Tubular Erythropoietin Receptor Expression Mediates Erythropoietin-Induced Renoprotection

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Abstract: Erythropoietin (EPO) has been shown to have tissue protective properties by binding to its receptor (EPOR) which is also expressed on non-haematopoietic cells. The mechanisms underlying this protection have not yet been elucidated and the renal cell types mediating these effects remain ill-defined. This study aimed to identify the EPOR expression in human tubular epithelial cells (hTECs) and in rat kidney and to investigate the role of EPOR in EPO-mediated renoprotection. Male Wistar rats were treated with saline or EPO (3000 U/kg, i.p.) 24h prior to sham-operation or 30 min bilateral renal ischemia. Renal morphology and function, tubular regeneration, apoptosis and expression of EPOR, heme-oxygenase-1 (HO-1) and hepatocyte growth factor (HGF) were analyzed. Primary cultures of human proximal (PTC) and distal/collecting duct (DTC) tubular cells were incubated with EPO (5-50-500 ng/mL) either or not in the presence of soluble EPOR. Total RNA was extracted and mRNA expression of HO-1 was investigated by quantitative RT-PCR. EPOR mRNA could be demonstrated in hTECs and in cortical tubules of the rat kidney. Furthermore, EPOR protein was expressed at the membrane and as intracellular vesicles in hTECs. *In vivo*, EPO treatment attenuated histological and functional renal damage, decreased both cell necrosis and apoptotic cell death, enhanced tubular regeneration and resulted in an upregulation of HO-1 and HGF mRNA. *In vitro*, EPO administration resulted in an early upregulation of HO-1 mRNA which was restricted to PTC and inhibited by simultaneous addition of supra-equivalent amounts of soluble EPOR. These data strongly suggest that the EPO-mediated renoprotection results from direct interaction of EPO with EPOR on tubular cells.

Key Words: Erythropoietin, erythropoietin receptor, human proximal tubular cells.

INTRODUCTION

It is now widely accepted that erythropoietin (EPO) is not solely a hormone charged with regulating the proliferation and differentiation of erythroid progenitor cells. A series of recent studies have provided evidence that EPO administration exerts significant tissue protective effects during renal ischemia reperfusion injury (IRI) [1-12]. A potential role for the non-haematopoietic activities of EPO in the kidney was suggested by the identification of the EPOR protein expression throughout the kidney, including both proximal and distal tubular cells [13]. Since the specificity of several anti-EPOR antibodies for Western blot and/or immunohistochemistry has recently been questioned [14,15], the exact localization of the EPOR in the kidney, and with it the main target of its renoprotective action, remains disputable. Moreover, with exception of the abundant data concerning the beneficial effects of EPO in several animal models of IRI, little is currently known about EPO-induced protective effects in the human kidney. Although several hypotheses have been put forward to explain the mechanisms behind EPO-related renal protection, such as pro-angiogenic, anti-apoptotic and anti-inflammatory effects, the exact biological mechanisms remain unknown. Abundant evidence has accumulated in studies of brain, heart and kidney injury that EPO has an anti-apoptotic effect in these tissues. Hepatocyte growth factor (HGF) is a potent regenerative factor for the liver, and besides having regenerative effects in renal tubular cells [16,17], it has been reported that HGF also attenuates renal injury by its anti-apoptotic [17-20] and anti-inflammatory [21,22] properties. Interestingly, EPO treatment increases serum HGF levels in chronic hemodialysis patients [23], but whether or not EPO has any direct effect on HGF expression in the kidney following injury has not been investigated. Similarly, certain members of the family of heat shock proteins (HSPs) are known to play a tissue protective role in a variety of renal injury models and a few reports have connected HSPs (HSP70, HSP32) to EPO-induced tissue protection [1,24]. Heme-oxygenase-1 (HO-1 or HSP32) induction and its cytoprotective effects are frequently seen in several models of renal injury [25-30]. Although EPO-mediated HO-1 upregulation has been recently described in cultured endothelial cells [24], it has not yet been reported whether or not EPO-mediated HO-1 upregulation is present in renal tubular epithelial cells and plays a role in EPO-induced tissue protection during acute renal failure. These studies aimed to investigate (i) the expression of EPOR in both human and rat kid-

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ney and (ii) the role of this receptor in the induction of important protective pathways (HO-1 and HGF).

MATERIALS AND METHODOLOGY

In Vivo Experiments

Experimental Design

All procedures were carried out in accordance with the *NIH Guidelines for the Care of Laboratory Animals* No. 85-23 (1985) following approval of the Antwerp University Ethical Committee. Saline or EPO (3000 U/kg, epoetin alfa - Eprex®, Janssen Cilag, Beerse, Belgium) was administered intraperitoneally 24h before male Wistar rats (225-250 g, Iffa Credo, Brussels, Belgium) were subjected to sham-operation or bilateral renal artery occlusion for 30min. Six animals per experimental group were sacrificed at 2, 6, 12, 24, 48 and 72h and 10 days after reperfusion. All sham-operated animals were sacrificed 24h after the sham-operation. Preoperatively and before sacrifice, blood samples were taken to measure hematocrit and serum creatinine levels. At sacrifice, both kidneys were removed and sagittal slices were either fixed in formol calcium or frozen in liquid nitrogen.

Morphological Analysis of Tubular Injury, Regeneration, Cell Proliferation and Apoptosis

Renal morphology and cell proliferation were evaluated on proliferating cell nuclear antigen (PCNA)/periodic acid Schiff (PAS) stained sections. For renal morphology, forty proximal tubules (PT) in the outer stripe of the outer medulla (OSOM) of each animal were assigned as (1) tubules with a normal appearance; (2) tubules with signs of sublethal injury; (3) tubules with signs of acute tubular necrosis and (4) tubules with signs of regeneration. Proliferation was measured by counting the number of PCNA+ cells in 20 fields in the OSOM. Apoptotic cells were examined by the TUNEL (terminal deoxynucleotide transferase uridine triphosphate nick-end labeling) assay using the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Chemicon International, Temecula, CA, USA). Apoptotic bodies were counted in 50 fields in both cortex and OSOM.

RNA Extraction and Quantitative RT-PCR

Total RNA of right rat kidneys from saline and EPO treated animals was extracted using the TRIzol® reagent (Invitrogen, Merelbeke, Belgium) and RNA extracts of each group were then pooled. cDNA was synthesized using the High-Capacity cDNA Archive kit (ABI) and EPO-mediated mRNA expression of renal EPOR, HO-1, HGF and Bcl-2 in sham-operated animals was evaluated by quantitative RT-PCR on the ABI Prism 7700 Sequence Detection System (ABI, Nieuwerkerk a/d IJssel, The Netherlands) using the fluorescent Taqman methodology and ready-to-use primer and probe sets predesigned by ABI. The mRNA quantities of the target genes were analyzed in triplicate, normalized against GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as an endogenous control housekeeping gene and expressed in relation to a calibrator sample. The saline treated sham-operated group served as the calibrator sample which was given a gene of interest/GAPDH mRNA expression ratio value of 1. Results are expressed as relative gene expression using the Pfaffl method [31].

Laser Capture Microdissection (LCM), Small Sample RNA Extraction and Quantitative RT-PCR

Snap-frozen rat kidneys of sham-operated animals were embedded in Neg-50™ (Richard-Allan Scientific, Kalamazoo, MI USA), 8 µm thick cryosections were cut, caught on a PEN-membrane, stretched on a metal frame, and fixed with ethanol. Sections were stained with Cresyl-Violet (LCM Staining Kit, Ambion, Huntingdon, UK); frames were sandwiched with an RNase-free slide and mounted on the LCM microscope (SL µCut, MMI, Glattbrugg, Switzerland). Three fractions of cells were isolated (about 5000-8000 cells for each cell population mentioned): cells of glomerular origin, cells of cortical tubular origin and cells originating from tubules from the inner stripe of the outer medulla (ISOM). RNA was extracted using the RNA Picopure™ Kit (Arcturus, Mountain View, CA, USA), the quality and concentration was assessed with the Agilent 2100 BioAnalyzer (Agilent Technologies, Santa Clara, CA, USA) and it was reverse transcribed using the Sensiscript RT Kit (Qiagen, Hilden, Germany). EPOR mRNA expression was analyzed by quantitative PCR as described above.

In Vitro Experiments

Primary Antibodies

Mouse anti-human leucine aminopeptidase (LAP) monoclonal antibody (clone AD1) was previously characterized in our laboratory [32]. Rat anti-human epithelial membrane antigen (EMA) monoclonal antibody (clone ICR.2) was purchased from Seralab (Leicestershire, UK). For the detection of EPOR, the rabbit polyclonal M-20 EPOR antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used.

Cell Culture

Human renal tubular epithelial cells (hTECs) were isolated as previously described [33,34]. Briefly, normal human kidney tissue, available through nephrectomies performed for oncological indications, was collected and processed in a sterile manner. The use of this tissue for the purpose of cell culture was approved by the local ethical committee. Tissue from cortex and OSOM was dissected, cut into pieces of approximately 1 mm^3 and digested in collagenase D solution (Roche, Ottweiler, Germany) supplemented with DNAse (Sigma, St Louis, Missouri, USA). The suspension was shaken vigorously for 2h at 37°C and sieved through a 120 µm sieve. The resulting single-cell suspension was loaded on top of a discontinuous Percoll (Amersham, Uppsala, Sweden) gradient with densities of 1.04 and 1.07 g/mL. After centrifugation, cells from the intersection were carefully aspirated, washed, and either brought into culture as a mixed population or subjected to further purification via flow cytometric sorting. For sorting, cells were incubated for 30min at 4°C with anti-human LAP and EMA monoclonal antibodies. Phycoerythrin-labeled rabbit F(ab')2 anti-mouse Ig (Dako, Glostrup, Denmark) and FITC-labeled goat F(ab')2 anti-rat Ig (SBA, Birmingham, UK) secondary antibodies were then added to the cell suspensions. Labeled cells were sorted into distinct proximal (PTC; LAP+/EMA-) and distal (DTC; LAP-/EMA+) populations by using a FACStar Plus flow cytometer (Becton Dickinson, San Diego, CA, USA). Mixed and pure cultures of PTC and DTC were grown until

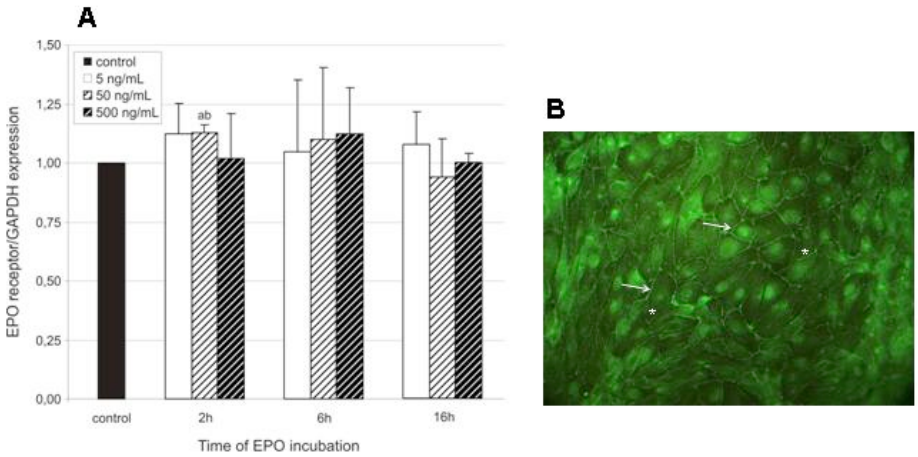

Fig. (1). EPO receptor expression in human tubular epithelial cells and rat kidney.
(**A**) EPOR relative gene expression in mixed hTECs incubated for 2, 6 or 16h with various concentrations of EPO (5-50-500 ng/mL). GAPDH was used as endogenous control housekeeping gene. EPOR mRNA was present in mixed hTECs and in general, EPOR mRNA expression was not influenced by EPO administration (means of triplicate measurements from two runs ± SD). [a] $P < 0.006$ vs. control; [b] $P < 0.05$ vs. 16h.
(**B**) EPOR localization in mixed hTECs was investigated by confocal microscopy. In addition to its expression at the membrane of hTECS (arrows), EPOR was also expressed as intracellular vesicles (asterisks). In negative control sections in which the primary antibody was omitted no signal was observed. Original magnification 100x.

confluence on 24-well plates (Costar, Cambridge, UK) or on polycarbonate Transwell filters (pore size 0.4 μm; Costar) in α-minimal essential medium (Life Technologies, Rockville, Maryland, USA) modified according to Gibson d'Ambrosio [35] supplemented with 10% fetal calf serum.

EPO / Soluble EPOR Competition Experiment

Confluent mixed and purified PTC and DTC were incubated with EPO (0-5-50-500 ng/mL darbepoetin alfa - Aranesp®, Amgen) either or not in the presence of soluble EPOR (kindly provided by Amgen) for 2, 6 or 16h in serum-free medium. Taking into account that one EPOR molecule binds two molecules of EPO, soluble EPOR was administered in different concentrations resulting in a 0x, 2.5x, 5x and 10x excess compared to the molar concentration of EPO. The cell cultures used as controls were incubated with serum-free culture medium containing neither EPO nor soluble EPOR. To exclude that soluble EPOR in itself had any upregulatory effect on the expression of HO-1, two additional controls were included, i.e. culture medium without EPO but supplemented with 2.5x or 10x soluble EPOR.

RNA Extraction and Quantitative RT-PCR

Total RNA from hTECs was extracted using the High Pure RNA Isolation kit (Roche, Basel, Switzerland) and optimization of RNA extraction was performed using the RNeasy® Mini Kit (Qiagen). To evaluate the EPO-mediated mRNA expression of EPOR and the effect of soluble EPOR on EPO-mediated HO-1 upregulation in hTECs, quantitative real-time RT-PCR was used as described above. Control cell cultures (i.e. cells incubated with culture medium without EPO) served as the calibrator sample which was given an EPOR or HO-1/GAPDH mRNA expression ratio value of 1.

Immunological Detection of EPOR Protein

hTECs grown on polycarbonate Transwell filters, were fixed in 4% formaldehyde for 10min, washed and blocked with normal goat serum. Cells were incubated overnight with the M-20 EPOR antibody and subsequently for 2h with FITC labeled goat anti-rabbit Ig (Southern Biotechnology, AL) secondary antibody. Fluorescent signals were imaged by a confocal laser scanning microscope (LSM 510-Meta; Zeiss, Germany).

Statistics

Data are presented as mean ± SD or as percentages. Data from quantitative RT-PCR analyses are presented as the mean of triplicate determinations from 2 runs ± SD. Statistical analysis was performed using the statistical software package SPSS 11.0. For animal, image analysis, and quantitative RT-PCR data non-parametric tests were used: Kruskall-Wallis H test and Mann-Whitney U test, with Bonferroni correction for multiple comparisons. Data from the morphological evaluation were analyzed using Pearson's 2-test. Values of $P < 0.05$ were considered significant.

Results

EPO Receptor Expression in Human Tubular Epithelial Cells and Rat Kidney

The presence of renal (tubular) EPOR expression was confirmed in primary hTECs cultures both at mRNA and protein level. Quantitative RT-PCR analysis revealed that mixed hTECs express EPOR mRNA under basal conditions. Mean Ct values for GAPDH and EPOR were 16.55 ± 0.29 and 28.65 ± 1.14 respectively. EPO administration did not consistently influence EPOR mRNA expression (Fig. **1A**). At protein level, besides the expected membranous EPOR expression, EPOR immunostaining also showed an intracellular, vesicular expression pattern (Fig. **1B**). In rat kidney, EPOR mRNA could be detected in cortical and medullary tubules and was absent in glomerular tissue (Ct values of 37.36 and 38.53 for cortical and medullary tubules, respectively).

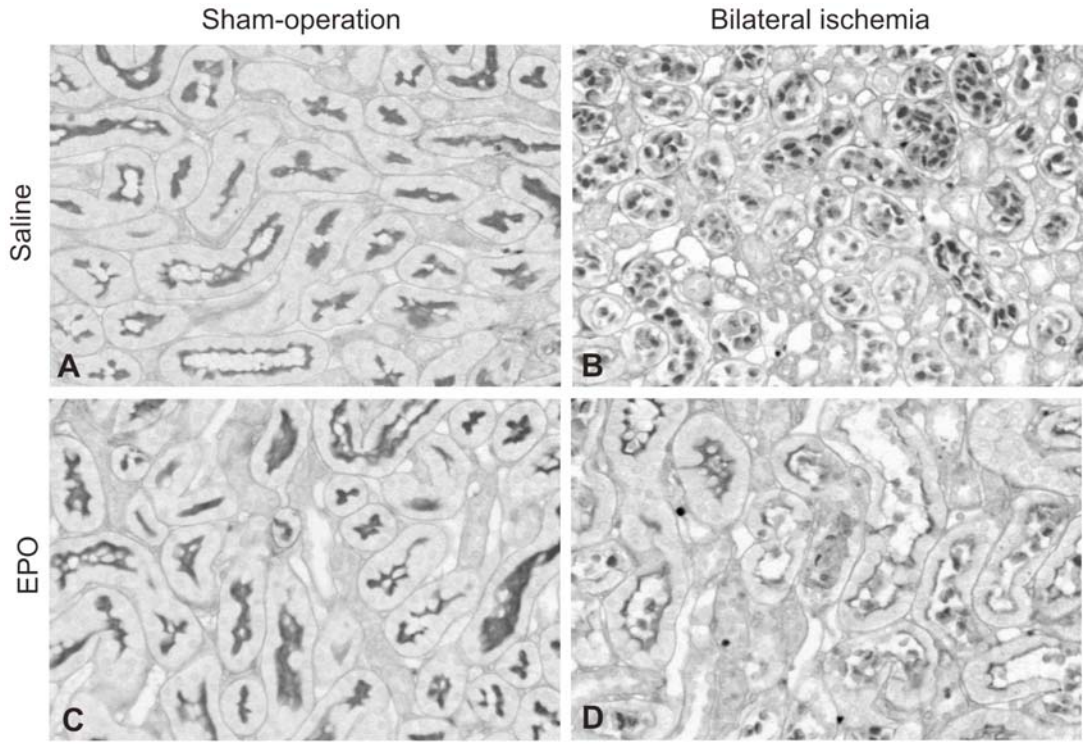

Fig. (2). EPO attenuates renal histological alterations following renal IRI.
Representative PAS-PCNA stained renal sections from saline and EPO treated sham-operated rats (**A** and **C**, respectively) and 12h following reperfusion in IRI rats (**B** and **D**, respectively), demonstrating that EPO preconditioning preserved renal tissue morphology. In negative control sections in which the primary antibody was omitted no signal was observed. Magnification 250x.

Effects of EPO Pretreatment on Histological Damage and Renal Function

Renal morphology. When compared to sham-operated animals (Fig. **2A** and **C**), rats subjected to bilateral renal IRI (Fig. **2B** and **D**) demonstrated significant changes in renal histology within 6h. EPO administration markedly attenuated the histological features of renal injury (Fig. **2D**) as compared to saline treated IRI animals (Fig. **2B**). EPO pretreatment resulted in significantly less necrosis 6h (38 vs. 75 %, $P < 0.01$), 12h (59 vs. 90 %, $P < 0.05$) and 24h (3 vs. 15 %, $P < 0.05$) after reperfusion (Fig. **3C**).

Tubular regeneration. The number of PCNA+ cells was significantly higher in the EPO treated group as compared to the saline treated group 24h following reperfusion (1004.62 ± 276.01 vs. 547.33 ± 171.77 PCNA+ cells/mm², $P < 0.01$) (Fig. **4**).

Apoptosis. Apoptotic bodies were found more frequently in OSOM as compared to cortex in both saline and EPO treated animals. IRI-induced apoptotic cell death was significantly abrogated by EPO both in cortex and OSOM 24h after reperfusion: 9.89 ± 15.33 vs. 39.56 ± 9.89 apoptotic bodies/mm² cortical tissue and 29.67 ± 7.95 vs. 107.69 ± 2.17 apoptotic bodies/mm² OSOM in EPO and saline treated animals, respectively ($P \leq 0.002$) (Fig. **5**).

Renal function. Serum creatinine gradually increased to a maximum in both groups 12h after reperfusion (1.62 ± 0.39 vs. 1.15 ± 0.46 mg/dL), ($P = 0.077$) and serum creatinine values were consistently lower in the EPO treated group (Fig. **6**). Hematocrit levels in EPO treated rats were not significantly different from saline treated animals (33.84 ± 3.37% vs. 34.73 ± 4.09%, NS).

EPO-mediated mRNA Expression of HO-1, HGF and Bcl-2 in the Rat Kidney

Administration of EPO, 24h before rats were subjected to sham-operation, resulted in a significant upregulation of HO-1 (3.95 fold), HGF (1.93 fold) and Bcl-2 (1.28 fold) mRNA levels (Fig. **7**). Furthermore, all of the investigated genes were upregulated after IRI, independent of EPO. HO-1 mRNA was already upregulated in the very early phase after renal IRI with a maximum expression level at 2h after reperfusion (19.4 fold). On the other hand, HGF and Bcl-2 mRNA showed maximal upregulation at day 10 following reperfusion, when the kidney was already regenerating (2.72 and 1.35 fold respectively).

EPO-mediated HO-1 mRNA Expression in hTECs was Inhibited by Soluble EPOR

Preconditioning of mixed hTECs with EPO resulted in a significant early (within 2h) upregulation of HO-1 mRNA compared to control cells (1.57 fold; $P < 0.01$) (Fig. **8A**). Furthermore, incubation of purified PTC and DTC during 2h with various concentrations of EPO resulted in a dose-dependent upregulation of HO-1 mRNA which was predominantly restricted to PTC (Fig. **8B**). Administration of soluble EPOR in excess to the EPO concentration resulted in a dose-dependent abolishment of the EPO-induced HO-1 upregulation (Fig. **9**).

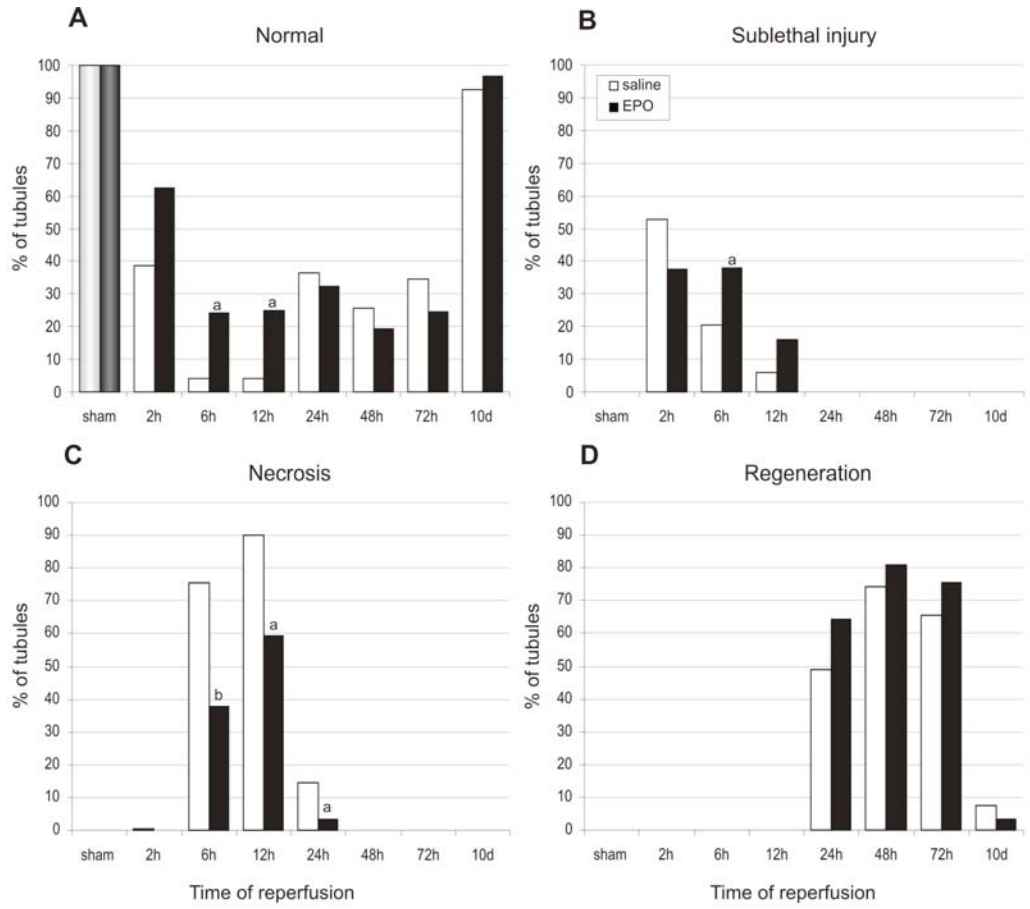

Fig. (3). EPO attenuates renal histological alterations following renal IRI.
Morphological injury was evaluated on PAS-PCNA stained tissue sections. Forty proximal tubules in each animal were scored as follows: morphologically normal (**A**), sublethally injured (**B**), showing necrosis/cell loss (**C**), or showing signs of regeneration (**D**). In the saline treated group, the percentage of normal tubules dropped drastically, toward a minimum at 12h after reperfusion. Part of the damaged tubules only showed sublethal injury to the brush border, whereas others lost their epithelial continuity due to necrosis or detachment of tubular cells. After 24h regenerating tubules started to re-appear, which coincided with a gradual increase in the percentage of normal tubules. EPO pretreatment reduced necrotic cell death both at 6 and 12h after reperfusion. [a] $P < 0.05$; [b] $P < 0.01$ saline vs. EPO treated group.

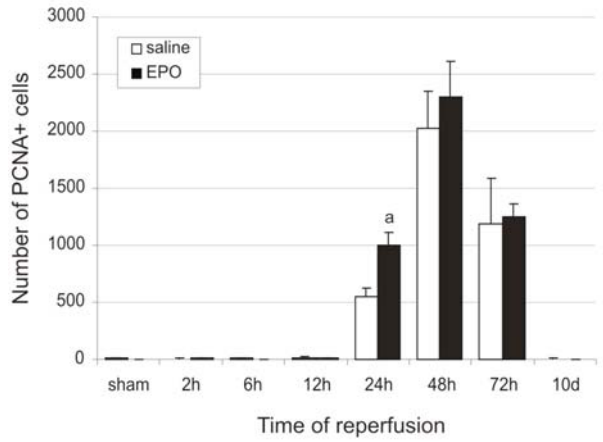

Fig. (4). EPO augments tubular epithelial proliferation in OSOM during renal IRI.
Evaluation of the proliferation of tubular epithelium was performed by counting the number of PCNA positive cells in 20 fields of the OSOM. The amount of PCNA positive cells was significantly higher 24h after reperfusion in the EPO treated group compared to the saline treated group. [a] $P < 0.05$ saline vs. EPO treated group.

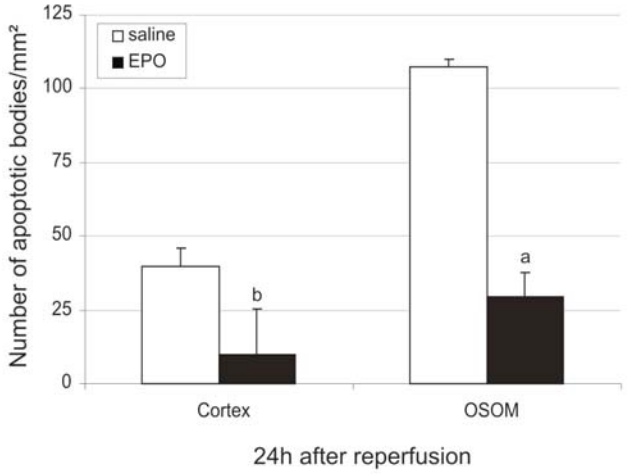

Fig. (5). EPO inhibits apoptotic cell death in renal IRI.
Histological sections of cortex and OSOM were evaluated for apoptosis on TUNEL-stained sections. Apoptotic cell death induced by bilateral IRI was significantly abrogated by EPO both in cortex and OSOM 24h after reperfusion. [a] $P = 0.002$; [b] $P = 0.001$ saline vs. EPO treated group.

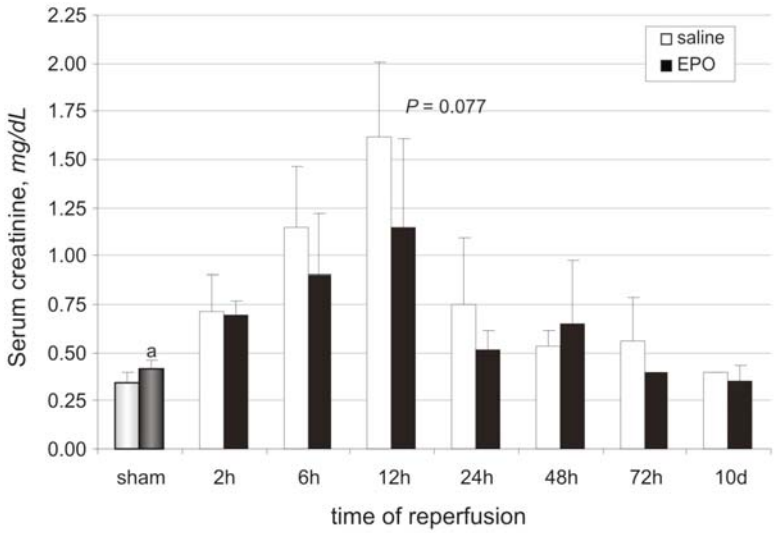

Fig. (6). EPO pretreatment attenuates serum creatinine rise in renal IRI.

Serum creatinine levels peaked 12h after reperfusion and serum creatinine values were consistently lower in the EPO treated group (3000 U/kg, intraperitoneally) as compared to saline treated animals (P = 0.077, 12h after reperfusion). Data are means ± SD. [a] $P < 0.05$ saline vs. EPO treated group.

DISCUSSION

In the bone marrow EPO exerts its erythropoietic effects through direct interaction with the EPOR on erythropoietic precursor cells. As far as its renoprotective effects are concerned, it is not known whether these effects are the result of a direct interaction of EPO with renal TECs and if this renoprotection is mediated through interaction with the "classical" EPOR [36] which has already been identified in homogenates of human and rat kidney tissue and in renal cell lines [13]. In line with these results, in the present study, both EPOR mRNA and protein could be detected in mixed hTECs. EPOR immunostaining of hTECs using the M-20 EPOR antibody, which has been described as the most suitable for immunohistochemistry [15], showed both membranous and intracellular EPOR expression. In addition, EPOR immunostaining was performed on rat kidney tissue sections, but no EPOR positive signals could be visualized. As an alternative technique to investigate the localization of EPOR expression in the rat kidney, laser capture microdissection (LCM) has been used in combination with quantitative RT-PCR. In this way, EPOR expression in the rat kidney could be confined to cortical tubules, and to a lesser extent to tubules from ISOM. In contrast to recent findings of Echigoya *et al.* [37] no glomerular EPOR expression could be found in our studies. In our hands, the administration of EPO as a single dose before the onset of ischemia clearly attenuated renal IRI. Based on morphological criteria and the TUNEL method, tubular epithelial cell death during ARF was characterized as a combination of necrosis and apoptosis and both were clearly decreased following EPO pretreatment. Our finding that EPO inhibited apoptotic cell death and necrosis after renal IRI is in agreement with previous data [1,3-5,7]. In addition to these findings however, this study demonstrates the beneficial effect of EPO on renal necrosis and apoptosis at several reperfusion time points ranging from 2h to 10 days. Bcl-2 has been noticed to contribute to the anti-apoptotic effects of EPO in several models of brain [38-40] and kidney [1,3,4,7] injury. In the present study, Bcl-2 mRNA expression was also significantly upregulated in EPO treated sham-operated rats. In addition to the EPO-mediated activation of proteins of the Bcl-2 family, there is evidence

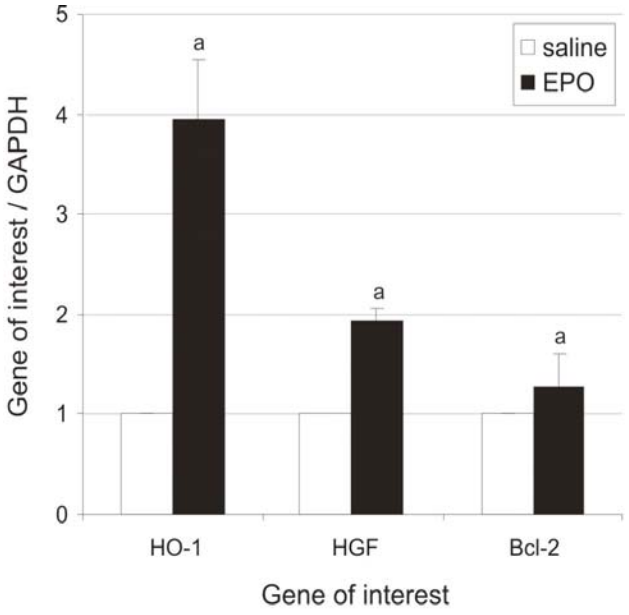

Fig. (7). EPO pretreatment induces the expression of HO-1, HGF and Bcl-2 in sham-operated animals.

Saline or EPO (3000 U/kg, intraperitoneally) was administered 24h prior to sham-operation and rats were sacrificed 24h after sham-operation. Real-time RT-PCR analysis revealed that HO-1, HGF and Bcl-2 mRNA were upregulated following EPO preconditioning (3.95 fold, 1.93 fold and 1.28 fold respectively) (means of triplicate measurements from two runs ± SD). [a] $P < 0.05$ saline vs. EPO treated animals.

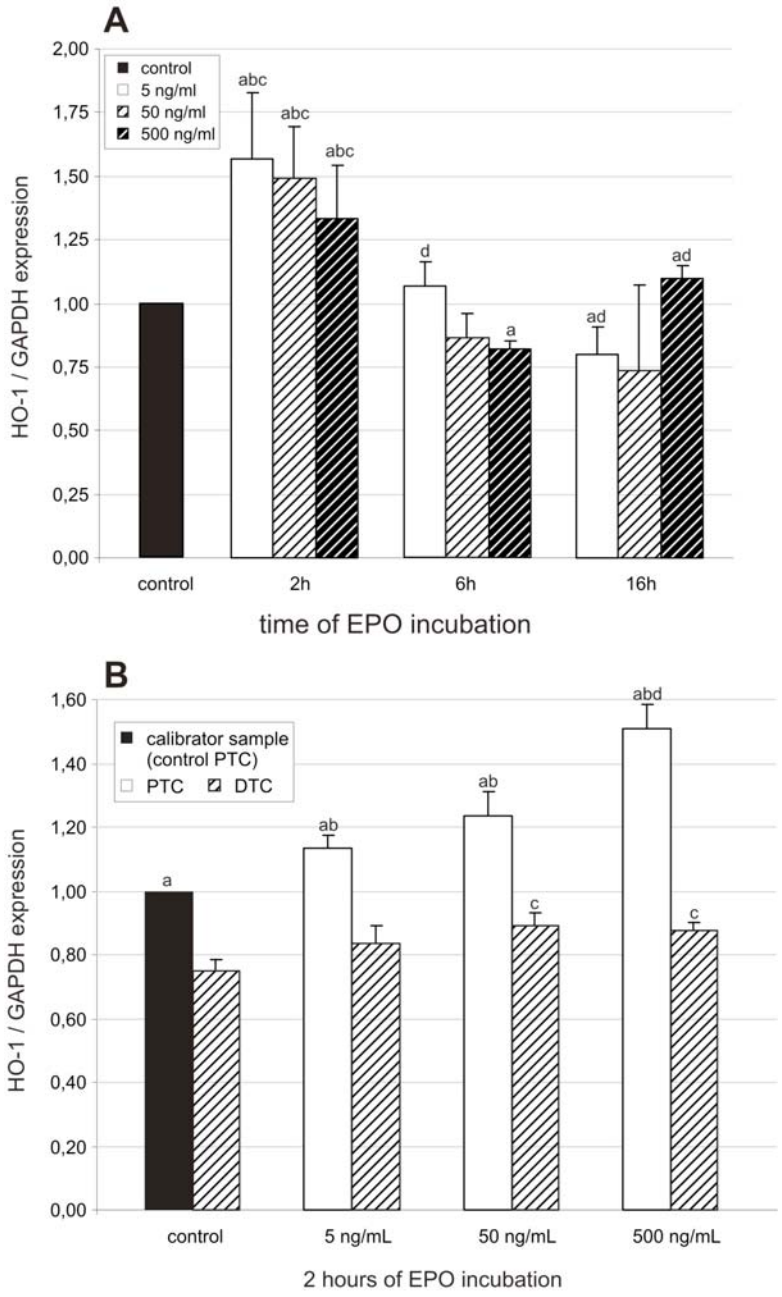

Fig. (8). EPO-mediated HO-1 upregulation is dose-dependent and predominantly restricted to proximal tubular cells.
(**A**) HO-1 relative gene expression in mixed hTECs incubated for 2, 6 and 16h with various concentrations of EPO (5-50-500 ng/mL). GAPDH was used as endogenous control housekeeping gene. Incubating the cells with EPO resulted in a significant early upregulation of HO-1 mRNA (1.57 fold) (means of triplicate measurements from two runs ± SD). [a] $P < 0.01$ vs. control; [b] $P < 0.01$ 2h vs. 6h; [c] $P < 0.05$ 2h vs. 6h; [d] $P < 0.05$ 2h vs. 16h.
(**B**) HO-1 relative gene expression in purified proximal (PTC) and distal tubular/collecting duct (DTC) epithelial cells incubated for 2h with various concentrations of EPO (5-50-500 ng/mL). GAPDH was used as endogenous control housekeeping gene. EPO-mediated HO-1 upregulation was dose-dependent and predominantly restricted to proximal tubular cells (means of triplicate measurements from two runs ± SD). [a] $P < 0.05$ PTC vs. DTC; [b] $P < 0.05$ vs. PTC control; [c] $P < 0.05$ vs. DTC control.

that EPOR signaling activates the ERK/STAT pathways and culminates in pro-survival Akt signaling which could also explain the anti-apoptotic effects of EPO [41]. More importantly, EPO administration resulted in enhanced tubular cell regeneration as indicated by a significant increase in the amount of regenerating cells in the injured regions within the kidney. Although EPO has previously been shown to stimulate proliferation of PTC and to have dose-dependent mitogenic effects *in vitro* [4,9], it is not clear from these studies whether EPO in itself is a growth factor for tubular cells and/or whether EPO acts through induction of other growth factors. In this regard we found EPO to result in a significant increase of HGF mRNA expression. HGF has been shown to have renotrophic activity, to stimulate renal regeneration and

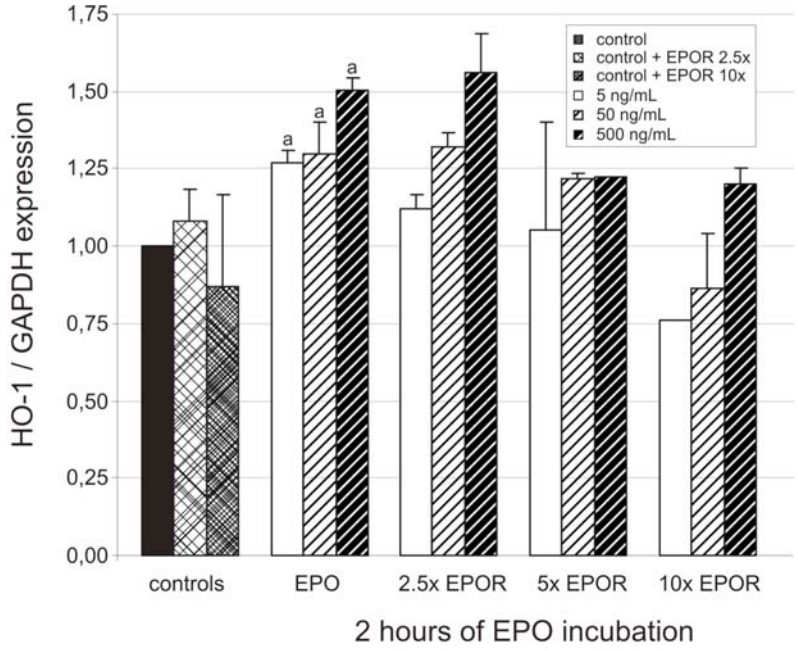

Fig. (9). Simultaneous addition of supra-equivalent amounts of soluble EPOR inhibits EPO-induced HO-1 upregulation.
HO-1 relative gene expression in mixed human renal tubular epithelial cells incubated for 2h with various concentrations of EPO (5-50-500 ng/mL) and soluble EPOR (0x, 2.5x, 5x or 10x excess compared with the molar EPO concentration; Amgen). GAPDH was used as endogenous control housekeeping gene. Soluble EPOR in itself had no effect on HO-1 mRNA expression. Incubating the cells with EPO resulted in a significant dose-dependent upregulation of HO-1 mRNA which was inhibited by high soluble EPOR concentrations (means of triplicate measurements from two runs ± SD). [a] $P < 0.05$ vs. control.

accelerate renal recovery during ARF [18,19,42,43]. Although it has been reported that serum HGF levels are increased after EPO treatment in chronic haemodialysis patients [23], to the best of our knowledge, this is the first report in which an EPO pretreatment-induced rise in HGF has been demonstrated at the site of injury in a rat model in which EPO provides injury attenuation. Many investigators have elegantly demonstrated the protective role of HO-1 in liver, heart and kidney IRI [25-30,44-47]. We could clearly show that EPO preconditioning of sham-operated animals resulted in a four-fold increase of HO-1 mRNA. Again, although increased mononuclear cell HO-1 gene expression and improved plasma anti-oxidant levels have been described in chronic haemodialysis patients after EPO treatment [48], in the present study it was demonstrated for the first time that EPO pretreatment induced a direct increase in HO-1 at the site of injury in a rat model in which EPO provides injury attenuation. Since little is currently known about the protective effects of EPO on human renal tissue, we studied EPO-induced HO-1 upregulation in primary hTECs. EPO administration resulted in an early and dose-dependent upregulation of HO-1 mRNA which was predominantly restricted to PTC. Furthermore, simultaneous addition of supra-equivalent amounts of soluble EPOR inhibited the EPO-mediated HO-1 mRNA upregulation, suggesting that EPO exerts its HO-1-mediated renoprotective effects through interaction with its receptor. Taking into account that (i) renal PTC are the most vulnerable cells during ARF, (ii) HO-1 is expressed in these cells, (iii) HO-1 mRNA is increased after EPO administration and (iv) EPOR is expressed in these cells, one may reasonably suggest an important role of HO-1 and EPOR in the mechanism underlying the renoprotective effects of EPO.

CONCLUSION

In summary, our *in vivo* study confirms the renal protective effect of EPO during renal IRI and the *in vitro* experiments for the first time clearly show that hTECs themselves play a central role in the EPO-mediated upregulation of the cytoprotective protein HO-1. Secondly, the presence of EPOR at both the mRNA and protein level in hTECs together with the results of the *in vitro* experiments using soluble EPOR suggests a direct action of EPO onto renal tubular cells through its "classic" EPOR. Finally, our study shows that HO-1 and HGF are important mediators in EPO-induced renal protection. Our findings may contribute to the development of a novel therapeutic approach in preventing acute kidney injury.

AUTHOR'S CONTRIBUTIONS

A.D.B. designed and performed research, analyzed data and wrote the paper; A.V. and M.H. provided extensive intellectual contribution and reviewed the manuscript; G.S. performed laser capture microdissection experiments; M.D.B. designed research and reviewed the manuscript; D.Y. supplied technical help with surgical routines; P.D. designed research and reviewed the manuscript.

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REFERENCES

[1] Yang CW, Li C, Jung JY, *et al*. Preconditioning with erythropoietin protects against subsequent ischemia-reperfusion injury in rat kidney. FASEB J 2003; 17: 1754-5.

[2] Gong H, Wang W, Kwon TH, *et al*. EPO and alpha-MSH prevent ischemia/reperfusion-induced down-regulation of AQPs and sodium transporters in rat kidney. Kidney Int 2004; 66: 683-95.

[3] Sharples EJ, Patel N, Brown P, *et al*. Erythropoietin protects the kidney against the injury and dysfunction caused by ischemia-reperfusion. J Am Soc Nephrol 2004; 15: 2115-24.

[4] Vesey DA, Cheung C, Pat B, Endre Z, Gobe G, Johnson DW. Erythropoietin protects against ischaemic acute renal injury. Nephrol Dial Transplant 2004; 19: 348-55.

[5] Patel NS, Sharples EJ, Cuzzocrea S, *et al*. Pretreatment with EPO reduces the injury and dysfunction caused by ischemia/reperfusion in the mouse kidney *in vivo*. Kidney Int 2004; 66: 983-9.

[6] Ates E, Yalcin AU, Yilmaz S, Koken T, Tokyol C. Protective effect of erythropoietin on renal ischemia and reperfusion injury. ANZ J Surg 2005; 75: 1100-5.

[7] Spandou E, Tsouchnikas I, Karkavelas G, *et al*. Erythropoietin attenuates renal injury in experimental acute renal failure ischaemic/reperfusion model. Nephrol Dial Transplant 2006; 21: 330-6.

[8] Vaziri ND, Zhou XJ, Liao SY. Erythropoietin enhances recovery from cisplatin-induced acute renal failure. Am J Physiol 1994; 266(3 Pt 2): F360-F366.

[9] Bagnis C, Beaufils H, Jacquiaud C, *et al*. Erythropoietin enhances recovery after cisplatin-induced acute renal failure in the rat. Nephrol Dial Transplant 2001; 16: 932-8.

[10] Johnson DW, Forman C, Vesey DA. Novel renoprotective actions of erythropoietin: new uses for an old hormone. Nephrology (Carlton) 2006; 11: 306-12.

[11] Imamura R, Moriyama T, Isaka Y, *et al*. Erythropoietin protects the kidneys against ischemia reperfusion injury by activating hypoxia inducible factor-1alpha. Transplantation 2007; 83: 1371-9.

[12] Okada T, Sawada T, Kubota K. Asialoerythropoietin has strong renoprotective effects against ischemia-reperfusion injury in a murine model. Transplantation 2007; 84: 504-10.

[13] Westenfelder C, Biddle DL, Baranowski RL. Human, rat, and mouse kidney cells express functional erythropoietin receptors. Kidney Int 1999; 55: 808-20.

[14] Elliott S, Busse L, Bass MB, *et al*. Anti-Epo receptor antibodies do not predict Epo receptor expression. Blood 2006; 107(5): 1892-5.

[15] Kirkeby A, van BJ, Nielsen J, Leist M, Helboe L. Functional and immunochemical characterisation of different antibodies against the erythropoietin receptor. J Neurosci Methods 2007; 164: 50-8.

[16] Matsumoto K, Nakamura T. Hepatocyte growth factor: renotropic role and potential therapeutics for renal diseases. Kidney Int 2001; 59: 2023-38.

[17] Liu Y. Hepatocyte growth factor promotes renal epithelial cell survival by dual mechanisms. Am J Physiol 1999; 277(4 Pt 2): F624-F633.

[18] Kawaida K, Matsumoto K, Shimazu H, Nakamura T. Hepatocyte growth factor prevents acute renal failure and accelerates renal regeneration in mice. Proc Natl Acad Sci USA 1994; 91(10): 4357-61.

[19] Fiaschi-Taesch NM, Santos S, Reddy V, *et al*. Prevention of acute ischemic renal failure by targeted delivery of growth factors to the proximal tubule in transgenic mice: the efficacy of parathyroid hormone-related protein and hepatocyte growth factor. J Am Soc Nephrol 2004; 15: 112-25.

[20] Liu Y, Sun AM, Dworkin LD. Hepatocyte growth factor protects renal epithelial cells from apoptotic cell death. Biochem Biophys Res Commun 1998; 246: 821-6.

[21] Gong R, Rifai A, Tolbert EM, Biswas P, Centracchio JN, Dworkin LD. Hepatocyte growth factor ameliorates renal interstitial inflammation in rat remnant kidney by modulating tubular expression of macrophage chemoattractant protein-1 and RANTES. J Am Soc Nephrol 2004; 15: 2868-81.

[22] Min JK, Lee YM, Kim JH, *et al*. Hepatocyte growth factor suppresses vascular endothelial growth factor-induced expression of endothelial ICAM-1 and VCAM-1 by inhibiting the nuclear factor-kappaB pathway. Circ Res 2005; 96: 300-7.

[23] Borawski J, Mysliwiec M. Serum hepatocyte growth factor is associated with viral hepatitis, cardiovascular disease, erythropoietin treatment, and type of heparin in haemodialysis patients. Nephrol Dial Transplant 2002; 17: 637-44.

[24] Katavetin P, Inagi R, Miyata T, *et al*. Erythropoietin induces heme oxygenase-1 expression and attenuates oxidative stress. Biochem Biophys Res Commun 2007; 359: 928-34.

[25] Akagi R, Takahashi T, Sassa S. Fundamental role of heme oxygenase in the protection against ischemic acute renal failure. Jpn J Pharmacol 2002; 88: 127-32.

[26] Wagner M, Cadetg P, Ruf R, Mazzucchelli L, Ferrari P, Redaelli CA. Heme oxygenase-1 attenuates ischemia/reperfusion-induced apoptosis and improves survival in rat renal allografts. Kidney Int 2003; 63: 1564-73.

[27] Blydt-Hansen TD, Katori M, Lassman C, *et al*. Gene transfer-induced local heme oxygenase-1 overexpression protects rat kidney transplants from ischemia/reperfusion injury. J Am Soc Nephrol 2003; 14: 745-54.

[28] Goncalves GM, Cenedeze MA, Feitoza CQ, *et al*. The role of heme oxygenase 1 in rapamycin-induced renal dysfunction after ischemia and reperfusion injury. Kidney Int 2006; 70(10): 1742-9.

[29] Shimizu H, Takahashi T, Suzuki T, *et al*. Protective effect of heme oxygenase induction in ischemic acute renal failure. Crit Care Med 2000; 28: 809-17.

[30] Takahashi T, Morita K, Akagi R, Sassa S. Protective role of heme oxygenase-1 in renal ischemia. Antioxid Redox Signal 2004; 6: 867-77.

[31] Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29: e45.

[32] Deng JT, Hoylaerts MF, Nouwen EJ, De Broe ME, Van Hoof V. Purification of circulating liver plasma membrane fragments using a monoclonal antileucine aminopeptidase antibody. Hepatology 1996; 23: 445-54.

[33] Helbert MJ, Dauwe S, De Broe ME. Flow cytometric immunodissection of the human nephron *in vivo* and *in vitro*. Exp Nephrol 1999; 7: 360-76.

[34] Helbert MJ, Dauwe SE, Van der Biest I, Nouwen EJ, De Broe ME. Immunodissection of the human proximal nephron: flow sorting of S1S2S3, S1S2 and S3 proximal tubular cells. Kidney Int 1997; 52: 414-28.

[35] Gibson-D'Ambrosio RE, Samuel M, Chang CC, Trosko JE, D'Ambrosio SM. Characteristics of long-term human epithelial cell cultures derived from normal human fetal kidney. *In Vitro* Cell Dev Biol 1987; 23: 279-87.

[36] Brines M, Grasso G, Fiordaliso F, *et al*. Erythropoietin mediates tissue protection through an erythropoietin and common beta-subunit heteroreceptor. Proc Natl Acad Sci USA 2004; 101: 14907-12.

[37] Echigoya MH, Obikane K, Nakashima T, Sasaki S. Glomerular localization of erythropoietin receptor mRNA and protein in neonatal and mature mouse kidney. Nephron Exp Nephrol 2005; 100: e21-e29.

[38] Emir M, Ozisik K, Cagli K, *et al*. Effect of erythropoietin on bcl-2 gene expression in rat cardiac myocytes after traumatic brain injury. Transplant Proc 2004; 36: 2935-8.

[39] Sattler MB, Merkler D, Maier K, *et al*. Neuroprotective effects and intracellular signaling pathways of erythropoietin in a rat model of multiple sclerosis. Cell Death Differ 2004; 11 (Suppl 2): S181-S192.

[40] Kumral A, Genc S, Ozer E, *et al*. Erythropoietin downregulates bax and DP5 proapoptotic gene expression in neonatal hypoxic-ischemic brain injury. Biol Neonate 2006; 89: 205-10.

[41] Bahlmann FH, Song R, Boehm SM, *et al*. Low-dose therapy with the long-acting erythropoietin analogue darbepoetin alpha persis-

tently activates endothelial Akt and attenuates progressive organ failure. Circulation 2004; 110: 1006-12.

[42] Miller SB, Martin DR, Kissane J, Hammerman MR. Hepatocyte growth factor accelerates recovery from acute ischemic renal injury in rats. Am J Physiol 1994; 266(1 Pt 2): F129-F134.

[43] Dai C, Yang J, Liu Y. Single injection of naked plasmid encoding hepatocyte growth factor prevents cell death and ameliorates acute renal failure in mice. J Am Soc Nephrol 2002; 13: 411-22.

[44] Amersi F, Buelow R, Kato H, *et al*. Upregulation of heme oxygenase-1 protects genetically fat Zucker rat livers from ischemia/reperfusion injury. J Clin Invest 1999; 104: 1631-9.

[45] Kato H, Amersi F, Buelow R, *et al*. Heme oxygenase-1 overexpression protects rat livers from ischemia/reperfusion injury with extended cold preservation. Am J Transplant 2001; 1: 121-8.

[46] Katori M, Buelow R, Ke B, *et al*. Heme oxygenase-1 overexpression protects rat hearts from cold ischemia/reperfusion injury via an antiapoptotic pathway. Transplantation 2002; 73: 287-92.

[47] Tsuchihashi S, Fondevila C, Kupiec-Weglinski JW. Heme oxygenase system in ischemia and reperfusion injury. Ann Transplant 2004; 9: 84-7.

[48] Calo LA, Stanic L, Davis PA, *et al*. Effect of epoetin on HO-1 mRNA level and plasma antioxidants in hemodialysis patients. Int J Clin Pharmacol Ther 2003; 41: 187-92.

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