Asialoerythropoietin Prevents Contrast-Induced Nephropathy

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ABSTRACT

Strategies to prevent contrast-induced nephropathy (CIN) are suboptimal. Erythropoietin was recently found to be cytoprotective in a variety of nonhematopoietic cells, so it was hypothesized that the nonhematopoietic erythropoietin derivative asialoerythropoietin would prevent CIN. Nephropathy was induced in rats by injection of the radiocontrast medium Ioversol in addition to inhibition of prostaglandin and nitric oxide synthesis. Administration of a single dose of asialoerythropoietin before the induction of nephropathy significantly attenuated the resulting renal dysfunction and histologic renal tubular injury. Contrast-induced apoptosis of renal tubular cells was inhibited by asialoerythropoietin both in vivo and in vitro, and this effect was blocked by a Janus kinase 2 (JAK2) inhibitor in vitro. Furthermore, phospho-JAK2/signal transducer and activator of transcription 5 (STAT5) and heat-shock protein 70 increased after injection of asialoerythropoietin, suggesting that the effects of asialoerythropoietin may be mediated by the activation of the JAK2/STAT5 pathway. Overall, these findings suggest that asialoerythropoietin may have potential as a new therapeutic approach to prevent CIN given its ability to preserve renal function and directly protect renal tissue.


Contrast medium–induced nephropathy (CIN) is an important problem in clinical practice. With the increasing use of contrast media in diagnostic and interventional procedures, CIN has become the third leading cause of hospital-acquired acute renal failure, accounting for 10 to 25% of all acute renal failure cases,1–4 despite the introduction of newer and safer contrast media, the improvement of hydration protocols, and the introduction of additional preventive strategies (e.g., acetylcysteine).5 Patients with CIN have been reported to have increased frequency of clinical adverse events, including permanent impairment of renal function, longer hospital stay, and increased 1-yr mortality rate6,7; therefore, there is a great requirement for new, effective strategies for prevention of CIN.

Erythropoietin (EPO), a cytokine that was originally identified as a hematopoietic factor, has been widely used for the treatment of anemia in patients with chronic kidney diseases for nearly 20 yr.8 More recently, EPO was found to interact with its receptor, expressed in a great variety of nonhematopoietic cell types (e.g., neurons,9 endothelial cells,10 vascular smooth muscle cells,11 cardiomyocytes,12 mesangial cells, renal proximal tubular cells13) to induce a range of cytoprotective cellular responses (mitogenesis,13 angiogenesis,14 promotion of vas-
cular repair,\(^{15}\) and inhibition of apoptosis\(^{16,17}\), which are independent of EPO’s effects on erythropoiesis.\(^{18}\) Indeed, EPO was reported to attenuate renal injury in acute renal failure models in experimental animals with ischemia-reperfusion,\(^{19,20}\) cisplatin,\(^{21}\) and radiocontrast medium injuries.\(^{22}\) Furthermore, several engineered EPO derivatives have been developed.\(^{23}\) Desialylated EPO (asialoEPO), one of these derivatives, has EPO receptor–binding affinity similar to native EPO,\(^{24}\) but it has an extremely short half-life \textit{in vivo} and reduces the magnitude of the hematopoietic response; however, it has been reported to retain neuroprotective activities \textit{in vivo}.\(^{25,26}\) Therefore, we hypothesized that asialoEPO can also attenuate renal injury in experimental acute renal failure. In this article, we provide evidence that systemically administered asialoEPO inhibits contrast medium–induced renal dysfunction and renal tubular apoptosis in a rat model of CIN as potently as native erythropoietin.

**RESULTS**

**Effects of EPO and AsialoEPO on Renal Dysfunction Caused by Contrast Medium**

In the rat model of CIN, induced by the administration of Ioversol after the inhibitions of prostaglandin and nitric oxide synthesis, the animals exhibited elevations in serum creatinine (SCr) and blood urea nitrogen (BUN) and a decrease in creatinine clearance compared with the control group at 24 h after the injection of Ioversol (Table 1). Pretreatment with a single intravenous bolus of EPO (80 ng/g) at 1 h before the injection of Ioversol significantly attenuated the Ioversol-induced renal dysfunction in comparison with the control group (Table 1). Similarly, administration of asialoEPO (80 ng/g) significantly improved the Ioversol-induced renal dysfunction in comparison with the Ioversol group (Table 1). The administration of EPO or asialoEPO did not produce any effects on body weight or hematocrit in either the control or the Ioversol group (Table 1).

**Effects of EPO and AsialoEPO on Histologic Alterations Caused by Contrast Medium**

The Ioversol group presented marked alterations in renal histology compared with the control group (Figure 1, A and C). Specifically, the most severe and pronounced alterations were observed in the renal tubular architecture, including severe cytoplasmic vacuolar changes, intratubular cast formation, and luminal congestion (Figure 1, C and H). Kidney sections

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Control + AsialoEPO</th>
<th>Ioversol</th>
<th>Iversol + EPO</th>
<th>Iversol + AsialoEPO</th>
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<tr>
<td>(n)</td>
<td>7</td>
<td>7</td>
<td>15</td>
<td>4</td>
<td>11</td>
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<tr>
<td>Body weight (g)</td>
<td>260.7 ± 3.5</td>
<td>268.6 ± 3.4</td>
<td>290.3 ± 4.6</td>
<td>245.0 ± 17.6</td>
<td>290.0 ± 10.4</td>
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<tr>
<td>SCr (mg/dl)</td>
<td>0.217 ± 0.010</td>
<td>0.224 ± 0.014</td>
<td>0.317 ± 0.020(^{P})</td>
<td>0.202 ± 0.019</td>
<td>0.233 ± 0.014</td>
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<tr>
<td>BUN (mg/dl)</td>
<td>11.43 ± 0.85</td>
<td>11.70 ± 0.59</td>
<td>24.19 ± 2.83(^{P})</td>
<td>11.125 ± 1.89</td>
<td>13.56 ± 1.37</td>
</tr>
<tr>
<td>Ccr (ml/min)</td>
<td>1.96 ± 0.12</td>
<td>1.86 ± 0.15</td>
<td>1.44 ± 0.09(^{P})</td>
<td>1.662 ± 0.18</td>
<td>1.90 ± 0.09</td>
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<td>Hematocrit (%)</td>
<td>49.9 ± 2.0</td>
<td>50.0 ± 1.7</td>
<td>52.4 ± 1.4</td>
<td>51.4 ± 2.1</td>
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\(^{a}\)Data are means ± SEM. Ccr, creatinine clearance.

\(^{b}\)\(^{P}\) < 0.05 versus other groups.

Figure 1. EPO or asialoEPO attenuates the contrast medium–induced morphologic changes. The marked tubular injuries caused by Ioversol (C and H) are diminished by pretreatment with EPO (D and I) or asialoEPO (E and J). Representative photomicrographs of HE-stained kidney sections are presented. (A and F) Control rat. (B and G) Control rat pretreated with asialoEPO. (C and H) Rat treated with Ioversol. (D and I) Rat treated with Ioversol after EPO treatment. (E and J) Rat treated with Ioversol after asialoEPO treatment. Figures are representative of four to 10 rats in each group. Magnifications: \(\times 100\) in A through E; \(\times 400\) in F through J.
from the ioversol groups that were pretreated with EPO or asialoEPO demonstrated markedly diminished histologic features of each of these renal tubular injuries (Figure 1, D, E, I, and J).

**Effects of EPO and AsialoEPO on Renal Tubular Apoptosis Caused by Contrast Medium**

Upon evaluation of renal tubular cell apoptosis by terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) immunostaining to localize DNA fragmentation in kidney sections, the kidneys from the ioversol group showed markedly increased number of TUNEL-positive tubular cells (Figure 2C), but the number was markedly decreased in the rats that were pretreated with EPO or asialoEPO (Figure 2, D and E). Quantitative analysis of TUNEL-positive cells also revealed that pretreatment with EPO or asialoEPO significantly decreased the apoptotic cell numbers in the ioversol group (Figure 2F).

**Effects of EPO and AsialoEPO on Caspase-3 Activity and Protein-Cleaved Caspase-3 from Rat Kidneys**

In further evaluation of contrast medium–induced renal cell apoptosis with the measurements of caspase-3 activity and its protein cleavage, caspase-3 activity in renal homogenates from the ioversol group was significantly elevated compared with the control group (Figure 3A). This elevation was significantly reduced by EPO or asialoEPO administration (Figure 3A). Immunohistochemical staining using a specific antibody for the cleaved fragment of caspase-3, which indicates caspase-3 activation, revealed increased cytoplasmic staining in the renal tubular cells after ioversol administration (Figure 3D), but this staining was markedly reduced in rats that were pretreated with EPO or asialoEPO (Figure 3, E and F).

**Effects of EPO and AsialoEPO on Contrast Medium–Induced Apoptosis in Cultured Renal Proximal Tubular Cells (LLC-PK1 Cells)**

In LLC-PK1 cells, 30-min exposure to ioversol significantly increased the caspase-3 activity at 24 h after exposure when compared with control cells (Figure 4). The 1-h preincubation with EPO or asialoEPO (25 ng/ml) significantly suppressed the ioversol-induced caspase-3 activation, indicating that both agents directly inhibit contrast medium–induced apoptosis (Figure 4).

**Effects of EPO and AsialoEPO Pretreatment on Signal Transductions in Vitro and In Vivo**

Next we examined the effects of EPO and asialoEPO on the signal transduction pathways in the kidney, which are thought to be related to antiapoptotic effects of EPO.16–18 In LLC-PK1 cells, 5-min exposure to asialoEPO or EPO significantly increased the phosphorylation of Janus kinase 2 (JAK2)/signal transducer and activator of transcription 5 (STAT5). The expression of heat-shock protein 70 (HSP70) protein was also enhanced 1 h after asialoEPO or EPO exposure (Figure 5, A and B). The asialoEPO- or EPO-induced phosphorylation of JAK2/STAT5 and expression of HSP70 protein were markedly inhibited by a JAK2 inhibitor (AG490; 50 μM; data not shown). Furthermore, the inhibitory effect of asialoEPO on ioversol-induced caspase-3 activation was reversed by JAK2 inhibitor (Figure 5C), suggesting that asialoEPO attenuates contrast medium–induced apoptosis in the renal tubular cells via the JAK2 signaling pathway. In the rat kidneys, the phosphorylation of JAK2/STAT5 was markedly increased within 5 min after EPO or asialoEPO administration, and the expression of HSP70 was enhanced 1 h after injection of these agents (Figure 6).
DISCUSSION

In this study, we found that both EPO and a nonhematopoietic EPO derivative, asialoEPO, prevent the development of renal dysfunction as assessed by biochemical parameters in a rat model of CIN. We also demonstrated that either EPO or asialoEPO markedly suppressed renal tubular injuries as assessed by histologic examination when administered as a single intravenous bolus injection 1 h before the injection of contrast medium. Furthermore, either can directly prevent the contrast medium–induced apoptosis of renal tubular cells observed both in vivo and in vitro.

We used both in vivo and in vitro models of radiocontrast-induced nephropathy in this study. In our study, injection of rats with contrast medium at the dosage of 2.9 g/kg iodine reproducibly produced renal dysfunction. Although this injected dosage is higher than that for clinical use (1.5 g/kg iodine in routine angiographic practice), several previous studies of CIN in rats used similar dosages of contrast medium.27,28 We also selected the in vitro concentration of Ioversol to induce proximal tubular cell injury (100 mg/ml iodine) to mimic the renal tubular concentration observed in clinical practice. Although the routinely used injection volume of contrast medium results in plasma concentrations of approximately 10 to 20 mg/ml iodine,29 proximal tubule concentrations are significantly higher as a result of the reabsorption of water/solutes in this structure.30 In previous studies, urinary iodine concentration ranged from 125 to 200 mg/ml iodine in rats after injection at clinical dosages of Iohexol (1.6 g/kg iodine).31

Although the pathogenic mechanism of CIN is controversial, the direct toxic effect of contrast medium on the renal tubular cells is known to play an important role in its development.1 Pathologic changes of renal tubular cells (e.g., cytoplasmic vacuolar changes, cell death/necrosis, as shown in this study), have been regarded as a characteristic pattern of contrast medium–induced direct tubular injury.32 Several studies have already shown that contrast medium can directly induce cytotoxic effects or caspase-dependent apoptosis in cultured renal tubular cells.29,33 Indeed, we also found that Ioversol can activate caspase-3, resulting in apoptosis in cultured LLC-PK1 cells. Furthermore, the preincubation with EPO or asialoEPO can directly inhibit this Ioversol-induced activation of caspase-3 in these cells, with concomitant increments of the phosphorylation of JAK2/STAT5 and expression of HSP70. We also noted that bolus injection of EPO or asialoEPO can rapidly increase the phosphorylation of JAK2/STAT5 and ex-

Figure 3. Administration of EPO or asialoEPO inhibits the activation of caspase-3 caused by contrast medium. In homogenates of rat kidneys 24 h after injection of Ioversol, caspase-3 activity was significantly increased compared with control rats. (A) EPO or asialoEPO administration significantly reduced caspase-3 activities. Data are means ± SE for four to 10 rats in each group. The exposure to Ioversol also increases cytoplasmic staining in the renal tubular cells at 24 h after exposure, but the staining for caspase-3 is markedly reduced in the Ioversol-treated groups that were pretreated with EPO or asialoEPO (B through F). Representative photomicrographs of kidney sections immunohistochemically stained with anti–active cleaved caspase-3 antibody from control rat (B), control rat pretreated with asialoEPO (C), rat treated with Ioversol (D), rat treated with Ioversol after EPO treatment (E), and rat treated with Ioversol after asialoEPO treatment (F). Magnification, ×100.

Figure 4. EPO or asialoEPO directly inhibits Ioversol-induced apoptosis in cultured LLC-PK1 cells. The cells were incubated with Ioversol for 30 min with or without pretreatment with EPO or asialoEPO for 1 h, and the activities of caspase-3 were measured at 24 h after Ioversol exposure. Data are means ± SEM (n = 4).
role in the prevention of contrast medium–induced renal tubular cell apoptosis observed in this study. Because EPO receptors are known to be expressed in renal tubular epithelial cells, these results indicate that native EPO and asialoEPO might have antiapoptotic effects on renal tubular cells mediated by the similar signaling pathways (JAK2, STAT5, and HSP70), both in vivo and in vitro; therefore, we speculate that EPO and asialoEPO can directly inhibit renal tubular cell apoptosis caused by contrast medium, thereby preventing the renal dysfunction induced by contrast medium.

To our knowledge, this is the first report documenting that asialoEPO can prevent renal injury in experimental models of acute renal failure (i.e., CIN). We found that asialoEPO prevented contrast medium–induced renal dysfunction and improved renal morphologic tubular injuries and apoptosis as potently as native EPO. These results indicate that the renoprotective effects of EPO might be independent of its erythropoietic function, because asialoEPO is a nonerythropoietic derivative of EPO. Goldfarb et al. also reported that native EPO can prevent contrast medium–induced renal dysfunction, but it does not prevent renal tubular injury related to CIN. The dosages of EPO that they used were 3000 and 600 U/kg at 24 and 2 h, respectively, before the CIN protocol. We selected the single injection of EPO and asialoEPO (10,000 U/kg, 80 ng/g) at 1 h before the exposure to contrast medium, identical to the protocol used by Wang et al. in their study showing that the single injection of asialoEPO can prevent ischemic neuronal damage, which might account for the dif-

Figure 5. Administration of asialoEPO or EPO activates intracellular signals in cultured proximal tubular cells. Cultured LLC-PK1 cells were incubated with asialoEPO (25 ng/ml) or EPO (25 ng/ml). Phosphorylated/total JAK2, STAT5, and HSP70 expressions in the LLC-PK1 cells were determined by immunoblot analysis. (A) Phosphorylated and total JAK2 and STAT5 after 5-min exposure to asialoEPO or EPO were determined with immunoblot analysis with the indicated antibody. The expressions of HSP70 and β-actin after 1-h exposure were also determined with immunoblot analysis. Blots are representative of at least four independent experiments. (B) Each set of immunoblots was quantitatively evaluated with densitometric analysis. (C) Pretreatment with JAK2 inhibitor (AG490; 50 μM) for 1 h significantly reversed the inhibitory effects of asialoEPO on Ioversol-induced caspase-3 activation.

expression of HSP70 in the kidneys in vivo. Indeed, HSP70 has already been reported to be among a subset of stress-responsive proteins regulated by activation of the JAK/STAT pathway, and the induction of HSP70 expression has been shown to promote renal tubular cell survival by inhibiting apoptosis in both in vivo and in vitro models of acute renal failure. Furthermore, it has been reported that the EPO-induced expression of HSP70 might contribute the EPO-related tissue protection (e.g., against myocardial infarction or renal ischemia-reperfusion injury) therefore, the induction of HSP70 expression at 1 h after stimulation with EPO or asialoEPO both in vitro and in vivo might have played an important

Figure 6. Administration of asialoEPO or EPO activates intracellular signals in rat kidneys in vivo. After bolus injection of asialoEPO (80 ng/g) or EPO (80 ng/g) in rats, the renal homogenates were subjected to immunoblot analysis. (A) Phosphorylated/total JAK2 and STAT5 after 5-min exposure to asialoEPO or EPO and the expression of HSP70 after 1-h exposure to asialoEPO or EPO in the kidney samples. Blots are representative of at least four rats. (B) Each set of immunoblots was quantitatively evaluated with densitometric analysis.
different effects of EPO on renal tubular injury associated with CIN that we observed in our study. Our experimental protocol, a single injection of a high dosage of EPO, did not raise the hematocrit of the test animals and would similarly be expected to have little effects in humans; however, the adverse effects of a high dosage of EPO (polycythemia-hyperviscosity syndrome, hypertension, and vascular thrombosis) cannot be completely ignored in clinical use. AsialoEPO has an extremely short half-life in vivo and cannot increase the hematocrit; therefore, asialoEPO might be theoretically safer than native EPO for clinical use for prevention of CIN.

In conclusion, our study suggests that asialoEPO administration in a single dose before the induction of CIN exerts significant renoprotective effects in a rat model of CIN. The results indicate that asialoEPO could represent a novel and effective preventive approach for CIN as a result of its capacity to preserve renal function and directly protect renal tissue. Investigation with additional experimental studies and clinical trials is required to confirm our experimental findings to ascertain the clinical safety of asialoEPO and to optimize dosage and administration schedule for prevention of the development of CIN.

CONCISE METHODS

Materials

EPO and AsialoEPO were from Chugai Pharmaceutical Co., Ltd. (Shizuoka, Japan). The iodinated radiographic contrast agent used in this study, Ioversol (Optiray 320; 320 mg/ml iodine), is a nonionic monomeric iodinated contrast medium with osmolality approximately twice that of saline and viscosity of 5.8 mPa/s (data obtained from Tyco Healthcare Japan, Tokyo, Japan). Anti–cleaved caspase-3 (ASP175), anti–phospho-JAK2, anti–phospho-STAT5, anti-JAK2, and anti-STAT5 antibodies were from Cell Signaling Technology (Beverly, MA). Anti-HSP70 antibody was from Stressgen (Victoria, BC, Canada), and anti–β-actin antibody was from Santa Cruz Biotechnology (Santa Cruz, CA).

Animal Experiments

The protocol was approved by animal experiment ethics review committees of the Research Center for Animal Life Science of Shiga University of Medical Science. Protocol 1 was designed to investigate the effect of pretreatment with EPO or asialoEPO on subsequent CIN, and protocol 2 was designed to determine signal transduction in the rat kidneys in vivo after injection of these agents.

Protocol 1: CIN in Rats

Mature male Sprague-Dawley rats, weighing approximately 250 to 300 g at the start of the experiment, were housed in individual cages under controlled conditions of light (12 h/12 h light/dark cycle) and temperature (21 to 23°C) and allowed free access to standard laboratory diet and tap water. After 7 d of acclimation to the experimental area, rats were randomized to receive EPO (80 ng/g), asialoEPO (80 ng/g; equivalent to 10,000 U/kg native EPO), or PBS 1 h before the induction of CIN. Rats were subjected to the previously detailed CIN protocol, consisting of indomethacin (10 mg/kg; Wako Pure Chemical, Osaka, Japan), followed at 15 and 30 min, respectively, by N-ω nitro-ω-arginine methyl ester (10 mg/kg; Wako Pure Chemical) and by Ioversol (2.9 g/kg organically bound iodine). Controls were administered an injection of PBS alone at each time. All drugs were injected from a tail vein under light sevoflurane anesthesia. The rats were allowed to recover in the metabolic cages for an additional 24 h, and the 24-h urine samples were collected for determination of urinary creatinine excretion. After the blood samples were collected for determination of SCr, BUN, and hematocrit, the rats were killed under pentobarbital (50 mg/kg) anesthesia. The kidneys were removed and bisected in the equatorial plane, the right kidney was divided for protein assay and evaluation of caspase-3 activity, and the left kidney was fixed in buffered 10% formalin and prepared for routine histologic examinations.

Protocol 2: Effects of EPO and AsialoEPO on Intracellular Signaling in the Rat Kidney

The phosphorylation of JAK2/STAT5 and expression of HSP70 were examined in the rat kidneys after the bolus injection of EPO or asialoEPO (80 ng/g). The treated rats were killed at indicated times as described for protocol 1. The kidneys were removed, and kidney homogenates were subjected to immunoblot analysis. Kidneys from four rats were used at each time point.

Evaluation of Blood and Urine Samples

Hematocrit was assessed using a hematocrit reader (Terumo, Tokyo, Japan). Mean SCr and urinary creatinine concentrations were measured by enzymatic method, using the respective assay kits (Kainos, Tokyo, Japan). Mean BUN concentration was determined by the diacetylmonoxime method using an assay kit (Wako Pure Chemical). Creatinine clearance during 24 h after the injection of contrast medium was calculated as ml/min.

Histologic Examination of Renal Tissues

The left kidney fixed in 10% formalin was dehydrated in a graded ethyl alcohol series and embedded in paraffin. Kidney blocks cut into 4-μm sections were subjected to hematoxylin and eosin (HE) staining for morphologic analysis and TUNEL staining and immunohistochemistry (cleaved caspase-3) for cell apoptosis. HE-stained tissue sections were viewed using light microscopy in 20 microscope fields per section zone at ×100 magnification. For TUNEL staining, sections were stained using ApopTag Peroxidase In situ Apoptosis Detection Kit (Takara, Shiga, Japan) according to the manufacturer’s protocol for paraffin-embedded sections. Numbers of TUNEL-positive tubular cells were counted at random in 20 nonoverlapping cortical fields under ×400 magnification and averaged. For immunohistochemistry of cleaved caspase-3, sections were deparaffinized and rehydrated before staining by the peroxidase-antiperoxidase method. Nonspecific binding of peroxidase or antibodies was blocked with 0.3% H2O2 in methanol followed by incubation in diluted normal swine serum. After blocking with 10% goat serum (Nichirei, Tokyo, Japan), anti–cleaved caspase-3 (ASP175) antibody (diluted 1:100 in TBS-T/1% BSA) was applied at 4°C overnight. Samples were washed
in buffer and incubated with the secondary antibody, using Histofine Simple Stain Rat MAX PO kit (Nichirei) for 30 min, followed by the dianisobenzidine-peroxidase reaction for 10 min. Rinsed with PBS, sections were lightly counterstained with hematoxylin and then dehydrated in xylene and mounted in Depex. Immunohistochemistry tissue sections were viewed using light microscopy in 20 microscope fields per section zone at ×100 magnification. All slides were observed by two blinded examiners independently.

**Cell Culture**

LLC-PK1 cells (American Type Culture Collection, Rockville, MD) were grown in a 10-cm² dish (Corning, Corning, NY) and maintained in Medium 199 (Life Technologies Invitrogen Corp., Grand Island, NY) supplemented with 10% FBS (Biosource, Rockville, MD), penicillin (100 U/ml), and streptomycin (100 µg/ml) in an atmosphere of 5% CO₂ in air at 37°C as previously reported. For this experiment, the cells were seeded on six-well plastic plates (Falcon; Becton Dickinson Co., Ltd., Franklin Lakes, NJ) at density of 1.0 × 10⁵ cells/cm² and cultured at 37°C for 24 h. After starvation with serum-free medium for 24 h, the cells were treated with or without EPO or asialo-EPO for 1 h and then exposed to 100-mg/ml-iodine Ioversol for 30 min, followed by incubation for 24 h without Ioversol. Apoptosis was evaluated by determination of caspase-3 activity.

**Assay for Caspase-3 Activity**

Caspase-3 activity was determined colorimetrically according to the manufacturer’s protocol in vivo and in vitro by using a caspase-3 activity assay kit (BioVision, Mountain View, CA) and expressed per microgram of protein. The protein concentration was measured by the Bradford method using Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA). Preincubation with JAK2 inhibitor (AG490; Calbiochem, Darmstadt, Germany; 50 µM) was carried out 1 h before treatment with asialoEPO.

**Immunoblot Analysis**

For the analysis of JAK2, STAT5, and HSP70, the kidneys of the rats from the four groups and the cultured LLC-PK1 cells were lysed in ice-cold lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl (pH 8.0), 0.1% SDS, and 1% Nonidet P-40. The homogenized renal tissues or the cell lysates were centrifuged at 15,000 rpm for 10 min after homogenization and were subjected to immunoblot analysis as described previously. For this experiment, the cells were seeded on six-well plastic plates (Falcon; Becton Dickinson Co., Ltd., Franklin Lakes, NJ) at density of 1.0 × 10⁵ cells/cm² and cultured at 37°C for 24 h. After starvation with serum-free medium for 24 h, the cells were treated with or without EPO or asialo-EPO for 1 h and then exposed to 100-mg/ml-iodine Ioversol for 30 min, followed by incubation for 24 h without Ioversol. Apoptosis was evaluated by determination of caspase-3 activity.

**Statistical Analysis**

Results are expressed as means ± SE. ANOVA with subsequent Scheffe test was used to determine the significance of differences in multiple comparisons. P < 0.05 was considered statistically significant.

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**DISCLOSURES**

None.

**REFERENCES**


