Increased superoxide formation induced by irradiation preconditioning triggers kidney resistance to ischemia-reperfusion injury in mice

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Submitted 7 October 2008; accepted in final form 2 February 2009

Kim J, Park J, Park KM. Increased superoxide formation induced by irradiation preconditioning triggers kidney resistance to ischemia-reperfusion injury in mice. Am J Physiol Renal Physiol 296: F1202–F1211, 2009. First published December 23, 2008; doi:10.1152/ajprenal.90592.2008.—One of the obstacles in irradiation therapy is cytoresistance, acquired by activation of self-defense systems, such as antioxidant or molecular chaperone systems, to cope with stress. We investigated whether irradiation preconditioning (IP) rendered resistance of the kidney against subsequent ischemia-reperfusion (I/R) and attempted to elucidate any such protective mechanisms. Mice were irradiated with a total of 4, 6, or 8 Gy using a cesium-137 source irradiator and then, 6 days later, were subjected to 28 min of bilateral renal ischemia followed by reperfusion. Eight Gy of IP significantly attenuated the increases in plasma creatinine (PCr) and blood urea nitrogen (BUN) concentration, structural damage, lipid peroxidation, superoxide formation, expression and activity of NADPH oxidase (NOX)-2, nitrotyrosine level, and hydrogen peroxide production after I/R in kidney tissues, indicating that IP protects the kidneys from I/R injury. IP markedly increased the activity of NOX, resulting in increased superoxide formation, manganese superoxide dismutase (MnSOD) activity and expression, and heat shock protein (HSP)-27 expression in kidneys. However, it did not change expressions of catalase, copper-zinc superoxide dismutase (CuZnSOD), and HSP-72. To investigate whether the protection afforded by IP was associated with increases in MnSOD and HSP-27 expression triggered by superoxide formation after IP, we administered manganese (III) tetrakis(1-methyl-4-pyridyl)porphyrin, a superoxide scavenger, to IP mice. This administration blocked superoxide formation and subsequent increases in MnSOD and HSP-27 expression and accelerated the post-I/R increases in PCr and BUN. In conclusion, IP renders kidney resistance to I/R injury, and this resistance is mediated by increased superoxide formation, which activates MnSOD activity and expression as well as HSP-27 expression.

manganese superoxide dismutase; ROS; oxidative stress; preconditioning; acute renal failure; ARF

A number of studies have demonstrated that mild stress induced by invasive insults such as ischemia-reperfusion (I/R) or noninvasive damages such as those instigated by pharmacological reagents, renders cells or tissues resistant to subsequent strong injury by activating their intracellular defense systems (26, 33, 35). One well-defined surgical method is ischemic preconditioning, where I/R preconditioning renders the organ resistant to subsequent I/R injury. We have also demonstrated that previous I/R insults protects the kidneys against subsequent I/R injury induced 8 days later and this resistance remains for at least 12 wk (32).

I/R in kidneys causes acute kidney injury, the high mortality and morbidity of which have not changed significantly over the last several decades (42). Ischemia causes ATP depletion, which induces necrotic and apoptotic cell death. Following reperfusion, cell damage is worsened to an extent greater than ischemia itself, due to the production of excessive ROS including superoxide anions, hydroxyl radicals, hypochlorous acid, hydrogen peroxide, and peroxynitrite (15, 34, 42). The ROS levels are excessively increased beyond the antioxidant capacity of the cells. This disrupts cell polarity, the cellular cytoskeleton, and cellular integrity by denaturation of proteins, lipid peroxidation, and DNA breakdown (44), finally resulting in renal cell death and loss of renal functions (34). Many studies including ours have shown that an attenuation of oxidative stress reduces kidney injury induced by I/R insult (14).

We thus hypothesized that the activation of cellular defense systems induced by irradiation protected the kidney against subsequent I/R injury within the antioxidant and HSP pathway. In this study we report that irradiation preconditioning (IP) protects mouse kidneys against subsequent I/R injury and that this protection is triggered by superoxide formation, enhancing manganese superoxide dismutase (MnSOD) activity and expression and HSP-27 expression.

MATERIALS AND METHODS

Animal preparation. Experiments were performed in age-matched (8 wk old) C57BL/6 male mice. In all cases, studies were approved by the Kyungpook National University Institutional Animal Care and Use Committee. Mice were irradiated with 4, 6, or 8 Gy by a single dose, or a total of 6 (2 Gy/day from day 0 to day 2, 2 Gy × 3) or 8 Gy (2 Gy/day from day 0 to day 3; 2 Gy × 4) by a fractionated dose using a cesium-137 source irradiator on day 0. Six days after the irradiation, mice were subjected to either 20, 28, or 32 min of bilateral renal ischemia followed by reperfusion (I/R) or a sham operation (sham) under anesthesia with pentobarbital sodium [60 mg/kg body wt (BW) ip, Sigma, St. Louis, MO], as previously described (33). Some mice were administrated either manganese (III) tetrakis (1-methyl-4-pyridyl)porphyrin (MnTMPyP; 5 mg/kg BW ip, Calbiochem, San Diego, CA), a superoxide scavenger, or 0.9% saline (vehicle) daily for 4 days beginning 1 h after 8 Gy of irradiation on...
day 0, and then subjected to either 28 min of bilateral renal ischemia followed by 4 or 24 h of reperfusion or sham operation on day 6. Kidneys were either perfusion-fixed in PLP (4% paraformaldehyde, 75 mM t-lysine, 10 mM sodium periodate, Sigma) for histological study or snap-frozen in liquid nitrogen for biochemical study 4, 24, or 48 h after the reperfusion. For histological studies, kidneys fixed in PLP were washed with PBS three times for 5 min each, embedded in otyxteracycline compound (Sakura FineTek, Torrance, CA) at −20°C or in paraffin at room temperature, and then cut into 4-μm cryosections and 2-μm paraffin sections using a cryotome (CM1850, Leica, Bensheim, Germany) and a microtome (RM2165, Leica), respectively. Paraffin sections were stained with periodic acid-Schiff (PAS). PAS staining was performed using the manufacturer’s protocol.

Renal functional parameters. Blood samples were taken from the retrocruov vein plexus at the times indicated (see the figures). Plasma creatinine (P) concentration was measured using a Beckman Analyzer II (Beckman, Fullerton, CA). Blood urea nitrogen (BUN) concentration was measured using a BUN assay kit (ASAN PHARM, Gyeonggi-do, Korea) according to the manufacturer’s protocol, as described previously (11). To obtain urine, mice were placed in mouse-specific metabolic cages for 18 h beginning at 6 h after I/R or sham. The value for creatinine clearance was considered as glomerular filtration rate (GFR). The formula for measurements of GFR was as follows: GFR = (urine creatinine × urine volume)/plasma creatinine concentration/time of urine collection/BW (33).

Histological score. Histological damage levels were scored as described previously (14, 39). Briefly, 50 tubules in the outer medulla of the kidney were analyzed by using a score as follows: 0, no damage; 1, mild damage with rounding of epithelial cells and dilated tubular lumen; 2, severe damage with flattened epithelial cells, loss of nuclear staining, dilated lumen, and congestion of lumen; and 3, destroyed tubules with flat epithelial cells lacking nuclear staining and congestion of lumen. Four kidneys from each experimental condition were used (n = 5). Ten fields per slide were used for the counting.

Measurement of catalase, copper-zinc superoxide dismutase, and MnSOD activities. Mitochondrial or cytosolic fractions of kidneys were prepared as described previously (14). The cytosolic fraction was used to measure the activity of catalase or copper-zinc superoxide dismutase (CuZnSOD). The mitochondrial fraction was used to measure the activity of MnSOD as described previously (14, 24).

Measurement of NADPH oxidase activity. NADPH oxidase (NOX) activity was determined as the oxidation of NADPH measured at 340 nm in the reaction mixture containing 50 mM Tris, 50 mM 2-(N-morpholino)ethanesulfonic acid (MES; pH 7.0), 1 mM KCN to inhibit low levels of mitochondrial oxidase activity (18), and 150 mM NADPH (Sigma) for 1 min at 37°C (28).

Measurement of lipid peroxidation and hydrogen peroxide levels in kidneys. Thiobarbituric acid-reactive substances (TBARS) were determined as a measure of lipid peroxidation. Samples were evaluated for malondialdehyde (MDA) production using a spectrophotometric assay for TBARS (20). Hydrogen peroxide (H2O2) concentrations were determined using a ferric-sensitive dye, xylenol orange, as described previously (14).

Determination of superoxide formation using nitroblue tetrazolium. Cryosections were incubated in 1 mg/ml of nitroblue tetrazolium (NBT; Sigma) in PBS, pH 7.4, for 2 h at 37°C and washed with PBS. Signals were observed using a light microscope. NBT-positive areas were analyzed using Image Acquisition and Analysis LabWorks Software 4.5 (Ultra-Violet Products, Upland, CA).

Western blot analysis. To prepare protein samples from kidneys, kidneys were homogenized with a Dounce homogenizer, and the homogenate was prepared for Western blot analysis, as previously described (33). Immunoblot analyses were performed with anti-MnSOD (Calbiochem), -CuZnSOD (Chemicon, Temecula, CA), -catalase (Fitzgerald, Concord, MA), -HSP-27 (Upstate, Lake Placid, NY), -HSP-72 (Sigma), - gp91phox (NOX-2; BD Biosciences, San Jose, CA), -NOX-4 (Novus, Littleton, CO), -nitrotyrosine (Cayman, Ann Arbor, MI), or -β-actin (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies.

Statistics. Results were expressed as means ± SE. Statistical analysis was carried out using two-way ANOVA with postoperation (sham and I/R) and preconditioning (nonirradiation and irradiation); period (0, 20, 28, and 30 min of ischemia) and preconditioning; postoperation and dose (0, 4, 6, and 8 Gy); and postoperation and treatment (vehicle and MnTMPyP) as factors. Differences among groups were evaluated with an unpaired Student’s t-test. Differences were considered statistically significant at a P value of < 0.05.

RESULTS

IP protected the kidney against I/R induced 6 days later. To investigate whether IP rendered the kidney resistant against injury caused by I/R, mice were exposed to 8 Gy of irradiation in a single dose on day 0, and then subjected to either 28 min of bilateral renal ischemia followed by reperfusion or sham on day 6. PCr concentrations were significantly increased 4, 24, and 48 h after I/R (Fig. 1A). The post-I/R increases in PCr in mice preconditioned by 8 Gy of irradiation were significantly lower than those in non-IP mice (Fig. 1A). Consistent with PCr concentrations, the post-I/R increases in BUN concentrations were lower in IP mice than in non-IP mice (Fig. 1B). GFR was dramatically decreased 24 h after I/R, but IP significantly attenuated the post-I/R decrease of GFR (Fig. 1C). The histological damage to kidneys in PAS-stained tissues 24 h post-I/R included severe dilatation, flattening and congestion of tubules, as well as an accumulation of leukocytes (Fig. 1D). These post-I/R histological changes were much milder in IP mice than in non-IP mice (Fig. 1, D and E). Six days after exposure of the mice to 8 Gy of irradiation, significant histological changes were not observed compared with the kidneys of non-IP mice (Fig. 1, D and E).

To investigate whether IP protected the kidneys against different strengths of ischemia, ischemic periods were extended to 32 min or shortened to 20 min. Twenty minutes of ischemia followed by 24 h of reperfusion significantly increased PCr and BUN concentrations in non-IP mice, whereas these were unchanged in IP mice (Fig. 2, A and B). When ischemic periods were extended to 32 min, the post-I/R increases in PCr and BUN in IP mice were significantly lower than those in non-IP mice, respectively (Fig. 2, A and B).

To evaluate whether the irradiation dose and irradiation method were associated with IP-induced protection, mice were preconditioned with doses totaling 4, 6, and 8 Gy in a single irradiation on day 0, and a total of 6 (2 Gy × 3) and 8 Gy (2 Gy × 4) doses in fractioned irradiation, and then subjected to 28 min of bilateral renal ischemia followed by 24 h of reperfusion on day 6. Preconditioning by 6 and 8 Gy, but not 4 Gy, in a single dose significantly prevented the increases in PCr and BUN concentrations in non-IP mice, whereas these were unchanged in IP mice (Fig. 2, A and B). When ischemic periods were extended to 32 min, the post-I/R increases in PCr and BUN in IP mice were significantly lower than those in non-IP mice, respectively (Fig. 2, A and B).

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Irradiation preconditioning reduced oxidative stress after I/R. Since I/R injury involves oxidative stress, and since irradiation is linked to ROS production and antioxidant enzyme activation, we investigated the mechanism of involvement with redox systems on protection against oxidative stress. Superoxide formation was determined using NBT staining 24 h after I/R and was seen to have dramatically increased in the kidneys (Fig. 3, A and B). The post-I/R superoxide formation was less in IP mice than non-IP mice (Fig. 3, A and B). Superoxide formation in the kidneys of IP mice was greater than that in the kidneys of non-IP mice 24 h after sham (Fig. 3, A and B). When the expressions of NOX-2 (phagocyte-specific protein) and -4 (kidney-specific protein) and the activity of NOX were measured after I/R, there was no significant difference in NOX-2 expression between IP and non-IP mice 24 h after sham (Fig. 3, C and E), whereas NOX-4 expression and NOX activity were significantly increased in IP mice than in non-IP mice (Fig. 3, C, E, and F). The post-I/R increases in NOX-2 expression and NOX activity were significantly inhibited in IP mice (Fig. 3, C, D, and F). These data show that the changes in the activity and expression of NOX after I/R ± IP are consistent with the changes in the degree of superoxide formation.

I/R significantly increased the levels of H_2O_2 in the kidneys (Fig. 4A). The post-I/R increase in H_2O_2 levels in IP mice was significantly lower than that in non-IP and IP mice after sham (Fig. 4, A–C). Table 1 showed that SOD activity was increased predominantly 1 day after irradiation, and this increase was maintained until 6 days after irradiation. Although catalase activity was significantly decreased 6 days after irradiation (Table 1), the H_2O_2 level was not increased (Fig. 4A). It has been well recognized that two forms of SOD convert superoxide to H_2O_2; however, numbers of antioxidant enzymes such as glutathione peroxidase (GPx) and radical scavengers such as glutathione also regulate H_2O_2 levels in vivo (4). Therefore, this discrepancy, i.e., the nonsignificant increases in H_2O_2 levels in the condition of decrease of catalase activity, may be due to the variety of productive and scavenging system of H_2O_2 in vivo. In fact, it has been reported that the activity and synthesis of GPx, which identically acts with the catalase, are increased after irradiation (47).

Fig. 1. Effect of irradiation preconditioning (IP) on renal dysfunction and histological damage after ischemia-reperfusion (I/R) injury. C57BL/6 male mice were irradiated at 8 Gy in a single dose (irrad) or nonirradiated (non-irrad) on day 0 and then subjected to either 28 min of bilateral renal ischemia followed by reperfusion or sham operation (sham) on day 6. A and B: concentrations of plasma creatinine (PCr) and blood urea nitrogen (BUN) were measured before, 1, 3, and 6 days after IP, and 4, 24, and 48 h after I/R. C: glomerular filtration rate (GFR; creatinine clearance) was measured 24 h after I/R. D and E: sections of kidney harvested 24 h after I/R were periodic acid-Schiff (PAS) stained. D: histological damages were scored. Values are means ± SE; n = 6–8. In an unpaired Student’s t-test, *P < 0.05 vs. respective sham on day 6; #P < 0.05 vs. non-irrad and I/R at each time point. In 2-way ANOVA, P values for postoperation, preconditioning, and the interaction of these 2 factors, respectively, were <0.001, 0.013, and 0.008 (A); <0.001, 0.017, and 0.017 (B); <0.001, 0.010, and 0.039 (C); and <0.001, 0.006, and 0.006 (E).
The endogenous glutathione level is also increased after irradiation (17).

Superoxide produces peroxynitrite from nitric oxide (NO), which is involved in I/R injury (4, 32). The peroxynitrite reacts with tyrosine to produce nitrotyrosine (4, 10). To study the involvement of NO in the IP-induced protection, we determined the peroxynitrite level by Western blot analysis using an anti-nitrotyrosine antibody. I/R significantly increased the level of nitrotyrosine in the kidneys (Fig. 4, B). This suggests that post-I/R injury is associated with peroxynitrite production. There were, however, no significant differences in nitrotyrosine levels between non-IP and IP mice 24 h after I/R (Fig. 4, B and C). Twenty-four hours after I/R, lipid peroxidation, one of the end products resulting from the reaction between ROS and the cells, was significantly greater in the kidneys of non-IP mice than the kidneys of IP mice (Fig. 4D). Lipid peroxidation levels were not different between non-IP and IP mice 24 h after sham (Fig. 4, B and C). Twenty-four hours after I/R, lipid peroxidation, one of the end products resulting from the reaction between ROS and the cells, was significantly greater in the kidneys of non-IP mice than the kidneys of IP mice (Fig. 4D). Lipid peroxidation levels were not different between non-IP and IP mice 24 h after sham (Fig. 4D). This suggests that IP may attenuate cell death in terms of the inhibition of excessive lipid peroxidation, nitrotyrosine, and H$_2$O$_2$ production induced by I/R.

After IP, the superoxide levels were shown to have significantly increased (Fig. 3, A and B), but H$_2$O$_2$, nitrotyrosine, and lipid peroxidation levels were not (Fig. 4, A–D). These results also showed that the increased levels of superoxide formation did not reach levels that induced lipid peroxidation and cell damage, as monitored by PAS staining for histological changes (Fig. 1C), and by immunofluorescence staining for the actin cytoskeleton (Fig. 4E). Excessively produced free radicals disrupt the actin cytoskeleton in tubular epithelial cells (38).
IP increased HSP-27 expression in the kidneys. HSP expression is highly linked to resistance against I/R injury and oxidative stress (33, 34). We also previously reported that HSP-27 expression is positively correlated with kidney resistance to I/R injury (33, 35). Therefore, we evaluated the expression of HSP-27 and HSP-72 in the kidneys 6 days after 8 Gy of irradiation. In the kidney of IP mice, HSP-27 expression significantly increased, whereas that of HSP-72 did not (Fig. 7, A and B), indicating that IP stimulated HSP-27-related defense systems.

Administration of MnTMPyP, a superoxide scavenger, abolished the kidney resistance afforded by IP. To investigate whether the increases in MnSOD expression and activity and HSP-27 expression were triggered by the superoxide produced by IP, mice were irradiated with a single dose of 8 Gy on day 0 and then administered either MnTMPyP, which reduces superoxide (20), or vehicle for daily for 4 days, beginning 1 h after the irradiation. Three days after the last administration of MnTMPyP, on day 6, the mouse kidneys were harvested and used for the study. When superoxide formation was analyzed by NBT staining, superoxide formation was found to be significantly lower in MnTMPyP-administered IP mice compared with that in vehicle-administered IP mice (Fig. 8, A and B), indicating that MnTMPyP effectively attenuated IP-induced superoxide formation. In antioxidant enzymes, MnTMPyP administration to IP mice inhibited the increase in MnSOD expression (Fig. 8, C and D). However, the expression of CuZnSOD and catalase was unchanged (Fig. 8, C and D). The increase in HSP-27 expression induced by IP was also prevented by the concurrent administration of MnTMPyP (Fig. 8, E and F). There were no changes in HSP-72 expression by MnTMPyP administration (Fig. 8, E and F). These results indicate that IP increases the activity and expression of MnSOD and the expression of HSP-27 through the mild production of superoxide.

In addition, the administration of MnTMPyP abolished the kidney resistance afforded by IP, as seen in the results of functional studies; PCr and BUN levels were significantly higher in MnTMPyP-administered IP mice than that in vehicle-administered IP mice 4 and 24 h after I/R (Fig. 9, A and B). This suggests that increased formation of superoxide by IP renders resistance to the kidney against I/R injury.

Fig. 3. Levels of superoxide formation (A and B), expressions of NADPH oxidase (NOX)-2 and -4 (C–E) and NOX activity (F) 24 h after 28 min of bilateral renal ischemia followed by reperfusion in IP or non-IP mice. C57BL/6 male mice were irradiated at 8 Gy in single doses or nonirradiated on day 0 and then subjected to either 28 min of bilateral renal ischemia followed by 24 h of reperfusion or sham on day 6. A: cryosections were subjected to nitroblue tetrazolium (NBT) staining. The brown color indicates the area of superoxide formation. B: areas of superoxide formation were measured using Image Acquisition and Analysis LabWorks Software 4.5. Values are means ± SE expressed as fold-changes vs. sham and nonirradiation. C–E: expression of NOX-2 (C and D) and -4 (C and E) were determined by Western blot analysis. β-Actin was used as an equivalent protein loading marker. N and I, nonirradiation and irradiation, respectively. D and E: densities of blots were measured using Image Acquisition and Analysis LabWorks Software 4.5 and are expressed as the fold-changes vs. nonirradiation and sham. F: NOX activity was determined in the kidneys as described in MATERIALS AND METHODS. Values are means ± SE; n = 4–8. OD, optical density. In an unpaired Student’s t-test, *P < 0.05 vs. respective sham; #P < 0.05 vs. respective nonirradiation. In 2-way ANOVA, P values for postoperation, preconditioning, and the interaction of these 2 factors, respectively, were <0.001, 0.005, and <0.001 (B); <0.001, 0.023, and 0.001 (D); <0.001, 0.045, and <0.010 (E); and <0.001, 0.044, and <0.001 (F).
In this present study, we demonstrated that IP rendered the kidney resistant following I/R injury. We also showed that this protection was inhibited by the treatment of animals with MnTMPyP, indicating that IP-rendered protection was mediated by an increase in MnSOD activity and expression and HSP-27 expression triggered by IP-induced superoxide production. Based on the knowledge that cells activate their intracellular defense systems to protect themselves from a variety of abnormal conditions, many investigators have established a variety of pharmacological, noninvasive, or surgical preconditioning methods and explored cellular defense mechanisms with the aim of designing novel therapeutic agents (19, 33, 36). In the kidney, we previously established an ischemic preconditioning model where previous ischemia protected kidneys against subsequent I/R insults induced 8 days later (32, 33, 35). Although the protective effect of IP was mild, IP itself did not result in a significant change in morphology and function of the kidney, compared with ischemic preconditioning, which itself

Fig. 4. Levels of hydrogen peroxide (H$_2$O$_2$) production (A), nitrotyrosine expression (B and C), lipid peroxidation (D), and F-actin staining (E) 24 h after 28 min of bilateral renal ischemia followed by reperfusion in IP or non-IP mice. C57BL/6 male mice were irradiated at 8 Gy in a single dose or nonirradiated on day 0 and then subjected to either 28 min of bilateral renal ischemia followed by 24 h of reperfusion or sham. The kidneys were used for measurement of H$_2$O$_2$ production, nitrotyrosine expression, and lipid peroxidation as described in MATERIALS AND METHODS. B: nitrotyrosine expression was determined by Western blot analysis. β-Actin was used as an equivalent protein loading marker. C: densities of blots were measured using Image Acquisition and Analysis LabWorks Software 4.5 and are expressed as fold-changes vs. non-irradiation and sham. E: cryosections were immunostained using the phalloidin conjugated with TRITC, and images were taken using a fluorescence microscope. Values are means ± SE; n = 6–8. In an unpaired Student’s t-test, *P < 0.05 vs. respective sham; #P < 0.05 vs. respective nonirradiation. In 2-way ANOVA, P values for postoperation, preconditioning, and the interaction of these 2 factors, respectively, were <0.001, 0.036, and 0.020 (A); <0.001, 0.023, and 0.001 (C); and <0.001, <0.001, and 0.002 (D).

Table 1. Activities of antioxidant enzymes in the kidneys of mice exposed to 8 Gy of irradiation

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<tr>
<th>Enzyme Activity</th>
<th>Time (day) After Irradiation</th>
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<tr>
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<tr>
<td>MnSOD, units/mg protein</td>
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<tr>
<td>CuZnSOD, units/mg protein</td>
<td>7.39±0.25</td>
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<tr>
<td>Catalase, units/mg protein</td>
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Values are means ± SE. MnSOD, manganese superoxide dismutase; CuZnSOD, copper-zinc superoxide dismutase. MnSOD activity was determined in the mitochondrial fraction of kidneys (n = 5). CuZnSOD and catalase activities were determined in the cytosolic fraction of kidneys (n = 5). In unpaired Student’s t-test, *P < 0.05 vs. nonirradiation. In 1-way ANOVA, P values for time were 0.018 (MnSOD); <0.001 (CuZnSOD); and 0.012 (catalase).
induces significant renal functional and morphological impairments (11, 32, 33, 35).

Previously, we reported that the protection afforded by ischemic preconditioning correlated with the occurrence of ischemia and the intervals between preconditioning and I/R insult (32, 33). In the present study, the levels of IP-induced protection correlated with the dose of irradiation. When the total dose of irradiation was the same, the protection afforded by irradiation by a single dose was stronger than that by a fractionated dose, suggesting that the single irradiation dose more strongly stimulated kidney resistance than the fractionated irradiation. When the totaling radiation amounts were the same, the effects of a single large dose was greater than that of fractioned doses (43).

In the present study, MnSOD activity dramatically increased from 24 h after IP (150% of baseline) and remained at high levels until 6 days after IP. In contrast, the levels of catalase and CuZnSOD enzymes did not change 24 h after IP but showed a slight decrease 6 days later (43).

Fig. 5. Activities of manganese superoxide dismutase (MnSOD; A), copper-zinc superoxide dismutase (CuZnSOD; B), and catalase (C) 24 h after 28 min of bilateral renal ischemia followed by reperfusion in IP or non-IP mice. C57BL/6 male mice were irradiated at 8 Gy in a single dose or nonirradiated on day 0 and then subjected to either 28 min of bilateral renal ischemia followed by 24 h of reperfusion or sham in IP or non-IP mice. The kidneys were used for measurement of activities of MnSOD, CuZnSOD, and catalase as described in MATERIALS AND METHODS. Values are expressed as means ± SE; n = 4. In an unpaired Student’s t-test, *P < 0.05 vs. respective sham; #P < 0.05 vs. respective nonirradiation. In 2-way ANOVA, P values for postoperation, preconditioning, and the interaction of these 2 factors, respectively, were <0.001, <0.001, and 0.048 (A); <0.001, 0.153, and 0.047 (B); and <0.001, 0.139, and 0.026 (C).

In the present study, MnSOD activity dramatically increased from 24 h after IP (~150% of baseline) and remained at high levels until 6 days after IP (~150% of baseline). In contrast, the levels of catalase and CuZnSOD enzymes did not change 24 h after IP but showed a slight decrease 6 days later (~83% of baseline).

Fig. 6. Expressions of MnSOD, CuZnSOD, and catalase enzymes in the kidneys 6 days after IP or non-IP. C57BL/6 male mice were irradiated at 8 Gy in a single dose or nonirradiated on day 0. Kidneys were harvested 6 days after preconditioning and subjected to Western blot analysis (n = 3). A: the mitochondrial fraction was used for MnSOD expression and the cytosolic fraction for CuZnSOD, catalase, and β-actin expression. β-Actin was used as equivalent protein loading marker. B: densities of blots were measured using Image Acquisition and Analysis LabWorks Software 4.5 and are expressed as fold-changes vs. nonirradiation. *P < 0.05 vs. respective nonirradiation by unpaired Student’s t-test.

Fig. 7. Expressions of heat shock protein (HSP)-27 and HSP-72 in the kidneys 6 days after IP or non-IP. C57BL/6 male mice were irradiated at 8 Gy on day 0. Kidneys were harvested 6 days after the irradiation and subjected to Western blot analysis (n = 3). A: Western blot analysis was carried out using the cytosolic fraction of the kidneys as described in MATERIALS AND METHODS. β-Actin was used as an equivalent protein loading marker. B: densities of blots were measured using Image Acquisition and Analysis LabWorks Software 4.5 (n = 3). Values are means ± SE expressed as fold-changes vs. nonirradiation. *P < 0.05 vs. respective nonirradiation by unpaired Student’s t-test.
and 82% of respective baselines). In a similar manner, the expression level of MnSOD, but not catalase and CuZnSOD, was dramatically increased 6 days after IP. The large increase in MnSOD activity and expression is postulated to be a cellular response to protect the kidney from ROS stress induced by irradiation. It has previously been reported that irradiation induced an increase in mRNA levels and activity of MnSOD in mammalian systems (1, 30).

Ionizing irradiation-induced DNA damages occur as a result of oxidative stress (27, 37). Ionizing radiation induces the alteration of mitochondrial permeability transition and an accumulation of ROS in treated cells (20). Numerous studies have demonstrated that, although ROS in high concentrations are toxic (41), low levels of ROS serve as intracellular signals stimulating defense mechanisms that prevent tissue injury (6, 40). In the present study, IP of 8 Gy slightly increased superoxide formation; however, it did not result in significant increases in lipid peroxidation and PCr and BUN concentration, suggesting that the mild increase in superoxide is not sufficient to exert toxicity but may have been sufficient enough to activate cellular defense systems. These reports suggest that cells exposed to mild ROS stress successfully activate antioxidant enzymes.

Pajovic et al. (31) reported that irradiation reduced the activity of CuZnSOD, whereas it quickly and dramatically increased the activity of MnSOD in lymphocytes. Han et al. (9) reported that sublethal whole-body irradiation marginally increased the expression of MnSOD mRNA and protein in mice spleens but significantly decreased catalase and CuZnSOD activity. In this study, the authors suggested that MnSOD

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**Fig. 8.** Inhibition of IP-induced increases in superoxide formation and MnSOD and HSP-27 expression by the administration of manganese (III) tetrais(1-methyl-4-pyridyl)porphyrin (MnTMPyP; a superoxide scavenger). C57BL/6 male mice were irradiated at 8 Gy in a single dose on day 0 administered with either MnTMPyP or 0.9% saline (vehicle) daily for 4 days from 1 h after the irradiation, and then they were euthanized on day 6. A and B: superoxide formation was evaluated by nitroblue tetrazolium staining, and the area of superoxide was measured using Image Acquisition and Analysis LabWorks 4.5 Software. C–F: expression of MnSOD (C and D) in the mitochondrial fraction and CuZnSOD (C and D), catalase (C and D), β-actin (C and E), HSP-27 (E and F), and HSP-72 (E and F) in the cytosolic fraction were determined by Western blot analysis. β-Actin was used as an equivalent protein loading marker. D and F: densities of blots were measured using Image Acquisition and Analysis LabWorks 4.5 Software (n = 3). Values are means ± SE expressed as fold-changes compared with vehicle. *P < 0.05 vs. respective nonirradiation by unpaired Student’s t-test.

**Fig. 9.** Inhibition of IP-induced protection by MnTMPyP. C57BL/6 male mice were irradiated at 8 Gy in a single dose on day 0, administered either MnTMPyP or vehicle daily for 4 days from 1 h after the irradiation, and then subjected to either 28 min of bilateral renal ischemia followed by 4 or 24 h of reperfusion or sham on day 6. PCr (A) and BUN (B) concentrations were determined 4 and 24 h after I/R. Values are means ± SE; n = 5–6. In unpaired Student’s t-test, *P < 0.05 vs. respective sham. In 2-way ANOVA, P values for postoperation, treatment, and the interaction of these 2 factors, respectively, were 0.004, <0.001, and 0.003 (A) and 0.015, <0.001, and 0.002 (B).
played an important role in radiation-induced cellular damage and that the increase in MnSOD activity may be used as a rapid predictive assay of radiosensitivity in the clinical setting (31).

To explore whether the protection afforded by irradiation was associated with increased superoxide formation, we administered MnTMPyP, a superoxide scavenger, to mice daily for 4 days beginning 1 h after irradiation. After a 3-day interval, the mice were subjected to 30 min of bilateral renal ischemia followed by reperfusion. Since MnSOD expression showed a greater increase than CuZnSOD and catalase expressions in IP mice, we used MnTMPyP to inhibit this increase induced by superoxide. The administration of MnTMPyP into the irradiated mice significantly reduced superoxide formation and expression of MnSOD in the kidneys 3 days after the last administration. Post-I/R increases in PCr and BUN concentrations in vehicle-administered IP mice were significantly greater in the MnTMPyP-administered IP mice, indicating that irradiation increased superoxide formation. The increased superoxide formation stimulated MnSOD activity and expression, and this rendered the kidney resistant to I/R injury. It is well demonstrated that the activation of antioxidant enzymes and scavenging of ROS reduces I/R injury in many organs, including the kidneys (13, 34). MnSOD is the major antioxidant enzyme located in the mitochondria and is essential for life. Genetic deletion of the MnSOD gene has been shown to be lethal (21). Asmakis et al. (2) reported that post-I/R functional recovery in the heart was more sensitive to a partial deficiency of MnSOD (SOD2+/−) than a partial deficiency of CuZnSOD (SOD1+/−). Mice expressing only 50% of the normal complement of MnSOD demonstrate increased susceptibility to oxidative stress and severe mitochondrial dysfunction. On the other hand, upregulation of MnSOD enzyme renders cells and tissues resistant to I/R injury (8). We also recently reported that MnSOD mimetic reduced kidney susceptibility to I/R injury (14). Jones et al. (13) reported that MnSOD overexpression in the heart protects it from I/R injury. Studies on the ischemic preconditioning phenomenon have reported that the scavenging of oxygen free radicals attenuates the protection afforded by ischemic preconditioning (8, 40). Yamashita et al. (48) reported that MnSOD was involved in the delayed phase of ischemic preconditioning in dog hearts.

Ionizing irradiation activates the induction of a variety of genes, including HSPs, which protect cells from oxidative stress (29, 36). Recently, we demonstrated that HSP-27 mediated the ischemic preconditioning phenomenon (33, 35), and therefore we investigated the implication of HSP-27 in the IP phenomenon. In the present study, we showed that IP significantly increased the expression of HSP-27, which stabilizes the actin cytoskeleton, suggesting that HSP-27 expression is involved in IP-induced protection. The increase of HSP-27 after IP was significantly inhibited by MnTMPyP treatment. This suggests that the expression of HSP-27 after IP is triggered by superoxide. It has been reported that ROS activates HSP expression (22, 23). Overexpressed HSP-27 increases glutathione production (3), which is the most abundant and important thiol-mass antioxidant. In radiotherapy, it has been reported that an increase in the expression of HSPs after irradiation renders the target cells resistant to irradiation therapies (3), reducing the therapeutic effects of irradiation. In conclusion, although the cytoresistance induced by irradiation may be undesirous for some treatments such as cancer therapy, IP may be a useful tool in development with the aim of preventing I/R injury induced by various conditions. Also, the IP phenomenon may be helpful for better understanding the intracellular defense mechanisms and for developing means of reducing I/R damage that occurs during surgical treatments such as kidney transplantation.

GRANTS
This work was supported by the Korea Science and Engineering Foundation (F01-2006-000-10034-0 to K. M. Park) and the stem cell research program of the Ministry of Science and Technology (M10641450010-06N4145-00110 to K. M. Park).

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