Original Article

Endostatin expression in the murine model of ischaemia/reperfusion-induced acute renal failure

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SUMMARY:

Background: Renal ischaemia-hypoxia is a leading cause of acute renal failure, a clinical condition associated with rapid loss of renal function and high rates of mortality. Renal proximal tubular cells are the most severely injured during renal ischaemia, caused by the breakdown of the extracellular matrix of the tubular basement membrane. Endostatin is the C-terminal fragment of collagen XVIII generated by proteolytic cleavage and it is well-known as being an inhibitor of angiogenesis. *In vitro*, endostatin inhibits endothelial cell proliferation and migration, as well as tubule formation. *In vivo*, it has a potent inhibitory effect on tumour growth. In this study, we analysed endostatin gene expression in C57BL/6 mouse kidneys subjected to ischaemia/reperfusion.
Methods: Ischaemic renal failure was induced via 45 min of bilateral occlusion of the renal artery and vein, followed by 12 h or 24 h of reperfusion. Whole-kidney homogenate and total RNA were extracted for examination by western blot analysis and quantitative polymerase chain reaction. The immunohistological examination revealed increased endostatin expression in injured kidney, mainly in the proximal tubule and collecting ducts.
Results: Endostatin/collagen XVIII mRNA and protein expression increased during ischaemia and within 12 h of reperfusion. In the western blot assay, we identified increased expression of the 30 kDa endostatin-related fragment and of matrix metalloproteinase-9. CD31 was significantly expressed during reperfusion (*P* < 0.05). Immunohistological examination revealed glomerular and tubulointerstitial expression of endostatin.
Conclusion: These data suggest the local synthesis of a 30 kDa endostatin-related fragment following acute renal failure and suggest its role in the modulation of renal capillary density.

KEY WORDS: acute renal failure, endostatin, gene expression, ischaemia/ reperfusion injury.

Acute renal failure (ARF) is a major clinical problem that affects up to 5% of all hospitalized patients. In most cases, ARF is caused by acute renal failure accompanied by a (typically reversible) loss of renal function resulting from ischaemic injury.[1] Mortality rates from ARF are reported to range from 30% to 80%. Despite these high rates, ARF is largely reversible in surviving patients and complete restoration of renal function is often observed.

Experimental ischaemia/reperfusion-induced ARF (irARF) is well documented as disrupting tubular function

and inducing vasoconstriction of arterioles to glomeruli.[2,3] Damage to the renal vascular endothelium, resulting in altered regulation of vascular tone and decreased blood flow in the peritubular capillaries, has been demonstrated within minutes of reperfusion injury.[4] The importance of endothelial dysfunction in the genesis of ischaemia/reperfusion injury is further suggested by the observation that administration of endothelial nitric oxide synthase-expressing endothelial cells will hone to the kidney and preserve blood flow and loss of function following ischaemia/reperfuion.[5] In addition, damage to the renal vasculature results in a breakdown of barrier function and increased adhesiveness to inflammatory cells, both of which appear to contribute to the loss of function following ischaemic injury.[6,7]

Collagen XVIII, a member of the heparan sulphate proteoglycan family, is an extracellular matrix protein associated with basement membranes of endothelial and epithelial cells. The extracellular matrix consists of 11 non-collagenous (NC) domains interrupted by 10 collagenous protein regions.[8,9] The NC1 region contains three
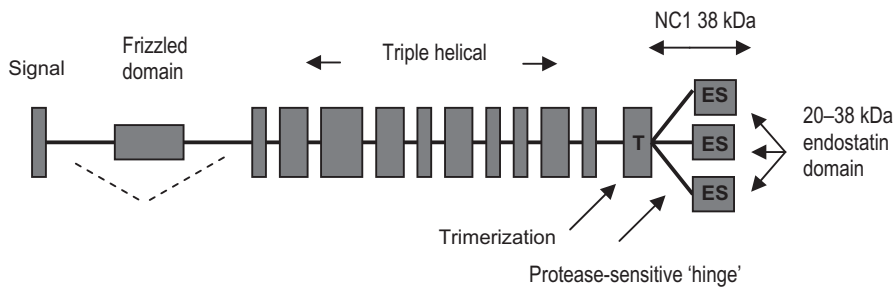
Fig. 1 Topology of collagen XVIII protein isoforms. Endostatin is derived from the C-terminal, common non-collagenous domain (NC1).

functionally distinct regions: an association domain (necessary for collagen XVIII oligomerization), a hinge domain (sensitive to proteases cleavage) and the endostatin domain (a 20 kDa fragment with potent antiangiogenic properties).[10,11]

Endostatin is an antiangiogenic factor isolated from haemangioendothelioma cells as a carboxyl-terminal segment of collagen XVIII. Although the mechanism through which endostatin suppresses angiogenesis remains unclear, it has been shown that endostatin induces endothelial cell apoptosis and inhibits endothelial migration. Several potential molecular targets of endostatin have been postulated. Recent evidence suggests that collagen XVIII is critical for basement membrane function in specific anatomical locations.

Within the NC1 domain of collagen XVIII (38 KDa), a non-triple helical trimerization domain redundantly trimerizes the globular COOH-terminal 20 kDa endostatin domains (Fig. 1). Several proteases and matrix metalloproteinases (MMP) may be involved in the cleavage of the C-terminal of NC1 of collagen type XVIII to produce endostatin.[12,13]

In vitro, endostatin regulates apoptosis, proliferation, migration and invasion of endothelial cells, as well as epithelial cell morphogenesis, suggesting that it plays a role in diverse physiological processes.[14,15] It has been shown that endostatin modulates numerous signal transduction pathways, downregulating integrin and Wnt-dependent signalling and inhibiting various MMP, as well as potentially disrupting focal adhesion and disassembling actin stress fibres.[16–18] The inhibitor likely exerts these effects through the binding of various membrane proteins, such as proteoglycans, αv and $\alpha 5$ integrins, or of the vascular endothelial growth factor receptor KDR/Flt-1.[19–22] In addition, the NC1 domain has been shown to regulate angiogenesis and cell motility.[23,24]

The aim of this study was to investigate endostatin expression in the mouse kidney after ischaemia/reperfusion and to characterize the potential role of endostatin in this experimental model.

METHODS

Animals

Experiments were conducted in adult C57BL/6 mice (2–5 months old, 20–32 g body weight). The animals were maintained under specific pathogen-free conditions at the National Pharmacology Institute, Federal University of São Paulo, São Paulo, Brazil.

Surgical procedure for ischaemia/reperfusion

Mice were placed on a heating pad to maintain body temperature. They were then anaesthetized with ketamine (125 mg/g body weight, Ketalar; Parke-Davis, Morris Plains, NJ, USA) and xylazine (12.5 mg/g body weight; Phoenix Scientific, Inc., St. Joseph, MO, USA). Both renal pedicles were prepared using a median dorsal skin incision and bilateral paramedian opening of the retroperitoneal space. In animals undergoing irARF, both pedicles were clamped off for 45 min using haemostatic microclips. After the clamps release, a 5–0 silk suture was used to close the incision into layers (a continuous suture closing the muscle layer and the skin) and animals were allowed to recover with free access to food and water. At 12 h or 24 h after ischaemia, the animals were reanaesthetized. Blood was collected by penetrating the retro-orbital plexus with a glass capillary and kidneys were harvested, snap-frozen or washed in sodium phosphate buffer (PBS) and then fixed in PBS-buffered 3.7% formalin. The experimental procedure was approved by the Federal University of São Paulo Committee for the Use of Live Animals in Teaching and Research.

Chemicals and renal function

From each animal, approximately 0.2 mL of blood were collected into ethylenediamine tetraacetic acid (EDTA) (used as an anticoagulant). Levels of blood urea nitrogen and plasma creatinine were determined using commercial kits (Urea UV Liquiform – Cat. 104 and Creatinina K – Cat. 96, respectively; Labtest Diagnostics, Santa Lagoa, Brazil).

Quantitative real-time polymerase chain reaction

Reverse transcription polymerase chain reaction (PCR) (RT-PCR) was performed for endostatin in RNA extracted from the kidneys of irARF mice. Total RNA was harvested from renal tissue using a reagent (TRIzol; Life Technologies, Rockville, MD, USA). One microgram samples of total RNA were used for the RT-PCR as follows. Quantitative real-time PCR was used to accurately detect changes in the numbers of endostatin gene copies. Expression of the target gene was normalized to the glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) levels. The primers for mouse endostatin were 5′-GCCCA GCTTCATCACACAGAGT-3′ (foward) and 5′-TGTTGAAAGAT GACTGGCTG-3′ (lower). The primers for mouse GAPDH were 5′-TCCCTCAAGATTGTCAGCAA-3′ (upper) and 5′-GATCCA CAACGGATACATT-3′ (reverse). Quantitative real-time PCR was carried out using a thermocycler (LightCycler; Roche Diagnostics Corp., Mannheim, Germany) and SYBR® Green PCR Master Mix (Applied Biosystems, Warrington Cheshire, UK) according to the

manufacturer recommendations. Cycle numbers obtained in the log-linear phase of the reaction were plotted against a standard curve prepared with serially diluted control samples.

Western blot analysis

Kidneys harvested from irARF mice were homogenized in warmed protein extraction buffer (0.1 M Tris, 0.01 M EDTA, 1% sodium dodecyl sulfate and 0.01 M dichloro-diphenyl-trichloro ethane at pH 8.0). After 2 min of incubation at 95°C, the homogenate was centrifuged (13000 r.p.m., 15 min). Supernatant was stored at −80°C. Samples (50 μg protein per lane) were run under reducing conditions on sodium dodecyl sulphate-polyacrylamide gels (4% stacking gel, 12% separating gel). Thereafter, gels were electroblotted onto nitrocellulose membranes. After blocking, blots were incubated overnight with a mouse monoclonal antiendostatin antibody (Upstate Biotechnology, Lake Placid, NY, USA) or anti-MMP-9 polyclonal antibody (Chemicon, Temecula CA, USA) or anti-mouse CD31 monoclonal antibody (BD Biosciences Pharmigen, LA, USA) and a horseradish peroxidase-conjugated goat anti-mouse antibody as the secondary antibody. Western blot signal was normalized by β-actin band density (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Bands were detected using enhanced chemiluminescence (ECL system; Amersham Bioscience, Piscataway, NJ, USA) in accordance with manufacturer guidelines.

Immunohistochemical staining for endostatin in mouse kidneys

Immunohistochemical analyses were performed at the Oncology Institute of the University of São Paulo School of Medicine. Paraffin sections (each 4 μm in thickness) were mounted onto SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA, USA), baked, deparaffinized and rehydrated. Heat-induced epitope retrieval was performed by immersion of the slides in 1 mM of EDTA (pH 8.0) and incubation in an electric pressure cooker (Decloaking Chamber; BioCare Medical, Walnut Creek, CA, USA) for 3 min at 25 psi. The slides were then placed in an automated immunohistochemical stainer (Ventana Medical Systems, Tucson, AZ, USA) to stain for endostatin. Endostatin labelling was performed using a monoclonal antibody (Upstate Biotechnology), at a dilution of 1:100 for 28 min at 42°C, an amplification kit and a basic diaminobenzidine detection system (Dako,

Carpinteria, CA, USA). The images were collected using a digital camera (DXM1200F; Nikon Instruments Inc., Melville, NY, USA) and analysed using the EclipseNet software for Nikon cameras.

Statistical analysis

Statistical analysis was performed between experimental groups by applying t-test. Significance was stated at $P < 0.05$. Data are expressed as mean ± SEM (standard error of the mean).

RESULTS

Renal function after ischaemia/reperfusion

Mice subjected to 45 min of ischaemia presented marked deterioration of renal function at 24 h after reperfusion. Blood urea nitrogen and plasma creatinine levels were significantly higher in ARF mice than in control mice (Fig. 2).

Quantitative real-time PCR

Based on quantitative real-time PCR assays, endostatin/collagen XVIII transcripts are upregulated as follow: 2.32-fold after 45 min of ischaemia, 1.34-fold after 12 h of reperfusion and 1.10-fold after 24 h of reperfusion (Fig. 3). There were no significant changes in endostatin signalling in the kidneys of control mice. In irARF mice, the GAPDH signal was not significantly altered.

Western blot analysis

Western blot analysis was performed to examine the presence of endostatin, MMP-9, an enzyme which converts collagen XVIII to endostatin and CD31, an endothelial cell marker. Western blot analysis of the total kidney proteins revealed an approximately 30 kDa endostatin isoform and
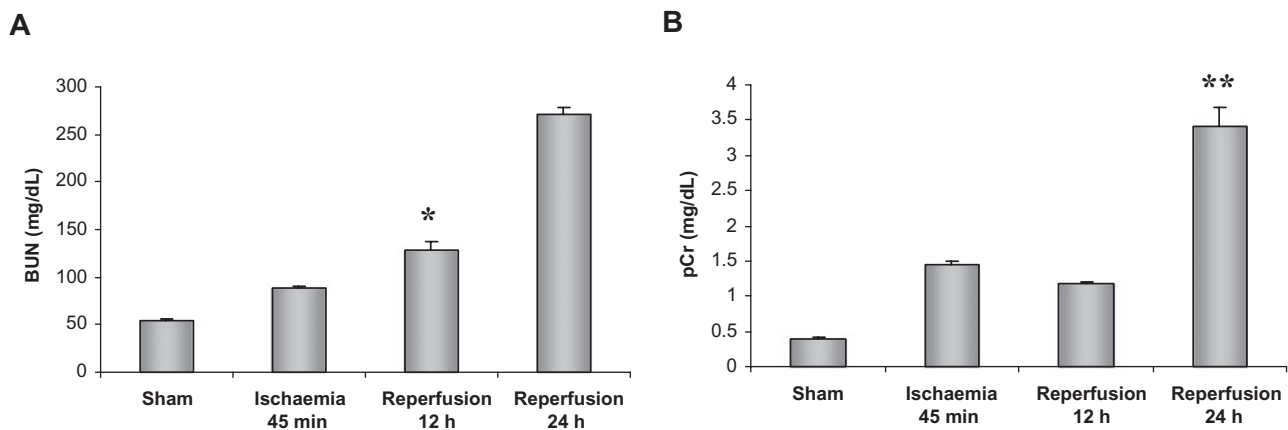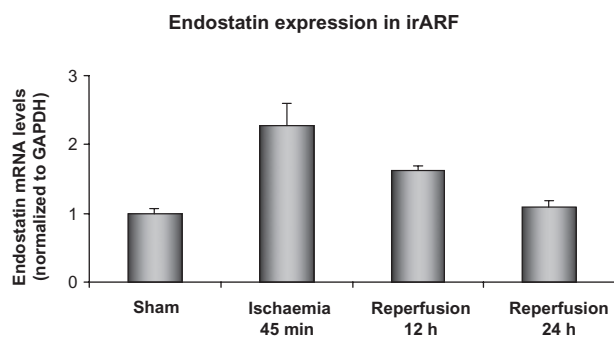
A

B

Fig. 2 Renal function measured by myeloperoxidase activity after 45 min of bilateral renal ischaemia, as well as at 12 h and 24 h after reperfusion, in C57BL/6 mice. Levels of (A) blood urea nitrogen (BUN) and (B) plasma creatinine (pCr) were determined ($n = 4$). *$P < 0.005$ and **$P < 0.05$.

Endostatin expression in irARF

Fig. 3 Endostatin/collagen XVIII mRNA expression during ischaemia/reperfusion-induced acute renal failure (irARF). Total RNA was isolated from mouse kidneys after 45 min of ischaemia followed by reperfusion, and endostatin/collagen XVIII mRNA expression was determined through quantitative real-time PCR using the LightCycler, as described in the Methods section ($n = 5$). $P < 0.05$.

demonstrated that endostatin expression was upregulated, being 2.14-fold during ischaemia, 1.74-fold after 12 h of reperfusion and 1.59-fold after 24 h of reperfusion (Fig. 4A,B). These findings corroborate the quantitative real-time PCR data (Fig. 3). The MMP-9 expression was significantly upregulated, 1.98-fold ($P < 0.05$), only in the ischaemic kidneys (Fig. 4C,D). The CD31 expression increased during the ischaemia/reperfusion, but the production of this endothelium marker was significantly different from sham-operated, only at 12 h (1.64-fold; $P < 0.05$) and 24 h of reperfusion (1.56-fold; $P < 0.05$) (Fig. 5).

Histological staining for endostatin in normal, ischaemic and irARF mouse kidneys

Immunohistochemical studies using polyclonal antibody showed increased endostatin expression in ischaemic kidneys and in kidneys reperfused for 12 h (Fig. 6D,E), respectively, consistent with the western blot analysis findings. It was found a positive staining in proximal tubule and collecting ducts (Fig. 6).

DISCUSSION

The pathophysiology of ischaemic ARF involves a complex interplay of tubular injury, inflammation and haemodynamic processes. Vascular endothelial cell injury and dysfunction play an important role in extending renal tubular injury.[4–7]

In normal kidneys, collagen XVIII is distributed throughout the tubular epithelium, Bowman's capsule, glomerular basement membranes and in the mesangial matrix.[25,26] Collagen XVII is processed by several proteases and MMP, including MMP-9, to generate endostatin.[12,13]

It is noteworthy that the regulation between endostatin and MMP occurs in both directions. Many proteases, including cysteine proteinase, serine proteinase and various MMP, are able to digest collagen XVIII, resulting in the release of endostatin-related fragments. Furthermore, it has been shown that endostatin is able to bind to some MMP, blocking their activity.[27]

In a study involving *in vitro* digestion assays, Heljasvaara *et al.* demonstrated that MMP are able to generate endostatin-specific cleavage sites of the full-length human collagen XVIII, and a recombinant C-terminal NC1 domain of human collagen XVIII.[27] These authors used MMP from different sources and observed that MMP-7, MMP-9 and MMP-13 each generated at least one fragment of approximately 30 kDa. Those fragments also demonstrated biological activity in the endothelial cell proliferation assay. In a recent study *in vivo*, expressions of MMP-2 and MMP-9 were observed in a rat model of ischaemia and reperfusion.[28] The immunofluorescence results obtained by these authors suggested that these MMP show the strongest intensities in the tubulointerstitial space of the outer medulla, where the peritubular capillaries are located. Ischaemia increased pro-MMP-9 and MMP-9 expression, as well as pro-MMP-2 expression, but not MMP-2 expression, in renal endothelial fractions.[29] On the other hand, it was demonstrated that endostatin can inhibit the activity of some MMP. Endostatin binds directly to MMP-2, blocking its activity, and it also inhibits the activity of MT1-MMP.[30,31] Additionally, the direct binding of endostatin and MMP-9 *in vitro* implies an interaction that could be important in the prevention of pro-MMP-9 activation.[32]

In this study, using RT-PCR and western blot analysis, we observed an increase in a 30 KDa endostatin expression in ischaemic and reperfused mouse kidneys. A significant increase in MMP-9 expression in ischaemic kidneys was also observed. The presence of this MMP is of interest with regard to its potential role in endostatin generation. We next examined the expression of the endothelial cell marker CD31, and we found a significant increase only at 12 h and 24 h of reperfusion. Also of interest is the opposite variation in protein expression between endostatin and CD31, suggesting an antiangiogenic effect of local endostatin ischaemic kidneys. The results of these studies raise the possibility that in irARF endostatin expression may be upregulated by hypoxia. The upregulation of endostatin has been described in the bladder of rats and the lung of mice submitted to hypoxia.[33,34] The results of such *in vivo* studies demonstrate that reduced oxygen supply produces a highly organ-specific pattern of endostatin fragments.

The immunohistological data show the presence of endostatin in the glomerulus and in the tubulointerstitium, suggesting that it plays a role in the vascular remodeling of glomerular and peritubular capillaries. This finding suggests that increased endostatin expression serves to direct the antiangiogenic effects of locally released endostatin towards these cells, although its precise mechanism remains unclear. Another antiangiogenic molecule, angiostatin, was detected after ischaemic ARF in a study involving rats.[34] The authors of that study used western blot assays to study angiostatin expression and found that the 38 kDa and 50 kDa forms of angiostatin were enhanced in the first 3 days following ischaemia.
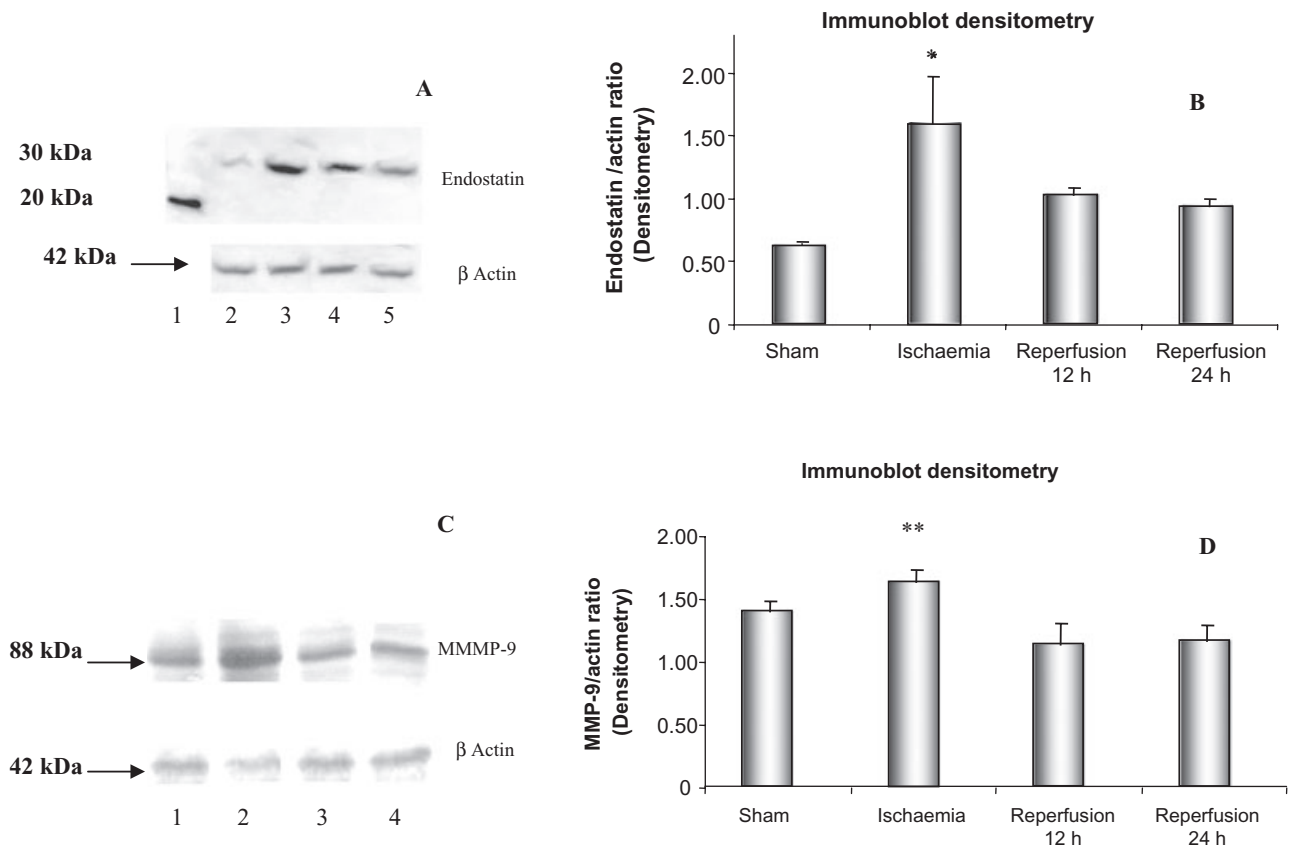
Fig. 4 Expression of endostatin and matrix metalloproteinase-9 (MMP-9) in kidney in response to acute renal failure induced by ischaemia/reperfusion. (A) Lane 1: Recombinant endostatin; Lane 2: Kidneys of sham-operated; Lane 3: Kidneys after 45 min of ischaemia; Lanes 4 and 5: Kidneys 12 h and 24 h of reperfusion (respectively). (B) Each band was scanned and subject to densitometry, intensities of endostatin bands are shown in relation to those obtained for β actin ($n = 4$). *$P < 0.05$. (C) Lane 1: Kidneys of sham-operated; Lane 2: Kidneys after 45 min of ischaemia; Lanes 3 and 4: Kidneys 12 h and 24 h of reperfusion (respectively). (D) Each band was scanned and subject to densitometry, intensities of MMP-9 bands are shown in relation to those obtained for β actin ($n = 4$). **$P < 0.05$.
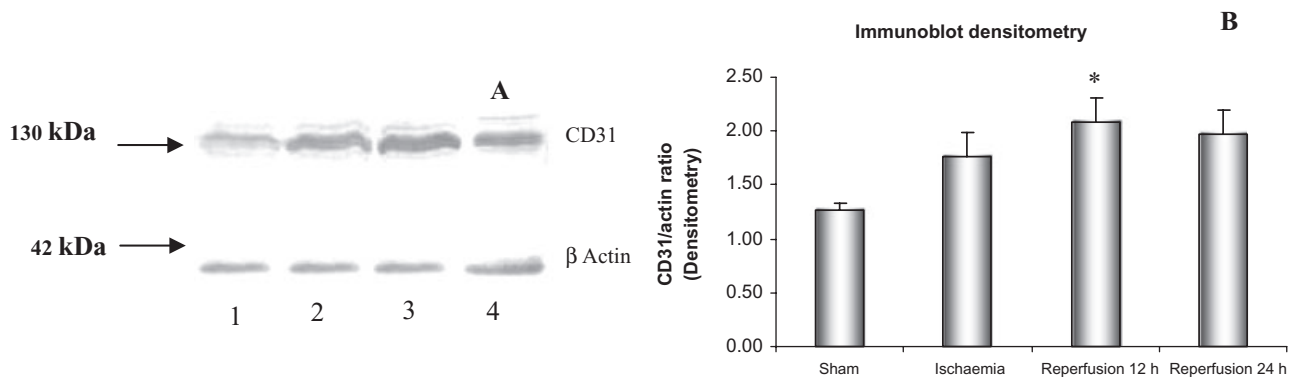
Fig. 5 Expression of CD31 in kidney in response to acute renal failure induced by ischaemia/reperfusion. (A) Lane 1: Kidneys of sham-operated; Lane 2: Kidneys after 45 min of ischaemia; Lanes 3 and 4: Kidneys after 12 h and 24 h of reperfusion (respectively). (B) Each band was scanned and subject to densitometry, intensities of CD31 protein are shown in relation to those obtained for β actin ($n = 4$). *$P < 0.05$.
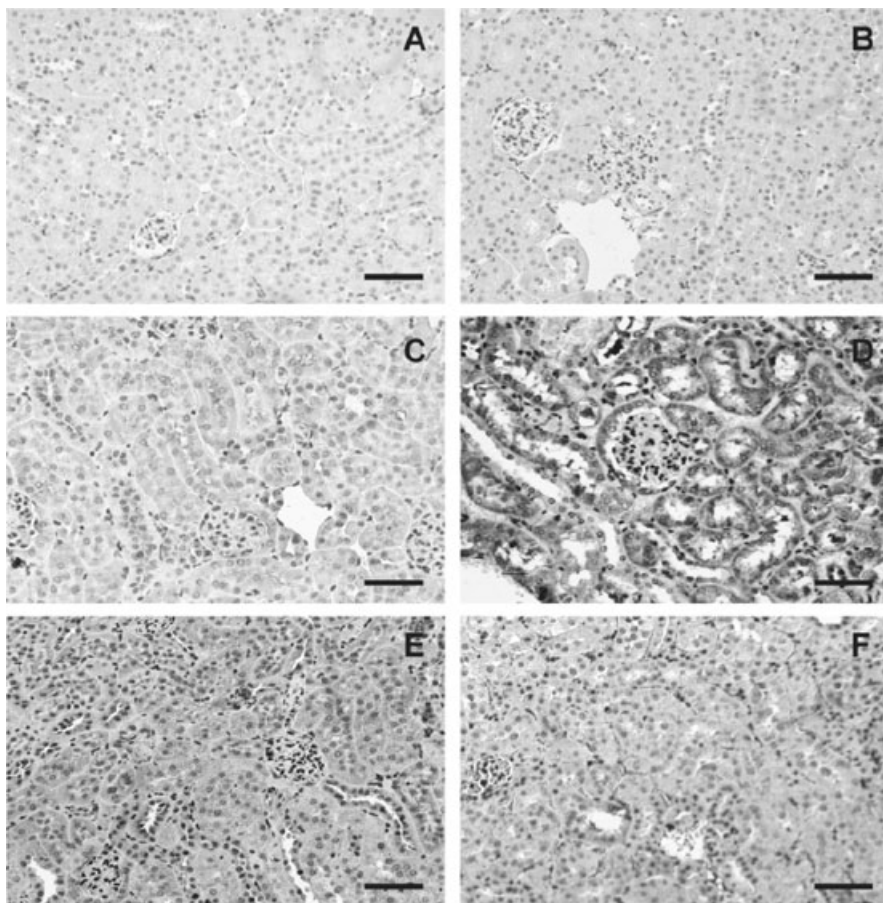
Fig. 6 Immunohistochemical staining for endostatin in normal mouse kidneys and in mouse kidneys subjected to ischaemia/reperfusion. (A) and (B) Negative control staining using normal rabbit serum instead of rabbit polyclonal antibody; (C) Normal kidney showing diffuse cytoplasmic staining for endostatin in collecting ducts and in the loop of Henle; (D) Kidney after 45 min of ischaemia, showing strong staining in the collecting duct and proximal tubule; (E) Kidney after 12 h of reperfusion, showing a weak staining for endostatin in the collecting duct cells and tubules; (F) Kidney after 24 h of reperfusion, showing weak and diffuse staining compared with renal tissue from normal kidneys, ischaemic kidneys and kidneys reperfused for 12 h (Original magnification ×100).

In the present study, we have demonstrated, for the first time, the presence and upregulation of endostatin mRNA and protein in a mouse model of ischaemia/reperfusion injury. In addition, our findings indicate the local synthesis of a 30 kDa endostatin-related fragment following ARF and suggest its role in the modulation of renal capillary density.

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