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L-NAME-treatment alters ectonucleotidase activities in kidney membranes of rats

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ABSTRACT

Aims: To investigate the effect of Nω-Nitro-L-arginine methyl ester (L-NAME) treatment, known to induce a sustained elevation of blood pressure, on ectonucleotidase activities in kidney membranes of rats.
Main methods: L-NAME (30 mg/kg/day) was administered to Wistar rats for 14 days in the drinking water. Enzyme activities were determined colorimetrically and their gene expression patterns were analyzed by semi-quantitative RT-PCR. The metabolism of ATP and the accumulation of adenosine were evaluated by HPLC in kidney membranes from control and hypertensive rats. PKC phosphorylation state was investigated by Western blot.
Key findings: We observed an increase in systolic blood pressure from 115 ± 12 mmHg (control group) to 152 ± 18 mmHg (L-NAME-treated group). Furthermore, the hydrolysis of ATP, ADP, AMP, and *p*-Nph-5′TMP was also increased (17%, 35%, 27%, 20%, respectively) as was the gene expression of NTPDase2, NTPDase3 and NPP3 in kidneys of hypertensive animals. Phospho-PKC was increased in hypertensive rats.
Significance: The general increase in ATP hydrolysis and in ecto-5′-nucleotidase activity suggests a rise in renal adenosine levels and in renal autoregulatory responses in order to protect the kidney against the threat presented by hypertension.

Introduction

Extracellular purines exert different responses in different organs and tissues. ATP and ADP act via activation of two major subfamilies of nucleotide receptors, P2X and P2Y (Yegutkin 2008). Currently, seven subtypes of P2X ligand-gated ion channel receptors ($P2X_{1-7}$) and eight subtypes of P2Y metabotropic G-protein coupled receptors ($P2Y_{1,2,4,6,11,12,13,14}$) are characterized and distributed across different cell types (Burnstock 2006; Burnstock and Knight 2004). Moreover, there are four subtypes of P1 receptors (Zimmermann 2001), A_1, A_{2A}, A_{2B}, and A_3 that respond to the nucleoside adenosine (Burnstock 2006).

Extracellular nucleotides are metabolized by ectonucleotidases (Lemmens et al. 2000), which can be located on the cell surface and in the interstitial medium or within body fluids (Zimmermann 2001). A number of enzymes play a role in the hydrolysis of nucleotides, including ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases), ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPPs), ecto-5′-nucleotidases, and alkaline phosphatases (Yegutkin 2008). In mammals, eight different genes encode members of the NTPDase family (Robson et al. 2006). NTPDase1, 2, 3 and 8 are cell surface-located; NTPDase5 and 6 exhibit an intracellular localization but can also be secreted; and NTPDase4 and 7 have an intracellular localization (Yegutkin 2008). The E-NPP family consists of seven members (NPP1-7), although only the first three (NPP1-3) are able to hydrolyze extracellular nucleotides (Goding et al. 2003). Finally, surface-associated ecto-5′-nucleotidase converts AMP to adenosine (Zimmermann 1992). The coordinated action of these enzymes regulates the nucleotide and adenosine levels under physio (patho)logical conditions (Agteresch et al. 1999).

The kidneys are important excretory and regulatory organs that maintain fluid and electrolyte homeostasis adapting renal excretion to bodily needs (Vallon 2008). They are composed of different cell types and display a complex pattern of expression for purinergic receptors, which are particularly found in glomeruli and tubules (Guan et al. 2007; Vitzthum et al. 2004; Turner et al. 2003). Ectonucleotidases are also clearly expressed along the rat nephron and can influence the

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activation of kidney purinoceptors (Vekaria et al. 2006a). ATP can be released by virtually all renal cells and it has been found in the tubular fluid and urine (Vekaria et al. 2006b; Schwiebert 2001). In the kidney, purinergic signaling is implicated in a wide range of processes, including glomerular hemodynamics, microvascular function, tubuloglomerular feedback (TGF) and tubular transport (Kishore et al. 2005).

Arterial hypertension is strongly associated with the development of vascular diseases (Whitworth 2003). The kidneys are important regulators of blood pressure since they control the sodium and water balance and, consequently, the extracellular volume (Rodrigues et al. 2006). Experimental evidence shows that renal autoregulation is impaired or the renal autoregulatory response is reduced in some animal models of hypertension (Imig and Inscho 2002; Persson 2002). Chronic inhibition of NO synthesis by L-arginine analogues such as Nω-Nitro-L-arginine methyl ester (L-NAME) produces volume-dependent elevation of blood pressure and renal vasoconstriction (Lerman et al. 2005).

The effects of purines on the kidneys as well as the considerable presence of purinoceptors and ectonucleotidases in these organs raises the question about the general balance of ectonucleotidase activities in a hypertensive condition. This study investigates the effect of L-NAME treatment on ectonucleotidases in kidney membranes of rats.

Materials and methods

Chemicals

Nω-Nitro-L-arginine methyl ester hydrochloride (L-NAME), *p*-Nitrophenyl thymidine 5′-monophosphate (*p*-Nph-5′-TMP), nucleotides, and Trizma Base were obtained from Sigma-Aldrich (St. Louis, MO, USA). Percoll was purchased from Pharmacia (Uppsala, Sweden) and was routinely filtered through Millipore AP15 prefilters to remove aggregated and incompletely coated particles. The creatinine dosage kit was obtained from Doles (Goiânia, GO, Brazil). TRIzol® Reagent, dNTPs, oligonucleotides, Taq polymerase, Low DNA Mass Ladder and SuperScript™ III First-Strand Synthesis SuperMix were purchased from Invitrogen (Carlsbad, CA, USA). Primers were obtained from Integrated DNA Technologies (Coralville, IA, USA) and GelRed™ was obtained from Biotium (Hayward, CA). Tetrabutylammonium chloride was purchased from Merck (Darmstadt,Germany).

Animals

Male Wistar rats (~250 g) from our breeding stock were maintained on a 12 h light/12 h dark cycle (lights on 7:00 a.m.) at a constant room temperature of 23 ± 1 °C. Unless otherwise mentioned, they received water and rat chow ad libitum. All protocols were performed in accordance with the guidelines of the Colégio Brasileiro de Experimentação Animal (COBEA), based on the Guide for the Care and Use of Laboratory Animals (National Research Council) and all efforts were made to minimize the number of animals used as well as their suffering.

Experimental protocols

In the first set of experiments, the animals were randomly divided in two groups, normotensive and hypertensive. Hypertension was induced as previously described (Balbinott et al. 2005). Briefly, the inhibitor of nitric oxide synthase (NOS), L-NAME (30 mg/kg body weight/day), was administered in the drinking water for 14 days (L-NAME-treated group). The normotensive group received standard tap water throughout the entire experiment (control group). The animals were euthanized on day 15.

In the second set of experiments, we performed a period of L-NAME washout. Thus, animals received tap water (control group) or 30 mg/kg/day L-NAME (L-NAME-treated group) for 14 days in the drinking water. The administration of the drug ceased for one week and animals were euthanized on day 22 for subsequent experiments.

Systolic blood pressure determination

Systolic blood pressure (SBP) and heart rate (HR) were measured in awake animals on three occasions during the treatment period, at approximately the same time of day, by tail-cuff plethysmography (RTBP1001 Rat Tail Blood Pressure System for rats and mice — Kent Scientific, Litchfield, USA). The rats were allowed time to adapt to the apparatus before taking the measurements. In the first set of experiments, SBP was recorded on day 0 (before the beginning of the treatment), on day 7 (7 days after starting L-NAME treatment) and on day 14 (the last day of the treatment). In the second set of experiments, SBP was measured on day 0 (before the beginning of the treatment), on day 14 (the day on which the L-NAME administration ceased) and on day 21 (7 days after L-NAME washout and one day before animals were euthanized). The heart rate values were derived from the pulsations detected from SBP.

Measurement of creatinine levels

Serum creatinine was measured in both control and L-NAME-treated animals using a colorimetric kit (Kit Creatinina, Doles, Goiânia, GO, Brazil) according to the manufacturer's instructions.

Preparation of kidney membranes

Kidney membranes were prepared essentially as previously described by Nagy and Delgado-Escueta (1984) with minor modifications. Briefly, about 1.0 g of both right and left kidneys was dissected on ice, washed and homogenized (20 strokes at 1500 rpm) in 10 volumes of a medium containing 0.32 M sucrose, 0.1 mM EDTA and 5.0 mM HEPES, pH 7.5 (Medium I), and then centrifuged at 1000 × *g* for 10 min. The supernatant was collected and centrifuged again at 12,000 × *g* for 20 min. The supernatant was discarded and the pellet was resuspended in 1.2 mL of Medium I, then an aliquot of 1.0 mL was mixed with 4.0 mL of 8.5% Percoll solution and layered onto an isoosmotic discontinuous Percoll/sucrose gradient (10%/16%). After a further centrifugation step at 15,000 × *g* for 20 min, the fractions that banded at the 10%/16% Percoll interface were collected with a wide-tip disposable plastic transfer pipette. The kidney membrane fraction was washed twice with Medium I at 15,000 × *g* for 20 min to remove the contaminating Percoll. The pellet from the second centrifugation was resuspended in order to reach a final concentration of 0.5–0.8 mg/mL. The membranes were prepared fresh daily and maintained at 0–4 °C throughout the experimental procedure.

Protein determination

Protein was measured by the Coomassie Blue method using bovine serum albumin as standard (Bradford 1976).

Assays of ecto-nucleoside triphosphate diphosphohydrolase (E-NTPDase) and ecto-5′-nucleotidase activities

For the measurement of ATP hydrolysis in membrane fractions, the reaction mixture employed contained 45 mM Tris–HCl, 5.0 mM KCl, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, 1.5 mM $CaCl_2$, 0.1 mM sodium azide, and 2.0 μg/mL oligomycin, pH 7.5, in a final volume of 200 μL. ADP hydrolysis was assessed using the same reaction mixture described above, except the fact that 1.0 mM $CaCl_2$ was used and neither sodium azide nor oligomycin was employed in this assay. The activity of ecto-5′-nucleotidase was determined in a reaction medium containing 100 mM Tris–HCl and 1.0 mM $MgSO_4$,

pH 7.5, in a final volume of 200 μL. Approximately 13 μg of kidney membrane protein were added per tube and were preincubated for 10 min at 37 °C. The enzyme reactions were started by the addition of nucleotide as substrate in a final concentration of 1.0 mM (ATP/ADP) or 2.0 mM (AMP). After 10 min of incubation, trichloroacetic acid (TCA) (5%, v/v, final concentration) was added to stop the reactions. Incubation times, protein concentrations, reaction mixtures, and substrate concentration were chosen according to a previously published study (Vieira et al. 2001). The amount of inorganic phosphate (Pi) released was determined using a colorimetric method as previously outlined by Chan et al. (1986). Controls to correct for the non-enzymatic hydrolysis of the substrate were performed by adding membrane proteins after the reactions had been stopped with TCA. All assays were performed in triplicate. Enzyme activities were generally expressed as nmol Pi released per minute per milligram of protein.

Assay of ecto-nucleotide pyrophosphatase/phosphodiesterase (E-NPP) activity

The phosphodiesterase activity was assessed using *p*-Nph-5′-TMP as an artificial substrate. Briefly, the reaction medium contained 50 mM Tris–HCl buffer, 5.0 mM KCl, 0.1 mM EDTA, 10 mM glucose, 225 mM sucrose, and 1.5 mM $CaCl_2$, pH 8.9, and was preincubated with approximately 13 μg per tube of kidney membrane protein for 10 min at 37 °C in a final volume of 200 μL. The enzyme reaction was started by the addition of *p*-Nph-5′-TMP to a final concentration of 0.5 mM. After 10 min of incubation, 200 μL of 0.2 N NaOH was used to stop the reaction. Incubation time and protein concentration were chosen in order to ensure the linearity of the reaction. The amount of *p*-nitrophenol released from the substrate was measured at 400 nm using a molar extinction coefficient of 18.8×10^{-3}/M/cm. Controls to correct for the non-enzymatic substrate hydrolysis were performed by adding kidney membrane protein after the reaction had been stopped with NaOH. All assays were performed in triplicate. Enzyme activity was generally expressed as nmol *p*-nitrophenol released per minute per milligram of protein.

Analysis of gene expression by semi-quantitative RT-PCR

Approximately 1.0 g of kidneys was isolated for total RNA extraction using the TRIzol® Reagent. RNA purity was quantified spectrophotometrically and assessed by electrophoresis in a 1.0% agarose gel containing ethidium bromide. The cDNA species were synthesized using SuperScript™ III First-Strand Synthesis SuperMix from 3 μg of total RNA according to the suppliers' instructions. For PCR assays, 1 μL cDNA was used as a template. cDNA was screened with specific primers for NTPDase1-3 and 8, 5′-nucleotidase, and NPP1-3. The β-actin gene was used for normalization as a constitutive gene. PCR was carried out in volume of 25 μL using a concentration of 0.2 μM of each primer, 200 μM $MgCl_2$ and 1 U Taq polymerase. The cycling conditions for all PCRs were as follows: Initial 1 min of denaturation at 94 °C, 1 min at 94 °C, 1 min of annealing (NTPDase1, 3, 8, NPP2, 3 and 5′-nucleotidase: 65 °C; NTPDase2: 66 °C; NPP1: 60 °C; β-actin: 58.5 °C), and 1 min of extension at 72 °C. These steps were repeated for 35 cycles. Finally, a 10 min extension step at was performed 72 °C. Primer sequences as well as the amplification products are listed in Table 1. Ten microliters of the PCR reaction were analyzed on a 1% agarose gel using GelRed™ and photographed under UV light. The Low DNA Mass Ladder was used as a molecular marker. The images of stained PCR products were analyzed by optical densitometry and semi-quantified (enzyme/β-actin mRNA ratio) using the computer software *Image J*.

Analysis of ATP metabolism by high performance liquid chromatography (HPLC)

Kidney membranes from control and L-NAME-treated animals were obtained as previously described. The same reaction mixture as well as the protein concentration was used to analyze the metabolism of ATP. However, the enzymatic reactions were started by the addition of 0.1 mM ATP (final concentration) in a final volume of 200 μL at 37 °C. Aliquots of the samples were collected at different times of incubation (0, 10, 30 and 60 min) with the reactions being stopped on ice. All samples were centrifuged at 14,000 ×*g* for 15 min. Aliquots of 40 μL were applied to a reversed-phase HPLC system using a 25 cm C_{18} Shimadzu column (Shimadzu, Japan) at 260 nm with a mobile phase containing KH_2PO_4 and 5.0 mM tetrabutylammonium chloride, pH 6.0, in 30% methanol as previously outlined by Voelter et al. (1980). The peaks of purines (ATP, ADP, AMP, adenosine and inosine) were identified by their retention times and quantified by comparison with standards. Results are expressed as nmoles of the different compounds per 200 μL of reaction for each time of incubation. All incubations were carried out in triplicate and the controls to correct for non-enzymatic hydrolysis of nucleotides were performed by measuring the peaks present for the same reaction medium without membranes.

Table 1
Primers sequences and PCR amplification products.

Enzyme	Primers sequences	Amplification product (bp)
β-actin	Rnβ-actinF 5′-TAT GCC AAC ACA GTG CTG TCT GG-3′ Rnβ-actinR 5′-TAC TCC TGC TTC CTG ATC CAC AT-3′	210
NTPDase1	RnNTPDase1F 5′-GAT CAT CAC TGG GCA GGA GGA AGG-3′ RnNTPDase1R 5′-AAG ACA CCG TTG AAG GCA CAC TGG-3′	543
NTPDase2	RnNTPDase2F 5′-GCT GGG TGG GCC GGT GGA TAC G-3′ RnNTPDase2R 5′-ATT GAA GGC CCG GGG ACG CTG AC-3′	331
NTPDase3	RnNTPDase3F 5′-CGG GAT CCT TGC TGT GCG TGG CAT TTC TT-3′ RnNTPDase3R 5′-TCT AGA GGT GCT CTG GCA GGA ATC AGT-3′	267
NTPDase8	RnNTPDase8F 5′-CCA CAC TGT CAC TGG CTT CCT TG-3′ RnNTPDase8R 5′-ACG AGG ATG TAT AGG CCT GAG G-3′	394
5′-nucleotidase	RnCD73F 5′-CCC GGG GGC CAC TAG CAC CTC A-3′ RnCD73R 5′-GCC TGG ACC ACG GGA ACC TT-3′	405
NPP1	RnNPP1F 5′- GAA TTC TTG AGT GGC TAC AGC TTC CTQ-3′ RnNPP1R 5′-CTC TAG AAA TGC TGG GTT TTG CTC CCG GCA-3′	410
NPP2[a]	RnNPP2F 5′-CCA TGC CAG ACG AAG TCA GCC GAC C-3′ RnNPP2R 5′-CCA AAC ACG TTT GAA GGC GGG GTA C-3′	512
NPP3	RnNPP3F 5′-GAG AAG ACA AAT TTG CCA TTT GGG AGG-3′ RnNPP3R 5′-TCT CAT TAT TTC CTT TGA TTG CGG GAG-3′	301

[a] Represents that the low mass (512 bp) splice isoform of NPP2 was found in kidney membranes.

Analysis of phospho-PKC by Western blot

Kidney membrane preparations were subjected to total protein extraction in a buffer containing 100 mM KCl, 10 mM HEPES, 3 mM $MgCl_2$, 5.0 mM EDTA, 10% glycerol, 1.0 mM DTT and 10% SDS. The lysate was then centrifuged at 14,000 ×*g* for 10 min at 4 °C. The resulting supernatant was collected and the protein was determined by Bradford assay (Bradford 1976). Fifty micrograms of protein were resolved by electrophoresis on 10% SDS-polyacrylamide gel. After the separation, proteins were transferred to a nitrocellulose membrane (Bio-Rad) and blots were blocked with 10% non-fat milk. Equal protein loading was confirmed by staining with Ponceau-S dye. The primary antibody against Phospho-PKCδ/θ (Ser643/676) (Cell Signaling Technology, Beverly, MA) diluted 1:500 was incubated overnight at 4 °C. The membrane was also probed for GAPDH (Cell Signaling Technology, Beverly, MA) as an internal control. Bound proteins were detected after the secondary antibody incubation (peroxidase-conjutaged IgG anti-rabbit, Jackson Immunoresearch, 1:10,000) by chemiluminescence reagents (Amersham Pharmacia Inc., Uppsala, Sweden).

Data analysis

Results are expressed as means ± standard deviation (S.D.). The comparison among groups was made by Student's *t* test for independent samples. Statistically significant differences between groups were considered at a $P \leq 0.05$.

Results

Blood pressure and serum levels of creatinine

The hypertension caused by the inhibition of nitric oxide biosynthesis is well established (Katsumi et al. 2007). In the present study, we found that oral administration of L-NAME (30 mg/kg/day for 14 days) lead to a marked increase in SBP in L-NAME-treated group. Moreover, after 7 days of L-NAME washout, blood pressure values returned to control levels. Additionally, no differences were observed in the HR between the experimental groups and the arterial hypertension induced by L-NAME treatment did not modify serum creatinine levels (Table 2).

Effect of L-NAME on ectonucleotidase activities in kidney membranes

Fig. 1 shows that oral administration of L-NAME for 14 days was associated with an increase in ATP, ADP, AMP and *p*-Nph-5′TMP hydrolysis in kidney membranes. The pattern of substrate hydrolysis reveals a ratio of ATP/ADP/AMP/p-Nph-5′TMP hydrolysis of approximately 2.3:1:1:1. Furthermore, ATP hydrolysis (A) was augmented by about 17%; ADP hydrolysis (B) was increased by 35%; the hydrolysis of AMP (C) rose by 27%; and the hydrolysis of the E-NPP substrate (D) by 20%. In order to determine if the effects on ectonucleotidase activities could be reversed by stopping the treatment, a 7-day period of L-NAME washout was performed and enzymatic activities were determined again. Fig. 2 shows that after 14 days of treatment with L-NAME plus 7 days of L-NAME washout, both the hypertension and the increase in ectonucleotidase activities were reversed to control levels.

In addition to the results presented above, an in vitro protocol was performed in order to assess the direct effect of L-NAME upon ectonucleotidase activities by incubating kidney membranes obtained from untreated animals with five different concentrations of L-NAME ranging from 0.1 to 1.0 mM. We found that L-NAME in vitro did not alter ectonucleotidase activities in kidney membranes at any of the concentrations tested (data not shown).

Effect of L-NAME treatment on ectonucleotidases mRNA expression

Since we found changes in nucleotide hydrolysis after L-NAME treatment, we further examined mRNA expression patterns of ectonucleotidases after such treatment. Using specific primers (listed in Table 1), mRNAs for NTPDase1,2,3 and 8, ecto-5′-nucleotidase, NPP1,2 and NPP3 (Fig. 3 and data not shown) were detected. Furthermore, an increase was observed in ectonucleotidase activities that may be a consequence of transcriptional control and/or post-translational regulation. Then, semi-quantitative RT-PCR analyses were performed when kinetic alterations were observed. The results of these analyses showed that the relative amounts of E-NTPDase2, 3 and E-NPP3 transcripts were significantly increased (26.4%, 16% and 11%, respectively) in kidneys of L-NAME-treated animals when compared to control animals (Fig. 3B, 3C and 3D, respectively).

Metabolism of ATP in kidney membranes of control and L-NAME-treated rats

As seen in Fig. 4, the control group (A) hydrolyzed extracellular ATP in a slower fashion than the L-NAME-treated group (B). This pattern of hydrolysis was clearly evident from the steady accumulation of adenosine and inosine in the hypertensive group, especially from 30 min of incubation. The final levels of adenosine and inosine were 2.45 and 10 times higher in the L-NAME-treated group, respectively.

Analysis of PKC activation

To test the possible activation of ecto-5′-nucleotidase by PKC, the phosphorylation state of PKC was evaluated by Western blot in kidney membrane extracts from control and L-NAME-treated rats. When activated, PKC can be displaced from the cytosol to the plasma membrane. Fig. 5 shows a strong signal for p-PKC in hypertensive animals that is almost absent in the control group. The phosphorylated state of PKC indicates that this enzyme is active and suggests the phosphorylation of ecto-5′-nucleotidase, contributing to the increase observed in this enzymatic activity.

Discussion

The hydrolysis of ATP, ADP, AMP, and *p*-Nph-5′-TMP was increased in hypertensive animals when compared to their respective controls (Fig. 1A–D, respectively). In addition, we found that ATP was

Table 2
Hemodynamic parameters and serum creatinine levels.

	After 14 days of treatment		After 14 days of treatment + 7 days of L-NAME washout	
	Control ($n=10$)	L-NAME ($n=13$)	Control ($n=5$)	L-NAME ($n=5$)
Body weight (g)	270 ± 28	255 ± 29	256 ± 21	249 ± 27
Systolic blood pressure (mmHg)	114.63 ± 12.38	152.43* ± 18.38	115.78 ± 14.93	116.47 ± 13.60
Heart rate (bpm)	369 ± 14.5	349 ± 16	368 ± 23	369 ± 1.27
Serum creatinine (mg/dL)	0.706 ± 0.010	0.682 ± 0.09	n.d.	n.d.

*Represents significant difference from the respective control group, $P \leq 0.05$ (n.d. — not determined).

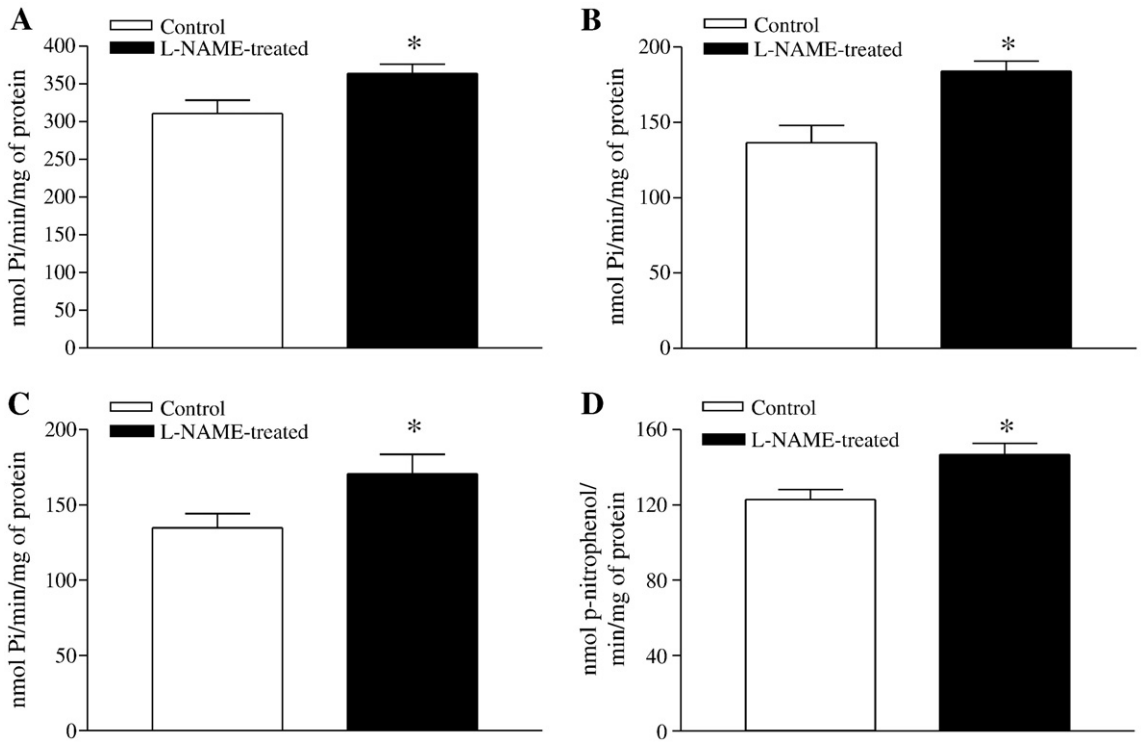

Fig. 1. Effect of L-NAME treatment on ectonucleotidase activities. ATP (A), ADP (B), AMP (C), and *p*-Nph-5′TMP (D) hydrolysis were increased in kidney membranes from animals orally treated with L-NAME for 14 days. Results are expressed as means ± S.D. ($n = 9$, 10, 13, and 6 for ATP, ADP, AMP, and *p*-Nph-5′TMP hydrolysis, respectively). The comparison among groups was made with Student's *t* test for independent samples. *Represents a statistically significant difference in comparison to the respective control group, considering $P \leq 0.05$.

hydrolyzed approximately 2.30 times more efficiently than other substrates, which is consistent with data previously reported in the literature for kidney membranes (Vieira et al. 2001). Several mechanisms can regulate ectonucleotidase activities, including transcriptional and/or post-translational modifications. A semi-quantitative RT-PCR analysis of the patterns of ectonucleotidase gene expression after L-NAME treatment showed a significant increase in mRNA transcript levels of NTPDase2 and 3, and NPP3 (Fig. 3B, C and D, respectively). Previous studies have already demonstrated that the enzymes able to hydrolyze extracellular nucleotides can be regulated at the transcriptional level following drug treatment (Vuaden et al. 2007; Pedrazza et al. 2008; Rico et al. 2008). Despite the fact that the semi-quantitative analysis of NTPDase1 expression did not show a significant increase in this parameter in the kidney of L-NAME-treated animals, it is noteworthy that there is a clear tendency towards an increase in the number of mRNA transcripts for this enzyme ($P = 0.07$) (Fig. 3A). Therefore, we believe that augmented expression of NTPDase2, NTPDase3, NPP3, and even NTPDase1, could contribute to the enhancement observed in ATP, ADP and *p*-Nph-5′-TMP hydrolysis in kidney membranes of L-NAME-treated animals.

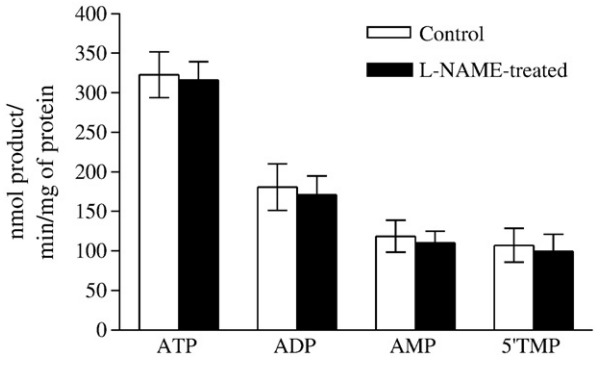

Fig. 2. Effect of L-NAME washout on ectonucleotidase activities. The increase observed in ectonucleotidase activities in kidney membranes was reversed after 7 days of L-NAME washout following the L-NAME treatment lasting 14 days. Results are presented as means ± S.D. of $n = 6$, 8, 9, and 5 for the substrates ATP, ADP, AMP, and *p*-Nph-5′TMP, respectively.

Conversely, the increase observed in AMP hydrolysis in the hypertensive group was not followed by an increase in ecto-5′-nucleotidase mRNA transcript levels (data not shown). However, this activity could be regulated by post-translational events, which might explain the increase found in AMP hydrolysis. Using NetPhosK, a tool for the kinase-specific prediction of protein phosphorylation sites at http://www.cbs.dtu.dk, we found that the ecto-5′-nucleotidase sequence contains at least two sites with a high possibility of phosphorylation by PKC located at threonine-432 and 479 residues. As demonstrated in Fig. 5, the phosphorylation state of PKC is significantly increased in L-NAME-treated rats. This result strongly supports the hypothesis that the activation of PKC by phosphorylation in hypertensive animals can promote the phosphorylation of ecto-5′-nucleotidase and, consequently, increase the hydrolysis of AMP by this enzyme.

Based on the different expression profiles of NTPDases, NPPs and ecto-5′-nucleotidase found in the kidney, it is possible to suggest that transcriptional and post-translational events act in a coordinated manner to up-regulate the activities of these enzymes in response to L-NAME treatment. Previous works have already described that nitric oxide (NO) is capable of alter ectonucleotidase activities. Kirchner et al. (2001) have shown that the in vitro metabolism of ATP, ADP and AMP were diminished by sodium nitroprusside, an NO donor, in rat hippocampal synaptosomes. In another study, Satriano et al. (2006) showed that NO decreased ecto-5′-nucleotidase in glomerular preparations, suggesting this mechanism as part of the adaptive phase of autoregulatory renal responses. Therefore, the inhibition of NO production performed in this study with the consequent increase in overall ectonucleotidase activities can also be seen as an adaptive response as it will be further discussed.

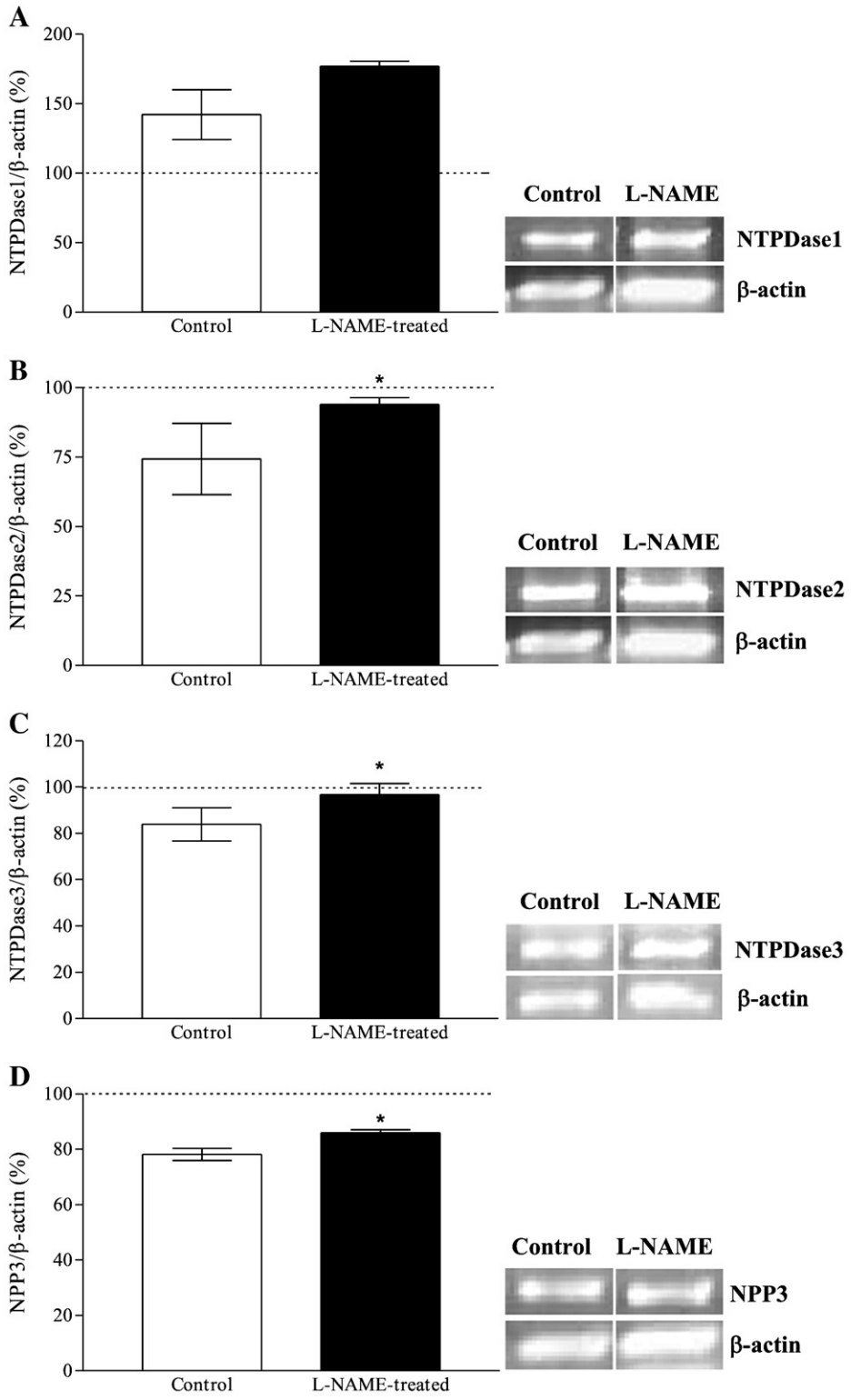

Fig. 3. Semi-quantitative RT-PCR analysis of patterns of ectonucleotidase gene expression after L-NAME treatment (30 mg/kg/day for 14 days). Kidneys were excised and total RNA was isolated. An increase in the number of transcripts for NTPDase2 (B), NTPDase3 (C) and NPP3 (D) was found in kidneys from hypertensive animals. Data on NTPDase1 (A) are also presented as this enzyme is the best characterized NTPDase ($P = 0.07$). The enzyme/β-actin mRNA ratios were obtained by optical densitometry. Data are expressed as means ± S.D. of at least 5 experiments, with entirely consistent results. *Represents a statistically significant difference in comparison to the respective control group, considering $P \leq 0.05$.

After the 7-day L-NAME washout period, both blood pressure and substrate hydrolysis (Fig. 2, Table 2) returned to control levels. In this sense, we can hypothesize that the results obtained in this study were probably due to the hypertensive condition since L-NAME in vitro had no direct effect upon nucleotide hydrolysis and the reversal in blood pressure levels led to the disappearance of the effect on ectonucleotidase activities.

Systemic hypertension is the main risk factor for the progressive loss of renal function. It is well known that in vivo administration of L-NAME leads to arterial hypertension (Lerman et al. 2005) and that

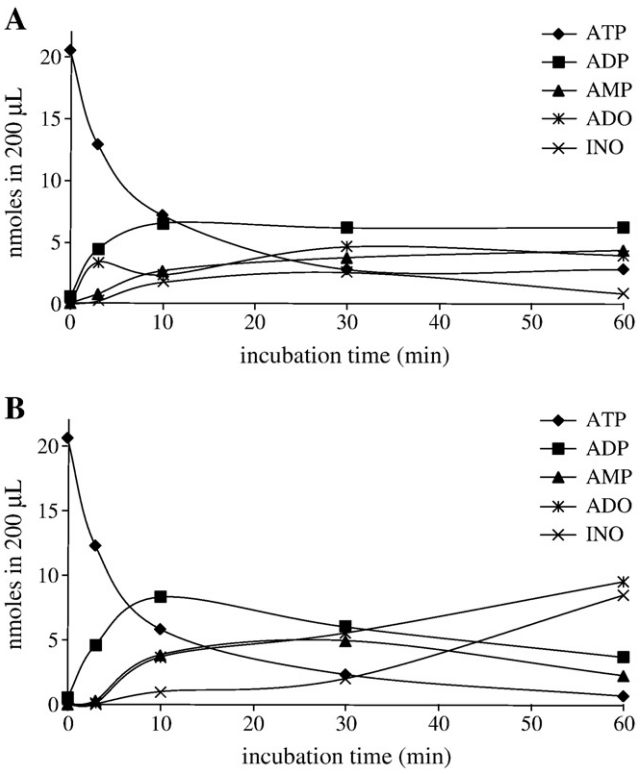

Fig. 4. Metabolism of ATP and product formation in kidney membranes from control and L-NAME-treated rats. Kidney membranes were incubated with 0.1 mM ATP. Data are representative of four independent experiments for control (A) and L-NAME-treated (B) groups. (ADO, adenosine–INO, inosine).

prolonged times of treatment as well as higher doses of L-NAME can cause renal complications (Zanfolin et al. 2006). However, the arterial hypertension induced by the treatment performed in this study did not cause renal damage since any significant differences in serum creatinine levels were detected among the experimental groups (Table 2). This was not surprising since the progression to kidney failure in primary hypertensive patients is a rare event, as previously reported (Tomson et al. 1991).

Extracellular purines are widespread molecules involved in a great number of patho-physiological processes. Despite the fact that only NTPDase2, NTPDase3, and NPP3 exhibited altered levels of mRNA in this study, we also detected the presence of NTPDase1 and 8, NPP1 and 2 (the low mass splice variant), and ecto-5′-nucleotidase in rat kidney (Fig. 3A and data not shown). In fact, this is in agreement with recent studies that have described the presence of several ectonucleotidases and purinoceptors along the nephron, and in the glomerulus and the renal vascular system (Vekaria et al. 2006a; Kishore et al. 2005; Vitzthum et al. 2004; Bailey et al. 2004; Turner et al. 2003; Unwin et al. 2003; Chan et al. 1998; Harahap and Goding 1988).

It is well known that afferent arteriolar resistance usually determines the degree of transmission of systemic pressure to the glomerular capillary network. The normal kidney develops an autoregulatory mechanism in order to adjust renal vascular resistance in response to changes in transmural pressure (Guan et al. 2007). Despite the fact that the kidney autoregulation process is not completely understood, it is generally recognized that this response involves a myogenic mechanism operating through the preglomerular vascular tree together with tubuloglomerular feedback (TGF), which enables the kidney to maintain blood flow and glomerular filtration rate (GFR) during fluctuations of systemic arterial pressure (Guan et al. 2007). Adenosine causes a biphasic response in the kidney involving both vasoconstriction and vasodilatation brought about through the activation of A_1 and A_{2A} receptors, respectively (Osswald et al. 1982). Extracellular ATP has been suggested as the major local autocrine and paracrine regulator of preglomerular microvasculature reactivity, especially through the constriction of smooth muscle cells of the afferent arterioles, through a mechanism involving both P2Y and P2X receptors (Inscho 2001; Inscho et al. 1992).

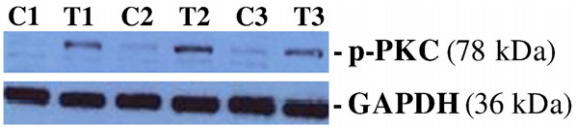

Fig. 5. PKC phosphorylation state. Western blot assay showing the band density of phospho-PKC in 3 control (C1, C2, C3) and 3 L-NAME-treated (T1, T2, T3) animals. Phospho-PKC is significantly increased in hypertensive animals. GAPDH was used as an internal control.

In the present study, we observed a general increase in ectonucleotidase activities in kidney membranes of L-NAME-treated rats. Several controversies have been raised about the role of ATP and/or adenosine as mediators of TGF. Recent studies have offered a solution to this question suggesting that both molecules are involved in this process. The hypothesis proposes that ATP released by macula densa cells is the primary mediator of TGF, being further converted to adenosine, which would then cause the constriction of afferent arterioles (Castrop 2007).

Conclusion

The results obtained in this study conform to the proposition stated above since they show an increase in ATP hydrolysis and an accumulation of adenosine in the kidney of hypertensive animals. Despite the fact that impairment in renal autoregulatory responses is reported in some animal models of hypertension (Imig and Inscho 2002; Persson 2002), our data may reflect a significant increment in renal autoregulatory responses as an adaptive mechanism developed in order to protect the kidney from the threat presented by an increase in blood pressure. It should be noted that increased concentrations of L-NAME as well as prolonged times of treatment could evoke different responses. As a result, further studies are necessary to determine the degree of participation of these enzymes in the renal response to arterial hypertension as well as to evaluate them as potential targets for the adjuvant treatment of this pathology.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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