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Research Article

Ischemia injury alters endothelial cell properties of kidney cortex: Stimulation of MMP-9

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Abstract

Although ischemia is the leading cause of acute renal failure in human, there is little information on the remodeling the kidney endothelium matrix during ischemic injury. In this study, we investigated the activity and expression of MMP-2 and MMP-9, in an isolated endothelial fraction following an acute in vivo reversible ischemia induced in rats by vascular clamping. Ischemia increased serum creatinine levels 1.4-fold, hallmark of acute renal failure. Isolation of the endothelial cell fraction was performed by affinity chromatography using an anti-PECAM-1 antibody. The isolated fraction was assessed by Western blotting analysis of endothelial cell markers. The positively selected fractions were enriched in the endothelial markers eNOS and PECAM-1 by 128-fold and 44-fold, respectively. Gelatin zymography showed that ischemia strongly stimulated proteolytic activity of proMMP-2 (1.8-fold), proMMP-9 (3-fold) and MMP-9 (4-fold) in the endothelial fractions. Western blot analysis indicated that TIMP-2 protein level increased by 3.2-fold in the endothelial fractions during ischemia. Surprisingly, TIMP-1 was absent from the endothelial preparations but was easily detected in the non-endothelial cells. Levels of the endocytic receptor LRP were increased by 2-fold during ischemia in the endothelial fractions. Occludin, a known in vivo MMP-9 substrate, was partly degraded in the endothelial fractions during ischemia, suggesting that the MMP-9 which was upregulated during ischemia was functional. These data suggest that ischemia in kidney could lead to the degradation of the vascular basement membrane and to increased permeability. This suggests new therapeutic approaches for ischemic pathologies by targeting MMP-9 and its regulators.

Keywords: Ischemia; Kidney cortex; Vascular endothelium; MMPs; TIMPs; Occludin; LRP

Introduction

Ischemia is a major focus of clinical and fundamental studies since it is the leading cause of acute renal failure in adults [1]. In vivo, ischemic acute renal failure is well documented as disrupting proximal tubule functions and inducing a vasoconstriction of afferent arterioles to glomeruli [2]. For instance, endothelial cell (EC) dysfunction in ischemic acute renal failure is related to the "no reflow" phenomenon where the renal vasculature undergoes an early swelling [3]. Overexpression of intracellular adhesion molecule-1 (ICAM-1) in renal ischemic endothelial cells has been demonstrated as playing a major role in ischemic acute renal failure [4]. Ischemic endothelial cells show a loss of polarity as revealed by the greater accessibility of Arg–Gly–Asp-binding (RGD-binding) integrins, similar to those observed in renal epithelial cells during ischemia [5]. The vascular endothelial cell injuries and dysfunctions can prolong ischemic effects by inducing vascular congestion, edema and further infiltration by inflammatory cells [6]. On the other hand, prolonged cold preservation promotes

Abbreviations: BSA, bovine serum albumin; ECM, extracellular matrix; EC, endothelial cell; EDTA, ethylenediamine-tetraacetic acid; ICAM-1, intracellular adhesion molecule-1; LRP, low-density lipoprotein receptor-related protein; MMPs, matrix metalloproteinases; PAI-1, plasminogen activator inhibitor-1; PBS, phosphate buffer saline; PVDF, polyvinylidene difluoride; SDS, sodium dodecyl sulfate; TBS, Tris-buffered saline; TBS-T, TBS with Tween 20; TIMPs, tissue inhibitors of metalloproteinases; tPA, tissue-type plasminogen activator; uPA, urokinase-type plasminogen activator; uPAR, uPA receptor; VEGF, vascular endothelial growth factor; ZO-1, zonula occludens-1.

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vascular injury, reducing tissue preservation in clinical kidney transplantation [7]. Finally, the kidney responds to ischemia by upregulating the expression of vascular endothelial growth factor (VEGF) which can initiate angiogenesis for the repair of injured tissues [8].

Vascular congestion, altered integrin exposure in endothelium, VEGF upregulation and angiogenesis are morphological and physiological changes suggesting that ischemic injury significantly alters cell–cell and cell-extracellular matrix (ECM) interactions in kidney. Matrix remodeling is mainly mediated by the metalloproteinases (MMPs) and serine proteases of the plasminogen system [9]. MMPs are Zn^{2+} endopeptidases that are secreted in latent forms and which require activation for proteolytic activity. Among MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) are distinctive members in that they possess unique structural features characterized by an additional fibronectin type-II-like domain. An important mechanism for regulation of MMP activity involves the binding of a family of homologous proteins, known as tissue inhibitors of metalloproteinases (TIMPs). TIMPs manifest several biochemical and physiological/biological activities other than the inhibition of active MMPs, including proMMP activation, inhibition of angiogenesis and the induction of apoptosis [10].

Ischemia, anoxia and ATP depletion have been reported to alter the functional integrity of tight junctions [11–13]. Protein components of the tight junctions, such as occludin and zonula occludens-1 (ZO-1), have been reported to be substrates for MMP-9 in endothelial cells [14] and during cerebral ischemia [13], suggesting that this MMP is in part responsible for the increased permeability. Moreover, occludin has been recently associated with the MMP-9-induced perturbation of the tight junction barrier in rat testes [15]. However, the renal ischemic effects on occludin levels in endothelial cells remain to be determined.

Urokinase-type plasminogen activator (uPA) and/or tissue-type plasminogen activator (tPA) cleave plasminogen into plasmin. Plasmin then activates several proMMPs, including proMMP-2 and proMMP-9, creating fully active enzymes [16]. The plasminogen activation system also includes the plasminogen activator inhibitor-1 (PAI-1) and the uPA receptor (uPAR). The low-density lipoprotein receptor-related protein (LRP) is an endocytic receptor that belongs to the low-density lipoprotein receptor family, whose members bind several structurally dissimilar ligands extracellularly. Receptor-mediated endocytosis involves immobilization of circulating ligands, such as MMPs and plasminogen system components, onto the cell surface followed by their internalization and degradation by lysosomes. For instance, LRP is a functional receptor for MMP-9 and it has been recently shown to play a role in modulating ECM remodeling by clearance of MMP-9 [17]. However, little is known about the effects of ischemia on the plasminogen activation system and LRP in renal ischemic injury and in particular in endothelial cells.

We recently reported the regulation of expression of MMP-2, MMP-9 and their inhibitors TIMP-1 and TIMP-2 in glomeruli after acute ischemia-reperfusion injury [18]. On the other hand, our laboratory has developed a method to isolate endothelial cells from kidneys [19]. Consequently, the purpose of the current study was to employ this method to isolate endothelial cells from kidney cortex following in vivo reversible unilateral ischemia in order to investigate its effects on some key matrix proteolytic enzymes that could be involved in tissue damages. Furthermore, the consequences of acute ischemia on the MMP inhibitors TIMP-1 and TIMP-2 and on the endocytic receptor LRP as well as on MMP-9 substrate occludin levels were investigated in the endothelial fraction.

Materials and methods

Materials

Male Sprague–Dawley rats (250–300 g body weight) were obtained from Charles River (Saint-Constant, Quebec). Acepromazine and ketamine HCl were from Ayerst Laboratories (Montreal, Quebec) and xylazine HCl was from Bayer (Etobicoke, Ontario). The AutoMACs magnetic cell sorting apparatus and the rat anti-mouse IgG microbeads were obtained from Miltenyi Biotec (Auburn CA). Type 3 collagenase was purchased from Worthington (Lakewood, NJ) while dispase II was from Roche Diagnostics (Indianapolis, IN). The monoclonal antibody directed against PECAM-1 (or CD-31) (557355) that was linked to microbeads was from Cedarlane (Hornby, ON) whereas goat polyclonal antibody anti-PECAM-1 (SC-1506) used in Western blotting analysis was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal antibodies against endothelial nitric oxide synthase (eNOS) (N30020) and CD-45 (C27220) were obtained from BD Biosciences (Mississauga, ON). A mouse monoclonal antibody against megalin (or GP330) was graciously provided by Dr. Klatt (Department of Cell Biology and Anatomy, Medical University of South Carolina, Charleston SC). A mouse monoclonal antibody against the 85-kDa subunit of LRP was purchased from RDI Research Diagnotics (Flanders, NJ) while a rabbit polyclonal antibody against occludin was from Zymed Laboratories (San Francisco, CA). Rabbit polyclonal antibodies against synthetic peptides based on the human sequences of MMP-2 (AB809), TIMP-1 (AB800), TIMP-2 (AB801) and a mouse monoclonal antibody against rat native MMP-9 (MAB13420) were purchased from Chemicon (Temecula, CA). Donkey anti-goat horseradish peroxidase-conjugated antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Donkey anti-rabbit and anti-mouse horseradish peroxidase-conjugated antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). A Western Lightning™ Chemiluminescence Reagent plus (ECL) detection system was from NEN™ Life Science

Products (Boston MA). Gelatin, casein, 1,10-phenanthroline, Brij-35, human uPA and a creatinine dosage kit were from Sigma (St. Louis, MO). Plasminogen was obtained from Boehringer Mannheim (Laval, Quebec).

Ischemic treatments

Approval for rat experiments was obtained from the Comité Institutionnel de Bonnes Pratiques Animales en Recherche de l'Université du Québec à Montréal. Rats were anesthetized by subcutaneous injection of acepromazine (2.5 mg/kg) followed by intraperitoneal injection of ketamine HCl (87 mg/kg) and xylazine HCl (7 mg/kg). Following a ventral incision was made, the renal artery and vein of the left kidney were dissected and a non-traumatic Schwartz vascular clamp was placed around them to induce acute ischemia for 30 min. Ischemic kidneys were decapsulated to prevent collateral circulation to the superficial cortex through capsular vessels. To avoid reflow after ischemia, the clamps were left until kidneys were removed. The animals were placed on a heating pad to maintain a constant body temperature (37°C). The sham groups were anesthetized and ventrally operated for 30 min. Immediately after treatment, blood was collected by intracardiac puncture. Rats were sacrificed by lethal exposure to carbon dioxide followed by decapitation. Treated kidneys were quickly removed and placed into ice-cold Ringer–Hepes solution (NaCl 150 mM, KCl 5.2 mM, $CaCl_2.2H_2O$ 2.2 mM, $MgCl_2$ 0.2 mM, $NaHCO_3$ 6 mM, Hepes 5 mM, D-glucose 2.8 mM). Three ischemic experiments were done with kidneys pooled from four rats per group. Serum was prepared following blood collection by coagulation.

Evaluation of renal failure by creatinine levels

Creatinine levels were measured using the manufacturer's directions. Briefly, 300 μl serum was mixed with an alkaline picrate solution. Samples were then incubated at room temperature for 10 min. Measurements were done with a spectrophotometer at a wavelength of 500 nm. In order to remove any interference, 100 μl of a mixture of sulfuric and acetic acid was added to samples which were then incubated for another 5 min and read again at 500 nm. Serum creatinine levels were calculated (in mg/l) for each ischemic animal (n = 12) and for each sham-operated control (n = 8).

Endothelial fraction isolation

Isolation of the endothelial cell fraction was performed with the method previously developed [19]. The kidney cortices were first minced with a scalpel and rinsed in Ringer–Hepes solution. Cortices were rinsed then homogenized in 5 ml of Ringer–Hepes solution per gram of cortex with a Polytron (Brinkmann Instruments, Rexdale, ON). Cortex homogenates were then incubated with Collagenase type 3 (10 mg/ml) and dispase II (1.6 mg/ml) in a volume of Ringer–Hepes solution for 45 min at 37°C with agitation. The suspension was filtered through Nitex (180 μm) followed by a second filtration through Nitex (30 μm). The second filtrates were centrifuged at 600 × g 10 min at 4°C and the pellets were washed three times in phosphate buffer saline (PBS) (NaCl 137 mM, KCl 2.7 mM, KH_2PO_4 1.3 mM, $Na_2HPO_4{\cdot}7H_2O$ 8.1 mM, $MgCl_2$ 0.3 mM, $CaCl_2$ 0.9 mM, NaN_3 0.02% (w/v)) containing 0.5% bovine serum albumin (BSA) and 2 mM ethylenediamine-tetraacetic acid (EDTA). The final pellets were resuspended in PBS with 0.5% BSA and 2 mM EDTA, cells were counted and PBS was added to obtain 10^7 cells/80 μl. Microbeads (100 μl) were preincubated with 10 μg of anti-PECAM-1 antibody for 1 h at 4°C. To remove uncoupled antibody, microbeads linked to anti-PECAM-1 antibody were positively selected with the AutoMACS magnetic cell sorting apparatus (Miltenyi Biotech, Auburn, CA). Microbeads linked to the anti-PECAM-1 antibody (100 μl for 5.7 × 10^7 cells) were added to the cell suspensions and incubated for 30 min at 4°C. The EC bound by the magnetic microbeads linked to the anti-PECAM-1 antibody were selected with the AutoMACs apparatus. The positive cell fractions retained by the column and the non-endothelial cell fractions (flow through of the column) were washed with PBS followed by centrifugation at 600 × g for 10 min at 4°C three times. The final pellets containing the endothelial fraction and the non-endothelial fraction were kept at −80°C until used.

Gelatin zymography

Cells from each fraction were solubilized in RIPA lysis buffer (NaCl 150 mM, Nonidet P-40 1% (v/v), deoxycholic acid 0.5% (w/v), sodium dodecyl sulfate (SDS) 0.1% (w/v), Tris 50 mM) and proteins were quantified using the bicinchoninic acid micro-assay [20] using a kit from Pierce (Rockford, IL). To characterize the metalloproteinases in cortex homogenates, endothelial and non-endothelial fractions during ischemic injury, proteins (10 μg) were separated on 7.5% polyacrylamide gels containing 0.1% (w/v) gelatin in the presence of SDS under non-reducing conditions. After electrophoresis, MMPs were refolded in 2.5% (v/v) Triton X-100 to remove the SDS, washed with distilled water then incubated in 50 mM Tris pH 7.6, 200 mM NaCl, 5 mM $CaCl_2$ and 0.02% (v/v) Brij-35 overnight at 37°C in the presence or absence of 0.5 mM 1,10-phenanthroline. Coomassie-blue-stained zymograms were scanned with a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA) to quantify the MMPs.

Gel electrophoresis and immunoblotting

Cortex homogenates, endothelial and non-endothelial fractions were incubated in sample buffer composed of 62.5 mM Tris/HCl pH 6.8, 10% (v/v) glycerol, 2% (w/v) SDS and 0.00625% (w/v) bromophenol blue, with or without 5% (v/v) β-mercaptoethanol. Samples were agitated for 15 min at room temperature or heated at 100°C for 3 min then

loaded onto polyacrylamide gels. SDS–PAGE was carried out according to Laemmli [21] at a constant voltage of 100 V. Proteins were transferred electrophoretically onto 0.45 μm pore diameter polyvinylidene difluoride (PVDF) membranes (Roche Molecular Biochemicals, Quebec, Canada) using a MilliBlot Graphite Electroblotter I apparatus (Millipore, Bedford, MA). The transfer buffer contained 96 mM glycine, 10 mM Tris and 10% (v/v) methanol. The transfer was carried out for 1.5 h at a constant current of 1 mA/cm^2. Hydrophobic or non-specific sites were blocked for 1 h at room temperature with 5% (w/v) powdered skim milk in Tris-buffered saline (TBS) (50 mM Tris and 137 mM NaCl, pH 7.5) containing 0.3% (v/v) Tween 20 (TBS-T). Membranes were washed three times for 15 min in TBS-T. PVDF membranes were incubated with antibodies in TBS-T, 3% (w/v) BSA and 0.03% (w/v) NaN_3 overnight at 4°C. Membranes were washed three times for 15 min and incubated for 1 h at room temperature with horseradish peroxidase-conjugated donkey anti-mouse IgG, donkey anti-goat or donkey anti-rabbit IgG (1:2000) in TBS-T containing 5% (w/v) milk powder. PVDF membranes were washed three times for 15 min and localization of immunoreactive proteins with ECL Plus was performed as described previously. Blots were exposed to Fuji films and autoradiograms were scanned with a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA).

Measurement of plasminogen activator activity by casein-plasminogen zymography

To characterize uPA and tPA in kidney fractions during ischemia-reperfusion injury, proteins (10 μg) were separated under non-reducing conditions on 10% polyacrylamide gels containing 0.04% (w/v) casein with or without 0.002% (w/v) plasminogen to distinguish their proteolytic activity from those due to other proteases. After migration, gels were washed in 2.5% (v/v) Triton X-100 to remove SDS then incubated in 50 mM Tris pH 7.4 with or without 1 mM 1,10-phenanthroline to inhibit metalloproteinase activities. Coomassie-blue-stained zymograms were scanned with a Personal Densitometer to quantify the amount of PAs.

Statistical analysis

The results are expressed as means ± SEM of three assays and analyzed with Student's *t* test. In the figures, only significant differences between treated fractions and their corresponding sham-treated kidneys are indicated.

Results

Serum creatinine level as indicator of acute renal failure

The hallmark of acute renal failure is a decreased glomerular filtration rate. We sought to monitor this condition by measuring the serum creatinine level. Serum creatinine was significantly increased during ischemia by 1.4-fold ($P < 0.01$). These data demonstrate that ischemia impairs renal function and thus validates our model (Fig. 1).

Marker enrichment for endothelial and non-endothelial fractions isolated from kidney following ischemia

Since it has been shown that eNOS was highly and mainly expressed in endothelial cells, this protein was used as a marker to assess the enrichment of endothelial cells in the isolated fractions [19,22,23]. eNOS (140 kDa) was only found in the endothelial fractions isolated from sham and ischemic kidneys (Fig. 2). Another endothelial marker, PECAM-1 (130 kDa), was also strongly detected in the endothelial fraction and was almost undetectable in the homogenates and non-endothelial fractions (Fig. 2). As expected, CD-45 which is a blood cell marker [24] was mainly found in the non-endothelial cell fractions (Fig. 2).

The quantity of each immunodetected marker was measured by laser densitometry in order to calculate an enrichment factor, by establishing the ratio of the quantity in the enriched cell fractions to that in homogenates. As expected, CD-45 was enriched by 2.8-fold in the non-endothelial fraction from ischemic rats and by 3.8-fold in sham-operated animals. For eNOS and PECAM-1, different amounts of proteins were used to have quantifiable signals while for CD-45 density was measured at equal protein loading (Table 1). eNOS was enriched by 136-fold in endothelial fractions from ischemic rats and by 128-fold of sham-operated controls. In addition, PECAM-1 was enriched by 59-fold during ischemia as compared to an enrichment of 44-fold in control rats in the endothelial fractions. This latter difference is likely due to the significant stimulation eNOS and PECAM-1 levels in endothelial cells isolated from kidney cortex following ischemia (Fig. 2 and Table 1). Together, these data clearly

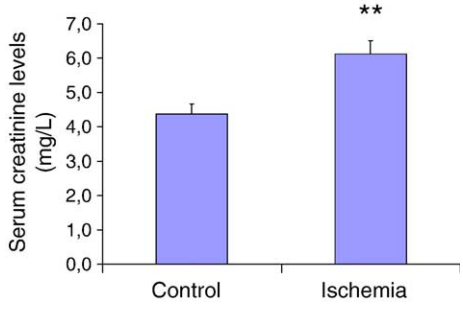

Fig. 1. Serum creatinine levels increase in ischemic animals. Immediately after ischemia, blood was collected by intracardiac punction and serum was prepared. Serum creatinine levels were measured for each animal ($n = 8$ for control; $n = 12$ for ischemia). These results are representative of three experiments and are means ± SEM. Significant differences (**$P < 0.01$) compared to sham-operated rats are indicated.

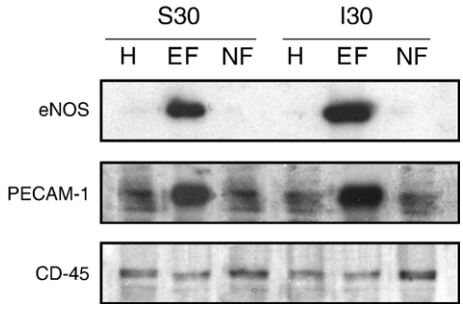

Fig. 2. Assessment of positive and negative markers for the endothelial and the non-endothelial fractions as compared to cortex homogenates. Endothelial (EF) and non-endothelial (NF) fraction and cortex homogenate (H) proteins (25 μg) were analyzed by Western blotting to immunodetect eNOS, PECAM-1 and CD-45. The treatments undergone by kidneys were as follows: S30, sham groups for 30 min; I30, ischemia for 30 min. These results are representative of three experiments.

demonstrated that the final positive fractions were highly enriched in endothelial cells.

Regulation of gelatinase activity in endothelial and non-endothelial cell fractions by renal ischemia

Having validated both the ischemic model and the procedure to enrich the endothelial fraction, the next step was to examine the regulation of gelatinases by ischemia. The presence of gelatinases, MMP-2 and MMP-9, was assessed by gelatin zymography and is shown in Fig. 3A. Four proteolytic activity bands were detected and their molecular weights corresponded to the active and precursor forms of MMP-2 and MMP-9 as demonstrated previously [18]. Both gelatinases were weakly present in cortex homogenates but there activities were clearly detected in the endothelial and non-endothelial cell fractions. Ischemia induced an increase in proMMP-9 levels in the endothelial fractions by 3-fold ($P < 0.001$) as compared with sham-operated controls (Fig. 3B). Moreover, ischemia induced an upregulation in the level of active MMP-9 by about 4-fold ($P < 0.001$) in the endothelial fractions (Fig. 3C). Similarly, proMMP-2 activity was increased by 1.8-fold ($P < 0.01$) in the endothelial fractions during ischemia (Fig. 3D) whereas the MMP-2 level itself remained stable during ischemia (Fig. 3E). On the other hand, there was a decrease in proMMP-9 and MMP-9 gelatinolytic activities by 29% ($P < 0.05$) and 40% ($P < 0.01$), respectively, in the non-endothelial fractions during ischemia in comparison with their controls (Figs. 3B and C). Similarly, proMMP-2 and MMP-2 levels decreased by 46% ($P < 0.001$) and by 90% ($P < 0.01$) in the non-endothelial fractions during ischemic treatment as compared with sham-operated controls (Figs. 3D and E). Thus, ischemia enhanced the level of both gelatinases in endothelial fractions while the same physiological insult reduced their amounts in the non-endothelial cell fractions. This suggests that the endothelium is highly sensitive to ischemia and rapidly responds by upregulating matrix MMPs as MMP-2 and MMP-9.

Modulation by kidney ischemia of TIMP-1 and TIMP-2 expression in endothelial and non-endothelial cell fractions

TIMP-1 and TIMP-2 inhibit MMP-2 and MMP-9 activities but TIMP-1 has more affinity for MMP-9 and TIMP-2 for MMP-2 [25]. TIMP-1 was not detected in either ischemic or control endothelial fractions but was found in the non-endothelial cell fractions (Fig. 4A). Again, this indicates that both cell populations exhibit specific phenotypes. However, TIMP-1 expression was significantly decreased in the non-endothelial fractions by 76% ($P < 0.001$) during reversible ischemia (Fig. 4B). TIMP-2 was observed in renal cortex homogenates but mostly in the non-endothelial fractions while it was barely detectable in the endothelial fractions (Fig. 4C). Compared to their sham-operated controls, ischemia significantly upregulated TIMP-2 expression in homogenates by 5.7-fold ($P < 0.001$), in the endothelial fractions by 3.2-fold ($P < 0.01$) and in the non-endothelial fractions by 2.3-fold ($P < 0.01$).

LRP expression is upregulated in the endothelial fractions in kidney submitted to ischemia

LRP is a cell surface receptor for multiple intracellular ligands and it has been recently shown to play a role in the clearance of MMP-9 and MMP-2 [17,26]. Thus, we investigated LRP expression in kidney following reversible ischemia. The transmembrane 85 kDa subunit of LRP was predominantly detected in the endothelial fractions (Fig. 5A). Protein expression of this endocytic receptor was increased 2-fold ($P < 0.05$) in the ischemic endothelial fraction as compared to the sham-operated controls (Fig. 5B). In contrast, another member of the LDL receptor family, megalin, was mainly expressed in the non-endothelial cell fractions in sham-operated rats (Fig. 5C) since this receptor is highly expressed in absorptive epithelial cells

Table 1
Enrichment of protein markers in endothelial and non-endothelial fractions isolated from ischemic and sham-operated control kidneys

	Endothelial fractions		Non-endothelial fractions	
	S30	I30	S30	I30
eNOS	127.7 ± 0.6	135.5 ± 0.5***	N/D	N/D
PECAM-1	43.5 ± 0.8	58.5 ± 0.7***	N/D	N/D
CD-45	0.8 ± 0.1	0.8 ± 0.1*	2.8 ± 0.1	3.8 ± 0.1***

Enrichment factors are ratios of relative amounts of proteins detected by Western blots in endothelial and non-endothelial fractions versus renal cortex homogenates. For Western blot analysis of eNOS and PECAM-1, protein amounts were adjusted to facilitate detection. Thus, 4 μg of proteins was used for the endothelial fractions whereas 100 μg was used for cortex homogenates. For CD-45 analysis, proteins were in equal quantities (10 μg). Enrichment factors are means ± SEM of three independent experiments.
N/D: Not detectable by densitometry.

* $P < 0.05$ during ischemia (I30) compared with their sham-operated controls (S30).

*** $P < 0.001$ during ischemia (I30) compared with their sham-operated controls (S30).

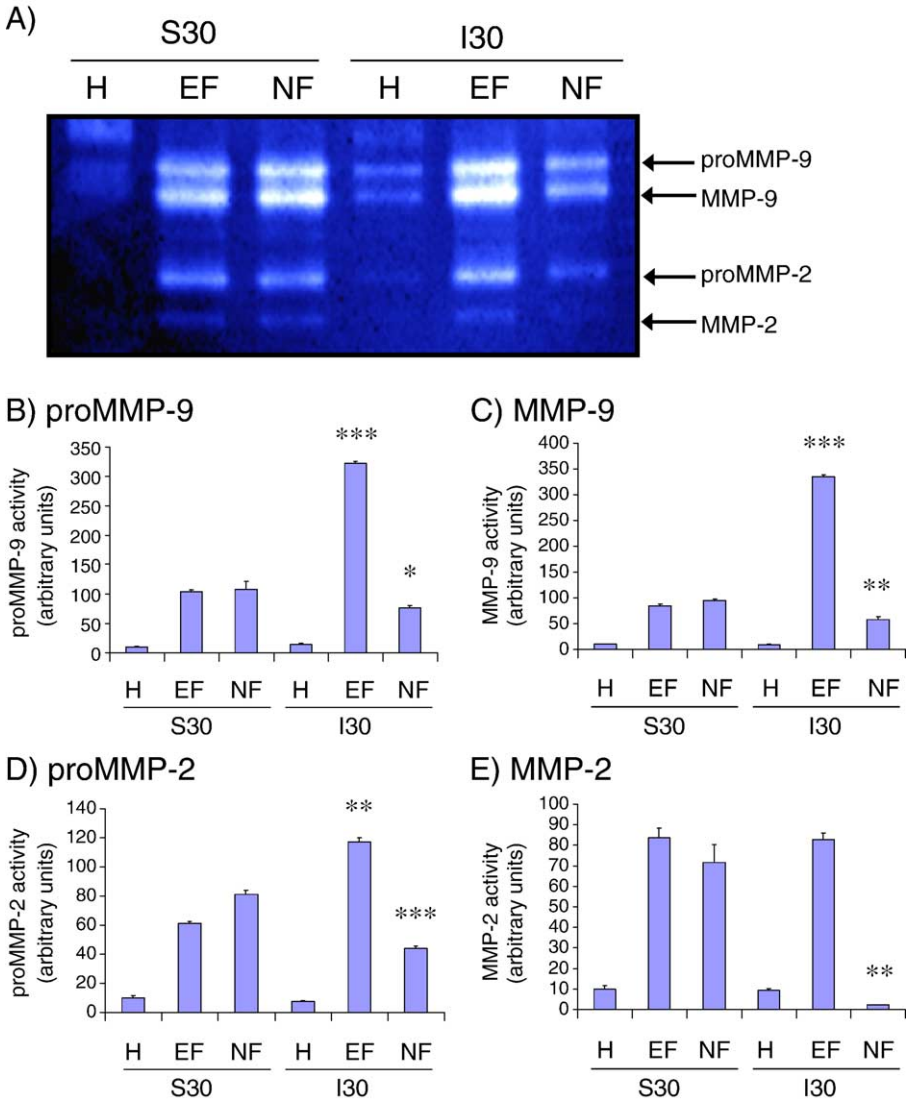

Fig. 3. MMP-2 and MMP-9 levels in kidney cortex fractions during ischemia as measured by gelatin zymography. Cortex homogenates (H), endothelial (EF) and non-endothelial fraction (NF) proteins (5 μg) were analyzed by gelatin zymography (A). The treatments undergone by kidneys were as follows: S30, sham groups for 30 min; I30, ischemia for 30 min. Gelatinolytic activities were quantified by densitometry for proMMP-9 (B), MMP-9 (C), proMMP-2 (D) and MMP-2 (E). The densitometric units were expressed relative to values for sham-operated cortex homogenates that are set at 10 units and are shown as means ± SEM of three experiments. Significant differences (*$P < 0.05$; **$P < 0.01$ and ***$P < 0.001$) compared to sham-operated kidneys are indicated.

such as those found in kidney [27]. Ischemia-modulated megalin levels with a significant rise of 1.4-fold ($P < 0.01$) in the endothelial fractions (Fig. 5D). Thus, these two receptors mediating endocytosis are similarly upregulated in endothelial cells by renal ischemia. In contrast, megalin expression was strongly diminished in the non-endothelial fractions by 57% ($P < 0.001$) after an ischemic treatment as compared to its sham control (Fig. 5D), in agreement with a previous study showing that renal brush border membranes are strongly disrupted by ischemia [28].

Effect of ischemia on expression of plasminogen activators

The expression of plasminogen activators in the endothelial and non-endothelial cell fractions was determined during ischemia, knowing that tPA and uPA play important roles in MMP activation [29,30]. As shown in Fig. 6A, two proteolytic activities of 41 and 62 kDa were detected by casein zymography done in the presence of plasminogen. The 41-kDa lysis activity represented uPA, as it was previously seen by zymography to have an apparent molecular weight of 40–45 kDa in rat [31,32]. The 62-kDa activity corresponded to tPA since it has been shown that rat tPA has a molecular weight of 57–65 kDa, which is different from human tPA [33]. While tPA activity was detected in renal cortex homogenates and in the endothelial fractions, it was almost absent from the non-endothelial fractions. On the other hand, uPA was present in all fractions but, in cortex homogenates, there was another proteolytic activity just below that of uPA (Fig. 6A). tPA activity

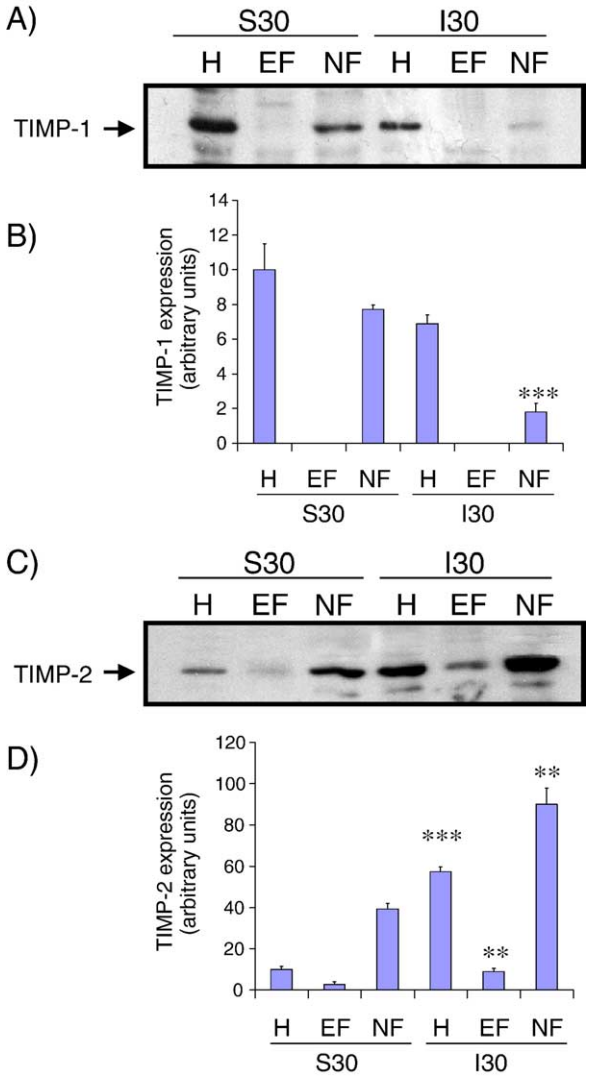

Fig. 4. Modulation of TIMP-1 and TMP-2 expression in kidney cortex fractions during ischemic injury. Cortex homogenate (H), endothelial (EF) and non-endothelial (NF) fraction proteins (20 μg) were analyzed by Western blot for immunodetection of TIMP-1 (A) and TIMP-2 (C). The treatments undergone by kidneys were as follows: S30, sham groups for 30 min; I30, ischemia for 30 min. The amounts of immunoreactive proteins were quantified by densitometry for TIMP-1 (B) and for TIMP-2 (D). The densitometric units were expressed compared to values for each sham-operated cortex homogenates that were set at 10 units and are shown as means ± SEM of three experiments. Significant differences (**$P < 0.01$ and ***$P < 0.001$) compared to sham-operated kidneys are indicated.

remained unaltered by ischemia in all kidney fractions (Fig. 6B). In contrast, uPA activity in both the endothelial and non-endothelial cell fractions was significantly decreased by 29% ($P < 0.01$) and 45% ($P < 0.01$), respectively, during ischemia in comparison to their sham-operated controls (Fig. 6C).

Occludin is degraded during kidney ischemia

Occludin is an extracellular component of tight junctions [34] and the increased permeability is a hallmark of tissue ischemia [35]. MMP-9 has been reported to degrade the endothelial basal lamina and to increase vascular permeability upon ischemia [36]. Furthermore, occludin proteolysis has been associated with MMP-9-induced perturbation of the tight junction barrier in rat testes [15]. Thus, we examined whether the upregulation of MMP-9 activity observed during ischemia could be correlated to occludin proteolysis. Occludin was more present in renal cortex homogenates and in the non-endothelial cell fractions than in the endothelial fractions (Fig. 7A). Ischemia provoked a significant reduction of occludin levels in the non-endothelial cell fractions by 71% ($P < 0.001$), along with lesser degradation in the endothelial fractions by 30% ($P < 0.05$) (Fig. 7B) as compared to their respective sham-operated

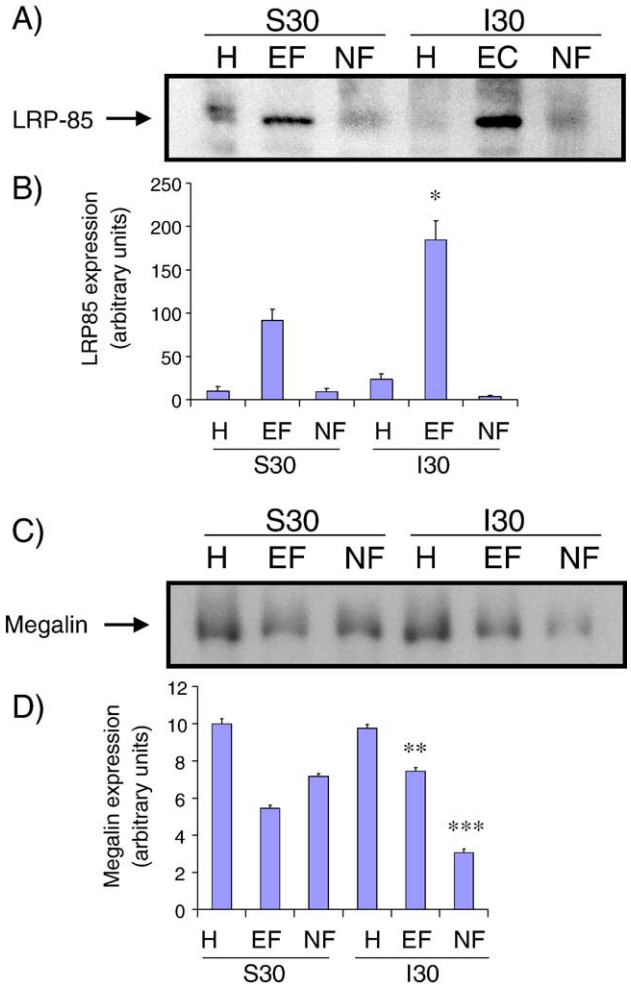

Fig. 5. LRP and megalin expressions are regulated in renal fractions during ischemia. Cortex homogenate (H), endothelial (EF) and non-endothelial (NF) fraction proteins (20 μg) were analyzed by Western blotting to detect the 85-kDa subunit of LRP (A) and megalin (C). The treatments undergone by kidneys are as follows: S30, sham groups for 30 min; I30, ischemia for 30 min. The relative amounts of LRP-85 (B) and megalin (D) were quantified by densitometry. The densitometric units were expressed compared to those for each sham-operated cortex homogenates that were set at 10 units and are shown as means ± SEM of three experiments. Significant differences (*$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$) compared to sham-operated kidneys are indicated.

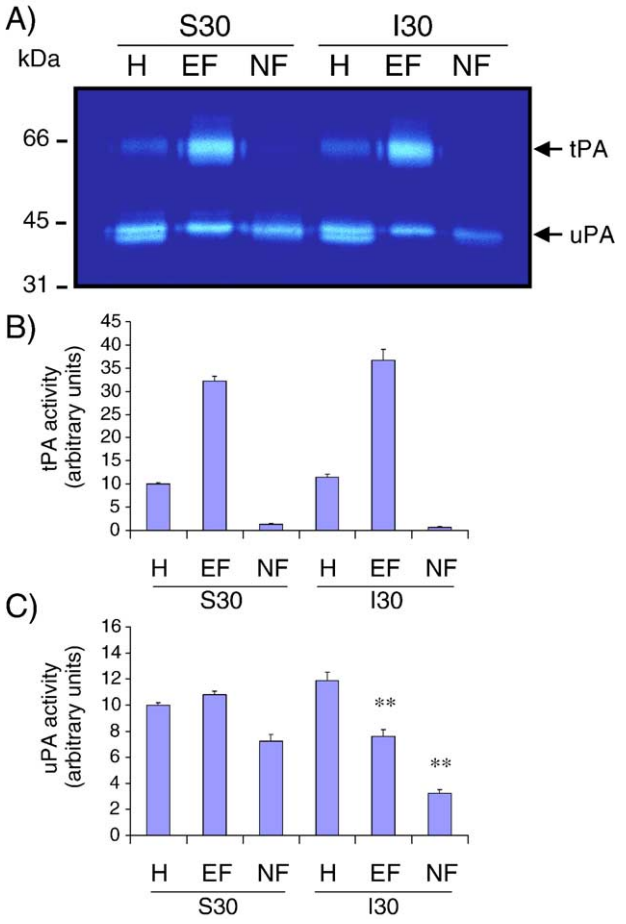

Fig. 6. Ischemia downregulates uPA levels in kidney cortex fractions. Cortex homogenate (H), endothelial (EF) and non-endothelial (NF) fraction proteins (10 μg) were analyzed by casein zymography in the presence of plasminogen following ischemia (A). The treatments undergone by kidneys were as follows: S30, sham groups for 30 min; I30, ischemia for 30 min. The proteolytic activities were quantified by densitometry for tPA (B) and uPA (C). The densitometric units were expressed relative to values observed for each sham-operated cortex homogenates that were set at 10 units and are shown as means ± SEM of three experiments. Significant differences (**$P < 0.01$) compared to sham-operated kidneys are indicated.

controls. We did not detect any occludin fragments in any cell fraction. These observations support the assumption that upregulated MMP-9 during ischemia in endothelial cells leads to the degradation of occludin and could affect tight junction structural integrity. However, another mechanism appears to be involved in the disappearance of occludin in the non-endothelial cell fractions since MMP-9 levels are also reduced during ischemia.

Discussion

The rat model used in this study employs acute renal ischemia for 30 min. This duration was selected because it is below the 45-min limit previously reported as the longest period of renal warm ischemia allowing reversible injury and compatible with survival [37]. A hallmark of acute renal failure is a decreased glomerular filtration rate [2]. This diminution was assessed by measuring serum creatinine level following 30 min ischemia. Under these conditions, serum creatinine level was increased by 1.4-fold. Previously, we reported similar changes in serum creatinine levels following renal ischemia [18,38]. In other studies where male Sprague–Dawley rats underwent this treatment, serum creatinine level was stimulated 4.8-fold following 55 min ischemia and 16 h reperfusion [39] and 7.5-fold for 45 min ischemia followed by 24 h reperfusion [40]. From the reversibility of 30 min ischemia and the weak induction of serum creatinine level, our ischemic model could be qualified as moderately severe.

In the current study, we applied a method that we previously developed and that is based on magnetic cell sorting to isolate endothelial cells from kidney cortex [19]. This time, the endothelial cells were isolated following acute ischemia to determine the effect on properties of vasculature during this injury. Indeed, different endothelial cell subpopulations are present in kidney cortex (glomeruli, venous, arteries) and they should express the cell surface marker PECAM-1 at various levels. In addition, ischemia also enhances the expression of PECAM-1 by 1.3-fold. To overcome this potential problem, protein amounts loaded on the magnetic beads were lower than the capacity of the column. Thus, despite the variation in PECAM-1 expression on the surface of endothelial cell due to ischemia or in

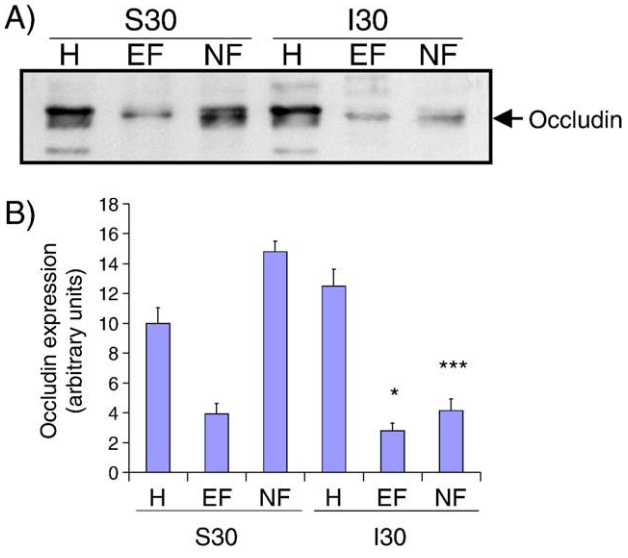

Fig. 7. Decreased levels of occludin in the endothelial and the non-endothelial fractions following reversible renal ischemia. Cortex homogenate (H), endothelial (EF) and non-endothelial (NF) fraction proteins (20 μg) were analyzed by Western blotting to immunodetect occludin (A). The treatments undergone by kidneys were as follows: S30, sham groups for 30 min; I30, ischemia for 30 min. The relative amounts of occludin were quantified by densitometry (B) and values were expressed relative to those obtained for each sham-operated cortex homogenates that were set at 10 units. Significant differences (*$P < 0.05$ and ***$P < 0.001$) compared to sham-operated kidneys are from means ± SEM of three experiments.

different subpopulations, one could expect that under these conditions most endothelial cells will be isolated because the magnetic beads were not saturated. This is supported by the observation that yields of endothelial cells were similar, as measured by recovered protein amounts, between sham-operated and ischemic rats. The strong increase in PECAM-1 and eNOS levels in the endothelial fractions relative to homogenates (between 44- and 136-fold) clearly indicates a marked enrichment of endothelial cells in this fraction.

Besides eNOS and PECAM-1, other proteins analyzed in this study further characterize both cell fractions. CD-45 is a common leukocyte antigen expressed on all nucleated hematopoietic cells and in dendritic cells. Accordingly, CD-45 levels are enriched in the non-endothelial cell fractions. Occludin, which is abundant in the tight junctions of epithelial cells [34], is mainly found in the non-endothelial cell fractions in sham rats, suggesting a depletion of epithelial cells from the endothelial fractions. However, the presence of some occludin in the endothelial fractions is not unusual since these cells also possess tight junctions, though to a lesser level than do the epithelial cells in kidney. Furthermore, in contrast to the endothelial fractions, TIMP-1 was easily immunodetected in the non-endothelial cell fractions. Finally, LRP is highly expressed in the renal endothelial fractions but not in the non-endothelial cell fractions. Together, these enrichments of various proteins in each cell populations clearly demonstrate that the positively selected fractions are enriched in endothelial cells and contained little contamination from other cell types.

Levels of both eNOS and PECAM-1 are significantly higher in endothelial cell fractions following 30 min ischemia. Previously, we reported a strong induction of the transcriptional factor HIF-1α in proximal tubules [38] indicating a hypoxic component in the ischemic response in our model. This suggests that hypoxia could be critical for the stimulation of eNOS and PECAM-1 levels in renal ischemic response. In fact, it is well documented that hypoxia regulates eNOS expression. For instance, it has been demonstrated that the human eNOS gene promoter contains two contiguous hypoxia-response elements and that hypoxia through the transcriptional factor HIF-2 stimulates transcription of the eNOS gene [41]. Similarly, it has been reported that intestinal ischemia increases PECAM-1 level [42] as we observed in kidneys. Moreover, it has been found that hypoxic conditions stimulate the phosphorylation of PECAM-1 in cultured endothelial cells [43]. Both enhanced PECAM-1 expression by ischemia and its phosphorylation by hypoxia are mediated by platelet-activating factor produced under low oxygen tension [42,43]. It remains to be established whether the platelet-activating factor mediates also the increased level of PECAM-1 during kidney ischemia.

The yields for the isolation of the endothelial and non-endothelial cell fractions are 11% and 32%, respectively, when comparing their protein contents with those found in renal cortex homogenates. Surprisingly, both cell preparations are highly enriched in MMP-2 and MMP-9 compared to renal homogenates. These observations suggest that MMP-2 and MMP-9 are predominantly associated with cells present in the endothelial and non-endothelial fractions rather than with the renal material (57%) that is lost during the washing steps to enrich cellular fractions. Interestingly, ischemia increases both proMMP-9 and MMP-9 as well as proMMP-2 but not MMP-2 in renal endothelial fractions. On the other hand, in the non-endothelial fractions, all forms of MMP-2 and MMP-9 decrease during ischemia. The significant increase in activity of MMP-9 in endothelial fractions (4-fold) observed in this study agrees with our previous data in glomeruli, showing a very strong induction of both proMMP-9 and MMP-9 forms (8- and 7-fold, respectively) by the same ischemic conditions [18]. Moreover, an MMP-9 antibody stained endothelial cells both within and at the periphery of the infarct regions during focal cerebral ischemia [8,44]. The direct effects of ischemia on endothelial MMP-2 have been rarely investigated. For example, it has been reported that prolonged hypoxia of endothelial cells enhances MMP-2 production and secretion [45]. MMP-2 levels appear after ischemia in the white matter of the corpus callosum [8] and in the ischemic-reperfused heart [46].

TIMP-1 and TIMP-2 inhibit the latent and active forms of MMPs, but they also form specific tight-binding complexes with the precursor forms proMMP-9 and proMMP-2 [25]. In contrast to TIMP-1, TIMP-2 is strongly induced in cortex homogenates, endothelial and non-endothelial fractions by ischemia. Similarly, TIMP-2 expression increases by more than 4-fold in ischemic glomeruli [18]. Thus, simultaneous MMP-2 and TIMP-2 upregulations during acute ischemia are similar to conditions seen in many cancers where both proteins increase in parallel [47]. The expression of TIMP-1 is reduced during ischemia in the non-endothelial cell fractions but TIMP-1 is not detectable in endothelial fractions. Following reversible ischemia, glomerular TIMP-1 levels are decreased by 38% [18]. Moreover, a decrease in the level of TIMP-1 has also been observed in ischemic myocardium [48]. Marked TIMP-1 decrease during ischemia could thus lead to selective activation of MMP-9 since its potential inhibition is lowered despite the reduced levels of the gelatinase in the non-endothelial cell fractions. The higher MMP-9 levels in endothelial fractions upon ischemia, while TIMP-1 is lacking, support the hypothesis that its activity is highly elevated in the injured endothelium.

Oxidative stress associated with ischemia through the generation of nitric oxide and reactive oxygen species including peroxynitrite can modify proteins during acute renal ischemia [49]. Peroxynitrite has been shown to activate the precursors of certain MMPs such as proMMP-1, -8 and -9 [50]. The molecular size of proMMP-9 does not change during a treatment with peroxynitrite [49,50] suggesting that its activation occurs without proteolytic

cleavage of the propeptide. Consequently, two mechanisms can enhance the activity of MMP-9 forms during ischemia. The first is a higher MMP-9/TIMP-1 ratio and the second the direct activation of proMMP-9 by peroxynitrite produced during renal ischemia.

While tPA levels remain stable, those of uPA were decreased by ischemia in both the non-endothelial and endothelial cell fractions but not in homogenates. This observation supports those regarding MMP-9, showing that these enriched fractions contain cell types with specific features as compared to cortex homogenates. Previously, it had been reported that uPA mRNA levels decrease in a rabbit model of renal ischemia/reperfusion [51]. PAI-1 and uPAR were not detectable by Western blot in all examined cortex fractions in rats (data not shown). Together, these data agree with the possibility that plasmin activity could be slightly reduced in ischemic kidney and suggest that the plasminogen system is a minor player in the activation of MMPs under these conditions.

LRP binds several extracellular, structurally dissimilar ligands and internalizes them for degradation by lysosomes. For instance, LRP is known to mediate the cellular uptake of MMP-9/TIMP-1, MMP-2/thrombospondin-2, tPA/PAI-1 and uPA/PAI-1/uPAR complexes, leading to their degradation [17,26,52]. Thus, the ischemia-induced upregulation of LRP in the endothelial fractions could contribute to the internalization of some uPA and at some degree to control the excess of MMP-9 induced by the injury. This process could partly overcome the TIMP-1 deficiency and cause a downregulation of MMP-9 in these cells. An alternative explanation is possible since receptor-mediated endocytosis by LRP and by the other members of the LDL-receptor family requires GTP and ATP [53], whose depletion is a feature of ischemia. Levels of these nucleotide triphosphates have been measured after 30 min of ischemia in rat kidneys, at which time ATP levels were at 4% and those of GTP at 11% of control values [54]. Thus, the increased levels of MMPs could result from a diminution of degradation caused by an ischemia-induced reduction of the endocytic internalization, in spite of a rise in LRP levels in the endothelial fraction, due to ATP and GTP depletion under these conditions. This latter suggestion is supported by the observation that levels of two LRP ligands, tPA and MMP-2, remain stable in endothelial cell fractions following kidney ischemia.

Occludin is an extracellular component of tight junctions and it has been identified as an in vivo MMP-9 target [14]. The enhanced activity of both forms of MMP-9 during ischemia is accompanied by a lower level of occludin in endothelial fractions. This result suggests that the induction of MMP-9 during kidney ischemia leads to an increased degradation of occludin. This conclusion is supported by the observation that ZO-1, a component of tight junctions, is also degraded during cerebral ischemia, but that proteolysis was reduced in MMP-9 knockout mice [13]. Moreover, occludin has been recently associated with the MMP-9-induced perturbation of the tight junction barrier in rat testes [15]. The strong decrease of occludin levels in the non-endothelial fractions during ischemia is likely not gelatinase dependent since all forms of MMP-2 and MMP-9 are also reduced. Occludin reduction likely reflects the disorganization and collapse of microvillosities during ischemia [28].

The physiological functions of upregulated proMMP-2 and, even more so, proMMP-9 and MMP-9 during ischemia in the endothelial fractions remain to be elucidated. However, the characterization of MMP-9 regulators in this study, such as the diminished levels of inhibitor TIMP-1, the upregulation of the endocytic receptor LRP and the proteolysis of the tight junction protein occludin which is an MMP-9 substrate, should help to define new therapeutical targets for ischemic pathologies including acute renal failure and kidney transplantation.

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