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Peptidase activities in rats treated chronically with N^{ω}-nitro-L-arginine methyl ester (L-NAME)

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Abstract

The chronic treatment of rats with N^{ω}-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide (NO) biosynthesis, results in hypertension. This inhibition of NO production results in activation of the renin-angiotensin system, with increased activity of the carboxypeptidase angiotensin I-converting enzyme (ACE). Since chronic NO inhibition increases ACE activity, we hypothesized that this inhibition could also affect the activities of other peptidases involved in cardiovascular functions. To test this possibility, we examined the activities of aminopeptidase M (APM), dipeptidyl peptidase IV (DPP IV), metalloendopeptidase 24.15 (MEP 24.15) and neutral endopeptidase 24.11 (NEP 24.11) in rat brain, heart, kidney, liver, lung and thoracic aorta. Male Wistar rats were treated chronically with L-NAME (80 mg kg^{-1} per day) administered in the drinking water for 4 weeks and their organs then removed and processed for the determination of peptidase activities. Treatment with L-NAME did not significantly alter the activities of the four peptidases in brain, heart, kidney, liver and lung. In contrast, in aorta, the activity of APM was slightly but significantly reduced whereas those of DPP IV and MEP 24.15 were markedly enhanced; NEP 24.11 was not detected in this tissue. Immunoblotting for DPP IV and MEP 24.15 showed increased expression in aortic tissue. Neither L-NAME (1–100 μM) nor the NO donors sodium nitroprusside and 3-morpholinosydnonimine (SIN-1; 1–100 μM) had any consistent effect on the activity of recombinant MEP 24.15 or renal DPP IV. The importance of MEP 24.15 in peptide metabolism was confirmed in pentobartibal-anesthetized rats pretreated with the MEP 24.15 inhibitor *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate (JA2), which significantly potentiated the hypotensive response to bradykinin. The altered peptidase activities seen in aorta may contribute to modulating vascular responses in this model of hypertension.

Keywords: Angiotensin I-converting enzyme; Chronic inhibition; Hypertension; Nitric oxide; Peptidases; Renin-angiotensin system

1. Introduction

Nitric oxide (NO) plays a major role in modulating regional blood flow and arterial blood pressure in animals and humans. The chronic administration of NO synthase (NOS) inhibitors such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) produces sustained arterial hypertension in rats [1] which may be accompanied by histopathological changes in cardiac and renal tissue [2]. L-NAME-induced hypertension is mediated to a large extent by increased formation of angiotensin II through increased activity of the renin-angiotensin system (RAS) since treating rats with angiotensin-converting enzyme (ACE) inhibitors such as enalapril [3], or with angiotensin II receptor antagonists such as losartan [1,2] restores blood pressure to near normal levels.

In addition to ACE, various other peptidases capable of metabolizing circulating peptides could potentially modulate blood pressure. These include membrane-bound

Abbreviations: ACE, angiotensin-converting enzyme; ANP, atrial natriuretic peptide; APM, aminopeptidase M; AT_1, angiotensin II type 1 receptor; BNP, brain natriuretic peptide; CNP, C-type natriuretic peptide; DPP IV, dipeptidyl peptidase IV; JA2, *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate; MEP 24.15, metalloendopeptidase 24.15; NEP 24.11, neutral endopeptidase 24.11; L-NAME, N^{ω}-nitro-L-arginine methyl ester; NO, nitric oxide; NOS, nitric oxide synthase; QFS, quenched fluorescent substrate; RAS, renin-angiotensin system; SIN-1, 3-morpholinosydnonimine; SNP, sodium nitroprusside; TBS, Tris-buffered saline

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enzymes such as aminopeptidase M (APM; EC 3.4.11.2) and neutral endopeptidase 24.11 (NEP 24.11; EC 3.4.24.11), which are capable of degrading peptides such as angiotensin, atrial natriuretic peptide (ANP), bradykinin, neurotensin and tachykinins [4–7]. Another important membrane-bound peptidase is dipeptidyl (amino)peptidase IV (DPP IV; EC 3.4.14.5) that cleaves peptides in which proline, and to a lesser extent, alanine or hydroxyproline, is the penultimate amino acid [8]. More recently, evidence has accumulated in support of a role for soluble neutral metallopeptidases 24.15 and 24.16 as regulators of endogenous peptide activity (reviewed in [9]). Metalloendopeptidase 24.15 (EC 3.4.24.15; MEP 24.15) is an important peptidase that degrades neurotensin, somatostatin, gonadotrophin releasing hormone, bradykinin, and various peptides with opioid activity [9].

Nitric oxide can modulate the expression and activity of a variety of peptides [10], receptors [11,12] and enzymes [13], including ACE, the activity and/or expression of which is increased in the tissues of rats treated chronically with L-NAME [14,15]. Based on the enhanced activity of ACE seen in this model, we hypothesized that the inhibition of NO biosynthesis could also affect the activities of other peptidases involved in regulating cardiovascular functions. To test this hypothesis, we examined the activities of four peptidases (APM, DPP IV, MEP 24.15 and NEP 24.11) in rats treated chronically with L-NAME.

2. Materials and methods

2.1. Reagents

Bovine serum albumin, *N*-dansyl-D-alanyl-glycyl, *N*-dansyl-D-alanyl-glycyl-*p*-nitrophenyl-alanylglycine, diprotin A, dithiothreitol (DTT), Hepes, leuhistin, L-Leu-*p*-nitroanilide, β-naphthylamine, *p*-nitroaniline, N^{ω}-nitro-L-arginine methyl ester, phosphoramidon and sodium nitroprusside were from Sigma. Acrylamide, ammonium persulfate, Coomassie brilliant blue R250, glycerol, Hybond-P PVDF membrane (0.45 μm), *N*,*N*′-methylene bis-acrylamide, sodium dodecyl sulphate (SDS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (Temed), Tris base and donkey anti-rabbit IgG-peroxidase conjugate were from Amersham Biosciences. SIN-1 (3-morpholinosydnonimine) was from Cassella AG. Gly-Pro-β-naphthylamide was from Bachem. 7-Methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-D-Lys-(2,4-dinitrophenyl) (QFS) was synthesized by Auspep and was a generous gift from Dr. A. Ian Smith (Baker Research Medical Institute, Australia), who also provided the MEP 24.15/24.16 inhibitor *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate (JA2). Purified human recombinant MEP 24.15 was prepared as described previously [16]. Rabbit anti-DPP IV polyclonal antibody was from Santa Cruz Technologies and antiserum specific for MEP 24.15 was produced in rabbits immunized with recombinant 24.15 as described [17]. Flat-bottomed 96-well plates were from Corning Inc., chemioluminescence kits (SuperSignal, West Pico) were obtained from Pierce and photographic film was from Kodak. Other reagents of analytical grade were obtained from Baker, Mallinkrodt or Merck.

2.2. Animals

Male Wistar rats (∼150 g at the start of the experiment) were obtained from the Central Animal House Services (UNICAMP) and were housed at 23 ± 1 °C on a 12 h light/dark cycle with food and water ad libitum. The experiments described here were done in accordance with the guidelines established by the Brazilian College for Animal Experimentation (COBEA).

2.3. Treatment with L-NAME and blood pressure measurements

Rats were treated with L-NAME (80 mg kg^{-1} per day) given in their drinking water for 4 weeks [1]. The amount of L-NAME ingested was calculated based on the water intake of the rats which was monitored daily. Control rats received tap water alone. Once a week, the rats were weighed and tail blood pressure was measured by a tail-cuff method [18].

2.4. NOS activity

Brain NOS activity was measured by the method of Förstermann et al. [19], as described by Faria et al. [20], using [^{3}H]-arginine as substrate. Enzyme activity was expressed as pmol of [^{3}H]L-citrulline formed/min/mg of protein.

2.5. Protein concentrations

Protein concentrations were determined by the method of Lowry et al. [21] using bovine serum albumin as standard.

2.6. Tissue preparation

After 4 weeks of treatment, the rats were anesthetized with sodium pentobarbital (>60 mg/kg, i.p.; Hypnol®) and perfused via the aorta with heparinized saline to wash out blood from the organs (brain, heart, kidney, liver, lung) which were then collected, snap frozen in liquid N_2, and stored at −80 °C until used. In the case of thoracic aorta, the rats were exsanguinated without perfusion in order to avoid damage to the endothelium and the vessels then rapidly removed and processed as described above. For measurement of enzymatic activities, tissues were homogenized (Ultraturrax, model T25, 24,000 rpm) at 4 °C in 5–10 volumes of 0.1 M Tris–0.32 M sucrose, pH 7.4. The homogenate was centrifuged at 3000 × *g* (20 min, 4 °C)

and the supernatant then collected and centrifuged at 20,000 × *g* (25 min, 4 °C). The resulting supernatant was used to measure MEP 24.15 activity and the precipitate was used for APM, DPP IV and NEP 24.11 activities. For aorta, preliminary experiments indicated that the most consistent results were obtained using only low speed centrifugation (3000 × *g*, 30 min, 4 °C) and all enzyme activities were assayed in the supernatant. Aliquots of these preparations were also used for immunoblotting.

2.7. Peptidase assays

2.7.1. Aminopeptidase M (APM)

APM activity was assayed using the chromogenic substrate L-Leu-*p*-nitroanilide [22]. The assay was done in 96-well plates containing 150–220 μl of 50 mM Tris–HCl, pH 7.4, 5–50 μl of sample, and 25 μl of substrate (final conc. 140 μM), to give a final volume of 250 μl/well. The increase in absorbance at 410 nm was followed for 20–30 min at 37 °C using a SpectraMax340 multiwell plate reader (Molecular Devices) and the amount of product formed was determined from a standard curve of *p*-nitroaniline. Enzyme activity was expressed as nmol of *p*-nitroaniline formed/min/mg of protein.

2.7.2. Dipeptidyl peptidase IV (DPP IV)

DPP IV activity was assayed using Gly-Pro-β-naphthylamide in an assay mixture containing 50–70 μl of 50 mM Tris–HCl, pH 8.0, 20 μl of substrate (final conc. 200 μM), and 10–30 μl of sample. After incubation for 15–30 min, the reaction was stopped by adding 900 μl of ammonium formate, pH 4.5 and the resulting fluorescence was measured (Hitachi F-2000 spectrofluorimeter) at 410 nm after excitation at 335 nm [23,24]. The amount of product formed was determined from a standard curve of β-naphthylamine and activity was expressed in nmol of β-naphthylamine formed/min/mg of protein. To examine the possibility of a direct effect of L-NAME and NO on DPP IV activity, renal DPP IV was assayed in the presence of L-NAME and the NO donors SNP and SIN-1 (1–100 μM). The activity was monitored as the increase in fluorescence over time.

2.7.3. Metalloendopeptidase 24.15 (MEP 24.15)

The activity of MEP 24.15 was determined fluorimetrically using the specific quenched fluorescent substrate (QFS) 7-methoxycoumarin-4-acetyl-Pro-Leu-Gly-Pro-D-Lys-(2,4-dinitrophenyl), as described [25]. The assays were done under conditions of linearity (zero-order kinetics), with <10% of the total substrate being consumed during the assay. The reactions were run for 10–30 min in a final volume of 100 μl consisting of TBS (0.025 M Tris–HCl, pH 7.4, 0.125 M NaCl), 10 μM QFS, 0.5 mM DTT and 10–50 μl of sample. The activity was expressed as the increase in arbitrary fluorescence units (AFU)/min/mg of protein.

To examine the possibility of a direct effect of L-NAME and NO on MEP 24.15 activity, recombinant MEP 24.15 (16 ng/well) was assayed in TBS (without DTT), in the presence of L-NAME and the NO donors SNP and SIN-1 (1–100 μM). The activity was monitored as the increase in fluorescence over time.

2.7.4. Neutral metalloendopeptidase 24.11 (NEP 24.11)

NEP 24.11 activity was assayed using *N*-dansyl-D-alanyl-glycyl-*p*-nitrophenyl-alanylglycine as substrate. The reaction mixture consisted of 170–190 μl of 0.1 M Hepes, pH 6.4, 10–30 μl of sample and 50 μl of substrate (final concentration, 200 μM). After incubation at 37 °C for 10–20 min, the reaction was stopped by boiling the samples for 5 min. The resulting fluorescence was measured at 562 nm after excitation at 342 nm [26] and the amount of product (*N*-dansyl-D-alanyl-glycyl) formed was determined from a standard curve constructed in Hepes buffer. Enzyme activity was expressed as nmol of product formed/min/mg of protein.

2.7.5. Specificity of assays

The specificities of the activities assayed was confirmed by using leuhistin [27], diprotin A [28], *N*-[1-(*R*,*S*)-carboxy-3-phenylpropyl]-Ala-Aib-Tyr-*p*-aminobenzoate (JA2) [29] and phosphoramidon [30] to inhibit APM, DPP IV, MEP 24.15 and NEP 24.11, respectively. The specificity of the inhibition was tested in all of the organs investigated in this study and was >80% in all cases ($n = 5$ for each enzyme; results not shown). In the MEP 24.15 assay, 0.5 mM DTT was included to inhibit MEP 24.16 [16].

2.8. SDS–PAGE and immunoblotting

Aliquots (10 μg) of aortic extracts prepared as described above were electrophoresed (100 V constant) in 10% polyacrylamide gels in the presence of SDS [31] and the proteins then transferred to Hybond-P PVDF membranes [32]. Peptidases were detected by blotting with rabbit polyclonal IgG antibodies against DPP IV (diluted 1:800) or MEP 24.15 (1:5000) and then detected by chemioluminescence. The immunoreactive bands were evaluated by densitometry using the software Scion Image® and the peptidase levels were expressed as arbitrary densitometric units.

2.9. Role of MEP 24.15/24.16 in bradykinin-induced hypotension

Male Wistar rats (~200 g) were anesthetized as described above. The trachea was cannulated to facilitate breathing, and the right carotid artery and left femoral vein were cannulated with polyethylene tubing for the measurement of arterial blood pressure and drug administration, respectively. The arterial pressure was recorded continuously via a pressure transducer (Abbott) coupled to a

computer-controlled data acquisition system (Transonic Systems). Bradykinin (0.3 and 3 μg/kg, i.v.) was administered after allowing 15 min for stabilization of the preparation. In some experiments, JA2 (10 mg/kg, i.v.) was given 10–15 min before repeating the above doses of bradykinin. Since JA2 alone caused a transient hypotension at the dose used, the possible involvement of NO in this response was examined by testing this inhibitor in rats treated with L-NAME as described above.

2.10. Statistical analysis

The data are presented as the mean ± S.D. Statistical analyses were done using Student's *t*-test or analysis of variance (ANOVA) followed by the Bonferroni test. Values of $P < 0.05$ were considered as significant.

3. Results

3.1. The efficacy of chronic treatment with L-NAME

Systolic blood pressure increased significantly ($P < 0.05$) in rats treated with L-NAME (191 ± 3 mmHg after 4 weeks, $n = 10$) compared to control rats (133 ± 4 mmHg, $n = 10$). Treatment with L-NAME for 4 weeks also inhibited the activity of brain constitutive NOS by >90% ($P < 0.05$).

3.2. Effect of chronic treatment with L-NAME on peptidase activities

With the exception of NEP 24.11, which was consistently detected only in kidney and lung (greatest activity in

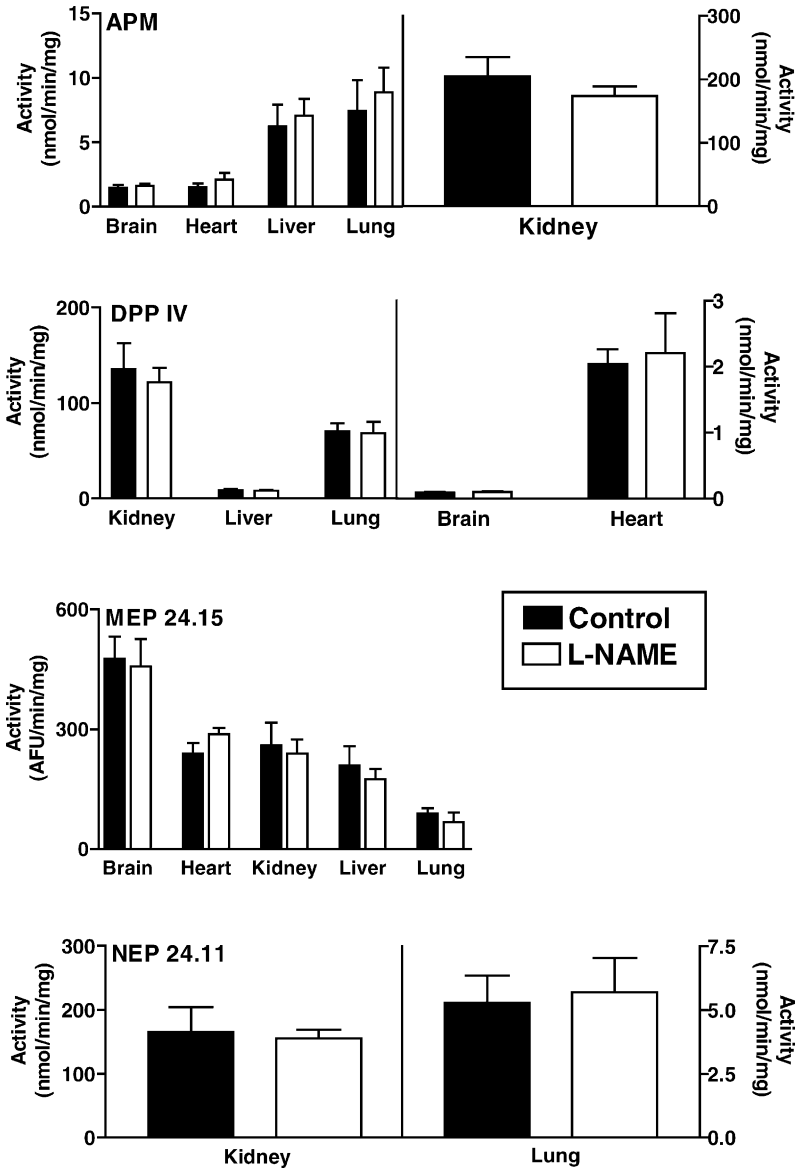

Fig. 1. APM, DPP IV, MEP 24.15 and NEP 24.11 activities in rat tissues after treatment with L-NAME for 4 weeks. The columns represent the mean ± S.D. of five rats each. There were no significant differences between control and L-NAME-treated rats. AFU, arbitrary fluorescence units.

the former), the other peptidases were detected in all of the tissues assayed. APM and DPP IV activities were highest in kidney and MEP 24.15 was highest in brain.

Fig. 1 shows that treating rats with L-NAME for 4 weeks did not significantly alter the enzyme activities of the four peptidases in any of the principal organs compared to the control rats. In contrast, the activity of aortic APM was slightly but significantly lower in L-NAME-treated rats whereas the activities of DPP IV and MEP 24.15 were markedly enhanced (Fig. 2). Since the levels of DPP IV and MEP 24.15 were increased in aortic tissue of L-NAME-treated rats, the expression of these enzymes was examined by western blotting. As illustrated in Fig. 3, immunoblots for DPP IV and MEP 24.15 showed increased expression of these enzymes in aortic tissue, which agreed with their enhanced enzymatic activity.

To further examine the influence of L-NAME and NO on the activity of DPP IV and MEP 24.15, renal membrane-bound DPP IV and purified recombinant MEP 24.15 were incubated with various concentrations of L-NAME or the spontaneous NO donors SNP and SIN-1. As shown in Figs. 4 and 5, neither L-NAME nor the NO donors SNP and SIN-1 had any consistent, marked effect on the activity of DPP IV and MEP 24.15 at concentrations up to 100 μM.

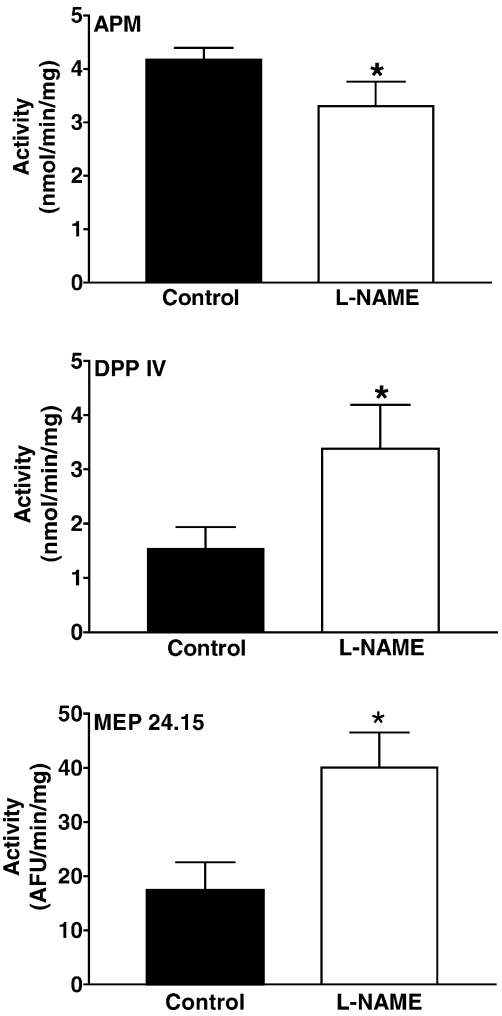

Fig. 2. APM, DPP IV and MEP 24.15 activities in rat aorta after treatment with L-NAME for 4 weeks. The columns represent the mean ± S.D. of five rats each. $^{*}P < 0.05$ compared to the corresponding control. AFU, arbitrary fluorescence units.

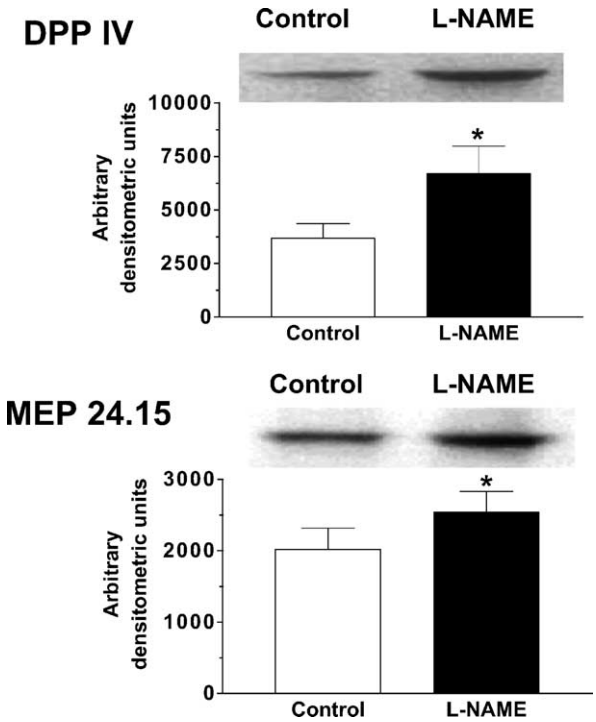

Fig. 3. Western blots for DPP IV (upper panel) and MEP 24.15 (lower panel) in rat aorta after treatment with L-NAME for 4 weeks. Representative blots and mean densitometric values for the expression of DPP IV and MEP 24.15 are shown for the control and L-NAME groups. Equal amounts of protein (10 μg) were applied to the gels in all cases. The columns represent the mean ± S.D. of four rats each. $^{*}P < 0.05$ compared to the corresponding control.

3.3. Involvement of MEP 24.15/24.16 in bradykinin-induced hypotension

To examine the role of MEP 24.15/24.16 in the hypotension induced by bradykinin, pentobarbital-anesthetized rats were pretreated with the non-selective inhibitor JA2. Administration of the inhibitor (10 mg/kg, i.v., 15 min before bradykinin) caused a transient but significant decrease in blood pressure that returned to normal within 5 min (Fig. 6A); a lower dose of JA2 (5 mg/kg, i.v.) had no such effect, nor did the vehicle solution (10% 2-hydroxypropyl-β-cyclodextrin in 0.9% saline). This hypotensive response to JA2 was attenuated in rats treated with L-NAME for 4 weeks. In normotensive Wistar rats, JA2 significantly enhanced the hypotensive responses to bradykinin (Fig. 6B). In these experiments, bradykinin was administered after the blood pressure response to JA2 had returned to basal values.

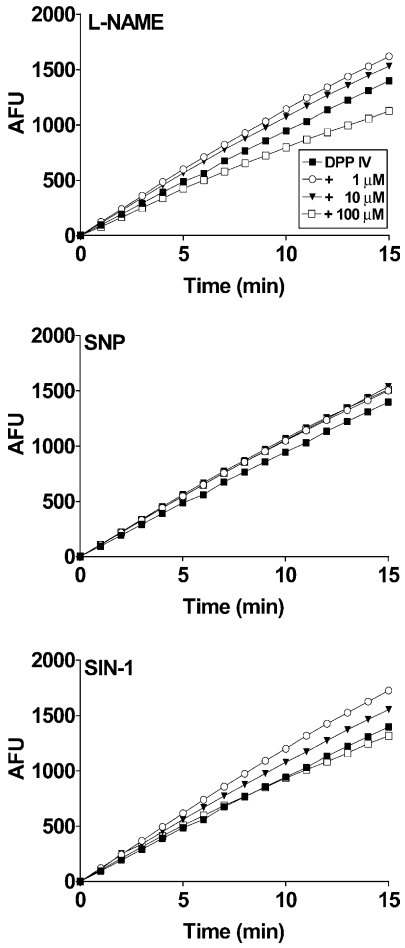

Fig. 4. DPP IV activity in the absence and presence of L-NAME, SNP and SIN-1. Peptidase activity using 5 μg of renal membrane preparation was assayed as described in Section 2. The points are the mean of two determinations, each in duplicate. AFU, arbitrary fluorescence units.

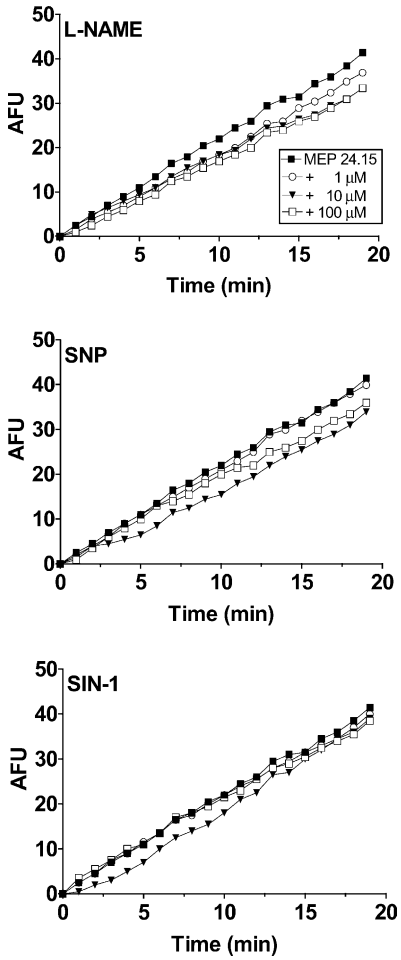

Fig. 5. MEP 24.15 activity in the absence and presence of L-NAME, SNP and SIN-1. Peptidase activity using 16 ng of purified enzyme was assayed as described in Section 2. The points are the mean of two determinations, each in duplicate. AFU, arbitrary fluorescence units.

4. Discussion

The chronic treatment of rats with L-NAME, an inhibitor of NO biosynthesis, leads to hypertension, with subsequent marked effects on the cardiovascular system (reviewed in [2]). To a large extent, the hypertension and vascular inflammatory responses observed in this model involve activation of the RAS since treating rats with ACE inhibitors or angiotensin II type 1 (AT_1) receptor antagonists reverts the above alterations [1,14,33,34]. In agreement with the involvement of the RAS, there is increased AT_1 receptor expression (mainly in the first week of treatment with L-NAME) [11] and increased ACE expression and activity in tissues, particularly heart and aorta [14,15,35]. The enhanced ACE activity contributes to vascular and myocardial damage associated with this model [36] and is mediated, at least partly, by oxidative stress, which is increased in L-NAME-treated rats [35]. In contrast to this role of ACE and angiotensin II in the hypertension resulting from chronic NO inhibition, little is known of the involvement of other peptidases in the cardiovascular responses in this model.

As shown here, treatment with L-NAME for 4 weeks did not significantly affect the activities of the four peptidases studied in brain, heart, kidney, liver and lung, but produced alterations in the levels of APM, DPP IV, and MEP 24.15 in aortic tissue. The altered activities seen in aorta agreed with the findings of Bouton et al. [37] for the serpin protease nexin-1 and of Takemoto et al. [14] and Gonzalez et al. [15] for ACE, all of whom reported increased activity of the corresponding enzyme in this vessel in this model. In addition, Takemoto et al. [14] demonstrated that, except in the heart, ACE activity was unaltered in other tissues examined (kidney, liver and lung). The finding of altered enzymatic activities in aorta, but not in other tissues, suggests that changes in peptidase activities in L-NAME-treated rats may be more associated with vascular than with non-vascular tissue or cell types. In this regard, it is possible that local changes in vascular peptidase activities within a given tissue may be masked when whole organ homogenates are used.

While there were no significant changes in APM activity in brain, heart, kidney, liver and lung after 4 weeks of treatment with L-NAME, a small but significant decrease

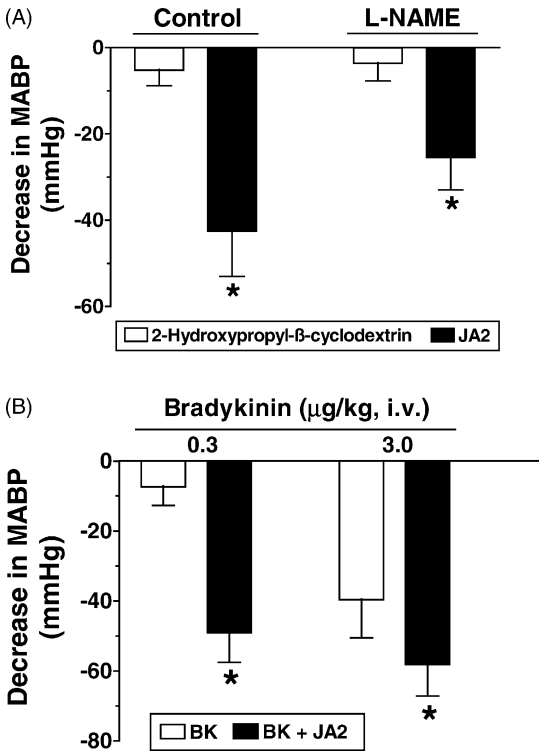

Fig. 6. Involvement of MEP 24.15/24.16 in the blood pressure response to bradykinin in pentobarbital-anesthetized male Wistar rats. (A) Effect of the dual MEP 24.15/24.16 inhibitor, JA2 (10 mg/kg, i.v.), on basal blood pressure in normotensive (control) rats and in rats treated with L-NAME for 4 weeks. JA2 alone produced transient hypotension in control and L-NAME-treated rats, with the response being attenuated in the latter. The vehicle solution alone (10% 2-hydroxypropyl-β-cyclodextrin in 0.9% saline) had minimal effect on the blood pressure. (B) Effect of MEP 24.15/24.16 inhibition by JA2 on the responses to bradykinin in anesthetized rats. Pre-treating the rats with JA2 potentiated the responses to bradykinin. Note that bradykinin was tested only after the blood pressure has returned to basal values following JA2 administration. The columns represent the mean ± S.D. of six rats each. $^{*}P < 0.05$ compared to the corresponding response seen with vehicle (2-hydroxypropyl-β-cyclodextrin) (A) or bradykinin (B) alone.

in this enzyme was seen in aorta. APM, or CD13, occurs in a variety of tissues, including aortic smooth muscle cells [38], heart and kidney. As shown here, the APM levels in kidney were consistently higher than in the other tissues in both control and L-NAME-treated rats. APM hydrolyzes the N-terminal of kallidin to form bradykinin [6,39] and can also degrade des(Asp^{1})angiotensin I, angiotensin III and enkephalins [4,39]. Since angiotensin III shares many properties with angiotensin II, including vasoconstrictor activity [40], APM could have an important role in attenuating the actions of the former peptide. Thus, the decrease in aortic APM activity in L-NAME-treated rats could represent a mechanism for exacerbating the hypertension mediated by angiotensins II and III in this model since there would be less degradation of the latter peptide; this change in enzyme activity may be greater in other vessels and microcirculatory beds, some of which are rich in APM [41].

DPP IV is a high molecular mass (>110 kDa) membrane-bound, serine-type peptidase with a wide distribution in rat tissues. This peptidase can cleave a variety of peptides and proteins, the most important of which, in terms of vascular activity, are substance P, neuropeptide Y and peptide Y ([42], reviewed in [43]). Important actions of DPP IV, additionally known as CD26, are also associated with the immunological system, particularly the activation of immunocompetent cells, as well as in cell–cell interactions [43,44]. Since the chronic inhibition of NO biosynthesis results in a cellular response involving the infiltration of inflammatory cells as well as necrosis and fibrosis in cardiac, renal and vascular tissues [2,14,33,34], we investigated whether there were any alterations in the activity of this peptidase following treatment with L-NAME.

As shown here, the only tissue in which there was a significant change in DPP IV activity was aorta, and this increase in activity appeared to involve increased expression of the peptidase rather than a direct action of L-NAME or NO donors on the enzyme itself (Figs. 3 and 4). The enhanced activity and expression of DPP IV most probably occurred at the level of the endothelium since vascular smooth muscle cells are apparently devoid of this enzyme [38]. Although little is known about the changes in neuropeptide levels in chronic L-NAME-induced hypertension, it is possible that increased vascular activity of DPP IV could serve to degrade substance P, thereby abolishing its vasodilator action and enhancing angiotensin II-induced vasoconstriction. Conversely, however, enhanced DPP IV activity would abolish the vasoconstrictor action of neuropeptide Y. Since neuropeptide Y stimulates hypertrophy in rat ventricular cardiomyocytes [45,46], and since cardiomyocyte hypertrophy is seen in L-NAME-induced hypertension, locally increased levels of vessel DPP IV could provide some protection against this phenomenon if neuropeptide Y levels are elevated in this model. This hypothesis would agree with evidence implicating DPP IV in angiogenesis and vascular remodeling [47], and with the histochemical demonstration of enhanced DPP IV activity in areas of fibrosis in cardiac tissue of rats with L-NAME-induced hypertension [48].

NEP 24.11, a membrane-bound enzyme that is identical to common acute lymphoblastic leukemia antigen (CALLA), also known as CD 10, occurs in endothelial cells, vascular smooth muscle cells, cardiomyocytes, fibroblasts and other cell types [49]. In the present study, NEP 24.11 was detected only in kidney and lung. This distribution agrees with other reports showing that these two organs are the principal sites of NEP 24.11 activity in the rat; the levels in brain are approximately 7–10-fold lower than in these two tissues and are undetectable in heart, liver, and some other tissues ([50], reviewed in [51]).

Treatment with L-NAME did not alter NEP 24.11 activity in rat tissues. This lack of effect of chronic treatment with L-NAME on NEP 24.11 activity is particularly interesting in view of the ability of dual ACE-NEP inhibitors, such as omapatrilat, in improving vascular function in a variety of cardiovascular conditions, including hypertension of

various origins (reviewed in [7]). These dual inhibitors act primarily by preventing the degradation of ANP and related peptides (BNP and CNP) by NEP 24.11. ANP levels are elevated after chronic inhibition with L-NAME [10]. Whilst this elevation involves increased ANP mRNA expression, a reduced activity of NEP 24.11 could also contribute to the higher levels of this peptide, although this seems unlikely based on the unaltered activities seen here.

NEP 24.11 can also produce angiotensin-(1–7) from angiotensin I, and this heptapeptide may antagonize the vasoconstrictor actions of angiotensin II through its vasodilator action [52]. However, the reduced production of NO, an important mediator of angiotensin-(1–7) action [53], and the increased activity of ACE, an important enzyme in degrading angiotensin-(1–7) to angiotensin-(1–5) [54], seen in L-NAME-treated rats could be important factors in limiting the action of angiotensin-(1–7) in this model. In addition to its action on ANP and angiotensin-(1–7), NEP 24.11 can degrade bradykinin by cleaving the terminal Pro-Phe bond that is also cleaved by ACE [55]. However, since the RAS is highly activated in L-NAME-induced hypertension, the levels of bradykinin in the circulation would be expected to be lower than in non-treated rats. These observations suggest that the role of NEP 24.11 in this model of hypertension may be limited, although this peptidase may be important locally in countering angiotensin II-mediated vasoconstriction in L-NAME-induced hypertension, particularly in the microcirculation and in non-aortic vessels.

Apart from DPP IV, the only other peptidase showing increased activity in this study was MEP 24.15 in aortic tissue. MEP 24.15 is an emerging target for the development of therapeutic drugs since this peptidase is involved in the degradation of a variety of vasoactive peptides, including bradykinin, and the generation of angiotensin-(1–7) from angiotensin I [9]. Angiotensin-(1–7) exerts a variety of physiological effects, including stimulation of NO and superoxide anion release from endothelial cells [53], potentiation of the vasodilatory action of bradykinin, and stimulation of prostaglandin and vasopressin release, diuresis and natriuresis (reviewed in [52,56]). Since ACE activity is augmented during chronic inhibition of NO biosynthesis, pathways leading to the formation of angiotensin-(1–7) may be activated to compensate for the increased action of angiotensin II, and this could involve MEP 24.15, although, as noted above, enhanced ACE activity could rapidly degrade angiotensin-(1–7).

Several studies in vitro and in vivo have demonstrated that MEP 24.15/24.16 have an important role in modulating the responses to vasoactive peptides, particularly bradykinin [9,57,58]. Our results confirm these findings for this peptide in normotensive rats, as shown by the marked potentiation of the hypotensive response after pretreating rats with JA2. These observations thus indicate a role for MEP 24.15/24.16 in cardiovascular regulation in rats, in agreement with a recent investigation in rat cerebral microvasculature [57] and a study in rabbits [59]. Whereas the enzyme assays in vitro were done in the presence of 0.5 mM DTT which selectively inhibits MEP 24.16 activity [16], the dual inhibitory capacity of JA2 means that the response in vivo probably reflected the action of MEP 24.15 and/or 24.16. Recent work has indicated that MEP 24.16 may be more important than MEP 24.15 in peptide metabolism by cultured endothelial cells [58]. However, it is possible that the relative contribution of these two peptidases may vary depending on the model, the animal species, and the vascular bed studied. The transient hypotension seen in response to JA2 alone could reflect an immediate action of this inhibitor on MEP24.15/24.16 or could be caused by a direct, non-specific action of this compound on the vasculature, independent of an interaction with MEP24.15/24.16. The reduced response seen in L-NAME-treated rats suggests that this phenomenon is partially NO-dependent.

The mechanism responsible for the increase in the activities of aortic DPP IV and MEP 24.15 seen here is unclear. A direct effect of NO on these enzymes seems improbable because, unlike ACE [60], the activities of these peptidases were unaffected by NO-releasing compounds (SNP and SIN-1). The activities were also unaffected by incubation with L-NAME. A lack of a direct effect of NO-donors [*S*-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) and DETA NONOate] and an NOS inhibitor (N^G-monomethyl-L-arginine, L-NMMA) on enzyme activity has also been reported for matrix metalloproteinase (MMP)-9 [61].

The enhanced expression of DPP IV and MEP 24.15 seen in western blotting suggested that the increase in enzymatic activities was mediated by events during biosynthesis of the peptidases, probably at the level of expression. This conclusion agreed with studies showing that NO regulates the expression of many proteins, including enzymes, by cGMP-dependent and independent mechanisms [13,62]. Such regulation could involve the modulation by NO of intracellular kinase signalling pathways [63] or the interaction of NO with specific regulatory regions in the genes concerned, e.g. heme oxygenase-1 [64]. The proportionally lower increase in MEP 24.15 expression seen here compared to that seen for activity may indicate that enhanced expression is only part of the explanation. Other factors, such as the intracellular levels of free ATP [65] and the degree of MEP 24.15 phosphorylation by protein kinase A at serine 644 [66] could perhaps be important in modulating MEP 24.15 activity in this model of hypertension (enzyme activity is inhibited by increased free ATP and phosphorylation).

In conclusion, the results of this study show that while the chronic treatment of rats with L-NAME does not significantly alter the levels of APM, DPP IV, MEP 24.15 and NEP 24.11 in various rat organs, there are significant changes in vascular tissue. The extent to which

these changes are characteristic of other large vessels and the microcirculation, as well as their contribution to modulating the hypertension in this model remain to be established. These findings also demonstrate that although the RAS is the principal peptidase pathway altered in this model of hypertension, other peptidases may also be affected. The tissue-selective changes in some of the peptidases studied here are reminiscent of those for ACE, the aortic activity of which is elevated after treatment with L-NAME [14,15] while there are no marked changes in serum or tissue (kidney, liver, lung) activities [14].

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