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Research Paper

Protective Effects of Diallyl Sulfide, a Garlic Constituent, on the Warm Hepatic Ischemia–Reperfusion Injury in a Rat Model

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Purpose. The aim of this study is to evaluate the effects of diallyl sulfide (DAS) on the warm hepatic ischemia–reperfusion (IR) injury in a rat model.
Methods. Rats (n=8–10/group) were subjected to sham operation or warm ischemia (1 h)-reperfusion (3 h) preceded by a single intraperitoneal dose (1.75 mmol/kg) of DAS or vehicle, and relevant biochemical parameters were monitored.
Results. Warm IR injury caused a significant increase in the plasma markers of liver injury, which was attenuated by DAS. The hepatoprotective effects of DAS were associated with significant reductions in lipid peroxidation markers and *in situ* generation of superoxide in the liver and increases in the glutathione levels of the liver and bile, suggestive of an antioxidant effect for DAS. Additionally, DAS caused an almost twofold increase in the protein expression of the liver heme oxygenase-1, an enzyme that confers cytoprotection against oxidative stress. Whereas the total cytochrome P450 remained unchanged, the protein levels and activity of CYP2E1, which plays an important role in the generation of reactive oxygen species, significantly decreased by DAS pretreatment.
Conclusions. DAS protects the liver from warm IR injury by reducing oxidative stress through, at least in part, induction of heme oxygenase-1 and inhibition of CYP2E1.

KEY WORDS: CYP2E1; diallyl sulfide; heme oxygenase-1; hepatic ischemia–reperfusion injury; reactive oxygen species.

INTRODUCTION

Warm hepatic ischemia–reperfusion (IR) injury is a serious complication of many clinical situations such as major liver resection surgery (Pringle maneuver), hemorrhagic and septic shock, and liver transplantation (1–5). Although the complex pathophysiology of the disease is not completely understood, it is believed that several mediators, such as reactive oxygen species (ROS), proinflammatory cytokines, chemokines, adhesion molecules, and excess nitric oxide (NO) contribute to this injury (1,3,6–8). Therefore, pharmacologic agents that inhibit or attenuate the release of these mediators may reduce the IR injury (9).

It has been reported that garlic, its extracts, or its various components possess antioxidant properties (10–12). For example, it is shown that garlic extract dose-dependently scavenges hydroxyl radical *in vitro* and prevents hydroxyl radical-mediated lipid peroxidation in the rabbit liver homogenate (12). Additional *in vitro* studies have shown that organosulfur components of garlic oil significantly inhibit the release of NO by the lipopolysaccharide-stimulated macrophages (13). The decrease in the secretion of NO was due to inhibition of iNOS expression (13). Further work (14) showed that the organosulfur components of garlic oil also reduce the production of a number of cytokines by the activated macrophages. Therefore, garlic and its components may have a protective effect in warm hepatic IR injury by reducing the mediators that contribute to this injury.

Garlic oil contains more than 20 organosulfur compounds, which are believed to play a major role in the reported biological activities of garlic (15). One of the main components of these organosulfur compounds is diallyl sulfide (DAS), which has shown effectiveness in prevention of cancer (16,17) and chemically-induced hepatotoxicity (18,19). *In vitro* and/or *in vivo* studies have shown that DAS scavenges free radicals (20), induces the cytoprotective protein heme oxygenase-1 (HO-1) (21), increases the glutathione levels of hepatocytes (22), decreases NO and cytokine release (13,14), and reduces the activity and expression of CYP2E1 (18,23). Because all of these reported effects potentially result in a reduction in the oxidative stress and cell injury, we hypothesized here that DAS reduces the injury caused by the warm hepatic IR of the liver. Therefore, the effects of pretreatment with a single dose of DAS on the hepatic IR injury were studied in a well-established *in vivo* model of partial IR in rats.

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MATERIALS AND METHODS

Chemicals

The following kits and reagents were used in this study: aspartate aminotransferase (AST), alanine aminotransferase (ALT), and lactate dehydrogenase (LDH) assay kits from Teco Diagnostics (Anaheim, CA); lipid hydroperoxides (LPO) assay kit from Cayman chemical company (Ann Arbor, MI); 15-isoprostane F_{2t} (IsoP) assay kit (EA 84) from Oxford Biomedical Research (Oxford, MI); reduced glutathione (GSH), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), GSH reductase, purpald (4-amino-3-hydrazino-5-mercapto-1,2,4-triazole), *p*-nitrophenol, 4-nitrocathecol, isocitrate, isocitrate dehydrogenase, dihydroethidium (DHE), and sodium hydrosulfite from Sigma-Aldrich Co. (St. Louis, MO); NADPH from Calzyme Laboratories Inc. (San Luis Obispo, CA, USA); sulfosalicylic acid and 2,4-dinitrophenylhydrazine from Alfa Aesar (Ward Hill, MA); oxidized glutathione (GSSG), DAS, and potassium periodate from Acros Organics (Morris Plains, NJ, USA); 1-methyl-4-vinyl-pyridinium trifluoromethane sulfonate (M4VP) from OxisResearch (Portland, OR, USA); 2,3-*bis*(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide inner salt (XTT sodium) from Biotium (Hayward, CA, USA); hypoxanthine from Research Organics Inc. (Cleveland, OH, USA); Oasis HLB cartridges from Waters Corporation (Milford, MA, USA); and xanthine oxidase from MP Biomedicals, Inc. (Solon, OH,USA). All other reagents were analytical grade and obtained from commercial sources.

Animals

All the procedures involving animals in this study were consistent with the "Principles of Laboratory Animal Care" (NIH publication no. 85–23, revised 1985) and approved by our Intuitional Animal Care and Use Committee. Adult male Sprague–Dawley rats (225–275 g) were obtained from Charles River laboratories (Indianapolis, IN, USA) and housed under a 12-h day/night cycle in institutional animal facility with free access to food and water.

Experimental Design and Surgery

Three different groups of animals (n=8–10/group) were subjected to sham operation (SO) or ischemia–reperfusion preceded by vehicle (IR-Control) or DAS (IR-DAS) pretreatment. The IR-DAS animals were administered a single dose (1.75 mmol/kg) of DAS (1 ml/kg in corn oil) intraperitoneally 12–15 h before the induction of ischemia. This dose of DAS has been used previously in studies of the *in vivo* effects of DAS on chemically-induced hepatotoxicity in rats (18). The SO and IR-Control groups were injected with an equal volume of the vehicle only. After drug or vehicle injections, the animals were fasted, while having free access to water. The studies were planned in such a way that all the surgical procedures were done between 7 and 10 A.M. To induce ischemia, animals were anaesthetized with an intramuscular injection of ketamine/xylazine (80:8 mg/kg). The visceral organs were then exposed by a midline abdominal incision and kept moist by covering with sterile, wet cotton gauze pads. Ischemia was induced to the left and median lobes of the liver by occluding the left branches of the portal vein, hepatic artery, and bile duct for 60 min using a microvascular clamp. The blood flow to the right lobe and the two small caudate lobes was kept intact during the ischemic time. This model causes ~70% ischemia without intestinal congestion. During ischemia, the body temperature of the animals was closely monitored and maintained at 37°C by a heating lamp. After one hour of *in vivo* partial ischemia, the clamp was removed to allow blood to the ischemic lobes, and the blood flow to the non-ischemic lobes was stopped by ligating vessels that supply blood to the right and caudate lobes. The ligation of non-ischemic lobes during reperfusion prevents "stealing" of blood flow by non-ischemic lobes from the ischemic lobes during the reperfusion period and has been used by a number of investigators (24,25). Five milliliters of sterile saline (37°C) was added to the peritoneal cavity to compensate for volume loss before closure of the abdomen.

In contrast to the IR-Control and IR-DAS groups, the blood flow to the entire liver was left uninterrupted in the SO group during the 1-h ischemic period. However, similar to the other two experimental groups, the blood supply to the right and caudate lobes of the SO animals was occluded during the reperfusion period. The reperfusion was allowed for 3 h in all the groups.

Sample Collection

During the last 15 min of reperfusion, bile was collected via a catheter in a preweighed microcentrifuge tube containing sulfosalicylic acid to prevent oxidation of GSH (26). At the end of reperfusion period, blood was collected from abdominal aorta into heparinized syringes and, after centrifugation, plasma was separated. A portion of the plasma samples was stored at 4°C for the analysis of ALT, AST, and LDH within a week, and the remainder of plasma was stored at −80°C. Median lobes of livers were used immediately for preparation of microsomes. Additionally, a portion of the left lobe was immersed in 10% (*v*/*v*) neutral-buffered formalin for subsequent histological analysis and the remaining parts of the left lobe was frozen by immersion into liquid nitrogen and stored at −80°C for biochemical analysis.

Determination of Plasma Markers of Liver Injury, TNF-α, and Nitric Oxide Metabolites

The plasma samples were diluted with distilled water and used to quantitate AST, ALT, and LDH levels using commercial kits obtained from Teco diagnostics (Anaheim, CA, USA).

The plasma concentrations of TNF-α were measured using an ultra sensitive ELISA kit (Biosource, Camarillo, CA, USA), according to the manufacturer's instructions. The assay has a sensitivity of <0.7 pg/ml and a range of 2.3–150 pg/ml and is suitable for measurement of TNF-α in the rat serum or plasma.

The plasma concentrations of the stable metabolites of NO (nitrate plus nitrite, NO_x) were quantitated using a commercial kit (Active Motif, Carlsbad, CA, USA) after protein removal. Plasma proteins were removed by ultrafiltration of the samples (150 µl) at 1,500 g for 10 min in an ultrafiltration device with a 30 kDa MW cut-off membrane (Centrifree; Millipore, Bedford,

MA, USA). Subsequently, 40 µl of the filtrate was used in the assay, which is based on nitrate reductase-mediated conversion of nitrate to nitrite before quantitation of nitrite using the Griess reagent. Standard curves were constructed by conversion of $NaNO_3$ to nitrite.

Determination of Reduced (GSH) and Oxidized (GSSG) Glutathione in the Liver Tissue and Bile

The GSH and GSSG contents of liver tissue and bile samples were estimated using a method recently developed in our laboratory (26). Briefly, the liver samples were selected from four different parts of the ischemic lobes and homogenized in cold 5% (*w*/*v*) sulfosalicylic acid (1:5) using a handheld homogenizer, and the homogenate was centrifuged at 14,000 rpm for 15 min at 4°C. Bile samples were also diluted (1:5) with sulfosalicylic acid before processing. The liver supernatant or diluted bile was used to estimate total glutathione based on an enzymatic recycling method using DTNB. Next, the GSSG contents were determined after masking free GSH in the sample using the reagent M4VP before the recycling reaction. Finally, the concentration of the reduced GSH was estimated by subtracting GSSG from the total glutathione content of the sample.

Determination of Superoxide Dismutase and Catalase in the Liver Tissue

Livers were homogenized (1:9) in 0.1% Triton-X in distilled water, and the homogenate was centrifuged at 4,000 rpm for 30 min. The supernatant was separated and used in the following assays. Superoxide dismutase (SOD) activity of the liver homogenates was estimated as the ability to inhibit the reduction of the tetrazolium dye XTT (27). The activity is expressed in relative units per milligram protein. One unit of SOD is defined as the amount of SOD required to inhibit XTT reduction by 50%. Catalase activity was determined as the ability to produce formaldehyde from methanol in the presence of optimal concentrations of hydrogen peroxide (28). The generated formaldehyde is then reacted with purpald to give a bicyclic heterocycle, which upon oxidation produces a purple color. The intensity of the color is proportional to the catalase activity, reported as units/mg protein.

Expression of Heme Oxygenase-1 in the Liver Tissue

The protein expression of heme-oxygenae-1 (HO-1) in the liver tissue was examined using Western blot analysis. Liver tissue was homogenized in an ice-cold lysis buffer (10 mM Tris HCl pH 7.4, 150 mM NaCl, 15% glycerol, 1% triton X100, 1 mM sodium orthovanadate, 10 mg/ml leupeptin, 10 mg/ml aprotinin, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride). Protein concentration was determined using Bio-Rad protein assay reagent based on the Bradford method. Equal amounts of proteins (20 µg per lane) were then resolved by 10% SDS polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and incubated with mouse monoclonal antibody against rat HO-1 (Assay Designs/Stressgen, Ann Arbor, MI, USA). Immunoreactive bands were visualized using an enhanced chemiluminescent Western blotting system according to the manufacturers' instructions (Pierce Biotechnology, Inc., Rockford, IL, USA). To ensure equal loading of proteins in all lanes, the blots were stripped and reprobed with anti-actin antibody. The relative quantity of HO-1 was determined with a VersaDoc MP 4000® (Bio-Rad Laboratories, Hercules, CA, USA) using Quantity One® image analyzing software and expressed in comparison with actin expression as the protein standard.

Determination of Lipid Peroxidation Markers in Plasma and/or Liver

As markers of lipid peroxidation, we used lipid hydroperoxides (LPO), malondialdehyde (MDA), and 15-isoprostane F_{2t} (IsoP). For determination of LPO content of the liver tissue, liver samples were homogenized (1:3) in deoxygenated cold methanol/chloroform mixture (1:2) containing 0.01% butylated hydroxy toluene. The homogenate was then centrifuged at 14,000 rpm for 10 min, and the organic layer was used in the LPO assay kit. 13-Hydroperoxy octadecadienoic acid was used as standard.

Malondialdehyde (MDA) content of the livers and plasma samples was estimated using a previously reported HPLC method (29) with minor modifications. The livers were first homogenized in cold PBS (1:5), and after centrifugation at 14,000 rpm for 30 min (4°C), the supernatant was collected. To hydrolyze the protein-bound MDA, an aliquot (250 µl) of supernatant or plasma was incubated with 50 µl of 6 M NaOH for 30 min in a water bath (60°C). The proteins were precipitated by addition of 125 µl of 35% (*w*/*v*) perchloric acid, and, after vortex-mixing and centrifugation, the supernatant was separated. The MDA content of the sample was then derivatized by incubation of the supernatant (250 µl) with 25 µl of 5 mM 2,4-dinitrophenylhyrazine for 30 min at room temperature with protection from light. Tetraethoxypropane hydrolyzed with 1 mM HCl was used for construction of calibration standards. An aliquot (50 µl) of the reaction mixture was then injected onto a Partisil 5 ODS-3 column (4.6×100 mm, Whatman, Florham Park, NJ, USA), and the samples were eluted with a mobile phase of 0.2% acetic acid (*v*/*v*)/acetonitrile (68:32) at a flow rate of 1 ml/min.

For determination of free 15-isoprostane F_{2t} (IsoP), the livers were homogenized (1:4) in 50 mM phosphate buffer pH 7.4 containing 0.05% butylated hydroxyl toluene. Subsequently, 250 µl of homogenate was mixed with 50 µl 12 M hydrochloric acid to precipitate proteins. After vortex-mixing and centrifugation (4,000 rpm for 10 min), the supernatant was separated and extracted with 5 ml ethyl acetate. The organic layer was separated by centrifugation at 4,000 rpm for 10 min and dried under nitrogen stream. This extract was reconstituted in 2 ml water (pH 3.0), and processed by a solid-phase extraction procedure adapted from Tong *et al.* (30). The dried extract, obtained following solid-phase extraction, was reconstituted in the dilution buffer and used for the determination of IsoP according to the kit's instruction.

Preparation of Microsomes and Determination of Total P450 Content and CYP2E1 Content and Activity

Microsomes were prepared according to the established ultracentrifugation methods (31), used by us recently (32).

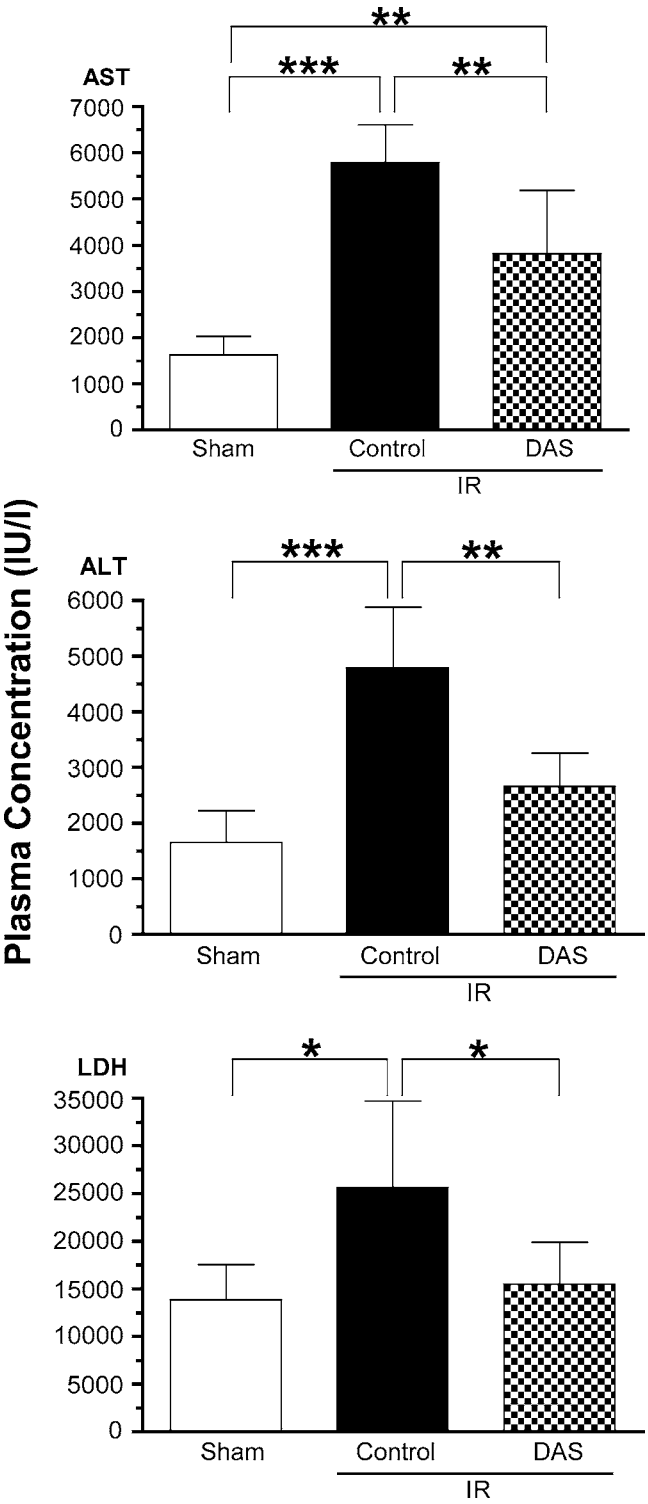

Fig. 1. Plasma concentrations of AST (*top*), ALT (*middle*), and LDH (*bottom*) in the Sham, IR-Control, and IR-DAS animals. *Columns* and *error bars* represent mean and SD values, respectively. Significant differences at $*p<0.05$, $**p<0.01$, or $***p<0.001$, based on ANOVA, followed by Fisher's *post hoc* analysis.

The final pellet was dispersed in storage buffer (150 mM KCl containing 50 mM Tris buffer pH 7.4, 1 mM EDTA, and 20% glycerol) and stored at −80°C. Total protein content was measured by Bradford assay using bovine serum albumin as standard. Spectral analysis of total CYP content was performed using the method of Omura and Sato (33).

The microsomal content of CYP2E1 protein was determined by western immunoblot analysis according to standard methods, explained above for HO-1 analysis. The amount of microsomal protein used was 5 μg, and the goat antirat primary antibody for CYP2E1 was from Gentest (Woburn, MA, USA).

CYP2E1 activity was assessed by hydroxylation of *p*-nitrophenol to 4-nitrocathechol, as described by us recently (34).

Evaluation of Superoxide Generation in the Liver Tissue

Superoxide generation in the livers was estimated as described by Minamiyama *et al.* (35). Superoxide oxidizes the non-fluorescent probe dihydroethidium (DHE) to ethidium bromide, which is excited at 488 nm with an emission of 610 nm. The intensity of ethidium bromide fluorescence is proportional to the capacity of superoxide generation in the tissue.

Liver sections (10 μm) were prepared from unfixed frozen livers and placed on glass slides. The surface of the

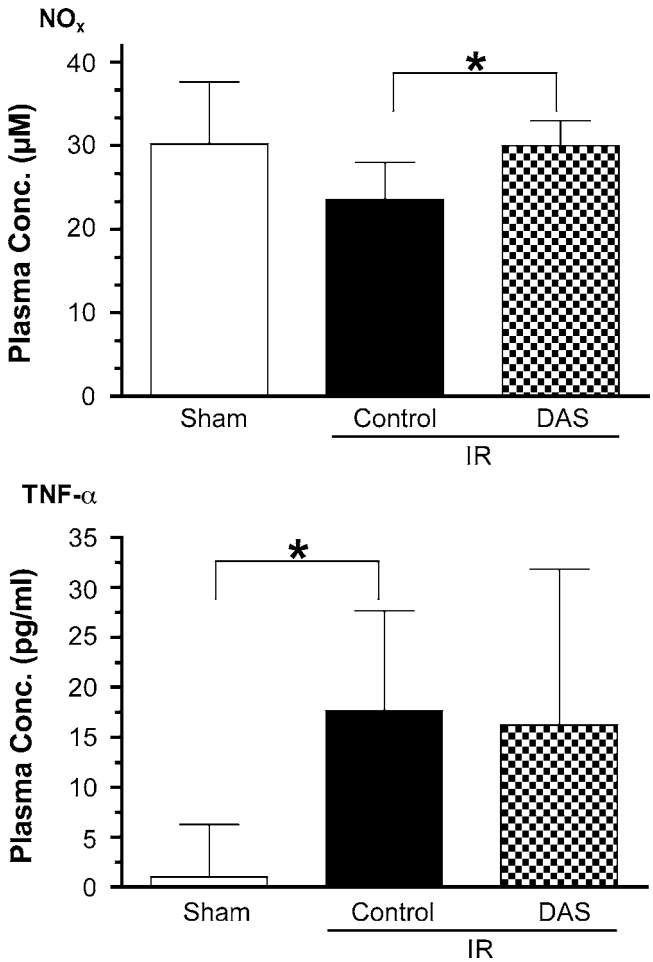

Fig. 2. Plasma concentrations of NO_x (*top*) and TNF-α (*bottom*) in the Sham, IR-Control, and IR-DAS animals. *Columns* and *error bars* represent mean and SD values, respectively. Significant difference at $*p<0.05$, based on ANOVA, followed by Fisher's *post hoc* analysis.

Table I. Mean (±SD) Values of Total, Reduced (GSH), and Oxidized (GSSG) Glutathione Concentrations and GSH/GSSG Ratio in the Liver of Rats Subjected to Sham operation (SO) or Ischemia–Reperfusion with Vehicle (IR-Control) or DAS (IR-DAS) Pretreatment

Parameter	SO	IR Control	IR-DAS
Total glutathione (μmol/g)	4.14±0.62	3.33±1.05	4.70±1.28[a]
GSH (μmol/g)	3.87±0.65	3.01±0.99	4.30±1.28
GSSG (μmol/g)	0.136±0.016	0.158±0.057	0.196±0.041
GSH/GSSG ratio	29.0±7.7	21.6±6.5	27.5±8.6

[a] Significantly different from the IR-Control group: $p<0.05$, ANOVA, followed by Fisher's *post hoc* analysis

sections were treated with 40 μM DHE and 1 mM NADPH, and the treated slides were incubated at 37°C in a light protected, humid chamber for 30 min. Extra DHE was removed, and the sections were observed under a fluorescent microscope (Olympus IX81, Center Valley, PA, USA). As negative controls, liver sections were treated with DHE in the absence of NADPH.

Histology

Formalin (10%)-fixed liver samples were embedded in paraffin, and 5-μm sections were prepared and stained with hematoxylin and eosin for histological evaluation. The sections were examined by a pathologist who was blinded to the treatment groups.

Statistical Analysis

The differences among different groups were tested statistically using ANOVA, followed by Fisher's post hoc analysis of means. A p value of ≤0.05 was considered significant. The results are presented as mean ± SD.

RESULTS

Plasma Markers of Liver Injury, TNF-α, and Nitric Oxide Metabolites

The plasma concentrations of the hepatic injury enzymes after 180 min of reperfusion in the SO, IR-Control, and IR-DAS groups are shown in Fig. 1. Ischemia–reperfusion caused substantial injury to the liver as demonstrated by significant increases in the plasma concentrations of AST, ALT, and LDH, relative to those in the SO group. A single dose of DAS significantly reduced the IR-induced elevations in the plasma concentrations of all three enzymes (Fig. 1). Indeed, for ALT and LDH, the enzyme levels in the IR-DAS group were not significantly different from those in the SO animals (Fig. 1).

Figure 2 depicts the plasma concentrations of NO_x and TNF-α in different groups of animals. A relatively small (22%) decline in the NO_x concentrations in the IR-Control group, compared with the SO group, did not reach statistical significance ($p=0.06$; Fig. 2, top). However, DAS pretreatment significantly, but modestly (28%), increased the plasma concentrations of NO_x, compared with the IR-Control group (Fig. 2, top). As for TNF-α, our model of IR caused a significant ($p<0.05$) increase in the plasma concentrations of TNF-α, relative to the SO animals, an observation that was not affected by DAS pretreatment (Fig. 2, bottom).

Hepatic and Biliary Concentrations of Reduced (GSH) and Oxidized (GSSG) Glutathione

The liver concentrations of total, reduced (GSH), and oxidized (GSSG) glutathione, along with GSH/GSSG concentration ratios, are presented in Table I. Our IR model did not significantly affect any of these parameters. However, DAS treatment significantly ($p<0.05$) increased the total glutathione levels in the livers subjected to IR by ~40% (Table I).

The bile flow and the biliary concentrations of total glutathione, GSH, and GSSG, along with GSH/GSSG concentration ratios, are presented in Table II. There was a 64% reduction ($p<0.0001$) in the bile flow rate due to IR injury (Table II). DAS pretreatment significantly ($p<0.05$) attenuated the IR-induced decrease in the bile flow. However, the bile flow in the IR-DAS group was still lower ($p<0.01$) than that in the SO livers (Table II). The biliary concentrations of total and reduced glutathione were significantly ($p<0.01$) reduced by IR

Table II. Mean (±SD) Values of Bile Flow Rate and Total, Reduced (GSH), and Oxidized (GSSG) Glutathione Concentrations and GSH/GSSG Ratio in the Bile of Rats Subjected to Sham Operation (SO) or Ischemia–Reperfusion with Vehicle (IR-Control) or DAS (IR-DAS) Pretreatment

Parameter	SO	IR Control	IR-DAS
Bile flow rate (ml/h)	1.20±0.34	0.429±0.126[a]	0.70±0.15[a,b]
Total glutathione (μmol/ml)	3.06±0.96	1.50±0.72[a]	2.25±0.54
GSH (μmol/ml)	2.67±0.81	1.25±0.69[a]	1.79±0.57
GSSG (μmol/ml)	0.196±0.088	0.125±0.043	0.229±0.151
GSH/GSSG ratio	14.4±2.9	10.3±5.0	10.5±5.4

[a] Significantly different from the SO group: $p<0.05$, ANOVA, followed by Fisher's *post hoc* analysis
[b] Significantly different from the IR-Control group: $p<0.05$, ANOVA, followed by Fisher's *post hoc* analysis

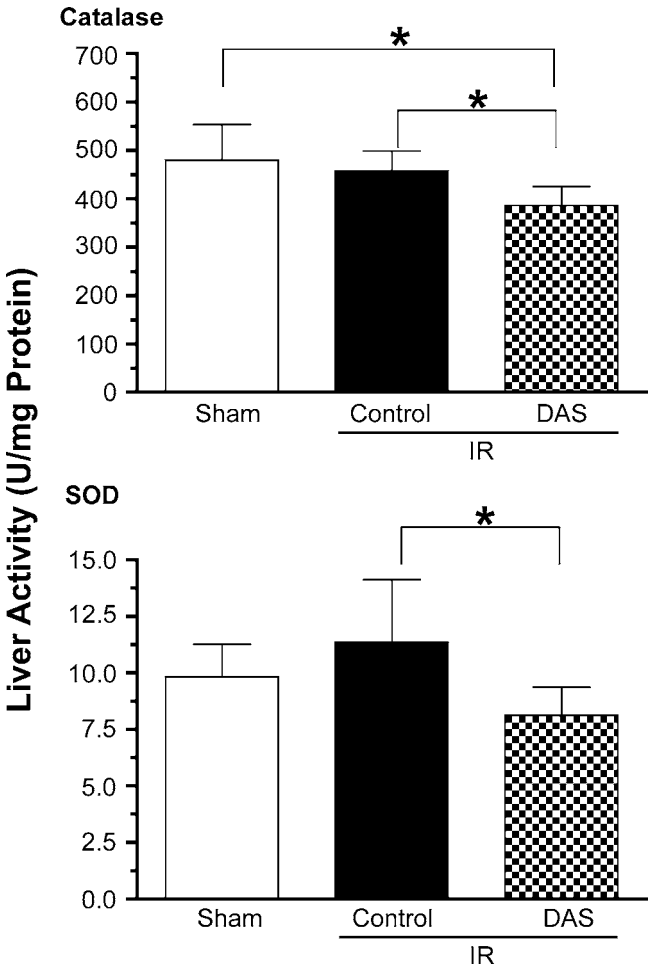

Fig. 3. Activity of catalase (*top*) and superoxide dismutase (SOD, *bottom*) in the liver tissues of the Sham, IR-Control, and IR-DAS animals. *Columns* and *error bars* represent mean and SD values, respectively. Significant difference at $*p<0.05$, based on ANOVA, followed by Fisher's *post hoc* analysis.

injury, an effect that was normalized by DAS pretreatment (Table II). On the other hand, the oxidized glutathione levels and GSH/GSSG ratios were not influenced by either IR or DAS pretreatment.

Hepatic Activities of Superoxide Dismutase and Catalase

The activities of the liver antioxidant enzymes catalase and SOD are presented in Fig. 3. Ischemia-reperfusion did not affect the activities of either enzyme. However, DAS pretreatment marginally, but significantly, reduced the activities of both enzymes, compared with the IR-Control groups. Additionally, catalase activity after DAS pretreatment was significantly lower than that in the SO group (Fig. 3).

Protein Expression of HO-1 in the Liver

Figure 4 demonstrates the expression of HO-1 protein in the liver tissue of different groups of animals. IR injury caused a 90% increase in the HO-1 protein ($p<0.001$). Treatment of rats subjected to IR with DAS further increased ($p<0.001$) the expression of HO-1 by an additional 90%, resulting in a 180% increase in the HO-1 protein in the IR-DAS group, compared with the SO animals (Fig. 4).

Concentrations of Lipid Peroxidation Markers in Plasma and/or the Liver Tissue

The concentrations of lipid peroxidation markers in the liver and plasma are presented in Fig. 5. Ischemia–reperfusion injury caused a 1.7-fold increase in the liver concentrations of lipid hydroperoxides (LPO). Pretreatment of rats with DAS normalized the LPO levels in the liver, as there was no difference between the Sham and IR-DAS groups in their LPO concentrations (Fig. 5A). A similar pattern was also observed with the liver concentrations of free IsoP; a twofold increase in the liver concentrations of free IsoP in the IR-Control group, compared with the SO animals, was normalized after pretreatment of animals with DAS (Fig. 5B). In contrast to LPO and IsoP, our IR model did not significantly affect the liver or plasma concentrations of MDA (Fig. 5C and D).

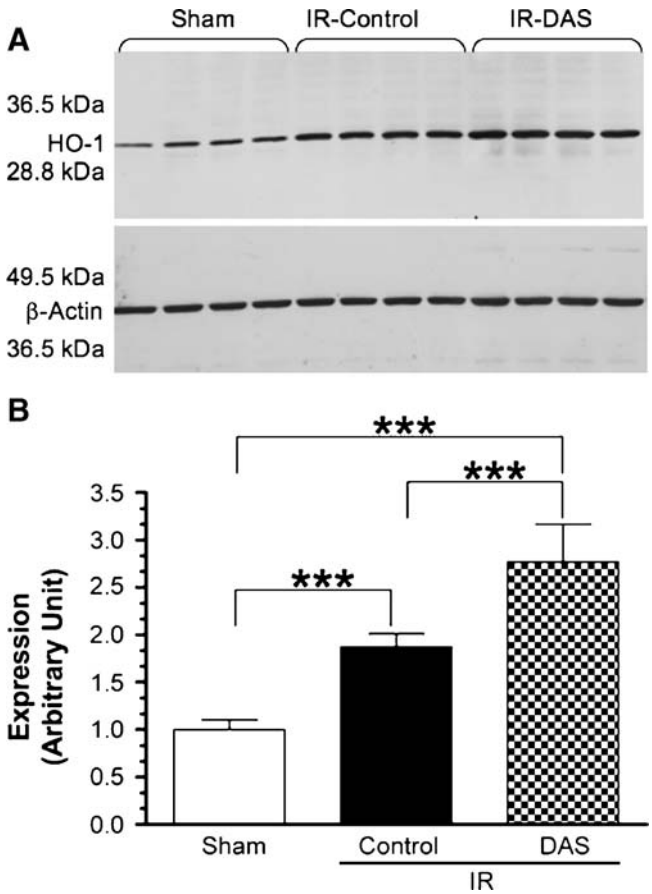

Fig. 4. Hepatic protein expression of heme oxygenase-1 (HO-1) in the Sham, IR-Control, and IR-DAS animals. **A** Representative blots of HO-1 and β-actin. **B** Expression of HO-1 based on densitometric analysis of immunoblots and correction for protein loading using β-actin. Corrected densitometric values are expressed as fold increase over Sham. *Columns* and *error bars* represent mean and SD values, respectively. Significant difference at $***p<0.001$, based on ANOVA, followed by Fisher's *post hoc* analysis.

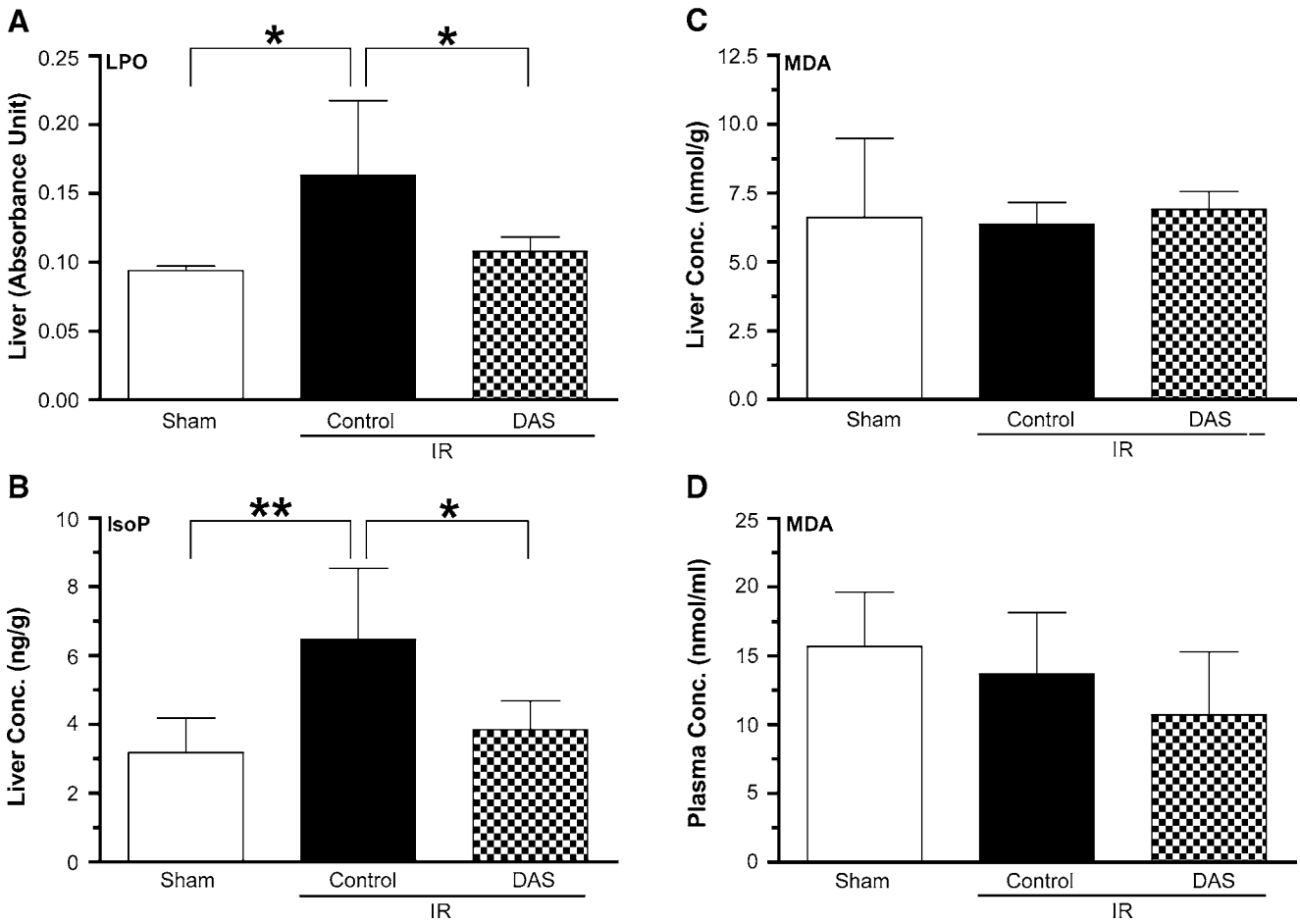

Fig. 5. Concentrations of liver LPO (**A**), liver IsoP (**B**), liver MDA (**C**), and plasma MDA (**D**) in the Sham, IR-Control, and IR-DAS animals. *Columns* and *error bars* represent mean and SD values, respectively. Significant differences at $*p<0.05$ or $**p<0.01$, based on ANOVA, followed by Fisher's *post hoc* analysis.

Microsomal P450 Content and CYP2E1 Content and Activity

The total CYP content and CYP2E1 protein and activities of the liver microsomes are presented in Fig. 6. No significant changes in the total CYP content were observed in either IR-Control or IR-DAS groups, when compared with the SO group (Fig. 6A). On the other hand, the CYP2E1 activity of the microsomes was significantly reduced by 17% due to IR (Fig. 6B). Additionally, DAS pretreatment of rats subjected to IR caused a further 25% reduction in the CYP2E1 activity, compared with that in the IR-Control group (Fig. 6B). Although IR by itself did not have any effect on the CYP2E1 protein, DAS pretreatment significantly reduced CYP2E1 protein by ~20%, compared with the SO or IR-Control groups (Fig. 6C and D).

In Situ Generation of Superoxide

Representative fluorescent images of liver sections demonstrating *in situ* superoxide production by oxidation of DHE to ethidium bromide are shown in Fig. 7. Compared with the sections from the SO livers (Fig. 7A), there was a high intensity fluorescence in the IR-Control sections, with strongest fluorescence observed around the central veins (Fig. 7B). The fluorescence intensity was substantially reduced in all the sections prepared from the animals pretreated with DAS (Fig. 7C), indicating DAS-mediated reduction in superoxide generation. Additionally, negative control sections, which were prepared from the IR-Control livers in the absence of NADPH did not show any significant fluorescence (Fig. 7D).

Histological Findings

Photomicrographs of representative rat livers, stained with hematoxylin–eosin, are presented in Fig. 8. As expected, the SO livers had normal lobular and cellular structures (Fig. 8A). However, IR resulted in substantial injury (Fig. 8B), presented as focal areas of acute coagulative hepatocellular necrosis and vacuolation of viable hepatocytes peripheral to foci of necrosis. DAS pretreatment of the livers subjected to IR substantially improved these IR-induced pathological changes (Fig. 8C).

DISCUSSION

Because of the advances in surgical procedures, the number of surgeries requiring interruption of blood supply to part or all of the liver, such as resection of liver tumors or living-donor liver transplantation, has increased dramatically during the recent

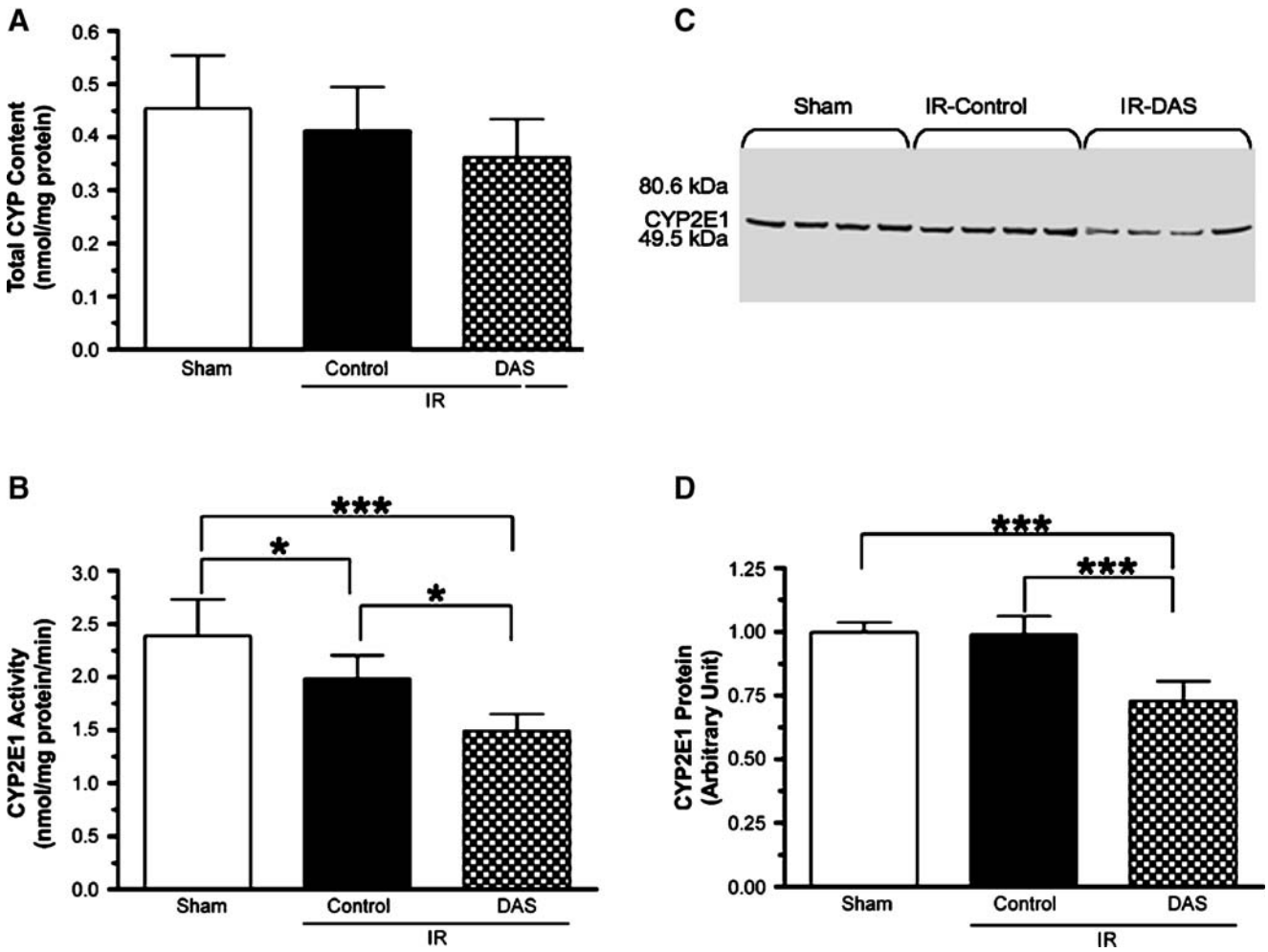

Fig. 6. Microsomal cytochrome P450 expression and activity in the Sham, IR-Control, and IR-DAS animals. **A** Total cytochrome P450 content. **B** CYP2E1 activity. **C** Representative blots of CYP2E1. **D** Expression of CYP2E1 based on densitometric analysis of immunoblots, expressed as a fraction of Sham. *Columns* and *error bars* represent mean and SD values, respectively. Significant differences at $*p<0.05$ or $***p<0.001$, based on ANOVA, followed by Fisher's *post hoc* analysis.

years (36). Interruption of blood supply to the warm liver causes ischemia, which results in a decline in the energy supply of the metabolically active organ and severe damage. Paradoxically, the reinstatement of blood flow after ischemia induces additional damage due to production of ROS, leading to inflammation, cell death, and organ failure (2–4). Indeed, severe cases of warm hepatic IR injury may lead to a systemic inflammatory response syndrome and multiorgan failure (2). Therefore, it is important to devise strategies to reduce IR injury and its consequent morbidity and mortality.

Major approaches to reduce warm IR injury include ischemic preconditioning, gene therapy, and pharmacologic intervention (9). Among the pharmacologic approaches, the use of antioxidants has been an attractive area of research in recent years because of the major role of oxidative stress in warm IR injury (9). Therefore, we hypothesized that DAS, which is known to have antioxidant properties, would reduce warm hepatic IR injury. Indeed, the significant reductions in the plasma markers of hepatic injury observed in our studies after administration of a single dose of DAS in an experimental model of the injury (Fig. 1) is in agreement with this hypothesis.

Based on the previous reports on the actions of DAS and related garlic components, several mechanisms could have potentially contributed to the hepatoprotective effects of DAS observed in our studies. The significant reductions in the concentrations of early markers of lipid peroxidation in the liver, such as LPO and IsoP (Fig. 5), and *in situ* generation of superoxide in the liver (Fig. 7) suggest that the protective effects of DAS are via reduction of oxidative stress. Therefore, additional studies were carried out to investigate the effects of DAS on the major pathways that maintain the antioxidant/oxidant homeostasis.

Among antioxidant pathways, HO-1 plays a pivotal role in the protection of cells from oxidative stress (37). The enzyme, which is the inducible isoform of HO, catalyzes the rate-limiting step in the conversion of heme to iron, bilirubin, and CO, with the latter two products exerting strong antioxidant, anti-inflammatory, and signaling activities. In fact, bilirubin is reported to scavenge superoxide and inhibit chemically-induced lipid peroxidation. Consequently, increased expression of HO-1 by hemin (38), isoflurane (39), cobalt protoporphyrin (40), or gene therapy (41) has been shown to reduce hepatic IR injury in different animal models.

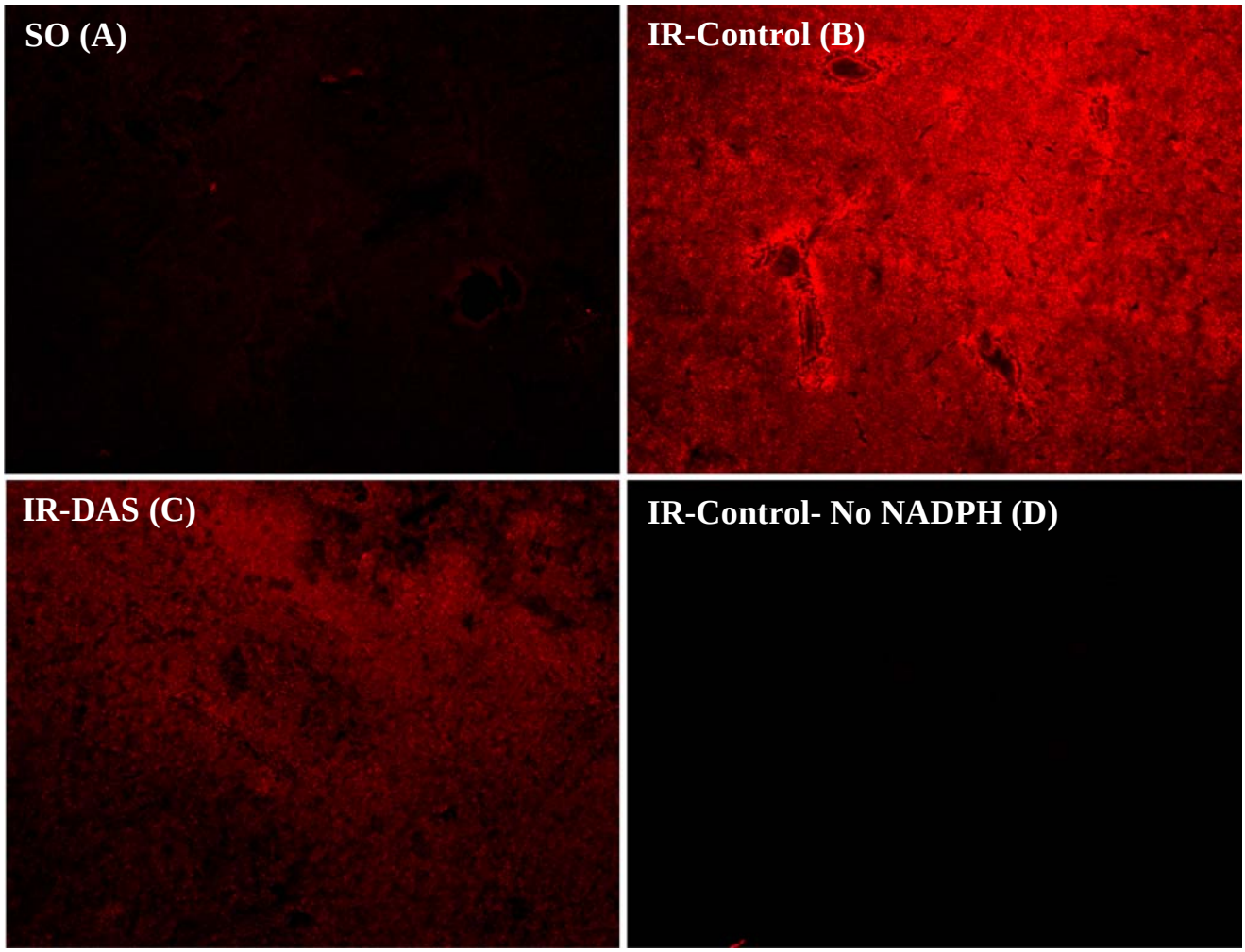

Fig. 7. Representative fluorescence images depicting the *in situ* generation of superoxide in the SO (**A**), IR-Control (**B**), and IR-DAS (**C**) liver tissue slices. For comparison, the fluorescence image in an IR-Control liver in the absence of NADPH is also shown as the negative control (**D**).

The induction of HO-1 in our IR-Control livers, compared with the SO animals (Fig. 4), is consistent with previous studies, showing that IR injury by itself induces HO-1 expression as a cytoprotective mechanism (42,43). Additionally, the further increase in the expression of the enzyme by DAS pretreatment in our *in vivo* model (Fig. 4) is consistent with two *in vitro* studies (21,44), demonstrating induction of HO-1 mRNA and protein by DAS and/or other organosulfur components of garlic in HepG2 cells. However, to the best of our knowledge, our study is the first to show the *in vivo* induction of HO-1 by DAS. Assuming a correlation between HO-1 expression and activity, our results (Fig. 4) suggest that the DAS-mediated improvement in the hepatic IR injury (Figs. 1 and 8) might be mediated, at least in part, by its ability to induce HO-1 expression in the liver.

Gong *et al.* (21) showed that 24-h incubation of HepG2 cells with 0.25 to 1.0 mM concentrations of DAS resulted in a concentration-dependent increase in both mRNA and protein expression of HO-1 by 1.8- to 6.6-fold. Additionally, they showed that the maximum induction of protein occurs between 8 to 16 h after the incubation, with substantial induction still remaining at 24 h. The 1.9-fold induction of HO-1 protein in our IR-DAS livers (Fig. 4), compared with the IR-Control livers, at 16–19 h after the *in vivo* administration of DAS is in good agreement with this *in vitro* study. In contrast to Gong *et al.* (21), the experiments reported by Chen *et al.* (44), while showing significant induction of HO-1 mRNA with diallyl disulfide and diallyl trisulfide, failed to show an induction by DAS in the same HepG2 cell line. However, this discrepancy is most likely due to the fact that Chen *et al.* used a concentration of 0.1 mM in their studies, as opposed to concentrations of 0.25–1 mM used by Gong *et al.* Nevertheless, our studies show that a single 1.75 mmol/kg dose of DAS significantly induces the HO-1 expression *in vivo* in rats subjected to warm hepatic IR.

Another mechanism contributing to the DAS-mediated reduction of oxidative stress and injury in our studies could be its ability to inhibit CYP2E1 (Fig. 6). In fact, the reported ability of DAS to inhibit chemical toxicity and carcinogenesis has been mostly attributed to its CYP2E1 inhibitory activity (45). CYP450-mediated metabolism of DAS at the sulfur atom results in sequential production of diallyl sulfoxide and diallyl sulfone (23). Whereas DAS and its metabolites are all competitive inhibitors of CYP2E1, diallyl sulfone is a suicide inhibitor of the enzyme. In our study, administration of DAS resulted in reductions in both protein content and activity of

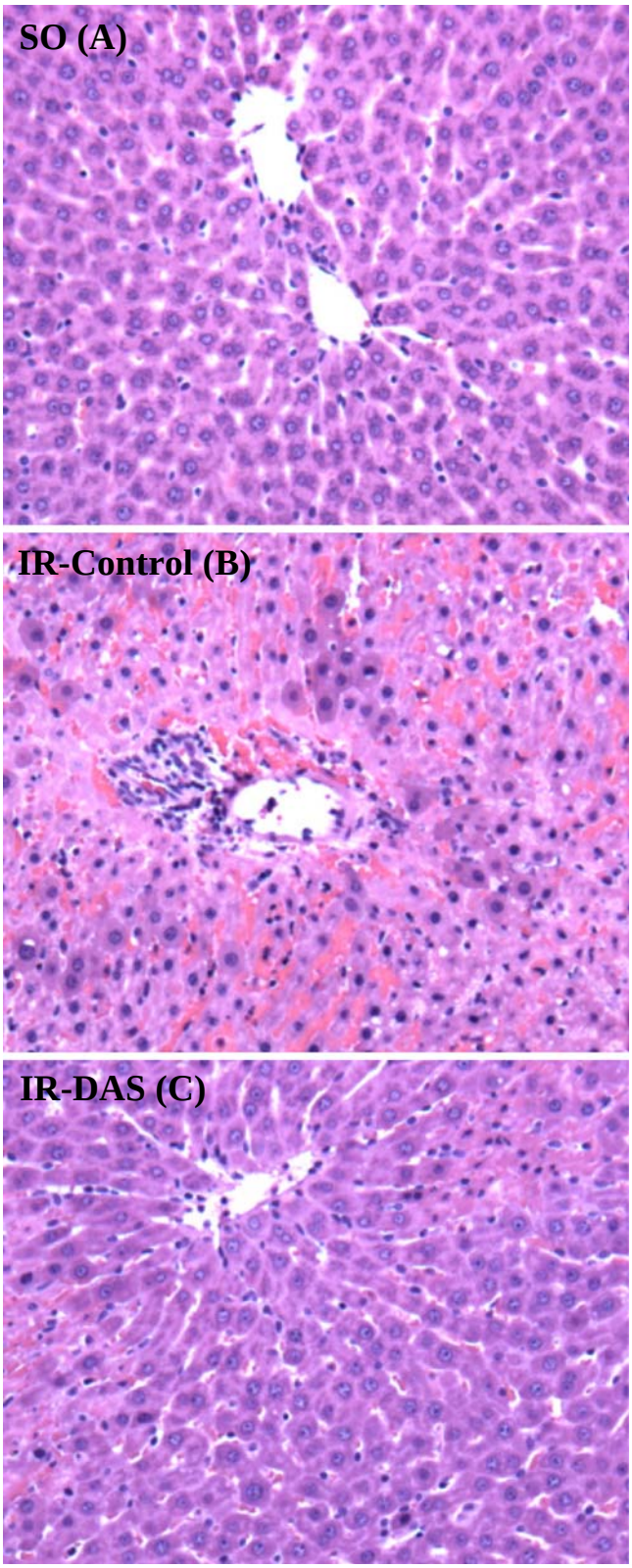

Fig. 8. Hematoxylin–eosin stained photomicrographs of representative rat livers from the SO (**A**), IR-Control (**B**), and IR-DAS (**C**) rats.

CYP2E1 (Fig. 6). It has been suggested that cytochromes P450, and in particular CYP2E1, generate significant amounts of ROS via uncoupling, even in the absence of substrates (46,47). Therefore, it is likely that the reduction in the protein and activity of CYP2E1 by DAS (Fig. 6) has contributed to its hepatoprotective activity observed in our IR injury model (Fig. 1).

Other mediators involved in IR are NO and proinflammatory cytokines, such as TNF-α. Whereas the damaging effects of proinflammatory cytokines in IR are relatively clear, NO may have damaging or protective effect depending on its source or concentrations (48,49). Generally, it is believed that the relatively low levels of NO produced by the constitutive endothelial nitric oxide synthase (eNOS) are hepatoprotective. However, the large amounts of NO synthesized by the inducible NOS (iNOS) may contribute to the hepatic injury by reacting with superoxide to produce the relatively toxic peroxynitrite (50). Although IR injury induces iNOS, we did not observe an increase in the plasma concentrations of NO_x in our model (IR-Control *vs.* SO, Fig. 2). This is most likely due to the fact that iNOS induction after IR insult takes longer than the reperfusion time in our model (51). In contrast, it has been shown that the plasma concentrations of NO decrease shortly after reperfusion (51) because of the significant release of arginase into the circulation (52). The trend in the NO_x data in our IR-Control and SO groups (22% reduction in NO_x in IR Control, $p=0.06$; Fig. 2) is in agreement with these reports.

Recent *in vitro* studies (13,14) have shown that DAS inhibits the expression of iNOS in lipopolysaccharide (LPS)-stimulated macrophages. Incubation of stimulated cells with relatively low concentrations (1–10 μM) of DAS for 24 h significantly reduced the formation of nitrite by the cells via inhibition of iNOS. Because the induction of iNOS does not occur within the time frame of our study (i.e., 3 h of reperfusion), a reduction in NO_x by DAS is not expected in our studies. Indeed, DAS caused a 28% increase in the NO_x concentrations in our study (Fig. 2), which may be explained by the antioxidant activity of DAS, reducing ROS, thus increasing the availability of constitutively-generated, protective NO.

As for other cellular antioxidant components, the DAS-mediated improvements in the levels of glutathione in the liver (Table I) and bile (Table II) are consistent with its overall hepatoprotective effects observed in our study. However, the small, but significant, negative impact of DAS pretreatment on the activities of the antioxidant enzymes catalase and SOD (Fig. 3) is in opposite direction to its overall antioxidant property. Chen *et al.* (53) also showed a 22% decrease in the catalase activity of the rat liver 24 h after the administration of a single 200 mg/kg dose of DAS. This is in agreement with a 16% decline in the activity observed in our studies 16–19 h after administration of an identical dose (Fig. 3). The decrease in the catalase activity was attributed to a DAS-mediated decrease in the cytosolic protein content of catalase. They also showed that the inhibitory effect of DAS on the protein and activity of the liver catalase increases with an increase in dose or chronic dosing. However, Chen *et al.* did not observe any effect of DAS on the SOD activity in the liver after chronic dosing. This is in contrast to our single dose study, which showed slightly lower activity of SOD due to DAS pretreatment (Fig. 3). The discrepancy between the results of Chen *et al.* (53) and ours with regard to SOD may be due to differences between the chronic and single dose regimens of DAS. Nevertheless, because our IR model did

not affect the liver activities of catalase and SOD, the slight DAS-mediated decreases in their activities may not have had a significant detrimental impact on the overall antioxidant activity of DAS.

Whereas our IR model caused a significant increase in the concentrations of the liver peroxidation markers LPO and IsoP, the concentrations of MDA were not affected (Fig. 5). This apparent discrepancy is most likely due to the fact that whereas LPO and isoprostanes are primary (54) and specific (30) markers of lipid peroxidation, respectively, MDA is considered a secondary marker formed by oxidation of LPO (54,55). The formation of MDA from LPO is dependent on a number of factors, such as the concentrations of transitional metals and heme proteins and partial oxygen pressure in the tissue (55). Additionally, MDA is produced only from the hydroperoxides of polyunsaturated fatty acids; hydroperoxides from the other abundant lipids such as cholesterol and linoleic acid do not form MDA (56). Therefore, it has been suggested that in the rat liver IR injury, secondary markers of lipid peroxidation (such as MDA) are less sensitive than primary markers (i.e., lipid hydroperoxides) to the oxidative stress (55).

Recently, it was reported that an aqueous garlic extract reduces liver injury in a rat model of total hepatic ischemia (45 min), followed by one h of reperfusion (57). The authors stated that the compounds present in their aqueous extract were *S*-allyl-cysteine, *S*-allyl-mercaptocystein, *S*-allyl-cysteinesulfoxide, and allicin. Therefore, the relevance of that study using the aqueous garlic extract to our study with purified DAS, which is a component of garlic oil, is not apparent. Nevertheless, that study suggests that garlic components other than DAS may also be hepatoprotective. Further studies on other purified garlic components are needed to verify this suggestion.

CONCLUSION

Administration of a single intraperitoneal dose of DAS to rats prior to the induction of ischemia significantly improved hepatic injury in a rat model of warm ischemia-reperfusion by decreasing liver enzymes, lipid peroxidation markers, and histological damage. The hepatoprotective effects of DAS appear to be due to a reduction in oxidative stress through, at least in part, the induction of heme-oxygenase-1 and inhibition of CYP2E1 in the liver. Further studies are needed to confirm the protective effects of DAS in other experimental models of IR injury.

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