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ORIGINAL ARTICLE

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Regulation of renin gene expression in kidneys of eNOS- and nNOS-deficient mice

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Abstract Our study aimed to assess the roles of nitric oxide derived from endothelium NO-synthase (eNOS) and macula densa neuronal NO-synthase (nNOS) in the regulation of renal renin expression. For this purpose renin mRNA levels and renin content were determined in kidneys of wild-type (wt), nNOS-deficient (nNOS–/–), and eNOS-deficient (eNOS–/–) mice, in which the renin system was suppressed by feeding a high-salt diet (NaCl 4%), or was stimulated by feeding a low-salt (NaCl 0.02%) diet together with the converting-enzyme inhibitor ramipril (10 mg kg^{-1} day^{-1}). In all mouse strains, renin mRNA levels were inversely related to the rate of sodium intake. In eNOS–/– mice renin mRNA levels and renal renin content were 50% lower than in wt mice at each level of salt intake, whilst in nNOS–/– mice renin expression was not different from wt controls. Administration of the general NO-synthase inhibitor nitro-L-arginine methyl ester (L-NAME, 50 mg kg^{-1} day^{-1}) to mice kept on the low-salt/ramipril regimen caused a decrease of renal renin mRNA levels in wt and nNOS–/– mice, but not in eNOS–/– mice. These observations suggest that neither eNOS nor nNOS is essential for up- or downregulation of renin expression. eNOS-derived NO appears to enhance renin expression, whereas nNOS-derived NO does not.

Key words mRNA · NO synthase inhibition · NO synthase knockout · Renin · Renin content

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Introduction

A possible role for nitric oxide (NO) in the control of renin synthesis and secretion has been investigated in a number of in vivo and in vitro studies. Although some experimental discrepancies remain, the majority of acute or subacute in vivo studies utilizing NO donors or NO-synthase (NOS) inhibitors suggest that NO exerts a stimulatory effect on renin secretion and renin gene expression [1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12]. Enhancement of the renin-activating effect of various stimulators by NO may be explained by cGMP-mediated inhibition of cAMP degradation in renal juxtaglomerular granular (JG) cells [13]. Regulation of renin secretion and synthesis by NO seems plausible because JG cells, the major renal cell type responsible for renin synthesis and secretion, are surrounded by cells with a high capacity for NO formation. Nevertheless, understanding the functional connection between NO and the renin system is complicated by the facts that at least two cell types in the vicinity of the JG cells could be the source of NO, and that these two cell types express different NOS isoforms. Endothelial cells possess endothelial NOS (eNOS) [14, 15], whereas tubular macula densa cells express the neuronal form of NOS (nNOS) [16, 17]. Despite several attempts, it has not been possible to clearly distinguish between the contributions of macula-densa-derived and endothelium-produced NO in the overall effect of NO on renin production.

The availability of mice with genetic ablations in specific NOS isoforms offers an opportunity to obtain direct information about the role of particular NOS subtypes in renin synthesis and renin secretion. Mice with null mutations in either nNOS or eNOS have been generated recently. Initial reports have shown that renal renin mRNA levels in eNOS–/– mice under control conditions are moderately decreased whereas plasma renin activities are increased [18]. Furthermore, nNOS-deficient mice have been reported to have a reduced renal renin content under basal conditions, but to show an essentially normal increase of renal renin content in response to a low-sodi-

um intake [19]. The present experiments on eNOS- and nNOS-deficient mice were performed to define the roles of specific NO-synthases in the control of the renin system. In particular, the study aimed to differentiate between the importance of nNOS and eNOS for the regulation of the renal renin system. For this purpose nNOS–/– and eNOS–/– mice were subjected to interventions known to stimulate or suppress renal renin mRNA levels. Our findings suggest that neither eNOS nor nNOS activity controls the changes in renal renin expression. However, eNOS, but not nNOS, appears to enhance renin synthesis in vivo, and thus to determine the amplitude of changes of the renin system in response to a regulatory challenge.

Materials and methods

Animals

The experiments were conducted using homozygous nNOS (nNOS–/–) or eNOS knockout mice (eNOS–/–) of both sexes [19, 20]. As controls, wild-type (wt) littermates (nNOS+/+ or eNOS+/+) were used. The strain background (129/SvEv × C57Bl/6) of nNOS–/– and +/+ animals, and (C57Bl/6 × CBA F1) of the eNOS+/+ and -/- animals were identical for each knockout strain.

Animal experiments

nNOS–/– and eNOS–/– and their respective littermates were divided into groups of five to seven animals and subjected to four different experimental treatments:

Group 1: Control animals received normal chow (0.6% NaCl) and remained untreated.

Group 2: Animals were maintained for 7 days on mouse chow balanced in all respects except for a high-salt content (4% NaCl, Altromin).

Group 3: Animals were kept on a low-salt diet (0.02% NaCl, Altromin) for 7 days. On the last 3 days of the treatment week, mice also received the angiotensin-converting-enzyme inhibitor ramipril (10 mg kg^{-1} day^{-1}) in the drinking water.

Group 4: Animals were treated with a low-salt diet plus ramipril as in group 3. In addition, mice received the NOS inhibitor nitro-L-arginine methyl ester (L-NAME, 50 mg kg^{-1} day^{-1}) for the last 3 days of the diet. L-NAME was applied in the drinking water.

At the end of the treatment week animals were killed by decapitation and the kidneys were rapidly removed and frozen in liquid nitrogen. The organs were stored at –80°C until isolation of total RNA, which was extracted from the frozen kidneys as described by Chomczynski and Sacchi [21].

Determination of preprorenin mRNA by RNase protection assay

Renin mRNA was measured by an RNase protection assay. To detect preprorenin mRNA, an antisense RNA probe suitable for detecting mRNA levels from both renin genes (*Ren-1* gene and *Ren-2* gene) was generated by in vitro transcription of the plasmid vector pSP73 (Promega-Serva, Heidelberg, Germany) containing a PCR-derived fragment of mouse preprorenin cDNA [22]. The 194-bp fragment, amplified by an upstream primer (5'-ATG AAG GGG GTG TTC TGT GGG GTC-3'; binding at 810–832 bp) and a downstream primer (5'-ATG CGG GGA GGG TGG GCA CCT G-3'; binding at 981–1003 bp), was cloned in a *Bam*HI/*Eco*RI-digested pSP73 vector using standard protocols. Linearization with *Hin*dIII and in vitro transcription with SP6 RNA polymerase yielded a 248-bp fragment. Hybridization, RNase digestion, phenol/chloroform extraction and acrylamide electrophoresis are described in detail elsewhere [4].

Determination of cytosolic β-actin by RNase protection assay

The presence of cytosolic β-actin mRNA was measured by an RNase protection assay. The upstream primer (5'-CCA ACT GGG ACG CAT G-3'; binding at 152–168 bp) and downstream primer (5'-TGG CGT GAG GGA GAG CAT-3'; binding at 428–445 bp) were used to amplify a 293-bp fragment of mouse β-actin cDNA [23]. Cloning in a *Bam*HI/*Eco*RI-digested pSP73 plasmid, linearization with *Hin*dIII, and in vitro transcription with SP6 RNA polymerase yielded a 347-bp antisense RNA transcript. β-Actin mRNA was used as a standard RNA for controlling the quality of the RNA preparation. Total RNA (1 µg) was hybridized under the conditions described previously [4].

Determination of renal renin content

Renal renin content was determined by measuring the capacity of homogenized kidneys to generate angiotensin I (ANG I). The frozen kidneys (150–200 mg) were homogenized in 8 ml phosphate buffer and centrifuged at 20,000 *g* for 45 min. The supernatants were removed, frozen at –20°C and then thawed three times by changing the temperature from –20 to 4°C. For the generation of ANG I, 50-µl aliquots of 1:50 supernatant dilutions were incubated with saturating concentrations of rat renin substrate and the generated ANG I was assayed with a commercially available radioimmunoassay kit (Sorin Biomedica, Düsseldorf, Germany). Values are given as micrograms of ANG I generated per hour of incubation per milligram dry weight of the kidney.

Blood pressure measurements

Mice were anesthetized with urethane (1.5 g/kg, i.p.). The throat was incised and a catheter of stretched PE tubing filled with phosphate-buffered saline containing 50 U heparin/ml was inserted into the exposed right carotid artery. Pulsatile blood pressure was recorded on a Gould recorder. Data were recorded on an analog plotter and transferred simultaneously to a personal computer. Mean blood pressures were calculated from the data collected for 10 min starting 30 min after the onset of anesthesia. Animals were kept on a 37°C warming plate during data collection.

Statistics

Significance levels between animal groups were calculated using the ANOVA test. A value of $P<0.05$ was considered significant.

Results

Using an RNase protection with a cRNA probe able to protect different length fragments of *ren-1* and *ren-2* mRNA, *ren-1* mRNA was detected with 20 µg of total kidney RNA in all animals. In contrast, in none of the mice examined in the course of this study were significant amounts of *ren-2* mRNA found. For semiquantification of *ren-1* mRNA abundance, the hybridization signals were related to those obtained for β-actin mRNA

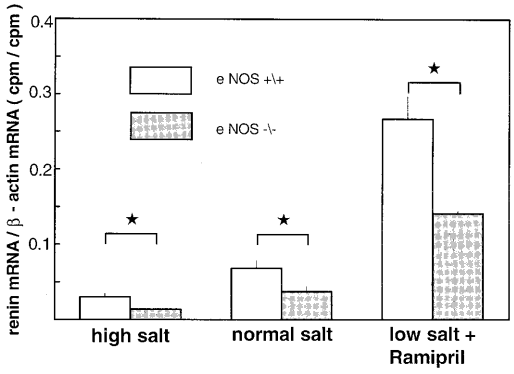

Fig. 1 Renin mRNA/β-actin mRNA ratio for mice deficient in endothelial nitric oxide synthase (*eNOS–/–*) and their wild-type controls kept on a normal or high-salt diet or on a low-salt diet with ramipril. Data are means ±SEM of seven mice in each group. *Asterisks* indicate $P<0.05$

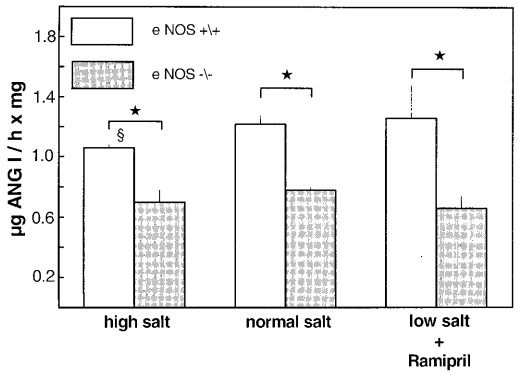

Fig. 3 Renal renin content in eNOS–/– mice and their wild-type controls subjected to different experimental maneuvers such as normal and high-salt diet, a low-salt diet plus additional treatment with ramipril. Data are means ±SEM of seven mice in each group. §$P<0.05$ between animals on a normal and a high-salt diet within the same mouse strain

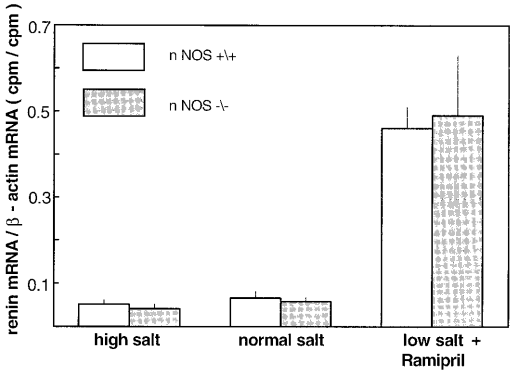

Fig. 2 Renin mRNA/β-actin mRNA ratio for mice deficient in neuronal NOS from the macula densa (*nNOS–/–*) and their wild-type controls kept on a normal or high-salt diet or on a low-salt diet with ramipril, an angiotensin converting enzyme inhibitor. Data are means ±SEM of five mice in each group. *Asterisks* indicate $P<0.05$

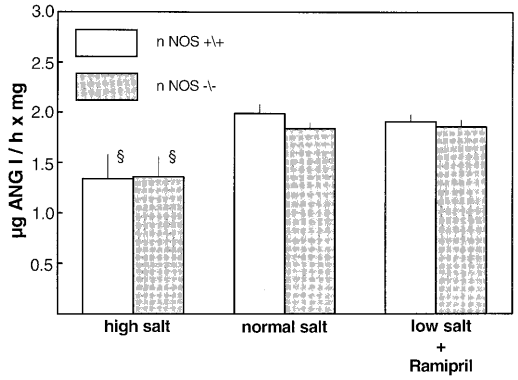

Fig. 4 Renal renin content in nNOS–/– mice and their wild-type controls subjected to different experimental maneuvers such as a normal and a high-salt diet, a low-salt diet plus additional treatment with ramipril. Data are means ±SEM of five mice in each group. §$P<0.05$ between animals on a normal and a high-salt diet within the same mouse strain

serving as a highly abundant and constitutively expressed mRNA. In fact, β-actin mRNA levels in the kidneys were invariable among the different experimental conditions as well as between wt, nNOS–/– and eNOS–/– mice (not shown).

In mice kept on a diet with standard NaCl content, the *ren-1* mRNA/β-actin mRNA ratio was decreased by about 50% in eNOS–/– mice compared to their littermate controls (Fig. 1). In contrast, there was no difference in *ren-1* expression corrected for β-actin between wt and nNOS–/– mice (Fig. 2).

A low-salt diet plus converting enzyme inhibition (ramipril) increased renin mRNA abundance by about 500% both in wt, nNOS–/– and eNOS–/– mice, the absolute levels being again significantly lower in eNOS–/– than in wt or nNOS–/– mice (Figs. 1 and 2). In mice maintained on a high-salt diet renin mRNA levels were suppressed to about 60% of their respective controls. Again, absolute renin mRNA levels in eNOS–/– mice were significantly lower than in wt or nNOS–/– animals (Figs. 1 and 2).

As an indicator of the amount of stored renin in the kidneys we determined total renin activity in the kidney tissue. As shown in Figs. 3 and 4, after 10 days on the respective salt diets the renal renin content was not markedly changed. However, the renin content of eNOS–/– mice was under all conditions significantly lower than that of their respective wild types (Fig. 3). In contrast, renal renin content in nNOS–/– mice was not different from that found in wt controls (Fig. 4).

To confirm the assumption that the altered expression of renin in eNOS–/– mice was primarily due to the lack of endothelial NO formation, we examined the effects of the pharmacological NOS inhibitor L-NAME on renin expression in eNOS–/– mice and their wt controls. In animals in which the renin system was stimulated by a low-salt diet plus conversion enzyme inhibitor, the addition of L-NAME to the drinking water caused a significant re-

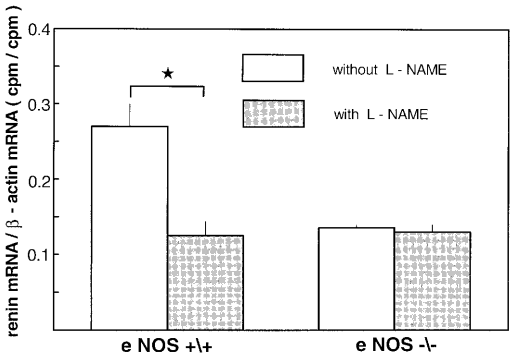

Fig. 5 Renin mRNA/β-actin mRNA ratio for eNOS–/– mice and their wild-type controls kept on a low-salt diet with ramipril with or without additional treatment with the general NO-synthase inhibitor nitro-L-arginine methyl ester (*L-NAME*). Data are means ±SEM of five mice in each group. *Asterisks* indicate $P<0.05$

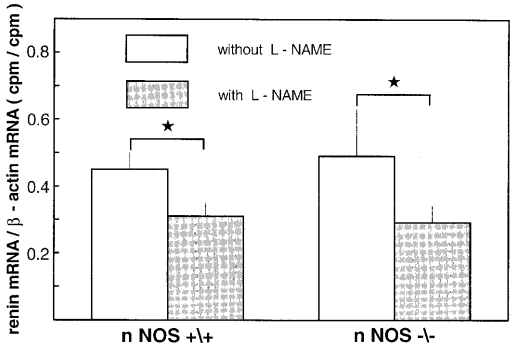

Fig. 6 Renin mRNA/β-actin mRNA ratio for nNOS–/– mice and their wild-type controls kept on a low-salt diet with ramipril with or without additional treatment with L-NAME. Data are means ±SEM of five mice in each group. *Asterisks* indicate $P<0.05$

duction of renin mRNA in littermate controls of both wt and nNOS–/– mice (Figs. 5 and 6). In contrast, L-NAME exerted no effect on renin mRNA in eNOS–/– animals (Fig. 5). In parallel to the changes of renin mRNA levels, renal renin content decreased by 20% in wt and nNOS–/– mice but not in eNOS–/– mice under treatment with L-NAME.

It has been found repeatedly that mean arterial blood pressure is elevated in eNOS–/– mice, whereas it is normal in nNOS–/– mice [18, 20, 24, 25]. Since the blood pressure is considered to be a determinant of renin synthesis, we examined whether the changes of renin expression in eNOS–/– mice paralleled changes in blood pressure. Mean arterial blood pressure in wt animals kept on a normal salt diet was on average 98 mmHg (Table 1). Blood pressure in eNOS–/– animals on a normal salt diet was on average elevated to 135 mmHg (Table 1). The high-salt diet did not change blood pressure in either mouse strain (Table 1). A low-salt diet combined with an angiotensin I converting enzyme (ACE) inhibitor (for 3 days) reduced mean arterial pressure to 57 mmHg both in wt and eNOS–/– groups (Table 1). Additional treatment of those animals with the NOS inhibitor L-NAME did not significantly increase blood pressure in either strain (64 mmHg in wt and 59 mmHg in eNOS–/–) (Table 1). Thus, the clear differences of renin expression between wt and eNOS–/– kept on a low-salt diet plus ACE inhibitor cannot be attributed to changes of blood pressure.

Table 1 Data are means ±SEM of six mice in each group. *Asterisks* indicate $P<0.05$ between eNOS+/+ and eNOS–/–. (*eNOS+/+* Wild-type mice with endothelial nitric oxide synthase, *eNOS–/–* eNOS-deficient mice)

	Mean arterial pressure (mmHg)	
	eNOS+/+	eNOS–/–
High salt (4% w/w)	99±4	131±6*
Normal salt (0.6%)	98±4	135±6*
Low salt (0.02%) + ramipril	57±3	56±5
Low salt + ramipril+ L-NAME	64±6	59±5

Discussion

Studies of the effects of pharmacological inhibitors of NOS have indicated that NO is an important regulator of renin synthesis and secretion. Local actions of NO at the level of the juxtaglomerular apparatus seemed plausible, since NO-generating enzymes are expressed both in endothelial and in macula densa cells, two cell types in the immediate vicinity of the renin-producing granular cells. The aim of the present study was to discriminate between the roles of endothelium- and of macula-densa-derived NO in renin production by using transgenic mice with null mutations in either eNOS or nNOS.

In mice lacking eNOS activity, basal renin mRNA abundance as well as renin content were found to be significantly lower than in wt mice suggesting that NO derived from endothelial cells acts as a tonic stimulator of renin synthesis. This observation is in accordance with a previous study that reported lower renal renin mRNA levels in eNOS–/– mice kept on a normal salt diet [18]. In nNOS–/– mice, in contrast, renal renin content or renin mRNA levels were not significantly different from values found in the wt animals. The finding that renal renin content was comparable between nNOS–/– and wt mice is in conflict with a previous report in which nNOS deficiency was associated with 45–60% lower levels of renal renin when compared to wt mice of the 129/SvEv and C57BL/6 progenitor strains [19]. It is currently unclear whether these different results are due to differences in the genetic background of the control animals used or to a role of nNOS in renin control mechanisms not mediated by the macula densa. Failure to detect a reduction in renin expression in the nNOS–/– mice is also unexpected in view of the findings that 7-nitroindazole (7-NI), used as a more selective inhibitor of nNOS activity, has been reported to attenuate the stimulation of renin gene expression in angiotensinogen knockout mice [26] and to block the acute renin secretory effect of fu-

rosemide in rats, a response presumably mediated through the macula densa [6]. Thus, the effects of acute or chronic interference with macula densa NO production on renin expression may be different. On the other hand, recent studies suggesting inhibition of eNOS by 7-NI indicate that the specificity of this compound to inhibit nNOS is not absolute [27].

As has been demonstrated earlier, renin mRNA levels in both rats and mice change inversely with the rate of salt intake, being moderately decreased in animals on a high-salt diet and strongly increased in sodium-depleted animals treated with a converting enzyme inhibitor [19, 24]. This well-described regulation of renal renin expression was essentially conserved inNOS-deficient mice suggesting that the basic physiological mechanisms responsible for regulating renin gene expression are not dependent upon the presence of either nNOS or eNOS activity. A similar conclusion was reached previously, following the observation that sodium depletion causes the same increase of renal renin content in nNOS–/– and in wt mice [19]. The maintenance of the regulated abundance of renin mRNA levels and renal renin contents was more complete in nNOS–/– mice, and there were no discernible differences between knockout and wt animals. In eNOS–/– mice, on the other hand, renin expression at each level of stimulation was reduced by about 50% compared to wt animals.

It has been found repeatedly that mean arterial blood pressure is elevated in eNOS–/– mice, whereas it is normal in nNOS–/– mice [18, 20, 25, 28]. The data of the present study confirm these observations (Table 1). Since blood pressure is a negative determinant of renin expression – at least in the range below normal renal perfusion pressures, an increase in blood pressure may account for the lower renin mRNA levels in eNOS–/– mice. However, in animals kept on a low-sodium diet plus converting enzyme inhibition, blood pressure was reduced to the same extent in eNOS–/– and eNOS+/+ animals, whilst renin mRNA levels were quite different. Treatment of mice kept on a low-salt diet plus converting enzyme inhibitor with the NOS inhibitor L-NAME did not significantly change blood pressure but decreased renin mRNA in eNOS+/+ but not in eNOS–/– mice. Apparently, the differences of renin expression between eNOS+/+ and eNOS–/– mice are not obligatorily linked to changes of blood pressure. Although from our data we cannot exclude the possibility that increased blood pressure in eNOS–/– mice theoretically contributes to the decreased renin expression observed during the normal or high-salt intake, we conclude that the marked reduction in renin mRNA and renal renin content in eNOS–/– mice is not primarily a consequence of differences in blood pressure. The regulation of renal renin expression in chronic eNOS–/– mice is similar to that seen in rats during acute pharmacological blockade of NO-synthases, where the activity of the renin system was depressed regardless of whether the renin system was prestimulated [4, 9, 24, 29, 30] or suppressed by a high-salt intake [11, 12, 31]. Thus, NO constitutively generated by eNOS appears to enhance renal renin expression without being a direct regulator in only a selected set of circumstances. Renin regulation was similar in eNOS–/– animals and in intact animals with pharmacological NOS inhibition. This is indirect evidence favoring the hypothesis that the endothelium makes a greater contribution to the NO pool than the macula densa in the control of renin synthesis. Predominance of endothelial- over macula-densa-produced NO is directly supported by our observations that pharmacological inhibition of NOS activity by L-NAME did not further decrease renin expression in eNOS–/– mice, and was not significantly different between nNOS–/– and wt animals.

It is noteworthy that renal renin expression was not affected by deficient NO production in macula densa cells, even under conditions where macula densa nNOS expression is known to be markedly enhanced such as in animals on a low-salt diet [32, 33]. In fact, from several observations showing that the activity of the renin system changes in parallel with the expression of nNOS in the macula densa, but not with the expression of eNOS in the endothelium [32, 33, 34, 35, 36], it has been concluded that macula-densa-produced NO may be more relevant to the regulation of renin production and renin secretion than endothelium-derived NO [7, 32]. In light of the present data it would appear that the parallel changes of macula densa nNOS expression and of renin expression that occur in a number of instances [32, 33, 34, 35, 36] may not be the result of a functional link between nNOS and renin.

In summary, the findings of the present study support the concept that the constitutive production of NO generally enhances renal renin expression, and that the endothelium is the predominant source of the NO involved in affecting the level of renin expression.

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