Potentiation of urinary atrial natriuretic peptide interferes with macula densa function

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Abstract

Objectives: Neutral endopeptidase (NEP) inhibition potentiated the renal action of Atrial Natriuretic Peptide (ANP) and was associated with appearance of the peptide in the urine, providing evidence of protection of the filtered peptide along the course of the nephron. The macula densa, composed of epithelial cells, receives ionic information from the urinary compartment via Na–K–2Cl cotransport and influences renin secretion by the myoepithelio-od cells in the afferent arteriole. bNOS constitutively expressed in the epithelial cells of the macula densa is involved in this feed-back. NEP inhibition was associated with the absence of any increase in renin secretion. The hypothesis is that potentiation of urinary ANP by NEP inhibition could limit renin secretion by directly or indirectly targeting the macula densa in vivo. Methods and results: We tested the interaction between NEP inhibition (candoxatril) and Na–K–2Cl inhibition (bumetanide) on electrolyte and ANP urinary excretion, renin secretion, macula densa activity (NADPH diaphorase activity and bNOS mRNA) and TSC-1 mRNA expression in the renal cortex and BSC-1 in the renal medulla of rats treated for 5 days. Bumetanide increased urinary electrolyte excretion whereas candoxatril did not. Candoxatril increased urinary ANP and cyclic GMP excretion. Bumetanide increased renin and aldosterone secretion whereas candoxatril decreased renin secretion. This effect on renin release was associated with an increase in macula densa NADPH diaphorase activity in the bumetanide-treated group which was blunted by candoxatril. Lastly, bumetanide increased TSC-1 mRNA expression in the cortex and this effect was blunted by candoxatril. Conclusion: These results suggest that potentiation of ANP by NEP inhibition could interfere with tubular function at different levels and limit renin secretion by a urinary pathway involving macula densa activity. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Gene expression; Natriuretic peptide; Renal function; Renin angiotensin system

1. Introduction

Atrial and brain natriuretic peptides are bioactive peptides, synthesised and secreted by the heart, vehicled in the plasma and targeting on renal function [1,2]. In the heart, ANP release is stretch-dependent, sensing in this way the body fluid status and participating in its homeostasis [3,4]. ANP metabolism is partly dependent on the kidney (15%). In the kidney, the hydrosoluble, filtered atrial natriuretic peptide (ANP) is degraded by neutral endopeptidase (NEP) [5] an ecto-enzyme mainly expressed in the apical brush border of the epithelial cells of the proximal tubule [6,7]. Therefore NEP inhibition increases the natriuretic response to ANP probably by potentiating the endo-urinary action of the filtered peptide. The appearance of the peptide in urine during NEP inhibition [8,9] gives evidence of such a pharmacological urinary protection, and suggests a possible urinary action.

In the kidney, the macula densa appears to be a unique type of chemo-sensor for body fluid homeostasis [10]. The macula densa, composed of epithelial cells, senses urinary ionic load via the Na–K–2Cl cotransport and modulates the secretion of renin by the myoepithelio-od cells in the afferent arteriole. Neuronal NO synthase (bNOS) activity has been demonstrated to be present in the macula densa and is involved in the intercellular communications be-
between the macula densa, at the urinary pole, and renin secretion by the afferent arteriole within the juxtaglomerular apparatus [11]. ANP and NEP inhibition are pharmacological factors which are reputed to decrease renin secretion [12,13], but the mechanisms by which ANP potentiation by NEP inhibition may decrease renin release remain unclear. A direct action of ANP on renin secreting cultured cells has been demonstrated in vitro [14]. But early studies have shown that intrarenal infusion of ANP did not inhibit renin release in nonfiltering kidney [15], and conversely ANP did not inhibit furosemide-induced renin release from isolated afferent arterioles [16].

The hypothesis of the present study was therefore that potentiation of filtrated ANP by NEP inhibition could, directly or indirectly by changing ionic load, interfere in vivo, in the distal part of the nephron, with different functions of the epithelial cells, particularly in the macula densa. For this purpose we have used bumetanide as a blocker of the Na–K–2Cl cotransport, acting via an endo-urinary pathway, increasing renin secretion by dissociating the urinary ionic load of Na⁺ and Cl⁻ from their epithelial intracellular signalling and intercellular communications in the juxtaglomerular apparatus [17]. We tested in vivo in rats, the interaction of NEP inhibition by candoxatril with bumetanide-induced renin secretion. Because neuronal NO synthase is expressed in macula densa and involved in macula densa feedback [11], NADPH diaphorase activity was used as a specific marker of macula densa involvement [18] in the interaction between ANP potentiation by NEP inhibition and renin secretion.

2. Methods

2.1. Experimental design

Adult normotensive male Wistar rats (IFFA CREDO, France) weighing 145–170 g, were used in this study. Rats were provided standard rat laboratory diet and water ad libitum. All the rats were placed in metabolic cages for at least 3 days for adaptation before experimentation and randomly allocated to four groups. One control group received distilled water, and three treated groups received the drug solutions, bumetanide (Leo, les Ulis, France) at 10 mg/kg, candoxatril (Pfizer, Sandwich, UK) at 50 mg/kg, and bumetanide+candoxatril at 10 and 50 mg/kg respectively. The bumetanide drug was administered by intraperitoneal injection and the candoxatril drug was administered by oral gavage at a volume of 5 ml/kg once a day during 6 days.

The influence of circadian rhythm was avoided by starting all experiments at 09:00 A.M. Immediately after gavage and intraperitoneal injection; the rats were individually housed in metabolic cages and allowed an ad libitum access to water and food. Urine was collected in graduated cylinders during the first 4 h and the next 20 h and volume and electrolyte contents were measured during 5 days.

Rats were killed on the sixth day, 4 h after bumetanide and candoxatril administration. The blood was drawn and rapidly transferred into an ice-cold tube containing the following protease inhibitors: 1 mg of EDTA, 500 kallikrein inhibitor units of aprotinin, and 4.25 mg of phenylmethylsulfonylfluoride (PMSF) at 10⁻⁵ M per ml of blood. The blood was immediately centrifuged (4000 rpm) at 4°C for 15 min, and plasma was kept frozen at −30°C until subsequently analyzed. The kidneys of all rats were excised, immediately dissected macroscopically into cortex and medulla, frozen in liquid nitrogen, and kept at −80°C until used in RT–PCR assays.

All experimental protocols were performed in accordance with the recommendations of the French Accreditation of Laboratory Animal Care (authorization N° 00577).

2.2. Biochemical assays

Urinary volume was determined gravimetrically and urinary flow (UV) was expressed in ml/4 h and ml/20 h. Urine samples were supplemented with 100 μl containing 5 mg of EDTA and were then frozen at −30°C. Electrolyte concentrations were measured in urine samples (100 μl) using an ion-selective electrode. The excretion rate for Na⁺ (UNa), K⁺ (UK), and Cl⁻ (UCI) were calculated and expressed in micromoles per 4 and 20 h respectively and pooled over 5 days.

Urinary cGMP (U cGMP V) was measured by commercial radioimmunounoassay kit (Amersham, Amersham, UK) in diluted urine (1/50–1/200) with [³H]cGMP (assay range from 0.5 to 8 pmol) [9].

ANP was extracted by passing the plasma and urine samples (1 ml) through Sep Pak C₁₈ cartridges (Millipore, Milford, MA, USA) which were equilibrated by 0.2% ammonium acetate, pH 4 (4 ml), and eluted by 60% of acetonitrile in ammonium acetate, pH 4. The supernatant was transferred to a fresh tube and the extract was evaporated to dryness under a stream of nitrogen at 60°C. The dried extracts were dissolved in assay buffer and urinary ANP (U ANP V) was measured by radioimmunoassay with [¹²⁵]I-(1-28)ANP (assay range from 3 to 200 pg) [19].

Urinary aldosterone was extracted from urine by use of Bond Elut Phenyl Silice (Bondelut PH, Varian, Prolabo, Harbour City, CA). The Bond Elut were pre-equilibrated with 100% ethanol and bidistilled water solution before the urine was applied. After elution and evaporation to dryness under a stream of air, the residue was reconstituted in assay buffer. The urinary aldosterone (U Aldo V) was measured by radioimmunoassay by passing [³H]aldosterone as tracer (assay range from 12.5 to 400 pg) [20] and was determined by liquid scintillation counting using a Packard Tri-carb liquid scintillation analyzer.

Blood samples were taken for plasma renin activity...
(PRA) measurements. The plasma renin activity (PRA) was measured by radioimmunoassay of the angiotensin I produced after incubation of 25 µl of plasma sample and was expressed in nanograms of angiotensin I per milliliter per hour (AI/ml/h) [21].

Renal renin content was determined in renal cortical extracts by its capacity to generate angiotensin I (AI) using a specific radioimmunoassay for AI [22]. Plasma of bilateral nephrectomized rats was used as renin substrate. Cortical renin activity was calculated using AI standards and expressed as micrograms of AI generated per hour per milligram of cortical protein (µg AI/mg protein). Proteins of cortical extracts were determined according to the method of Bradford, with bovine serum albumin as standard using a Bio-Rad Kit Laboratories.

2.3. Histochemical staining of the macula densa

For the histochemical demonstration of NADPH diaphorase activity, 5 µm-thick cryostat kidney sections were incubated in 100 mM Tris/HCl buffer, pH 7.7 containing nitroblue tetrazolium (NBT, 0.2 mM), β-NADPH (1 mM) and triton X-100 (0.2%). The exposure time was 30 min at 25°C [18]. For semi-quantitative evaluation the density of NADPH diaphorase positive macula densa were expressed per 100 glomeruli [18].

Immunohistochemistry of the neuronal bNO synthase (bNOS) was performed on 7 µm paraffin sections [18]. After warming sections for 15 min in a microwave oven, they were incubated with protein blocking reagent (DAKO) to minimize spurious background staining. The sections were then incubated with the primary rabbit polyclonal antibody against bNOS (from Transduction Laboratories) or with a non-immune IgG control (diluted 1:100). After washing sections, an avidin–biotin peroxidase system kit (LSAB2 from DAKO) was used. The reaction was stopped in water and the sections coverslipped using Aquamount.

2.4. Semiquantitative analysis of rBSC1, rTSC1, bNOS and rat renin mRNA expression

The relative quantification of rat thiazide-sensitive Na–Cl cotransporter (rTSC1), renin and neuronal NO synthase (bNOS) mRNA expressions were assessed in renal cortex by comparative Reverse Transcriptase–Polymerase chain reaction (RT–PCR). Rat bumetanide-sensitive Na–K–2Cl cotransporter (rBSC1) mRNA was similarly quantified in the renal medulla.

2.5. Reverse transcription

Extraction of total RNA from medulla and cortex of individual rat kidneys was performed according to the manufacturer’s directions with Trizol reagent (Gibco-BRL, Life Technologies Inc) and a polytron homogenizer. RNA quality was determined by running samples on 0.7% agarose gels stained with ethidium bromide, and RNA concentration and purity were determined by the ultraviolet (UV) light absorbance at 260 and 280 nm (Spectrophotometer U-2001, HITACHI).

Reverse transcription (RT) was carried out using 300 ng of total RNA from renal cortex or medulla of each rat. Prior to RT reaction, the total RNA was primed with 1 µg of oligo d(T)12-18 (Pharmacia Biotech) and was heated at 65°C for 10 min; then, the tubes were chilled on ice for 1 min. RT was performed at 37°C for 60 min in a total volume of 18 µl, using 200 U of Moloney murine leukemia virus reverse transcriptase (M-MLV reverse transcriptase, Gibco-BRL, Life Technologies), 20 U RNase inhibitor, 1X RT buffer (75 mM KCl, 3 mM MgCl₂, 50 mM Tris–HCl, pH 8.3), 10 mM dithiothreitol (DTT) and 0.5 mM of each dNTP (Sigma).

2.6. Polymerase chain reaction

Part of the reverse transcription solution (3 µl) was mixed with the PCR mix containing, 1X Taq-buffer (1.5 mM MgCl₂, 50 mM KCl, and 10 mM Tris–HCl, pH 8.3), 1.25 U Taq DNA polymerase (Gibco-BRL, Life Technologies), 0.05 mmol/l dNTP (Pharmacia), 10 pmol of sense and antisense primers and 4×10⁷ cpm of a ³²P radiolabelled mix of both primers. The samples were overlaid with 30 µl mineral oil, and PCR cycles were performed in a DNA thermal cycler (Technne GENIUS) with the following profile: initial denaturation 4 min at 94°C, annealing 1 min at 62°C for rBSC1, rTSC1, and bNOS, and 1 min extension step at 72°C. The last cycle was followed by 1 min at 62°C and a final extension step of 10 min at 72°C. The annealing temperature for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers was 55°C.

Preliminary studies were performed to determine the optimum number of cycles for quantification. From this study, we chose the optimal number of cycles for each primer pair as follows: in renal cortex, 21 for rTSC1, 31 for rNOS I, 23 for rRenin and 21 for GAPDH; in renal medulla, we chose 21 for rBSC1, and 21 for GAPDH. A negative control sample (distilled water) was used for each set of samples to check the reverse transcription and the PCR amplification reagents for any contamination. PCR amplification was verified to be exponential.

To analyze the PCR products, each reaction was electrophoresed in an 8% acrylamide/dihydroxyethylene bisacrylamide (29:1, vol:vol) gel in 1×TBE buffer with a miniprotein II cell apparatus (Bio-Rad Laboratories). Bands were stained with ethidium bromide and observed under UV light, cut out, suspended in 0.75 ml of periodic acid at 25 mM (ACROS ORGANIGS), incubated for 2 h at 50°C, and the incorporated radioactivity was determined in 4 ml of liquid scintillation using Packard Tri-carb liquid scintillation analyzer.
Table 1
Primer sequences derived from rat cDNAs, used in the RT–PCR assays

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences</th>
<th>Amplification size</th>
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<tbody>
<tr>
<td>BSC-1</td>
<td>Upstream 1605 5'-TCAAGATGTCAGCATGAGCC-3' 209 bp</td>
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<tr>
<td></td>
<td>Downstream 1813 5'-CCCTTGGGAAGAAGCTGAAG-3'</td>
<td></td>
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<tr>
<td>TSC-1</td>
<td>Upstream 2303 5'-ACTTCAACTACGGGGYGTGC-3' 349 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Downstream 2651 5'-ATGTCAGGAAGGACGTGGAC-3'</td>
<td></td>
</tr>
<tr>
<td>bNOS</td>
<td>Upstream 3987 5'-CTGGCTCAACAGAATACAGGCT-3' 293 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Downstream 4280 5'-GCAGTGTACAGCTCTCTGAAGA-3'</td>
<td></td>
</tr>
<tr>
<td>Renin</td>
<td>Upstream 754 5'-GCAGTGACCCTCAACATTACC-3' 451 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Downstream 1204 5'-CGATGCGATTGTTATGCC-3'</td>
<td></td>
</tr>
<tr>
<td>GAPDH</td>
<td>Upstream 5 5'-GTGAAGGTCGGAGTCAACG-3' 302 bp</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Downstream 5-GGTGAAGACGCCAGTACTCTC-3'</td>
<td></td>
</tr>
</tbody>
</table>

The primer sequences for rBSC1, rTSC1, bNOS, rat renin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) used in RT–PCR assays are reported in Table 1. These primers were custom obtained from Cybergene. The rBSC1, rTSC1, bNOS and rat renin mRNA expressions were calculated by normalizing to that of the ‘housekeeping’ gene GAPDH mRNA.

2.7. Statistical analysis

The results were expressed as means±S.E.M. The experimental protocol was designed for using two-way Analysis of Variance (ANOVA, Statview software). One factor was the NEP inhibitor effect; the second factor was the bumetanide effect. This statistical approach permitted us to test for an independent effect of each factor (NEPI and bumetanide) and for an interaction between the two factors. When the interaction was significant, comparison between groups was performed by the Bonferroni method. When one parameter was measured several times during the experimental protocol an ANOVA for repeated measures was used. P<0.05 was considered significant.

3. Results

3.1. Diuresis and urinary electrolyte excretions

Fig. 1 shows the mean effect of candoxatril and bumetanide on urinary excretion of water (Fig. 1A) and electrolytes (Fig. 1B–D) in the first 4 h after drug administration and in the following 20 h for the 5 days of the experiment. Candoxatril significantly increased diuresis during the first 4 h whether associated or not with bumetanide (F=12, P<0.05, two-way ANOVA for repeated measures). Administration of bumetanide also increased diuresis during the first 4 h (F=70, P<0.05). There was no interaction between candoxatril and bumetanide on diuresis, showing only a small additive effect. During the following 20 h only bumetanide induced a small increase in diuresis (F=5.2, P<0.05).

The urinary excretion of sodium, potassium, and chloride is represented in panels Fig. 1B–D. Bumetanide increased significantly the rate of urinary excretion of all electrolytes, (Na^+, K^+ and Cl^-), during the first 4 h (two-way ANOVA, P<0.05). Candoxatril did not significantly increase natriuresis but significantly increased K^+ excretion (two-way ANOVA, F=14, P<0.05) during the first 4 h following administration of the compound. Candoxatril alone had no effect on Cl^− excretion but slightly increased bumetanide-induced chloruria in the first 4 h (interaction; F=4.1, P=0.05). During the following 20 h, bumetanide had no additive effect on natriuresis and chloruresis; only kaliuresis remained significantly elevated under bumetanide (two-way ANOVA, F=5, P<0.05). Candoxatril had no effect on electrolyte excretion during the subsequent 20 h.

3.2. Urinary excretion of ANP and cyclic GMP

The urinary excretion of atrial natriuretic peptide (ANP) and cyclic guanosine monophosphate (cGMP) was measured on the first, the third, and the fifth days of treatment and expressed per 24 h. Fig. 2 shows that candoxatril induced a significant increase in urinary excretion of ANP (two-way ANOVA, F=175, P<0.05). Moreover there was a significant interaction between candoxatril and bumetanide (F=9.2, P<0.05), bumetanide alone did not modify basal ANPuria, whereas it reduced the ANPuria induced by candoxatril (interaction, F=9, P<0.05). Similarly, candoxatril increased urinary cyclic GMP (F=8.7, P<0.05) whereas bumetanide had no effect (Fig. 2). There was no interaction between the two treatments. Plasma
ANP was also significantly increased by candoxatril ($F = 17$, $P < 0.05$) and did not change with bumetanide.

### 3.3. Renin–angiotensin–aldosterone system

Rats treated with bumetanide showed an increase in 24 h urinary excretion of aldosterone (ANOVA, $F = 4.5$, $P < 0.05$) that reached a peak after 4 days of treatment (time effect, $F = 9$, $P < 0.05$) (Fig. 3). No significant effect and no interaction on aldosteronuria was observed with candoxatril.

The plasma renin activity (PRA) was measured at sacrifice in all the animals 4 h after the last administration of treatment. The results are represented in Fig. 3. Bumetanide significantly increased (ANOVA, $F = 13$, $P < 0.05$) whereas candoxatril decreased PRA (ANOVA, $F = 5$, $P < 0.05$). Therefore the plasma renin activity did not differ between controls and rats treated with the association of candoxatril + bumetanide. No significant difference was observed between groups for renal renin content. Nevertheless, we observed with bumetanide that the renin content showed a tendency to decrease rather than to increase, suggesting an acute increase in renin release (Fig. 3).

Similarly, the expression of renin mRNA in renal cortex demonstrated no difference between groups 4 h after the last administration of compounds (Fig. 3).

### 3.4. NADPH activity and bNOS in macula densa

In the bumetanide group, NADPH diaphorase activity appeared qualitatively more intense. Particularly, the area and the intensity of each stained macula densa appeared larger in the bumetanide group compared to controls (Fig. 4A and B). Semiquantitative evaluation showed that bumetanide alone significantly increased the density of NADPH diaphorase activities in the macula densa (ANOVA, $F = 86$, $P < 0.05$). There was a powerful significant interaction between bumetanide and candoxatril (interaction, $F = 31$, $P < 0.05$) demonstrating that the macula densa response to bumetanide was blunted by candoxatril. These data were confirmed by the specific immunohistochemistry of the neuronal NOS (Fig. 4C).

Two-way ANOVA showed also a strong interaction between the two treatments on bNOS mRNA expression in the renal cortex (interaction, $F = 12$, $P < 0.05$). Bumetanide alone increased bNOS mRNA expression as compared to
4. Discussion

The present study was undertaken in order to test the hypothesis that urinary ANP potentiation induced by NEP inhibition could directly or indirectly alter epithelial function within the renal cortex, via modulation of the ionic urinary load delivered to the distal nephron. Because bumetanide is a specific inhibitor of Na–K–2Cl cotransport [23], and because Na–K–2Cl (BSC1) is expressed at the apical pole of the epithelial cells directly sensing the urinary electrolyte load in the tubule lumen [24], we investigated the pharmacological interaction between NEP inhibition by candoxatril and bumetanide in vivo. We have also used markers of epithelial function in the cortex, focusing our attention on the macula densa-dependent renin secretion.

As previously described, NEP inhibition is associated with a marked increase in ANP and cyclic GMP urinary excretion [8,9,25,26]. In the present study, bumetanide alone had no effect on ANP and cGMP urinary excretion. Nevertheless, bumetanide significantly interacted with the NEP inhibitor to decrease urinary ANP excretion. This result probably reflects the decrease in ANP secretion by the heart and the consequent decrease in ANP filtration by the glomeruli in relation to the bumetanide-induced decrease in body fluid volume. Similarly, we have recently described an increase in urinary ANP excretion proportional to ANP secretion in congestive heart failure during NEP inhibition [27].

ANP targets the particulate guanylate cyclases in the renal epithelial cells, inducing cyclic GMP production. Guanylyl cyclase-coupled ANP receptors have been described in the different segments of the nephron with varying density [28]. The particulate guanylate cyclase appears to be expressed not only at the basolateral pole of the epithelial cells but also at the apical pole [29], suggesting that urinary ANP, generated by glomerular filtration, and prevented from proximal degradation by NEP inhibition, could act on epithelial cell function by an apical interaction. In contrast with acute inhibition of NEP in rats during which plasma ANP did not increase [9], chronic administration of candoxatril induced a significant rise in plasma ANP, not completely excluding a role for plasma ANP in the regulation of renin secretion. Nevertheless, although a direct action of ANP on renin-secreting cultured cells has been shown [14], it has also been demonstrated that ANP did not influence renin secretion from intact, isolated, rabbit afferent arterioles in basal conditions as well as after in vivo stimulation by furosemide, another Na–K–2Cl inhibitor [16]. Similarly, evidence of a direct effect of circulating ANP on small arteriole function has never been established in vivo.
Fig. 3. Renin–aldosterone system parameters: 24 h aldosteronuria (UAldo V), plasma renin activity (PRA) at sacrifice, renal renin content (RRC), and cortical renin mRNA/GAPDH mRNA ratio. Bumetanide ($) increased aldosteronuria ($P<0.05$) independently of candoxatril which has no significant effect. Bumetanide ($) increased ($P<0.05$) whereas candoxatril (*) decreased ($P<0.05$) plasma renin activity without significant interaction. The variations in renal renin content and mRNA did not reach statistical significance; $n=$number of rats.

[30,31]. Therefore, we proposed the concept of the compartmented biodisponibility of ANP in vivo [32,33].

As expected, bumetanide, which blocks Na–K–2Cl cotransport mainly in the thick ascending limb (TAL), induced a marked rise in electrolyte excretion. Candoxatril alone had a small additive effect on diuresis, no effect on natriuresis, a small additive effect on chlorouria and a significant, bumetanide-independent effect on kaliuresis. This could be due to a direct or indirect modulation of $K^+$ conductance [34–36]. These data show an escape from ANP-dependent natriuresis during chronic inhibition of NEP. That acute inhibition of renal NEP potentiates the natriuretic effect of ANP has been largely documented [25,26,37], but the effect of chronic administration of NEP inhibitors on natriuresis is less documented. As in congestive heart failure [38], characterized by a chronic increase in ANP secretion [39,40], chronic NEP inhibition-induced ANP potentiation probably induced escape from the natriuretic effect of ANP. The mechanisms of ANP escape during chronic NEP inhibition remain to be explored but in congestive heart failure both the induction of phosphodiesterase activity [41–43] and Na–K–2Cl overexpression [44] have been reported.

As expected, bumetanide increased the plasma level of renin activity 4 h after the last injection of the compound. Na–K–2Cl, the molecular target of bumetanide, is expressed in the TAL but also in the macula densa [45]. In the latter, Na–K–2Cl is probably the main molecular effector of the link between luminal urinary ionic concentration and juxta-glomerular apparatus function, controlling renin secretion [10]. Our results confirmed that bumetanide, by blocking the apical Na–K–2Cl activity in the macula densa [17,46], increased renin secretion, slightly decreased renin storage, and probably increased renin synthesis in a delayed manner not assessed in the present study. These data fit well with the time course of renal renin secretion and biosynthesis in response to furosemide [47] and to other agents [48].

In contrast, NEP inhibition significantly decreased renin secretion and prevented the increase induced by bumetanide. This observation suggests that urinary ANP potentiation by NEP inhibition could influence directly or indirectly, via the modulation of urine electrolyte concentration, probably mainly of chloride, the intracellular signaling in the macula densa and intercellular communications through the juxta-glomerular apparatus. These
Fig. 4. Macula densa activity: NADPH diaphorase staining in control (A) and bumetanide (B) treated rats, and neuronal NOS immunohistochemistry in bumetanide treated rats (C). Bumetanide significantly increased both NADPH diaphorase activity and bNOS mRNA expression ($P<0.05$). Candoxatril significantly ($P<0.05$) interacted with bumetanide in decreasing both NADPH activity and bNOS mRNA expression. Therefore only the bumetanide group ($) differed from the other three groups (Bonferroni, $P<0.05$); $n=$ number of rats.

The present data fit well with an early study showing that intrarenal infusion of ANP in the nonfiltering kidney, lacking a functional macula densa, did not change renin secretion whereas it decreased renin secretion in filtering dog kidney [15]. It also fits well with the more recently published results of the interactions between ANP knock-out, salt sensitivity and renin secretion [49,50] in mice. In these studies, despite the absence of alterations in cumulative urinary excretion of electrolytes, Melo et al. [49,50] showed a lack of inhibition of renin secretion in response to high salt intake in ANP knock-out mice. Therefore, our results of the combined treatment group with functional
effects on renal excretory function (similar to bumetanide) despite a normalisation of PRA suggested that potentiation of urinary ANP by NEP inhibition could directly or indirectly interact with the macula densa.

In order to provide further support for this hypothesis, NADPH diaphorase activity and neuronal NO synthase expression were chosen as specific markers of macula densa activity [18,51]. bNOS activity is correlated with renin under various stimuli [52] and possibly involved in the regulation of renin secretion [53]. It has been recently shown that nitro-arginine blocks the renin secretion in response to furosemide in humans [54]. Our present study confirmed these data, showing that bNOS expression was significantly increased by bumetanide, paralleling the increase in renin secretion. Similarly, as NEP inhibition decreased renin secretion, it also decreased neuronal NO synthase expression preventing the increase due to bumetanide. These data suggest that NEP inhibition influenced directly or indirectly epithelial function in the macula densa.

Lastly we tested the interaction of NEP inhibition and bumetanide on rBSC1 expression in the medulla and rTSC1 in the cortex. BSC1 and TSC1 are differentially expressed along the rat nephron [55]. BSC1 is mainly expressed in the medulla and TSC1 in the cortex. NEP inhibition significantly interacted with the bumetanide-induced overexpression of rTSC1 in the renal cortex. These cortical overexpression of rTSC1 appeared to be a more distal compensating mechanism in response to the chronic blockade of Na–K–2Cl cotransport in the TAL.

The mechanism by which NEP inhibition could prevent this bumetanide-induced TSC1 overexpression in the cortex remains to be explored. In contrast, as in congestive heart failure [44] we observed an upregulation of Na–K–2Cl expression in the medulla in response to chronic NEP inhibition suggesting a more distal counter regulation to the proximal action of ANP along the nephron.

In conclusion, using the NEP inhibitor candoxatril as a pharmacological agent capable of potentiating the urinary action of ANP, and bumetanide as a pharmacological blocker of the Na–K–2Cl cotransporter, the results of this study suggest, that urinary ANP could directly or indirectly interfere with epithelial function at different levels of the nephron. By altering macula densa activity, urinary ANP potentiation by NEP inhibition could directly or indirectly modulate renin secretion.

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