Endoglin regulates renal ischaemia–reperfusion injury

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Abstract

Background. Renal ischaemia–reperfusion (I–R) can cause acute tubular necrosis and chronic renal deterioration. Endoglin, an accessory receptor for Transforming Growth Factor-β1 (TGF-β1), is expressed on activated endothelium during macrophage maturation and implicated in the control of fibrosis, angiogenesis and inflammation.

Methods. Endoglin expression was monitored over 14 days after renal I–R in rats. As endoglin-null mice are not viable, the role of endoglin in I–R was studied by comparing renal I–R injury in haploinsufficient mice (Eng+/−) and their wild-type littermates (Eng+/+). Renal function, morphology and molecular markers of acute renal injury and inflammation were compared.

Results. Endoglin mRNA up-regulation in the post-ischaemic kidneys of rats occurred at 12 h after I–R; endoglin protein levels were elevated throughout the study period. Expression was initially localized to the vascular endothelium, then extended to fibrotic and inflamed areas of the interstitium. Two days after I–R, plasma creatinine elevation and acute tubular necrosis were less marked in Eng+/− mice than in Eng+/+ mice. Significant up-regulation of endoglin protein was found only in the post-ischaemic kidneys of Eng+/− mice and coincided with an increased mRNA expression of the TGF-β1 and collagen IV (α1) chain genes. Significant increases in vascular cell adhesion molecule-1 (VCAM-1) and inducible nitric oxide synthase (iNOS) expression, nitrosative stress, myeloperoxidase activity and CD68 staining for macrophages were evident in post-ischaemic kidneys of Eng+/− mice, but not Eng+/+ mice, suggesting that impaired endothelial activation and macrophage maturation may account for the reduced injury in post-ischaemic kidneys of Eng+/− mice.

Conclusions. Endoglin is up-regulated in the post-ischaemic kidney and endoglin-haploinsufficient mice are protected from renal I–R injury. Endoglin may play a primary role in promoting inflammatory responses following renal I–R.

Keywords: endoglin; fibrosis; inflammation; renal ischaemia–reperfusion; TGF-β1

Introduction

Ischaemia–reperfusion (I–R) injury is an important cause of acute renal failure in humans, especially following renal transplant surgery and renal artery stenosis. The precise origin/cause of injury is difficult to delineate but the combination of events occurring during the ischaemic phase and following reperfusion synergize to produce acute renal failure. I–R injury has been correlated with the incidence of acute rejection in several clinical series, and evidence suggests that I–R injury is an antigen-independent risk factor for chronic renal allograft failure [1]. The inflammatory reaction, tubular necrosis and fibrosis that characterizes I–R injury in renal transplantation may cause delayed initiation of graft function and contribute to decreases in long-term graft viability. On the other hand, significant differences in the incidence of delayed graft function are found between kidneys derived from cadaver and living donors, in which the most obvious differential is the relative lengths of warm and cold ischaemic times [2,3].

Endoglin (CD105), an accessory component of the (TGF-β) receptor system, is a 180 kDa homodimeric membrane glycoprotein identified thus far on a limited number of cell types. It is constitutively expressed on the vascular endothelium and on other cell types such as macrophages, vascular smooth muscle cells and mesangial cells [4–7]. Endoglin-null embryos die at 10–11.5 days due to vascular and cardiac abnormalities and, therefore, animal models focused on endoglin rely on the use of the haploinsufficient heterozygote, which in some mouse strains serves as a model for...
the autosomal-dominant vascular disorder, human hereditary telangiectasia-1 [8,9]. Endoglin-haploinsufficient mice have a reduced expression of the eNOS gene and a reduced reliance upon nitric oxide (NO) in the maintenance of vascular tone [10].

Several in vitro studies demonstrated that endoglin is up-regulated by TGF-β1 and is able to modulate some cellular responses to TGF-β1, mainly those related to extracellular matrix synthesis and accumulation [6,7]. Other observed effects of endoglin overexpression upon TGF-β1 signalling in vitro include the reversal of TGF-β1-mediated inhibition of the proliferation and migration of the U937 monocyte cell line and inhibition of TGF-β1-induction of PAI-1 and fibronecstin expressions in NCTC929 fibroblasts [11,12]. Endoglin antagonized TGF-β1-mediated suppression of the growth and migration of cultured human endothelial cells while it enhanced TGF-β1-induced inhibition of smooth muscle cell migration following arterial injury [13,14].

Endoglin is up-regulated in biopsies of patients with chronic renal disease [15]. We have reported endoglin up-regulation during renal fibrosis induced by renal mass reduction in rats [16] and in the obstructed kidneys of mice following unilateral ureteral obstruction [17]. Moreover, TGF-β1, angiotensin II and hypoxia are known to be of importance in I–R injury and all of these mediators can up-regulate endoglin expression [18–20]. Interestingly, the induction of endoglin occurs during the activation of the endothelium and during macrophage activation [4], both of which are instrumental events in post-ischaemic renal damage. These findings led us to hypothesize that endoglin could play a role in the pathogenesis of renal I–R injury.

To study this hypothesis, we have used a rat model to assess if endoglin expression was increased in renal I–R injury. Subsequently, we examined the possible role of endoglin in I–R injury by comparing the post-ischaemic renal injury in endoglin-haploinsufficient (Eng−/−) mice and their wild-type littermates (Eng+/+).

Subjects and methods

Animals

Animals were used in experimental protocols in accordance with the regulations of the following institutions: Conseil de l’Europe (published in the Oficial Daily No. L358/1–358/6, 18 December 1986) and Spanish Government (published in Boletín Oficial del Estado No. 67, pp. 8509–8512, 18 March 1988 and Boletín Oficial del Estado No. 256, pp. 31349–31362, 28 October 1990).

I–R induction and sample collection in rats

Male Wistar rats weighing between 250 and 300 g were housed within a temperature and humidity-controlled facility on a 12 h light–12 h dark cycle with free access to food (standard rat chow containing 20% protein by weight) and water provided ad libitum. Anaesthesia was induced by inhalation of isoflurane. Following midline laparotomy, I–R injury was induced by 45 min microvascular clamping of the left renal pedicle. The right kidney was removed 5 min before the end of the ischaemia period. Sham-operated (S) rats had their left renal pedicle freed by blunt dissection and a right-sided unilateral nephrectomy. Rats were sacrificed at the indicated time points post-ischaemia (between 12 h and 14 days). Analyses were performed in the left kidneys of S rats and in the left ischaemic (I) kidney of rats subjected to I–R.

At the time of sacrifice, blood was collected from the tail vein into heparinized capillaries (150 μl) and plasma was separated by centrifugation at 10 000 g for 10 min. Then, all rats were terminally anaesthetized using triple-strength sodium pentobarbital. Via the abdominal aorta, rats were in situ perfused with physiological saline to clear the left kidney of blood.

Renal tissue was harvested for different assays. Pieces for morphological and immunohistochemical studies, including cortex and medulla, were trimmed and fixed by immersion in 4% buffered formalin for 24 h. The blocks thus obtained were dehydrated in a graded series of ethanol and embedded in paraffin. Then in subsequent analyses, 3 μm thick sections were used. For Northern and Western blotting, kidney slices were snap frozen in liquid nitrogen and then, pulverized on dry ice to obtain homogenous samples, which were stored at −80°C until use.

I–R induction and sample collection in mice

Eng+/− mice were generated in the laboratory of Dr Michelle Letarte (Hospital for Sick Children, Toronto, Canada) as previously described [8] and backcrossed onto the C57BL/6 (B6) background. Male C57BL6 Eng+/− mice of the N6 generation and their Eng+/+ littersmate controls with normal endoglin expression weighing between 25 and 30 g were used in the present studies. All mice were housed within a temperature/humidity-controlled room on a 12 h light–12 h dark cycle. Groups of six mice were allocated per large mouse cage with free access to food (standard mouse chow) and water supplied ad libitum.

Induction of I–R injury was established using the following protocol. Mice were anaesthetized by intraperitoneal injection of 65 mg/kg sodium pentobarbital. After laparotomy, the left renal pedicle was identified and cleaned. A microvascular clamp was then placed on the pedicle for 25 min. The protocol then proceeded as described earlier for the rat, including a contralateral nephrectomy at the end of the ischaemic period. In our hands, this manoeuvre provokes a reversible acute renal failure with a recuperation of the normal function after 7 days [21–23]. As we have consistently found maximal damage between 12 and 48 h, we chose to obtain renal samples 48 h after I–R.

Animals were sacrificed and samples collected following the same protocol described for rats, with in situ perfusion carried out via the left ventricle. Analyses were performed in the left kidneys of S mice, and in the I kidney of mice undergoing I–R.

Plasma creatinine determination

Creatinine was measured using a Hitachi 717 automatic analyser in a reaction based on the Jaffé reaction (reagents from Boehringer Mannheim, IN, USA).
Histological studies

Sections (3 μm) were mounted on glass slides and counterstained with haematoxylin–eosin or Masson's trichrome for light microscopy analysis as previously described [16,17]. For immunohistochemistry, sections were deparaffinized in xylene and rehydrated in a graded series of ethanol before processing with a peroxidase–anti-peroxidase method. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide.

Tissue sections were incubated with the following antibodies: monoclonal anti-endoglin (CD105) (1/400 dilution; clone SN6h, DAKO, USA); monoclonal anti-CD68 (1/50 dilution; clone KPI, DAKO, USA) and polyclonal anti-vascular cell adhesion molecule 1 (VCAM-1) (1/50 dilution; C-19, Santa Cruz Biotechnology, USA). Sections were then washed in phosphate-buffered saline (PBS), and thereafter incubated with a LSAB+–HRP 2 system (DAKO, USA) using 3,3′-diaminobenzidine as the chromogenic substrate. Sections were lightly counterstained with haematoxylin, dehydrated and cover slipped. Negative controls were prepared without primary antibody.

Northern blot

Total renal RNA was obtained using a guanidine-isothiocyanate-phenol-chloroform-based protocol. A sample (20 μg) was loaded and separated on a 1% denaturing formaldehyde gel. RNA was transferred to a positively charged nylon membrane (Amersham Iberica, Madrid, Spain) for 16 h and then cross-linked using UV light. Hybridization was performed at 60°C for 12–16 h and the membranes were washed for 30 min twice in washing buffer 1 (2× sodium citrate solution (SSC), 1% Sodium dodecyl sulfate (SDS)) at 60°C and then for 1 h at room temperature in washing buffer 2 (0.2% SSC, 0.1% SDS). Bands were subsequently quantified using the MacBAS 2.2 program.

Northern probes used in the study were as follows: a 360 bp SacI/SacII fragment of rat endoglin cDNA cloned into the PUC18 (AmpR) plasmid (Dr Calvin Vary, Maine Medical Center Research Institute, Scarborough, Maine, USA); a 938 bp BamHI/AccI fragment of mouse endoglin inserted into pBluescript M13þ plasmid; a 451 bp fragment of TGF-β1 cloned into the pGEMT plasmid (Dr Jean Pierre Girolami, U388 INSERM, Toulouse, France); and a 1.6 kB probe was a 1.5 kB EcoR1 fragment digested from human de Henares, Madrid, Spain). The internal loading control using 18S signal to correct for loading variations.

Western blot

Membrane-enriched fractions were prepared for the detection of endoglin expression. 100 mg of powdered tissue was disrupted in 1 ml buffer (0.25 M Sucrose, 10 mM ethylene diamine tetraacetate (EDTA), 10 mM Tris, pH 7) containing a protease inhibitor cocktail (aprotinin, leupeptin, pepstatin, all from Sigma, Madrid, Spain). Samples were cleared by centrifugation at 4000 g for 15 min (4°C). The supernatant was collected and centrifuged at 210,000 g for 2 h (4°C). The pellet was suspended in 20 μl of Western blot lysis buffer (140 mM NaCl, 10 mM EDTA, 10% glycerol, 1% NP40, 20 mM Tris pH 8.0, plus protease inhibitor cocktail). For all other Western blots, total cellular protein extract was prepared from 100 mg of tissue in western blot lysis buffer. Protein concentrations were determined using a kit assay (Bio-Rad, Madrid, Spain).

For analysis, 100 μg of protein was mixed with an equal volume of 2× sample buffer (4% SDS, 20% glycerol, 0.01% bromophenol blue, 125 mM Tris, pH 6.8) and samples were boiled for 5 min. When it was necessary to separate proteins under reducing conditions, the 2× sample buffer contained 4% beta-mercaptoethanol. Proteins were separated by electrophoresis in 7.5–10% polyacrylamide gels and transferred to 0.45 mm pore nitrocellulose membranes (Bio-Rad, Madrid, Spain) for 2 h at 400 mA in a transfer buffer (190 mM glycine, 20 mM Tris, pH 8.3). Membranes were washed in distilled water blocked overnight and serially incubated with primary antibodies and horseradish peroxidase-conjugated secondary antibodies (Bio-Rad, Madrid, Spain).

Specific primary antibodies for the detection of specific proteins are the following: for rat endoglin, we used a rabbit anti-human endoglin anti-serum (1/1000 dilution); murine endoglin was detected with the supernatant of the cultured MJ178 hybridoma containing rat anti-mouse antibodies (1:2 dilution; gift from Dr Butcher, Stanford University Medical School, CA, USA); murine VCAM-1 was detected with a goat anti-VCAM-1 (1/1000 dilution; SC1504, Santa Cruz Biotechnology, USA); murine inducible nitric oxide synthase (iNOS) was detected using a rabbit anti-iNOS (1/500 dilution; #320300, Transduction Laboratories, USA); and mouse tyrosine nitrosylated proteins were detected with a rabbit anti-nitrotyrosine (1/1000 dilution; Upstate Biotechnology, USA). Appropriate secondary antibodies were diluted at 1/1000 in 1% bovine serum albumin and incubated for 1 h.

A chemiluminescence-based detection system was used to reveal bands (ECL detection reagents, Amersham Iberica, Madrid, Spain). Autoradiographs were scanned and quantified using the National Institute of Health image analysis programme.

Renal myeloperoxidase activity

Myeloperoxidase activity was used as an index of renal monocyte and macrophage infiltration. Renal samples were processed and activity measured, using O-dianisidine-dihydrochloride as a substrate, as previously described [23]. Activity was expressed as the change in absorbance per minute monitored over 5 min and normalized for each sample by Bradford assay measurement of protein concentration.

Statistics

All values are expressed as mean ± either the SD or SEM. All multiple comparison data have been analysed using one-way ANOVA with post hoc Bonferroni and Scheffes Tests. Direct group to group comparisons were carried out using independent Student's t-tests with prior Levene's tests for equality of variances. A value of P < 0.05 was considered statistically significant.
Results

Assessment of renal injury following renal I–R in rats

Plasma creatinine did not change significantly in S rats. In the case of animals subjected to renal I–R, plasma creatinine concentrations increased at 12 h post-I–R, rising to a maximum increase of 8-fold over control levels by 2 days. Five days after I–R, plasma creatinine had returned to almost normal levels with complete recovery occurring by day 14 (Figure 1A).

Normal renal structure was observed in the left kidneys of S rats when stained with haematoxylin–eosin (Figure 1B, a). Twelve hours after I–R, the I kidneys showed cortical and medullary hyperaemia with areas of tubular necrosis found in the deep and, to a lesser extent, in the superficial cortex. Tubular casts were observed in some of the damaged nephrons (Figure 1B, b). After 5 days, I–R-induced tubular damage worsened particularly in the deep cortex. Glomerular structure was altered in numerous corpuscles. Inflammatory infiltrate was visible in perivascular regions. After 5 days after I–R, the I kidneys continued to present evidence of hyperaemia but to a lesser extent than that seen at 12 h (Figure 1B, c). After 14 days post-I–R, tubular and glomerular lesions and levels of inflammatory infiltrate were worse than after 5 days. Some discrete areas of the renal parenchyma appeared to be well conserved (Figure 1B, d).

Masson’s trichrome revealed fibre staining only in the basement membranes and the glomerular mesangium of S kidneys at all time points studied (Figure 1B, e). After 12 h of I–R, the I kidneys did not show signs of renal fibrosis (Figure 1B, f). The situation was similar after 5 days with the exception of some slightly increased staining in I kidneys of several rats (Figure 1B, g). After 14 days following I–R, I kidneys

Fig. 1. Plasma creatinine and morphological injury following renal I–R injury in rats. (A) Plasma creatinine values (mean ± SD) in rats before surgery (basal, grey bars), after right uninephrectomy without ischaemia (sham, black bars) and in rats undergoing 45 min of left-sided renal ischaemia followed by contralateral nephrectomy and immediate reperfusion (I–R, unfilled bars). *P < 0.05 I vs basal and #P < 0.05 I–R vs S, at the same time (n = 3–6 per group). (B) Sections of rat kidney (3 μm) stained with haematoxylin–eosin for representative light microscopy (a–d) and with Massons trichromic to visualize extracellular matrix accumulation (e–h). Captions are as follows: kidney of S rat 5 days after right sided nephrectomy (a, e); ischaemic (I) kidneys at 12 h following I–R (b, f); I kidneys 5 days following I–R (c, g); I kidneys 14 days following I–R (d, h). Bar = 100 μM in (a–e) and 200 μM in (f–h).
showed a generalized interstitial fibrosis that varied slightly in severity between animals (Figure 1B, h).

**Expression of endoglin following renal I–R injury in rats**

Twelve hours after I–R, the renal endoglin mRNA (2.7 kB) expression showed a 2-fold increase in I kidneys over levels in S kidneys (Figure 2A). Up-regulation of endoglin protein (198 kDa) expression was also observed at 12 h post-I–R; these levels in I kidneys were maintained 3–4-fold higher than in S kidneys during the first 2 days following I–R, prior to a fall at day 3. Further overexpression over S values was noted between days 5 and 7 post-ischaemia with a final drop at 14 days (Figure 2B).

In S rats, renal endoglin expression was limited occasionally to interstitial capillaries (Figure 3a). 12 h after ischaemia, there was evidence of increased expression in interstitial capillaries of I kidneys (Figure 3b). After 5 days, the expression in the interstitial capillaries was more intense and staining began to appear in isolated areas of the interstitium (Figure 3c). Expression was undetectable in large vessel endothelium with strong expression appearing in adventitial layers. At 14 days post-I–R, strong endoglin expression appeared in the fibrotic areas of the interstitium and associated capillaries as well as in the large vessel adventitia (Figure 3d).

**Fig. 2.** Endoglin mRNA and protein expression in the kidneys of rats after renal I–R. Representative northern blot (A) and western blot (B) of endoglin mRNA and protein expression in the left kidney of rats over a 14 day period following I–R (I, n=3, unfilled bars) or sham operation (S, n=3, black bars). Bars represent mean ± SEM of endoglin/18S RNA or endoglin peptide signals for northern and western analyses, respectively. *P < 0.05: I vs S at the same time point.
Expression of mRNAs for TGF-β1 and collagen IV (α1) following renal I–R injury in rats

We examined the mRNA expression profiles for both, TGF-β1 and the TGF-β1 responsive gene coding for the basement membrane collagen IV (α1) monomer, in kidneys after I–R or sham operation. I kidneys showed a biphasic response with a significant 2.5-fold increase of TGF-β1 mRNA levels over S kidneys observed at 12 h post-ischaemia, returning to S values by 2 days post-I–R. A second up-regulation in I over S kidneys was observed between days 3 and 7 after I–R. TGF-β1 mRNA levels were normalized to those of S animals by day 14 after I–R (Figure 4A).

We observed a similar profile for collagen IV (α1) mRNA expression: an acute 2.5-fold increase was found to occur in I kidneys 12 h after I–R; levels then, decreased at 2 days with further significant increases of 5- and 2.5-fold occurring between days 3 and 7 after I–R. Levels normalized to values similar to those of S by 14 days post-ischaemia (Figure 4B).

Expression of endoglin 2 days after renal I–R in Eng+/+ and Eng−/− mice

As expected, endoglin mRNA (2.9 kB) expression was significantly lower in the renal tissue of S kidney in Eng−/− when compared with those of Eng+/+ mice. No significant up-regulation of endoglin mRNA was noted in I kidneys of either type of mouse 2 days after I–R (Figure 5A).

Two days after I–R, endoglin protein (198 kDa) expression was up-regulated by ~2-fold in I kidneys of Eng+/− mice. No significant up-regulation was noted in the I kidneys of Eng+/+ mice (Figure 5B).

Kidneys of S Eng+/+ mice expressed endoglin in glomerular and interstitial capillaries (Figure 6c and d). In kidneys of S Eng−/− mice, endoglin expression was almost absent in glomerular capillaries (Figure 6a), being limited to the occasional peritubular capillaries (Figure 6b). I kidneys of Eng+/− mice showed increased endoglin expression in the glomerular and peritubular capillaries and the mesangium (Figure 6e). Furthermore, a focal expression was detected at some sites of interstitial inflammation (Figure 6f). The pattern of endoglin expression in I kidneys of Eng+/+ was similar, but markedly more intense (Figure 6g and h).

Assessment of renal injury 2 days after renal I–R in Eng+/+ and Eng−/− mice

Analysis of plasma creatinine showed no significant differences between Eng+/+ and Eng−/− mice before the operation (control, C) or in S animals. I–R induced a 4.25-fold increase in Eng+/+ mice at 2 days post-I–R.
The corresponding increment in creatinine levels in \( \text{Eng}^{+/−} \) mice was significantly lower (1.6-fold) (Figure 7A).

Normal renal structures were observed in \( \text{S Eng}^{+/−} \) (Figure 7B, a and b) and \( \text{S Eng}^{+/+} \) animals (Figure 7B, c and d) when stained with haematoxylin–eosin. I kidneys of \( \text{Eng}^{+/−} \) mice showed severe alterations such as tubular dilation and hyaline casts that were observed in superficial cortex and in collecting tubules of the deep medulla. Extensive tubular necrosis was found in the cortico-medullary region, and inflammatory infiltrate observed particularly in perivascular regions (Figure 7B, g and h). I kidneys of \( \text{Eng}^{+/−} \) mice showed an almost normal histology with only a light cortical and medullary hyperaemia. Inflammatory cell infiltration was almost absent in these animals (Figure 7B, e and f).

Expression of mRNAs for TGF-β1 and collagen IV (α1) 2 days after renal I–R in \( \text{Eng}^{+/+} \) and \( \text{Eng}^{+/−} \) mice

No differences were noted in mRNA levels for TGF-β1 and collagen IV (α1) between the S kidneys of \( \text{Eng}^{+/+} \) and \( \text{Eng}^{+/−} \) mice (Figure 8A and B). Renal I–R up-regulated the expression of the mRNA for TGF-β1 in the I kidneys of both, \( \text{Eng}^{+/+} \) and \( \text{Eng}^{+/−} \) mice, but the magnitude of induction was significantly higher in \( \text{Eng}^{+/+} \) than in \( \text{Eng}^{+/−} \) mice (2.85-fold and 1.49-fold increase in I and S, respectively) (Figure 8A).
of renal vessels (Figure 10a–d). Immunohistochemistry also revealed a stronger staining for VCAM-1 in the I renal tissue of Eng+/+ (Figure 10d) compared with Eng+/− mice (Figure 10c).

Renal myeloperoxidase activity was studied in the renal tissue as an index of neutrophil and macrophage infiltration. Post-ischaemic kidneys of Eng+/+ mice had a significant 1.7-fold higher level of activity than that found in I kidneys of the Eng+/− group (0.032 ± 0.0039 vs 0.019 ± 0.0047 absorbance units per minute per milligram, respectively; n = 4, P = 0.04). Immunohistochemistry (Figure 10e–h) showed an abundant macrophage infiltration in the interstitium of I renal tissue of Eng+/+ mice (Figure 10h) as compared with the I kidneys of Eng+/− mice (Figure 10g). Macrophage staining was minimal in S kidneys of both strains of mice (Figure 10e and f).

Expression of iNOS and protein tyrosine nitrosylation 2 days after renal I–R in Eng+/+ and Eng+/− mice

iNOS expression is known to be related to the inflammatory response to renal injury and to play a deleterious role via the generation of peroxynitrite species and, at least in part, via protein nitrosylation [24]. No significant differences in iNOS expression were found between S renal tissue of Eng+/+ and Eng+/− mice (Figure 9B). The same was the case with protein tyrosine nitrosylation measured through the detection of a representative 160 kDa protein (Figure 9C). Significantly, higher iNOS expression and increased levels of tyrosine nitrosylation were found in the I kidneys of Eng+/+ as compared with the I kidneys of Eng+/− mice.

Discussion

Our data demonstrate that following renal I–R injury, endoglin was up-regulated in rats at both, the mRNA and protein levels. Endoglin up-regulation was localized to the microvasculature, interstitium and adventitial layer of large vessels. A specific role for endoglin in the activated/injured endothelium and in activated macrophages has already been demonstrated [4,25]. Thus, endoglin expression on small vessels and in areas of inflamed tubulo-interstitium is suggestive of a role for endoglin in the vascular damage and in the inflammatory response to renal I–R injury. Expression on the adventitial layer of large vessels may be reflective of the previously described up-regulation of endoglin on activated fibroblasts and, to some extent, on proliferating smooth muscle cells of the outer tunica media [26].

A biphasic post-ischaemic overexpression was noted in endoglin mRNA, in the mRNAs of its ligand, TGF-β1, and in the TGF-β1-responsive gene, collagen IV (α1). The kinetics of endoglin expression in the time-course study of renal tissue of rats after I–R, may be indicative of a TGF-β1-mediated control of

Markers of renal inflammation 2 days after renal I–R in Eng+/+ and Eng+/− mice

No difference in the amount of VCAM-1 assessed by Western blot, was seen between the left kidneys of S Eng+/+ and S Eng+/− mice. I–R-induced increases in VCAM-1 expression in I kidneys of Eng+/+ mice (2-fold over S) but not in I kidneys of Eng+/− mice (Figure 9a). VCAM-1 was localized in the endothelium
Fig. 6. Endoglin immunohistochemistry in the kidneys of $Eng^{+/+}$ and $Eng^{+/−}$ mice 2 days after renal I–R. Sections of mouse kidney (3 μm) for endoglin immunohistochemical detection. Captions are as follows: kidneys of sham-operated $Eng^{+/+}$ mice (a, b) and $Eng^{+/−}$ mice (c, d); ischaemic kidneys of $Eng^{−/−}$ mice (e, f) and $Eng^{++/+}$ mice (g, h) at 2 days following I–R. Bar = 50 μm.
endoglin expression as TGF-β1 has previously been described to induce the expression of endoglin. The biphasic increase in TGF-β1 expression might be explained by an early activation by hypoxia associated with tissue ischaemia, and a second increase dependent on its production from activated macrophages that infiltrate the renal tissue following I–R [14,20]. Endoglin can be up-regulated by hypoxia and angiotensin II, two stimuli relevant to renal ischaemia [19,20]. Angiotensin II initiates and sustains many pathophysiological processes by inducing the expression of TGF-β1, a major stimulator of endoglin expression. In addition, hypoxia is known to synergize with TGF-β1 to cause maximal endoglin expression [20]. Therefore, angiotensin II and hypoxia can initiate TGF-β1 and endoglin up-regulation in I–R damage.

In order to get an idea of the possible role of endoglin upregulation in I–R-induced renal damage, we compared post-ischaemic renal injury in endoglin-haploinsufficient mice (Eng+/−) and their wild-type littermates (Eng+/+) 2 days after I–R injury, a period which coincides with maximal renal failure. Northern blotting demonstrated that haploinsufficiency for endoglin was associated with a significantly lower renal endoglin mRNA expression. Two days after I–R, endoglin was up-regulated at the protein level in the renal tissue of Eng+/− mice. Endoglin overexpression was localized to the endothelium of glomerular and interstitial capillaries in areas of inflamed interstitium, mainly in kidneys from Eng+/− mice.

The major purpose of these studies in mice was to assess if differences in endoglin expression would have any impact upon renal injury following I–R. When we compared plasma creatinine levels and renal architecture at 2 days post I–R injury in ischaemic kidneys from Eng+/+ and Eng+/− mice, we consistently found evidence of less renal injury in the endoglin-haploinsufficient mice. We have identified a number of changes that could be crucial mediators of the reduced damage in these animals. Peri-tubular capillary damage has been observed after I–R and plays a major role in subsequent chronic renal failure.
Expression of VCAM-1 on endothelial cell surfaces is a consequence of capillary damage and a pre-requisite for lymphocyte and monocyte infiltration into sites of tissue injury, representing a key step in post-I–R inflammatory response [27]. Here, we have shown that renal I–R injury causes an increase in VCAM-1 expression in the I kidney of Eng+/+ mice whereas in the I kidney of Eng+/− mice, VCAM-1 remained almost unchanged compared with S kidneys. This corroborates previous data from our laboratory demonstrating VCAM-1 overexpression after I–R in rats and a relationship between decreases in VCAM-1 expression and the amelioration of post-I–R renal injury [22].

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Given that early changes in endoglin expression occur on the endothelium of the renal microvasculature, it is possible that reduced endoglin expression might be associated with or lead to a reduced expression of VCAM-1, causing reductions in inflammation and, ultimately, reduced renal injury. Supporting this concept, endoglin has been shown to be overexpressed...

Fig. 8. TGF-β1 and collagen IV (α1) mRNA expression in the kidneys of Eng+/+ and Eng+/− mice 2 days after renal I–R. Representative northern blot of TGF-β1 (A) and collagen IV (α1) (B) in the left ischaemic (I) kidney of Eng+/+ and Eng+/− mice at 2 days following I–R (I, unfilled bars, n=3–6) or sham operation (S, black bars, n=2). Bars represent mean ± SEM of individual mRNA/18S signal ratios. *P < 0.05: I vs S Eng+/+; *P < 0.05: I Eng+/− vs I Eng+/+.

Fig. 9. Expression of injury marker proteins in the kidneys of Eng+/+ and Eng+/− mice 2 days after renal I–R. Representative western blot of VCAM-1 (A), iNOS (B) and tyrosine nitrosylation (C) in the left ischaemic (I) kidney of Eng+/+ and Eng+/− mice at 2 days following I–R (I, unfilled bars, n=3) or sham operation (S, black bars, n=3). A positive control (C+) for protein detection consisting of renal homogenate derived from the obstructed kidney of mice after 3 days of unilateral ureteral obstruction was included. Bars represent mean ± SEM. *P < 0.05: I vs S Eng+/+; *P < 0.05: I Eng+/− vs I Eng+/+. 
Fig. 10. Immunohistochemistry for the macrophage marker CD-68 and VCAM-1 in the kidneys of Eng\(^{+/+}\) and Eng\(^{+/-}\) mice 2 days after renal I–R. Sections of mouse kidney (3 \(\mu\)m) stained for VCAM-1 (a–d) and the macrophage marker CD68 (e–h). Captions are as follows: kidneys of sham-operated Eng\(^{+/+}\) mice (a, e) and Eng\(^{+/-}\) mice (b, f); ischaemic kidneys of Eng\(^{+/-}\) mice (c, g) and Eng\(^{+/+}\) mice (d, h) at 2 days following I–R. Bar = 100 \(\mu\)m.
on microvessels and to be associated with inflammatory infiltration during dermal wound healing [25] and to be up-regulated in blood vessels of skin involved in psoriatic plaques. Much opinion is based on the belief that the majority of the damage in renal I–R injury is immune-mediated and is initiated by endothelial activation [28]. Hence, reductions in endoglin may exert a protective effect by reducing endothelial cell activation and adhesion molecule expression.

Increase in myeloperoxidase activity measured in renal tissue after I–R is a good indicator of neutrophil and monocyte–macrophage infiltration. Here we have shown a reduction in the activity of this enzyme in the post-ischaemic renal tissue of endoglin-haploinsufficient mice as compared with that of wild-type littermates. In agreement with these data, morphological analysis showed less inflammatory infiltrate and immunohistochemistry revealed reduced in the post-ischaemic renal tissue of endoglin and monocyte–macrophage infiltration. Here we

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