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Sustained hypertension despite endothelial-specific eNOS rescue in eNOS-deficient mice

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ABSTRACT

The aim of the study was to evaluate the possible contribution of non-endothelial eNOS to the regulation of blood pressure (BP). To accomplish this, a double transgenic strain expressing eNOS exclusively in the vascular endothelium (eNOS-Tg/KO) has been generated by endothelial-specific targeting of bovine eNOS in eNOS-deficient mice (eNOS-KO). Expression of eNOS was evaluated in aorta, myocardium, kidney, brain stem and skeletal muscle. Organ bath studies revealed a complete normalization of aortic reactivity to acetylcholine, phenylephrine and the NO-donors in eNOS-Tg/KO. Function of eNOS in resistance arteries was demonstrated by acute i.v. infusion of acetylcholine and the NOS-inhibitor L-NAME. Acetylcholine decreased mean arterial pressure in all strains but eNOS-KO responded significantly less sensitive as compared eNOS-Tg/KO and C57BL/6. Likewise, acute i.v. L-NAME application elevated mean arterial pressure in C57BL/6 and eNOS-Tg/KO, but not in eNOS-KO. In striking contrast to these findings, mean, systolic and diastolic BP in eNOS-Tg/KO remained significantly elevated and was similar to values of eNOS-KO. Chronic oral treatment with L-NAME increased BP to the level of eNOS-KO only in C57BL/6, but had no effect on hypertension in eNOS-KO and eNOS-Tg/KO. Taken together, functional reconstitution of eNOS in the vasculature of eNOS-KO not even partially lowered BP. These data suggest that the activity of eNOS expressed in non-vascular tissue might play a role in physiologic BP regulation.

1. Introduction

Among NO-synthases (NOS) the endothelial isoform (eNOS) significantly contributes to the regulation of BP as hypertension is the most obvious phenotype in 4 different strains of eNOS-deficient mice (eNOS-KO) suggesting that other physiologic systems regulating BP cannot compensate for the absence of eNOS [1–4]. Likewise, hypertension develops following treatment with NOS-inhibitors such as monomethyl arginine (L-NMMA) or L-nitroarginine (L-NA) in rabbits [5], mice [1,6], and humans [5,7]. Based on these findings it is generally assumed that the lack of vasodilation by endothelial NO is an important underlying cause [1,2], i.e. that hypertension in eNOS-KO is caused by the lack of endothelium-dependent NO-induced vasodilation.

Anaesthetized mice of one eNOS-KO strain showed a paradoxical decrease in blood pressure (BP) in response to i.p. administration of the NOS-inhibitor L-NA suggesting a role for non-endothelial isoforms of NOS in maintaining BP [1]. Alternatively, diminution in the activity of the renin-angiotensin system and the autonomic nervous system, which serve as a defense against hypertension and/or involvement of NOS in establishing the baroreceptor setpoint were proposed as an explanation of hypertensive phenotype [1]. However, studies on nNOS-deficient mice revealed that they are normotensive [8]. Furthermore, triple e/i/nNOS knockouts have hypertension and the degree of hypertension is similar to that in the eNOS-gene disrupted single and double e/nNOS-KO [9]. These results clearly demonstrate that among NO-synthases eNOS plays by far the most important role in BP regulation. However, in contrast to large arteries of eNOS-KO where no compensation for the lack of eNOS is observed [1,6,10], other endothelium-dependent vasodilators such as prostaglandins, nNOS or endothelium-derived hyperpolarizing factor compensate for the lack of eNOS in coronary, femoral, mesenteric, cerebral and skeletal resistance arteries [11–16]. These data indicate that different vascular-bed specific mechanisms regulate arterial tone in

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the absence of vascular eNOS and raise the question to what extent hypertension in eNOS-KO is caused by the lack of endothelial eNOS expression.

The aim of this study was to evaluate the contribution of non-vascular eNOS expression and function in physiologic regulation of BP. To distinguish the relevance of endothelial and non-endothelial eNOS, we generated a novel double transgenic mouse model lacking eNOS in all organs except the vascular endothelium (eNOS-Tg/KO) and found that these mice were as hypertensive as eNOS-KO. These data establish the necessity of functional non-endothelial eNOS expression for BP regulation.

2. Methods

2.1. Transgenic mice

We generated a double transgenic mouse strain expressing wild-type eNOS only in vascular endothelial cells by crossing mice with an endothelial-specific overexpression of bovine eNOS driven by the Tie-2 promoter on a C57BL/6J background (eNOS-Tg) [17] with eNOS knockouts (eNOS-KO) [2] on a C57BL/6J background. All experiments were performed using 3–4 months old male mice. Permission for the animal studies was provided by the regional government of Germany (AZ 50.05-230-3-94/00, AZ 50.05-230-18/06, AZ 8.87-51.04.20.09.383), the experiments were performed according to the guidelines of German „Tierschutzgesetz“ and the „Guide for the Care and Use of Laboratory Animals“ of the U.S. National Institutes of Health.

2.2. Measurement of systolic blood pressure

Systolic blood pressure and heart rate was measured in awake C57BL/6, eNOS-KO, and C101A/eNOS-KO mice using an automated tail cuff system (Visitech Systems, Apex, North Carolina) as described [6]. In some experiments establishment of basal blood pressure was followed by L-NA treatment (100 mg L-NA/kg BW/day) with the drinking water and blood pressure recordings were continued for 3 weeks [6,17,19] (refer also to Fig. S1).

2.3. Acute infusion of L-NAME and acetylcholine

Measurements of mean arterial (MAP), systolic (sBP) and diastolic (dBP) blood pressure (BP) and L-NAME-induced changes of BP were performed invasively [18]. Briefly, eNOS-Tg/KO, eNOS-KO and C57BL/6 mice were anesthetized by i.p. injection with ketamine (100 mg/kg) and xylazine (5 mg/kg) and placed on a heating plate to ensure constant body temperature (36–37 °C). Right carotid artery was cannulated for continuous measurement of MAP, sBP and dBP. Right jugular vein was cannulated for drug administration and changes in BP were detected using a pressure transducer and PowerLab data acquisition system (ADInstruments, Spechbach, Germany). Hypotensive responses to acetylcholine (0.02–2 μM/kg BW) were recorded as a transient fall in MAP and calculated as % fall in MAP with respect to baseline MAP before each response. In a separate set of mice, hypertensive responses to a bolus application of NOS-inhibitor L-NAME (32 mg/kg BW) were evaluated.

2.4. Vasorelaxation studies

Isolated aortic arteries were cannulated and pressurized as described previously [6,19,20]. Function of the endothelium was examined in aortic rings of C57BL/6, eNOS-KO and C101A/eNOS-KO by cumulative addition of acetylcholine (0.01–10 μmol/L) after submaximal precontraction with 0.2 μmol/L phenylephrine [20]. Thereafter, vasorelaxation to the NO donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP, 1–10 μmol/L) or diethylamine/nitric oxide (DEA/NO 1–10 μmol/L) was measured. In some experiments the NOS inhibitor L-NAME (1 mM) or the colloidal $Fe(DETC)_2$ spin trap (0.5 mM) were administrated 30 min before application of acetylcholine.

2.5. Real-time PCR

RNA of frozen tissues was extracted by RNeasy® Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany) using the TissueRuptor® (Qiagen, Hilden, Germany). Synthesis of cDNA was performed with 500 ng RNA using the QuantiTect® Reverse Transcription Kit (Qiagen, Hilden, Germany). Quantification of bovine eNOS mRNA was carried out with bovine-specific primer (Bt03217671_m1, TaqMan®, Life Technologies GmbH). No cross-reactivity for this bovine primer with Mus musculus was detected in wild type probes. Experiments were performed using Abi Prism 7900HT Sequence Detector System (Applied Biosystems, Weiterstadt, Germany). Samples were analyzed in triplicate and normalized to the housekeeping gene RPL0 (Mm01974474_gH (TaqMan®, Life Technologies GmbH) as described [21].

2.6. SDS–PAGE and immunoblotting

Western blots for eNOS, nNOS, ecSOD, sGCβ1, sGCα1, nitrotyrosine residues, and actin were performed in different mouse tissues using standard techniques as described previously [6,17,19,20,22].

2.7. Immunohistochemistry

Endothelium specific overexpression was analyzed by eNOS/CD31-double staining as described previously [17].

2.8. Statistics

All data are expressed as mean ± s.e.m. of n individual samples. Statistical comparisons between groups were performed by t-tests, Newman–Keuls multiple comparisons post-hoc test following one-way ANOVA (more than two groups) or two-way ANOVA (concentration–response curves). $P < 0.05$ was considered statistically significant.

3. Results

3.1. eNOS expression in eNOS-Tg/KO mice

Real-time PCR using bovine-specific primers confirmed eNOS mRNA expression in aorta and brain stem of eNOS-Tg/KO, while no signal for bovine eNOS mRNA was detectable in C57BL/6 (Fig. S2a). Western blot demonstrated eNOS protein expression in a variety of organs of eNOS-Tg/KO as depicted in Fig. 1A. However, in each organ of eNOS-Tg/KO expression of eNOS was significantly lower than in C57BL/6 (Fig 1A, Fig. S2b–f). There was no Tie-2-driven eNOS protein expression in circulating or bone marrow mononuclear cells or in whole bone marrow extracts (data not shown) and only an infinitesimal eNOS mRNA content not exceeding 1% of that detected in aortic tissue was found in blood and bone marrow mononuclear cells (Fig. S3). These findings are consistent with previous data [17,22,23]. In addition, we comparatively analyzed the expression of several other proteins which were shown to be associated with vascular bioavailability or function of NO such as the vascular NO receptor soluble guanylyl cyclase (sGC) β1- and α1-subunits, extracellular superoxide dismutase (ecSOD) protecting NO from interstitial

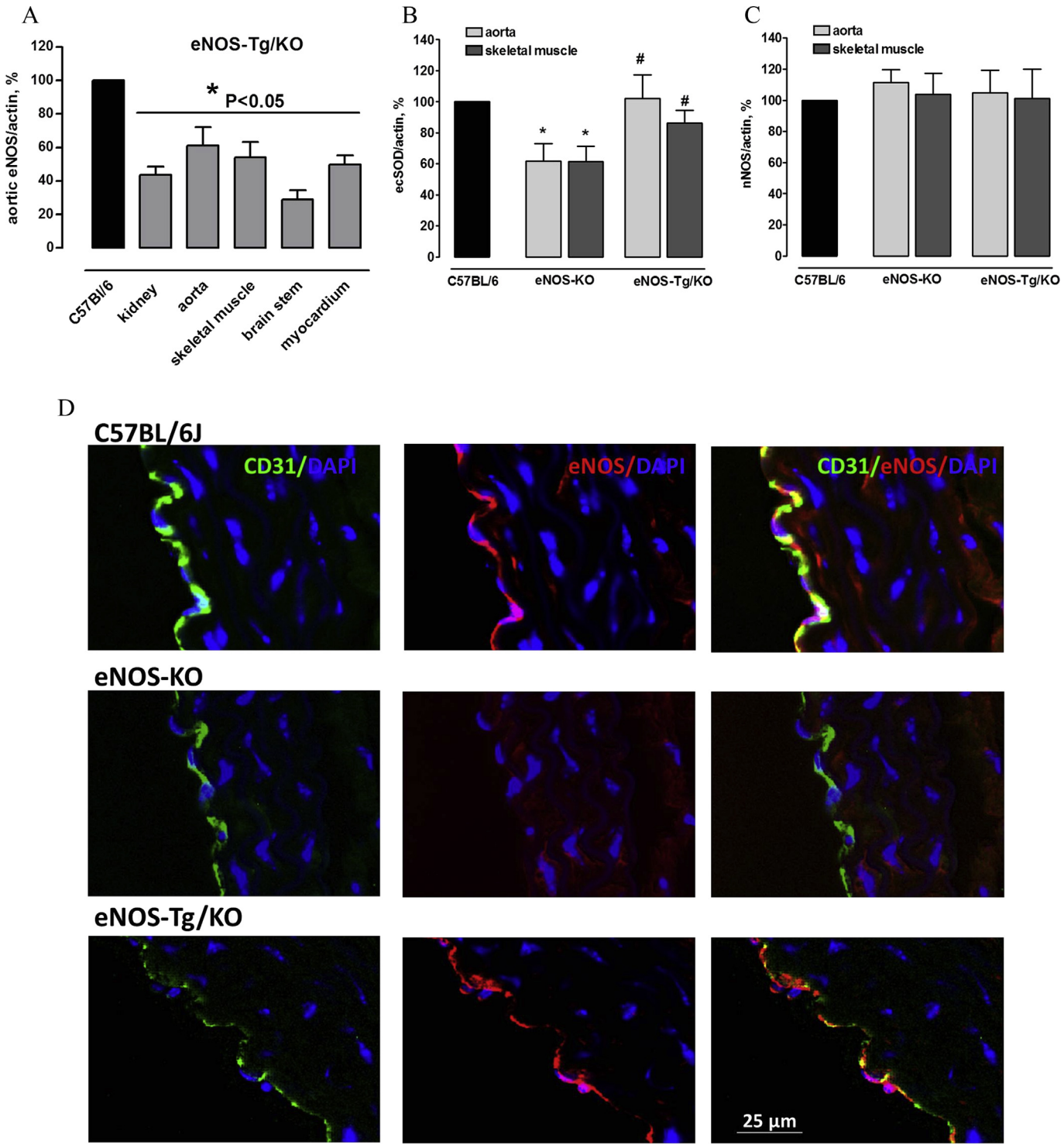

Fig. 1. Characterization of eNOS-Tg/KO mice. (A) Protein expression of eNOS in aorta (n = 11), skeletal muscle (n = 10), kidney, brain stem and myocardium (n = 4, each) of eNOS-Tg/KO (*P < 0.05 vs. C57BL/6). The control values referring to eNOS protein expression in every tissue of C57BL/6 was set to 100% and is reflected by just 1 bar. (B) Aortic and skeletal muscle ecSOD expression (*P < 0.05 vs. C57BL/6, #P < 0.05 vs. eNOS-KO n = 4–7, One-way ANOVA). (C) nNOS protein in aorta (n = 5) and skeletal muscle (n = 7) of eNOS-Tg/KO, eNOS-KO and C57BL/6 mice (P > 0.05, One-way ANOVA). (D) Images showing immunofluorescent staining for eNOS (red) and CD31 (pseudocolored in green) in the aorta of C57BL/6, eNOS-KO and C101A/eNOS-KO mice. Nuclei are stained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

oxidation [24], neuronal NOS (nNOS) and protein nitrotyrosine residues. Expression of ecSOD was significantly higher in aorta and skeletal muscle of eNOS-Tg/KO then in eNOS-KO and similar to that of C57BL/6 (Fig. 1B). There were no other expression changes in these tissues comparing C57BL/6, eNOS-Tg/KO and eNOS-KO (Fig. 1C; Fig. S4). The localization of eNOS-Tg was restricted to endothelium as evidenced by the overlay with an endothelial-specific marker CD31 (Fig. 1D).

3.2. Conductance vessel reactivity of eNOS-Tg/KO

Aortic relaxation in response to acetylcholine was identical in C57BL/6 and eNOS-Tg/KO, while a slight vasoconstriction occurred in eNOS-KO (Fig. 2A). Preincubation of aortic rings with the NOS-inhibitor L-NAME abolished acetylcholine-induced vasodilation in eNOS-Tg/KO and C57BL/6 and induced a slight vasoconstriction as observed in eNOS-KO (Fig. 2B). Likewise, the cell membrane permeable NO-scavenger $Fe(DETC)_2$ completely inhibited acetylcholine-induced aortic dilation of eNOS-Tg/KO and C57BL/6 suggesting that it was mediated by NOS-derived NO (Fig. 2C). Furthermore, the strongly increased sensitivity of aortic tissue of eNOS-KO or C57BL/6 treated with L-NAME to alpha-adrenergic stimulation by phenylephrine [6] was completely reversed in eNOS-Tg/KO which showed a concentration-dependent vasoconstriction similar to C57BL/6 (Fig. 2D). We also observed that the hypersensitivity of aortic rings of eNOS-KO to NO-donors [6] was completely abolished in eNOS-Tg/KO (Fig. 2E and F). Taken together,

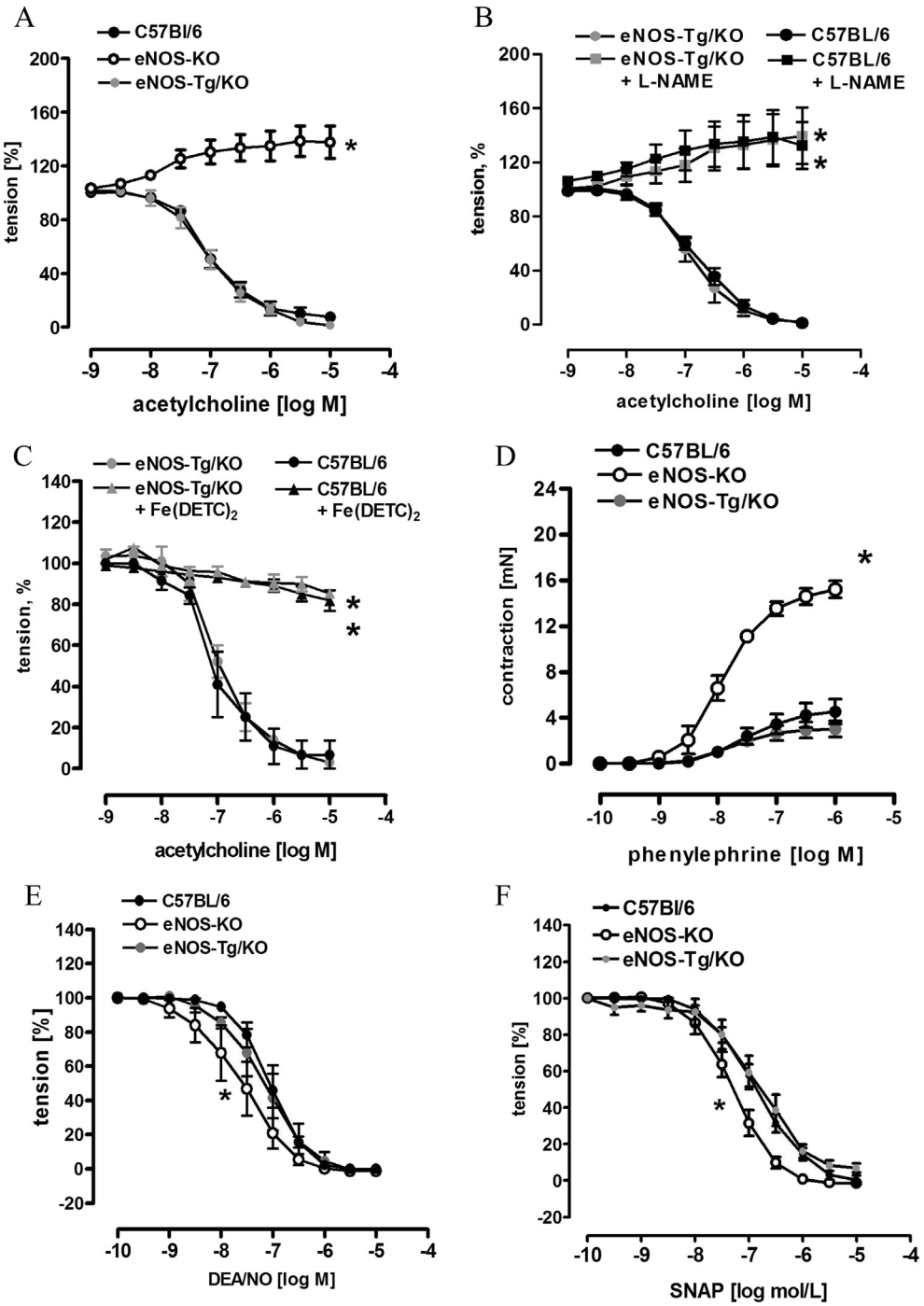

Fig. 2. Aortic reactivity of eNOS-Tg/KO. (A) Aorta of eNOS-Tg/KO (n = 8) showed a complete restoration of endothelium-dependent relaxation to acetylcholine (P = 0.311 vs C57BL/6, n = 11). (B) Effects of the NOS-inhibitor L-NAME (1 mM), and (C) the NO-scavenger $Fe(DETC)_2$ (0.5 mM) on acetylcholine-induced relaxation of aortic rings of eNOS-Tg/KO and C57BL/6 (*P < 0.05 vs. C57BL/6, Two-way ANOVA, n = 4). Aortic reactivity to (D) phenylephrine (*P < 0.0001, n = 5–7, Two-way ANOVA), (E) NO-donors diethylamine/NO (*P < 0.0001, n = 4–5, Two-way ANOVA) and (F) S-nitroso-N-acetyl-D,L-penicillamine (*P < 0.0001, n = 11, Two-way ANOVA) vs. C57BL/6 in all investigated strains.

these data suggest a normal function of eNOS in conductance vessels of eNOS-Tg/KO.

3.3. Resistance vessel reactivity of eNOS-Tg/KO

In all mice i.v. acetylcholine caused a dose-dependent and substantial reduction of MAP but the sensitivity to acetylcholine was significantly different between the strains (Fig. 3A). The fall of MAP occurring during infusion of 0.0002–0.02 μg/kg BW of acetylcholine is likely eNOS-dependent since it is absent in eNOS-KO. Higher concentrations of acetylcholine induced BP reduction in eNOS-KO as well. Data analysis by Two-way ANOVA revealed a significant decrease in sensitivity to acetylcholine-induced hypotension in eNOS-KO ($P = 0.0003$). We observed a hypotensive response to acetylcholine in eNOS-Tg/KO that was almost similar to C57BL/6 ($P = 0.376$) but significantly more sensitive than that of eNOS-KO ($P = 0.012$, Fig. 3A). In accordance, i.v. injection of the NOS-inhibitor L-NAME caused a rapid increase of sBP, dBP and MAP in C57BL/6 and eNOS-Tg/KO but not in eNOS-KO. Original tracings and mean values are shown in Fig. 3B–E. These data suggest that reintroduced eNOS in resistance arteries of eNOS-Tg/KO was as functional as in aortic rings.

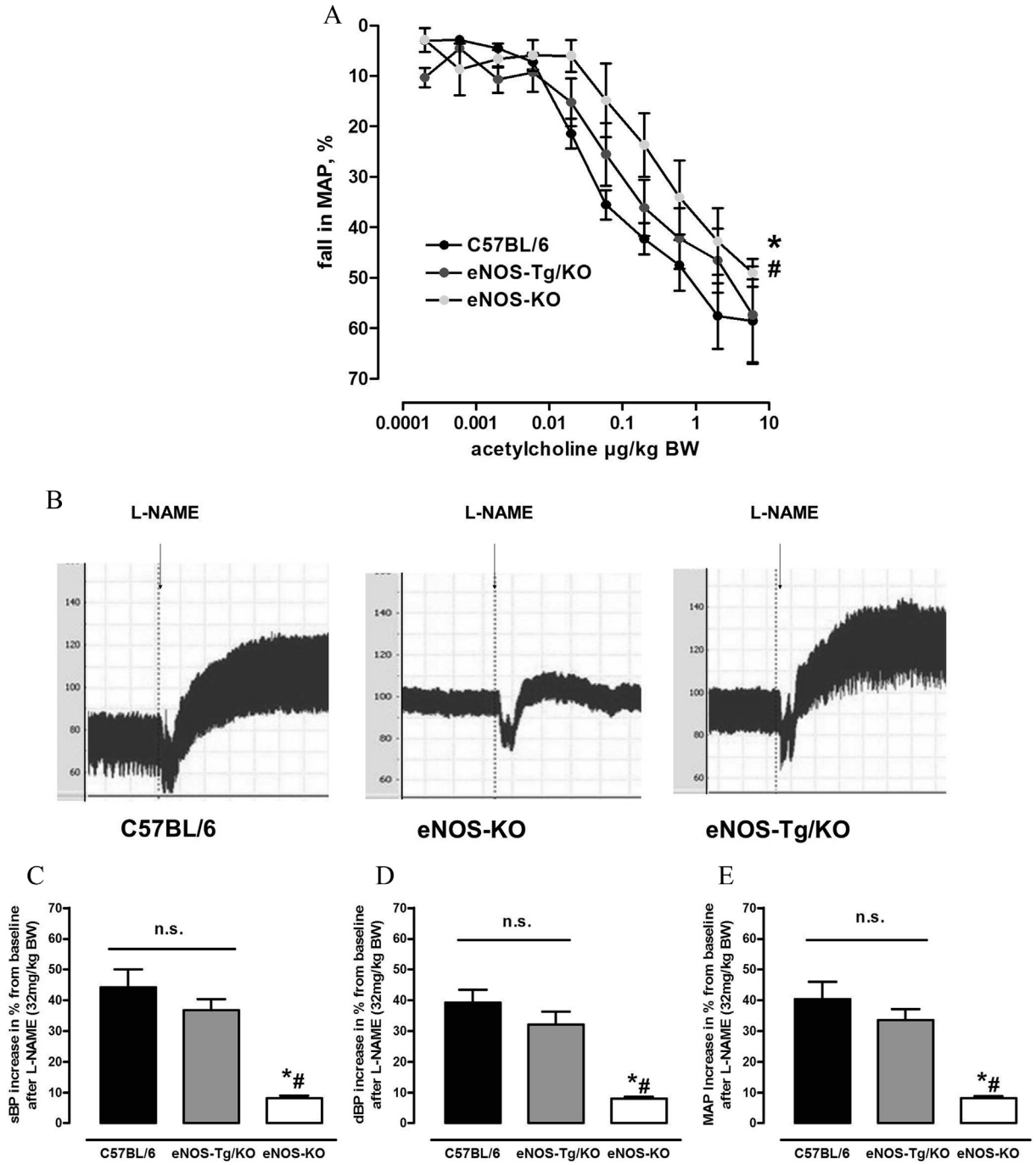

Fig. 3. Resistance vessel reactivity of eNOS-Tg/KO. (A) Acetylcholine-induced fall in mean arterial pressure (MAP) measured invasively in C57BL/6, eNOS-KO and eNOS-Tg/KO (*P < 0.05 vs. C57Bl/6, #P < 0.05 eNOS-Tg/KO vs. eNOS-KO, n = 3–4, Two-way ANOVA). (B) Representative original recordings of L-NAME-induced increase of MAP in C57BL/6, eNOS-KO, eNOS-Tg/KO and mean summary of L-NAME-induced changes of (C) systolic (sBP), (D) diastolic (dBP) and (E) MAP (*P < 0.001 vs. C57BL/6, n = 4, #P < 0.001 vs. eNOS-Tg/KO, n = 6, One-way ANOVA).

3.4. Hypertension and bradycardia in eNOS-Tg/KO

Our findings demonstrate that bovine eNOS of eNOS-Tg/KO is functional in both conductance and resistance arteries. Thus, we expected that hypertension occurring in eNOS-KO would not be a phenotype of eNOS-Tg/KO but this was not the case. Using a well-established tail-cuff system [19,25], we observed hypertension in eNOS-Tg/KO that was indistinguishable from eNOS-KO (Fig. 4A). In addition, bradycardia occurring in eNOS-KO was evident in eNOS-Tg/KO as well (Fig. 4B). The finding of sustained hypertension in eNOS-Tg/KO was confirmed by invasive BP measurement with a pressure transducer placed into the right carotid artery (Fig. 4C). Long-term oral treatment with L-NA caused an increase of BP only in C57BL/6 so that there was no BP difference between the 3 strains anymore (Fig. S5a). Likewise, the significant reduction of heart rate induced by L-NA was similar in eNOS-KO and eNOS-Tg/KO but more pronounced in C57BL/6 (Fig. S5b). These data suggest that endothelial-specific reintroduction of functionally active eNOS is not sufficient to rescue hypertension and bradycardia of eNOS-KO.

4. Discussion

Accumulating evidence, including observations that mice lacking the eNOS gene are hypertensive, has demonstrated that eNOS-derived NO plays an important role in the regulation of BP. Although it is generally assumed that hypertension in eNOS-KO is caused by the lack of endothelium-dependent NO induced vasodilation, there is in fact no evidence from knockout models clearly linking eNOS activity in endothelial cells to the regulation of BP. To address this question we have reintroduced eNOS into eNOS-KO in an endothelium-specific manner. Surprisingly, we found not even a partial reduction of BP in eNOS-KO. Therefore, our data suggest that the activity of eNOS expressed in non-vascular tissue might play a role in physiologic BP regulation.

Endothelial-specific reintroduction of eNOS in eNOS-KO resulted in a lower eNOS protein expression in different tissues of eNOS-Tg/KO. Therefore, we investigated vascular eNOS activity by acetylcholine-induced endothelium-dependent vasodilation in conductance and resistance vessels, i.e. in aortic rings and in mice in-vivo. We observed no aortic response to acetylcholine in eNOS-KO [1,6] but a complete and indistinguishable dilation in C57BL/6 and eNOS-Tg/KO. Thus, aortic eNOS in eNOS-Tg/KO appears to be functionally active and the lower level of expression didn't impair the magnitude and sensitivity of endothelium-dependent aortic relaxation. Previous investigations have shown that the sensitivity of the NO-receptor sGC likely adapts to variations of eNOS expression and tonic vascular NO generation inasmuch as NO can induce nitrosylation of sGC which inhibits its activity. This was demonstrated in smooth muscle and endothelial cells upon treatment with the NO-donors or vascular endothelial growth factor [26] and in aortic and lung tissues of eNOS-Tg or of C57BL/6 treated with L-NA [17]. Such sGC S-nitrosylation might also underlie the hypersensitivity of aortic rings of eNOS-KO to organic nitrates such as glycerol trinitrate [6], and the spontaneous NO-donor spermine-

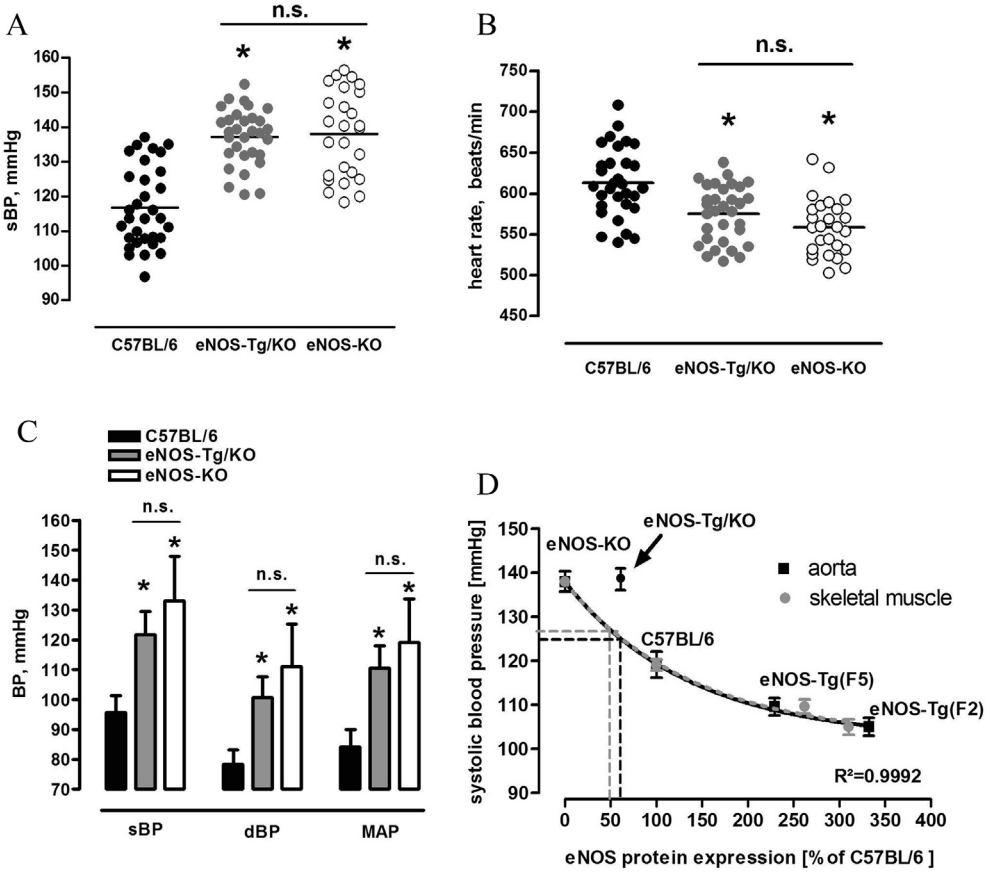

Fig. 4. Sustained hypertension in eNOS-Tg/KO. (A) sBP and (B) heart rate measured by tail-cuff method in eNOS-Tg/KO (n = 31), eNOS-KO (n = 26) and C57BL/6 (n = 32, $P < 0.001$ vs.C57BL/6, One-way ANOVA) (C) sBP, dBP and MAP measured invasively in all strains (*$P < 0.05$, n = 4–7, One-way ANOVA). (D) Non-linear correlation between aortic and skeletal muscle eNOS protein expression and sBP measured by tail cuff method in eNOS-KO, C57BL/6 and two different colonies of eNOS-Tg (F2 and F5). Endothelial-specific targeting of eNOS in eNOS-KO to 61.1% of the aortic level of C57BL/6 was expected to lower sBP in eNOS-Tg/KO to appr. 124.5 mmHg (black dotted lines and an arrow). 54.2% of the skeletal muscle eNOS expression (as compared to C57BL/6) was expected to reduce sBP to 126 mmHg (grey dotted line).

NONOate [27]. Thus, correction of the NO-hypersensitivity occurring in eNOS-KO can be viewed as further evidence for normal aortic eNOS function in eNOS-Tg/KO. In accordance, the increased sensitivity of aortic tissue of eNOS-KO to alpha-adrenergic stimulation by phenylephrine as described previously [6] was completely reversed in eNOS-Tg/KO which showed a concentration-dependent vasoconstriction similar to C57BL/6. Taken together, these data suggest a normal function of eNOS in conductance vessels of eNOS-Tg/KO.

To investigate endothelium-dependent vasodilation of resistance arteries in-vivo we invasively monitored BP of mice subjected to increasing doses of i.v. acetylcholine as performed previously in rabbits [5] and rats [18]. Acetylcholine produced a dose-dependent and strong fall of BP in all strains including eNOS-KO albeit with a lower sensitivity to acetylcholine. Therefore, other endothelium-dependent vasodilators appear to compensate for the lack of eNOS in resistance arteries. For example, the dilatory response of coronary arteries to acetylcholine is preserved by compensatory generation of e.g. cyclooxygenase products [11,12]. Furthermore, preservation of endothelium-dependent vasodilation has been demonstrated in femoral and mesenteric vessels [13], cerebral arteries [14,16] and small skeletal muscle arterioles [15] of eNOS-KO mice. While these data indicate compensation for the lack of endothelial generation of NO in eNOS-KO, our data suggests that endothelial-specific reintroduction of eNOS to eNOS-KO partially reverses such compensation since there was no statistically significant difference in sensitivity to acetylcholine between eNOS-Tg/KO and C57BL/6. Furthermore, the strong and comparable increase of systolic, diastolic and mean arterial pressure to i.v. L-NAME in C57BL/6 and eNOS-Tg/KO suggests the reversion of compensatory mechanisms and indicates a functional eNOS enzyme in resistance arteries of eNOS-Tg/KO as observed in aortic rings.

In view of the hypotensive effect of endothelial-specific overexpression of eNOS [17,19,28] we correlated skeletal muscle eNOS expression with sBP including data on colony 5 of eNOS-Tg [17] which was used to generate eNOS-Tg/KO (Fig. 4D). We found a highly significant one phase exponential decay (R^2 =0.992) described by the equation $Y = SPAN \cdot e^{(-K \cdot X)} + Plateau$ (Y = sBP; SPAN = 35.56; X = Expression; K = 0.00720; Plateau = 102.3) and similar values were found for the correlation of aortic eNOS expression and sBP (R^2 =0.9992; SPAN = 35.92; K = 0.00727; Plateau = 102.0). Using these equations the expected sBP calculated for eNOS-Tg/KO was 126.0 and 124.5 mmHg, respectively. Hence, the predicted reduction of ≥12 mmHg of sBP in eNOS-Tg/KO did not occur. Moreover, we observed not even a part of this expected reduction of sBP regardless of the method of measurement suggesting that endothelial-specific and thus vascular expression of eNOS alone appears not sufficient to reduce BP in eNOS-KO.

It has been shown that both expression and activity of vascular extracellular SOD (ecSOD) are dependent on bioavailability of vascular endogenous NO and are largely reduced in eNOS-KO [24,29]. Likewise, upregulation of eNOS by exercise triggers induction of ecSOD expression [24]. In striking contrast to lower expression levels of ecSOD in eNOS-KO, the expression of ecSOD in eNOS-Tg/KO was elevated to the levels observed in aorta and in skeletal muscle of C57BL/6. We observed also no difference in the expression of other important proteins which may modify and/or compensate for the activity of vascular NO. For example, there was no change in the expression of the NO receptor sGCβ1- and α1-subunits which confirms previous results on sGC expression and activity in eNOS-KO [6] as well as eNOS-Tg [17]. Despite the small amount of nNOS present in blood vessel nerves, it has been reported that low levels of nNOS-derived NO could compensate for the lack of eNOS when eNOS activity is compromised. For instance, nNOS-cGMP-dependent pathways dilated pial arterioles [14] and nNOS–derived NO contributed to the flow-induced responses in coronary arteries of eNOS-KO [30]. However, there was no compensatory upregulation of nNOS protein in aortic and skeletal muscles of eNOS-Tg/KO as compared to C57BL/6 suggesting that such effects unlikely contributed in eNOS-Tg/KO to the effects of acetylcholine and L-NAME in aortic rings and in vivo. Finally, we investigated whether eNOS-Tg/KO show an increase of protein tyrosine nitration which likely reduces vascular NO-bioavailability [31] and found no differences between the strains. Thus, examination of the expression levels of important proteins associated with vascular NO-activity showed no alterations which would call into question a normal vascular NO activity.

Taken together our data demonstrate a previously unrecognized obligatory role of extra-endothelial eNOS in the physiologic regulation of BP. They open the perspective that non-endothelial eNOS impairment may contribute to the pathogenesis of hypertension which is complex, multifactorial and incompletely understood [32]. Several extra-endothelial locations of eNOS might be of particular importance [33–35]. For example, there is considerable evidence that NO in the central nervous system affects sympathetic nerve activity and modulates BP and heart rate [36,37]. Our novel mouse model might also be a useful tool to study whether current antihypertensive treatments are similarly effective if non-endothelial eNOS is missing.

Conflict of interest

The authors have declared that no competing interests exist.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.152.

Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.152.

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