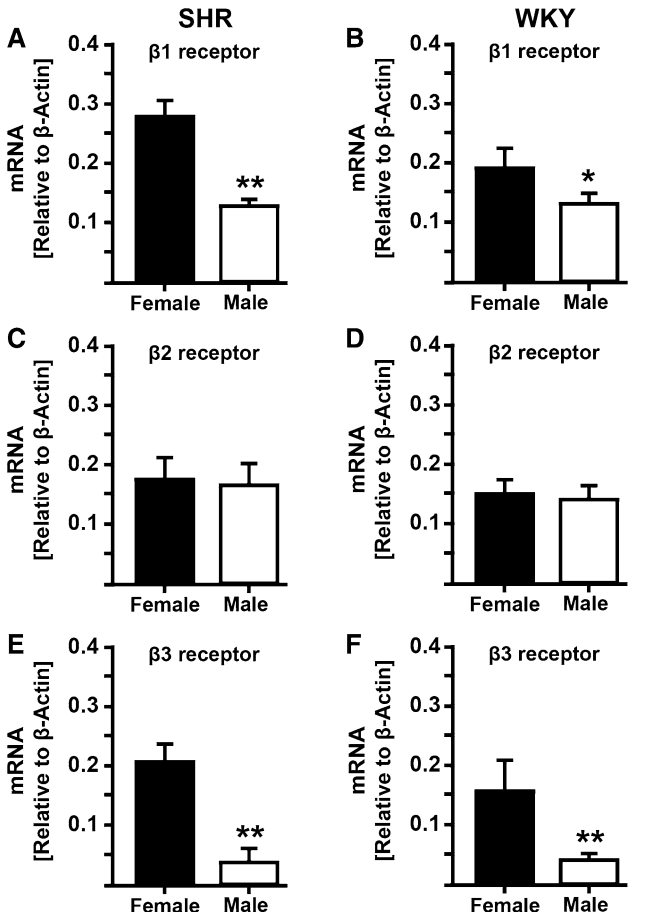

Fig. 4 β-Adrenoceptor mRNA levels in 12-week-old female and male rats. **a** β_1- **b** β_2- and **c** β_3-adrenoceptor mRNA levels in SHRs and WKYs. β_1-adrenoceptor mRNA level was lower in male than in female rats, in SHRs as well as WKYs. Level of β_2-adrenoceptor was neither different in SHRs nor in WKYs. β_3-adrenoceptor mRNA levels were lower in male compared to female rats, in SHRs as well as WKYs. *$P < 0.05$ versus female, **$P < 0.01$ versus female. $n = 6$

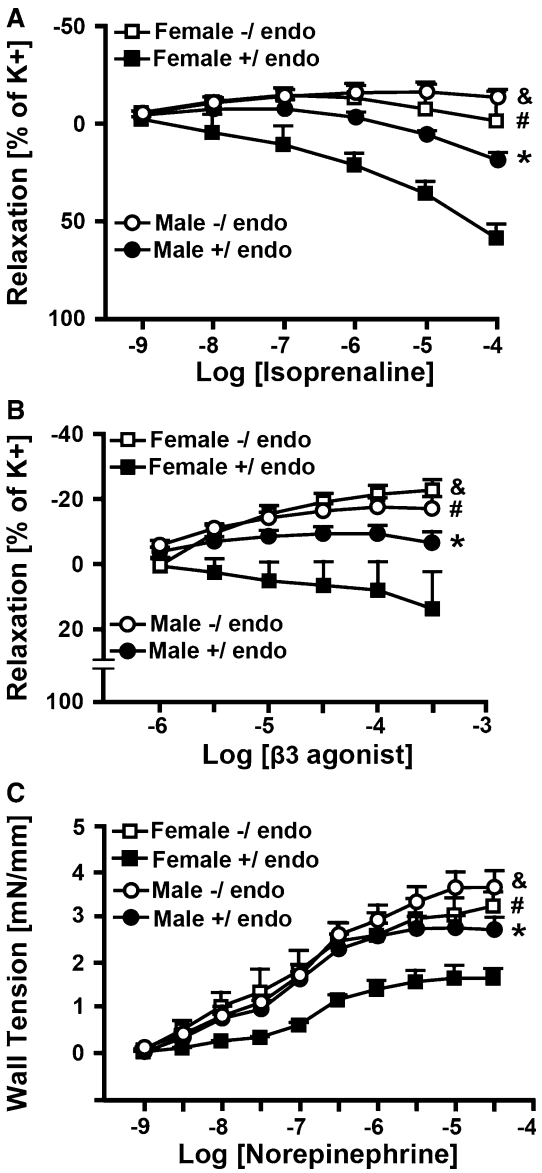

Fig. 5 Effects of endothelial removal on isoprenaline-induced aortic relaxation in 12-week-old female and male SHRs. **a** Isoprenaline-induced aortic relaxation with and without endothelium in female and male SHRs. **b** β_3-agonist-induced aortic relaxation with and without endothelium in female and male SHRs. **c** Norepinephrine-induced aortic tension with and without endothelium in female and male SHRs. Removal of endothelium diminished isoprenaline-induced aortic relaxation both in female and male SHRs. Removal of endothelium also abolished β_3-adrenoceptor agonist-induced aortic relaxation in female SHRs, whereas it did not have any effect in male SHRs. In female SHRs, removal of endothelium profoundly increased wall tension development of aorta in response to norepinephrine. A smaller difference was observed in aorta of male SHRs. $n = 6$

β_1-adrenoceptor mRNA levels between males and females, while the difference in β_3-adrenoceptor mRNA levels persisted ($P < 0.01$, Fig. 8c). In WKYs, all age groups showed significant sex-differences in aortic mRNA levels of β_1—($P < 0.05$) and β_3—($P < 0.01$) adrenoceptors (Fig. 8d–f). There was no sex-specific difference in mRNA levels of β_2-adrenoceptors in any age group of SHRs and WKYs (Fig. 8a–f).

Measurements of SBP showed that 5-week-old SHRs and WKYs were normotensive in both sexes, whereas 12-week-old SHRs where hypertensive (Table 5). However, SBP of female SHRs in this age group was ~12 mmHg lower ($P < 0.05$) compared to males. In the age of 29 and 36 weeks both female and male SHRs had profoundly higher SBP ($P < 0.01$ for both sexes) compared to their WKY counterparts.

Human mammary arteries of women constrict less in response to norepinephrine than arteries of men

We analyzed data from ex vivo experiments performed on mammary arteries donated in the frame of coronary bypass

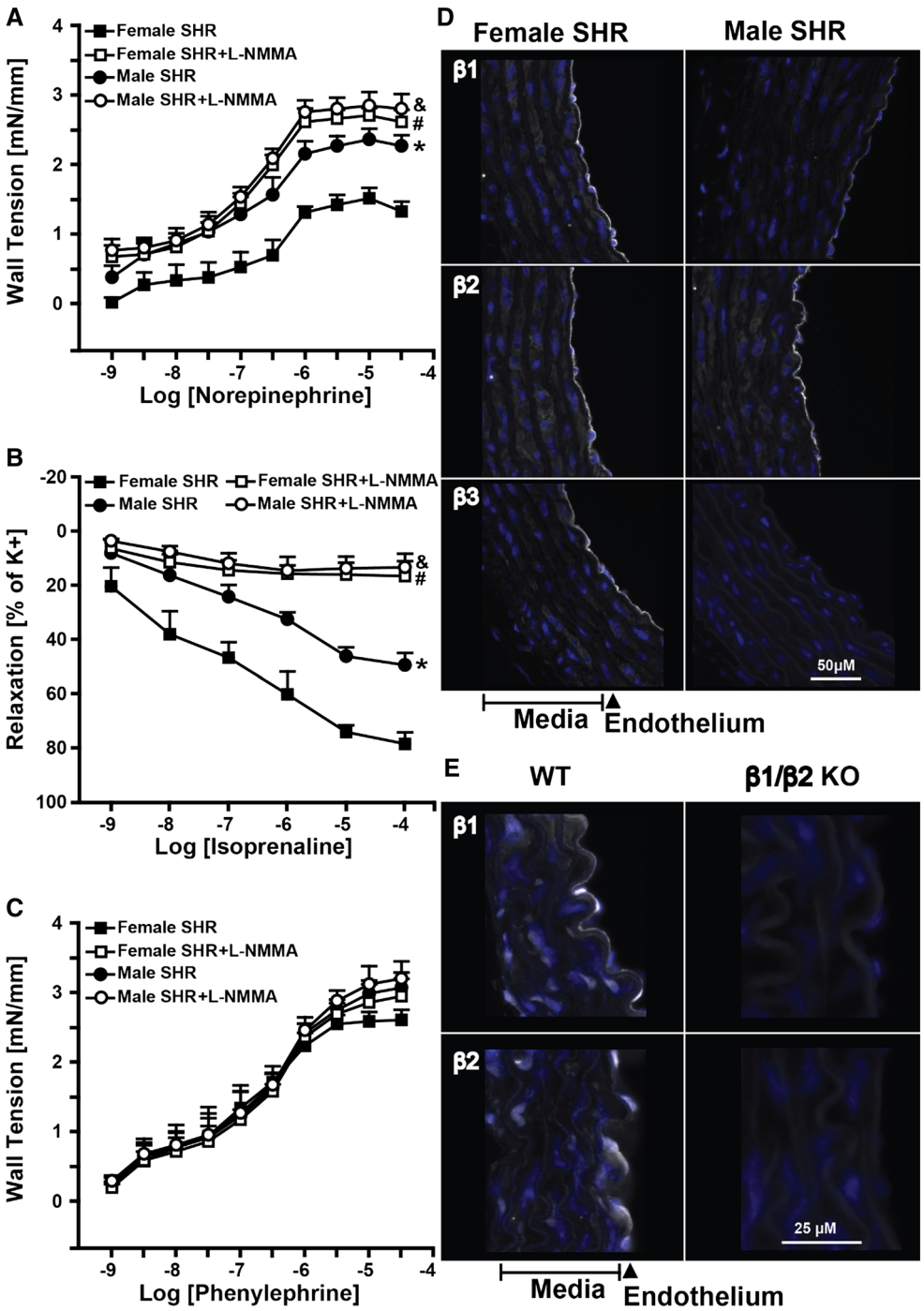

Fig. 6 Effects of NO blockade on sex-specific difference in aortic reactivity toward norepinephrine and isoprenaline, respectively, in 12-week-old female and male SHRs. **a** Norepinephrine-induced aortic tension with and without L-NMMA in female and male SHRs. **b** Isoprenaline-induced aortic relaxation with and without L-NMMA in female and male SHRs. **c** Phenylephrine-induced aortic tension with and without L-NMMA in female male SHRs. **d** Immunostaining for β_1-, β_2-, and β_3-adrenoceptors in aorta of female and male SHRs. **e** Control stainings for specificity of antibodies and autofluorescence in WT and β_1-/β_2-double KO mice. Removal of endothelium diminished isoprenaline-induced aortic relaxation both in female and male SHRs. Removal of endothelium also abolished β_3-adrenoceptor agonist-induced aortic relaxation in female SHRs, whereas it did not have any effect in male SHRs. In female SHRs, removal of endothelium profoundly increased wall tension development of aorta in response to norepinephrine. A smaller difference was observed in aorta of male SHRs. Immunostainings for β-adrenoceptors in aorta of female and male SHRs show that fluorescence signals for β_1-, β_2- and β_3-adrenoceptors were primarily located on the endothelium. While the signal for β_3-adrenoceptor is visible in aorta of female SHRs, there was no clear fluorescence signal detected for β_3-adrenoceptor in aorta of male SHRs. $^{*}P < 0.01$ versus female SHR without L-NMMA, $^{\#}P < 0.01$ versus female SHR without L-NMMA, $^{\&}P < 0.05$ versus male SHR without L-NMMA. $n = 6$

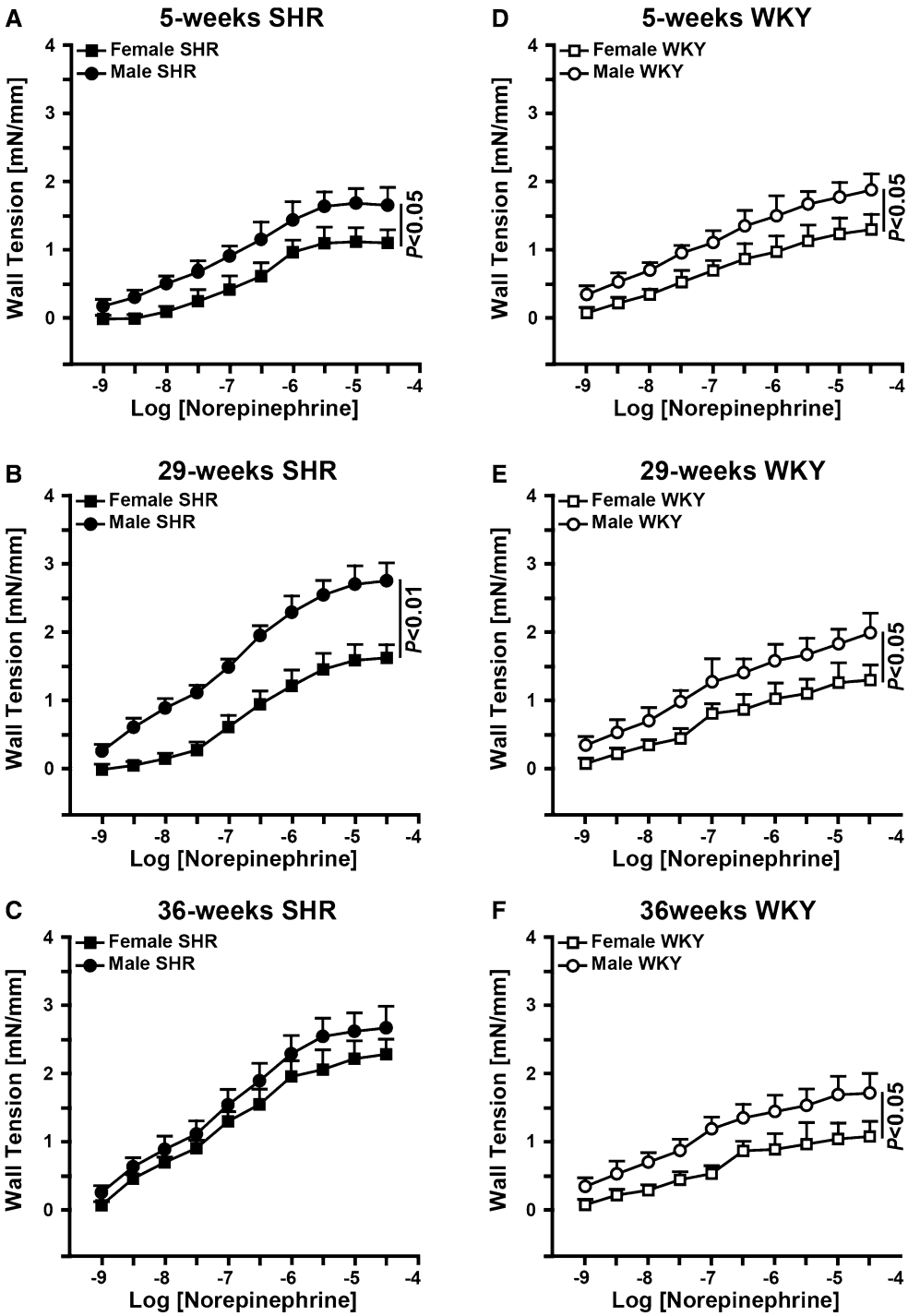

Fig. 7 Development of α-adrenergic wall tension in aorta of young and old SHRs and WKYs. **a**, **d** Development of aortic wall tension in response to norepinephrine in 5-, **b**, **e** 29- and **c**, **f** 36-week-old female and male SHRs and WKYs. Aortas of 5-week-old female SHRs showed less tension in response to norepinephrine than aorta of males. The difference was more pronounced in 29-week-old SHRs and abolished in 36-week-old SHRs. In WKYs of all age groups a similar sex-related difference in aortic constriction to norepinephrine is found. $n = 6$

surgery. In these experiments, stimulation with norepinephrine was used to pre-constrict vessels to assess ACh-dependent relaxation. While the major findings of the study have been reported [17], sex-specific reactivity to norepinephrine stimulation had not been analysed. The retrospective analysis of these measurements shows, that mammary artery from men developed significantly ($P < 0.01$) stronger tension in response to norepinephrine than arteries from women when averaged over the entire age group (45–85 years; Fig. 9a). Development of tension in response to KCl was not different between sexes (Fig. 9b). Because we observed that in SHRs sex-specific reactivity to norepinephrine showed an age-dependent pattern, we also addressed the responsiveness of human mammary artery to norepinephrine with respect to age. While arteries of men in age groups of 45–55 and 56–65 years developed significantly higher tension in response to norepinephrine than arteries of women, this difference was no longer observed in the age

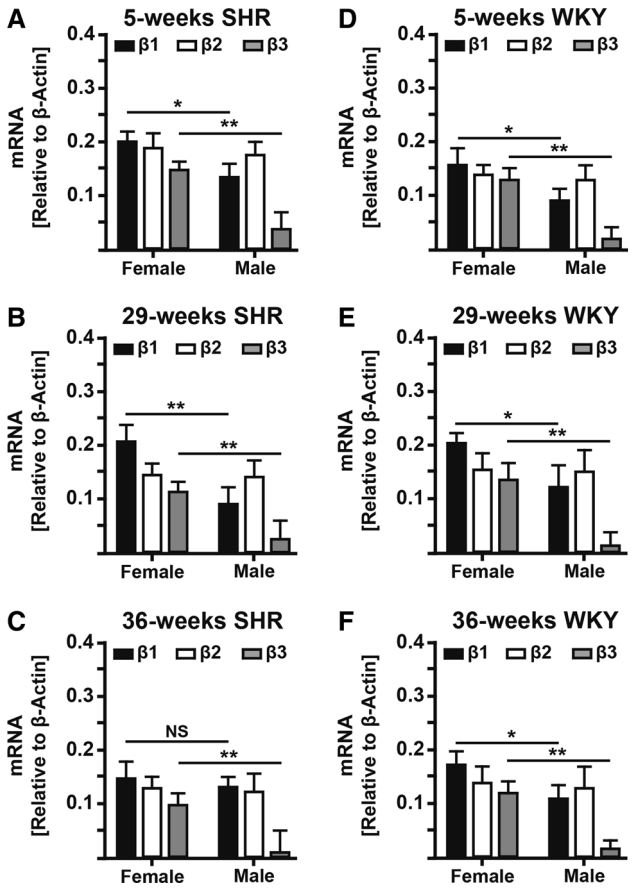

Fig. 8 β-Adrenoceptor mRNA levels in young and old SHRs and WKYs. **a**, **d** mRNA levels of aortic β-adrenoceptors in 5-, **b**, **e** 29- and **c**, **f** 36-week-old female and male SHRs and WKYs. β_1- and β_3-Adrenoceptor mRNA levels were lower in 5-week-old male SHRs compared to females. 29-week-old SHRs showed a more pronounced difference in mRNA levels of β_1- and β_3-adrenoceptors. At the age of 36-weeks there was no difference in β_1- and β_2-adrenoceptor mRNA levels between female and male SHRs, but β_3-adrenoceptor mRNA levels were still different. In all age groups of WKY, both β_1- and β_3-adrenoceptor mRNA levels were lower in males than in females. Levels of β_2-adrenoceptor mRNA was not different between males and females neither in SHRs nor in WKYs of any age group. $^*P < 0.05$, $^{**}P < 0.01$. $n = 6$

Table 5 Systolic blood pressure (mmHg)

Age (weeks)	Female		Male	
	SHR	WKY	SHR	WKY
5	115 ± 3	117 ± 3	119 ± 2	116 ± 9
14	139 ± 4*	119 ± 2	151 ± 4	113 ± 5
29	190 ± 4	101 ± 9	195 ± 6	118 ± 12
36	184 ± 9	104 ± 9	189 ± 3	120 ± 7

$n = 6$

* $P < 0.05$ versus age-matched male SHR

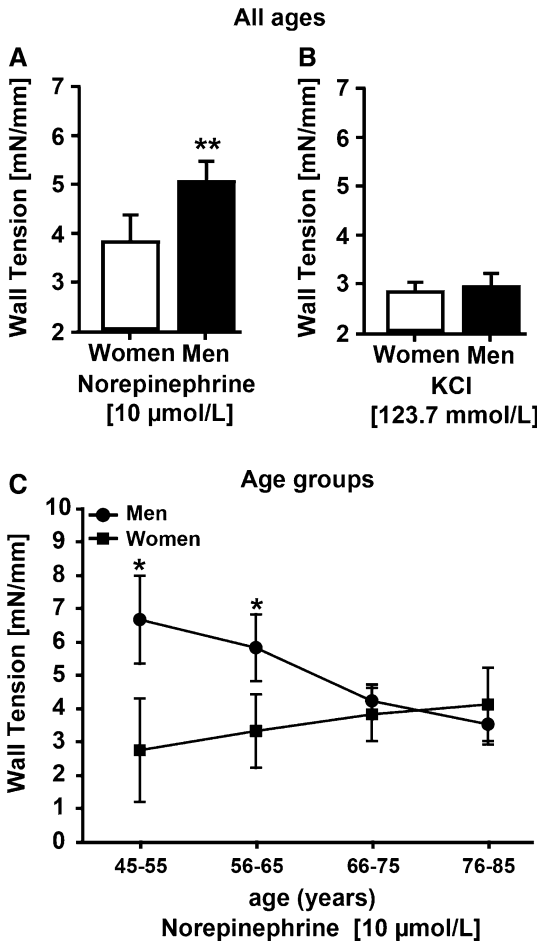

Fig. 9 Development of α-adrenergic wall tension in human mammary artery. **a** Aortic wall tension in response to norepinephrine and **b** KCl in women and men of 45–85 years. **c** Age-dependent development of wall tension in response to norepinephrine. In the age group of 45–85 years, mammary arteries of men developed stronger tension than arteries of women. Different age groups show stronger wall tension development in ages of 45–55 and 56–65 years, while there were no differences in age groups of 66–75 and 76–85 years. $^*P < 0.05$ versus age-matched women, $^{**}P < 0.01$ versus women. $n = 103$ men (45–85 years), $n = 30$ women (45-85 years), $n = 12$ men, $n = 3$ women (45–55 years), $n = 18$ men, $n = 4$ women (56–65 years), $n = 53$ men, $n = 15$ (66–75 years), $n = 20$, $n = 8$ (76–85 years)

groups of 66–75 and 76–85 years. The findings in mammary artery (Fig. 9c) are consistent with finding in rat aorta.

Discussion

Our study demonstrates that there is a sex-specific difference in expression and function of β_1- and β_3-adrenoceptors in aorta of female and male rats. This sex-specific

difference, which is unanimously found in SHR and WKY strains, is of functional importance. Higher expression of dilatory β-adrenoceptors in aorta of female SHRs stronger counteracts the constrictive effects of α-adrenoceptors, as well as it causes a stronger vessel relaxation in female SHRs compared to males. Our findings may explain the pronounced sex-differences in aortic reactivity to adrenergic stimulation in SHRs. Prominent endothelial localization of β-adrenoceptors reveals the endothelium as a major determinant of this sex-related difference. We also demonstrate that the sex-specific difference of β-adrenoceptor expression is most pronounced in young adult SHRs, whereas the difference is attenuated with aging and chronic hypertension. Finally, we show that the findings on rat aorta may be translated to humans.

Previous reports have demonstrated a difference in vascular reactivity to adrenergic stimulation between female and male rats, including SHRs [12, 16, 19, 21, 22]. Further studies also indicated the important role of estrogen in modulation of vascular reactivity to adrenergic stimulation in SHRs [5, 10, 27, 29, 44]. Here, we propose that the sex-specific difference in α-adrenoceptor-mediated aortic constriction may be explained by a sex-specific alteration in the balance between constrictive α- and dilatory β-adrenoceptor-mediated effects. Our concept is supported by findings demonstrating that selective blockade of β_1- or β_3-, but not β_2-adrenoceptors equalized both constriction and relaxation of aorta by shifting the effects in female SHRs, but not of males (Figs. 2, 3). The findings of functional experiments are supported by the results from quantification of mRNA levels of β-adrenoceptors. Three times higher mRNA levels of β_1- and β_3-adrenoceptors were found in aortas of young adult female SHRs than in males. A functional role of β-adrenoceptors in the sex-specific difference in norepinephrine-induced aortic constriction is also supported by our findings using the selective α_1-agonist phenylephrine (Fig. 6). In contrast to constrictive effects seen with norepinephrine those of the selective α_1-agonist did not differ between sexes. This indicates that because phenylephrine does not stimulate β-adrenoceptors over a wide concentration range, it was unable to invoke sex-related differences of vessel tone modulation, which depend on β-adrenoceptor activations. Our data also conclusively demonstrate that the sex-related difference is specific to adrenoceptor-induced regulation of vessel tone, because constriction was identical with KCl (Table 3; Fig. 9b). Taken together these findings provide comprehensive evidence for a quantitatively and functionally relevant difference in vascular β_1- and β_3-adrenoceptors between female and male SHRs.

Generally, it is assumed that norepinephrine acts via adrenoceptors located on vascular smooth muscle cells [26]. Our work provides evidence that β-adrenoceptors, which determine the sex-difference in α-adrenoceptor-mediated aortic constriction and β-adrenoceptor-mediated relaxation are localized on the endothelium. In support, our findings show that β_1/β_2-mediated relaxation (with isoprenaline) was strictly endothelium- and NO-dependent (Figs. 5, 6). Furthermore, the sex-related differences in α-adrenoceptor-mediated aortic constriction were abolished by removal of the endothelium or blockade of NO production (with L-NMMA, Figs. 5, 6). Moreover, immunostainings revealed a clear localization of β-adrenoceptors on endothelial cells (Fig. 6). Taken together this suggests, that β-adrenoceptors located on aortic endothelial cells, via NO functionally counteracted the constrictive effects of α-adrenoceptors on aortic smooth muscle cells. This may reveal another example of paracrine control of vascular smooth muscle by the endothelium. Based on our findings, we propose that the underlying mechanism of the sex-difference in aortic reactivity to norepinephrine and isoprenaline may invoke endothelial β_1- and β_3-adrenoceptors, which act via NO. Our findings are in line with previous reports [14, 25, 28] who provided evidence of β-adrenoceptor-mediated NO production and that reduction in NO bioavailability or blockade of NO production with L-NAME increases α-adrenergic constriction of aorta. It should be noted that Loria et al. demonstrate that 12- to 13-week-old male SHRs have decreased aortic endothelial NO production compared to age matched females and consequently show that males react stronger to α-adrenergic constriction than females [28]. To support our concept that the sex-specific difference in α-adrenergic constriction is rather due to β-adrenoceptor expression differences than a sex-specific decrease in NO production, we demonstrate that there was no sex-specific difference in endothelium-dependent aortic relaxation in response to ACh in young SHRs (Table 4). Of note, in rat aorta ACh acts through release of NO from the endothelial cells independent of β-adrenoceptors [9]. Therefore, these results indicate that both sexes had preserved and similar endothelial function including unaltered NO production.

With increasing age, the response toward ACh was diminished first in aortas of male (29 weeks) and later (36 weeks) in female SHRs (Table 4). This transient sex-specific alteration of ACh-mediated aortic relaxation goes along with an augmentation of the sex-specific difference in α-adrenergic constriction of aorta, as well as declining aortic mRNA levels of β_1- and β_3-adrenoceptors (Figs. 7, 8). This suggests that with advancing age (29 weeks), the sex-specific difference in α-adrenergic constriction of aorta is augmented on the expense of impaired NO production in male SHRs. Table 4 also shows that at an even more advanced age (36 weeks) ACh-mediated relaxation is severely diminished in female SHRs, as well as males,

along with abolished sex-specific differences in α-adrenergic constriction of aorta and aortic mRNA levels of β_1-adrenoceptors. This further supports the concept that β-adrenoceptor-triggered NO production may causally be involved in mediating the sex-specific difference in adrenergic constriction at younger age. Loss of ACh-mediated relaxation was specifically seen in SHRs, but not in WKYs (Table 4; Fig. 7) suggesting that age-associated changes observed in SHRs may at least in part be related to arterial hypertension. Finally, estrogen may play an important role in regulation of these receptors because, with estrogen levels declining with aging, postmenopausal women show decreased vascular sensitivity to β-adrenergic stimulation [19], while our data shows that mRNA levels for β_1-/β_3-adrenoceptors are decreased with aging.

Our work demonstrates that the sex-specific differences in α-adrenergic constriction of aorta is not strain dependent, but found both in SHRs and WKYs (Fig. 1). In support, both models reveal sex-specific differences in β_1- and β_3-adrenoceptor mRNA levels (Fig. 4). Moreover, the sex-related difference does not seem to require a hypertension-associated cardiovascular adaptation, because arterial blood pressure was in the normotensive range in WKYs at all ages (Table 5). Most interestingly, it appears that the age-dependent variability of α-adrenergic constriction of aorta and β_1- and β_3-adrenoceptor mRNA levels is considerably lower in WKYs as compared with SHRs (Figs. 7, 8). It is thus concluded that the differences in endothelial β-adrenoceptor expression and related functional effects are primarily sex-related, whereas functional differences may be changed in an age-related manner by either strain or blood pressure-related effects.

In addition to the comprehensive analysis of sex-specific differences of endothelial β-adrenoceptor effects on adrenergic vessel tone control in two rat strains, we addressed the potential translation to human (patho)physiology. Our findings on human mammary artery clearly demonstrate a similar sex-specific pattern in α-adrenergic constriction. The arteries of men constricted stronger than arteries of women (Fig. 9). Most interestingly, the effects of age on the sex-specific difference in α-adrenergic constriction were evident. It is currently unknown whether this pattern represents a general feature of arterial macrovessels. Nevertheless, the effects seen in human mammary artery are similar to those of rat aorta. Results obtained in human forearm model indicate that β-adrenoceptor stimulation causes a stronger vasodilatation in women than in men. However, previous human forearm perfusion studies did not address an altered NO production between women and men [19, 21]. It thus remains open whether the difference seen in these studies was primarily due to differences in adrenoceptor expression or resulted from differences of NO production. To the best of our knowledge, the involvement of endothelial β-adrenoceptors in this effect has never been explored to date.

In conclusion, our study provides conclusive evidence that there is a sex-specific difference in expression of endothelial β_1- and β_3-adrenoceptors in rat aorta. This difference is not strain dependent, but exists similarly in WKYs and SHRs. However, hypertension-associated factors seem to affect the response toward β-adrenoceptor activation, in part by blunting endothelial NO production at older age. Our data suggest that a higher expression of endothelial β_1- and β_3-adrenoceptors may play a causal role for the lower incidence of vascular diseases in premenopausal women as compared with men of the same age. Our explorative analysis of α-adrenergic constriction of human mammary artery indicates that the sex-specific differences objectified in rat aorta may also be pertinent to human macrovessels.

Study limitations

Using selective block of NO-synthases with L-NMMA we provide evidence for the involvement of NO release in the endothelium-dependent mechanism that blunts α-adrenergic vessel constriction. We would like to indicate that we did not assess eNOS expression, eNOS phosphorylation or NO release. Therefore, we do not provide direct evidence of greater NO production in female rats compared to males. However, our concept that the sex-differences depend on endothelial β-adrenoceptors, which act via NO, is supported by the work of Figueroa et al. which demonstrate that enhanced production of NO in response to adrenergic stimulation is mediated via activation of β-adrenoceptors [14]. While we provide evidence for sex-related differences of β-adrenoceptor localization at 12 weeks of age in SHRs (Fig. 6), we have not performed immunostainings for β-adrenoceptors in older age groups. This may be another limitation of the present study. However, we would like to point to the comprehensive analysis of β-adrenoceptor expression for the different age groups (Fig. 8), which indicates that sex-related differences existed over a large age span. Finally, we acknowledge that we did not provide direct evidence to support an estrogen-dependent regulation of endothelial β_1- and β_3-adrenoceptors.

Compliance with ethical standards

Disclosures None declared.

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Conflict of interest The authors declare that they have no conflict of interest.

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