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Renal ischemia-reperfusion increases endothelial VEGFR-2 without increasing VEGF or VEGFR-1 expression

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Renal ischemia-reperfusion increases endothelial VEGFR-2 without increasing VEGF or VEGFR-1 expression.

Background. Hypoxia is a potent stimulus to angiogenesis. Expression of the angiogenic growth factor vascular endothelial growth factor (VEGF) and its receptors (VEGFR-1 and VEGFR-2) is up-regulated by hypoxia in a variety of organs and cell lines. We have previously reported that VEGF expression is not increased in renal ischemia-reperfusion injury, although tubular cells concentrate VEGF at their basolateral surface. In this study we assess whether altered VEGF receptor expression compensates for the lack of VEGF regulation during renal ischemia-reperfusion injury.

Methods. VEGFR-1 mRNA expression was assessed by Northern blotting and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR). VEGFR-2 mRNA expression was analyzed by Northern blotting and in situ hybridization (ISH), while VEGFR-2 protein expression was studied using immunohistochemistry. VEGF mRNA expression was assessed by ISH.

Results. VEGFR-2 mRNA and protein expression were up-regulated without an increase in VEGF or VEGFR-1 expression. Normal kidneys showed low-level VEGFR-2 mRNA and protein expression in glomerular and peritubular endothelium. Following ischemia and ischemia-reperfusion, a marked increase in VEGFR-2 mRNA and protein expression was seen (2- to 4-fold). Most prominent was VEGFR-2 mRNA up-regulation in the glomerulus although, surprisingly, increased protein was not demonstrated here. ISH showed that VEGF mRNA was not up-regulated in this model, confirming our previous findings for VEGF.

Conclusion. VEGF and VEGFR-1 expression are not increased by renal ischemia and ischemia-reperfusion injury. Instead, endothelial expression of VEGFR-2 is increased. VEGFR-2 up-regulation in renal ischemia-reperfusion may be important in mediating the mitogenic and anti-apoptotic actions of VEGF on endothelial cells, thereby preserving the integrity of the endothelium and the potential for blood supply to ischemic tissues.

Vascular endothelial growth factor (VEGF) is a potent multifunctional cytokine that promotes angiogenesis, increases vascular permeability and is chemotactic for monocytes [1, 2]. VEGF has been shown to have a role in a wide variety of situations including embryogenesis, placental growth, tumor growth, diabetes, wound healing, inflammatory responses and tissue remodeling [2, 3]. There are two well established receptors for VEGF previously described as flt-1 and KDR/flk-1, and now designated VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2), respectively [4–6]. VEGF also binds to the co-receptors neuropilin-1 and neuropilin-2 [7, 8]. VEGFR-1 and VEGFR-2 are type III tyrosine kinases predominantly expressed on endothelial cells. VEGF acts via these receptors stimulating anti-apoptotic actions [9, 10] and endothelial cell division and migration [11–13].

Studies performed in vitro have demonstrated similarities between the regulation of VEGF, VEGFR-1 and erythropoietin (EPO). The expression of VEGF mRNA has been shown to be markedly increased in a variety of cells when they are made hypoxic or when hypoxia-mimicking techniques are used [14–20]. Evidence suggests that this occurs via the activation of a heme-containing, oxygen-sensing receptor that ultimately leads to the induction of the transcription factor hypoxia-inducible factor-1 (HIF-1) [21]. Studies performed in vivo also have shown that VEGF mRNA increases in response to hypoxia in most organs [19, 22–24]. The kidney appears to differ in this regard as we [1] and others [25, 26] have previously demonstrated. We recently reported that VEGF mRNA and protein expression were not increased in acute renal ischemia and ischemia-reperfusion injury [1]

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in contrast to the findings in other organs. The reason for this difference is unknown.

Like VEGF, the VEGFR-1 promoter region contains a heptamer sequence matching the HIF-1 consensus-binding site. Mutations of this site decrease transcriptional activation by hypoxia [27]. Although the VEGFR-2 promoter region has no HIF-1 consensus, it does contain potential nuclear factor-κB (NF-κB) and activator protein-2 (AP-2) sites [28]. While the hypoxia-responsiveness of VEGFR-2 remains contentious in vitro, this is not the case in vivo where several studies have shown that it is up-regulated. Up-regulation of mRNA for both VEGFR-1 and VEGFR-2 has been demonstrated in rat lungs exposed to acute and chronic hypoxia [24] and in rat hearts from a myocardial infarction model [29]. VEGFR-1 protein was increased in brain tissue from rat cerebral infarction models [30]. VEGFR-2 but not VEGFR-1 mRNA and protein have been reported to be up-regulated in a mouse model of ischemia-induced retinal neovascularization [31].

Despite several studies in a variety of organs, there are few reports in the literature examining VEGF receptor expression in renal hypoxia or ischemia-reperfusion. In addition, the lack of hypoxia-responsiveness demonstrated by VEGF in renal ischemia-reperfusion [1] suggests that there may be a response mediated at the receptor level. The present studies demonstrate that VEGFR-2 mRNA and protein expression are up-regulated in renal ischemia-reperfusion injury, while VEGF and VEGFR-1 expression remain unchanged. These data suggest that signaling through VEGFR-2 may be important in the survival of endothelial cells in the kidney following ischemic injury.

METHODS

Ischemia-reperfusion injury

Outbred male Sprague-Dawley rats were purchased from Monash University Central Animal Services (Clayton, Victoria, Australia) and given free access to food and water. The renal ischemia-reperfusion model was produced as previously reported [1]. Briefly, rats were anaesthetized with intraperitoneal injections of sodium pentobarbitone (6 mg/100 g body weight; Nembutal, Abbott Laboratories, North Ryde, NSW, Australia) and a midline peritoneal incision was made. The left renal pedicle was clamped for 40 minutes using non-traumatic vascular clamps. Variable periods of reperfusion followed (0, 20, 40 and 80 min) and the left kidneys were then harvested and processed. The 0 minute time point, therefore, refers to the ischemic kidneys that were not reperfused, whereas the 20, 40, and 80 minute time points indicate 40 minutes of ischemia followed by the stated periods of reperfusion. The right contralateral kidneys were removed simultaneously with the left and used for comparison. Four rats were used at each time point, giving a total of sixteen left ischemia-reperfused (LIR) kidneys, and sixteen contralateral right normal (RN) kidneys. Four normal and two sham-operated kidneys were used also for comparison.

Synthesis of VEGF and VEGFR-2 riboprobes

These were performed as previously described [32, 33]. Briefly, sense and anti-sense RNA probes for VEGF and VEGFR-2 were generated by in vitro transcription (Promega, Madison, WI, USA). Linearized template (500 ng) was added to a reaction mixture of transcription buffer (final volume 20 μL) containing 6 mmol/L dithiothreitol (DTT), 333 μmol/L each of adenosine 5′-triphosphate (ATP), carbamylated protein (CTP) and guanosine 5′-triphosphate (GTP), 12 μmol/L uridine triphosphate (UTP), 100 μCi [^{33}P]UTP (2,000 Ci/mmol, New-Dupont, Boston, MA, USA), 20 U RNAsin (Boehringer-Mannheim, Mannheim, Germany) and 20 mU RNA polymerase (Boehringer-Mannheim). The reaction mixture was incubated at 37°C for 90 minutes, after which the DNA template was digested with 1 U RNAse-free DNAse for 15 minutes. The riboprobe was precipitated by ammonium acetate and ethanol using yeast tRNA as a carrier and then reconstituted in 100 μL of water. Purified riboprobe length was adjusted to approximately 150 bases by alkaline hydrolysis.

In situ hybridization for VEGF and VEGFR-2 mRNA

Four micrometer-thick sections were cut from paraformaldehyde-fixed paraffin-embedded kidney tissue. Tissue sections were dewaxed, rehydrated, and equilibrated in P buffer [50 mmol/L Tris-HCl, pH 7.5, 5 mmol/L ethylenediaminetetraacetic acid (EDTA)], and then incubated in 125 μg/mL Pronase E (Sigma Chemical Co., St. Louis, MO, USA) in P buffer for 10 minutes at 37°C. Sections were then washed in 0.1 mol/L sodium phosphate buffer (pH 7.2), briefly refixed in 4% paraformaldehyde for 10 minutes, rinsed in milliQ water, dehydrated in 70% ethanol, and air-dried. Hybridization buffer containing 2×10^4 cpm/μL riboprobe in 300 mmol/L NaCl, 10 mmol/L Tris-HCl (pH 7.5), 10 mmol/L Na_2HPO_4, 5 mmol/L EDTA (pH 8.0), 1 × Denhardt's solution, 50% formamide, 17 mg/mL yeast RNA, and 10% wt/vol dextran sulfate was heated to 85°C for five minutes. A 25 μL aliquot of this solution was then applied to each tissue section under coverslips. Hybridization of tissue to the riboprobe was performed overnight at 60°C in 50% formamide-humidified chambers. Sense probes were used on a further set of tissue sections as controls. After hybridization, slides were washed in 2 × standard sodium citrate (SSC) containing 50% formamide prewarmed to 50°C to remove coverslips. Sections were then washed for one hour at 55°C, rinsed three times in RNAse buffer (10 mmol/L Tris-HCl, pH 7.5,

1 mmol/L EDTA, pH 8.0, 0.5 mol/L NaCl), and then incubated with RNAse A (150 μg/mL) for one hour at 37°C. Sections were later washed in 2 × SCC for 45 minutes at 55°C, dehydrated in graded ethanol, air-dried, and exposed to Kodak X-Omat autoradiographic film (Eastman Kodak, Rochester, NY, USA) for one to three days. Slides were then dipped in Ilford K5 nuclear emulsion (Ilford, Mobberley, UK), stored in a light-free box with dessicant at room temperature for two to three weeks, and developed in Kodak D19, followed by fixation with Ilford Hypam. Sections were then stained with hematoxylin and eosin (H&E) for examination under light microscopy.

Isolation of total RNA

Total RNA was extracted from tissues using Trizol (Life Technologies, Gibco BRL, Melbourne, Australia). Whole tissue samples were homogenized in Trizol using a homogenizer (pro200; Proscientific Inc., Oxford, CT, USA). Sample RNA levels were quantitated by reading the absorbance at 260 nm. Final samples were stored at −70°C until required for Northern blot analysis and semiquantitative reverse transcription-polymerase chain reaction (RT-PCR).

Northern blotting

Vascular endothelial growth factor receptor-1 and VEGFR-2 cDNA inserts were PCR-amplified from reverse transcribed rat kidney RNA using primer sequences obtained from the known rat receptor sequences (Genebank accession nos. D28498 and U93306, respectively) [34, 35]. Primers for the VEGFR-1 insert (forward 5′-CAA GGGACTCTACACTTGTC-3′ and reverse 5′-CCGA ATAGCGAGCAGATTTC-3′) resulted in a 240 bp product corresponding to a portion of the extracellular domain (amino acid residues 305–384). Primers for the VEGFR-2 insert were as described by Wen and co-workers [34] (forward 5′-GCCAATGAAGGGGAACTGA AGAC-3′ and reverse 5′-TCTGACTGCTGGTGATG CTGTC-3′). These produced a 537 bp product corresponding to the intracellular, amino terminal end of the tyrosine kinase domain of the receptor (amino acid residues 870–1049). The PCR products were cloned into pGEM-T easy (Promega) and the DNA sequences confirmed by sequencing. The VEGFR-1 insert was excised from the vector using the restriction enzyme *EcoR1* (Promega), while the VEGFR-2 insert was excised using *NcoI* and *SalI* (Promega). A mouse GAPDH insert that cross-hybridizes with rat GAPDH was obtained as a 1.2 kb PstI fragment in clone pHcGAP [36].

Total RNA samples were fractionated on a 1% agarose-formaldehyde gel and transferred to Genescreen Plus membranes (NEN Life Sciences, Boston, MA, USA). Membranes were cross-linked using a Stratalinker (Stratagene, La Jolla, CA, USA) and then prehybridized for one hour at 65°C using Rapid Hyb buffer (Amersham International, Little Chalfont, Buckinghamshire, UK). Inserts were labeled using the Megaprime DNA Labeling System (Amersham) and added to fresh Rapid Hyb buffer at 2×10^6 counts/mL hybridization fluid. Membranes were hybridized for two hours at 65°C and then washed three times for 20 minutes each [first wash in (2 × SSC)/0.1% sodium dodecyl sulfate (SDS) at 65°C, second wash in (1 × SSC)/0.1% SDS at 65°C, third wash in (0.1 × SSC)/0.1% SDS at room temperature], prior to exposure to x-ray film (Kodak). Bands were analyzed by densitometry (Molecular Dynamics Computing Densitometer, Model 300A; Sunnyvale, CA, USA) using ImageQuant Software (version 3.0).

Semiquantitative RT-PCR

This assay was performed as previously described [1] using a modification of published methods [37, 38]. Briefly, first strand cDNA was synthesized from 1 μg of total RNA using AMV-reverse transcriptase (Promega). The subsequent PCR reaction used the VEGFR-1 primers already described for Northern blotting experiments, producing a 240 bp product. The PCR reaction was interrupted at five cycle intervals and the products were analyzed. Levels of VEGFR-1 mRNA expression were assessed by comparing the density of bands on 2% agarose gels, and the cycle of first appearance of PCR products. Amplification of a 254 base pair product representing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to check the efficiency of the reverse transcription and of the PCR reaction. GAPDH PCR profiles were compared and equalized. Duplicate amplifications were performed for each sample and the results were shown to be reproducible.

Immunohistochemical staining of rat kidney for VEGFR-2

Paraffin-embedded rat kidney tissue sections (4 μm-thick) were fixed in 4% paraformaldehyde and analyzed for the presence of VEGFR-2 protein. The primary antibody used was N-931 (Santa Cruz, Biotechnology Inc., Santa Cruz, CA, USA), a polyclonal anti-mouse VEGFR-2 antibody directed against amino acid residues 931–997. Endogenous peroxidase was quenched with 4% hydrogen peroxide in methanol. Non-specific binding was blocked using 10% swine serum prior to incubation with primary antibodies overnight at 4°C (concentration of 1 μg/mL). Negative controls used normal rabbit IgG (Sigma) in place of N-931, at the same concentration as the primary antibody. A peroxidase kit was used for subsequent steps (LSAB 2 Peroxidase kit; Dako, Carpinteria, CA, USA) followed by development of staining with diaminobenzidine (DAB; Dako). Counterstaining was performed with hematoxylin.

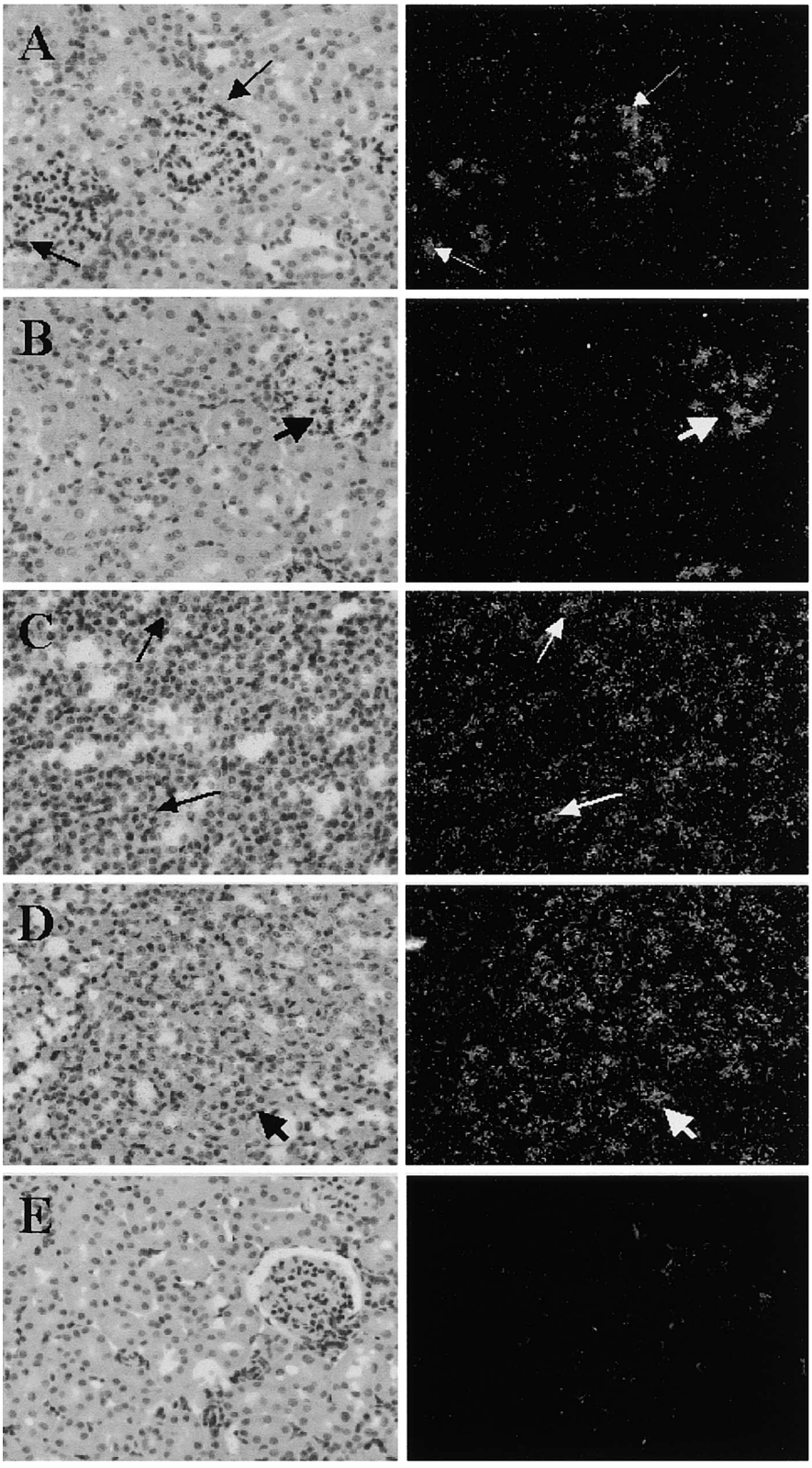

Fig. 1. In situ hybridization for vascular endothelial growth factor (VEGF) mRNA in normal, ischemic and control kidneys probed with anti-sense. Panels are in pairs; bright field photographs of tissues stained with hematoxylin and eosin on the left with corresponding dark field images on the right. A positive signal is shown by dark granules on bright field images, with bright granules on corresponding dark field photographs. (*A*) Normal rat cortex showing VEGF mRNA expression in podocytes (arrows) with low-level expression in tubular epithelial cells. (*B*) The cortex from ischemic kidneys with no reperfusion was similar to that of normal kidneys. (*C*) Medulla from normal rat kidneys showing VEGF mRNA expression in tubular epithelial cells (arrows). (*D*) The appearance in ischemic kidneys was similar. All ischemic, ischemia-reperfused (not shown), and control kidneys had a similar appearance. (*E*) Normal kidney probed with a sense riboprobe. There was no difference in VEGF mRNA expression by in situ hybridization between the various groups of kidneys. Magnifications: ×160.

Statistics

Northern blots were compared using ANOVA and Dunnett's multiple comparison test. A P value <0.05 was considered to be significant.

RESULTS

VEGF mRNA expression

We have previously reported the expression of VEGF protein (by immunohistochemistry and Western blotting analysis) and mRNA (by semiquantitative RT-PCR and Northern blotting analysis) in this model [1]. To confirm these findings, VEGF mRNA was assessed by in situ hybridization (Fig. 1). In normal rat kidney, VEGF mRNA was localized predominantly to glomerular epithelial cells (Fig. 1A) and medullary tubular epithelial cells (Fig. 1C). These areas showed prominent VEGF mRNA expression. Ischemic (Fig. 1B), ischemia-reperfused (Fig. 1D), and control kidneys (not shown) all demonstrated a similar appearance to the normal kidneys (Fig. 1, A, C). Use of a sense riboprobe showed no signal (Fig. 1E). These findings were consistent with results previously reported [1], indicating that there was no difference in VEGF mRNA expression between the various groups.

VEGFR-1 mRNA expression

VEGFR-1 mRNA expression was assessed by semiquantitative RT-PCR (Fig. 2) and Northern blotting analysis (Fig. 3). Both of these methods showed no difference in VEGFR-1 mRNA expression between the various groups of kidneys. By semiquantitative RT-PCR, the band representing VEGFR-1 mRNA first appeared at 35 cycles (Fig. 2). Bands from normal, ischemic and ischemia-reperfused kidneys showed no significant difference when assessed by densitometry. The PCR profiles for VEGFR-1 and GAPDH were similar for all kidneys. Semiquantitative RT-PCR experiments for VEGFR-1 mRNA expression were performed on three groups of rats and shown to be reproducible. Northern blotting analysis confirmed these findings (Fig. 3), with similar renal VEGFR-1 mRNA expression being demonstrated in all rats. Densitometric analysis (Fig. 3B) showed no change in VEGFR-1 mRNA expression following renal ischemia and ischemia-reperfusion. Northern blotting analysis was performed on three rats at each time point, with similar results.

VEGFR-2 mRNA expression

VEGFR-2 mRNA expression was assessed by Northern blotting analysis (Fig. 3) and in situ hybridization (Figs. 4 and 5). Both techniques showed VEGFR-2 mRNA expression to be markedly up-regulated in the ischemic and ischemia-reperfused kidneys compared

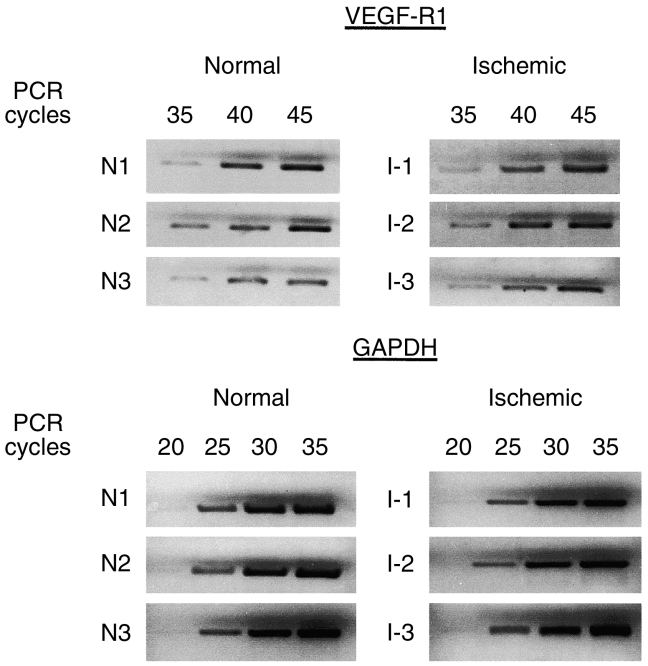

Fig. 2. Semiquantitative RT-PCR of mRNA, ischemia-reperfusion model RT-PCR profiles for VEGFR-1 mRNA (upper six panels) and GAPDH mRNA (lower six panels) from normal and ischemic kidneys (40 min ischemia, no reperfusion). N1, 2 and 3 represent different normal kidneys. I-1, -2 and -3 represent different ischemic kidneys. Samples were titrated to obtain equal intensity bands representing GAPDH mRNA at 25 PCR cycles. The PCR product representing VEGFR-1 mRNA is demonstrated in the upper six panels (size 240 bp). Bands representing VEGFR-1 mRNA first appeared at 35 PCR cycles with appearances between all groups being similar. Profiles for PCR products from the reperfused kidneys (not shown) had a similar appearance to the ischemic, normal and control kidneys.

with normal and control kidneys. Northern blotting analysis demonstrated a single mRNA species of approximately 6.8 kb (Fig. 3A), consistent with the known size for VEGFR-2 mRNA [4, 5]. Samples from normal and control kidneys showed only a faint band for VEGFR-2 mRNA. By contrast, ischemic and ischemia-reperfused kidneys showed a prominent band (Fig. 3A). Densitometric analysis of Northern blots demonstrated that VEGFR-2 mRNA expression in the ischemic and ischemia-reperfused kidneys was increased two- to fourfold compared to normal and control kidneys (Fig. 3B). The level of VEGFR-2 mRNA expression was similar in all the kidneys subjected to ischemia, regardless of the reperfusion time (0, 20, 40 or 80 min).

In situ hybridization demonstrated VEGFR-2 mRNA expression in normal glomeruli localized predominantly to the endothelial cells (Fig. 4 A, C, E). In the interstitium, peritubular capillaries also showed VEGFR-2 mRNA expression (Fig. 5A), although this was less prominent than that seen in glomerular endothelial cells. These appearances were consistent with the notion that VEGFR-2 is predominantly expressed on endothelial

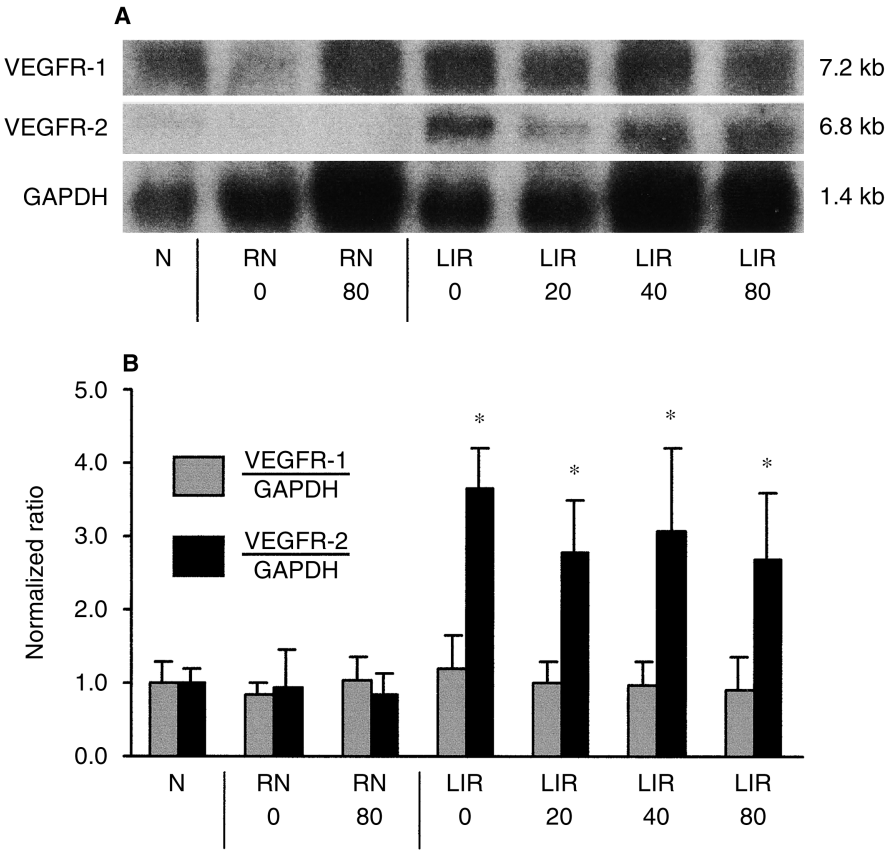

Fig. 3. Northern blotting analysis of total RNA samples from the ischemia-reperfusion model Northern blots demonstrating mRNA for VEGFR-1 and VEGFR-2 relative to GAPDH mRNA. A single mRNA species was identified for each receptor. VEGFR-1 was at 7.2 kb and VEGFR-2 at 6.8 kb. (*A*) Normal kidney (N; lane 1), representative contralateral right normal kidneys (RN; lanes 2 and 3), left ischemia-reperfused kidneys (LIR; lanes 4-7). 0, 20, 40 and 80 refer to reperfusion time in minutes. The Northern blot is representative of three separate experiments. VEGFR-1 expression appears similar in all samples whereas VEGFR-2 mRNA expression is increased in the LIR kidneys. (*B*) Densitometric analysis of the Northern blots in panel A. Symbols are: Results are from three separate rats, at each time point. Symbols are: (■) VEGFR-1 GAPDH; (■) VEGFR-2 GAPDH. VEGFR-2 mRNA levels are increased in the LIR kidneys (2- to 4-fold) compared to normal and control kidneys. VEGFR-1 mRNA expression is unchanged. Data are mean ± SD (* $P < 0.05$ vs. normal; Dunnett's multiple comparison test).

cells. A marked increase in VEGFR-2 mRNA expression was seen in the ischemic and ischemia-reperfused kidneys in both the glomerulus (Fig. 4 B, D, F) and renal interstitium (Fig. 5 B, C). Glomeruli from ischemic kidneys (Fig. 4 B, D, F) showed marked expression of VEGFR-2 mRNA compared to normal (Fig. 4 A, C, E) and control kidneys (not shown) in a pattern consistent with localization to the glomerular endothelial cells. However, the signal was so intense that it proved impossible to determine whether glomerular mesangial cells, in particular, expressed VEGFR-2 mRNA. The cortical (Fig. 5C) and medullary interstitium (Fig. 5B) of ischemic kidneys also showed increased VEGFR-2 mRNA expression compared to normal (Fig. 5A) and control kidneys (not shown), with localization predominantly to peritubular capillary endothelium. Tubular epithelial cells also demonstrated some evidence of VEGFR-2 mRNA expression, with some of the ischemic tubules clearly showing a signal overlying these cells rather than peritubular capillary structures (Fig. 5 B, C).

VEGFR-2 protein expression

VEGFR-2 protein expression was assessed immunohistochemically using the polyclonal antibody, N-931. It showed interstitial staining in normal, ischemic and ischemia-reperfused (data not shown) kidneys that was consistent with the in situ hybridization results (Fig. 6 D, F). Normal (Fig. 6 C, D) and control kidneys (not shown) demonstrated slight glomerular (Fig. 6C) and peritubular (Fig. 6D) endothelial cell staining. These appearances were similar to those that we have previously reported in normal rat kidney using the N-931 antibody [39]. It was difficult to identify any change in VEGFR-2 protein in glomeruli from ischemic (Fig. 6E) and ischemia-reperfused kidneys (not shown) by immunohistochemistry using the N931 antibody. In the interstitium of ischemic (Fig. 6F) and ischemia-reperfused kidneys (not shown), however, VEGFR-2 was increased and appeared to be predominantly expressed on peritubular capillary endothelium. In addition, there was some generalized staining of renal tubular epithelium in the kidneys from all groups, particularly in the medulla (Fig. 6 D, F).

The immunohistochemical appearances in control kidneys (right contralateral non-ischemic kidneys; not shown) resembled those of normal kidneys (Fig. 6 C, D). Negative controls demonstrated no significant staining (Fig. 6 A, B).

DISCUSSION

These studies demonstrate the up-regulation of VEGFR-2 mRNA and protein in a rat model of renal

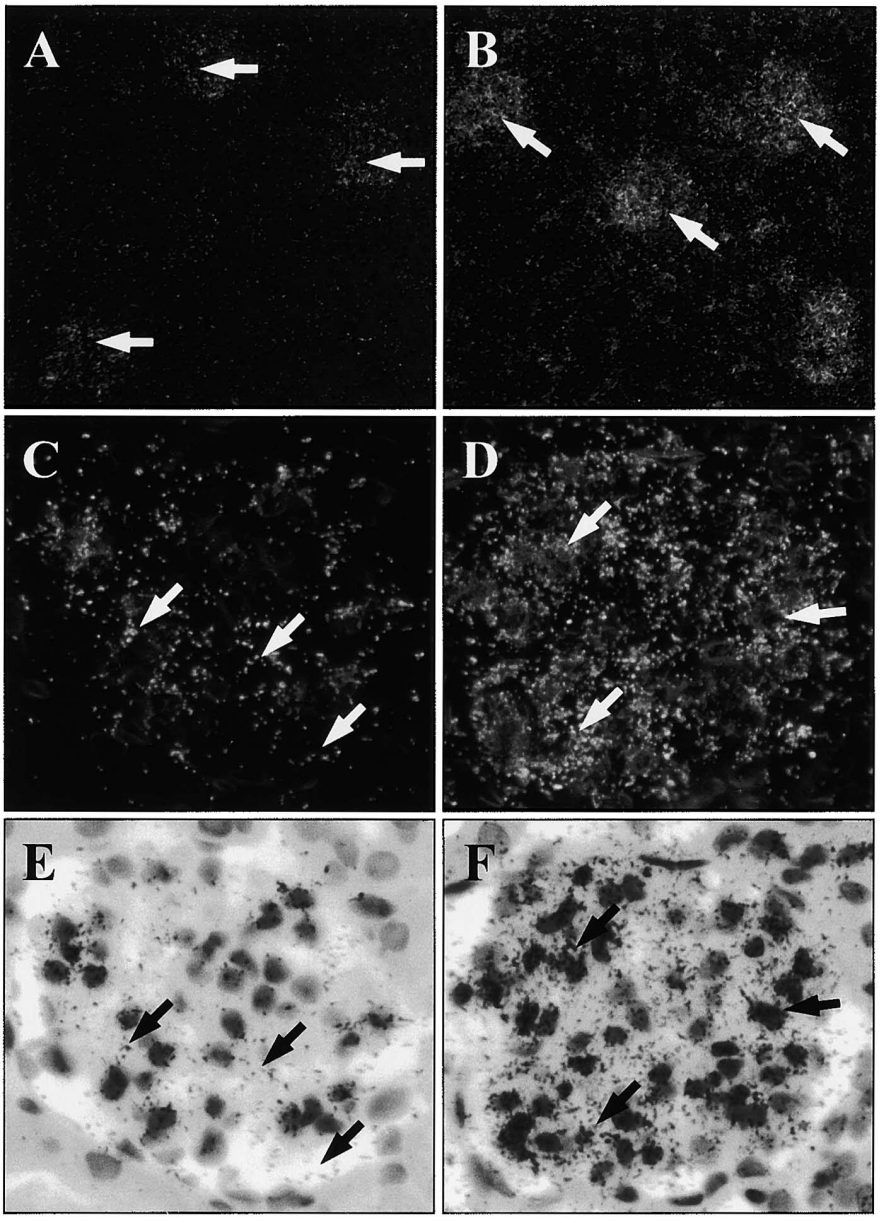

Fig. 4. In situ hybridization for VEGFR-2 mRNA in normal (*A*, *C* and *E*) and ischemic (*B*, *D* and *F*) rat kidney. Glomerular appearances. (A–D) Dark field images. (E and F) Bright field images stained with hematoxylin and eosin, corresponding to panels C and D, respectively. Normal glomeruli (A, C and E) showed low-level VEGFR-2 mRNA expression, localized to glomerular endothelium (arrows). By contrast, ischemic kidneys (B, D and F) showed a marked increase in VEGFR-2 mRNA expression (arrows). The appearances are consistent with localization to glomerular endothelium, although the intensity of the signal makes it difficult to say with certainty whether or not other glomerular cells are positive. All the ischemic kidneys in the figure were not subjected to reperfusion, although reperfused kidneys (not shown) had a similar appearance. Ischemic rat kidney probed with sense demonstrated virtually no signal (not shown). Magnifications: A and B ×150, C–F ×350.

ischemia-reperfusion injury. By contrast, VEGF and VEGFR-1 mRNA expression were not increased in response to the injury. These findings occurred despite the presence of HIF-1 binding sites in the promoter regions of the *VEGF* and *VEGFR-1* genes, and the lack of such a site in the promoter region of the *VEGFR-2* gene [17, 27]. We have previously reported the absence of increased VEGF mRNA and protein in this model [1], and have now confirmed this finding using in situ hybridization. These results are in contrast to the findings reported in other organs such as the lung and heart, which show up-regulation of both VEGF receptors [24] in addition to VEGF [22–24]. They are not consistent with models where renal hypoxia is induced with carbon monoxide or low ambient oxygen concentration [40]. In this circumstance, VEGF mRNA was increased in tubular cells but decreased in the glomerulus. VEGFR-2 was unchanged and VEGFR-1 was increased in the vasculature. These results suggest that there may be a different signaling pathway for hypoxia induced by reduced oxygen loading of hemoglobin in the lungs compared with ischemia, where local blood flow is severely reduced and leads, secondarily, to tissue hypoxia.

In situ hybridization studies suggested that VEGFR-2 mRNA expression in the interstitium was predominantly localized to endothelial cells, consistent with the known expression of this receptor [41, 42]. Peritubular capillary endothelium showed a marked increase in VEGFR-2

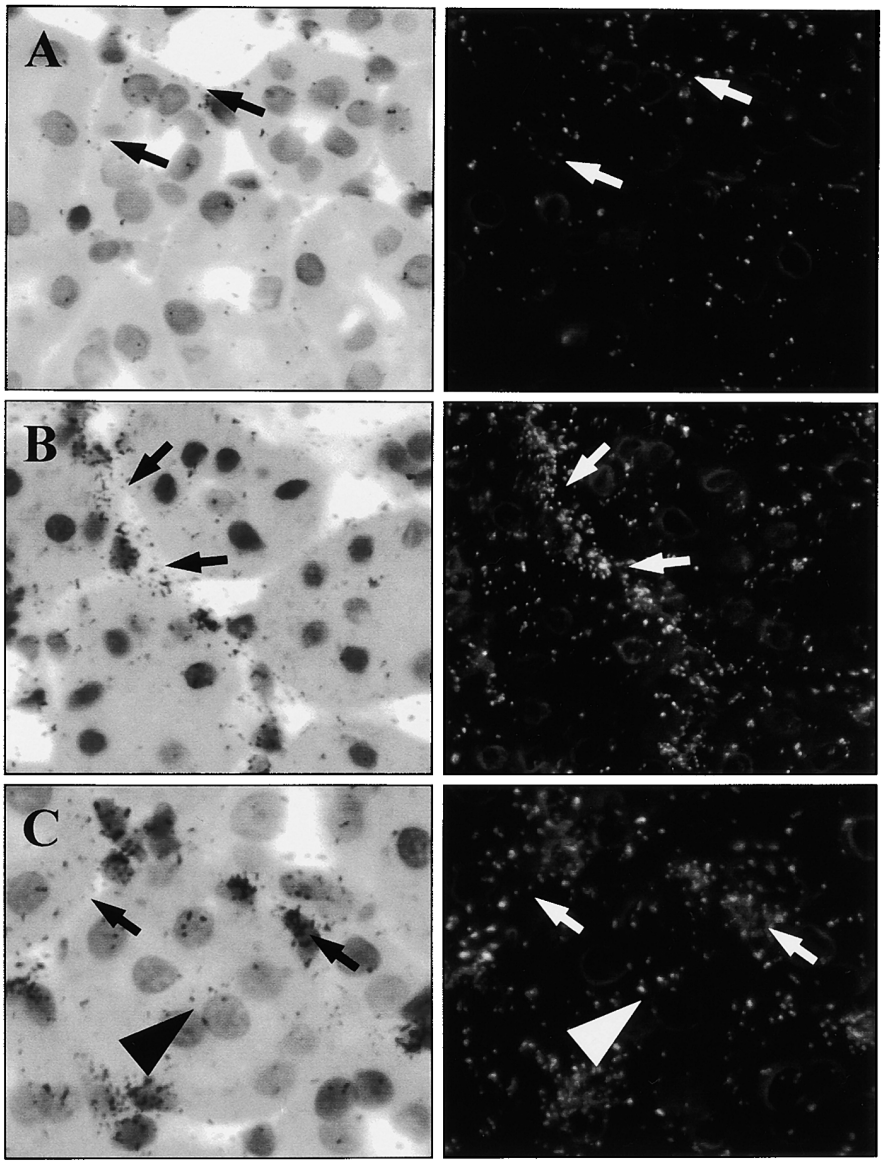

Fig. 5. In situ hybridization for VEGFR-2 mRNA in normal (*A*) and ischemic (*B* and *C*) rat kidney. Interstitial appearances. Panels are in pairs; bright field photographs on the left corresponding with dark field images on the right. Normal kidneys (A) showed low-level VEGFR-2 mRNA expression, localized to peritubular capillaries (arrows). Ischemic kidneys (B and C) showed a marked increase in VEGFR-2 mRNA expression in these structures (arrows). In addition, some of the ischemic tubules appeared to show a signal for VEGFR-2 mRNA (arrowheads, C), which was not evident in the negative controls. All the ischemic kidneys demonstrated in the figure were not subjected to reperfusion although reperfused kidneys (not shown) had a similar appearance. Ischemic rat kidney probed with sense (not shown) demonstrated virtually no signal. Magnifications: A ×350, B ×400, C ×500.

mRNA expression following ischemia, and ischemia-reperfusion. In addition, there appeared to be some evidence of VEGFR-2 mRNA expression in tubular epithelial cells, although this was much less prominent than the endothelial expression. We have previously shown evidence supporting VEGFR-2 expression by renal tubular epithelial cells both in vitro and in vivo [39]. From the current study, however, the in situ hybridization findings show that the endothelium is the predominant site of VEGFR-2 expression in the renal interstitium. Immunohistochemical studies using the N-931 polyclonal antibody showed increased VEGFR-2 expression in the peritubular capillaries, consistent with the in situ hybridization findings.

The function of increased expression of VEGFR-2 by peritubular capillaries is probably to direct the angiogenic effects of locally released VEGF to these cells. Although tubular cell production of VEGF protein is not increased during ischemia-reperfusion, we have previously reported that VEGF in tubular cells is redistributed to the basolateral surface of the cells [1]. This could act to protect the endothelium adjacent to viable tubular epithelial cells, so preserving their capillary supply when blood flow is re-established. Several reports have clearly demonstrated a role for VEGF in preventing endothelial cell apoptosis via stimulation of VEGFR-2 [9, 10]. This would be an obvious role for tubular secretion of VEGF, since survival of a tubular cell following hypoxic injury is likely to be critically dependent on the survival of the adjacent endothelium. Increased nitric oxide expression mediated through VEGFR-2 and endothelial nitric oxide synthase (eNOS) [43–46] also may be of importance in

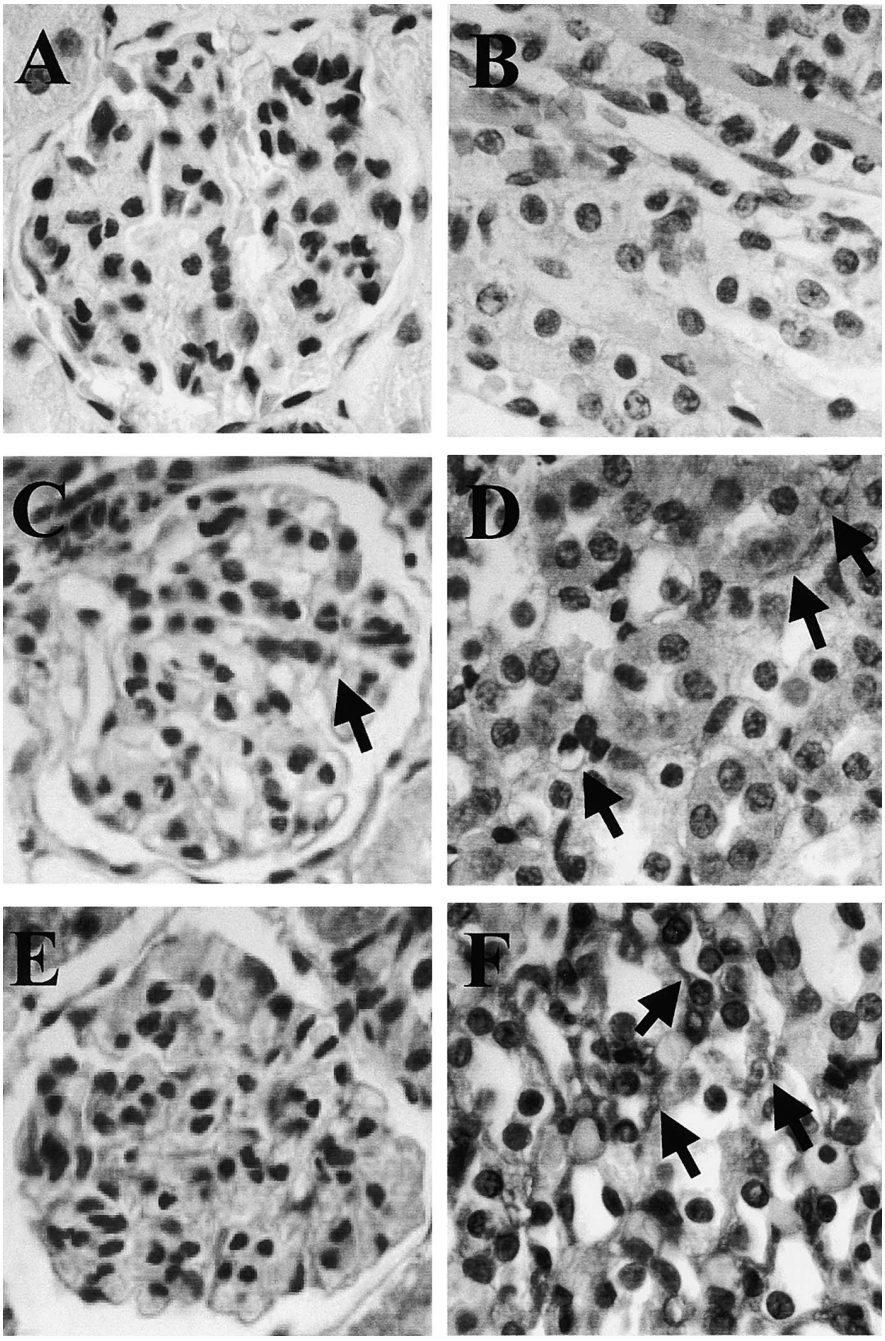

Fig. 6. Immunohistochemical staining for VEGFR-2 in normal and ischemic rat kidneys. Negative controls stained using normal rabbit serum instead of rabbit polyclonal antibody (*A* and *B*). Normal kidney cortex and medulla stained with N-931 antibody (*C* and *D*). There is staining consistent with peritubular capillaries (arrows) in the medulla (D) in normal kidneys and within the glomerular capillaries (C, arrows). Ischemic kidney showed little change in glomerular staining for VEGFR-2 (*E*) but more obvious increased staining in the peritubular regions (*F*, arrows), consistent with peritubular capillary expression. Magnifications: A, C, E ×350, B, D, F ×400.

altering renal hemodynamics. Furthermore, alterations in vascular permeability mediated through VEGFR-2 [47] may contribute to the extensive edema and extravasation seen in the model, although the precise mechanism for this effect remains unclear, as studies have demonstrated [48]. More recent reports have clearly demonstrated a protective role for VEGF on the peritubular endothelium in models of tubulointerstitial injury [49]. Results of in vitro studies suggest that this effect is likely to be mediated through VEGFR-2 [2, 50].

The glomerulus also showed a marked increase in VEGFR-2 mRNA expression following 40 minutes of ischemia, which persisted with reperfusion of up to 80 minutes. Interestingly, this was not associated with an apparent increase in VEGFR-2 protein expression by immunohistochemical staining with the N-931 polyclonal antibody. Such a profound up-regulation of VEGFR-2 mRNA within the glomerulus demonstrates that ischemia-reperfusion injury affects both glomerulus and tubulointerstitium, although most studies have focused on

the latter. The absence of any detectable increase in protein expression suggests that VEGFR-2 may not have a significant role within the glomerulus in the response to ischemia and ischemia-reperfusion, despite the increase in mRNA.

In summary, VEGFR-1 and VEGFR-2 expression were examined in normal rat kidney, and in a renal ischemia-reperfusion model. Despite several studies in a variety of organs, there are no reports examining VEGF receptor expression in this model. VEGFR-2 mRNA expression in the glomerulus and in peritubular capillary endothelium was shown to be markedly up-regulated by renal ischemia and ischemia-reperfusion, although we could not demonstrate increased VEGFR-2 protein expression within the glomeruli. By contrast, renal VEGFR-1 mRNA expression was unaltered. VEGFR-2 up-regulation in the renal tubules during renal ischemia-reperfusion injury may be important in directing the mitogenic, anti-apoptotic and vascular permeability actions of VEGF released by ischemic tubular epithelial cells to adjacent endothelial cells, thereby preserving the potential for capillary blood supply and promoting tubular cell survival and recovery.

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