Intermedin protects against renal ischemia-reperfusion injury by inhibition of oxidative stress

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Qiao X, Li RS, Li H, Zhu GZ, Huang XG, Shao S, Bai B. Intermedin protects against renal ischemia-reperfusion injury by inhibition of oxidative stress. Am J Physiol Renal Physiol 304: F112–F119, 2013. First published October 17, 2012; doi:10.1152/ajprenal.00054.2012.—Reactive oxygen species (ROS) play a critical role in renal ischemia-reperfusion injury (IRI). Intermedin (IMD) reportedly protected against myocardial IRI via its antioxidant effects; however, its protective role in renal IRI has not been investigated. We overexpressed IMD in rat kidneys and examined how the kidneys respond to renal IRI. Eukaryotic expression plasmid encoding the rat IMD gene or control empty vector was transfected into the left kidney using an ultrasound-microbubble-mediated delivery system. This method yielded high expression of IMD in kidney cells. Renal IRI was induced by clamping the left renal artery followed by reperfusion. In response to IRI, overexpression of IMD in the kidney significantly improved renal function and pathology compared with the kidney transfected with control plasmid. We investigated the mechanisms by which IMD protects against renal IRI. We examined renal superoxide dismutase (SOD) activity and malondialdehyde (MDA) content and found SOD activity was significantly increased, while MDA level was markedly decreased in kidneys transfected with IMD, suggesting ROS production and oxidative stress were reduced by IMD overexpression. We also measured myeloperoxidase (MPO) activity, tubular cell apoptosis, and the expression of intercellular adhesion molecule-1 (ICAM-1), P-selectin, and endothelin-1 (ET-1) in the kidney. Renal MPO activity and the expression of ICAM-1, P-selectin, and ET-1 stimulated by IRI were significantly inhibited by IMD overexpression. Moreover, IMD overexpression prevented kidney cells from apoptosis caused by IRI. Our results demonstrate that overexpression of IMD in the kidney protects against renal IRI, apparently by reducing oxidative stress, consequently suppressing inflammation and vasoconstrictor production and apoptosis.

Intermedin; transfection; renal IRI; reperfusion; oxidative stress

ACUTE KIDNEY INJURY following ischemia-reperfusion injury (IRI) is a frequent clinical problem associated with high morbidity and mortality (5, 35). Much has been learned about the pathophysiology of renal IRI, but fewer therapeutic strategies have been shown to reduce mortality (7, 36).

In clinical settings, evidence is emerging that reactive oxygen species (ROS) play an important role in the pathophysiology of renal IRI (3). ROS can stimulate adhesion molecules expression including intercellular adhesion molecule-1 (ICAM-1) and P-selectin; these two molecules have been shown to play a critical role in recruitment of leukocytes to injury sites and adherence to endothelium (2, 28). Furthermore, the infiltration of leukocytes can also enhance tissue damage in a positive feedback manner by increase the production of ROS (7). ROS increase the production of vasoconstric- tors, such as endothelin-1 (ET-1), and cause persistent vasoconstriction and hence a decreased glomerular filtration rate (GFR; Refs. 32, 19). In addition, ROS are able to react with proteins, lipids, nucleic acids, and carbohydrates to contribute to cell apoptosis and necrosis (7). Thus inactivating ROS might be an effective strategy to combat renal IRI. For example, Kojima et al. (18) demonstrated the protective effects of antioxidant therapies in renal IRI models.

Intermedin (IMD) is a newly discovered peptide that belongs to the calcitonin gene-related peptide (CGRP) family. Studies have been shown that IMD can ameliorate inflammation and tissue damage by inhibition of oxidative stress in DOCA-salt hypertensive rats (11). IMD shares structural and functional homology with adrenomedullin (30, 38), which can attenuate renal IRI (27). These two peptides elicit their biological actions via nonspecific interaction with different combinations of the calcitonin receptor-like receptor and the three receptor activity-modifying proteins (30, 38). IMD is distributed in a wide variety of tissues, including brain, heart, lung, gastrointestinal tract, pituitary, and kidney (33, 25). IMD reportedly protects against myocardial IRI (37), while its role in renal IRI is unknown. In kidney, IMD mainly expresses in tubular cells of cortex and medulla (33). Our previous studies indicated that IMD and its receptors were upregulated after renal IRI, suggesting IMD may involve in renal IRI. Based on these findings, we hypothesized that IMD can attenuate renal IRI by reducing oxidative stress. In this study, we examined how kidneys with overexpression of IMD respond to renal IRI and investigated the mechanisms leading to these effects of IMD.

MATERIALS AND METHODS

Ultrasound-mediated gene delivery into the kidney. The Experimental Animal Committee approved all animal protocols. Eukaryotic expression plasmid pcDNA3.1-IMD containing full-length cDNA sequence of rat IMD was successfully constructed in our previous study. Ten minutes after removal of the right kidney, pcDNA3.1-IMD plasmid or control empty vector was transfected into the left kidney of male Wistar rats via the renal artery using an ultrasound-mediated system as described by Lan et al. (20). Briefly, procedures of ultrasound-based gene transfer technique includes the following: 1) SonoVue (sulphur hexafluoride, an echocardiographic contrast microbubble; BRACCO Imaging B.V.) was dissolved with 0.9% sodium chloride to a final volume of 5 ml (45 μg/ml). Mixing pcDNA3.1-IMD plasmid or control empty vector with the above mixture in 1:1 vol/vol ratio and injecting the mixed solution containing 50 μg of designated plasmid in 0.4 ml into the left renal artery while temporarily clamping off the renal artery and vein (<5 min); 2) applying the ultrasound transducer (Xinzen Medical Electronic Apparatus, Shenyang, China) directly onto one side of the left kidney with a continuous-wave output of 0.95-MHz ultrasound at 5% power output for a total of 60 s with 30-s...
involvement of IMD in renal ischemia-reperfusion injury

In situ terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling assay. Fixed rat kidney sections were deparaffinized in xylene and rehydrated through a graded ethanol series to water. Terminal deoxynucleotidyl transferase biotin-dUTP nick end-labeling (TUNEL) staining (brown) was performed with a commercially available in situ cell death detection kit (Roche). For observation of the total number of cells in the field, kidney sections were also stained with hematoxylin (blue). TUNEL-positive nuclei were expressed as a percentage of total nuclei per field (13). Sections treated with DNase I or reaction omitting TDT enzyme serve as positive or negative control, respectively.

Caspase-3 activity assays. Renal tissues were pulverized under liquid nitrogen and homogenized in ice-cold lysis buffer. Caspase-3 activity in lysates was determined using a colorimetric caspase-3 assay kit according to the manufacturer’s instructions (United States Biological). Samples were read at 405 nm in a microplate reader.

Statistical analysis. Results are presented as means ± SD. The data were analyzed with one-way ANOVA for comparisons of mean values among multiple treatment groups. Student’s t-test was used for comparisons between two groups. *P < 0.05 was considered significant.

RESULTS

Efficacy of ultrasound-microbubble-mediated gene transfection. We examined the efficacy of ultrasound-microbubble-mediated gene transfection in kidney by semiquantitative RT-PCR and Western blot analysis. After 7 days of transfection, kidney sections from rats treated with pcDNA3.1-IMD plasmid exhibited significant increase in IMD expression compared with kidneys of rats treated with control empty vector (Fig. 1, A–D), indicating that IMD was transfected into the kidney successfully. To confirm this result, we performed immunohistochemistry and found that the kidney transfected with pcDNA3.1-IMD plasmid exhibited strong IMD immunostaining in both cortical and medullary tubular cells, glomerular cells, vascular and perivascular cells, and interstitial cells (Fig. 1, E and F). At a baseline, endogenous IMD also can be detected in tubular cells and endothelial cells of the glomerulus and vasa recta in the kidney transfected with control empty vector. Notably, the ultrasound itself did not cause any histological and functional abnormalities in kidney because there was no difference in serum creatinine concentrations and morphology between nontreated and ultrasound-microbubble-treated kidneys (data not shown). In addition, there is no overexpression of IMD in livers of transfected rats (Fig. 1, G and H), indicating the transfection is kidney specific.

Overexpression of IMD in kidney ameliorated renal function in rats with IRI. In rats without transfection with renal IRI, the serum creatinine markedly increased compared with the values in sham controls rats. Overexpression of IMD in kidney significantly decreased the level of serum creatinine in rats with renal IRI (Table 1). In rats transfected with empty plasmid, the levels of serum creatinine were also sharply increased following renal IRI, indicating the improved kidney function in rats transfected with pcDNA3.1-IMD plasmid was due to the overexpression of IMD (Table 1).

Overexpression of IMD prevented kidney from IRI-induced renal damages. Compared with the kidney of sham-operated rats, we found that there were severe pathological changes in the kidney with renal IRI. These included a loss of brush-border membranes, tubular dilatation, flattened tubular epithelium, cast formation, luminal debris, and interstitial infiltration (Fig. 2, A and B).
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In contrast, all these renal pathological abnormalities were significantly improved in the IRI kidney with overexpression of IMD (Fig. 2C). In the kidney transfected with empty vector, IMD produced similar pathological changes compared with kidney from nontransfected IRI group (Fig. 2D).

**IMD reduced oxidative stress stimulated by renal IRI.** Renal IRI stimulates ROS generation, which causes secondary damages (3). SOD inactivates ROS and, therefore, plays a protective role in IRI (22). To understand how overexpression of IMD protects against renal damages caused by IRI, we measured the activity of SOD. We found that renal IRI decreased SOD activity in kidney without transfection or transfected with empty vector and overexpression of IMD significantly increased SOD activity (Table 1). We assessed MDA content (a marker of oxidative stress). As expected, IRI significantly increased MDA content in kidney, but overexpression of IMD largely blocked the increase in MDA content caused by IRI (Table 1). These results suggested that IMD reduces oxidative stress perhaps by increasing SOD activity. We also investigated the effect of IMD transfection on baseline levels of SOD and MDA and found that IMD slightly increased SOD activity but did not alter MDA content at baseline (Table 2).

**Overexpression of IMD inhibited ICAM-1 and P-selectin expression.** Since ROS stimulate ICAM-1 and P-selectin, which are responsible for reperfusion caused tissue damages, inactivation of ROS should dampen the expression of ICAM-1 and P-selectin, as results ameliorating tissue damage caused by IRI (2, 28). We performed immunohistochemistry and quantitative PCR and found that renal IRI caused a robust induction of ICAM-1 (Fig. 3, B and F), and the increased ICAM-1 was

Table 1. Serum creatinine concentrations, SOD activity, MDA content, MPO activity and caspase-3 activity in kidneys

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum Creatinine, mg/dl</th>
<th>SOD Activity, U/mg</th>
<th>MDA Content, nmol/mg</th>
<th>MPO Activity, U/g</th>
<th>Caspase-3 Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sham group</td>
<td>0.55 ± 0.08</td>
<td>474.39 ± 93.20</td>
<td>12.05 ± 4.92</td>
<td>0.24 ± 0.04</td>
<td>1.00 ± 0.20</td>
</tr>
<tr>
<td>IRI group</td>
<td>0.91 ± 0.17*</td>
<td>316.09 ± 46.92*</td>
<td>20.30 ± 5.88*</td>
<td>0.77 ± 0.14*</td>
<td>1.58 ± 0.11*</td>
</tr>
<tr>
<td>IRI + IMD group</td>
<td>0.66 ± 0.12†</td>
<td>395.61 ± 97.35†</td>
<td>15.86 ± 5.49†</td>
<td>0.42 ± 0.13†</td>
<td>1.31 ± 0.01†</td>
</tr>
<tr>
<td>IRI + empty plasmid group</td>
<td>0.86 ± 0.09</td>
<td>329.39 ± 83.55</td>
<td>21.12 ± 5.12</td>
<td>0.77 ± 0.13</td>
<td>1.59 ± 0.03</td>
</tr>
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</table>

Values are means ± SD; n = 6/group. SOD: superoxide dismutase; MDA, malondialdehyde; MPO, myeloperoxidase; IRI, ischemia-reperfusion injury; IMD, intermedin. *P < 0.05 vs. sham control group. †P < 0.05 vs. IRI group.
Thus the protective effect of IMD may be due to repression of ICAM-1 and P-selectin. Therefore, the expression of ICAM-1 and P-selectin was not found to be significantly different between the kidneys transfected with empty plasmid and nontransfected kidney when they were subjected to renal IRI (Fig. 3, B and C). As a control, we did not find that the expression of ICAM-1 and P-selectin was significantly different between the kidneys transfected with empty plasmid and nontransfected kidney when they were subjected to renal IRI (Fig. 3, D and 3F and Fig. 4, D and F). Thus the protective effect of IMD may be due to repression of ICAM-1 and P-selectin.

**IMD reduced MPO activity.** Leukocyte, especially neutrophil, infiltration to the kidney also participates in renal IRI (24). ROS is thought to be a critical event in the recruitment and activation of leukocytes (26). We investigated MPO activity, a marker of neutrophil infiltration, in the kidney and found that renal IRI produced marked increase in MPO activity, indicating that neutrophils infiltration into kidney tissue was more pronounced compared with sham-operated animals (Table 1). Treatment of rats with IMD gene transfer before renal IRI produced a significant attenuation of MPO activity caused by renal IRI. However, pretreatment of rats with empty plasmid before renal ischemia did not have a significant effect on MPO activity (Table 1).

**Overexpression of IMD suppressed ET-1 expression.** ET-1, a vasoconstrictor, can trigger vasoconstriction and causes tis-

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**Table 2. Influence of IMD transfection on baseline levels of SOD, MDA, and caspase-3 activity in kidneys**

<table>
<thead>
<tr>
<th></th>
<th>Sham Group</th>
<th>Sham + IMD Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOD activity, U/mg</td>
<td>474.39 ± 93.20</td>
<td>496.21 ± 102.31</td>
</tr>
<tr>
<td>MDA content, nmol/mg</td>
<td>12.05 ± 4.92</td>
<td>11.92 ± 3.06</td>
</tr>
<tr>
<td>Caspase-3 activity</td>
<td>1.00 ± 0.20</td>
<td>1.03 ± 0.17</td>
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Values are means ± SD.
sue damage, especially during renal IRI (32). Since ROS is a potent stimulus for ET-1 expression and IMD can inactivate ROS, we examined if overexpression of IMD reduces ET-1. Immunohistochemistry for ET-1 revealed a weak staining in the tubules of kidney from sham rat (Fig. 5A). Renal IRI markedly increased the level of ET-1 (Fig. 5B and F). This response was suppressed by overexpression of IMD while empty plasmid had no effect on ET-1 expression (Fig. 5, D and F).

**IMD overexpression suppressed tubular cell apoptosis triggered by renal IRI.** Since tubular cell apoptosis is a major issue in renal IRI (6), we assessed the apoptotic cells using TUNEL assay and found that IMD overexpression significantly reduced the number of apoptotic tubular cells in kidney with IRI (Fig. 6C), as control, nontransfected kidney or kidney transfected with empty plasmid exhibited a massive apoptosis after renal IRI.
thereby protecting kidney from renal IRI. Suggested that IMD can suppress tubular cell apoptosis, activity in kidney of sham group (Table 2). These results stimulated by renal IRI; however, it did not influence the caspase-3 activity. IMD overexpression markedly reduced caspase-3 activity stimulated in kidney and kidney transfected with empty plasmid. Substantial increase in kidney caspase-3 activity in both non-transfected kidney and kidney transfected with empty plasmid. We found that overexpression of IMD in kidney significantly improved renal function and protected against renal damage caused by IRI, and these responses were associated with suppression of ICAM-1, P-selectin, and ET-1 and inhibition of tubular cell apoptosis (Figs. 6 and Table 1). In addition, MPO, a marker of neutrophil infiltration, was significantly decreased in the injured kidney with overexpression of IMD (Table 1). In investigating the mechanisms for these results we found increased SOD activity, which was confirmed by a decrease in MDA content (a marker of lipid peroxidation; Table 1). These findings indicate that enhancing IMD can inhibit oxidative stress and, therefore, protect against renal IRI.

Renal IRI is characterized by a burst of ROS formation and a reduction of antioxidants, resulting in increased oxidative stress and tissue damage (3, 15). Consistent with this concept, we found that renal IRI significantly reduced SOD activity and increased MDA content (Table 1). This increased oxidative stress was associated with impaired renal function and histological changes, as evidenced by a marked increase in serum creatinine and characteristic renal morphological changes. However, overexpression of IMD prevented severe depletion of SOD activity in injured kidney and inhibited IRI-induced lipid peroxidation and, therefore, improved kidney function and damage caused by renal IRI (Table 1 and Fig. 2).

Inflammation has been recognized as an important factor in the development and progression of renal IRI. Although the mechanisms involved in the generation of an inflammatory response following renal IRI are complex, evidence has shown that induction of ROS may trigger these responses (26). Ischemic injury upregulates the expression of adhesion proteins, such as ICAM-1 and P-selectin, in endothelial cells and leukocytes. In this study we observed intense staining of both P-selectin and ICAM-1 after IRI in the kidney. These factors facilitate the recruitment and infiltration of circulating leuko-

### DISCUSSION

Acute kidney injury caused by renal IRI is often associated with high morbidity and mortality and ROS play a critical role in this pathological process (3). ROS can trigger inflammation, increase vasoconstrictors production, and induce tubular cell apoptosis and necrosis (26, 1, 7). Thus agents that can reduce ROS have received considerable attention. As a secreted peptide, IMD was shown to inhibit oxidative stress (11, 37), suggesting that IMD might ameliorate renal IRI. We hypothesized that overexpression of IMD would protect kidney from IRI by reducing oxidative stress, which causes renal damage. It is reported that oxidative stress occurs early in the reperfusion phase and remains at a high level 24 h after reperfusion (22); however, the detrimental effects of oxidative stress, such as inflammation and renal damage, become obvious 24 h after reperfusion. Thus we examined the protective effects of IMD at 24 h after reperfusion.

To investigate the role of IMD in renal IRI, we transfected IMD locally into the kidney. Two categories of gene delivery are commonly used in animal models. Nonviral delivery has low transfection efficiency and transient expression of the gene product (34). Virus-mediated gene delivery significantly increases the efficacy of transfection; however, it still has many disadvantages such as local immunogenicity, production of neutralizing antibodies, and, sometimes, lethal toxicity (4). Recently, ultrasound-induced microbubble destruction has been proposed as a new technique for local delivery of genes to specific target tissues (10). Lan et al. (20) demonstrated that the ultrasound-mediated disruption of gas-filled microbubbles can be used to effectively transfect the naked plasmid DNA(s) into the kidney. In this study, we adopted this technique to deliver the pcDNA-IMD overexpression construct into the rat kidney and detected that both mRNA and protein of IMD were dramatically increased in the transfected kidney (Fig. 1). Notably, no abnormalities of immunoresponses, histology, and kidney function were found after transfection (data not shown). These results indicated that ultrasound-microbubble-mediated transfection is an efficient and safe approach for gene delivery into kidney.

We found that overexpression of IMD in kidney significantly improved renal function and protected against renal damage caused by IRI, and these responses were associated with suppression of ICAM-1, P-selectin, and ET-1 and inhibition of tubular cell apoptosis (Figs. 6 and Table 1). In addition, MPO, a marker of neutrophil infiltration, was significantly decreased in the injured kidney with overexpression of IMD (Table 1). In investigating the mechanisms for these results we found increased SOD activity, which was confirmed by a decrease in MDA content (a marker of lipid peroxidation; Table 1). These findings indicate that enhancing IMD can inhibit oxidative stress and, therefore, protect against renal IRI.

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**Fig. 6.** Determination of tubular cell apoptosis in situ by terminal deoxynucleotidyl transferase nick end-labeling (TUNEL) assay. Sham rats showed few TUNEL-positive cells (A). Rats subjected to ischemia-reperfusion injury (IRI) showed increased TUNEL-positive cells (B). Pretreatment with IMD gene transfer before renal IRI reduced TUNEL-positive cells (C). There was no significant deference between empty plasmid with IRI group and IRI group (D). TUNEL-positive nuclei were expressed as a percentage of total nuclei per field; 6–8 outer medulla and inner medulla fields per section and 2–3 sections per kidney were examined in each experiment. NC, negative control; PC, positive control. TUNEL-positive nuclei were counted and shown as a percentage of total nuclei per field (E). Magnification: ×200. Data in bar graphs are means ± SD (n = 6 for each group). *P < 0.05 vs. sham control group. **P < 0.05 vs. IRI group.

(6, B and D). Because TUNEL assay can label both apoptotic cells and necrosis cells, we further measured caspase-3 activity. As shown in Table 1, renal IRI exhibited a substantial increase in kidney caspase-3 activity in both non-transfected kidney and kidney transfected with empty plasmid. IMD overexpression markedly reduced caspase-3 activity stimulated by renal IRI; however, it did not influence the caspase-3 activity in kidney of sham group (Table 2). These results suggested that IMD can suppress tubular cell apoptosis, thereby protecting kidney from renal IRI.
cytes, resulting in rolling, adherence, and extravasation of leukocytes in the renal tissue (24). Our results indicated that with the upregulation of adhesion molecules, renal IRI markedly increases the activity of MPO in the kidney of rats with renal IRI, indicating that neutrophil infiltration into kidney tissue was more pronounced compared with sham-operated animals. This result was consistent with the results reported by De Greef et al. (9). Neutrophil-endothelial cell adhesion in the vasa recta in the outer stripe of the outer medulla leads to capillary plugging and vascular congestion (16). Furthermore, leukocytes release additional ROS, proteolytic enzymes, and cytokines that incite cell death (16). Several studies have demonstrated that blockades of adhesion molecules and leukocyte infiltration are beneficial for renal IRI (14, 31, 17). In this study, we found that overexpression of IMD can reduce adhesion molecule expression and MPO activity, probably due to its antioxidative properties leading to reduction in inflammation.

Oxidative stress can stimulate many vasoactive mediators that can affect renal function directly by causing renal vasoconstriction or decreasing the glomerular capillary ultrafiltration coefficient and, thus, reduce GFR (1). Of all the vasoconstrictive agents, ET-1 seems to be particularly important, because its level increased after ischemia and blockage of the ET pathway by endothelin A receptor antagonists protects against renal IRI (12). In the present study, the expression of ET-1 in kidney was increased following IRI but was suppressed by IMD overexpression, likewise, through the antioxidative ability of IMD.

Renal IRI increases renal tubular cell apoptosis, which plays an important role in the pathogenesis of IRI. In addition, inhibition of apoptosis is associated with reduction of postreperfusion damage and improved renal function and survival (6, 8). In this study, we observed a significant increase of TUNEL-positive tubular cells and caspase-3 activity in the rats with renal IRI compared with that in the sham control rats, indicating IRI can trigger the pathways leading to tubular cell apoptosis. ROS has been proposed to initiate apoptosis in renal IRI-induced cells (21). We demonstrate that IMD overexpression significantly reduced the number of TUNEL-positive cells and suppressed caspase-3 activation induced by renal IRI, suggesting that overexpression of IMD inhibits tubular cell apoptosis by reducing oxidative stress.

Collectively, our results demonstrate that overexpression of IMD in the kidney protects against renal IRI, apparently by reducing oxidative stress, consequently suppressing inflammation, vasoconstrictors production, and apoptosis. Targeting IMD could be a new therapeutic strategy to combat ischemia-mediated acute renal failure.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: X.Q. and R.-S.L. conception and design of research; X.Q., H.L., G.-Z.Z., X.-G.H., S.S., and B.B. performed experiments; X.Q. analyzed data; X.Q. interpreted results of experiments; X.Q. and H.L. prepared figures; X.Q. drafted manuscript; X.Q. and R.-S.L. edited and revised manuscript; R.-S.L. approved final version of manuscript.

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