Overexpression of kidney neutral endopeptidase (EC 3.4.24.11) and renal function in experimental cirrhosis

G. Sansoë, M. Aragno, R. Mastrocola, J. C. Cutrin, S. Silvano, G. Mengozzi, A. Smedile, F. Rosina, O. Danni, and M. Rizzetto


Neutral endopeptidase degrades atrial natriuretic peptide (ANP) and bradykinin and may generate endothelin-1 from big-endothelin. In advanced cirrhosis, sodium retention is accompanied by elevated plasma ANP levels, and infusion of ANP causes hypotension, but in normal humans the concentration of ANP through the inhibition of neutral endopeptidase, localized in renal proximal tubule cells, causes natriuresis without any arterial pressure drop. The purpose of this study was the assessment of kidney neutral endopeptidase expression and responses to candoxatril (a specific inhibitor of this enzyme) in rats with CCL-induced cirrhosis. Two groups of control rats (n = 5) were injected with vehicle or candoxatril. Three groups of cirrhotic rats with ascites (n = 10) received vehicle alone or 3 or 10 mg/kg candoxatril. In cirrhotic rats, Western blot analysis revealed a 170% increase in renal neutral endopeptidase protein content (P < 0.03), mainly in the proximal nephron and macula densa, and both candoxatril dosages increased plasma ANP levels, urinary volume, and plasma creatinine. A significant overexpression of this enzyme in renal cortex cannot fully account for the natriuresis of immersion by itself, due to other factors occurring in this setting, i.e., changes in renal-sympathetic nerve traffic and blunting of renin secretion (13). Because higher ANP levels occur in the setting of sodium retention, this indicates that ascitic patients with cirrhosis exhibit reduced natriuretic responses to this peptide, a finding confirmed by infusion of ANP in cirrhotic patients. Furthermore, ANP infusion determines hypotension in ascitic cirrhosis (17). The mechanisms of renal hyporesponsiveness to ANP so far identified are activation of the renin-angiotensin-aldosterone axis and sympathoadrenergic tone as a consequence of the arterial hypotensive effect of this hormone (49), downregulation of natriuretic peptide receptors A and B in the kidney (19), and increased activity of cGMP phosphodiesterase, the enzyme degrading the second messenger of ANP (4). The occurrence of renal overexpression of NEP as a possible cause of renal resistance to the effects of ANP has never been investigated in cirrhosis.

NEP is a membrane-bound Zn-metalloendopeptidase of the brush-border membrane of kidney proximal tubule cells, of lung myofibroblasts and epithelial cells, B lymphoid progenitors, and glial cells (2, 25). On the surface membrane of these cells, NEP degrades bradykinin (11), bombesin-like peptide (9), substance P (15), ANP (23), adenomedullin (29), endothelin-1 (ET-1) (1), and angiotensin II (15). Not only does NEP degrade the above peptides but it produces the vasoconstrictor polypeptide ET-1 from circulating precursors (i.e., big-ET-1 and ET-11-31) (16, 32).

NEP is specifically inhibited by candoxatril (34), its prodrug candoxatril (3), and by thiorphan (44), sinorphan (21), and phosphoramidon (15). In healthy subjects, NEP inhibitors increase the concentrations of ANP and cause natriuresis (21, 34) without affecting blood pressure (21, 34, 39). Actually, both candoxatril (3) and candoxatril (31) have been reported to raise blood pressure in normotensive human subjects. Because NEP inhibitors potentiate the effects of ANP but do not cause hypotension in other groups of patients (such as those with cardiac failure) (34), we tested the hypothesis that NEP expression is increased in cirrhosis and that intravenous candoxatril would have a natriuretic effect without causing hypotension.

METHODS

Studies were performed in anesthetized adult male Wistar rats with ascitic cirrhosis and anesthetized adult male Wistar control rats. Both the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
groups were fed ad libitum with standard chow and water. Cirrhosis was induced by CCl4 (Riedel de Haen, Sigma, Seelze, Germany) administered by gavage twice weekly (37). Cirrhotic rats were studied between 9 and 12 wk after the start of the cirrhosis induction program, when ascites was fully developed. Control rats were studied following a similar period of standardized diet. Experiments in rats were performed in compliance with the procedures outlined in the Italian Ministry of Health guidelines (no. 86/609/EEC) and according to the Principles of Laboratory Animal Care (National Institutes of Health Publication no. 85–23, revised 1985). Candoxatrilat, a specific NEP inhibitor (3, 34), was provided by Pfizer Central Research (Sandwich, Sussex, UK).

Animal groups. Candoxatrilat was dissolved in 5% glucose solution as diluent to obtain two different solutions to be administered intravenously to the rats in the same volume of fluid (1 ml), producing two different bolus doses, 3 or 10 mg/kg body wt.

The rats were divided into 5 groups: 5 control rats receiving diluent alone, 5 control rats receiving 3 mg/kg body wt candoxatrilat, 10 cirrhotic rats receiving diluent alone, 10 cirrhotic rats receiving 3 mg/kg body wt candoxatrilat, and 10 cirrhotic rats receiving 10 mg/kg body wt candoxatrilat.

Study protocol. The rats were anesthetized with a mixture of Ketavet 100 (Farmaceutici Gellini, Sabaudia, Italy) and Rompun (4:1, vol/vol, Xilazina, Bayer, Leverkusen, Germany) by intraperitoneal injection (0.5 ml mixture/200 g body wt). Blood was sampled (time 0) by cardiac puncture (0.5 ml), and 10% inulin (wt/vol, Laevosan-Gesellschaft, Linz/Donau, Austria) plus 20% PAH (wt/vol, Nephrotest, BAG, Munich, Germany) were administered intravenously into the caudal vein as a priming bolus (0.14 and 0.03 ml/kg, respectively) followed by a continuous infusion of 0.09 ml-kg⁻¹-h⁻¹ inulin and 0.025 ml-kg⁻¹-h⁻¹ PAH for 150 min to allow for glomerular filtration rate (GFR) and renal plasma flow (RPF) at different times by means of their respective steady-state plasma clearances (Cin and CPRPF) (10, 30). After 90 min of inulin plus PAH infusion (i.e., once their steady-state plasma concentrations were reached) (10), a laparotomy was performed and the urinary bladder was emptied; a clamp was positioned on the urethral orifice. Cardiac blood was then sampled (time 1) to assess basal values of Cin and CPRPF, and then either diluent alone or candoxatrilat (F3 or F10) was injected as a single bolus into the right femoral vein. Cardiac blood was then sampled (0.5 ml) into tubes containing 1 mM PMSF, 1 mM sucrose, 1 mM PMSF, pH 7.5) and centrifuged at 1,000 g for 10 min. The supernatant was centrifuged at 10,000 g for 10 min and the pellet was dissolved in Tris buffer (50 mM Tris, 2 mM MgCl2, 0.25 M sucrose, 1 mM PMSF, pH 7.5) and centrifuged at 1,000 g and 4°C for 10 min. The supernatant was centrifuged at 10,000 g for 10 min and at 100,000 g for 45 min. The pellet was dissolved in Tris buffer (50 mM Tris, 2 mM MgCl2, 80 mM NaCl, 1 mM PMSF, pH 8.0), and the protein content was determined using a modified Bradford assay (Bio-Rad) using BSA as a standard. Protein (15 μg) was loaded on vertical SDS-polyacrylamide gels (4% stacking gel and 10% resolving gel) and transferred to a membrane (Hybond-PVDF, Amersham Lifescience) by electrophoretic transfer (48 mM Tris, 39 mM glycine, 0.037% SDS, 20% methanol). Nonspecific binding was blocked with blocking solution (PBS, 0.05% Tween 20.95% nonfat dry milk, 5% fetal calf serum), and blots were incubated with a rabbit polyclonal NEP antibody (CD 10, Santa Cruz Biotechnology) and an antibody against the receptor-associated protein (RAP) at a dilution of 1:1,000 in PBS for 1 h at room temperature. After being washed three times for 15 min in PBS, blots were incubated with the secondary antibody (goat IgG, Sigma) for 30 min at room temperature. Three further washing steps (15 min each) in PBS were followed by detection using an ECL detection kit (ECL Western blotting detection, Amersham Pharmacia Biotech). Densitometric quantification was performed using RAP expression as an internal standard: before any comparison was made, the net intensity of NEP bands in each experiment was normalized to the intensity of the corresponding RAP band, used as an internal standard to evaluate the degree of nonspecific protein expression in the homogenate (26).

**NEP immunostaining.** Tissue samples were embedded in paraffin, and standard immunohistochemistry procedures were applied using the labeled streptavidin-biotin (LAB-SA) and the AEC-chromogen-producing red staining (Zymed Laboratories) methods. After fixation, endogenous peroxidase activity was quenched with 1% H2O2. Sections were incubated with a primary anti-CD 10-antibody for 16 h (rabbit polyclonal, 2 μg/ml, sc-9149, Santa Cruz Biotechnology). Staining with a nonspecific IgG control antibody (Sigma) served as control.

**Plasma and urine analyses.** Plasma and urinary concentrations of electrolytes were measured by flame photometry. Inulin and PAH concentrations in plasma were determined colorimetrically (40, 46). AYP systemic concentrations were measured in EDTA plasma by RIA (Vasopressin Direct RIA, Buhlmann Laboratories, Postfach, Switzerland). Urine and plasma ANP was measured using a specific RIA (ANP Shionoria, Cis Bio International, Gif-sur-Yvette Cedex, France). PRA was determined using RIA for angiotensin I after an incubation period of 2 h (Renin Maia Kit, Biodata, Rome, Italy). ET-1
plasma concentrations were measured using a commercially available kit (Endothelin-1 Radioimmunoassay, Peninsula Laboratories, King of Prussia, PA). Urinary concentrations of cGMP were determined using a commercial RIA kit (Immunotech, Marseille, France).

**Calculations.** Sodium clearance (C\textsubscript{Na}) and potassium clearance (C\textsubscript{K}) were calculated through the formula

\[ C_x = \frac{U_x \times V}{P_x} \]

where \( U_x \) is the urinary concentration of \( x \), \( P_x \) is the plasma concentration of \( x \), and \( V \) is the urinary output (ml/min). C\textsubscript{In} and C\textsubscript{PAH} were calculated through the steady-state plasma clearance formula as

\[ C_x = \frac{\text{Infusion rate}}{\text{ssP}_x} \]

where ssP\(_x\) is the steady-state plasma concentration of \( x \). C\textsubscript{In} and C\textsubscript{PAH} were taken as measures of GFR and RPF (30, 31). Filtered sodium load (F\textsubscript{Na}) was derived, following Boer et al. (6), as

\[ \text{FlNa} = \text{P}_{\text{Na}} \times C_{\text{in}} \]

Filtration fraction (FF) was calculated from the formula

\[ \text{FF} = \frac{\text{GFR/RPF} \times 100}{\text{FF}} \]

Fractional sodium excretion (FE\textsubscript{Na}) and fractional potassium excretion (FE\textsubscript{K}) were calculated, respectively, from the ratios of C\textsubscript{Na} and C\textsubscript{K} to C\textsubscript{in} \times 100.

Tubular solute-free water reabsorption (TFWR) was calculated, following Rose and Post (41), through the formula

\[ \text{TFWR} = C_{\text{osm}} - V \]

where \( V \) is urinary output (ml/min), and \( C_{\text{osm}} \) is the osmolar clearance, which was computed via the usual formula

\[ C_{\text{osm}} = \frac{(U_{\text{osm}} \times V)}{P_{\text{osm}}} \]

where \( U_{\text{osm}} \) and \( P_{\text{osm}} \) are urine and plasma osmolalities, respectively.

All renal function parameters measured after diluent or candoxatrilat administration were derived by computing the mean of three determinations of osmolarity, inulin, PAH, sodium, and potassium in plasma during the 60-min urine collection period (blood sampling times 2–4).

Mean arterial pressure (MAP) was calculated from the formula

\[ 1/3(\text{systolic blood pressure} - \text{diastolic blood pressure}) + \text{diastolic blood pressure} \]

**Morphological liver studies.** Livers were removed from 20 rats submitted to CCl\(_4\) intoxication, and hepatic tissue samples for light microscopy were placed in buffered 4% formaldehyde solution (pH 7.4). The sections were stained with hematoxylin and eosin to assess fibrosis. Silver-impregnated liver sections were used to observe portal-central or central-central bridging fibrosis.

**Statistical analysis.** The main comparisons were between renal or hormonal parameters measured after administration of candoxatrilat or after diluent alone. Results are expressed as means ± SD. All comparisons between groups of rats were made by a nonparametric statistical method, the Wilcoxon rank sum test. Correlation coefficients were derived using Spearman’s rank correlation. Significance is accepted at the 5% probability level.

**RESULTS**

Liver morphological studies. Micronodular cirrhosis with hepatocellular necrosis and microvacuolar steatosis was found in all 20 livers removed from ascitic rats (data not shown).

**Renal NEP expression.** Neutral endopeptidase appeared significantly overexpressed in the membrane fraction of renal tissue homogenate from cirrhotic animals, without differences in expression between candoxatrilat-treated and untreated animals (Figs. 1 and 2). Immunohistochemical determination of NEP in renal tissue slices showed more intense positive NEP staining in the proximal convoluted tubules, the macula densa, Bowman’s capsule, and the mesangium in kidneys from rats with liver cirrhosis compared with controls (Fig. 3).

Hormonal status and mean arterial pressure. Infusion of candoxatrilat caused a significant increase in plasma ANP concentrations in both control and cirrhotic rats but had no

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**Fig. 2. Relative densities of NEP bands.** Values are means ± SD of 5 rats/group.

**Fig. 3.** Immunohistochemical determination of NEP in kidney cortex from a control (A) and a cirrhotic rat (B). A more intense positive reaction was observed in proximal convoluted tubules, macula densa (arrow), Bowman’s capsule, and mesangium in kidneys from rats with liver cirrhosis (×400, hematoxylin counterstained).
NEP inhibition and renal function in experimental liver cirrhosis

Table 1. Hormonal plasma and urine determinations in different groups of rats

<table>
<thead>
<tr>
<th></th>
<th>G1 (n = 5)</th>
<th>G2 (n = 5)</th>
<th>G3 (n = 10)</th>
<th>G4 (n = 10)</th>
<th>G5 (n = 10)</th>
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<tr>
<td>Plasma AVP, pg/ml</td>
<td>100±58</td>
<td>88±31</td>
<td>90±12</td>
<td>85±15</td>
<td>99±26</td>
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<td>Plasma ANP, pg/ml</td>
<td>1.50±0.03</td>
<td>2.22±0.31†</td>
<td>2.16±0.21†</td>
<td>2.50±0.33‡</td>
<td>2.56±0.11†</td>
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<td>Plasma ET-1, fmol/ml</td>
<td>0.68±0.23</td>
<td>0.74±0.13</td>
<td>0.58±0.40</td>
<td>0.71±0.41</td>
<td>0.64±0.47</td>
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<tr>
<td>PRA, mg·ml⁻¹·h⁻¹</td>
<td>92±5</td>
<td>95±12</td>
<td>137.7±9.9‡</td>
<td>140±21†</td>
<td>114±147†</td>
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<tr>
<td>Urinary ANP, pg/min</td>
<td>0.22±0.31</td>
<td>0.19±0.12</td>
<td>0.033±0.03‡</td>
<td>0.23±0.19†</td>
<td>0.11±0.05‡</td>
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<tr>
<td>Urinary cGMP, pM/min</td>
<td>8.52±8.06</td>
<td>13.30±10.01</td>
<td>9.81±7.21‡</td>
<td>15.49±4.73*</td>
<td>22.44±3.71‡</td>
</tr>
<tr>
<td>Urinary cGMP/Naₑ⁻¹ ratio/(pM/μeq)</td>
<td>14.3±5.8*</td>
<td>15.9±10.4*</td>
<td>27.7±4.7†</td>
<td>19.1±3.8*</td>
<td>21.4±1.8*</td>
</tr>
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Values are means ± SD, n. No. of rats; G1, control rats receiving diluent alone; G2, controls receiving 3 mg/kg body wt candoxatrilat; G3, cirrhotic animals receiving 3 mg/kg body wt candoxatrilat; G4, cirrhotic animals receiving 10 mg/kg body wt candoxatrilat; ET-1, endothelin-1; PRA, plasma renin activity. *P < 0.05 vs. G3. †P < 0.05 vs. G1. ‡P < 0.03 vs. G3. §P < 0.05 vs. G2 (Wilcoxon rank sum test).

**Effect on plasma renin activity, ET-1, or AVP levels (Table 1). Both candoxatrilat dosages significantly raised urinary excretion of ANP (Fig. 4) and cGMP in cirrhotic animals. Cirrhotic rats infused with candoxatrilat had a significantly lower urinary cGMP/sodium ratio, suggesting that potentiation of natriuretic peptide function is not the sole mechanism that increases natriuresis after NEP inhibition. Candoxatrilat had no effect on blood pressure [89 ± 27 vs. 86 ± 37 mmHg (P = 0.37)] in cirrhotic rats.

Renal function. In cirrhotic rats, both 3 and 10 mg/kg candoxatrilat caused marked diuresis and increased absolute (Fig. 5) and fractional excretion of sodium; a significant reduction of tubular solute-free water reabsorption and urinary osmolality was observed after 10 mg/kg candoxatrilat (Table 2). Because FE₉Na was also moderately decreased in the control group receiving vehicle alone, a certain degree of reduction of the circulating fluid volume due to the experimental procedures cannot be ruled out; the increased urine osmolality and tubular free water reabsorption observed in the cirrhotic group confirm the occurrence of free water retention in those rats. Candoxatrilat had no effect on RPF, GFR, or FF.

Correlations. In both controls and cirrhotic rats, there were significant correlations between FF and diuresis (respectively, r = 0.61, P < 0.05 and r = 0.43, P < 0.03) and between urinary excretions of cGMP and natriuresis (respectively, r = 0.68, P < 0.05 and r = 0.70, P < 0.001) (Fig. 6). However, there was a significant correlation between ANP and cGMP urinary excretions only in the control group receiving diluent alone (r = 0.79, P < 0.05) but in none of the other groups of rats. We found a significant correlation between urinary excretion of sodium and ANP only in control or cirrhotic rats given diluent alone (respectively, r = 0.84, P < 0.05 and r = 0.79, P < 0.001) but not in candoxatrilat-treated groups.

DISCUSSION

In advanced cirrhosis, splanchnic vasodilatation activates the renin-angiotensin-aldosterone axis, sympathetic nervous system, and nonosmotic hypersecretion of AVP, with ensuing renal sodium and water retention and ascites development (8, 22). This sodium and water retention is treated with diuretic drugs that determine a further stimulation of renin secretion and may even lead to prerenal azotemia. Therefore, a drug able to enhance exclusively the renal vasodilator, natriuretic, and AVP-antagonistic actions of ANP but be devoid of the systemic hypotensive effects of this hormone would be of outstanding importance in treating cirrhotic ascites.

The effects of NEP inhibitors in cirrhosis have been evaluated in two previous studies. Dussaule et al. (12) administered single oral doses of sinorphan to 16 patients with ascitic cirrhosis and observed increases in plasma ANP, a natriuretic response, a significant reduction of plasma renin activity, and no effects on arterial pressure. Park et al. (36) observed that thiorphan caused natriuresis but did not alter cardiac output and systemic vascular resistance in cirrhotic rats. Neither study evaluated the effects of NEP inhibition on renal perfusion and free water metabolism, AVP plasma levels, and ANP urinary...
between urinary excretion of cGMP and that of sodium.

Fig. 6. Cirrhotic rats (30 animals). There was a significant direct correlation between urinary excretion of cGMP and that of sodium.

The increase in FENa produced by candoxatrilat, without any change in GFR, suggests exclusive tubular actions of this drug.

Further tubular diuretic mechanisms of candoxatrilat probably exist: 1) NEP inhibitors may reduce intrarenal levels of sodium-releasing factors (45) because enhancement of ANP tubular content by NEP inhibition limits renal secretion by targeting the macula densa (12, 23); 2) urinary ANP and natriuresis were correlated only when control or cirrhotic rats were given vehicle, but not in the candoxatrilat-treated groups; 3) compared with cirrhotic rats receiving vehicle, the candoxatrilat-treated cirrhotic groups had higher urinary sodium excretions for any given value of cGMP urinary excretion (Table 1); and 4) when inhibition of NEP is accompanied by administration of bradykinin or adrenomedullin antagonists, blunted natriuretic effects are found (11, 29). Indeed, in the model of dogs with ascites due to chronic constriction of the supradiaphragmatic vena cava (chronic caval dogs), it was postulated that, during NEP inhibition, increased delivery of both kinins and ANP distally to the cortical collecting duct allowed a natriuresis in the animals resistant to the sole infusion of exogenous ANP (27). Last, NEP inhibition might also account for decreased degradation of kaliuretic peptides derived from ANP prohormone (48), because candoxatrilat determined a potent tendency toward increased absolute potassium excretion and FENa (Table 2).

In the kidneys of cirrhotic rats, we found overexpression of NEP (Figs. 1 and 2), mainly in the proximal convoluted tubules, macula densa, Bowman’s capsule, and mesangium (Fig. 3). Consequently, ANP urinary excretion in cirrhotic rats was significantly lower than in either group of healthy animals (Table 1) despite similar GFR values (Table 2). This finding, previously described in chronic caval dogs with ascites (27), confirms enhanced tubular degradation of ANP in cirrhosis. Accordingly, the lower candoxatrilat dose we used caused a significant increase in absolute sodium excretion and FENa only in rats with cirrhosis (Table 2 and Fig. 5). NEP overexpression has been found to contribute to ANP resistance also in rats with congestive heart failure (26).

The cause of NEP overexpression in cirrhotic rat kidneys is unknown; however, several cytokines whose systemic levels are increased in liver cirrhosis (47), interleukin-1, tumor necrosis factor-α, transforming growth factor-β, and interleukin-6, enhance the in vitro expression of NEP on the surface of fibroblasts and bronchial and kidney tubular epithelial cells (3, 9).

In agreement with the findings of Park et al. (36), in our study NEP inhibition did not cause significant changes in ET-1 plasma levels in cirrhotic rats. Previous studies have reported widespread results: Newaz et al. (33) found a reduction in plasma ET-1 after candoxatril administration in arterial hypertensive rats. Asaad et al. (5) reported that NEP did not con-
tribute to the in vivo clearance of ET-1 in rats, whereas in a study in rabbits Grantham et al. (20) demonstrated a reduction in aortic tissue concentration of ET-1, despite plasma concentrations of ET-1 being increased, after NEP inhibition. These variable results are explained by the curious behavior of NEP, an enzyme that, depending on location and available substrates, can inactivate ET-1 or produce this hormone via hydrolysis of big-ET-1 and ET-1(1-31) (32).

Our study has identified a new target for future therapeutic regimens aimed at treating patients with liver cirrhosis and sodium and water retention. Furthermore, improved understanding of the subtle biohumoral modifications occurring in the kidney after inhibition of neutral endopeptidase may provide a valid occasion for knowledge of this clinical syndrome to advance further.

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GRANTS

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REFERENCES


