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HEPATIC ISCHEMIA-REPERFUSION INDUCES RENAL HEME OXYGENASE-1 (HO-1) VIA NF-E2-RELATED FACTOR 2 (Nrf2) IN RATS AND MICE

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Running Title : Renal heme oxygenase-1 induction after hepatic ischemia

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ABBREVIATIONS

IR, ischemia-reperfusion; TNFα, tumor necrosis factor α; HO-1, heme oxygenase-1; Nrf2, NF-E2-related factor 2; NQO1, NAD(P)H:quinone oxidoreductase 1; 15-d-PGJ2, 15-deoxy-Δ12,14-prostaglandin J2; IL1β, interleukin1β; NFκB, nuclear factor-κB; AP-1, activator protein 1; $GdCl_3$, Gadolinium chloride (III) hexahydrate; bDNA, branched DNA signal amplification; BCA, bicinchoninic acid; NPSH, non-protein sulfhydryls; Keap1, Kelch-like ECH associated protein 1

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ABSTRACT

Hepatic ischemia-reperfusion (IR) results in Kupffer cell activation and subsequent tumor necrosis factor (TNF) α release, leading to localized hepatic injury and remote organ dysfunction. Heme oxygenase (HO)-1 is an enzyme that is induced by various stimuli, including proinflammatory cytokines, and exerts anti-oxidative and anti-inflammatory functions. Up-regulation of HO-1 is known to protect against hepatic IR injury, but the effects of hepatic IR on the kidney are poorly understood. Thus the purpose of this study was to determine whether hepatic IR and resultant Kupffer cell activation alters renal HO-1 expression. Male Sprague-Dawley rats and wild-type and NF-E2-related factor 2 (Nrf2)-null mice were subjected to 60 min of partial hepatic ischemia, and at various times thereafter blood, liver, and kidneys were collected. After reperfusion: 1) creatinine clearance decreased, 2) HO-1 mRNA and protein expression in liver and kidney markedly increased, 3) renal NAD(P)H:quinone oxidoreductase 1 (NQO1) mRNA expression was induced, 4) serum TNFα levels increased, 5) Nrf2 translocation into the nucleus of renal tissue increased, and 6) renal and urinary 15-deoxy-Δ12,14-prostaglandin J2 (15-d-PGJ2) levels increased. Kupffer cell depletion by pretreating with gadolinium chloride: 1) attenuated increased mRNA expression of HO-1 in kidney, 2) attenuated the increase in TNFα, 3) inhibited the increase in Nrf2 nuclear translocation, and 4) tended to attenuate renal 15-d-PGJ2 levels. Whereas renal HO-1 mRNA expression increased in wild-type mice, it was attenuated in Nrf2-null mice. These results suggest that renal HO-1 is induced via Nrf2 to protect the kidney from remote organ injury after hepatic IR.

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Introduction

Hepatic ischemia-reperfusion (IR) injury is an unavoidable process in liver transplantation that often results in inflammatory liver injury or dysfunction (Lemasters and Thurman, 1997), but can also lead to remote organ injury (Colletti et al., 1990). In the initial reperfusion period, proinflammatory cytokines, such as tumor necrosis factor-α (TNFα) and interleukin (IL)1β, are induced in liver via Kupffer cell activation (Jaeschke and Farhood, 1991). The underlying mechanisms by which TNFα causes hepatocellular injury are not fully defined, but may cause cell death by inducing mitochondrial injury via apoptotic or necrotic signalling pathways (Leist et al., 1995). Serum TNFα levels increase shortly after reperfusion and are thought to cause remote organ dysfunction. The most notable example of remote injury following hepatic IR is neutrophilic infiltration, edema, and intra-alveolar hemorrhage in the lung (Colletti et al., 1990). Clinical studies have indicated that renal dysfunction following liver transplantation is also common (Braun et al., 2003). However, only one study has reported that hepatic ischemia-reperfusion causes mild histological renal damage and dysfunction in rodent models (Wanner et al., 1996).

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Heme oxygenase (HO) is the rate-limiting enzyme in heme catabolism, resulting in the formation of biliverdin, carbon monoxide, and iron. HO-1, the inducible isozyme of heme oxygenase, is ubiquitously expressed, and can be induced by various noxious stimuli, including proinflammatory cytokines, endotoxin, hypoxia, heat shock, heavy metals, and other pathophysiological responses, involving oxidative stress (Maines, 1988; Morse and Choi, 2002).

In recent years, much attention has been paid to the anti-oxidative and anti-inflammatory functions of HO-1 (Maines, 1988). HO-1 deficiency in humans is associated with susceptibility to oxidative stress and an increased pro-inflammatory state with severe endothelial damage, which is thought to be mediated by TNFα and IL1β (Terry et al., 1998). HO-1 induction has been implicated as a protective gene in numerous clinically relevant disease states including hypertension, atherosclerosis, diabetes, lung injury, Alzheimer's disease, endotoxic shock, transplantation, and ischemia-reperfusion injury (Amersi et al., 1999; Hayashi et al., 2001; Juan et al., 2001; Motterlini et al., 1998; Otterbein et al., 1996; Premkumar et al., 1995; Yet et al., 1997). HO-1 is induced in several models of renal injury, including glycerol-induced renal failure, cisplatin nephrotoxicity, and ischemia-induced renal failure (Agarwal et al., 1995;

Maines et al., 1993; Nath et al., 1992). Human patients with HO-1 deficiencies present with persistent proteinuria and hematuria, which are changes consistent with renal injury (Ohta et al., 2000).

In animal models there have been several lines of evidence suggesting that HO-1 protects organs from toxicity. For example, endotoxin administration to HO-1 null mice causes more end-organ damage and higher mortality than in wild-type mice (Wiesel et al., 2000). HO-1 induction may exert anti-inflammatory activity through generation of carbon monoxide, which subsequently inhibits TNFα expression, aiding in protection (Otterbein et al., 2000). Mice treated with a HO-1 inhibitor, tin-mesoporphyrin, have exacerbated renal dysfunction during acute ischemic renal failure, but pretreatment with tin chloride, an HO-1 inducer, reduces renal dysfunction (Toda et al., 2002). Pretreatment with cobalt protoporphyrin or hemin before transplantation alleviates hepatic and renal injury during IR by increasing HO-1 expression. Also, transgenic modulation of HO-1 prolongs the survival of mice undergoing hepatic, renal, and cardiac allografts (Amersi et al., 1999; Blydt-Hansen et al., 2003; Braudeau et al., 2004). Thus HO-1 may be a target for therapeutic intervention to minimize the effects of organ transplantation.

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NF-E2-related factor 2 (Nrf2) is a member of the leucine zipper transcription factor family, and mediates transcriptional activation of genes in response to oxidative and electrophilic stress. Nrf2 coordinately mediates cytoprotective enzyme induction of genes such as glutathione-*S*-transferase, quinone reductase, and HO-1, via Nrf2/Maf binding to antioxidant responsive elements in promoter sequences in these genes (Ishii et al., 2002). Furthermore, Nrf2 is important in regulating the process of acute inflammation (Itoh et al., 2004; Mochizuki et al., 2005), and a recent study demonstrated that LPS-induced HO-1 expression in human monocytic cells requires Nrf2 (Rushworth et al., 2005).

Whereas a study demonstrating HO-1 expression is induced in rat heart after renal IR (Raju and Maines, 1996), reports of remote organ injury after IR are few. Therefore, the purpose of this study was to investigate the effects of hepatic IR on renal HO-1 expression, and to determine whether alterations in HO-1 expression during reperfusion are mediated via Kupffer cell activation. To clarify whether altered expression of HO-1 following IR is associated with changes in Nrf2, translocation and expression of Nrf2 were determined. Furthermore, Nrf2-null mice were utilized to determine whether renal HO-1

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induction occurs via Nrf2 activation.

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MATERIALS AND METHODS

Materials. Gadolinium chloride (III) hexahydrate ($GdCl_3$) and all other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). Serum and urine creatinine levels were quantified by colorimetric assay (Stanbio, Boerne, TX). Serum urea nitrogen was assayed using a colorimetric assay (Biotron diagnostics Inc, Hemet, CA). Serum TNFα levels as well as renal and urinary concentrations of 15-d-PGJ2 were quantified by ELISA kits (R&D Systems, Minneapolis, MN).

Liver Ischemia-Reperfusion operations. Male Sprague-Dawley rats (290-340 g, N= 4 or 5 ; Sasco, Wilmington, MA) were acclimated for 7 days before any surgical procedures. The animals were divided into two surgical groups: IR (hepatic ischemia 60 min) and sham. Rats were anesthetized with sodium pentobarbital (50 mg/kg), a midline incision was made, the liver exposed, and branches of the hepatic artery and portal vein supplying blood to the left lateral and median lobes of the liver were occluded with an atraumatic Glover bulldog clamp. After 60 min of partial hepatic ischemia, the clamp was removed to initiate hepatic reperfusion. Sham-operated control rats underwent the same surgical procedure without vascular occlusion. At the indicated times of

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reperfusion (0, 3, 6, 24 and 48 h), blood samples were obtained, and the ischemic lobes of liver were harvested as well as kidneys. In a separate experiment, pretreatment with $GdCl_3$ (20 mg/kg i.v. in saline to restrained animals, 24 h prior to sham or IR operation) or saline was administered in a volume of 3 ml/kg before hepatic ischemia or sham surgery (290-350g, N= 4-8). $GdCl_3$ is a toxicant known to destroy Kupffer cells, which are the resident macrophages in liver, and thus limits cytokine release (Hardonk et al., 1992). Blood was taken and the ischemic lobes of liver as well as kidney were harvested at 6 and 24 h after reperfusion. The same procedure was conducted in wild-type and Nrf2-null mice. Male wild-type and Nrf2-null mice on a mixed C57BL/6 and AKR background (aged 8-10 weeks and 20-25g, N= 4) were obtained from Dr. Jefferson Chan (University of California, Irvine). Blood was taken and the ischemic lobes of liver as well as kidney were harvested at 6 and 24 h after reperfusion and stored at -80°C. Animals received humane care as outlined in the Guide for the Care and Use of Laboratory Animals (NIH publication 86-23, revised 1985).

Evaluation of renal function by creatinine clearance in rats. Blood samples were collected 24 h after reperfusion. For urine sampling, rats were placed in

metabolic cages and urine was collected for 24 h starting right after reperfusion. Serum and urine creatinine concentrations were determined using a creatinine assay kit in accordance with the manufacturer's protocols. Creatinine clearance (ml/min/100g) was calculated using the following formula: [urine volume (ml/min) / body weight (100g)]×[urine creatinine (mg/dl) / serum creatinine (mg/dl)].

Serum urea nitrogen. Serum urea nitrogen (BUN) levels were detemined using enzymatic-colorimetricmethod. The absorbsnce wavelength used was 540 nm (Tel Test Inc., Friendswood, TX).

Branched DNA Signal Amplification (bDNA) Assay. Total RNA was isolated using RNA Bee reagent (Tel Test Inc., Friendswood, TX) according to the manufacturer's protocol. Rat HO-1 and NAD(P)H:quinone oxidoreductase 1 (NQO-1) and mouse HO-1 mRNA were quantified using the branched signal amplification assay (QuantiGene®, High Volume bDNA Signal Amplification Kit; Genospectra, Fremont, CA) as previously described (Cherrington et al., 2002). Oligonucleotide probe sets specific to rat HO-1 and NQO-1, as well as mouse HO-1 mRNA transcripts were designed as previously described (Aleksunes et al., 2005; Cherrington et al., 2002; Heijne et al, 2004).

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Western blot analyses. Liver and kidney tissues were homogenized in ice-cold homogenization buffer (10mM Tris-HCl, pH 7.4, 1.5mM EDTA, pH 8.0, 30μM phenylmethylsulfonyl fluoride). Microsomal fractions were obtained by centrifuging the homogenate at 20,000 g for 20 min at 4°C and the resultant supernatant at 105,000 g for 1 h at 4°C. Nuclear proteins were prepared from rat liver and kidney using the NE-PER kit (Pierce, Rockford, IL). Microsomal and nuclear protein concentrations were determined with a bicinchoninic acid (BCA) assay kit (Pierce, Rockford, IL). For Western immunoblot analysis, 20 μg of microsomal fractions or 50 μg of nuclear extract were mixed with sample loading buffer and heated at 95°C for 5 min. The samples were electrophoretically resolved using Bio-Rad Criterion 12.5% or 10% Tris-glycine gels and then transblotted overnight at 4°C onto nitrocellulose (Bio-Rad) with Tris-glycine buffer containing 20% methanol. The blots were then blocked for 1 h in 5% nonfat dry milk in TBS-Tween buffer, incubated overnight at 4°C with rabbit anti-rat HO-1 (1:1000) (Stressgen Biotechnologies, Victoria, BC Canada) or Nrf2 (1:500) (Santa Cruz Biotechnology, Santa Cruz, CA) polyclonal antibodies diluted in 5% nonfat dry milk, and then washed three times for 5 min in TBS-Tween buffer. The blots incubated for 1 h at room temperature with

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donkey anti-rabbit (1:5000) (Amersham Biosciences Inc., Piscataway, NJ) IgG conjugated with horseradish peroxidase-conjugated antibodies. The immune complexes were detected using ECL® Western blotting reagents (Amersham Biosciences Inc.), and resulting autoradiographs were exposed to Fuji medical X-ray film (Fisher Scientific, Springfield, NJ) and quantified by densitometric analysis using Scion Image (Scion, Frederick, MD).

Kidney tissue glutathione (GSH). Kidney samples from rats were homogenized (20% w/v) in a 5% trichloroacetic acid/ethylenediamine tetra-acetic acid solution (TCA/EDTA). Homogenates were centrifuged at 1,500g for 15 min. Non-protein sulfhydryls (NPSH) in supernatants were determined as an indicator of reduced GSH following the colorimetric procedure of Ellman (1959). NPSH concentration was quantified by comparison with a GSH standard curve.

Serum, tissue, and urine ELISA assay. Serum concentrations of rat TNFα were determined per manufacturer's instruction. Briefly, 50 µl of serum and assay diluent were added to TNFα-coated 96-well plates and incubated for 2 h. After washing, 100 µl of conjugate was added to each well and incubated for 2 h, after which 100 µl of substrate solution was added and then incubated for 30 min in the dark. Spectrophotometric absorbance was read at 450 nm.

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Cytoplasmic extract isolated from 50 mg of rat kidney was prepared using the NE-PER kit (Pierce, Rockford, IL) as per the manufacturer's recommendations. Similarly concentrations of rat renal and urinary 15-d-PGJ2 were quantified per manufacturer's instruction. Renal and urinary 15-d-PGJ2 were normalized to mg protein and mg creatinine, respectively.

Statistical analysis. Statistical differences between sham and IR groups were determined using Student's t test with significance set at $p< 0.05$. Pretreatment with $GdCl_3$ to sham and IR operated rats and wild-type and Nrf2-null mice were analyzed by analysis of variance, followed by Duncan's multiple range post hoc test. Significance was set at $p< 0.05$. Bars represent mean ± S.E.M.

RESULTS

Effect of hepatic IR on creatinine clearance.

Hepatic IR causes remote renal dysfunction (Wanner et al., 1996), thus creatinine clearance was utilized to assess renal function. Creatinine clearance was measured 24 h after hepatic IR, and creatinine clearance was decreased compared with sham-operated animals (Figure 1).

Time-course of hepatic IR on HO-1 and NQO-1 mRNA expression in liver

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and kidney.

The expression of the Nrf2 target genes HO-1 and NQO-1 in liver and kidney was determined in male Sprague-Dawley rats following IR. HO-1 mRNA expression in liver was increased 3 and 6 h post-reperfusion by 99 and 73%, respectively, and surprisingly HO-1 expression was also increased in shams at these time-points. Renal HO-1 mRNA expression was increased markedly by 3420 and 4540% at 3 and 6 h after reperfusion, respectively (Figure 2). The mRNA expression of NQO-1 in liver was increased in both sham and IR at 24 h after reperfusion, but there was no significant difference between sham and IR. NQO-1 in kidney was increased at 3 and 6 h post-reperfusion by 89 and 137%, respectively (Figure 2).

Time-course of hepatic IR on protein expression of HO-1 in liver and kidney.

To determine whether changes in mRNA expression correspond with protein levels, western blotting was conducted in liver and kidneys (Figure 3). Rat hepatic HO-1 protein expression increased 307, 108, 19, and 140% over sham-operated animals after 3, 6, 24, and 48 h of IR, respectively. Renal protein expression markedly increased by 4090, 5220, 1180 and 622% over

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sham-operated animals after 3, 6, 24, and 48 h of IR, respectively.

Kidney tissue GSH levels.

Tissue GSH concentration in kidneys did not change in any group compared to sham saline control at 6 or 24 h after reperfusion (Figure 4A).

Serum cytokine levels.

IR injury causes activation of Kupffer cells, which results in the production and release of proinflammatory cytokines during the initial reperfusion period. The serum levels of TNFα were increased at 6 and 24 h following IR. Pretreatment with $GdCl_3$ blocked the increase in serum TNFα at 6 h (Figure 4B).

Effect of $GdCl_3$ pretreatment on HO-1 mRNA expression in liver and kidney.

The involvement of Kupffer cells in hepatic and renal HO-1 up-regulation after IR was examined after $GdCl_3$ treatment. The mRNA expression of HO-1 in liver tended to increase at 6 h after reperfusion, but was not statistically significant. Renal HO-1 mRNA was increased at 6 h after reperfusion by 801%. $GdCl_3$ pretreatment tended to block the increase in HO-1 mRNA levels in liver 6 h after IR, but it was not statistically significant. However in kidney, $GdCl_3$ markedly attenuated renal HO-1 mRNA levels at 6 h post-IR (Figure 5). $GdCl_3$ treatment by itself (Gd Sham group) had no effect on HO-1 expression. These results

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suggest that Kupffer cell activation is involved in the IR-mediated increase in HO-1 mRNA levels in kidneys.

Effect of $GdCl_3$ pretreatment on serum urea nitrogen.

To determine whether the decreased renal function observed after hepatic I/R (Figure 1) is alleviated by $GdCl_3$ pretreatment, serum urea nitrogen levels were quantified after $GdCl_3$ treatment. Serum levels of urea nitrogen were increased 24 h after reperfusion, and $GdCl_3$ pretreatment completely attenuated this increase (Figure 6).

Effect of $GdCl_3$ pretreatment on Nrf2 protein expression in kidney.

Expression of the Nrf2 target genes HO-1 and NQO-1 increased in kidney after hepatic IR (Figure 2), suggesting Nrf2 may be involved in protection of a remote organ. To test this hypothesis, Nrf2 mRNA and Nrf2 protein translocation to the nuclei were determined. Nrf2 mRNA expression in liver and kidney was not altered at any time point after reperfusion (data not shown), but Nrf2 protein expression in nuclei of kidneys was increased 6 h after reperfusion, and $GdCl_3$ pretreatment inhibited this increase (Figure 7).

Renal and urinary 15-deoxy- Δ12,14-prostaglandin J2 levels

Nrf2 activation may occur in response to serum TNFα secreted from Kupffer

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cells through generation of prostaglandins (Figure 4 and 7). Cyclooxygenase (COX) enzymes, such as COX-2, are anti-inflammatory due to their ability to produce cyclopentenone prostaglandins, including 15-d-PGJ2 (Gilroy DW et al., 1999) Acute inflammation after IR injury may stimulate COX activity and generate 15-d-PGJ2, a prostaglandin which strongly activates the Nrf2 pathway (Itoh et al., 2004). Thus renal and urinary 15-d-PGJ2 levels were quantified by ELISA, and 15-d-PGJ2 concentrations were increased in rat kidney tissue 6h post-reperfusion. $GdCl_3$ pretreatment tended to attenuate this increase in renal 15-d-PGJ2 levels ($p=0.056$) (Figure 8A). Furthermore, urinary 15-d-PGJ2 levels increased in rats whose livers underwent IR (Figure 8B). These data suggest that 15-d-PGJ2 may activate Nrf2 in kidney after hepatic IR.

Effects of hepatic IR on mouse HO-1 mRNA expression in livers and kidney of Nrf2-null mice.

Nrf2-null mice were used to determine whether HO-1 expression in kidneys was induced via Nrf2. In liver after IR, HO-1 mRNA expression increased 6h post-reperfusion in wild-type mice, but was similarly increased in Nrf2-null mice, suggesting Nrf2 independence in liver (Figure 9). In contrast to liver, HO-1 mRNA expression in kidney increased after 6h of reperfusion in wild-type mice

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that underwent IR, but HO-1 induction was ablated in Nrf2-null mice after IR (Figure 9).

Effects of hepatic IR on mouse HO-1 protein expression in kidney of Nrf2-null mice.

Renal protein expression of HO-1 was quantified 24h after reperfusion, and in wild-type mice HO-1 was markedly induced, yet expression was completely attenuated in Nrf2-null mice after IR, corresponding well with the mRNA data (Figure 10).

Effect of hepatic IR on serum urea nitrogen of Nrf2-null mice.

To determine whether Nrf2 protects the kidney from the remote organ injury and loss-of-function after hepatic IR, serum urea nitrogen levels were quantified in wild-type and Nrf2-null mice. Serum levels of urea nitrogen were increased in wild-type mice 24 h after reperfusion, whereas serum urea nitrogen levels were increased to a greater extent in Nrf2-null mice. These findings suggest Nrf2 protects kidney from remote organ injury caused by hepatic IR (Figure 11).

DISCUSSION

Hepatic ischemia-reperfusion (IR) injury is a common event in hepatic

resectional surgery and liver transplantation (Lemasters and Thurman, 1997), and organs undergoing transplantation often suffer a certain degree of injury caused by IR. Moreover, hepatic IR can lead to remote organ injury, or in some severe cases, dysfunction (Braun et al., 2003; Colletti et al., 1990; Wanner et al., 1996). The present study demonstrates that creatinine clearance decreases after hepatic IR, and the data in total suggests that hepatic IR can lead to renal dysfunction.

Heme oxygenase-1 (HO-1) is induced during IR injury (Maines et al, 1993; Yamaguchi et al., 1996). HO-1 overexpression exerts cytoprotective functions in a number of IR injury transplant models, including heart, liver, and kidney (Amersi et al., 1999; Blydt-Hansen et al., 2003; Braudeau et al., 2004). However, little is known about the relationship between IR and HO-1 expression in remote organs (Raju and Maines, 1996). In a present study, HO-1 expression in liver and kidney from rats and mice that underwent hepatic IR was examined. The results show that liver HO-1 mRNA and protein expression is increased after hepatic IR in rats, which is in agreement with a previous study (Yamaguchi et al., 1996). However, HO-1 induction in liver is modest. Conversely, our present experiment demonstrates that after hepatic IR, a

marked increase in HO-1 expression occurs in kidney, suggesting a strong defense to protect the kidney from organ dysfunction (Maines, 1988).

Potential mechanisms that may lead to remote kidney damage include oxidative stress and serum cytokines, either independently, or in combination (Morse and Choi, 2002; Terry et al., 1998). Thus, kidney GSH levels were examined as an indicator of oxidative stress, yet these levels were not altered, suggesting that HO-1 induction may be independent of the oxidative condition. During the initial phase of hepatic ischemia-reperfusion injury, Kupffer cells are activated and begin to release proinflammatory cytokines, including tumor necrosis factor-alpha (TNFα), which can lead to remote injury (Colletti et al., 1990; Jaeschke and Farhood, 1991; Wanner et al., 1996). This was indirectly demonstrated, as LPS administration, which is known to cause strong proinflammatory activity, induces HO-1 expression and activity in liver, lung, and kidney (Suzuki et al., 2000). The present study demonstrates that serum TNFα levels increase after IR, and that $GdCl_3$ pretreatment inhibits this process. $GdCl_3$ pretreatment did not block the increase in HO-1 in liver, but in kidney, HO-1 was markedly increased 6 h post-IR, and $GdCl_3$ pretreatment attenuated this induction. These results suggest that serum cytokines, including TNFα

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secreted from activated Kupffer cells may mediate HO-1 induction in kidneys 6 h after IR. Furthermore, $GdCl_3$ pretreatment protected the decreased renal function after hepatic IR, suggesting that cytokines released by the Kupffer cell during hepatic IR causes renal dysfunction.

The regulatory region of the HO-1 gene contains various putative binding sites for transcription factors that regulate pro-inflammatory gene expression, including NFκB, AP-1, and Nrf2 response elements. Although the precise mechanism of HO-1 induction is controversial, NFκB, AP-1, and Nrf2 are all thought to play a role in inflammation and HO-1 regulation (Kurata et al., 1996). Two distal enhancers in the 5'-flanking region of mouse HO-1 contain an AP-1 binding site that is thought to be crucial for LPS-mediated HO-1 up-regulation (Camhi et al., 1998). Furthermore, in human monocytic LPS induced HO-1 in human monocytic cells is via Nrf2 activation, thus suggesting a multifaceted response to LPS that occurs through a variety of transcription factors (Rushworth et al., 2005).

In the present study, NQO-1 and HO-1, two prototypical Nrf2 target genes, were induced in kidney 3 and 6 h post-reperfusion, yet no changes in Nrf2 mRNA expression occurred in liver or kidney at any time point examined (data

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not shown). However, nuclear localization of Nrf2 was not changed in liver, but Nrf2 nuclear levels were increased in kidney at 6 h post-IR. Furthermore, renal HO-1 induction was ablated in Nrf2-null mice. Therefore, the present data indicates that after hepatic ischemia, Nrf2 translocates into the nucleus, and transcriptionally activates HO-1 and NQO-1, thus Nrf2 is critical in driving HO-1 expression in kidney after hepatic IR.

Kupffer cells may also play a supporting role in mediating renal HO-1 expression after IR injury. $GdCl_3$, a selective Kupffer cell toxicant, inhibits Nrf2 translocation in kidney after IR injury, suggesting that Kupffer cells may be involved in remote organ injury and HO-1 regulation. There are two plausible mechanisms for how this activation occurs. The first is that Kupffer cells release cytokines, including TNFα, which activates Nrf2 (Yang et al., 2005), which then induces HO-1 expression. The second is that inflammation increases the production of cyclopentenone prostaglandins, including 15-d-PGJ2 in kidneys, which binds to Keap1 in a manner that allows for Nrf2 release (Itoh et al., 2004). 15-d-PGJ2 has already been implicated in protecting the lung from acute injury through activation of the Nrf2 pathway (Mochizuki et al., 2005). To gain insight into the plausibility of this mechanism, renal and

urinary 15-d-PGJ2 levels were quantified in the present study. Hepatic IR increased renal 15-d-PGJ2, and pretreatment with $GdCl_3$ tended to inhibit the increase in renal 15-d-PGJ2 levels (Fig. 7). Furthermore, urinary 15-d-PGJ2 levels increased in the IR group. Therefore increased renal 15-d-PGJ2 may explain renal Nrf2 activation after hepatic IR, and that Kupffer cell activation may be involved in this increase.

In summary, hepatic IR increases HO-1 expression in kidney and liver. Depletion of Kupffer cells by $GdCl_3$ largely blocked or attenuated the induction of HO-1 in kidney, suggesting a role for cytokines, including TNFα, in this remote organ regulation of HO-1 expression. Furthermore, cytokines released from activated Kupffer cells cause remote injury in kidney. The induction of HO-1 may exert anti-inflammatory functions through generation of carbon monoxide, and may inhibit the expresson of cytokines (Otterbein et al., 2000). The COX enzymes may be a critical in increasing 15-d-PGJ2 levels in kidney, which may lead to subsequent Nrf2 activation, and the subsequent renal up-regulation of HO-1. Thus, Nrf2 may play an important role in the resolution of renal injury after hepatic IR via as the downstream molecule of 15-d-PGJ2 mediated activation (as summarized in Fig. 12). Because pre-transplant renal failure is a

well-known risk factor that adversely affects the prognosis after liver transplantation (Braun et al., 2003), increased renal HO-1 expression may protect kidneys from injury from hepatic IR. Thus, activation of Nrf2 or HO-1 might be a useful therapeutic approach for protection from renal dysfunction during liver transplantation.

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Figure Legends

Fig. 1. Creatinine clearance 24h after IR or sham operation . Creatinine clearance ($ml \cdot min^{-1} \cdot 100\ g^{-1}$) was calculated as described in MATERIALS AND METHODS. Data are presented as mean ± S.E.M. (each group, n = 6 animals). Asterisks (*) represent a statistically significant difference ($p < 0.05$) between sham and IR groups.

Fig. 2. Time course of HO-1 and NQO-1 mRNA expression in liver and kidney after IR. Total RNA was isolated from both sham and IR rat liver and kidney, and analyzed by the bDNA signal amplification assay as described in MATERIALS AND METHODS. Data are presented as mean RLU ± S.E.M. (each group, n = 4 or 5 animals). Asterisks (*) represent statistically significant differences ($p < 0.05$) between sham and IR groups.

Fig. 3. Time course of HO-1 protein expression in liver and kidney after IR. Representative Western blot of liver and kidney microsomal fractons stained with antibodies that detect rat HO-1 (20 μg protein/lane). Immunoreactive bands were semiquantitated by a densitometric analysis. Data are presented as

mean ± S.E.M. (each group, n = 4 or 5 animals). Asterisks (*) represent statistically significant differences ($p < 0.05$) between sham and IR groups.

Fig. 4. A; Effects of $GdCl_3$ pretreatment prior to IR on kidney GSH levels. B; effects of $GdCl_3$ pretreatment prior to IR on serum TNFα. $GdCl_3$ (20mg/kg) or saline was administered 24 h prior to sham or IR operation. Data are expressed as mean ± S.E.M. (each group, n = 3-7 animals). SAL Sham, saline administered to sham operated rats; SAL IR, saline administered to IR operated rats; Gd Sham, $GdCl_3$ administered to sham operated rats; Gd IR, $GdCl_3$ administered to IR operated rats. Asterisks (*) represent a statistically significant difference ($p < 0.05$) from the SAL Sham group; a single dagger (†) represents a statistically significant difference ($p < 0.05$) from SAL IR group; a double dagger (‡) represents a statistically significant difference ($p < 0.05$) from Gd Sham group.

Fig. 5. Effects of $GdCl_3$ pretreatment prior to IR on HO-1 expression in liver and kidney. $GdCl_3$ (20mg/kg) or saline was administered 24 h prior to sham or IR operation. Data are expressed as mean RLU ± S.E.M. (each group, n = 7 or 8

animals). SAL Sham, saline administered to sham operated rats; SAL IR, saline administered to IR operated rats; Gd Sham, $GdCl_3$ administered to sham operated rats; Gd IR, $GdCl_3$ administered to IR operated rats. Asterisks (*) represent a statistically significant difference ($p < 0.05$) from SAL Sham group; a single dagger (†) represents a statistically significant difference ($p < 0.05$) from SAL IR group.

Fig. 6. Effects of $GdCl_3$ pretreatment prior to IR on serum urea nitrogen level. $GdCl_3$ (20mg/kg) or saline was administered 24 h prior to sham or IR operation. Data are expressed as mean ± S.E.M. (each group, n = 4-7 animals). SAL Sham, saline administered to sham operated rats; SAL IR, saline administered to IR operated rats; Gd Sham, $GdCl_3$ administered to sham operated rats; Gd IR, $GdCl_3$ administered to IR operated rats. Asterisks (*) represent a statistically significant difference ($p < 0.05$) from SAL Sham group; a single dagger (†) represents a statistically significant difference ($p < 0.05$) from SAL IR group.

Fig. 7. Effects of $GdCl_3$ pretreatment prior to IR on Nrf2 nuclear protein expression in liver and kidney. Representative Western blot of liver and kidney

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nuclear proteins stained with antibodies that detect rat Nrf2 (50 µg protein/lane). Immunoreactive bands were semiquantitated by a densitometric analysis. $GdCl_3$ (20mg/kg) or saline was administered 24 h prior to sham or IR operation. Data are expressed as mean ± S.E.M. (each group, n = 7 or 8 animals). SAL Sham, saline administered to sham operated rats; SAL IR, saline administered to IR operated rats; Gd Sham, $GdCl_3$ administered to sham operated rats; Gd IR, $GdCl_3$ administered to IR operated rats. Asterisks (*) represent a statistically significant difference ($p < 0.05$) from SAL Sham group; a single dagger (†) represent a statistically significant difference ($p < 0.05$) from SAL IR group.

Fig. 8. A; Effects of $GdCl_3$ pretreatment prior to IR on renal 15-deoxy-Δ12,14-Prostaglandin J2 levels. $GdCl_3$ (20mg/kg) or saline was administered 24 h prior to sham or IR operation. Data are expressed as mean ± S.E.M. (each group, n = 7 or 8 animals). SAL Sham, saline administered to sham operated rats; SAL IR, saline administered to IR operated rats; Gd Sham, $GdCl_3$ administered to sham operated rats; Gd IR, $GdCl_3$ administered to IR operated rats. Asterisks (*) represent a statistically significant difference ($p < 0.05$) from SAL Sham group. B; urinary 15-deoxy- Δ12,14-Prostaglandin J2 levels after IR

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or sham operation. Data are presented as mean ± S.E.M. (each group, n = 6 animals). Asterisks (*) represent a statistically significant difference ($p < 0.05$) between sham and IR groups.

Fig. 9. Change of HO-1 mRNA expression in liver and kidney from wild-type and Nrf2-null mice after hepatic IR. Data are expressed as mean RLU ± S.E.M. (each group, n = 4 animals). Asterisks (*) represent a statistically significant difference ($p < 0.05$) from wild-type sham group; a single dagger (†) represents a statistically significant difference ($p < 0.05$) from wild-type IR group.

Fig. 10. Changes in HO-1 protein expression in kidney from Nrf2 wild-type and null mice at 24h after hepatic IR. Representative Western blot of kidney microsomal fractons stained with antibodies that detect mouse HO-1 (40 µg protein/lane). Immunoreactive bands were semiquantitated by densitometric analysis. Data are presented as mean ± S.E.M. (each group, n = 4 animals). Asterisks (*) represent a statistically significant difference ($p < 0.05$) from wild-type sham group; a single dagger (†) represents a statistically significant difference ($p < 0.05$) from wild-type IR group.

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Fig. 11. Serum urea nitrogen levels in wild-type and Nrf2-null mice at 24h after hepatic IR. Data are expressed as mean ± S.E.M. (each group, n = 4 animals). Asterisks (*) represent a statistically significant difference ($p < 0.05$) from wild-type sham group; a single dagger (†) represents a statistically significant difference ($p < 0.05$) from wild-type IR group.

Fig. 12. Schematic diagram of 15-deoxy- Δ12,14-Prostaglandin J2- and Nrf2-mediated activation of HO-1 gene in kidney after hepatic IR. Hepatic ischemia-reperfusion activates Kupffer cell which releases cytokines, especially TNFα. TNFα may induce inflammation and injury in kidney. COX-2 may be induced by TNFα induced inflammation and generate 15-deoxy- Δ12,14 Prostaglandin J2. 15-deoxy- Δ12,14 Prostaglandin J2 activates the Nrf2-Keap1 pathway, perhaps via covalent binding to Keap1. Nrf2 induces HO-1 and NQO-1 expression as well as other ARE-regulated genes. These enzymes cooperatively serve to reduce inflammation and injury.

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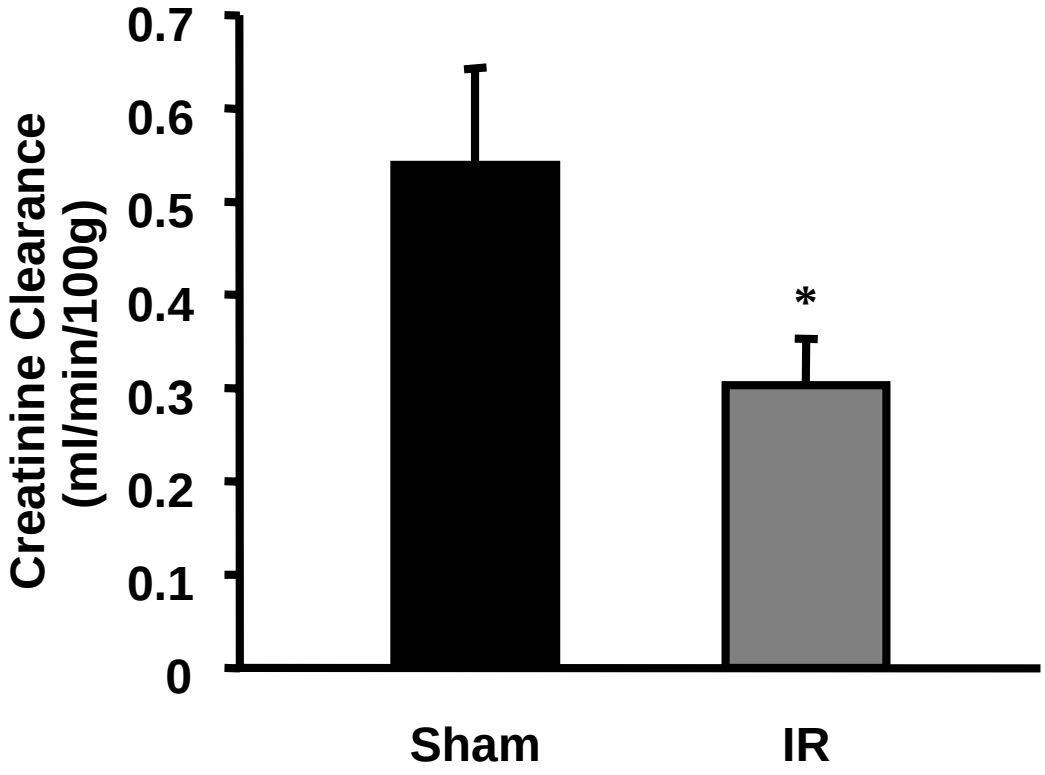

Fig 1

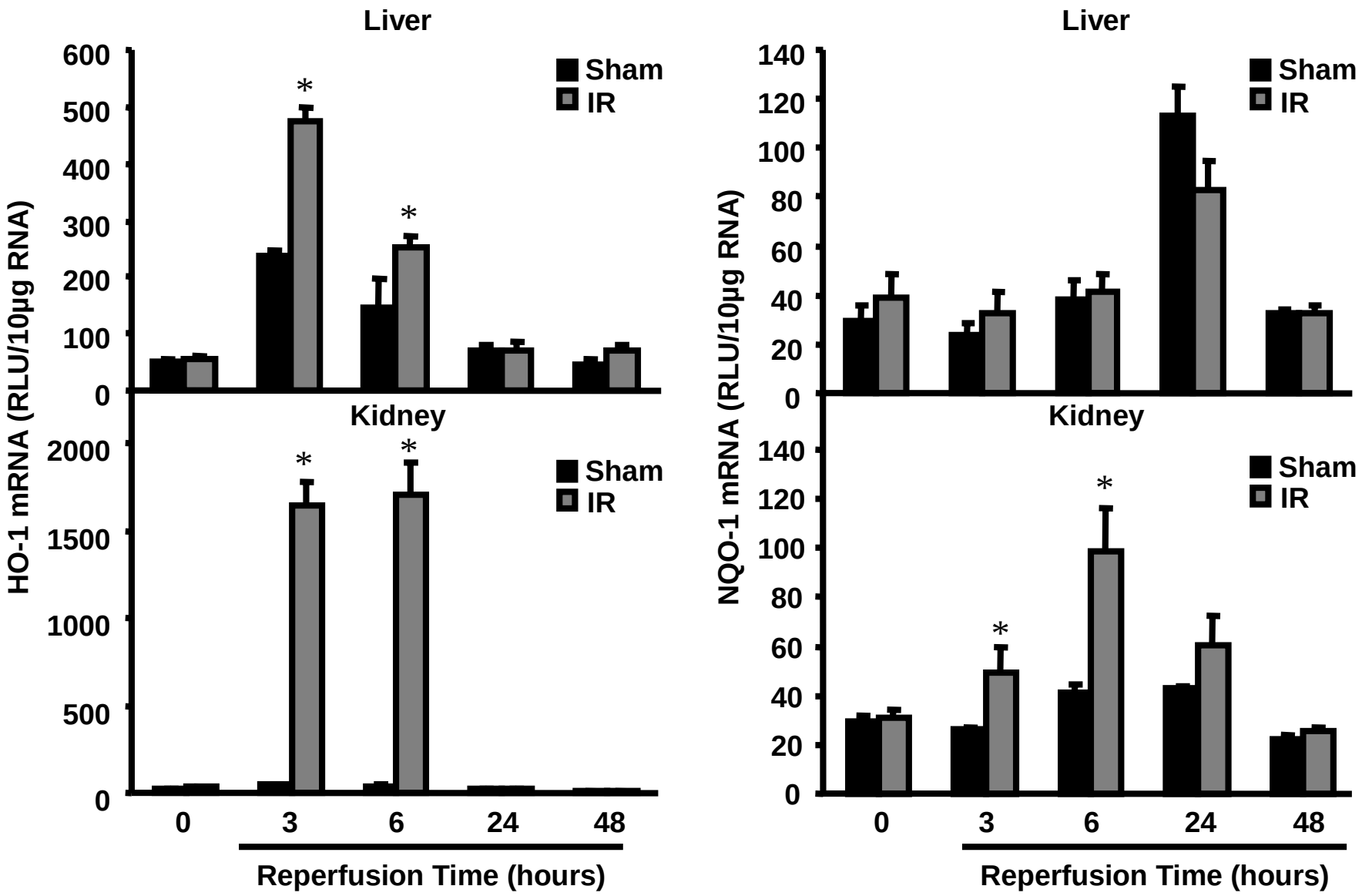

Fig 2

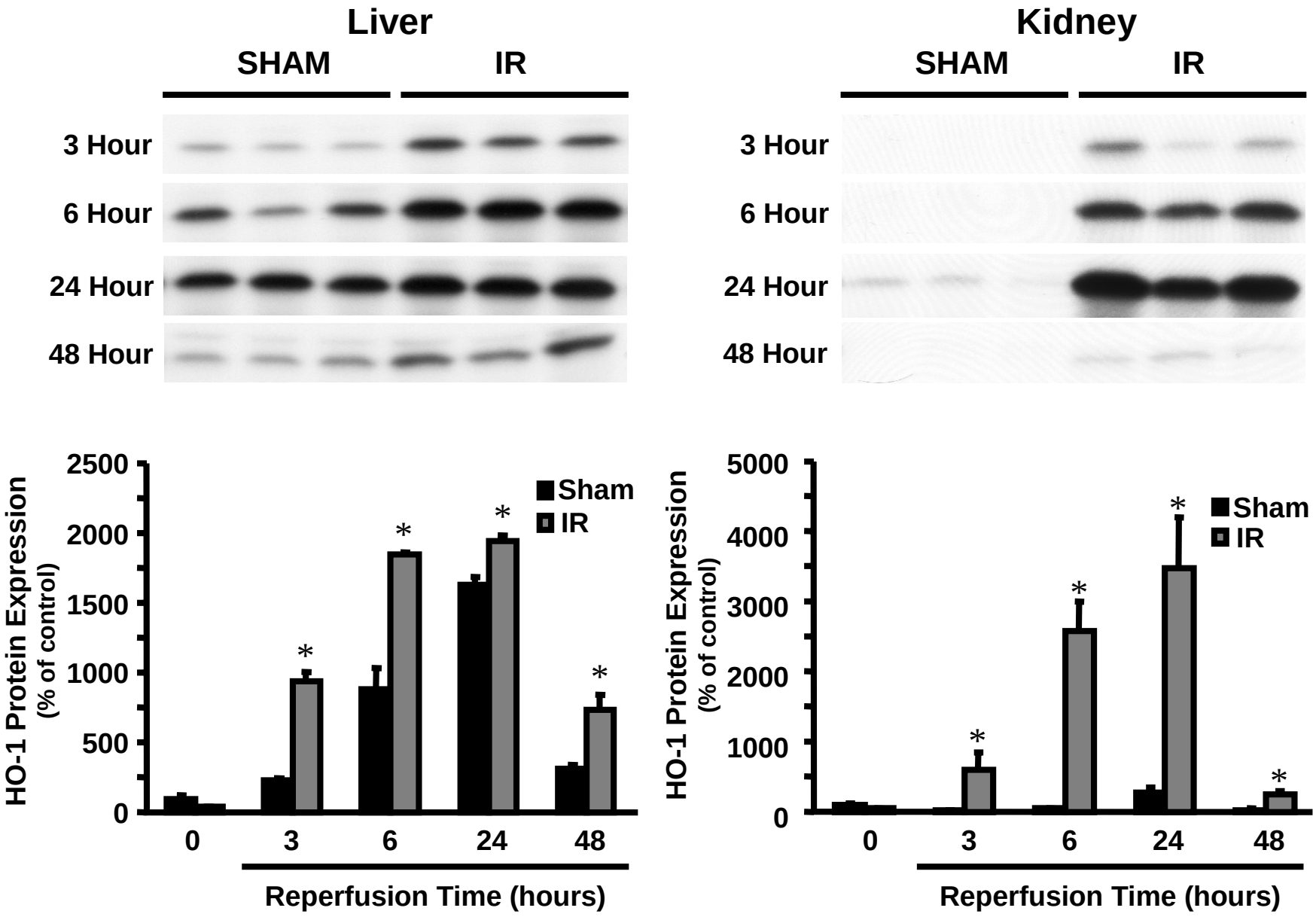

Fig 3

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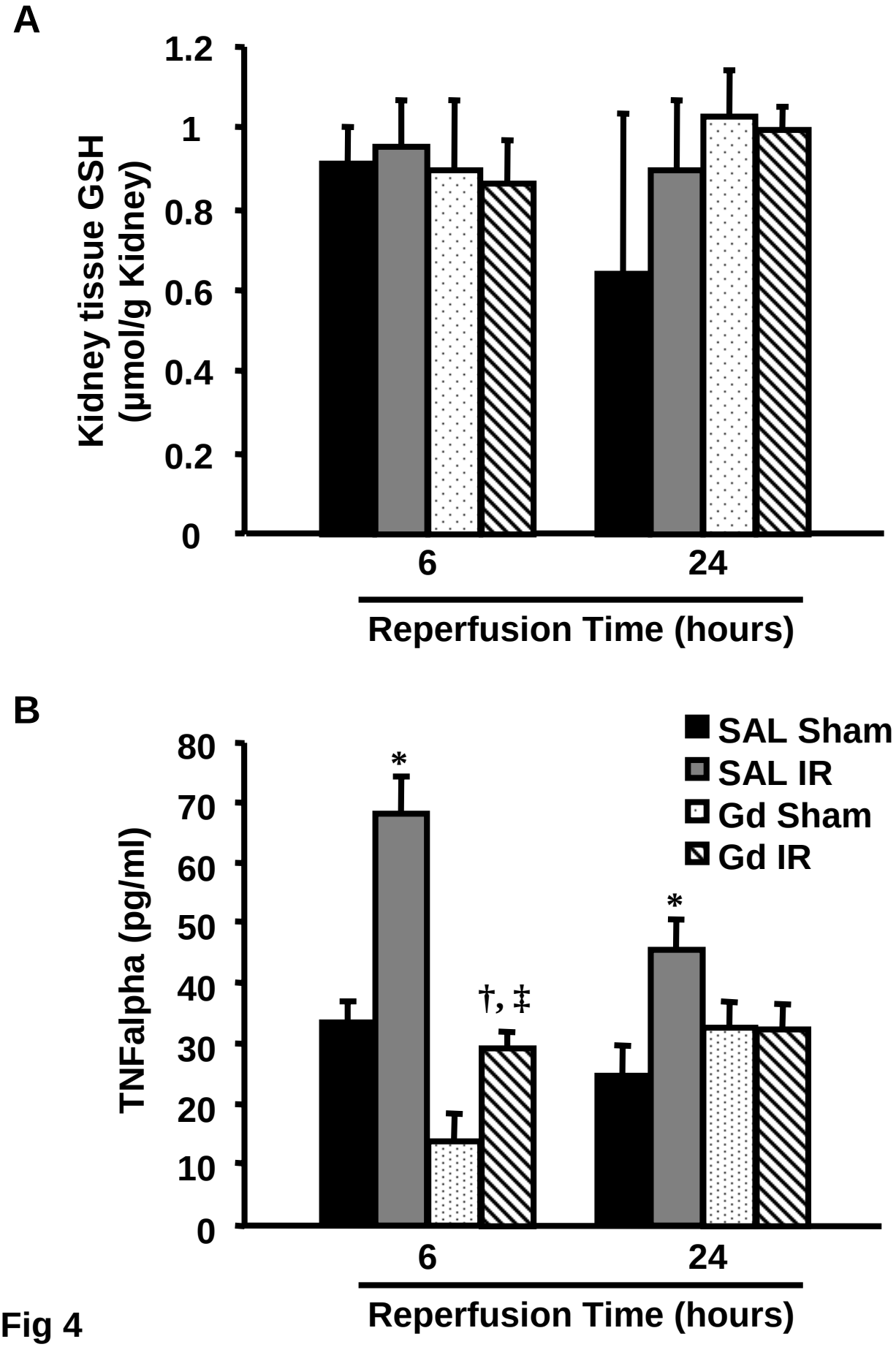

Fig 4

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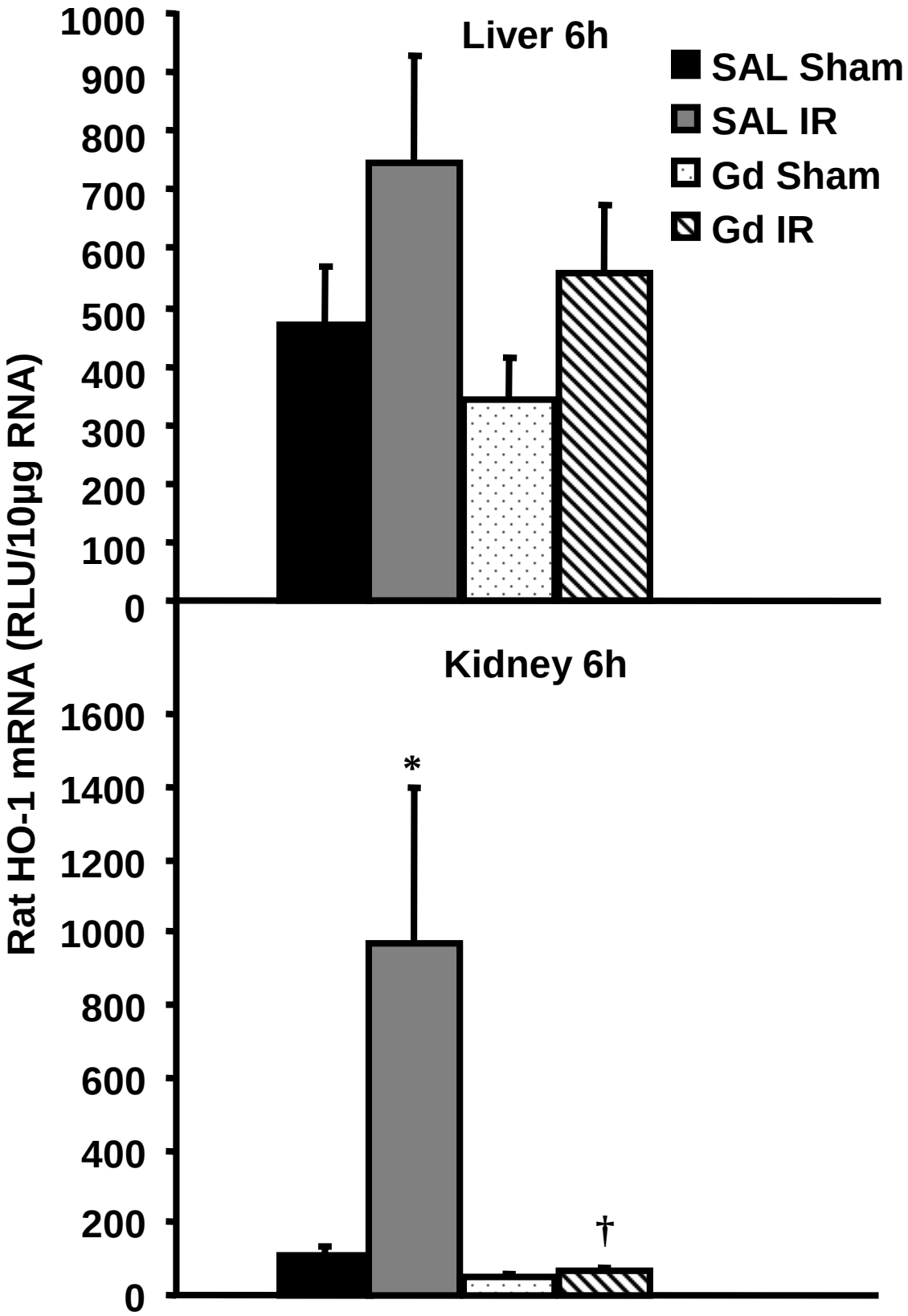

Fig 5

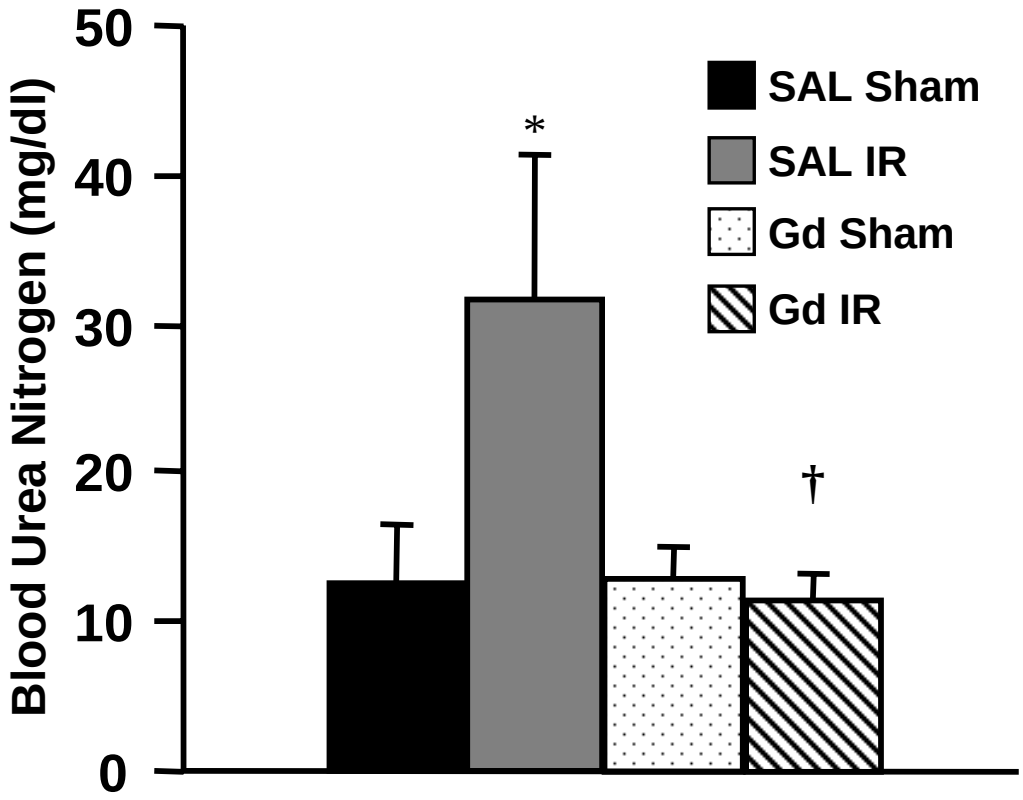

Fig 6

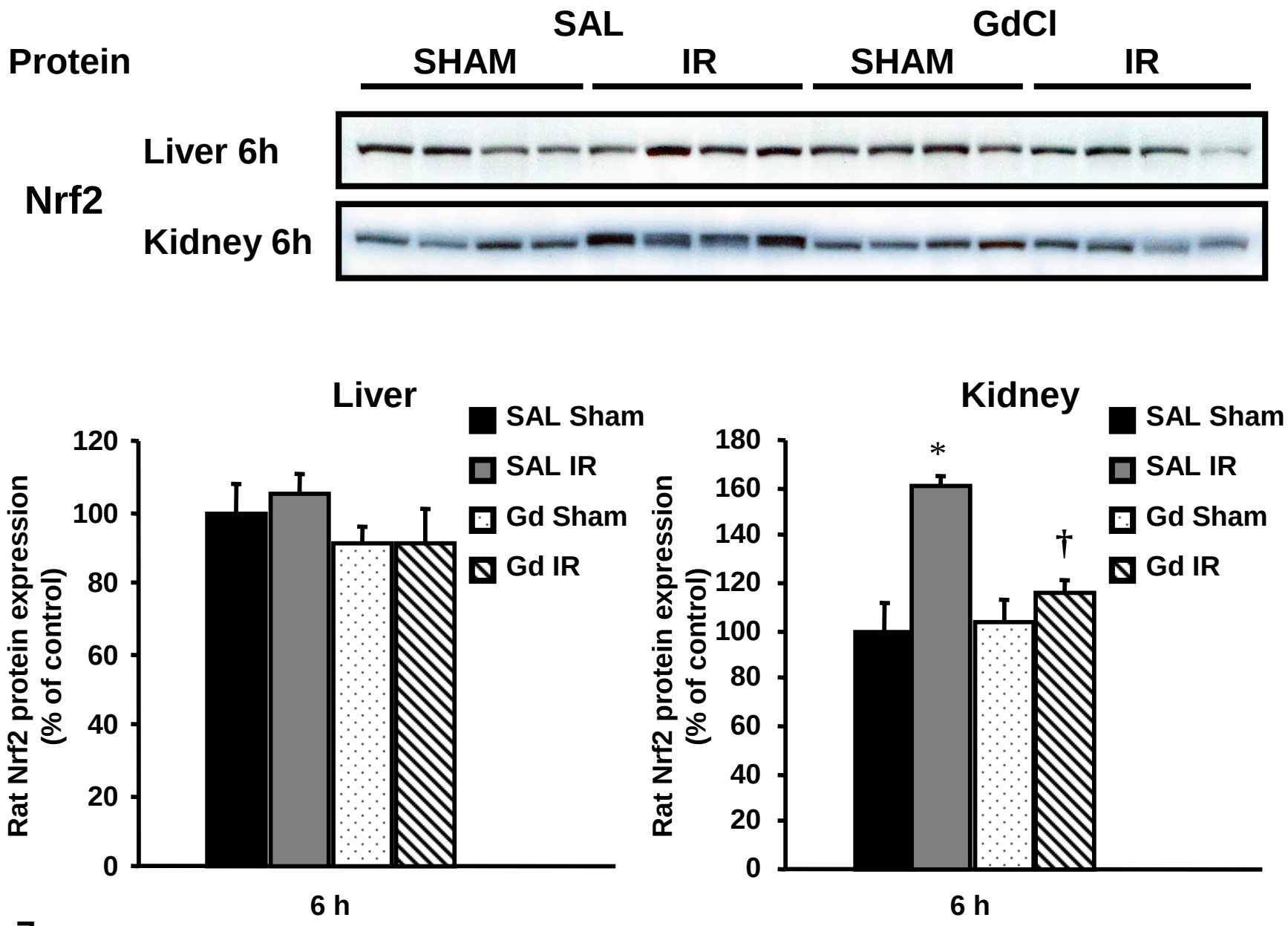

Fig 7

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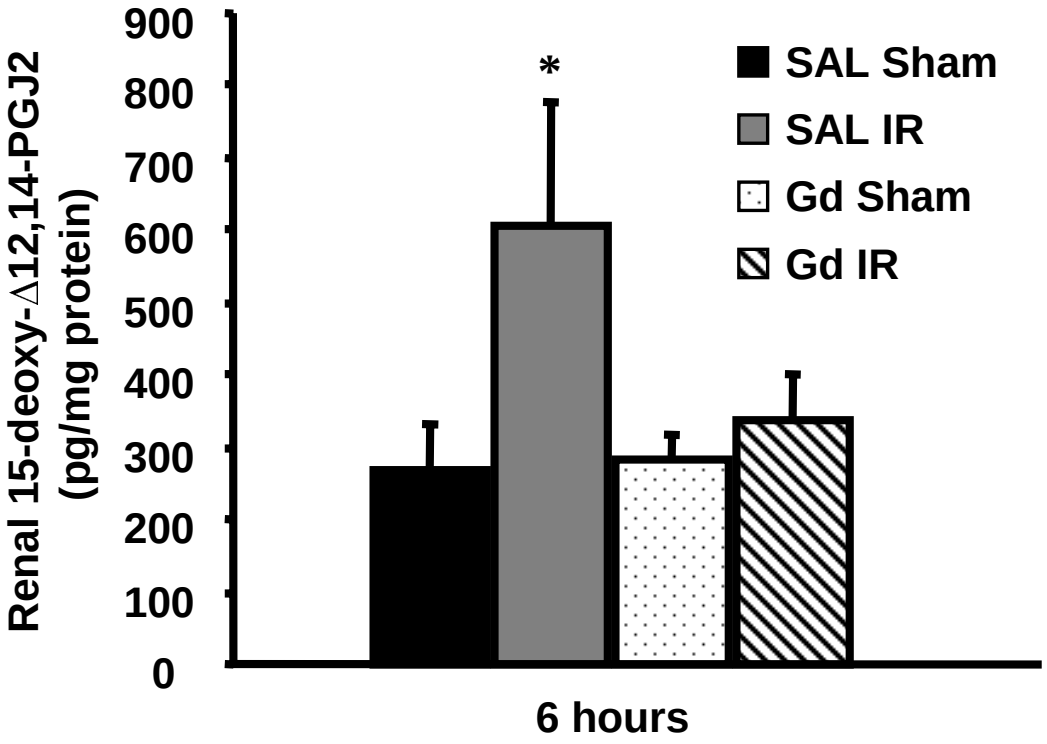
A



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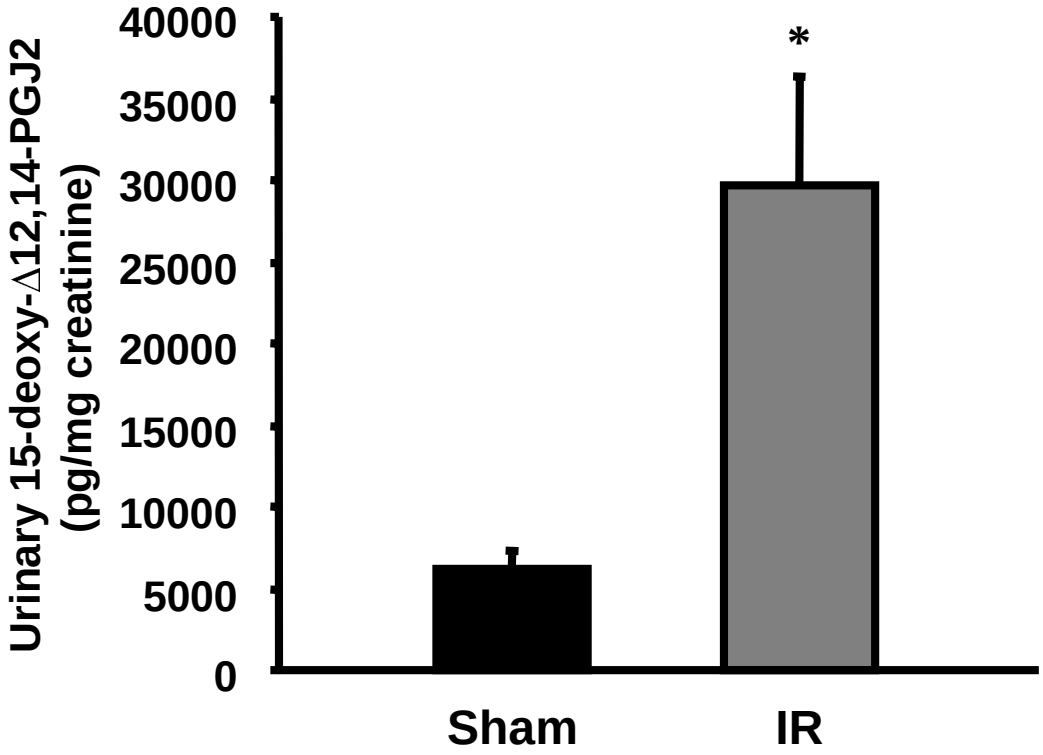

Fig 8

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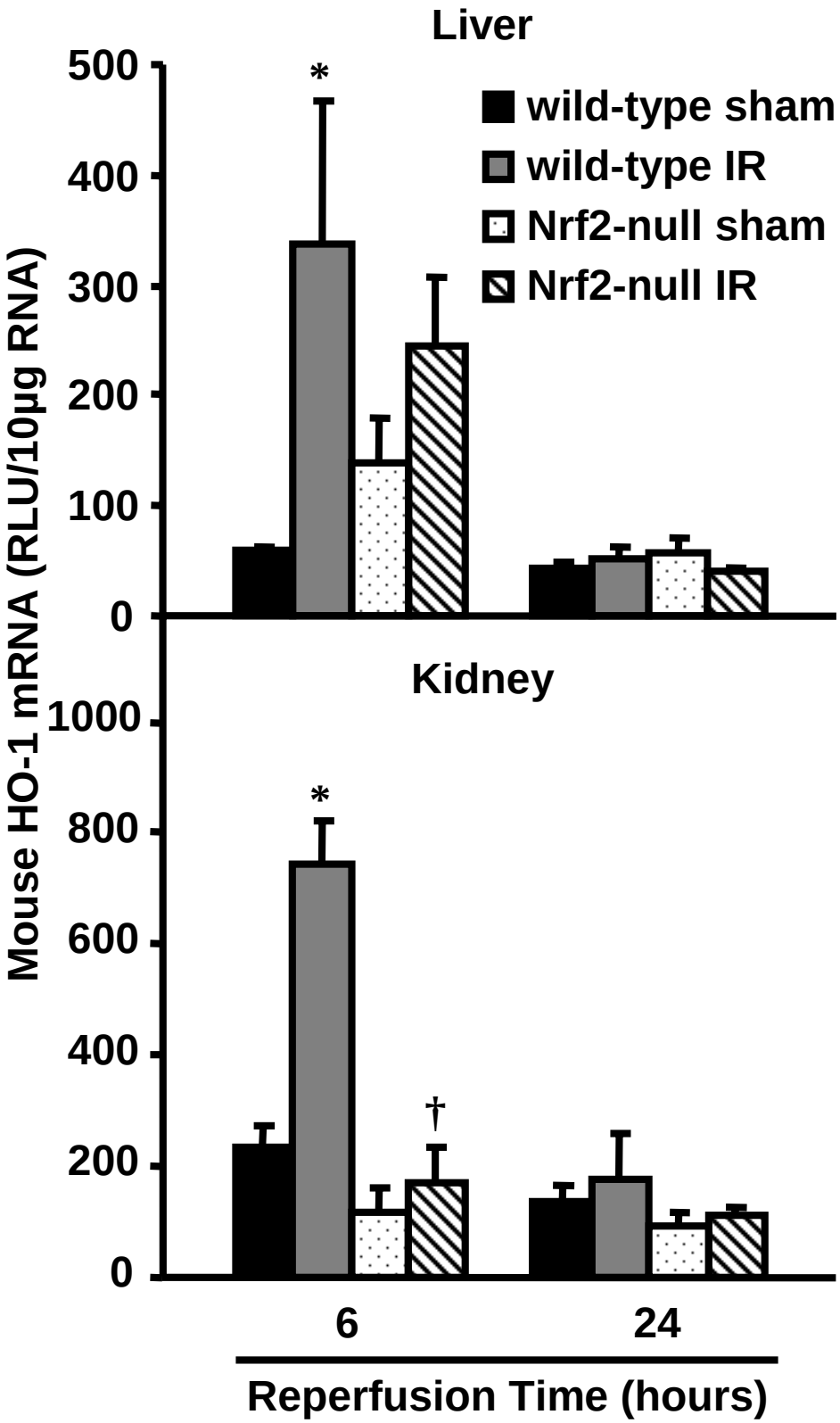

Fig 9

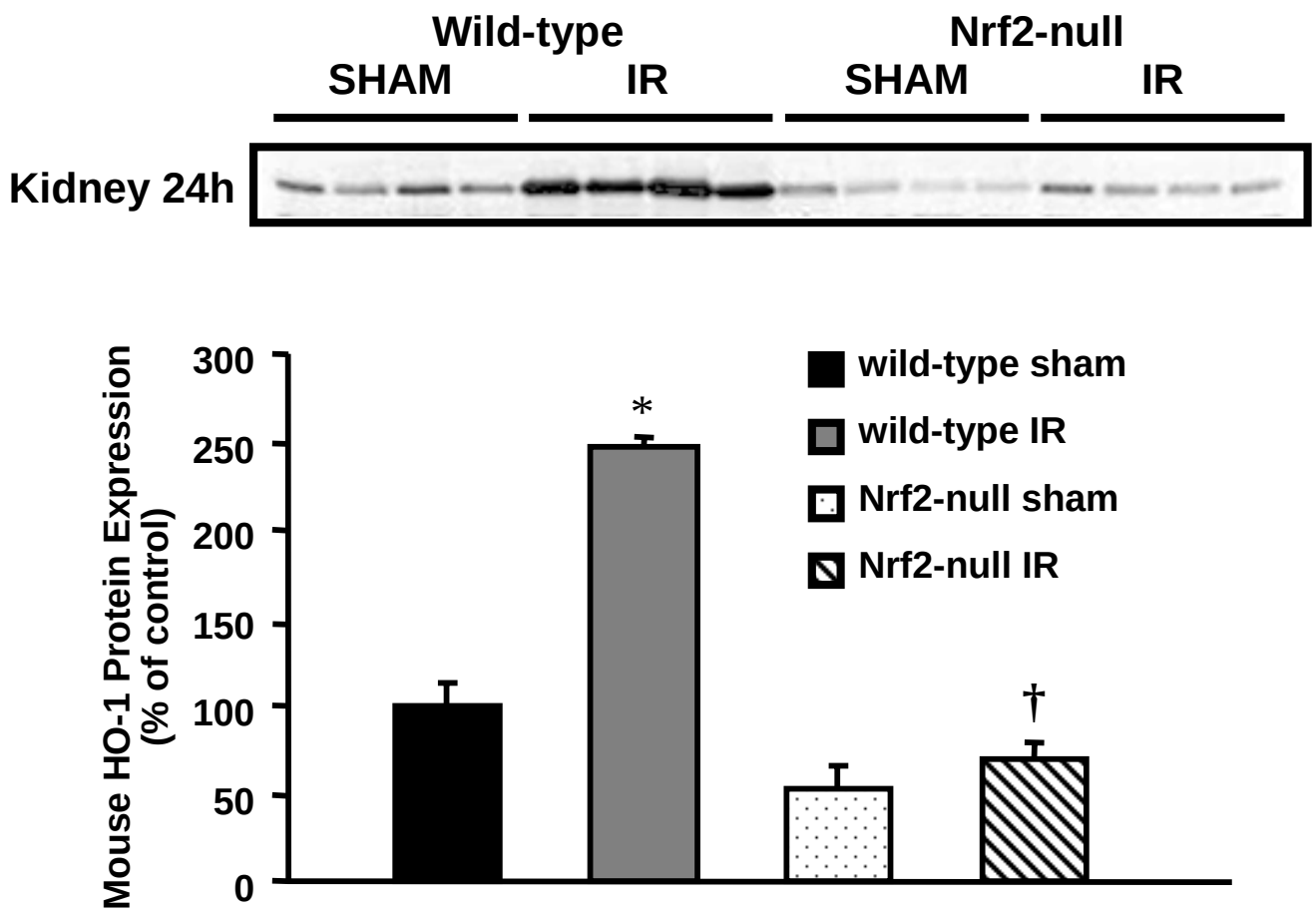

Fig 10

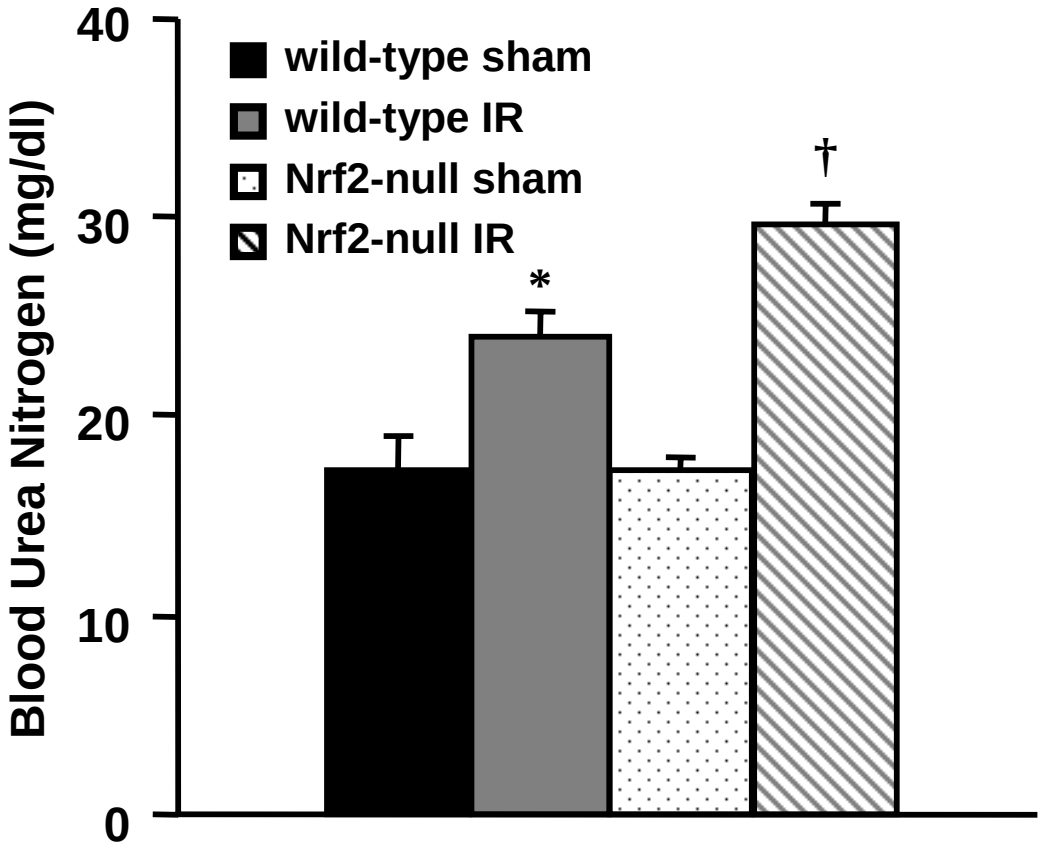

Fig 11

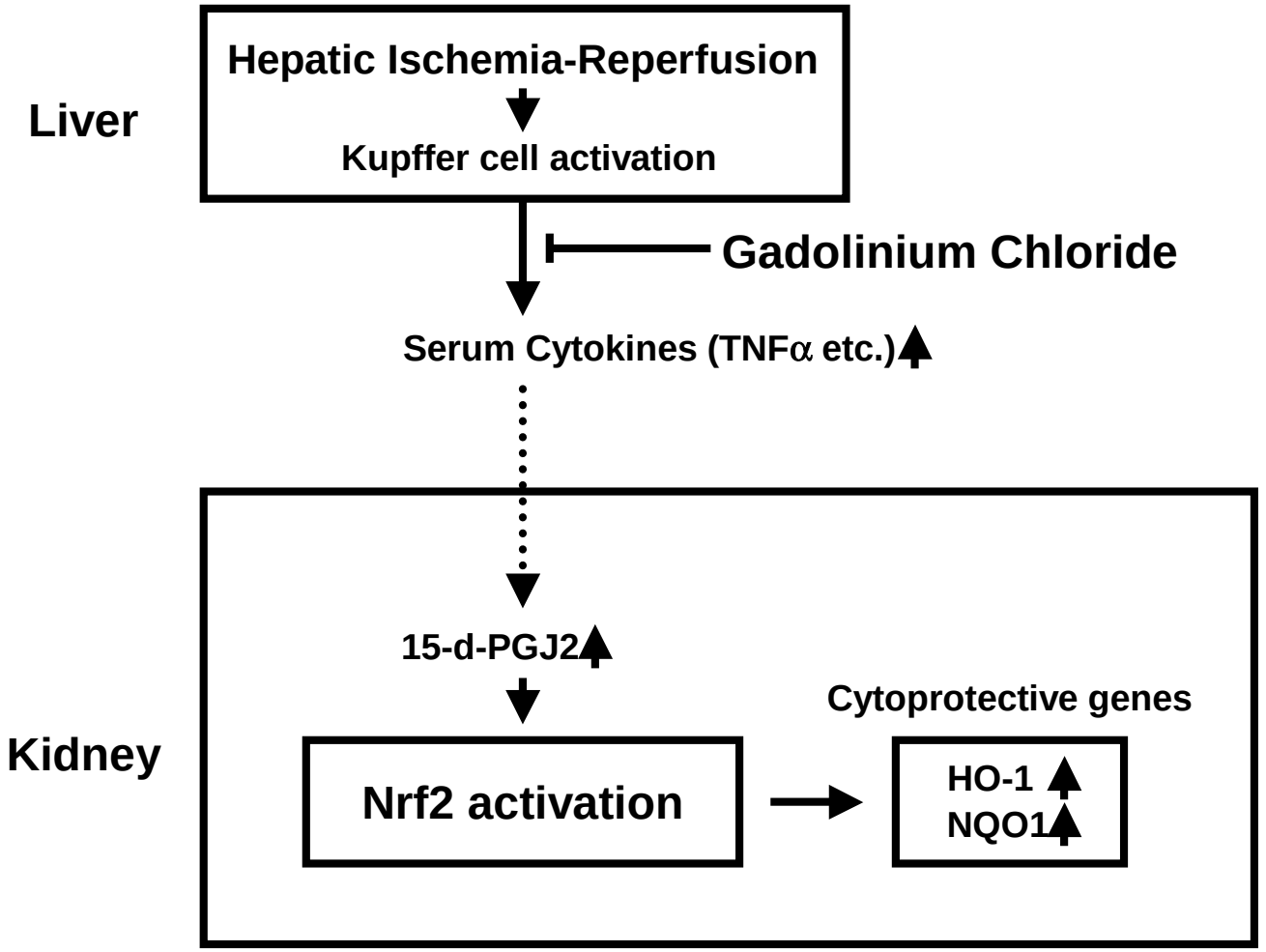

Fig 12