Kidney International, Vol. 50 (1996), pp. 962–972

An orally active ET_A/ET_B receptor antagonist ameliorates proteinuria and glomerular lesions in rats with proliferative nephritis

DULCENOMBRE GÓMEZ-GARRE, RAQUEL LARGO, XUE-HUI LIU, SYLVIA GUTIERREZ, M. JOSÉ LÓPEZ-ARMADA, ITZIAR PALACIOS, and JESÚS EGIDO

Renal Research Laboratory, Fundación Jiménez Díaz, Universidad Autónoma, Madrid, Spain

An orally active ET_A/ET_B receptor antagonist ameliorates proteinuria and glomerular lesions in rats with proliferative nephritis. The proliferation of mesangial cells and the extracellular matrix expansion constitute the most outstanding morphological aspects of the majority of progressive glomerular diseases. *In vitro*, endothelin-1 (ET-1) is mitogenic for mesangial cells and induces matrix protein synthesis. We studied the possible participation of ET-1 in the pathogenesis of renal damage in a normotensive model of proliferative nephritis. Coincidentally with maximal proteinuria and glomerular lesions, an increase was found in the glomerular mRNA expression of preproET-1 and the ET_A receptor (10 and 6 times compared to controls, respectively), but not of the ET_B receptor, and in ET-1 urinary excretion (217 ± 33 vs. 84 ± 4 pg ET-1/24 hr, $N = 4$ to 5, $P < 0.05$). By *in situ* hybridization, an increase in preproET-1 mRNA expression in glomerular endothelial, epithelial and mesangial cells, and in some tubular cells was observed. The administration of bosentan, an ET_A/ET_B receptor antagonist, had a beneficial effect on the evolution of nephritis, preventing the appearance of intense proteinuria (76 ± 35 vs. 380 ± 77 mg/24 hr, $N = 4$ to 5, $P < 0.05$), the morphological lesions and the renal function impairment (creatinine clearance 367 ± 46 vs. 268 ± 33 μl/min/100 g, $N = 4$ to 5). Simultaneously, there was a decrease in ET-1 urinary excretion (88 ± 14 vs. 217 ± 33 pgET-1/24 hr, $N = 4,5$, $P < 0.05$) and in the renal preproET-1 mRNA expression. The mean systolic blood pressures remained in the normal range in all animals. These data indicate that ET-1 participates in the pathogenesis of proteinuria and glomerular injury in a model of proliferative nephritis. The nonpeptidic orally active ET_A/ET_B receptor antagonists could be useful in the treatment of some human nephritis.

The progressive glomerular diseases are characterized by mesangial proliferation and extracellular matrix accumulation. Both aspects have been associated with the onset of proteinuria, glomerulosclerosis and renal failure [1]. During the last years, several studies have suggested the participation of a large array of cytokines, lipid mediators and vasoactive hormones in the pathogenesis of renal injury [2].

The potential implication of endothelin-1 (ET-1) in the renal pathology has recently emerged. ET-1 is a potent vasoconstrictor peptide initially isolated from the supernatant of cultured endothelial cells [3], although it can be synthesized by numerous cells, including various renal cell types [4]. Besides its effects on renal and systemic hemodynamics, ET-1 elicits many other different biological actions [4]. *In vitro*, ET-1 promotes cell proliferation and synthesis of extracellular matrix proteins and induces the expression of protooncogenes and early genes in different cell types [4, 5], suggesting that an alteration of ET could contribute to the onset and progression of renal failure. In different models of renal injury, such as renal mass ablation, puromycin nephrosis, diabetic rats and lupus nephritis, an up-regulation of renal ET-1 gene expression, correlating with the progression of the disease, has been found [6–9]. Further implication of ET-1 in those settings has been supported by the beneficial effect of a specific ET_A receptor antagonist on renal disease progression [10, 11]. Since systemic hypertension is often seen in animals with these models of renal injury, the beneficial effects observed with the ET_A receptor antagonists could be due to their antihypertensive actions.

In this work we have studied the potential participation of ET-1 in the pathogenesis of renal damage in a normotensive model of immune complex proliferative nephritis. This model shows a similar histological pattern to that of lupus and mesangiocapillary nephritis in humans [12, 13]. It is characterized by intense mesangial cell proliferation, matrix expansion and glomerular and interstitial infiltration of mononuclear cells [12, 13]. The absence of hypertension in those animals [14] allows us to separate the ET-1 effects on systemic hemodynamics from those exerted in an autocrine or paracrine manner on renal cells. Therefore, we investigated the steady-state mRNA expression of ET-1 and its receptors, as well as the mature protein ET-1 level in the kidneys of rats with proliferative nephritis. We also tested whether or not an orally-active dual ET_A/ET_B receptor antagonist protected nephritic rats against functional and structural renal damage.

Methods

Immune complex nephritis

All studies were performed in female Wistar rats initially weighing between 220 and 250 g. During the entire study animals were given a standard diet and had free access to water.

Received for publication August 15, 1995
and in revised form April 17, 1996
Accepted for publication April 18, 1996

Immune complex nephritis was induced according to a previously described protocol [15]. The animals received an initial subcutaneous injection of 5 mg ovalbumin (Sigma Chemical Company, St. Louis, MO, USA) diluted in 250 μl isotonic saline (0.9% NaCl) emulsified with 250 μl complete Freund's adjuvant (Difco Laboratories, Detroit, MI, USA). After three weeks, the animals received a subcutaneous booster injection consisting in the same dose of ovalbumin in incomplete Freund's adjuvant (Difco Laboratories). One week later, daily intraperitoneal administration of 10 mg ovalbumin was started.

From the third week of daily ovalbumin injection, proteinuria was measured by the sulfosalicylic acid method to monitor the course of kidney disease. By week 4, a group of rats was killed in the early stage of the disease (mild nephritis; proteinuria < 15 mg/24 hr and mesangial deposits [13]). By around week 5, when proteinuria reached 30 to 60 mg/24 hr, the animals were randomly distributed into two groups. One group was treated with the nonpeptidic ET_A/ET_B receptor antagonist bosentan (Ro 47-0203; 4-tert-butyl-N- [6-(2-hydroxy-ethoxy]-5-(2-methoxy-phenoxy)-2,2'-bipyrimidin-4-yl]-benzene-sulfonamide, Hoffmann-La Roche Ltd, Basel, Switzerland) during 21 days. For its administration, a daily bosentan suspension (100 mg/kg) in a dissolution of 5% arabic rubber was administered by gastric gavage with a cannula. The other group received a dissolution of 5% arabic rubber for 21 days and served as non-treated group (severe nephritis or late nephritis). A control group of healthy animals was used for the duration of the study.

At the time of sacrifice, diseased animals and healthy controls were anesthesized with 5 mg/100 mg sodium pentobarbital. Blood was drawn, kidneys were removed to obtain a sample for histological studies, *in situ* hybridization and immunohistochemistry, and cortex was dedicated to isolation of glomeruli.

Biochemistry and processing of renal tissue

At the end of the period of study, serum levels of creatinine, albumin, triglycerides and cholesterol were determined by standard methods using an autoanalyzer (Technicon SMAC System). Creatinine clearance was calculated from an urine sample taken 24 hours before the animal sacrifice.

For light microscopy analysis, *in situ* hybridization and immunohistochemistry, renal tissue was fixed in 10% buffered formalin and embedded in paraffin. All these studies were performed without the observer knowing to which group animals belonged (double blind study).

Blood pressure measurement

Systolic arterial blood pressure was measured in conscious, restrained rats by tail-cuff sphyngomanometer (NARCO Biosystems, CO, USA). The blood pressure value for each rat was calculated as the average of four separate measurements at each session.

Renal histopathological studies

For light microscopy, sections (4 μm thick) were prepared and stained with hematoxylin and eosin, and Masson trichrome. For each animal, around 30 glomeruli were examined and the histopathology was graded from 0 to 4+ by a semiquantitative score according to the following criteria: 0, normal; 1+, mild mesangial cell proliferation and matrix expansion; 2+, more extensive mesangial proliferation and matrix expansion; 3+, diffuse glomerular proliferation with basement membrane thickening; and 4+, all the above changes associated with wire loop lesions, glomerular sclerosis and interstitial fibrosis.

Isolation of glomeruli

Glomeruli were isolated according to a previously published sieving technique [16], washed and used for mRNA expression experiments.

Endothelin extraction and assessment in plasma and urine

At sacrifice, a sample of blood was collected by aortic puncture into cold tubes containing EDTA (2 mg/ml) and aprotinin (500 U/ml), two protease inhibitors. As soon as possible, the samples were centrifuged at 2500 rpm for 15 minutes, the plasma collected and stored at −70°C until the ET was extracted. Urine was collected during the 24 hours prior to the sacrifice of animals. Four milliliter aliquots were separated and frozen at −70°C until use.

ET was extracted from a 1 ml aliquot of plasma and 4 ml aliquots of urine using a Sep-Pak C_{18} disposable column (Waters, Milford, MA, USA), as previously described [17]. In both cases, the eluted fractions were evaporated in a N_2 atmosphere and the residues stored at −20°C until radioimmunoassay (RIA) was performed.

ET-1 was measured in each sample by a commercial RIA (Peninsula Laboartories, Inc., Belmont, CA, USA). The samples were resuspended in RIA buffer immediately before the assay, those of plasma in 150 μl and those of urine in 400 μl. The ET-1 antibody used in the RIA had a 7% cross-reaction with ET-2 and ET-3 and 17% with the Big-ET (human). The cross-reactivity with nonrelated peptides (ANP, BNP, Ang II, vasopressin and VIP) was 0%.

Reverse transcription (RT)

From each animal, total RNA was obtained from isolated glomeruli by the acid-guanidinium-phenol-chloroform method [18], precipitated with isopropanol and the pellet washed with 70% cold ethanol. The resultant pellet was dissolved in diethylpyrocarbonate-treated H_2O and the concentrations of total RNA were then calculated by spectrophotometric measurements at 260 nm.

To obtain cDNA for the polymerase chain reaction (PCR), 1 μg of RNA from each animal was transcribed in a final volume of 20 μl which contained 5 mM $MgCl_2$, RT buffer (10 mM Tris-HCl, 50 mM KCl and 0.1% Triton X-100), 1 mM deoxynucleotide mixture (dNTP), 20 units RNasin^R, 15 U of reverse transcriptase of the avian Moloney virus (AMV) and 50 ng random primer. The reaction mixture was incubated at 42°C for 45 minutes. At the end of the incubation, the samples were heated at 95°C to eliminate the transcriptase activity and to denaturalize the RNA-cDNA hybrides. Four microliters of cDNA templates were used for each PCR reaction of preproET-1, ET_A and ET_B receptors and 2 μl for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

PCR and analysis of its products

PCR was performed with rat preproET-1, ET_A and ET_B receptors and GAPDH specific oligonucleotides primers (Ramon

Cornet, Madrid, Spain). PreproET-1 sense primer 5′-TGATCT-TCTCTCTGCTGTTTGTGG-3′ corresponded to nucleotides 17 to 40, and the antisense primer 5′-TCTTTTACGCCTTTCTG-CATGGTAC-3′ corresponded to nucleotides 401 to 425 of the published sequence [19]. ET_A receptor sense primer 5′-GAAGTCGTCCGTGGGCATCA-3′ corresponded to nucleotides 495 to 514, and the antisense primer 5′-CTGTGCT-GCTCGCCCTTGTA-3′ corresponded to nucleotides 691 to 710 of the published sequence [20]. ET_B receptor sense primer 5′-TTACAAGACAGCCAAAGACT-3′ corresponded to nucleotides 801 to 820, and the antisense primer 5′-ACGATGAGGA-CAATGAGATT-3′ corresponded to nucleotides 1345-1365 of the published sequence [21]. We performed RT-PCR of GAPDH as an internal standard. GAPDH sense primer 5′-AATGCATC-CTGCACCACCAA-3′ corresponded to nucleotides 439 to 458, and the antisense primer 5′-GTAGCCATATTCATTGTCATA-3′ corresponded to nucleotides 934 to 954 of the published sequence [22]. The cDNA amplification products were predicted to be 409, 216, 565 and 516 base pairs (bp) in length. Ten picomoles of sense and antisense primers, 0.5 μCi dCTP-α^{32}P (>3000 Ci/mmol, Amersham International, Buckinghamshire, UK) and 1.5 U *Taq* DNA polymerase were used per reaction. The reaction mixture (20 μl) was overlaid with mineral oil. The tubes were placed on a Thermal Cycler^R (Perkin Elmer Cetus, Emeryville, CA, USA), which was programmed as follows: incubation at 92°C for 30 seconds; and then, 30 cycles of the following sequential steps: (a) preproET-1, 92°C for 30 seconds, 59°C for one minute, 73°C for one minute; (b) ET_A receptor, 92°C for one minute, 60°C for one minute, 72°C for one minute; (c) ET_B receptor, 92°C for one minute, 57°C for one minute, 72°C for one minute; (d) GAPDH: 92°C for 30 seconds, 54°C for one minute, 73°C for one minute; finally, incubation was done at 72°C for seven minutes. The optimum number of amplification cycles used for quantitative RT-PCR was chosen based on pilot experiments that established the exponential range of the reaction. In all experiments, the presence of possible contaminants was checked by control reactions in which amplification was carried out in the absence of reverse transcriptase. PCR amplified products were saved and kept at −20°C until analysis.

The PCR products were size-fractionated with 1.5% agarose gel electrophoresis and the DNA bands visualized with ethidium bromide staining. PCR products were blotted to Gene Screen (DuPont New England Nuclear, Boston, MA, USA) in 0.4 N NaOH. After the transfer, the membrane was washed in excess 2 × SSC for one to two minutes and the blotted DNA was fixed to the membrane by ultraviolet-irradiating for two to three minutes. The dried membranes were exposed to X-Omat AR films and intensifying screens at −70°C. The optical density of mRNA for PCR products of preproET-1, ET_A and ET_B receptor and GAPDH on the autoradiograph was quantified using scanning densitometry (Molecular Dynamics, Sunnyvale, CA, USA).

In situ hybridization

Digoxigenin-labeled single-strand RNA probes of preproET-1 were prepared using a nonradioactive RNA labeling Kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. Sense and antisense preproET-1 riboprobes were synthesized from a linearized plasmid PMAM neoblue containing a fragment of the rat preproET-1 as run-off transcripts with T3 and T7 RNA polymerases in the presence of digoxigenin-11-UTP.

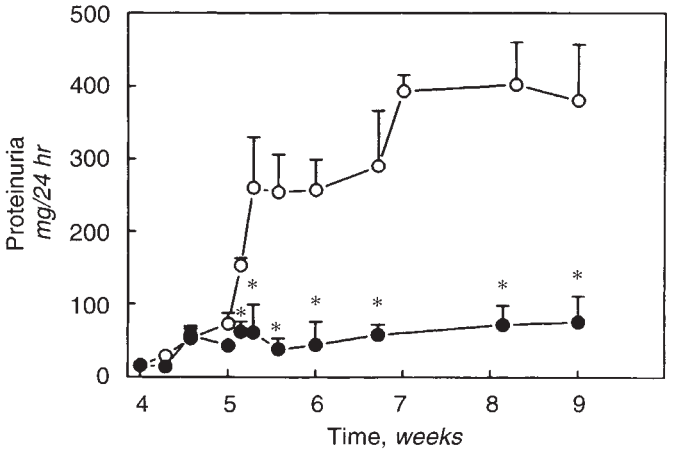

Fig. 1. *Urinary protein excretion in rats with non-treated nephritis (○) and rats treated with bosentan (100 mg/kg/day orally; ●).* Data represent mean ± SEM, $N = 4$ to 5, $^*P < 0.05$ respect to non-treated animals at the corresponding time.

Paraffin-embedded renal tissue was cut at 4 μm and floated onto APES (Sigma) coated slides. The hybridization was performed with modifications of a previously described protocol [23]. The tissue sections were heated at 65°C overnight and then fixed with 1.5% paraformaldehyde-1.5% glutaraldehyde. After dewaxing, the tissues were incubated in 5 mM levamisole. Deproteinization was carried out in 0.2 N HCl and followed by digestion with proteinase K to better expose the RNA target. After digestion, all sections were post-fixed as above and dehydrated and air dried at room temperature. The slides were hybridized with 10 ng/μl denatured digoxigenin-11-UTP-labeled riboprobes in hybridization buffer [2 × SSC, 1 × Denhardt's solution, 0.1 M sodium phosphate (pH 6.5), 10% dextran sulfate (in formamide)]. Sealed cover slips were placed over the tissue sections and the hybridization was allowed to occur overnight at 42°C in a moisturized chamber. The cover slips were removed and the slices were washed in 2 × SSC for one hour at room temperature. This was followed by incubation in 1 × SSC for one hour at room temperature, 0.5 × SSC for 45 minutes at 37°C and, finally, 0.2 × SSC for 30 minutes at 37°C. The sections were incubated with an anti-digoxigenin antibody conjugated with alkaline phosphatase (Boehringer Mannheim) for 30 minutes at 37°C. Colorimetric detection of RNA-RNA hybrids was performed with nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolylphosphate (X-phosphate) in the dark for three to eight hours. The color reaction was stopped with 10 mM Tris-HCl, 1 mM EDTA (pH 8) and then cover slips applied with 60% glycerol before microscopic examination. The negative controls included: (1) hybridization with the sense probe, (2) RNAse treatment before hybridization, and (3) omission of the RNA probe.

Table 1. Serum and renal parameters in control, nephritic and bosentan-treated rats

	Proteinuria *mg/24 hr*	Cholesterol *mg/dl*	Triglycerides *mg/dl*	Albumin *g/dl*	Creatinine clearance *μl/min/100 g*
Control	3 ± 1	73 ± 6	30 ± 5	3.8 ± 0.1	379 ± 55
Mild nephritis	8 ± 2[b]	62 ± 12[b]	38 ± 8	3.5 ± 0.1	338 ± 23
Severe nephritis	380 ± 77[a]	255 ± 63[a]	344 ± 148	2.1 ± 0.5	268 ± 33
Bosentan-treated nephritis	76 ± 35[b]	32 ± 4[b]	55 ± 13	3.3 ± 0.7	367 ± 46

The values show the mean ± SEM of the individual values of animals from each group.
[a] $P < 0.05$ with respect to healthy control rats
[b] $P < 0.05$ with respect to rats with severe nephritis

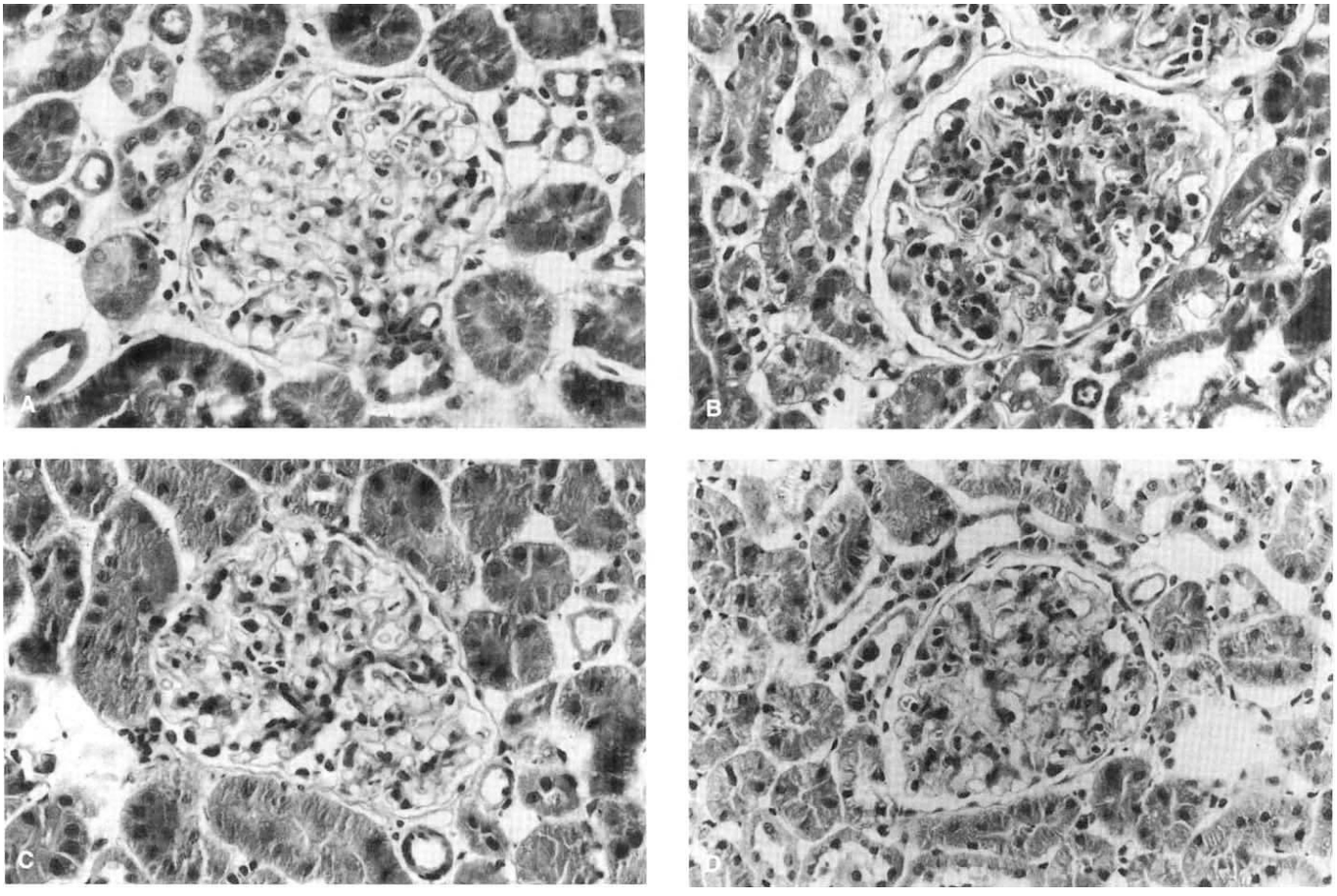

Fig. 2. *Histological analysis showing pathological changes in glomeruli of healthy control rats (***A***), non-treated nephritic rats (***B***), mild nephritis rats (***C***) and bosentan-treated rats (***D***).* Microphotographs showing Masson's trichrome staining of the glomeruli (magnification × 400).

Tissue localization of endothelin-like immunoreactivity

Paraffin-embedded renal tissue was cut at 4 μm and mounted on poly-L-lysine-coated slides. Immunoperoxidase staining was performed by the avidin-biotin-peroxidase complex method [24]. The slides were incubated overnight at 65°C and deparaffinized with graded concentrations of xylene and ethanol. The slides were quenched in methanol containing 3% H_2O_2 at 25°C for 30 minutes. The sections were washed and incubated in trypsin (0.1% trypsin in 0.1% $CaCl_2$ wt/vol) to activate antigenic sites. They were subsequently incubated with 5% normal swine serum for 30 minutes at room temperature to reduce nonspecific background staining and then incubated overnight at 4°C with rabbit polyclonal anti-ET-1 antibody (Peninsula Laboratories) diluted 1:1000 in PBS containing 5% normal swine serum. The control slides were treated with diluted normal rabbit serum. After being washed in PBS, the sections were incubated with biotinylated swine antirabbit IgG (Dako A/S, Glostrup, Denmark) at a dilution of 1:200, and after further washing, incubated with avidin-biotin-peroxidase complex (Dako A/S) diluted 1:100 in PBS for 30

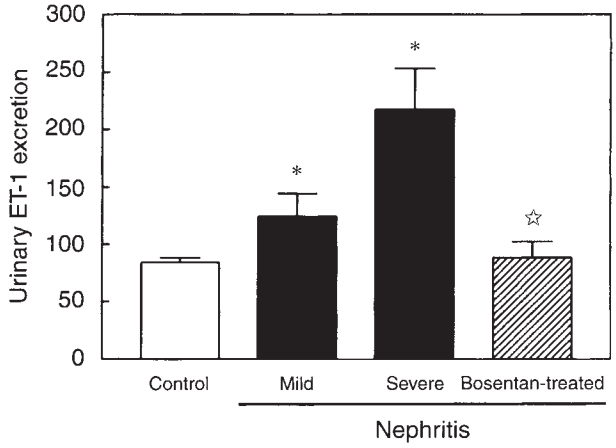

Fig. 3. *Urinary endothelin (ET-1) excretion in control and nephritic (non-treated and bosentan-treated, 100 mg/kg/day) rats.* Mean ± SEM, $N = 4$ to 5. $^*P < 0.05$ respect to control animals, $\star P < 0.05$ respect to the animals with severe nephritis.

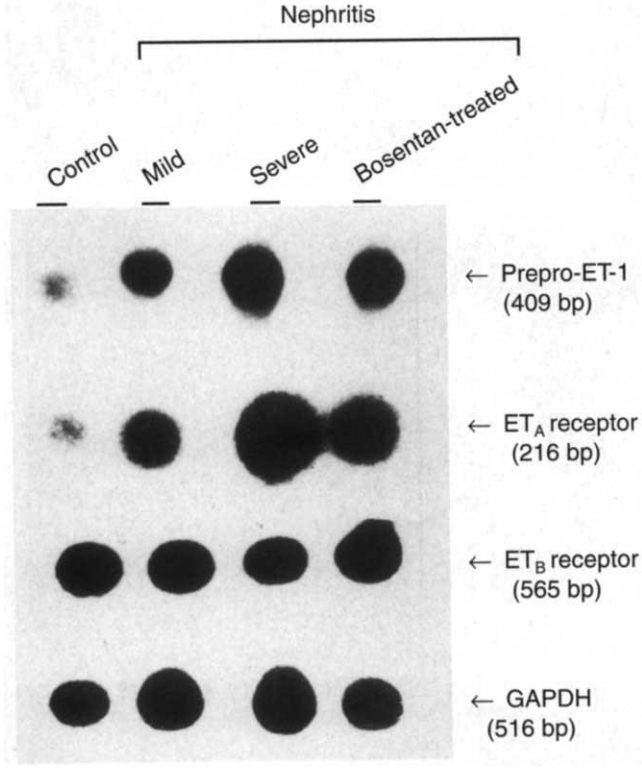

Fig. 4. *Gene expression of preproET-1 and ET_A and ET_B receptors in glomeruli of control and nephritic (non-treated and bosentan-treated, 100 mg/kg/day) rats.* A representative animal of each group is shown. Arrows indicate the expected size for each PCR product.

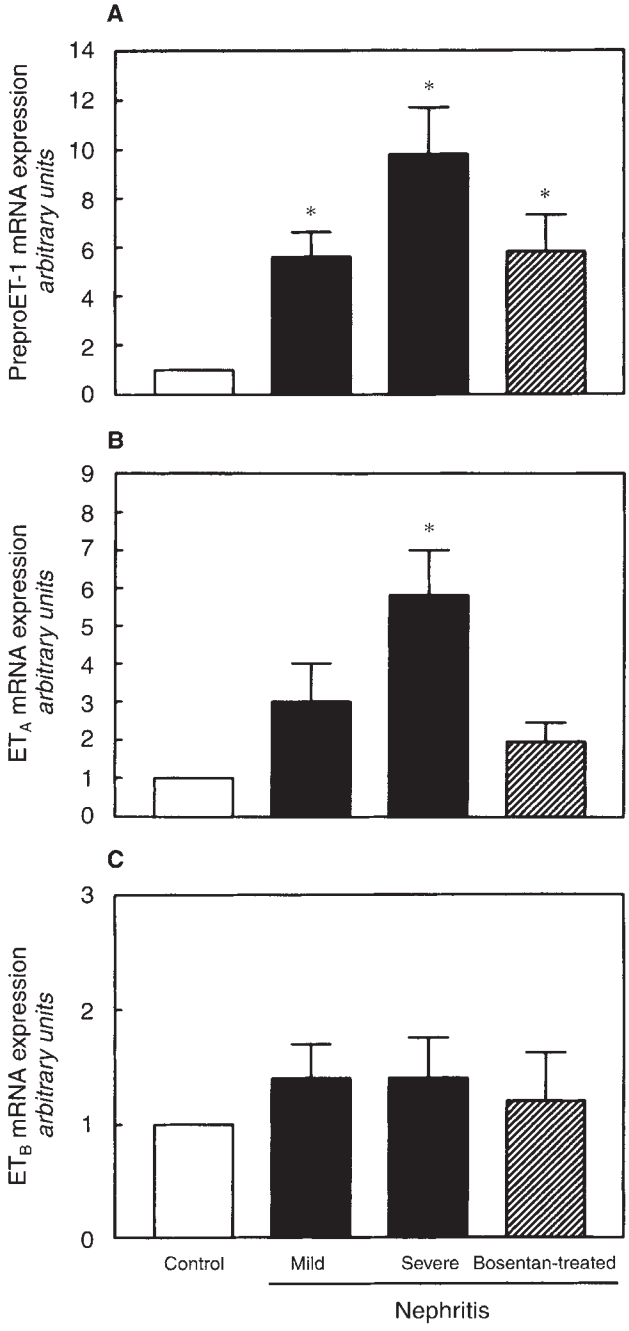

Fig. 5. *Densitometry analysis of mRNA glomerular expression of preproET-1* (**A**) *and ET_A* (**B**) *and ET_B* (**C**) *receptors in control and nephritic (non-treated and bosentan-treated, 100 mg/kg/day) rats.* Results are expressed as the fold-increase of healthy control rats. Data are mean ± SEM of 4 to 5 animals in each group, $^*P < 0.05$ versus control rats.

minutes. The sites of peroxidase activity were visualized with 0.05% 3,3′-diaminobenzidine (Dako A/S) in 0.01% H_2O_2 for 10 minutes. The sections were counterstained with Mayer's hematoxylin (Sigma).

Around 15 glomeruli from each animal were examined and the immunostaining was graded from 0 to 4+ by a semiquantitative score according to the following criteria: 0, no staining; 1+, minimal staining; 2+, mild staining; 3+, moderate staining; 4+, marked staining.

Statistical analysis

Results are expressed as mean ± SEM. Significance at the 95% level was established using ANOVA and Student's *t*-test. The

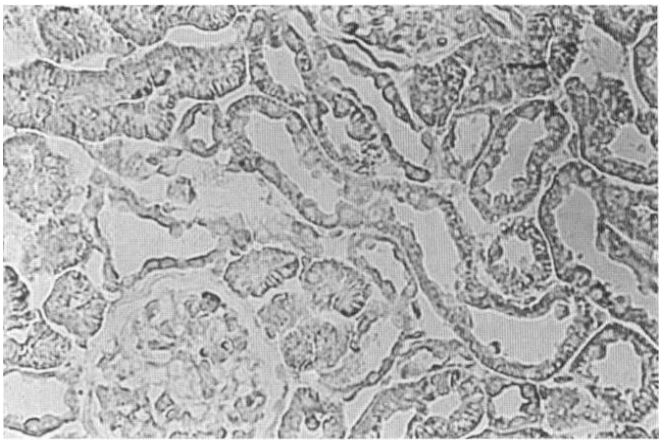

Fig. 6. *Absence of labeling in renal rat tissue hybridized with a sense riboprobe for preproET-1 (magnification × 100).*

Kolmogorov-Smirnov test was used to assess the normal distribution of data. The Mann-Whitney U test for nonparametric values was used for histological data.

Results

Effect of bosentan on proteinuria, renal function and morphological lesions

In this model of proliferative glomerulonephritis, immune deposits are initially restricted to the mesangium, and urinary protein excretion is within normal limits (< 15 mg/24 hr; mild nephritis) [12, 13, 25]. Thereafter, immune deposits extend to the glomerular capillary wall and proteinuria rises above normal. At this stage randomly assigned to received either bosentan 100 mg/kg/day (bosentan-treated group) or vehicle (non-treated group) for 21 days. Proteinuria at the start of therapy was 43 ± 9 mg/24 hr and 53 ± 14 mg/24 hr, respectively (P = NS). In non-treated rats, proteinuria increased over three weeks of study, progressing to a full-blown nephrotic syndrome (Fig. 1). The administration of bosentan avoided the development of intense proteinuria (at the end of the study, 76 ± 35 vs. 380 ± 77 mg/24 hr; $N = 4$ to 5, $P < 0.05$; controls 3 ± 1 mg/24 hr). Concomitantly, bosentan-treated animals showed an improvement in the renal function (measured as creatinine clearance) with respect to nephritic rats (367 ± 46 vs. 268 ± 33 μl/min/100 g, $N = 4$ to 5; controls 379 ± 55 μl/min/100 g) and in serum lipids (cholesterol and triglycerides) and albumin (Table 1).

Mean systolic blood pressure in all rats was in the normotensive range throughout the three weeks of study, and the administration of bosentan did not significantly modify the blood pressure (control rats 107 ± 11; nephritic rats 115 ± 11; bosentan-treated rats 104 ± 8 mm Hg, $N = 4$ to 5, P = NS in all group).

Studies from our group have detailed the morphological aspects of glomerular lesions in this model [14, 15]. At the end of the three weeks of study, non-treated rats showed glomerular hypercellularity, matrix expansion, and mononuclear cell infiltration in the glomeruli and in the interstitium (Figs. 2 and 9). By contrast, the bosentan-treated animals showed a significant diminution in the glomerular structural damage, with discrete evidence of mesangial hypercellularity and matrix expansion (Figs. 2 and 9).

Serum and urine ET-1 levels

Initially, we determined whether in this experimental model there existed an alteration in the ET synthesis which could cause an increase in the plasma and/or urinary levels of ET. Blood and urine samples were taken from the treated and non-treated nephritic animals and ET was extracted for quantification. The ET-1 plasma levels were similar in all groups studied (control 5.4 ± 1.9; mild nephritis 4.5 ± 0.7; severe nephritis 3.9 ± 0.1, bosentan-treated nephritis 4.7 ± 1.1 pg ET-1/ml plasma, $N = 4$ to 5, P = NS for all groups).

In spite of the absence of significant differences between plasma ET levels, an increase in the ET urinary excretion was already seen in animals with mild nephritis, with a further augmentation in those with severe nephritis (Fig. 3). Surprisingly, bosentan significantly reduced the urinary ET-1 excretion to values similar to those of control animals (Fig. 3).

Renal preproET-1 and ET receptors mRNA expression

The steady state mRNA levels for preproET-1 and its receptors in the glomeruli of control and nephritic rats (treated and non-treated) were determined by RT-PCR. The expected size of each PCR product was apparent: preproET-1 409 bp, ET_A receptor 216 bp, ET_B receptor 565 bp, and GAPDH 516 bp. The amplification product of GAPDH served as internal standard for RT-PCR reaction. Figure 4 shows an autoradiograph of all PCR products of glomeruli from a representative animal from each group.

The densitometric analysis of preproET-1 and ET_A receptor mRNA signals shows an increase in the expression according to the progression of the disease, although no change in ET_B receptor mRNA levels was observed (Fig. 5). With respect to healthy control rats, the preproET-1 and ET_A receptor expression was already increased in early phases of nephritis (6- and 3-fold, respectively) with a further augmentation in rats with severe nephritis (10- and 6-fold, respectively).

Animals receiving bosentan had a certain decrease in the preproET-1 expression, though not significant, in relation to rats with severe nephritis (Fig. 5). Bosentan reduced the ET_A mRNA expression in bosentan-treated rats although it had no effect on ET_B mRNA expression (Fig. 5).

In situ hybridization for preproET-1 mRNA

The cellular distribution of preproET-1 mRNA in the kidneys of animals from different groups was investigated by *in situ* hybridization using digoxigenin-labeled riboprobes. In control experiments, hybridization with the sense riboprobe (Fig. 6), RNAse treatment before hybridization or the omission of the RNA probe did not label any renal structures. By contrast, hybridization of kidney sections from normal rats with the antisense riboprobe yielded a patchy labeling in glomerular capillary walls, mesangial cells and parietal and visceral glomerular epithelial cells, as well as in the luminal pole of some proximal tubular cells and in some vessels (Fig. 7A). A nearly identical labeling pattern of the renal structures was observed in rats with nephritis, although the intensity was stronger (Fig. 7B). The administration of bosentan decreased the mRNA expression of preproET-1 in all renal structures, principally in proximal tubules (Fig. 7C). Previous studies in our laboratory showed that nephritic rats have an

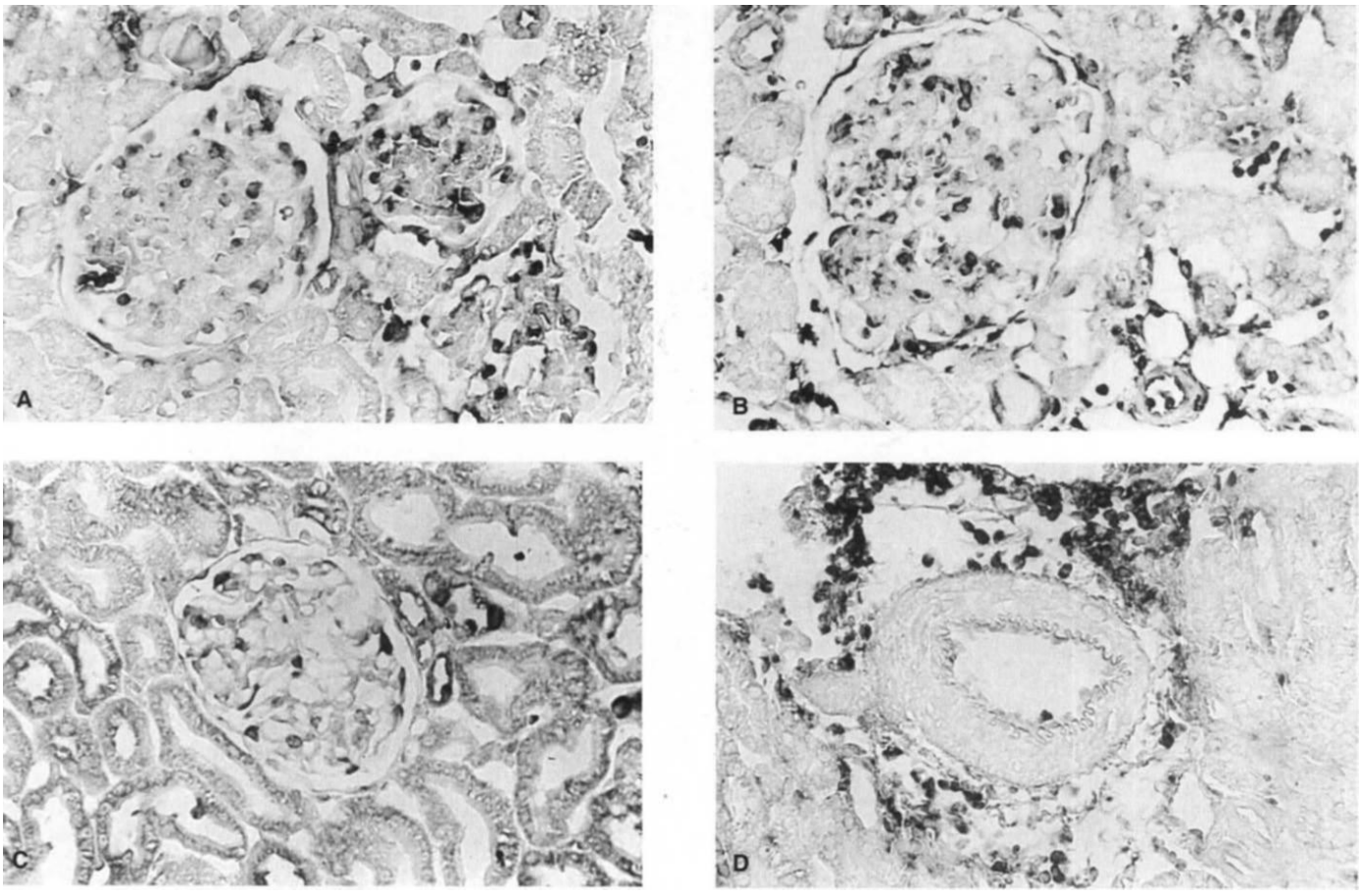

Fig. 7. *Cellular distribution of preproET-1 mRNA in control rats* (**A**)*, rats with severe nephritis* (**B**) *and rats with nephritis treated with bosentan* (**C**). (**D**) Mononuclear cells around a vessel showing an intense preproET-1 mRNA expression. Note that no staining was seen in the endothelial surface of this medium-sized vessel. A digoxigenin-labeled antisense riboprobe specific for preproET-1 was hybridized under high-stringency conditions with kidney sections (magnification × 100).

important interstitial infiltrate of monocytes/macrophages and T lymphocytes [14, 15]. By *in situ* hybridization the mononuclear cells intensively took the labeled preproET-1 probe (Fig. 7D).

Tissue localization of endothelin immunoreactivity

Endothelin immunoreactivity was identified in the renal cortex and medulla of control rats. No immunoperoxidase staining was observed in the samples from control and nephritic rats (treated and non-treated) when a non-immune serum was used (not shown). At the glomeruli, immunostaining was localized in a patchy distribution in endothelial and epithelial cells and mesangial regions (Fig. 8A, and Fig. 9). Very marked immunostaining was seen in the endothelium of peritubular capillaries. Focal immunostaining was also noted in the brush border of some proximal tubules (not shown). In the animals with severe nephritis, the distribution of the immunostaining was similar, but with a greater intensity (Fig. 8B and Fig. 9). The administration of bosentan to nephritic rats diminished the immunostaining in an appreciable manner, but it remained above control rats (Fig. 8C and Fig. 9).

Discussion

In this work we provide evidence that ET-1 is implicated in the pathogenesis of glomerular injury in a normotensive model of immune complex proliferative nephritis. We demonstrate that rats with nephritis have an increase in the renal gene expression of preproET-1 and its ET_A receptor, in the mature ET-1 peptide as well as in its urinary excretion, coinciding with the major glomerular lesions. This study also shows that the administration of an orally active ET_A/ET_B receptor antagonist decreased proteinuria and attenuated the morphological lesions in this model.

Our results clearly suggest that the ET-1 generated in the kidney may participate in the generation of glomerular damage in a proliferative model of immune complex nephritis. In fact, a good correlation between urinary ET-1 excretion and the renal expression of preproET-1 mRNA and its protein was noted, while no appreciable changes existed in the blood levels of the peptide. These results are in agreement with that previously observed in the renal ablation model [17], and indicate that urinary ET-1 is probably originated in the kidney. Indeed, less than 0.3% of ET

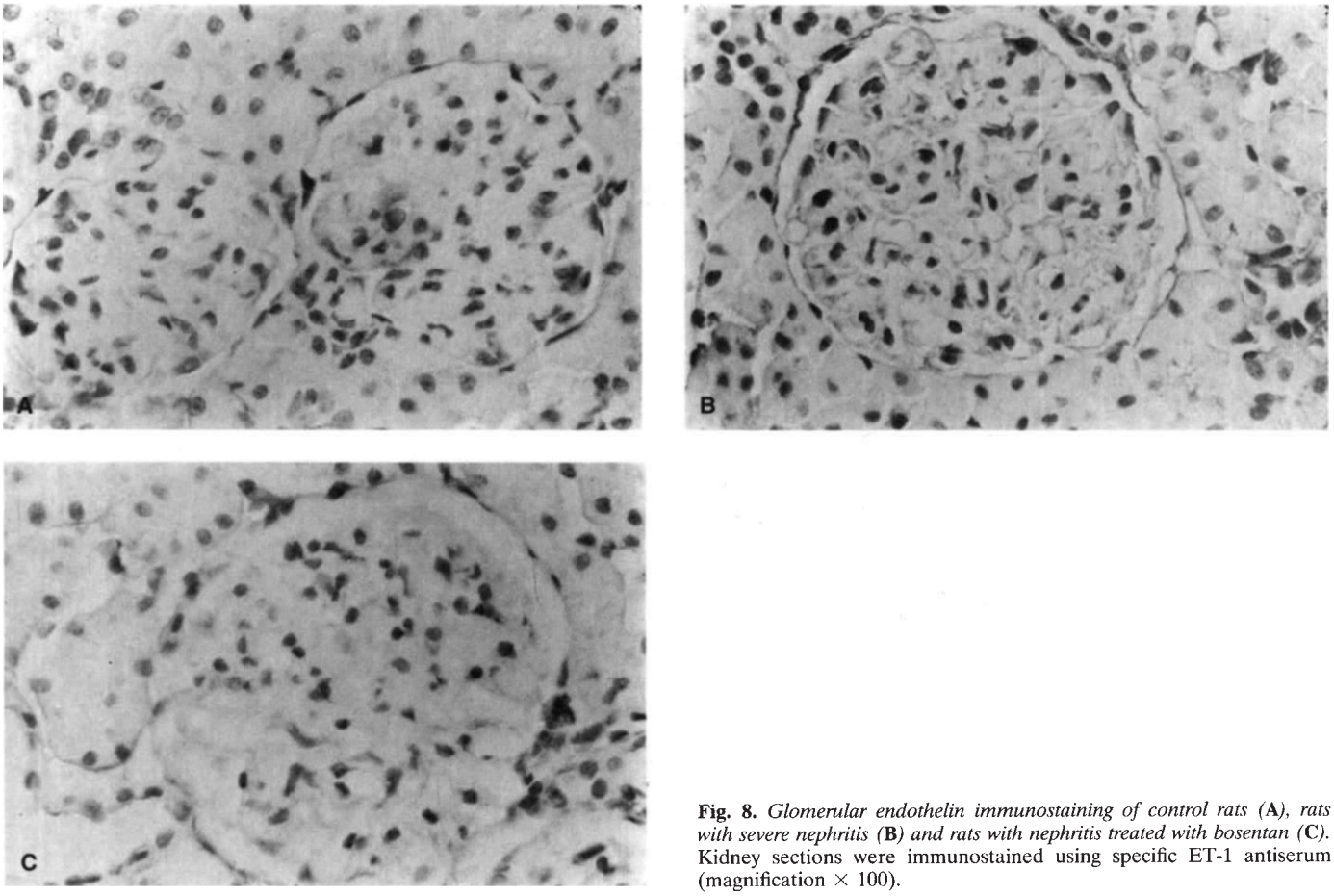

Fig. 8. *Glomerular endothelin immunostaining of control rats* (**A**), *rats with severe nephritis* (**B**) *and rats with nephritis treated with bosentan* (**C**). Kidney sections were immunostained using specific ET-1 antiserum (magnification × 100).

was recovered in the urine after the infusion of radiolabeled ET, indicating that ET formed in the systemic circulation is not likely to be excreted in the urine in a significant manner [17]. Interestingly, the ET-1 overexpression was already seen in the early phase of nephritis, suggesting its direct implication in the glomerular lesions in this model. By *in situ* hybridization, in control animals the preproET-1 gene expression was observed in a patchy distribution in the glomerular capillary walls, glomerular mesangial and epithelial cells, in the luminal pole of some proximal tubules and in some vessels. In rats with severe nephritis, there was an important increase in the gene expression in all these areas, but with the same localization. Previous *in situ* hybridization studies had localized ET-1 mRNA in the medulla with a distribution along the vasa recta, but not in glomeruli. However, the autoradiography technique employed by these authors [26] is severalfold less sensitive than the digoxigenin labeled probe used in the present work [27]. Our results are in agreement with *in vitro* studies in which mesangial, glomerular epithelial and endothelial cells can express the ET-1 gene and synthesize the protein under appropriate stimuli [28–33]. Interestingly, an intense preproET-1 expression was seen in the cells infiltrating the interstitium of rats with severe nephritis. Although double immunostaining studies were not performed in the present study, it has been demonstrated that resting or stimulated cultured human macrophages produce and release abundant mature ET-1 [34]. Therefore, we cannot discard that these cells may also contribute to the kidney content of ET-1 and participate in tissue injury.

The mechanisms of ET-1 overexpression in the kidney of animals with immune injury are unclear. In several models of glomerulonephritis, including the model used in this work, an increase in the glomerular expression and synthesis of growth factors and cytokines, such as transforming growth factor β (TGFβ) and tumor necrosis factor α (TNFα), was observed coinciding with the major injury [9, 14, 15]. *In vitro*, glomerular cells can synthesize ET-1 in response to numerous proinflammatory stimuli, including TGFβ and TNFα, two cytokines that are implicated in the pathogenesis of kidney diseases [2, 16, 28–33, 35]. It is tempting to speculate that during renal injury, both infiltrating inflammatory and resident cells may release those mediators, stimulating mesangial cells to increase ET-1 synthesis that could further induce cell proliferation and matrix protein production [4, 5, 35]. ET-1, in turn, also increases the expression and synthesis of several cytokines, including PDGF and TGFβ, that could mediate the ET-1 effects on renal cells [4, 36].

Recently it has been demonstrated that mRNA of both subtypes of ET receptors can be localized in the glomerulus by

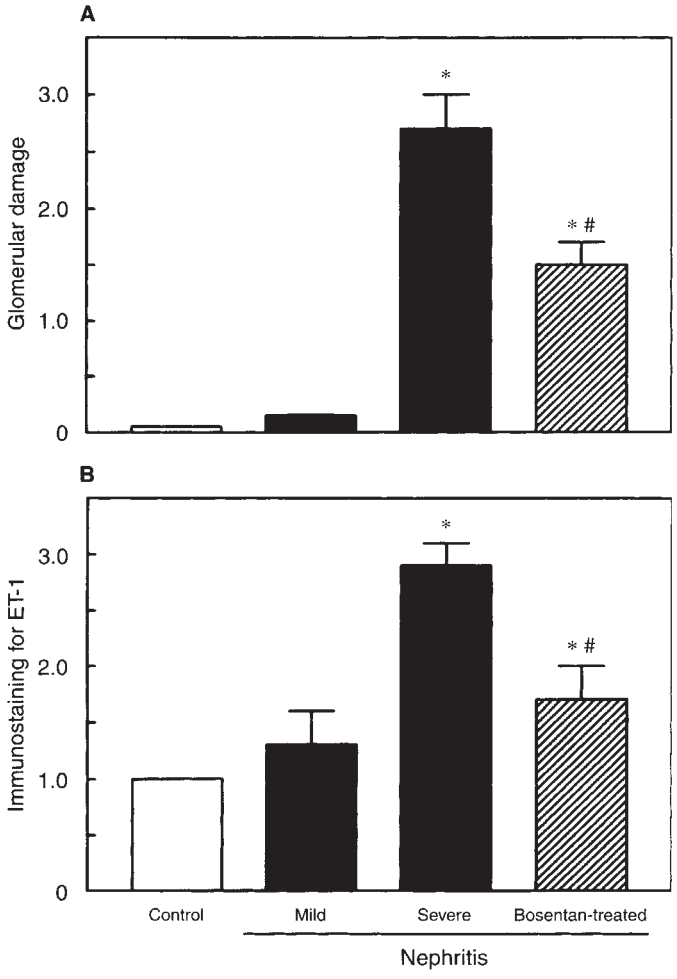

Fig. 9. *Histological score by light microscopy* (**A**) *and glomerular ET-1 immunostaining score* (**B**) *of control* (□), *non-treated* (■) *and bosentan-treated (100 mg/kg/day;* ▨) *rats.* Mean ± SEM of 4 to 5 animals in each group. $*P < 0.05$ versus control rats; $\#P < 0.05$ versus rats with severe nephritis.

RT-PCR [37]. ET_A and ET_B receptors have different structures, function and distribution [38]. However, the regulation and expression of both receptors in renal tissue during injury are hardly known. In this work, we have observed an increase in the ET_A receptor mRNA expression during the progression of the disease, without changes in the mRNA levels of ET_B receptor. Several authors have described that ET-1 produced by cells in culture can down-regulate ET receptors by an autocrine mechanism [39, 40]. However, an up-regulation of ET receptors associated with increased preproET-1 mRNA levels have been observed in various diseases [9, 41, 42]. Since ET_A has been associated with cell proliferation and matrix protein synthesis [4, 35], the increase in the ET_A receptor mRNA expression in the kidney from animals with severe nephritis, in the presence of unaltered ET_B receptor levels, would further enhance these effects of ET-1 in renal cells.

To demonstrate in a more direct manner the participation of ET-1 in this nephritis, a group of animals received bosentan, a new orally-active ET_A and ET_B receptor antagonist [43]. Though bosentan is a competitive antagonist of both receptors, functional and binding studies have shown that it is more specific for ET_A than for ET_B [43]. The administration of bosentan to animals with established nephritis (proteinuria 30 to 60 mg/24 hr) during 21 days diminished the renal permeability to proteins, limited glomerular injury and prevented renal function deterioration. Recent studies have shown that an ET_A receptor antagonist protected against injury in a remnant kidney model and in mice with lupus nephritis [10, 11], two situations commonly associated with elevated systemic blood pressure. Therefore, the beneficial effect of the ET_A receptor antagonists could be due to the antihypertensive actions of those compounds. Since blood pressure remained normal in all animals with immune nephritis during the period of study [14], and bosentan did not modify it in a significant manner, our data suggest that the improvement observed with this therapy may be due to the blockade of direct ET-1 effects on the kidney. However, we cannot discard that bosentan ameliorated intraglomerular hypertension in the immune nephritis, though, to our knowledge, that has not been determined in this model.

Since the ET_A receptor mediates constriction, cell proliferation and matrix protein production by cultured mesangial cells [4, 5], one can speculate that blocking ET_A receptor would limit the biological consequences of ET-1. The role of the ET_B receptor blockade in the improvement observed by bosentan in our model is uncertain. The ET_B receptor mRNA is also abundant in the rat kidney [4] and its expression was found increased in the remnant kidney [44]. In this model of progressive renal disease, a similar amelioration in proteinuria and renal function was noted with both ET_A and ET_A/ET_B receptor antagonists. Therefore, the authors suggested that ET_B receptor was only marginally involved in ET-related renal disease progression [44].

Rats treated with bosentan had also a diminution in the ET-1 urinary excretion and in the preproET-1 mRNA expression, as could be verified by RT-PCR and *in situ* hybridization. The reasons of this phenomenon are not clear. As ET-1 induces its own expression and synthesis in several cells through the ET_B receptor [4, 45], one can speculate that bosentan, by blocking the ET-1 binding to both ET_A and ET_B receptors, could modify the synthesis of a large array of inflammatory mediators and that of ET-1 itself. This could theoretically be an additional advantage of dual (ET_A and ET_B) versus specific ET_A receptor antagonists, but deserves further study.

On the whole, this work presents strong evidence that ET-1 is involved in the pathogenesis of glomerular damage in a normotensive model of immune injury. The beneficial effect of bosentan, a nonpeptidic oral ET_A/ET_B receptor antagonist, suggests that those kinds of compounds could eventually be used in human nephritis.

Acknowledgments

This work has been partially supported by grants from Fondo de Investigaciones Sanitarias de la Seguridad Social (FIS) (94/370) and Ministerio de Educación y Ciencia (PB 92/42; PB 94/211; PM 95/93) and Fundación Iñigo Alvarez de Toledo. DGG is a postdoctoral fellow of the Fundación Iñigo Alvarez de Toledo, RL is a fellow of FIS, XHL is a visiting fellow from the University of Beijing, Peoples Republic China, and SG, MJL-A and IP are fellows of Fundación Conchita Rábago. We thank to Dr. Derek Nuñez and Dr. Martine Clozel for providing the ET-1 cDNA probe for the *in situ* hybridization and bosentan, respectively. The authors thank Dr. Antonio Barat and Dr. Julia Blanco for their help in the histopathological studies.

Reprint requests to Jesús Egido, M.D., Renal Research Laboratory, Fundación Jiménez Díaz, Avda Reyes Católicos, 2, 28040 Madrid, Spain. Email: egido@alpha2.ft.uam.es

References

1. KASHGARIAN M, STERZEL RB: The pathobiology of the mesangium. *Kidney Int* 41:524–529, 1992
2. ORTIZ A, GÓMEZ-CHIARRI M, ALONSO J, BUSTOS C, GÓMEZ-GUERRERO C, LÓPEZ-ARMADA MJ, GÓMEZ-GARRE D, PALACIOS I, RUIZ-ORTEGA M, GUTIERREZ S, GONZÁLEZ E, EGIDO J: The potential role of inflammatory and fibrogenic cytokines in the glomerular diseases. *J Lipid Mediators* 9:55–74, 1994
3. YANAGISAWA M, KURIHARA H, KIMURA Y, TOMOBE Y, KOBAYASHI M, MITSUI Y, YAZAKI Y, GOTO K, MASAKI T: A novel potent vasoconstrictor peptide produced by vascular endothelial cells. *Nature* (London) 332:411–415, 1988
4. SIMONSON MS: Endothelins: Multifunctional renal peptides. *Physiol Rev* 73:375–411, 1993
5. EGIDO J: Vasoactive hormones and renal sclerosis. (Nephrology Forum) *Kidney Int* 49:578–597, 1996
6. ORISIO S, BENIGNI A, BRUZZI I, CORNA D, PERICO N, ZOJA C, BENATTI L, REMUZZI G: Renal endothelin gene expression is increased in remnant kidney and correlates with disease progression. *Kidney Int* 43:354–358, 1993
7. NAKAMURA T, EBIHARA I, FUKUI M, OSADA S, TOMINO Y, MASAKI T, GOTO K, FURUICHI Y, KOIDE H: Modulation of glomerular endothelin and endothelin receptor gene expression in aminonucleoside-induced nephrosis. *J Am Soc Nephrol* 5:1585–1590, 1995
8. FUKUI M, NAKAMURA T, EBIHARA I, OSADA S, TOMINO Y, MASAKI T, GOTO K, FURUICHI Y, KOIDE H: Gene expression for endothelins and their receptors in glomeruli of diabetic rats. *J Lab Clin Med* 122:149–155, 1993
9. NAKAMURA T, EBIHARA I, FUKUI M, OSADA S, TOMINO Y, MASAKI T, GOTO K, FURUICHI Y, KOIDE H: Renal expression of mRNAs for endothelin-1 and endothelin-3 and endothelin receptors in NZB/W F1 mice. *Renal Physiol Biochem* 16:233–243, 1993
10. BENIGNI A, ZOJA C, CORNA D, ORISIO S, LONGARETTI L, BERTANI T, REMUZZI G: A specific endothelin subtype A receptor protects against injury in renal disease progression. *Kidney Int* 44:440–444, 1993
11. NAKAMURA T, EBIHARA I, TOMINO Y, KOIDE H: Effect of a specific endothelin A receptor antagonist on murine lupus nephritis. *Kidney Int* 47:481–489, 1995
12. SÁNCHEZ-CRESPO M, ALONSO F, BARAT A, EGIDO J: Rat serum sickness: possible role of inflammatory mediators allowing deposition of immune complexes in the glomerular basement membrane. *Clin Exp Immunol* 49:631–638, 1982
13. QUIRÓS J, GONZÁLEZ-CABRERO J, EGIDO J, HERRERO-BEAUMONT G, MARTÍNEZ-MONTERO JC: Beneficial effect of fibronectin administration on chronic nephritis in rats. *Arthritis Rheum* 33:685–692, 1990
14. RUIZ-ORTEGA M, GONZÁLEZ S, SERÓN D, CONDOM E, BUSTOS C, LARGO R, GONZÁLEZ E, ORTIZ A, EGIDO J: ACE inhibition reduces proteinuria, glomerular lesions and extracellular matrix production in a normotensive rat model of immune complex nephritis. *Kidney Int* 48:1778–1791, 1995
15. ORTIZ A, ALONSO J, GÓMEZ-CHIARRI M, LERMA JL, SERÓN D, CONDOM E, GONZÁLEZ E, EGIDO J: Fibronectin decreases glomerular lesions and synthesis of TNFα, PAF and fibronectin in proliferative nephritis. *Clin Exp Immunol* 101:334–341, 1995
16. GÓMEZ-CHIARRI M, ORTIZ A, LERMA JL, MAMPASO F, GONZÁLEZ E, EGIDO J: Involvement of tumor necrosis factor and platelet activating factor in the pathogenesis of experimental nephrosis in rats. *Lab Invest* 70:449–459, 1994
17. BENIGNI A, PERICO N, GASPARI F, ZOJA C, BELLIZI L, GABANELLI M, REMUZZI G: Increased renal endothelin production in rats with reduced renal mass. *Am J Physiol* 260:F331–F339, 1991
18. CHOMCZYNSKI P, SACCHI N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162:156–159, 1987
19. SAKURAI T, YANAGISAWA M, INOUE A, RYAN US, KIMURA S, MITSUI Y, GOTO K, MASAKI T: cDNA cloning, sequence analysis and tissue distribution of rat preproendothelin-1 mRNA. *Biochem Biophys Res Commun* 175:44–47, 1991
20. LIN HY, KAJI EH, WINKEL GK, IVES HE, LODISH HF: Cloning and functional expression of a vascular smooth muscle endothelin-1 receptor. *Proc Natl Acad Sci USA* 88:3185–3189, 1991
21. SAKURAI T, YANAGISAWA M, TAKUWA Y, MIYAZAKI H, KIMURA S, GOTO K, MASAKI T: Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor. *Nature* 348:732–735, 1990
22. FORT P, MARTY L, PIEZCHACZYK M, SABROUTY SE, DANI C, JEANTEUR P, BLANCHARD JM: Various rat adult tissues express only one mayor mRNA species from glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucl Acid Res* 13:1431–1442, 1985
23. MARTÍNEZ-MONTERO JC, HERRINGTON CS, STICKLAND J, SAWYER H, EVANS M, FLANNERY DMJ, MCGEE JO'D: Model system optimising mRNA non-isotopic in situ hybridization: Riboprobe detection of lysozyme mRNA in archival gut biopsy specimens. *J Clin Pathol* 44:835–839, 1991
24. WILKES BM, SUSIN M, MENTO PF, MACICA CM, GIRARDI EP, BOSS E, NORD EP: Localization of endothelin-like immunoreactivity in rat kidneys. *Am J Physiol* 260:F913–F920, 1991
25. NOBLE B, REN K, TAVERNE J, DIPIRRO J, VAN LIEW J, DIJKSTRA C, JANOSSY G, POULTER LW: Mononuclear cells in glomeruli and cytokines in urine reflect the severity of experimental proliferative immune complex glomerulonephritis. *Clin Exp Immunol* 80:281–287, 1990
26. MACCUMBER MW, ROSS CA, GLASER BM, SNYDER SH: Endothelin: Visualization of mRNAs by in situ hybridization provides evidence for local action. *Proc Natl Acad Sci USA* 86:7285–7289, 1989
27. DIRKS RW, VAN DE RIJKE FM, RAAP AK: *In situ* hybridization applicable to abundantly expressed mRNA species, in *Cell Biology* (vol 2), edited by CELIS JE, New York, Academic Press, Inc., 1994, pp 459–467
28. ZOJA C, ORISIO S, PERICO N, BENIGNI A, MORIGI M, BENATTI L, RAMBALDI A, REMUZZI G: Constitutive expression of endothelin gene in cultured human mesangial cells and its modulation by transforming growth factor-β, thrombin, and a thromboxane A_2 analogue. *Lab Invest* 64:16–20, 1991
29. SAKAMOTO H, SASAKI S, NAKAMURA Y, FUSHIMI K, MARUMO F: Regulation of endothelin-1 production in cultured rat mesangial cells. *Kidney Int* 41:350–355, 1992
30. KOHAN DE: Production of endothelin-1 by rat mesangial cells. Regulation by tumor necrosis factor. *J Lab Clin Med* 141:279–283, 1992
31. KASINATH BS, FRIED TA, DAVALATH S, MARSDEN PA: Glomerular epithelial cells synthesize endothelin peptides. *Am J Pathol* 141:279–283, 1992
32. OHTA K, HIRATA Y, IMAI T, KANNO K, EMORI T, SHICHIRI M, MARUMO F: Cytokine-induced release of endothelin-1 from porcine renal epithelial cell line. *Biochem Biophys Res Commun* 169:578–584, 1990
33. MARSDEN PA, DORFMAN DM, COLLINS T, BRENNER BM, ORKIN S, BALLERMANN BJ: Regulated expression of endothelin-1 in glomerular capillary endothelial cells. *Am J Physiol* 261:F117–F125, 1991
34. EHRENREICH H, ANDERSON RW, FOX CH, RIECKMANN P, HOFFMAN GS, TRAVIS WD, COLIGAN JE, KEHRL JH, FAUCI AS: Endothelins, peptides with potent vasoactive properties, are produced by human macrophages. *J Exp Med* 172:1741–1748, 1990
35. BORDER WA, NOBLE NA: Transforming growth factor-β in tissue fibrosis. *N Engl J Med* 331:1286–1292, 1994
36. GÓMEZ-GARRE D, RUIZ-ORTEGA M, ORTEGO M, LARGO R, LÓPEZ-ARMADA MJ, PLAZA JJ, GONZÁLEZ E, EGIDO J: Effects and interactions of endothelin-1 and angiotensin II on matrix protein expression and synthesis and mesangial cell growth. *Hypertension* 27:885–892, 1996
37. TERADA Y, TOMITA K, NONOGUCHI H, MARUMO F: Different localization of two types of endothelin receptor mRNA in microdissected rat nephron segments using reverse transcription and polymerase chain reaction. *J Clin Invest* 90:107–112, 1992
38. SAKURAI T, YANAGISAWA M, MASAKI T: Molecular characterization of endothelin receptors. *Trends Pharmacol Sci* 13:103–108, 1992

39. CLOZEL M, LÖFFLER BM, BREU V, HILFIGER L, MAIRE JP, BUTSCHA B: Down regulation of endothelin receptors by autocrine production production of endothelin-1. *Am J Physiol* 265:C118–C192, 1993
40. ROUBERT P, VIOSSAT I, LONCHAMPT M-O, CHAPELAT M, SCHULZ J, PLAS P, GILLARD-ROUBERT V, CHABRIER P-E, BRAQUET P: Endothelin regulation by endothelin synthesis in vascular smooth muscle cells: Effects of dexamethasone and phosphoramidon. *J Vas Res* 30:139–144, 1993
41. LI H, ELTON S, CHEN YF, OPARIL S: Increased endothelin receptor gene expression in hypoxic rat lung. *Am J Physiol* 266:L553–L560, 1994
42. NAKAMURA T, EBIHARA I, FUKUI M, OSADA S, TOMINO Y, MASAKI T, GOTO K, FURUICHI Y, KOIDE H: Modulation of glomerular endothelin and endothelin receptor gene expression in aminonucleoside-induced nephrosis. *J Am Soc Nephrol* 5:1585–1590, 1995
43. CLOZEL M, BREU V, GRAY GA, KALINA B, LÖFFLER B-M, BURRI K, CASSAL J-M, HIRTH G, MÜLLER M, NEIDHART W, RAMUZ H: Pharmacological characterization of bosentan, a new potent orally active non-peptide endothelin receptor antagonist. *J Pharmacol Exp Ther* 270:228–235, 1994
44. BENIGNI A, ZOJA C, CORNA D, ORISIO S, REMUZZI G: Role of renal endothelin type B receptor in experimental progressive renal disease. (abstract) *J Am Soc Nephrol* 5:573, 1994
45. IWASAKI S, HOMMA T, MATSUDA Y, KON V: Endothelin receptor subtype B mediates auto-induction of endothelin-1 in rat mesangial cells. *J Clin Invest* 270:6997–7003, 1995