Role of endothelin in the pathophysiology of renal ischemia-reperfusion in normal rabbits

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Role of endothelin in the pathophysiology of renal ischemia-reperfusion in normal rabbits. The present study addressed the acute effects of endothelin-1 on renal function and neutrophils accumulation in the setting of *in vivo* severe (60 min) acute ischemia/reperfusion. Ischemia/reperfusion decreased renal functional parameters and increased renal neutrophil accumulation and medullary congestion. All these parameters markedly improved with the intrarenal administration of anti-endothelin-1 antiserum. Comparatively, the intrarenal infusion of endothelin-1 decreased renal function and increased neutrophil accumulation. Abnormalities in renal histology were, however, less pronounced than with ischemia/reperfusion. In experiments using rabbit isolated perfused kidneys, endothelin-1 induced the accumulation of labeled neutrophils. This accumulation was similar to that observed in kidneys obtained after 60 minutes of ischemia plus 60 minutes of reperfusion. Both endothelin and ischemia/reperfusion effects were counteracted by an anti-endothelin antibody. In further *in vitro* studies, we found that endothelin-1-induced the expression of the CD18 antigens on the neutrophil surface. In subsequent experiments based on this effect of ET-1 on CD18 antigens, a blockade of both ischemia/reperfusion-induced and endothelin-1-induced neutrophil accumulation was obtained by infusion an anti-CD18 antibody. In conclusion, our experiments disclosed the critical role of endothelin-1 as a major promoter of early neutrophil accumulation after ischemia/reperfusion, which occurred through an integrin-mediated mechanism.

Ischemia/reperfusion (I/R) is a leading cause of organ damage in diverse clinical circumstances [reviewed in 1–3]. The mechanisms proposed to explain the I/R-induced damage include anoxia, neutrophil accumulation and release of oxygen free radicals during reperfusion and lytic enzymes [1–3]. Several approaches have been tried, such as: to restore blood flow by using vasodilatory drugs [4]; to reduce the effects of oxygen free radicals by means of scavenging agents [5, 6]; and to diminish neutrophil accumulation or block the activation of neutrophil-borne, tissue-damaging enzymes, by using antibodies against adhesive integrins or protease inhibitors [7, 8].

In recent years, considerable information has been gathered on the role of the vasoconstricting peptide, endothelin-1 (ET-1), in circulatory regulation [reviewed in 9]. Experimental evidence

from our and other laboratories amply supports the existence of a relevant role of ET-1 in renal ischemia [10–14].

In the past two years, several effects of ET-1-related mechanisms have been described as neutrophil properties, namely, Ca^{2+} mobilization [15], nitric oxide (NO) and $O_2^{·-}$ production [16, 17], adhesion to endothelial cells and heart tissue [18], aggregation [19] and neutrophil-dependent platelet activation [20]. However, data are needed examining the effects of ET-1 on neutrophils in pathological circumstances. In the case of ischemic injury, increased ET-1 formation and neutrophils accumulation are coexistent phenomena [21, 22], and the presence of a mechanistic relationship between them is worthy of exploration.

The aim of the present experiments was to test the hypothesis that ET-1 has a relevant role on I/R-related renal neutrophil accumulation. The complementary hypothesis, that the effects of kidney-borne ET-1 may extend to the systemic level, also was tested by examining neutrophil accumulation in the hearts of animals with kidney I/R or intrarenal ET-1 infusion.

Methods

In vivo studies

Animals instrumentation and experimental procedures. The study protocols were approved by the Institutional Review Board and performed according the international conventions on animal experimentation. All the *in vivo* and perfused organ studies were performed in male New Zealand rabbits, weighing 2.5 to 2.8 kg. All the animals had free access to food and water. For *in vivo* experiments, after sodium pentobarbital anesthesia (100 mg/kg body wt, i.p.), rabbits underwent femoral vein and artery cannulation and the abdominal cavity was midline opened. A continuous physiological saline solution (PSS, in mM: NaCl 137, KCl 2.6, KH_2PO_4 1.5, NaH_2PO_4 8, glucose 5.6, pH 7.4, 37°C, 0.25 ml/min) infusion was started and maintained throughout the entire experiment. After the extraction of blood samples for measuring femoral and right renal vein ET-1 plasma levels, right uninephrectomy was performed, the first branch of the renal artery and ureter were cannulated and heparin (100 I.U. kg body wt, i.v.) was administered. The extracted kidneys were frozen in liquid nitrogen, to serve as baseline controls of myeloperoxidase (MPO) activity. Mean arterial pressure was continuously monitorized (Letica polygraph; Scientific Instruments, Madrid, Spain) and

body temperature was monitorized by an intrarectal electrical thermometer (Elektrolaboratoriet, Copenhagen, Denmark) and maintained between 36° and 37°C to avoid possible interference in the ischemic phenomenon by temperature changes [23]. At the end of a 45-minute equilibration period, blood samples were taken for the determination of packed cell volume, creatinine and electrolytes. Thereafter, the left renal artery was occluded for 60 minutes with a nontraumatic clamp, as described previously [10]. At the end of this time, the clamp was released and reperfusion allowed for an additional 60 minutes. Once the reperfusion phase was finished, samples for ET-1 measurement were obtained from renal and femoral veins, the animals were killed by overdose of anesthesia, and the kidney and heart were extracted, washed with cold PSS and frozen in liquid nitrogen.

Measurement of renal function. Renal functional parameters were measured as previously described [10, 24], by administrating metoxi-[^{14}C]-IN (C_{IN}; loading dose, 3 μCi/ml; infusion, 0.62 μCi/ml in PSS, 3 ml/hr) and [^{3}H]-PAH (loading dose, 13 μCi/ml; infusion, 2.5 μCi/ml, 3 ml/hr). Two 30-minute urine collections were completed, with blood sampling (150 μl) done at the beginning and end of each clearance period. [^{3}H] and [^{14}C] activities were measured using a two channel liquid scintillation counter which corrected for the interference between isotopes. C_{IN} was used for calculating fractional Na excretion (FE_{Na}). Even though important changes in the renal handling of PAH may occur during ischemia, C_{PAH} was used as a comparative index of the putative changes in RPF occurring with each experimental maneuver. The absolute values of C_{PAH} should, however, be considered with the caveat that I/R may generate a systematic error. Creatinine and electrolytes were measured by an automatic analyzer (Astra IV Beckman).

Pathology studies. Kidney samples were fixed in 10% formaldehyde, stained with routine techniques and examined by optical microscopy in a blinded fashion by a renal pathologist (F.M.). The kidneys were specifically analyzed for the presence of tubular damage and medullary congestion patterns, as suggested by Solez et al [25] and classified using a numerical score to define the degree of tubular damage: 0 = no damage; 1 = unicellular, patchy isolated necrosis; 2 = tubular necrosis less than 25%; 3 = tubular necrosis between 25 and 50%; 4 = more than 50% tubular necrosis and presence of infarcted tissue. Degree of medullary congestion was defined by: 0 = no congestion; 1 = vascular congestion with identification of erythrocytes by ×400 magnification; 2 = vascular congestion with identification of erythrocytes by ×200 magnification; 3 = vascular congestion with identification of erythrocytes by ×100 magnification; 4 = vascular congestion with identification of erythrocytes by ×40 magnification.

Measurement of myeloperoxidase activity. MPO activity was measured in animals submitted to I/R with or without the different maneuvers detailed in the experimental protocols (see below), by assessing the change in absorbance of o-dianisidine hydrochloride, as described [18, 26], and after homogenizing the tissues in 50 mM potassium phosphate buffer, pH 6.0, containing 0.5% hexadecyltrimethylammonium bromide. In the conditions used, no interference could be expected from peroxidase activities other than MPO [26]. A standard *in vitro* curve was constructed using known numbers of neutrophils that showed a significant correlation between the number of neutrophils and MPO activity ($r = 0.92$, $P < 0.001$).

Measurements of plasma ET-1 levels. Blood samples were extracted in cold tubes containing EDTA (0.2 M), phenyl-methylsulphonyl fluoride (10^{-4} M) and leupeptin (10 μg/ml), centrifuged (3000 rpm, at 4°C for 15 min) and plasma stored at −70°C until determinations were performed. ET-1 levels were measured by enzymoimmunoassay (EIA; Cayman Chemical, MI, USA). The sensitivity of the assay was 7.8 pg/ml and the intra- and interassay variations were below 5.0 and 15%, respectively.

Experimental protocols for the in vivo studies. All of the solutions were freshly prepared in sterile PSS (37°C, pH 7.4), immediately before using. The different agents or vehicle were given as detailed below, by continuous intrarenal infusion (3.75 ml/hr) through the retrograde cannulated branch of the renal artery. All the infusions began immediately prior (2 min) to the initiation of reperfusion and lasted during the entire reperfusion phase. In the animals receiving no pharmacological agents, a similar volume of the vehicle was administered.

The rabbits were assigned to one of the following protocols.

Study I: (a) I/R group. A group of animals ($N = 10$) was submitted to I/R as detailed above, receiving only vehicle infusions.

(b) Anti-ET-1 group. To study the potential role of ET-1 in the initiation and maintenance of ischemic acute renal failure, a similar I/R procedure was carried out with the intrarenal administration of either anti-ET-1 monoclonal antibody (MoAb) (Peninsula Laboratories, Inc, London, UK; cross-reaction 100% with ET-1, less than 0.01% with ET-2 and ET-3, and 1% with porcine and human big ET-1, 1:10000; $N = 6$) [10]. The use of the anti-ET-1 MoAb was preferred instead of using the ET-1 antagonist BQ-123 for two reasons: (1) BQ-123 does not block ET-B receptors, which are instrumental in the renal vasoconstricting response of ET-1 [27]; instead, the MoAb blocks all the effects of ET-1; and (2) an effect of BQ-123 has been described as a blocker of angiotensin II-mediated effects, which could be a source of misinterpretation [28].

(c) Nonspecific IgG group. A nonspecific IgG (1:10000, $N = 3$) that had no cross reactivity with any of the endothelin isoforms was used for control purposes.

(d) Dopamine group. Dopamine, a non-NO-mediated vasodilator, was used at renal vasodilating dose (2 μg/kg body wt/min, $N = 3$) to assess whether any effect of the maneuvers mentioned above could be unspecifically attributed to vasodilation. In previous set-up experiments, we found that in control animals, this dose of dopamine increased C_{IN} and C_{PAH} by 30 ± 2 ($P < 0.01$) and 47 ± 3% ($P < 0.005$), respectively (Cernadas et al, unpublished results).

Study II. To further clarify the role of ET-1 on the pathophysiological mechanisms involved in I/R, the response to 60 minutes of intrarenal ET-1 infusion (0.027 μg/min, $N = 7$) was examined. In this case, the kidneys were not submitted to renal artery clamping and the ET-1 infusion period substituted for the renal ischemia.

In vitro studies

Neutrophils preparation. Rabbit neutrophils were obtained from peripheral blood by Ficoll-hypaque centrifugation, as previously described [15]. Neutrophils (95% pure, 98% viable by trypan blue exclusion) were resuspended (5 × 10^7 cells/ml) in 3 ml Hepes buffer containing, in mM: 131 NaCl; 4.7 KCl; 5 glucose and 20 Hepes, pH 7.4 and 2 μCi/ml $Na_2[^{51}Cr]$ O_4. After incubation

(37°C, 60 min) the cells were washed three times in Hepes and resuspended in Krebs-Henseleit buffer (in mM: NaCl 118, KCl 4.7, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, Cl$_2$Ca 2.5, NaHCO$_3$ 25, pH 7.4) at a density of 5 × 10^6 cells/ml.

The monoclonal antibodies anti-CD18 MoAb (TS1/18) and P3X63 used in the *in vitro* studies described below were provided by Prof. F. Sánchez Madrid (Hospital de la Princesa, Universidad Autónoma, Madrid, Spain) and prepared as reported [29]. P3X63 myeloma culture supernatant was used as a negative control. No *in vivo* studies could be done using the TS1/18 MoAb because of its limited availability.

Flow cytometry experiments. In an effort to clarify whether the effects of ET-1 on neutrophil accumulation were related to changes in the surface expression of adhesive integrins. For this purpose, rabbit neutrophils were incubated with ET-1 (10^{-7} M) for 30 minutes. After the incubation was completed, the neutrophils were incubated with the anti-CD18 MoAb followed by a second incubation with a fluorescein isothyocyanate-conjugated goat anti-mouse IgG (The Binding Site, Birmingham, UK). The fluorescence was measured as described [18] by using an EPICS flow cytometer/sorter (Coulter) at 480 nm excitation and 520 nm emission. A minimum of 5000 cells were analyzed for each sample. The data were displayed as one-parameter histograms plotting the logarithm of the fluorescence signal versus the cell number.

Isolated perfused kidney experiments. Experiments on isolated perfused kidneys were done as an *in vitro* probe for neutrophil accumulation in renal tissue. Perfusion of isolated rabbit kidneys was carried out according to modifications of the methods of Maack [30] and Little and Cohen [31]. Briefly, after a surgical preparation procedure similar to described above, the left kidney was perfused *in situ* (PSS infusion, 4°C, arterial 22-gauge needle) and rapidly removed and placed into a siliconyzed glass beaker (37°C, 95% O$_2$, 5% CO$_2$) which contained Krebs-Henseleit solution (pH 7.4) supplemented with 5 mM glucose, 5% fatty-acid free bovine serum albumin and 10 ml/liter of a 20 amino acid mixture (Nitrógeno 10; Ibys, Madrid, Spain). Flow was initially set at 10 ml/min and graduated to keep pressure at 100 mm Hg. A peristaltic pump (Harvard) was used for carrying out the perfusion; the perfusion system was closed, therefore allowing the perfusate to recirculate. Experiments were performed as follows: (a) in kidneys from normal animals, ET-1 (0.013 μg/min in Krebs-Henseleit solution, $N = 4$) or vehicle ($N = 4$) were perfused for 30 minutes at a rate of 0.1 ml/min. The ET-1 dose was selected according to previous reports, which have shown that doses in the above-mentioned range did not produce significant *in vivo* vasoconstrictor effects [32]. Two minutes after the beginning of the ET-1 infusion, ^{51}Cr-labeled rabbit neutrophils in sterile Krebs buffer (8 × 10^6 in 0.5 ml, 79,995 ± 10,354 cpm/8 × 10^6 cells) were injected as a bolus. The experiments were stopped by washing out the non adherent neutrophils with fresh Krebs-Henseleit buffer, the kidneys were homogenized in 10% TCA and radioactivity counted. (b) Experiments similar to those described in (a) were done, preincubating (10 min) the ^{51}Cr-labeled neutrophils with the MoAb TS1/18 or with the nonspecific antibody, P3X63. (c) Kidneys extracted after 60 minutes I/R were submitted to identical procedures. Infusions were carried out of either vehicle alone ($N = 4$), anti-ET-1 ($N = 4$) or nonspecific IgG ($N = 3$). Further studies (see below) were done in kidneys submitted to identical experimental procedures, to examine the role of anti-

Table 1. Renal functional parameters after 60 min I/R in uninephrectomized rabbits treated with different agents

	C$_{IN}$	C$_{PAH}$
	ml/min	
Baseline	3.76 ± 0.33	11.22 ± 2.55
Post-I/R	0.39 ± 0.13[a]	1.01 ± 0.55[a]
I/R+anti-ET-1	2.58 ± 0.12[ab]	8.52 ± 2.30[ab]
I/R+IgG	0.47 ± 0.05[a]	1.31 ± 0.09[a]

Values are mean ± SEM of two periods of 30 min each, after 60 min ischemia/60 min reperfusion ($N = 10$ animals) or in the presence of anti-ET-1 antibody (1:10000; $N = 6$) or nonspecific IgG ($N = 3$). Abbreviations are: ET-1, endothelin-1; I/R, 60 min ischemia/60 min reperfusion. A mean vlaue of all the animals was used for the baseline data represented in this table. This was done because no significant differences in the baseline C$_{IN}$ or C$_{PAH}$ were detected between the different groups in the baseline measurements, $P = $ NS, data not shown).
[a] Significant differences with respect to the baseline
[b] Significant differences with respect to ischemia alone

ET-1 in the functional behavior of the isolated perfused kidney preparation.

To discard a nonspecific trapping of neutrophils induced by a putative undetected vasoconstrictor effect of ET-1, two additional experiments were carried out: (a) neutrophils were incubated with the MoAb against the CD18 antigen, TS1/18, from 10 minutes before and throughout their infusion in the renal circulation. (b) To rule out any possible nonspecific interaction between anti-human TS1/18 and rabbit neutrophils, experiments were carried out using human neutrophils preincubated with TS1/18 before infusing them into the isolated rabbit kidney ($N = 3$). Human neutrophils were prepared and labeled following the same procedure than for rabbit neutrophils.

Additional experiments were done to determine the role of ET-1 antagonism by the ET-1 antibody in the functional behavior of the isolated perfused kidney preparation. For this purpose, experiments identical to those described in item 3 were performed, with the modifications that both renal vein and ureter were cannulated in addition to the renal artery, urine was collected every 20 minutes and inulin (Sigma, 250 mg/liter) was added to the perfusate. Infusions were carried out of either vehicle plus nonspecific IgG ($N = 3$) or anti-ET-1 ($N = 3$) in kidneys extracted after 60 minutes I/R; kidneys extracted without I/R ($N = 3$) were used for control purposes. Inulin was measured in simultaneous perfusate and urine samples by the anthrone method.

Statistics

Values are expressed as mean ± SEM. Changes in variables between groups were analyzed by ANOVA and subsequent Scheffé's and Fisher's test. Differences between two groups were evaluated by the unpaired Student's *t*-test and differences between two periods of the same animals by the paired Student's *t*-test. A value of $P < 0.05$ was considered significant.

Results

In vivo experiments

Study I. (a) Renal functional studies. The occlusion of the renal artery for 60 minutes in control uninephrectomized rabbits induced a decrease of C$_{IN}$, C$_{PAH}$ (Table 1) and urinary volume (UV), and an increase of FE$_{Na}$ in the first hour following unclamping of the renal artery ($P < 0.001$ with respect to the

Table 2. Diuresis and natriuresis after infusion of the anti-endothelin-1 antibody or nonspecific IgG in rabbits

	UV $\mu l/min$	$U_{Na} V$ $\mu Eq/min$	FE_{Na} %
Baseline	37.08 ± 0.28	5.70 ± 0.9	0.8 ± 0.3
Post-I/R	7.50 ± 0.25	5.43 ± 1.5	9.4 ± 3.4^a
I/R+anti-ET-1	53.07 ± 1.48^{ab}	13.3 ± 3.15^{ab}	3.7 ± 0.6^{ab}
I/R+IgG	10.24 ± 0.22^a	7.5 ± 1.35	10.2 ± 1.9^a

Values are expressed as mean $\pm$ SEM, after 60 min ischemia/60 min reperfusion ($N = 10$) or in the presence of anti-ET-1 antibody (1:10000, $N = 6$) or nonspecific IgG ($N = 3$). Abbreviations are: UV, urinary volume; $U_{Na}V$, urinary sodium excretion; FE_{Na}, fractional sodium excretion; I/R, 60 min ischemia/60 min reperfusion.
[a] Significant differences ($P < 0.001$) with respect to the baseline
[b] Significant differences ($P < 0.05$) with respect to IR alone

baseline; Table 2). A recovery to near baseline values (Table 1, $P < 0.001$) of the post-I/R decrease in C_{IN} and C_{PAH} was obtained with the infusion of the anti-ET-1 antibody (1:10000). The anti-ET-1 antibody infusion also induced significant changes on diuresis, natriuresis and FE_{Na} with respect to I/R alone (Table 2). No effects on C_{IN}, C_{PAH}, urinary volume or Na excretion were detected with the administration of the nonspecific antibody ($P = $ NS; Table 1).

The experiments using dopamine demonstrated only a minor effect on C_{IN} (0.79 ± 0.18 ml/min, $P = $ NS with respect to I/R alone) and a moderate effect on C_{PAH} (2.64 ± 0.94 ml/min, $P < 0.05$ with respect to I/R alone), UV (50.9 ± 5.4 $\mu l/min$, $P < 0.05$ with respect to I/R alone) and $U_{Na}V$ (9.9 ± 1.8 $\mu Eq/min$, $P < 0.02$ with respect to I/R alone). The FE_{Na} was, however, in a range similar to that in I/R ($8.9 \pm 0.9\%$, $P = $ NS). No major changes in systemic mean arterial pressure were observed with either of the experimental maneuvers ($P = $ NS; data not shown).

(b) Histology studies. I/R provoked a significant degree of medullary congestion and tubular damage, which was significantly improved by the infusion of the ET-1 antibody and not by the nonspecific IgG (Fig. 1). Histologic scores at baseline are not represented, since they were not different from zero. No significant increase in neutrophil accumulation was found at optical microscopy with any of the experimental maneuvers ($P = $ NS for all the samples with respect to the nonischemic controls, data not shown). In view of the MPO findings shown in the next paragraph, multiple samples from differents areas of the kidney were used to further ascertain the reliability of this observation.

(c) Measurement of myeloperoxidase activity. An increased renal MPO activity, a marker of neutrophil accumulation, was observed after I/R (Fig. 2). This increase was blunted by the administration of the anti-ET-1 antibody infusion (1:10000; Fig. 2). An increased MPO activity was also detected in the hearts of the animals with renal I/R (Fig. 2). Moreover, no effect on the increased MPO activity was observed in the animals submitted to I/R infused with dopamine (2 $\mu g/kg$ body wt/min, $N = 3$; MPO, 2.4 ± 0.4 vs. 2.1 ± 0.2 U/g tissue in I/R and I/R + dopamine, respectively; $P = $ NS). This phenomenon was also inhibited in the presence of the anti-ET-1 antibody infusion (1:10000; Fig. 2). No significant effects were detected with the administration of nonspecific IgG (1:10000; Fig. 2). Moreover, no effect on the increased MPO activity was detected in the animals submitted to I/R and infused with dopamine (2 $\mu g/kg$ body wt/min, $N = 3$; MPO, 2.4 ± 0.4 vs. 2.1 ± 0.2 U/g tissue in I/R and I/R + dopamine, respectively; $P = $ NS).

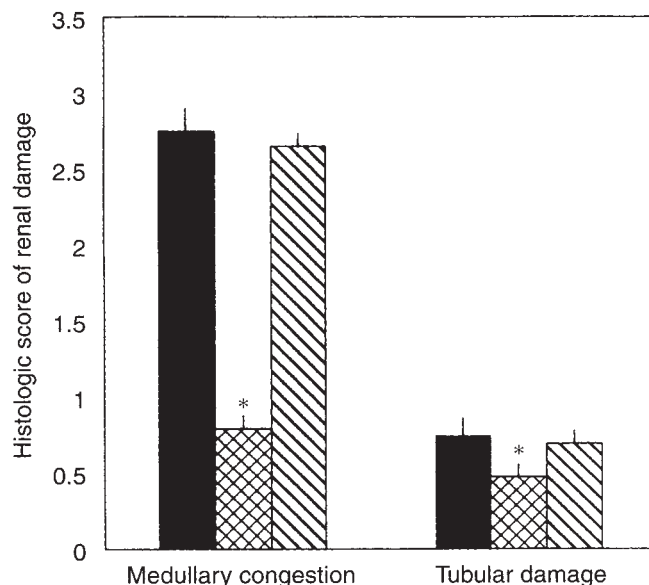No changes in baseline MPO activity were observed in either

Fig. 1. *Histologic score of vascular medullary congestion and tubular damage after 60 minutes of ischemia/60 minutes of reperfusion in rabbits.* Effects of anti-ET-1 infusion. * Represents significant differences ($P < 0.01$) with respect to I/R alone. Abbreviations are: I/R, 60 minutes of ischemia/60 minutes of reperfusion; ET-1, endothelin-1.
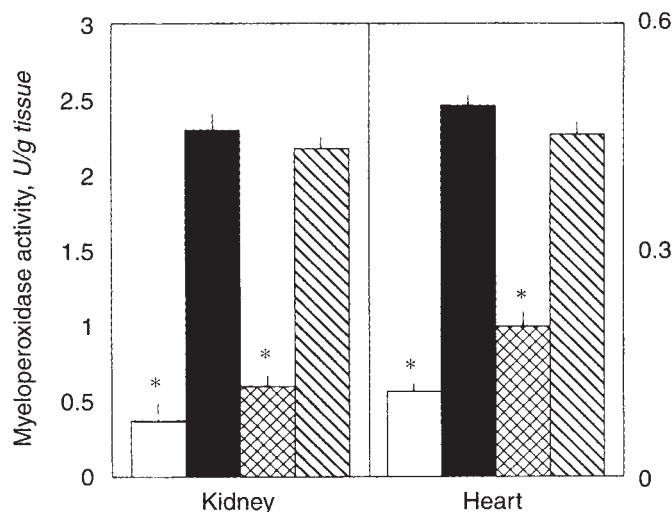

Fig. 2. *Effects of anti-endothelin-1 antibody and nonspecific IgG administration on the increased myeloperoxidase activity after renal I/R in rabbits.* *$P < 0.05$ with respect to increased renal and heart myeloperoxidase activity.

the kidney or the heart of sham-operated rabbits not submitted to I/R (MPO activity 0.47 ± 0.01 U/g kidney tissue and 0.17 ± 0.04 U/g heart tissue, $N = 3$, $P = $ NS). Moreover, no changes in MPO activity were detected in similar sham-operated animals submitted to an anti-ET-1 antibody infusion ($P = $ NS, $N = 3$; data not shown).

(d) Measurements of ET-1 plasma levels. The plasma ET-1 levels in the renal vein were in the limit of detection in control conditions (9.3 ± 1.2 pg/ml), but increased significantly after I/R in vehicle-treated rabbits (48.2 ± 6.8 pg/ml, $P < 0.01$ with respect

Table 3. Functional parameters after intrarenal endothelin-1 infusion in rabbits

	C_{IN}	C_{PAH}
	ml/min	
Baseline	4.38 ± 0.34	12.30 ± 0.67
ET-1	1.27 ± 0.22[a]	4.72 ± 1.05[a]

Values are expressed as mean ± SEM of two periods of 30 min each, after ET-1 infusion (0.027 μg/min; $N = 7$). Abbreviation is ET-1, endothelin-1.
[a] Significant differences ($P < 0.001$) with respect to the baseline

Table 4. Diuresis and natriuresis after intrarenal ET-1 infusion in rabbits

	UV μl/min	U_{Na} V μEq/min	FE_{Na} %
Baseline	37.08 ± 0.28	5.70 ± 0.9	0.8 ± 0.3
ET-1	13.03 ± 0.24[a]	0.31 ± 0.09[a]	0.2 ± 0.05[a]

Values are expressed as mean ± SEM, after ET-1 infusion (0.027 μg/min; $N = 7$). Abbreviations are: UV, urinary volume; U_{Na}V, urinary sodium excretion; FE_{Na}, fractional sodium excretion.
[a] Significant differences ($P < .001$) with respect to the baseline

Fig. 3. Myeloperoxidase activity (U/g tissue) in renal and heart tissue from intrarenal ET-1-infused rabbits (0.027 μg/min, $N = 7$). Results are represented as mean ± SEM. *$P < 0.05$ with respect to baseline. #$P < 0.05$ with respect to ET-1 infusion. Abbreviations are: ET-1, endothelin-1; I/R, 60 minutes of ischemia/60 minutes of reperfusion.

Fig. 4. Flow cytometry analysis of the expression of CD18 antigen on the rabbit neutrophil surface, unstimulated (1), or stimulated with 10^{-7} M ET-1 (2). The Figure reproduces the trace of one typical experiment. Abbreviation is ET-1, endothelin-1.

to the baseline). Also, a similar increase was detected in systemic venous blood (baseline 7.9 ± 1.5; I/R 45.7 ± 4.7 pg/ml, $P < 0.01$). No differences were observed in renal vein ET-1 between vehicle- and anti-ET-1 antibody-treated animals (42.2 ± 7.5 pg/ml).

Study II

Renal functional studies. The intrarenal ET-1 infusion caused a significant reduction of C_{IN} and C_{PAH} (Table 3), which was blocked by the anti-ET-1 antibody (Table 3). Also, a reduction in urinary volume and Na excretion was observed, with increased FE_{Na} (Table 4). No changes in systemic arterial pressure were detected with the administered dose (0.027 μg/min) of ET-1 (increase in mean arterial pressure 4 ± 1.8 mm Hg, $P = NS$).

Renal histology. In spite of the intense functional changes observed after ET-1 infusion, only a moderate degree (1.9 ± 0.4 histologic score, $N = 4$, $P < 0.02$ with respect to the control animals) of medullary congestion was detected. No signs of tubular damage were detected in any instance (score NS with respect to control).

Measurement of myeloperoxidase activity. The intrarenal administration of ET-1 significantly increased MPO activity within the rabbit kidney (Fig. 3). A significant increase of MPO activity in the rabbit heart was also detected in these conditions (Fig. 3).

In vitro experiments

Study IV. Flow cytometry studies. The flow cytometry experiments showed a 34.7 ± 3.6% increase in CD18 expression in ET-1 (10^{-7} M)-treated with respect to untreated neutrophils (Fig. 4).

Neutrophil accumulation in the isolated, perfused kidney. ET-1 infusion markedly increased the accumulation of rabbit neutrophils on the isolated, perfused rabbit kidney (neutrophil accumulation with vehicle infusion, 1.5 × 10⁶ ± 1.6 × 10⁵; with ET-1 infusion, 4.2 × 10⁶ ± 3.8 × 10⁵ neutrophils; $N = 5$ each; $P < 0.01$). No changes in the renal perfusion pressure were evident with the dose of ET-1 used (0.013 μg/min, perfusion pressure 100 ± 21 and 104 ± 26 mm Hg, in the absence or presence of ET-1, respectively; $P = NS$). TS1/18 inhibited ET-1-induced
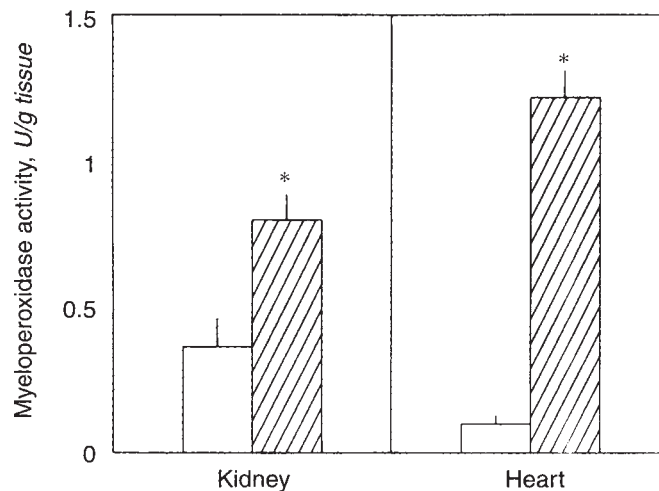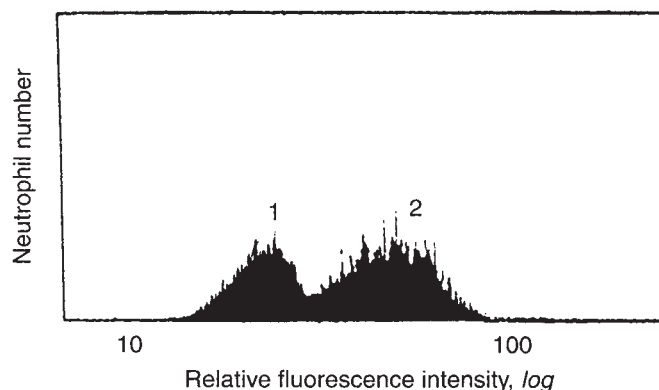
neutrophil accumulation by 75 ± 15% ($P < 0.01$), whereas only 1.2 ± 0.8 inhibition was obtained by preincubating the neutrophils with the non specific MoAb P3 × 63 ($P = NS$).

ET-1 stimulated human neutrophil accumulation in a similar degree than observed with rabbit neutrophils (increase of human neutrophils accumulation 3.01 × 10⁶ ± 3.01 × 10⁵ neutrophils, $N = 4$, $P < 0.05$). This effect was inhibited (73 ± 12% inhibition, $N = 3$, $P < 0.05$ with respect to ET-1 alone) by the simultaneous infusion of TS1/18.

An increased accumulation of ⁵¹Cr-labeled neutrophils was also observed in isolated, perfused kidneys extracted after one hour of *in vivo* ischemia, followed by one hour reperfusion (% accumulation 55.3 ± 3%, $N = 3$ I/R kidneys; 28.2 ± 4, $N = 3$ kidneys from sham operated animals). This increased accumulation was inhibited (83.5 ± 5% inhibition, $P < 0.05$) by the administration of anti-ET-1 antibody (1:10000) in the medium containing the ⁵¹Cr-labeled neutrophils. No inhibition of the I/R-stimulated neutrophil accumulation was found with the nonspecific IgG.

In further studies assessing the functional characteristics of kidneys submitted to identical experimental procedures but without the infusion of labeled neutrophils, we obtained the following data: as predictable, I/R provoked a marked reduction of C_{IN} (78.5 $\pm$ 5.8% reduction, $P < 0.01$ with respect to the control, non-I/R kidneys). This effect was markedly blocked in the presence of the anti-ET-1 antibody (16.4 $\pm$ 5.6% reduction, $P = NS$ with respect to the control, $P < 0.01$ with respect to the I/R + nonspecific IgG). The mean value of C_{In} in the control, non-I/R kidneys was 367 $\pm$ 37 $\mu l \cdot min^{-1} \cdot g^{-1}$ wet kidney weight. Moreover, different characteristics of intrarenal resistances were observed in the I/R kidneys compared to the I/R kidneys perfused with anti-ET-1 antibody and control kidneys, namely, the mean flow necessary for maintaining 100 mm Hg intrarenal pressure throughout the 60-minute perfusion period was 4.05 $\pm$ 0.7 ml/min in I/R versus 8.9 $\pm$ 0.9 ml/min in I/R + anti-ET-1 antibody versus 10.8 $\pm$ 0.3 ml/min in controls, $P < 0.05$ of I/R with respect to the other two groups.

Discussion

The rationale for the present study was based on previous evidence that separately implicated ET-1 as a relevant mediator in renal I/R [10, 11] and in neutrophil activation [18, 20]. The administration of the anti-ET-1-antibody revealed that ET-1 was a crucial mediator not only of renal functional derangement but also of post-I/R renal neutrophil accumulation. The specificity of this effect and the significant role of ET-1-related expression or neutrophil adhesive integrins were supported by the results obtained with the *in vivo* ET-1 infusions and flow cytometry isolated perfused kidneys experiments.

The accumulation of neutrophils after I/R is a well characterized phenomenon in different organs [3, 7, 8, 33–35]. At the present time, the prevalent opinion indicates that neutrophils may have a role in aggravating the severity of myocardial and mesenteric ischemia [33–35], but their pathogenetic potential in renal I/R is still controversial. Data have been obtained that either deny [7, 36] or favor [8, 37] the importance of neutrophils in renal I/R, and the debate is still alive. Our experiments suggested that a significant neutrophil accumulation occurred in the kidney in the first hour after I/R. This phenomenon was evidenced by the MPO method but not by the histological techniques. This apparent discordance between both methods therefore suggests that 60 minutes of I/R might be too short a time to detect a significant increase in neutrophil infiltration in the microscopical preparations. Accordingly, in this setting MPO might be a more sensitive technique. Similar to our findings, Thornton et al observed no histological evidence of neutrophil infiltration after a 60-minute ischemic period [7]. Moreover, Solez, Kramer and Fox have found that a significant presence of leukocytes could not be demonstrated by histology up to 36 hours after experimental acute renal failure [25]. The validity of the MPO method in this case is supported by the specific and high concentration of MPO within the azurophil bodies of the granulocytes, which makes any other resident or macrophage type cell unlikely to be responsible for any increase in MPO activity. An explanation for this apparent dissociation between the histological and MPO findings can be derived from data recently published by Saeki et al [38], who found that in conditions of neutrophil activation and massive degranulation, MPO is released outside the neutrophils and bounds to kidney cellular membranes. In these conditions, degranulated neutrophils would be not longer identified by histolog-

ical techniques, whereas MPO activity would be readily quantified by enzymatic assays.

It is necessary to re-emphasize, however, that our experiments were not specifically aimed at analyzing the role of neutrophils in the outcome of renal I/R. In fact, the present studies were circumscribed only to examine the role of ET-1 and the mechanisms intervening in neutrophil accumulation during renal I/R. In this regard, three aspects appeared with a defined profile: (a) the same maneuver that interfered with the renal functional consequences of I/R, namely, the anti-ET-1 antibody infusion, blocked neutrophil accumulation; (b) the specificity of this effect was demonstrated by several experimental findings, namely, the effect of the TS1/18 antibody in the *in vitro* perfusion experiments, the increase in CD18 expression by ET-1 detected in the flow cytometry studies and the lack of effect of the renal vasodilator, dopamine, in MPO levels. (c) ET-1 infusion *per se* favored the neutrophil accumulation in the kidneys, as we have previously observed in heart tissue [18]. The experiments showing that the anti-ET-1 antibody blocked the post-I/R neutrophil accumulation strongly suggested that ET-1 was a significant mediator of this phenomenon. The renal functional improvement observed in the same experiments further confirms that the anti-ET-1 antibody was effective in modifying the immediate outcome of renal I/R, even in the isolated perfused kidney. The findings on neutrophils adherence should, however, be mainly traced to an integrin-mediated mechanism, on the basis of the effect of the anti CD18 antibody. A hemodynamic-related mechanism could, however, be also operative, as judged by the results obtained with ET-1 blockade by the anti-ET-1 antibody. Of additional interest are the data showing an increased neutrophil accumulation in the hearts of rabbits with renal I/R and ET-1 infusion. The results obtained with intrarenal ET-1 and anti-ET-1 antibody infusions demonstrated a distant effect of ET-1 generated in the kidney during I/R. The potential pathophysiological implications of this finding need to be specifically addressed. In fact, these results suggest that myocardial effects may occur during ischemic episodes in distant organs. The possibility for the observed myocardial neutrophil accumulation to be pathologically relevant should be considered on the light of the proposed role of neutrophils on deranged myocardial function [39]. Preliminary data from Atanasova et al [40] have suggested that, in circumstances of NO-generation inhibition, renal I/R may provoke systemic circulatory complications.

The present results expand to neutrophil-mediated effects the possible actions of ET-1 as a pathogenetic agent in early I/R. However, since increased release of ET-1 can be expected as long as post-I/R endothelial persists, this finding may provide a clue to understanding the pathophysiological sequence leading to sustained neutrophil accumulation and prolonged post-ischemic renal damage, and perhaps for neutrophil infiltration in pathological situations involving ET-1 release other than I/R.

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Appendix

Abbreviations are: ET-1, endothelin-1; FE_{Na}, fractional sodium excretion; GFR, glomerular filtration rate; MPO, myeloperoxidase; PSS, physiological sterile saline; $U_{Na}V$, urinary sodium excretion; UV, urinary volume.

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