Hyperoxia-induced preconditioning against renal ischemic injury is mediated by reactive oxygen species but not related to heat shock proteins 70 and 32

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Objective. *Pre-exposure of rats to normobaric hyperoxia ($O_2 \geq 95\%$) may induce late preconditioning against renal ischemia-reperfusion (IR) injury. In this study we investigated probable mechanisms of IR injury such as the role of reactive oxygen species (ROS), renal antioxidant agents, and heat shock proteins (HSP) 32 and 70 during delayed hyperoxia-preconditioning (HO).*

Methods. *Fifty-two rats were divided into 7 groups: (A) IR, (B) HO + IR, (C) mercaptopropionyl glycine (MPG) + HO + IR, (D) MPG + IR, (E) HO + sham, (F) MPG + sham, and (G) sham. Rats in the following study groups (group B, C and E) were kept in a normobaric hyperoxic environment for 4 h/day for 6 consecutive days, after which they were subjected to 40 minutes of ischemia; animals in the control group (group A, D, F, and G) were kept in a normoxic cage. At the end of the preconditioning period, 24 hours of reperfusion was performed. Renal function was assessed by measuring serum creatinine (Cr), blood urea nitrogen (BUN), and creatinine clearance (CLCr). Induction of the antioxidant system was evaluated by measuring renal catalase (CAT) and superoxide dismutase (SOD) activities and glutathione (GSH) and malondialdehyde (MDA) content. The role of ROS was investigated by use of MPG (a ROS scavenger). HSP32 & 70 mRNA and protein also were determined.*

Results. *The hyperoxia-preconditioned IR group (B) had a lower plasma Cr and BUN and greater CLCr compared with the IR group (A) ($P \leq .016$). Administration of MPG led to an increase in plasma Cr and BUN and a decrease in CLCr in group C compared with the hyperoxia-preconditioned group B ($P \leq .004$). The hyperoxia-preconditioned IR group had a greater CAT activity and GSH level compared with the IR group A ($P \leq .007$), whereas the administration of MPG did not change the GSH level but led to a decrease in CAT activity in group D compared with group B ($P < .001$). SOD activity did not change in hyperoxia-preconditioned ischemic rats compared with ischemic rats. Hyperoxia preconditioning and MPG administration in ischemic animals did not result in any considerable change in MDA level compared with the IR group A. Also, there were no clinically relevant differences in HSP32 & 70 mRNA and protein between all groups.*

Conclusion. *The present study demonstrates that repeated pre-exposure to hyperoxia can decrease subsequent renal IR damage in this rat model of renal ischemia. Free radical production after hyperoxia appears to play a pivotal role in the hyperoxia-induced renal protection independent of HSP level. Antioxidant enzyme activities and especially catalase seem to be implicated in this renal protective mechanism. (Surgery 2015;■:■-■.)*

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Acute renal failure remains a serious medical condition, and its relatively high morbidity and mortality rates have not improved considerably.[1-3] Ischemia-reperfusion (IR) injury is one of the major causes of acute renal failure in the setting of various operative interventions, including urologic and cardiovascular operations and kidney transplantation. Among diverse procedures inducing tissue tolerance against IR damage, such as preconditioning induced by ischemia, heat stress, or hypoxia,[3-5] hyperoxic preconditioning is one of the most applicable methods in clinical practice.[6] Ischemic preconditioning consists of first an early phase, which provides protection for up to 3–4 hours after the preconditioning induction[7] and then a late phase, which plays its role 24–72 hours later.[8] The mechanisms of early and delayed preconditioning appear to be different.[9] Similar to ischemic preconditioning, hyperoxia also has an immediate and a delayed phase of protection with different mechanisms.[10]

Pretreatment with normobaric hyperoxia protects several tissues against subsequent IR injury, including the brain, spinal cord, heart, and kidneys.[3,4,9,11-13] In our previous dose-response study in rats, delayed renal protection was induced by exposing rats to a normobaric, hyperoxic environment (HO) (≥95% O_2), 4 hours per day for 6 consecutive days.[3] To the best of our knowledge, no study has explored the underlying mechanisms of delayed, hyperoxia-induced preconditioning in the kidney. Some studies have suggested that during hyperoxic preconditioning, large amounts of reactive oxygen species (ROS) are produced as a result of the high oxygen content in the inspired air,[3,14] which activate the cellular antioxidant system. Therefore, activation of the antioxidant enzymes can be one of the mechanisms which protects tissues from subsequent IR injury.[3,15] Heat shock proteins (HSPs), which primarily function as molecular chaperones, are expressed both constitutively and under stressful conditions.[16] HSPs, such as HSP32 and HSP70, play a prominent role in liver preconditioning by thermal and fasting stimulation.[17-20] Indeed, HSP32 expression may be critical for the recovery of cell function after oxidative damage in ischemic kidneys.[19]

The aims of our study were: (1) To elucidate the possible role of ROS in oxygen-induced renal protection by using mercaptopropionyl glycine (MPG) as a ROS scavenger; (2) To determine the role of some renal antioxidant enzymes, including catalase (CAT), superoxide dismutase (SOD), levels of glutathione (GSH), and malondialdehyde (MDA) in inducing protection; and (3) to determine the possible role of HSP32 and HSP70 mRNA and protein expressions in delayed renal preconditioning.

METHODS

Animals and experimental procedures. Fifty-two adult male Wistar rats (250 ± 50 g) were kept under a 12-hour:12-hour, light:dark cycle at room temperature (22 ± 2°C). Free access to food and water before and after operative intervention was allowed, and animal care was carried out in accordance with the National Institutes of Health guidelines for laboratory animal care. All experiments were performed under the permission of local authorities. Animals were assigned randomly to 7 groups as follows (Fig 1): (A) Normal saline + normoxia + ischemia (NS + NO + IR) (n = 8); (B) normal saline + hyperoxia + ischemia (NS + HO + IR) (n = 7); (C) (MPG + HO + IR) (n = 8); (D) (MPG + NO + IR) (n = 8); (E) (NS + HO + Sham) (n = 7); (F) (MPG + NO + sham) (n = 7); (G) (NS + NO + sham) (n = 7).

In the hyperoxic groups rats were exposed for 4 h/day to nearly pure oxygen (O_2 ≥95%) for 6 consecutive days until 24 h before intervention. Oxygen exposure was achieved by placing the rats in a chamber with continuous delivery of O_2 through a tube. Through a previously published pilot study,[3] the authors obtained the most effective protocol for inducing delayed renal protection against IR injury. To evaluate arterial O_2 concentration after placement in the hyperoxic chamber, a gas analyzer (Ciba-Corning 865; Ciba-Corning Diagnostic Corporation, Medfield, MA) was used in 2 separate groups of animals (n = 3 in each) immediately after 120 min of exposure to normoxia or hyperoxia. Rats in normoxia groups (A, D, F, and G groups) were kept in the same container with continuous delivery of normal air before intervention. MPG (150 mg/kg), a free radical scavenger, was injected every day before oxygen exposure in groups C, D, and F. Rats were anaesthetized with an intraperitoneal injection of sodium pentobarbital (Sigma Chemical Co., St. Louis, MO; 50 mg/kg) 24 hours after the last session of hyperoxia or normoxia. All anesthetized rats received 300 U of heparin (intraperitoneally) before beginning the operation, and their body temperature was maintained at approximately 37°C by placing them on a heating pad. Right nephrectomy was performed through a midline laparotomy incision. With an atraumatic vascular clamp, the left renal artery was clamped for 40 minutes. After we removed the clamp, the kidney was observed for an additional 20 minutes to

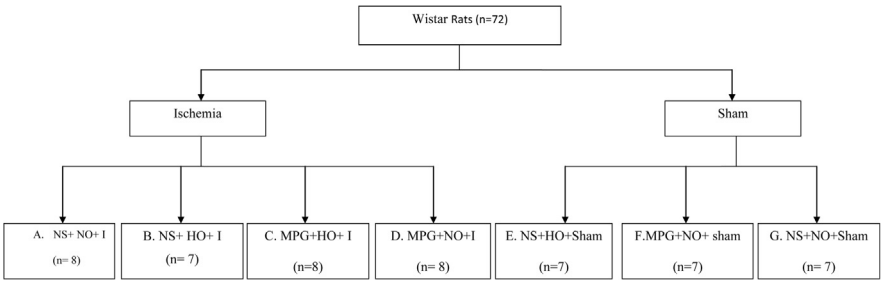

Fig 1. Diagram of experimental design.

ensure restoration of blood flow. Induction of this renal IR was ascertained not only by viewing color changes of the kidney but also by measuring renal blood flow using a Laser Doppler flow meter (Moor Instruments, Devon, UK). Sham-operated rats underwent the same operative procedures except for clamping of the left renal artery. After the operation, each animal was placed in a metabolic cage for a 24-hour urine collection. At 24 hours postoperatively, the rats were reanesthetized, and a blood sample was obtained by cardiac puncture after left nephrectomy. Plasma and urine samples were kept at 24°C for biochemical analysis. A piece of the left kidney was stored in 10% formaldehyde for histologic evaluation, and 3 pieces were washed immediately with normal saline (0.9% NaCl) and then frozen in liquid nitrogen for antioxidant content, PCR, and Western blot assay.

Laser Doppler flowmetry. Renal cortex microcirculatory blood flow was measured by a Laser Doppler flowmeter at three distinct defined locations (2 points in the upper and lower halves of anterior surface and 1 point on posterior surface of the kidney). The flow was measured in all groups in four different times: pre-ischemia (immediately before clamping), during ischemia and 20 minutes and 24 hours after reperfusion. The average of flow rates in these 3 locations was reported as the renal blood flow in each time in any individual rat.

Assessment of renal function. To measure plasma BUN and Cr levels, plasma or urine samples were processed in an automatic analyzer (Abbott Alcyon 300; Abbott Laboratories, Ltd, Maidenhead, UK). Plasma and urine sodium concentrations were determined using a flame photometer (fp20; Seac, Calenzano, Italy). CLCr (mL/d/kg) was calculated from its standard formula, and fractional excretion of sodium (FENa%) was obtained from the formula: $FENa\% = [U_{Na}*UF/P_{Na}*CLCr] \times 100$, in which U_{Na} is urine Na concentration, UF (urine flow) is 24-hour urine volume (mL/d/kg), and P_{Na} is plasma Na concentration.

Biochemical analysis. Frozen tissue samples were weighed and homogenized 1:10 in ice-cold 50 mM potassium phosphate buffer (pH 7.4) containing proteases inhibitors and 1 mM ethylenediaminetetraacetic acid. The homogenates were then centrifuged at 12,000*g* for 15 minutes at 4°C. The supernatants were taken and studied for enzymes activities, GSH, and MDA levels and protein assays.

MDA production was quantified according to the Ohkawa protocol.[3,20] The level of GSH was determined by the Tietz method.[21] SOD activity was determined according to the method of Sun et al. CAT activity in kidney tissue homogenates was measured by a colorimetric method as previously described by Cohen.[22] Protein concentration of the tissue homogenates was determined by the method of Bradford,[23] with the use of bovine serum albumin as the standard.

Histopathologic examination of renal tissues. Sections were prepared from fixed kidneys via routine histologic methods. Thin sections (4–5 μm) were prepared from paraffin-embedded kidney and stained with hematoxylin and eosin. Morphologic assessment of renal damage was performed by grading the extent of necrosis of the proximal tubules as described by Jablonski et al.[24]

Western blot analysis. Frozen kidney tissue was homogenized at 4°C for 15 seconds in a lysis buffer and centrifuged at 10,000*g* for 15 minutes. Protein concentration was measured by the Bradford method. Approximately 50–80 μg of total protein was mixed with loading buffer, boiled for 5 minutes, and loaded onto sodium dodecyl sulfate-polyacrylamide gel using Mini Protein 3 Cell. Proteins were transferred to polyvinylidenedifluoride membranes in the presence of glycine/methanol transfer buffer in Trans-Blot Semi-Dry Electrophoretic Transfer system. Membranes were

blocked with Western blocking solution (Sigma-Aldrich) for 1 hour at room temperature. Membranes were exposed subsequently to rabbit polyclonal HSP70 antibody (1:500), rabbit polyclonal HSP32 antibody (1:200), and rabbit polyclonal b-actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After washing with Tris-buffered saline Tween-20, membranes were incubated with horseradish peroxidase conjugated goat anti-mouse immunoglobulin G (Santa Cruz Biotechnology) for HSP70 and horseradish peroxidase–conjugated donkey anti-goat immunoglobulin G (Santa Cruz Biotechnology) for HSP32. The 3,3′,5,5′ tetramethylbenzidine reagent was used to visualize peroxidase reaction products. The membranes were scanned, and the intensities of bands were determined by the National Institutes of Health Image J program.

mRNA isolation and semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR). Total RNA was isolated from frozen renal tissue using a tissue grinder. Then, 1 mL of RNX was used to dissolve the grinded tissue and the solution was shaken well with 200 μL of chloroform for 15 seconds. Then the mix was incubated on ice for 5 minutes, and the sample was centrifuged at 12,000 rpm for 15 minutes at 4°C. The top aqueous layer (RNA) was transferred carefully into the new tube to which 1:1 volume of isopropanol was added and incubated on ice for 15 minutes and then centrifuged at 12,000 rpm for 15 minutes at 4°C. The supernatant was discarded, and 1 mL of 75% ethanol was added to the precipitate and centrifuged at 7,500 rpm for 8 minutes at 4°C.The supernatant was removed, and the precipitate was dissolved in 50–100 μL of diethylpyrocarbonate-treated water. The concentration of RNA was determined by using a spectrophotometer. In summary, cDNA synthesis from total RNA included an initial step in which 10 μL of RNA was incubated with 1.5 μL of oligodT and 6.5 μL of DEPC-treated water for 5 minutes at 70°C. Subsequently, 6 μL of buffer, 3 μL of dNTP, 1.5 μL of M-MuLV reverse transcriptase, and 1.5 μL of Ribolock (RNAase Inhibitor) were added and incubated for 60 minutes at 42°C, after which the reaction was inactivated by a 15-minute incubation at 70°C. A total of 2.5 μL of cDNA was amplified by PCR in a buffer consisting of 0.5 μL of dNTP, 1.25 μL of $MgCl_2$, 2.5 μL of 10x PCR buffer, 0.5 μL of Tag-polymerase enzyme with 15.25 μL of DEPC-treated water, 1.25 μL of sense primer, and 1.25 μL of antisense primer. Primers for HSP70, HSP32, and β-actin (MWG Biotech, Ebersberg, Germany) were as follows:

HSP70 (347 bp): 5′-GAGTCCTACGCCTTCAA TATGAAG-3′, 5′-ATCAAGAGTCTGTCTCTAGCCA A-3′; HSP32 (309 bp): 5′-CTTTCAGAAGGGT CAGGTGTTCCA-3′, 5′-CTGAGAGTCACCCAGGT AGCGG-3′; β-Actin (582 bp): 5′-TCATGCCATC CTGCGTCTGGACCT-3′, 5′-CCGGACTCATCGTA CTCCTGCTTG-3′.

PCR was performed on a Thermocycler. RT-PCRs were run in the following procedures:

HSP70: at 95°C for 4 minutes, 1 cycle; at 95°C for 30 seconds, at 55°C for 45 seconds, at 72°C for 75 seconds, 25 cycles; at 72°C for 10 minutes, at 30°C for 3 minutes, 1 cycle. HSP32: at 95°C for 3 minutes, 1 cycle; at 94°C for 30 seconds, at 60°C for 30 seconds, at 72°C for 30 seconds, 25 cycles; at 72°C for 6 minutes, at 30°C for 3 minutes, 1 cycle. β-Actin: at 95°C for 3 minutes, 1 cycle; at 94°C for 30 seconds, at 57°C for 42 seconds, at 72°C for 30 seconds, 35 cycles; at 72°C for 6 minutes, and at 30°C for 3 minutes, 1 cycle.

A 10-μL PCR product was placed onto agarose gel 1%, stained by ethidium bromide, and viewed under ultraviolet light. The images were then analyzed using the National Institutes of Health Image J program.

Statistical analysis. Data are shown as median (range). The Kruskal-Wallis test with post-hoc analysis was used for comparing groups of nonparametric data. The nonparametric Mann-Whitney *U* test was also used.

RESULTS

Cortical microcirculatory blood flow. Flow did not differ significantly at the different time points in sham groups (E, F, and G). Renal cortex flow during ischemia was less than pre-ischemia and 20 minutes after reperfusion in all IR groups (A, B, C, and D), indicating efficient induction of ischemia and successful reperfusion of the kidney ($P < .006$). Flows at 20 minutes and 24 hours after reperfusion were less than the pre-ischemic flow in group A ($P = .002$ and $P = .006$, respectively). No considerable change in groups B and D were observed between pre-ischemia, 20 minutes' reperfusion, and 24-hour reperfusion flows. Also, both 20 minutes' reperfusion and 24-hour reperfusion flows were considerably less than the pre-ischemia one in group C ($P < .01$) (Table I).

Blood PO_2 measurement. Arterial PO_2 in the normoxic group was 93 ± 2 mmHg, and increased to 433 ± 27 mmHg in the hyperoxic group immediately after 120 min exposure ($P = .05$).

Renal function tests. Renal function parameters among different groups are shown in Table II.

Table I. Renal cortex microcirculatory blood flow

Groups	*Preischemic flow (arbitrary number)*	*During-ischemic flow (arbitrary number)*	*20 min-reperfusion flow (arbitrary number)*	*24 h-reperfusion flow (arbitrary number)*
A: NS + NO + I	346 (226–504)	49.3 (31.4–76)*	278 (222–378.5)*	269 (198–437.5)*
B: NS + HO + I	323 (248–380)	51 (34–99)*	301 (200–354)	302 (297–398)
C: MPG + HO + I	352 (263–382)	60.5 (34–73)*	290 (260–346)*	314 (227–347)*
D: MPG + NO + I	345 (317–406)	58 (36–77)	307 (199–352)	327 (109–356)
E: NS + HO + SHAM	334 (277–460)	330 (285–410)†	333 (276–358)	289 (272–415)
F: MPG + NO + SHAM	321 (255–393)	310 (260–387)†	319 (254–389)	341 (312–390)
G: NS + NO + SHAM	330 (289–380)	349 (291–399)†	329 (292–364)	321 (309–347)

*Shows significant difference with preischemic flow.
†Sham groups in which flow measured as the same time as ischemia in other groups.
The flow was measured in 4 different times: preischemia, during ischemia, 20 min after reperfusion, and 24 h after reperfusion. Data are presented as Median (min-max).
HO, Hyperoxy; *I*, ischemia; *MPG*, mercaptopropionyl glycine; *NS*, normoxy.

Table II. Renal function tests in all groups

Groups	*PCr, mg/dL*	*BUN, mg/dL*	*CLCr, mK/day/kg*	*FENa%*
A: NS + NO + I	4.5 (3.1–5.5)*	137 (101–148)*	251 (27.6–653.2)*	3.43 (3.29–3.58)*
B: NS + HO + I	1.05 (0.9–2.4)†	27 (21–70)†	923 (678.2–1,525)†	0.85 (0.76–1.03)†
C: MPG + HO + I	5.3 (4.8–5.6)‡	137 (101–159)‡	10.7 (15.3–166.9)‡	1.06 (0.14–1.99)
D: MPG + NO + I	3.45 (2.6–3.9)	113 (86–140)	279 (108.4–587.4)	1.47 (0.85–2.80)
E: NS + HO + SHAM	0.95 (0.7–1.6)	31 (19–35)	2,123 (1,407–3,500)	0.45 (0.2–0.85)
F: MPG + NO + SHAM	0.8 (0.8–1)	22 (19–35)	3,131 (1,489.5–3,968.8)	0.45 (0.32–0.56)
G: NS + NO + SHAM	0.9 (0.7–0.9)	22 (21–28)	3,123 (2,573–3,322)	0.48 (0.32–0.76)

*Shows significant difference with normoxy sham group.
†Shows significant difference with normoxy ischemic group.
‡Shows significant difference with hyperoxia preconditioned ischemic group.
Data are presented as median (min-max).
BUN, Blood urea nitrogen; *CLCr*, creatinine clearance; *FENa*, fractional excretion of sodium; *MPG*, mercaptopropionyl glycine; *PCr*, plasma creatinine.

Group A had greater plasma levels of Cr and BUN, a greater FENa% and lesser CLCr compared with group G ($P \leq .01$). Hyperoxia-preconditioned group B had a lesser plasma Cr and BUN, a lesser FENa%, and a greater CLCr compared with group A ($P \leq .016$). The administration of MPG led to an increase in plasma Cr and BUN and a lesser CLCr in group C compared with group B (NS + NO + I; $P \leq .004$) with no change in FENa%. Moreover, plasma Cr and BUN, FENa% and CLCr were not different between groups E and F compared with group G (Table II).

Biochemical analysis. *Catalase activity.* Ischemia resulted in less CAT activity in group A compared with group G ($P < .001$). The hyperoxia-preconditioned IR group (B) showed a greater CAT activity compared with group A ($P < .007$). The administration of MPG decreased CAT activity in group C compared with group B ($P < .001$). Moreover, there was no significant difference between the groups (F and G) who received MPG (Table III).

SOD activity. Both hyperoxia and ischemia (groups A and E) increased SOD activity in comparison with group G ($P \leq .006$). SOD activity was not different in group B compared with group A. After MPG administration, SOD activity did not change significantly in group C compared with group B, nor was there any difference between groups F and G (Table III).

MDA level. The MDA level was greater in group A compared with group G ($P < .001$). Hyperoxia preconditioning and administration of MPG in ischemic animals (groups B and C) did not result in a considerable change in comparison with group A (Table III).

GSH level. GSH level was less in group A compared with group G ($P < .001$). Hyperoxia preconditioning in ischemic animals (group B) resulted in a greater level of GSH ($P < .002$). Administration of MPG (group C) did not alter in GSH levels compared with group B (Table III).

Western blot analysis. The level of HSP70 protein was greater in group A compared with group

Table III. Biochemical analysis

Groups	CAT, units/mg protein	SOD, units/mg protein	MDA, nmol/mg protein	GSH, nmol/mg protein
A: NS + NO + I	55.2 (50.97–57.54)*	58.7 (54.55–67.62)*	19.4 (15.6–24.9)*	29.4 (24.54–36)*
B: NS + HO + I	59.1 (54.89–63.80)†	57.5 (50.75–62.7)*	18.0 (14.62–19.20)*	36.8 (31.09–39.27)†
C: MPG + HO + I	51.0 (48.9–54.06)‡	53.1 (48.50–56.9)*	15.9 (14.28–17.7)	34.4 (27.82–39.27)
D: MPG + NO + I	55.7 (50.97–68.61)	56.9 (52–59.3)*	18.7 (17.51–20.31)	32.1 (29.3–36)
E: NS + HO + SHAM	72.2 (68.61–79)	59.3 (46.28–66.82)*	15.5 (9.55–18.29)	35.9 (29.45–44.18)
F: MPG + NO + SHAM	63.7 (60.30–69.10)	51.3 (44.20–53.9)	13.7 (9.7–17.27)	39 (34.36–42.54)
G: NS + NO + SHAM	69.9 (60.88–77.56)	49.2 (45.72–55.77)	13.6 (9.5–18.6)	37.6 (31.09–40.91)

*Shows significant difference with normoxy sham group.
†Shows significant difference with normoxy ischemic group.
‡Shows significant difference with hyperoxia-preconditioned ischemic group.
Data are presented as median (min-max).
CAT, Catalase; *GSH*, glutathione; *HO*, hyperoxy; *I*, ischemia; *MDA*, malondialdehyde; *MPG*, mercaptopropionyl glycine; *NS*, normoxy; *SOD*, superoxide dismutase.

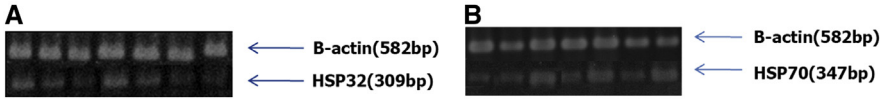

Fig 2. Western blot analysis. (*A*) HSP32 mRNA bands in different groups. (*B*) HSP70 mRNA bands in different groups.

G ($P < .016$); however, it did not differ significantly in group A and B. In addition, values were considerably greater in group E compared with group G ($P < .038$). Administration of MPG did not increase HSP70 expression in group C in comparison with groups B and A, nor in group F compared with group G. We could not see a band in the Western blots of HSP32, possibly due to very low level of protein.

HSP70 mRNA expression. IR resulted in a greater HSP70 mRNA expression in group A vs group G ($P < .016$), and hyperoxia preconditioning did not alter this result in Group B compared with group A. Administration of MPG had no significant effect on HSP70 mRNA expression in group C compared with group B. Similarly HSP70 mRNA expression was not different in group E compared with group G (Fig 2, *B*; and Fig 3).

HSP32 mRNA expression. IR, hyperoxic preconditioning, nor administration of MPG altered HSP32 mRNA expression in groups A, E, and F compared with group G. MRNA expression was similar in the 3 sham groups (E, F, and G) (Fig 2, *A*; and Fig 4).

DISCUSSION

In the present study, repeated exposure of rats to a normobaric, hyperoxic environment (O_2 ≥95%) for 4 h/day for 6 consecutive days ending 24 hours before renal IR resulted in protection against renal IR injury for a delayed phase of effect. Our study showed that ROS production during the hyperoxic reperfusion period is one of the initiators of the delayed phase of preconditioning. Repeated HO preconditioning induced an increase in the activities of the major antioxidant enzymes CAT and SOD and in GSH levels without affecting the level of MDA. According to our results, HSP32 and 70 had no significant role in this delayed phase of preconditioned tolerance to renal ischemia induced by repeated pre-exposure to hyperoxia.

Renal IR injury is unavoidable in renal transplantation and during some cardiovascular operations. Preconditioning is one of the most powerful protective interventions against IR injury in experimental studies.[25] Short-term exposure to hyperoxia has no appreciable side effects and seems to be useful as a practical method for induction of preconditioning (eg, by maximizing arterial oxygen saturation).[6] Hyperoxia preconditioning like other kinds of preconditioning can induce an early and late phase of ischemic tolerance.[3,6,26] To the best of our knowledge, few studies have investigated the underlying mechanisms mediating the delayed phase of hyperoxia preconditioning. Our study revealed that the tolerance against renal ischemia after HO pretreatment was attenuated by the administration of the ROS inhibitor, MPG

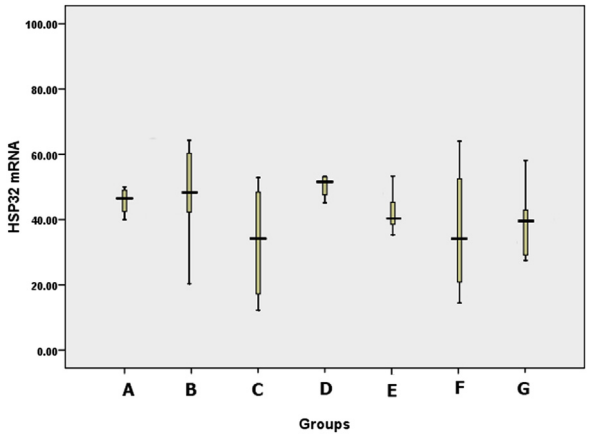

Fig 3. Semiquantitative RT-PCR of HSP70 mRNA in different groups. Values are median with interquartile range and an overall range. *HSP*, Heat shock protein; *RT-PCR*, reverse-transcription polymerase chain reaction.

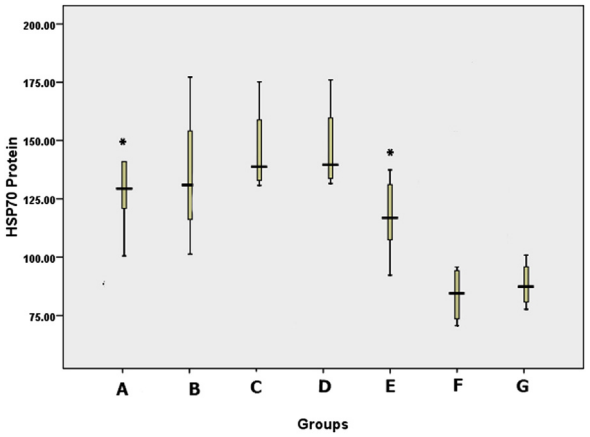

Fig 4. Semiquantitative RT-PCR of HSP32 mRNA in different groups. Values are median with interquartile range and an overall range. *Shows a significant difference with group G. *HSP*, Heat shock protein; *RT-PCR*, reverse-transcription polymerase chain reaction.

(a potent free radical scavenger). After MPG administration, not only was the protection in renal function provided by HO abolished, but the improvement in histopathologic score in the HO group was attenuated. These results suggest that the cellular ROS formation whereby hyperoxic preconditioning activates protective downstream cascades was blocked. Wada et al[27] demonstrated that pretreatment with hyperbaric oxygen once every other day for 3 or 5 sessions induced ischemic tolerance in gerbil hippocampus through the generation of O_2 radicals. Similarly, in 2004 Zhang et al[28] showed that the administration of a free radical O_2 scavenger, DMTU, given before inhaling 100% oxygen abolished the ischemia tolerance induced by pure O_2 preconditioning in the brain of rat models of ischemia.

ROS are involved in the regulation of a number of cellular processes.[29] Transcription factors, such as nuclear factor κB (NF κB) and activator protein 1, are regulated in part by oxidative stress.[30] These transcription factors initiate multiple programs of gene expression that generate mediators of protection, including antioxidants, HSPs, cytokines, and nitric oxide synthase.[14] Therefore, induction of an oxygen free radical-scavenging system appears to be a mechanism of hyperoxia-induced renal protection.

Nie et al[31] reported that hyperbaric oxygen preconditioning induced the up-regulation of antioxidant enzyme activities such as CAT and SOD, which play an important role in the formation of tolerance against rabbit spinal cord ischemia. Kim et al[32] abolished the infarct-limiting effect of hyperbaric oxygenation pretreatment completely in rat myocardium by using a catalase inhibitor suggesting that hyperbaric oxygenation–induced tolerance against IR injury was caused by catalase induction.

In our study, after 4 h/day of hyperoxia exposure for 6 days, catalase activity increased significantly but was abolished by the administration of the ROS scavenger, MPG. SOD and GSH levels showed similar changes, but the levels of MDA did not show any substantial changes between all groups. Overall, these findings suggest that 6 days of 4 h/day hyperoxic exposure before ischemia induces the endogenous antioxidant system, which explains in part the tolerance induction by hyperoxia.

HSPs are important cellular mediators induced under different stressful conditions. Oxidative stress, hypoxia, and heat shock up-regulate HSPs as cytoprotective molecular chaperones.[33] Wada et al[27] suggested that HSP72 plays a role in hyperbaric oxygen–induced neuroprotection. Yang et al[34] demonstrated that preconditioning with erythropoietin was associated with the induction of HSP70 in rat kidney. In contrast, Yu et al[15] showed that hepatic HSP70 expression did not change significantly after preconditioning of rat liver by one exposure to hyperbaric oxygen. The study by Hirata et al,[35] in which they examined the temporal profile of genomic responses in ischemic tolerance of rat brain induced by repeated exposure to hyperbaric oxygen, did not show any significant up-regulation of HSP. Lee et al[33] demonstrated that HSP32 is activated as a cytoprotective mechanism in human proximal

tubular cells through oxidant tolerance induction. Our study demonstrated that HSP32 and 70 mRNAs and proteins changes were not different between all groups. It can be postulated that HSP32 and 70 have no role in ischemic tolerance induction by HO in rat kidney.

In summary, the present study demonstrated that repeated exposure to hyperoxia of 4 h/day for 6 consecutive days ending 24 hours before renal IR can decrease subsequent renal IR injury. This beneficial effect of hyperoxia pretreatment was abolished by the free radical scavenger, MPG. These results suggest that hyperoxia induces some antioxidant enzymes such as catalase that play a role in tolerance to ischemia. The increased expression of HSP32 and HSP70 after IR was not required for induction of ischemic tolerance by hyperoxia in rat kidney.

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