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ORIGINAL ARTICLE

Reactive Oxygen Species Mediate Human Hepatocyte Injury During Hypoxia/Reoxygenation

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Increasing evidence shows that reactive oxygen species (ROS) may be critical mediators of liver damage during the relative hypoxia of ischemia/reperfusion injury (IRI) associated with transplant surgery or of the tissue microenvironment created as a result of chronic hepatic inflammation or infection. Much work has been focused on Kupffer cells or liver resident macrophages with respect to the generation of ROS during IRI. However, little is known about the contribution of endogenous hepatocyte ROS production or its potential impact on the parenchymal cell death associated with IRI and chronic hepatic inflammation. For the first time, we show that human hepatocytes isolated from nondiseased liver tissue and human hepatocytes isolated from diseased liver tissue exhibit marked differences in ROS production in response to hypoxia/reoxygenation (H-R). Furthermore, several different antioxidants are able to abrogate hepatocyte ROS–induced cell death during hypoxia and H-R. These data provide clear evidence that endogenous ROS production by mitochondria and nicotinamide adenine dinucleotide phosphate oxidase drives human hepatocyte apoptosis and necrosis during hypoxia and H-R and may therefore play an important role in any hepatic diseases characterized by a relatively hypoxic liver microenvironment. In conclusion, these data strongly suggest that hepatocytes and hepatocyte-derived ROS are active participants driving hepatic inflammation. These novel findings highlight important functional/metabolic differences between hepatocytes isolated from normal donor livers, hepatocytes isolated from normal resected tissue obtained during surgery for malignant neoplasms, and hepatocytes isolated from livers with end-stage disease. Furthermore, the targeting of hepatocyte ROS generation with antioxidants may offer therapeutic potential for the adjunctive treatment of IRI and chronic inflammatory liver diseases. ***Liver Transpl 16:1303-1313, 2010.*** © **2010 AASLD.**

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Most studies that have investigated the mechanisms of liver damage occurring as a result of ischemia/reperfusion injury (IRI) have focused on the setting of liver transplantation. It is equally possible that relatively hypoxic conditions can occur during other episodes of hepatic inflammation or established chronic disease. Hepatocytes exposed to a hypoxic microenvironment would therefore be potentially sensitized to cell death, although no studies have yet explored this process in primary human hepatocytes.

Despite considerable advances in surgical practice and more judicious use of immunosuppression, hepatic IRI continues to adversely affect allograft function and survival after orthotopic liver transplantation (OLT). Toledo-Pereyra et al.[1] in 1975 were among the first to note the detrimental effects of IRI during experimental liver transplantation. IRI is a proinflammatory, antigen-independent process that culminates in hepatocyte injury. The potential processes regulating

Abbreviations: 7-AAD, 7-aminoactinomycin D; ALD, alcoholic liver disease; Cy5, cyanine 5; DCF, 2′,7′-dichlorofluorescin; DPI, diphenyliodonium; FITC, fluorescein isothiocyanate; FL, fluorescence; FS, forward scatter; H-R, hypoxia/reoxygenation; HSEC, hepatic sinusoidal endothelial cell; IRI, ischemia/reperfusion injury; KC, Kupffer cell; MFI, mean fluorescence intensity; NAC, N-acetylcysteine; NADPH, nicotinamide adenine dinucleotide phosphate; OLT, orthotopic liver transplantation; PE, phycoerythrin; ROS, reactive oxygen species; Rot, rotenone; SS, side scatter.

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hepatic IRI have been summarized in recent reviews.[2,3] Recent studies have suggested that hepatic IRI accounts for up to 10% of early allograft failures and is associated with a higher incidence of both acute and chronic rejection.[4] The primary cellular target during IRI is the hepatocyte. Hepatocyte death seen during the hypoxic and reperfusion phases of IRI occurs within a relatively hypoxic environment, and hepatic IRI can be divided into 2 phases. The early phase is thought to involve the activation of Kupffer cells (KCs), which release proinflammatory cytokines and reactive oxygen species (ROS).[5,6] The late phase is characterized by increased expression of chemokines and adhesion molecules and hepatic recruitment of effector cells, which amplify the tissue damage. The latter phenomenon is a feature common to many other hepatic diseases. There is evidence suggesting that hepatocyte injury occurs during the relatively hypoxic early phase of IRI.[7] ROS release is one of the earliest and most important components of tissue injury after the reperfusion of ischemic organs and is a major contributor to hepatocyte death during reperfusion.[8] Moreover, diseases such as alcoholic liver disease (ALD) are characterized by the chronic accumulation of ROS. The source of hepatic ROS remains controversial. Numerous studies have suggested that hepatocyte damage is triggered by KC-derived ROS,[9,10] whereas others have shown that the absence or elimination of KCs does not prevent tissue damage in IRI.[11] This suggests that other cells within the liver, including hepatocytes, may be involved in the pathophysiological production of ROS during IRI and chronic hepatic inflammation.

Early work with rat hepatocytes suggested xanthine oxidase as the main generator of ROS.[12] However, the xanthine oxidase inhibitors used in these studies are now known to inhibit mitochondrial function, and mitochondria are now accepted as the main source of ROS within hepatocytes.[13] Furthermore, the inhibition of mitochondrial complexes I and III can ameliorate ROS production in human hepatoma cell lines[14] and rat hepatocytes.[15] Other enzymes, such as the flavoenzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, can also produce ROS in rat hepatocytes.[16] The accumulation of excess intracellular ROS induces cell death, and during hepatic IRI, hepatocytes undergo both apoptosis and necrosis.[17] Some studies have supported apoptosis as the primary mode of death,[18] and others have supported necrosis.[19] In reality, it is uncertain whether the apoptosis and necrosis observed in liver tissue are separate processes because the terms primarily represent morphological descriptions, and it has been suggested that apoptotic cells not effectively cleared from inflammatory sites may eventually assume a necrotic appearance (so-called secondary necrosis). This led Jaeschke and Lemasters[20] to propose the term *necroapoptosis*, and it may be that the 2 forms of cell death are in part related and share some common intracellular pathways.

Despite these observations, little is known about the relative contribution of endogenous hepatocyte ROS production and its potential impact on hepatocyte cell death after hypoxia and hypoxia/reoxygenation (H-R). Much of our understanding of hepatic IRI comes from studies of rodent hepatocytes and experimental models. However, the response of human hepatocytes to hypoxia and H-R is unknown. In addition, the research performed with human hepatocytes has used cells isolated from resected liver tissue from patients with neoplastic disease.[21] Whether cells from such normal sources can be considered to truly reflect normal hepatocytes has never to our knowledge been objectively studied.

The aim of our study was to characterize the responses of human hepatocytes isolated from normal liver tissue and diseased liver tissue to hypoxia and H-R. Specifically, we delineated intracellular ROS accumulation and cell death in primary human hepatocytes isolated from normal liver tissue and diseased liver tissue during hypoxia and H-R. Using an in vitro model of warm hepatocyte IRI, we show for the first time highly variable responses of primary human hepatocytes isolated from normal liver tissue, surgically resected liver tissue, and diseased liver tissue to hypoxia and H-R. Although these findings have obvious clinical implications within the transplant setting, they are equally important for diseases in which local cellular responses to hypoxia may shape the inflammatory and regenerative microenvironment. The apparent variable responses of primary hepatocytes isolated from normal tissue, resected normal tissue, and diseased tissue are also important to workers studying hepatocyte physiology and function ex vivo.

MATERIALS AND METHODS

Isolation of Human Hepatocytes

Liver tissue was obtained from fully consenting patients undergoing transplantation for a variety of end-stage liver diseases and from patients undergoing hepatic resection for liver metastasis; normal donor tissue exceeding surgical requirements was also used. Specifically, normal donor tissue was obtained from the in situ splitting of adult livers used for pediatric OLT. These patients were 19 to 31 years old. The left lateral segment was used for pediatric OLT, and this meant that tissue could be procured from the right lobe. Human hepatocytes were isolated from explanted diseased livers from patients with ALD, primary biliary cirrhosis, or primary sclerosing cholangitis. Hepatocytes were also isolated from tissue taken from patients who had undergone hepatic resection for liver metastasis from colorectal carcinoma. Liver tissue was obtained from surgical procedures carried out at Queen Elizabeth Hospital (Birmingham, United Kingdom). Ethical approval for the study was granted by the local research ethics committee (reference number 06/Q702/61).

Importantly, all liver tissue, including explants, normal donor tissue, and normal resected tissue, was obtained from patients with the same rigorous standard protocol. Briefly, each liver specimen was in circuit

and was supplied with blood under normoxic conditions until the liver was explanted, split, or resected. The only technical difference was that normal resected tissue involved the removal of a specific portion of the liver (usually right hemihepatectomy rather than the whole organ). When liver tissue was obtained from patients undergoing hepatic resection, all the patients had received preoperative chemotherapy. After liver explantation, splitting, or resection, all specimens were placed on ice, immediately transported, and processed within the laboratory. All hepatocyte isolation was carried out within 6 hours of surgical explantation, splitting, or resection. Strict adherence to the procurement and processing protocols ensured that any differences observed between hepatocytes were results of disease or altered physiology and were not explainable by differences in the method of surgical tissue procurement or subsequent processing.

Hepatocytes were isolated from fresh liver wedges (60-156 g) with a 2-step collagenase protocol. Each liver wedge was first perfused with a nonrecirculating wash buffer [10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (pH 7.2), Sigma, Dorset, United Kingdom] at 37°C with a flow rate of 75 mL/minute in order to remove remaining blood within the liver. After this, the wedge was perfused with a nonrecirculating chelating solution [10 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid and 0.5 mM ethylene glycol tetraacetic acid (pH 7.2), Sigma]. This was followed by further perfusion with a nonrecirculating wash buffer to remove any remaining ethylene glycol tetraacetic acid. After this, the tissue was perfused with a recirculating enzymatic dissociation solution (Hank's balanced salt solution, Gibco, Paisley, United Kingdom) with 5 mM calcium chloride (Sigma), 5 mM magnesium chloride (Sigma), 0.5% wt/vol collagenase (Roche, Hertford, United Kingdom), 0.25% wt/vol protease (Sigma), 0.125% wt/vol hyaluronidase (Sigma), and 0.05% wt/vol deoxyribonuclease (Sigma) at 37°C with a flow rate of 75 mL/minute for 1 to 7 minutes. After manual dissociation of the liver wedge, the suspension was passed through a 250-μm nylon mesh and then a 60-μm nylon mesh. The suspension was then washed at 50*g* for 10 minutes at 4°C in a supplemented medium (Dulbecco's modified Eagle's medium, Gibco) with 10% vol/vol heat-inactivated fetal calf serum (Gibco), 2 mM glutamine (Gibco), 20,000 U/L penicillin, and 20 mg/L streptomycin (Gibco). Immediately after the washing, the cell viability was determined by trypan blue dye exclusion. Hepatocytes were plated in a supplemented medium and left for 2 hours. After this period, the medium was changed to Williams' E medium (Sigma) with 2 μg/mL hydrocortisone, 0.124 U/mL insulin, 2 mM glutamine, 20,000 U/L penicillin, and 20 mg/L streptomycin. Cells were cultured for another 2 days before use.

Model of Warm H-R Injury

In experiments, hepatocytes were grown for 2 days at 37°C with 5% CO_2 in Williams' E medium (Sigma) on rat type 1 collagen-coated plates. Hepatocytes were maintained in normoxia, placed into hypoxia for 24 hours, or placed into hypoxia for 24 hours and then reoxygenated for 24 hours. Hypoxia was achieved by the placement of cells in an airtight incubator (RS Mini Galaxy A incubator, Wolf Laboratories, United Kingdom) flushed with 5% CO_2 and 95% N_2 until the oxygen content in the chamber reached 0.1%; this was verified with a dissolved-oxygen monitor (DOH-247-KIT, Omega Engineering, United Kingdom). No previous studies had evaluated the response of primary human hepatocytes to hypoxia and H-R. Therefore, we modified a well-established model of warm in vitro IRI.[22,23] In preliminary experiments, primary human hepatocytes were exposed to 5% or 1% oxygen for 2 or 24 hours, and no increase in ROS accumulation or cell death was noted (data not shown). Therefore, we used 0.1% oxygen in all subsequent experiments for 24 hours. Additionally, Williams' E medium was preincubated in the hypoxic chamber in a sterile container (which allowed gas equilibration) for 8 hours before experiments were carried out; this resulted in a final oxygen concentration of <0.1% as measured with the dissolved-oxygen meter. When it was appropriate, after 24 hours of hypoxia, the medium was aspirated and replaced with a fresh, warmed, oxygenated medium, and the cells were returned to normoxic conditions. This was defined as the beginning of reoxygenation. In experiments involving ROS inhibitors/antioxidants, all reagents were made fresh as stock solutions and were added with the correct dilutional factor to the relevant experimental wells. Specifically, 100 mM *N*-acetylcysteine (NAC; Sigma) was dissolved in molecular-grade water, 1 mM rotenone (Rot; Sigma) was dissolved in chloroform, and 1 mM diphenyliodonium (DPI; Sigma) was dissolved in dimethyl sulfoxide; they were diluted appropriately to produce working concentrations of 20 mM, 2 μM, and 10 μM, respectively. In experiments using inhibitors/antioxidants, solvent-alone controls were used to ensure no vehicle effects. In addition, in experiments using inhibitors/antioxidants, agents were added at the time of placement of the cells into hypoxia or reoxygenation.

Flow Cytometry

ROS production, apoptosis, and necrosis were determined with a 3-color reporter assay system. ROS accumulation was determined with the fluorescent probe 2′,7′-dichlorofluorescin diacetate.[24] This probe is cell-permeable and, once inside a cell, is cleaved by intracellular esterases into 2′,7′-dichlorofluorescin (DCF; Merck, Nottingham, United Kingdom), which is then rendered cell-impermeable. DCF is then able to react with intracellular ROS (specifically hydrogen peroxide) and produce a fluorescent signal detectable on the fluorescein isothiocyanate (FITC) channel. The signal is directly proportional to the level of intracellular ROS present.

Apoptosis was determined via the labeling of cells with annexin V (Molecular Probes, Paisley, United

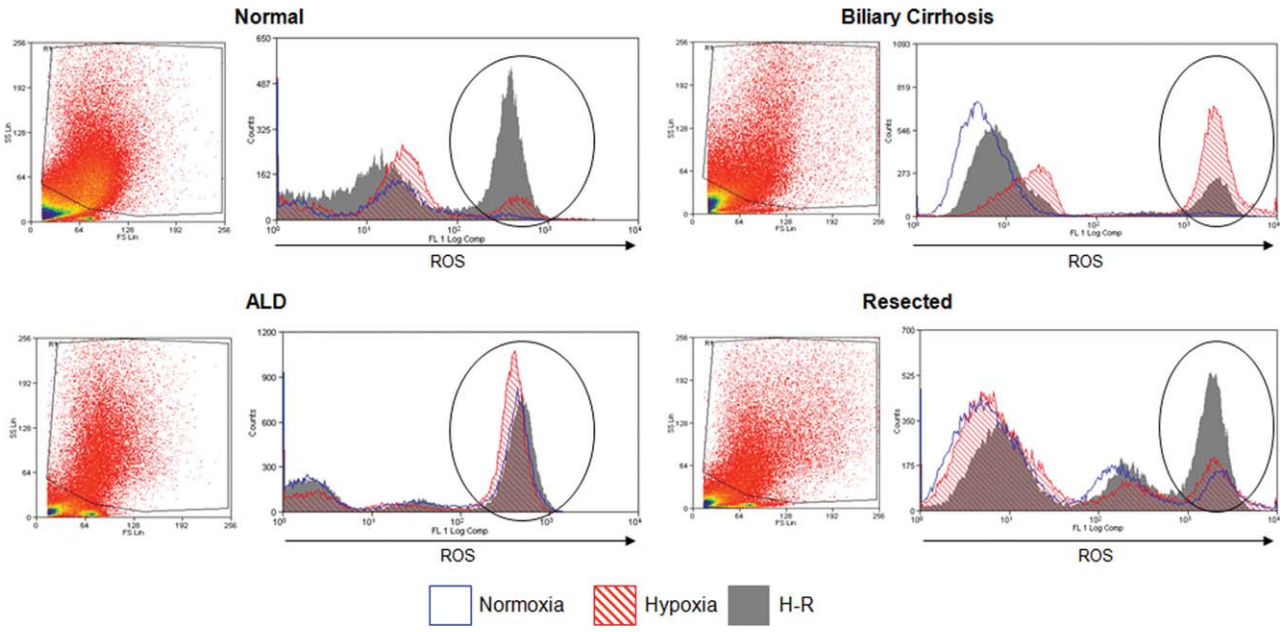


Figure 1. Hypoxia and H-R mediate ROS accumulation in human hepatocytes. Representative flow cytometry plots are shown to illustrate the effects of hypoxia and H-R on ROS accumulation in human hepatocytes isolated from normal liver tissue, diseased liver tissue, and normal resected liver tissue. Vertical ellipses mark the areas of interest within the plots. The area to the left of each ellipse represents cell debris. The reason that the cell debris is included within the plots is that human hepatocytes vary considerably in size; therefore, to include all viable human hepatocytes in the analysis, a large gate was required on the flow cytometer, and this by necessity included the cell debris. The gate is shown on the corresponding representative FS-SS plots located to the left of each flow cytometry plot. The FS-SS plots are from the H-R samples of each preparation, but similar plots were obtained during normoxia and hypoxia (data not shown). Although in the flow cytometry plot of the normal resected liver tissue there are 2 peaks to the left of the ROS peak, these both represent debris; this has been confirmed by 7-AAD staining and their size on the FS-SS plots. The data are representative of 4 normal hepatocyte preparations, 4 biliary disease preparations, 3 ALD preparations, and 9 resected normal liver preparations.

Kingdom), which detects exposed phosphatidylserine on the cell membrane. 7-Aminoactinomycin D (7-AAD; Molecular Probes) is a vital dye that binds to DNA, enters cells only once the cell membrane is disrupted, and is indicative of cellular necrosis. To ensure the consistency of the flow cytometry data, each human hepatocyte preparation was labeled with DCF alone, annexin V alone, and 7-AAD alone to ensure that the cells were labeled and that the flow cytometry data could be compensated for the crossover of fluorophore emission spectra. The same flow cytometry protocol was used for all experiments of the study; this meant that voltages for all markers were constant for all human hepatocyte preparations, so the internal consistency of the experiments was ensured.

After appropriate treatment of the cells, the medium was aspirated and replaced with Hank's balanced salt solution (Gibco) without calcium or magnesium. DCF (30 μM) was added, and the cells were incubated for 20 minutes in the dark at 37°C. Next, the cells were trypsinized and washed extensively in a fluorescence-activated cell sorting buffer [phosphate-buffered saline (pH 7.2) with 10% vol/vol heat-inactivated fetal calf serum, Gibco]. Cells were then labeled with annexin V and 7-AAD for 15 minutes while they were on ice, and samples were immediately subjected to flow cytometry. At least 20,000 events were recorded within the gated region of the flow cytometer for each human hepatocyte cell preparation under each experimental condition. Only the cells within the gated region were used to calculate the mean fluorescence intensity (MFI).

Statistical Analysis

Data analysis was carried out with SPSS software (version 13.0). All values are presented as means and standard errors unless otherwise noted. Statistical analysis was carried out with the Student *t* test.

RESULTS

Variable ROS Responses to Hypoxia and H-R of Human Hepatocytes Isolated From Patients With Different Liver Diseases

Figure 1 and Table 1 show ROS production and accumulation in primary human hepatocytes isolated from normal liver tissue, normal resected liver tissue, and diseased liver tissue. Hepatocytes isolated from normal livers, ALD liver tissue, and normal resected liver tissue showed similar and consistent responses to hypoxia and H-R. Interestingly, normal human hepatocytes had little basal intracellular ROS. However, after

TABLE 1. ROS Accumulation in Human Hepatocytes During Hypoxia and H-R

	Normal	Biliary Cirrhosis	ALD	Resected
Normoxia	30.3 (13.3-53.5)	11.9 (6.2-21.1)	226.5 (217.1-247.1)	352.8 (256.4-450.5)
Hypoxia	121.9 (39.6-166.2)*	255.2 (139.9-444.0)*	259.5 (229.2-281.2)	377.1 (284.3-547.2)
H-R	271.1 (217.7-370.0)*,†	102.6 (35.2-151.0)*	294.7 (278.2-311.1)	506.1 (332.2-874.0)

NOTE: The mean ROS accumulation for human hepatocytes isolated from normal liver tissue, diseased liver tissue, and normal resected liver tissue is shown for each of the 3 experimental conditions. Data are expressed as MFIs. Numbers in parentheses are the ranges of MFI readings for each experimental condition. The MFI values have been derived from the cells within the ellipses shown in Fig. 1. The data are representative of 4 normal hepatocyte preparations, 4 biliary disease preparations, 3 ALD preparations, and 9 normal resected liver preparations.
*$P < 0.05$ versus normoxia.
†$P < 0.05$ versus hypoxia.

exposure to hypoxia and H-R, normal human hepatocytes had significantly increased intracellular ROS accumulation. Hepatocytes isolated from ALD liver tissue and normal resected liver tissue showed similar responses with respect to ROS accumulation during hypoxia and H-R but had greater basal intracellular ROS contents; this possibly reflected their continual exposure to an inflammatory microenvironment.[25] Hepatocytes isolated from the tissue of patients with the biliary diseases, primary biliary cirrhosis and primary sclerosing cholangitis, had very low basal levels of ROS production similar to those detected in hepatocytes from normal livers, but they showed a 22-fold increase in ROS production during hypoxia and a reduction in ROS accumulation during H-R. This may be a reflection of increased engagement of hepatocyte cytosolic antioxidant defenses, which is possibly a result of cholestasis, a common feature of these diseases.

ROS Activation Mediated Hepatocyte Apoptosis and Necrosis

We next assessed the effects of hypoxia and H-R on human hepatocyte cell death. Previous in vitro studies have shown that human hepatoma cell lines[26] and murine[27] and rodent hepatocytes[28] undergo cell death during hypoxia and H-R. We have found that human hepatocytes isolated from normal liver tissue and diseased liver tissue experience increased apoptosis and necrosis during hypoxia and H-R (Fig. 2A,B). The level of human hepatocyte apoptosis and necrosis during hypoxia and H-R mirrored the level of intracellular ROS production within the particular type of hepatocyte. The decrease in ROS production observed in hepatocytes isolated from the tissue of patients with biliary diseases was accompanied by a concomitant decrease in apoptosis and necrosis; this confirmed the association of ROS with apoptotic and necrotic cell death. The highest levels of ROS were seen in hepatocytes isolated from normal resected liver tissue. Despite the increase in intracellular ROS in normal hepatocytes isolated from resected liver tissue, the level of apoptosis or necrosis did not increase; this suggested an important difference in the metabolic activity or protective mechanisms of these cells. Moreover, hepatocytes from normal resected tissue did have higher basal levels of both apoptosis and necrosis.

Effect of ROS Inhibitors on ROS Accumulation

Antioxidants and inhibitors of ROS generation have been shown to abrogate human hepatoma cell line death during hypoxia.[26] Figure 3 shows the effects of antioxidants, mitochondrial chain inhibitors, and NADPH oxidase inhibitors on primary human hepatocyte ROS production during H-R. Similar results were observed during normoxia and hypoxia (data not shown). NAC acts as a glutathione precursor; it enters cells and interacts with and detoxifies free radicals by nonenzymatic reactions. It is deacetylated to form cysteine, which supports the biosynthesis of glutathione, one of the most important components of the intracellular antioxidant system.[29] NAC almost completely inhibited ROS production in all hepatocytes during H-R. Rot, a mitochondrial complex I inhibitor, was also able to inhibit ROS production in hepatocytes isolated from all sources, and this confirmed mitochondria as a major source of endogenous ROS in human hepatocytes. The inhibition of ROS by Rot was substantial but not as great as that observed with NAC. The production of ROS in the presence of mitochondrial inhibition implies the involvement of other mechanisms in human hepatocytes. Accordingly, we found that the flavoenzyme NADPH oxidase was also involved in ROS production within the hepatocyte. The specific NADPH oxidase inhibitor DPI significantly decreased ROS production in all human hepatocytes. Thus, although the overall effect was not as great as that of Rot, NADPH oxidase is also an important source of ROS in human hepatocytes. In all cases, vehicle controls caused no inhibition of intracellular hepatocyte ROS levels (data not shown).

Effects of ROS Inhibitors on Human Hepatocyte Apoptosis and Necrosis

Because NAC, Rot, and DPI all inhibited ROS, we assessed whether this decrease in ROS affected

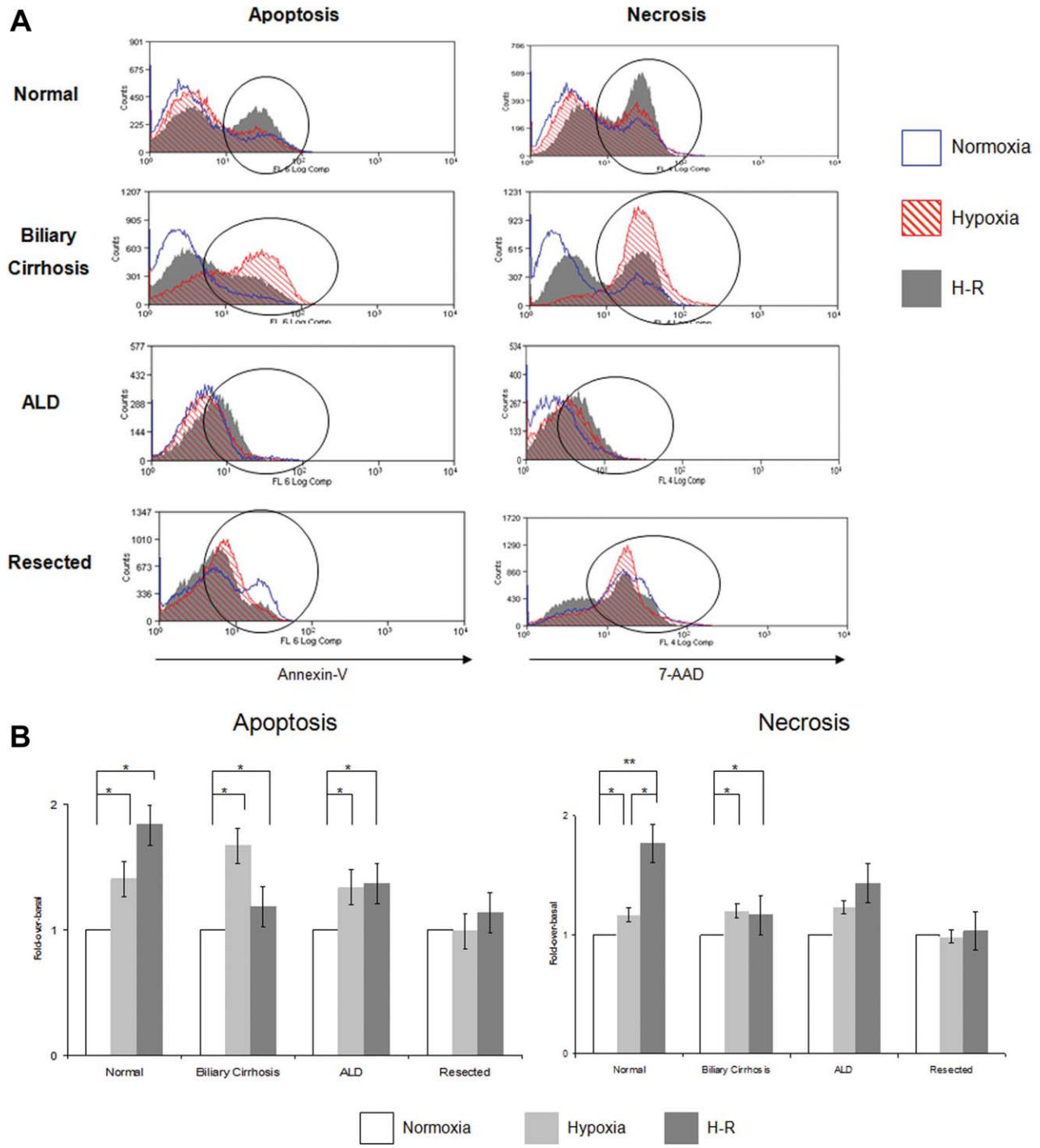


Figure 2. Hypoxia and H-R induce human hepatocyte apoptosis and necrosis. (A) Representative flow cytometry plots are shown to illustrate the effects of hypoxia and H-R on the apoptosis and necrosis of human hepatocytes isolated from normal liver tissue, diseased liver tissue, and normal resected liver tissue. The areas of interest within the plots are marked by vertical ellipses. Just as in the ROS plots shown in Fig. 1, the area to the left of each vertical ellipse represents cell debris. The data are representative of 4 normal hepatocyte preparations, 4 biliary disease preparations, 3 ALD preparations, and 9 normal resected liver preparations. (B) The bar charts show pooled data illustrating the level of apoptosis and necrosis in human hepatocytes isolated from normal tissue, diseased tissue, and normal resected tissue during hypoxia and H-R. The data are shown as fold over the basal level. The levels of apoptosis and necrosis occurring during normoxia have been taken as the basal levels. The data are expressed as means and standard errors. The data are representative of 4 normal hepatocyte preparations, 4 biliary disease preparations, 3 ALD preparations, and 9 normal resected liver preparations. *$P < 0.05$; **$P < 0.05$.

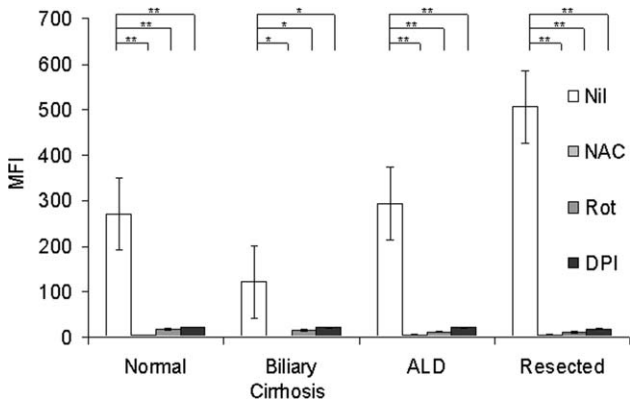


Figure 3. Antioxidants, mitochondrial chain inhibitors, and NADPH oxidase inhibitors reduce human hepatocyte ROS production during H-R. Human hepatocytes isolated from normal livers, diseased livers, and normal resected livers were treated with 20 mM NAC, 2 μM Rot, or 10 μM DPI during H-R. ROS accumulation was determined by flow cytometry as described in the Materials and Methods section. ROS production data are shown as MFIs and are based on the MFI readings taken from cells within the ellipse region shown in Fig. 1. The data are expressed as means and standard errors. The data are representative of 3 normal hepatocyte preparations, 3 biliary disease preparations, 3 ALD preparations, and 5 normal resected liver preparations. *$P < 0.01$; **$P < 0.01$.

human hepatocyte apoptosis and necrosis. Data for H-R are shown in Fig. 4; similar results were observed during normoxia and hypoxia (data not shown). The reduction in endogenous ROS levels in human hepatocytes treated with the various inhibitors led to significantly decreased hepatocyte apoptosis (Fig. 4A) and necrosis (Fig. 4B). In line with the more potent effect of NAC on ROS inhibition, the effects of NAC were the greatest. Rot and DPI also decreased apoptosis and necrosis during H-R. Moreover, ROS inhibition abrogated apoptosis in human hepatocytes isolated from tissue taken from patients with different types of hepatic diseases but only partially inhibited hepatocyte necrosis. These data provide clear evidence that endogenous ROS production by mitochondria and NADPH oxidase drives human hepatocyte apoptosis and necrosis during H-R.

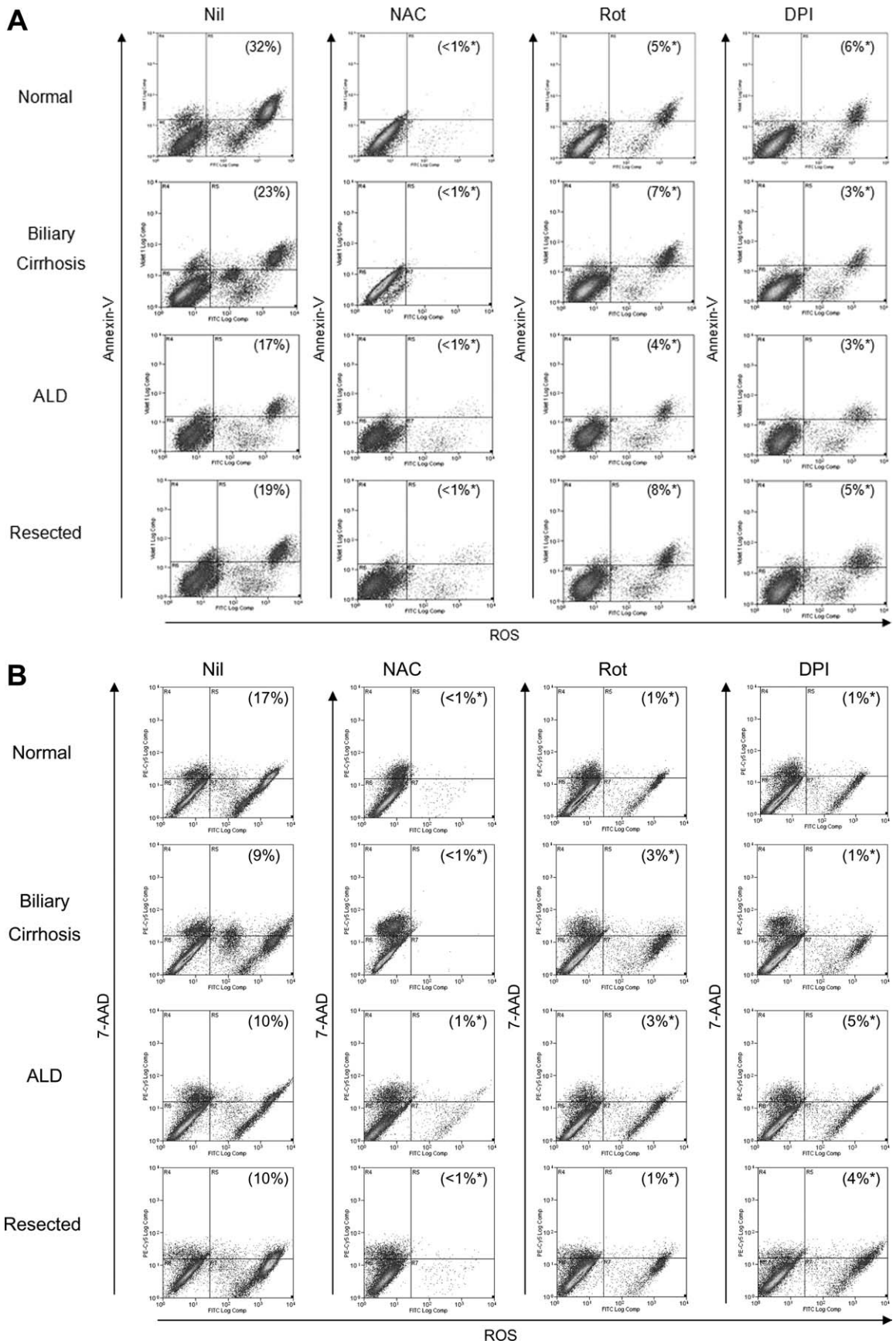
DISCUSSION

Although hypoxia is a feature of hepatic IRI associated with hepatic surgery, it may also occur during chronic liver inflammation or infection. Therefore, by defining the effects of hypoxia on liver physiology, we can obtain important information regarding the development of future therapeutics.

Hepatocytes have conventionally been considered relatively insensitive to IRI because of their extensive antioxidant defense mechanisms and their existence within the comparatively hypoxic hepatic environment.[30] However, numerous in vitro and in vivo studies have reported deleterious effects of IRI on hepatocytes, although the effects of hypoxia and H-R on human hepatocytes are unknown. Our data reveal that hepatocytes are susceptible to apoptosis and necrosis induced in response to hypoxia and H-R, and we report for the first time that human hepatocytes isolated from patients with different hepatic diseases show variable responses to hypoxia and H-R.

Hypoxia is known to cause dysfunction of the mitochondrial electron transport chain and thus lead to an increase in ROS; this is accentuated by reoxygenation and can result in cell death.[31] Hepatocytes isolated from normal donor livers, ALD livers, and uninvolved livers from hepatic resections exhibit this classic response to hypoxia and H-R. Interestingly, hepatocytes isolated from ALD livers and resected liver tissue have high basal levels of ROS production, and these are then augmented by hypoxia and H-R; however, normal hepatocytes and hepatocytes isolated from the livers of patients with biliary cirrhosis show very little basal ROS production. These differences are likely due to the particular inflammatory microenvironment and milieu to which each hepatocyte population has been exposed. For example, many mediators found in ALD patients are known to increase ROS,[25] and hepatocytes isolated from hepatic resections will have been exposed to chemotherapy, a treatment known to increase intracellular ROS.[32] Hepatocytes isolated from patients with biliary cirrhosis had lower levels of ROS during H-R, and this possibly reflected the up-regulation of the antioxidant defenses in these particular cells. The differential responses of hepatocytes isolated from patients with different hepatic diseases are unlikely to be attributable to the method of isolation because the liver tissues were procured and processed according to an identical and stringent protocol. The precise cellular mechanisms underlying hepatocyte responses to hypoxia and H-R remain the subject of ongoing research within our laboratory.

It has been suggested previously that hepatocytes are bystanders in IRI and are targeted by the inflammatory process.[2] We now propose that they have the capacity to actively participate in it primarily through the production of ROS. The functional relevance of ROS is emphasized by our finding that increased levels of endogenous ROS are clearly linked to hepatocyte apoptosis and necrosis. This has particular relevance for liver diseases in which hepatocytes are exposed to relative hypoxia and may be responsible for perpetuating injury. We have demonstrated that ROS can directly activate apoptosis and necrosis and thereby support the suggestion of Jaeschke and Lemasters[20] that common pathways may at least in part regulate both processes. Moreover, ROS derived from mitochondria and cytosolic NADPH oxidase are crucial for regulating both apoptosis and necrosis, and their inhibition significantly improves human hepatocyte viability during hypoxia and H-R. The final mode of cell death is likely to be dictated by the intracellular adenosine triphosphate content.[33] Interestingly, ROS inhibition had a greater effect on reducing human hepatocyte apoptosis and only partially inhibited hepatocyte necrosis. Although ROS does contribute to human hepatocyte necrosis, it is likely that a


A
Nil
NAC
Rot
DPI
Normal
Biliary Cirrhosis
ALD
Resected
Annexin-V
ROS
(32%)
(<1%*)
(5%*)
(6%*)
(23%)
(<1%*)
(7%*)
(3%*)
(17%)
(<1%*)
(4%*)
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(19%)
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ROS
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Figure 4.

number of factors, including calcium overload, calpain activation, and lysosome rupture, force the cell to undergo necrosis.[34] In contrast, Wang et al.[35] demonstrated that ROS derived from mitochondria directly activated apoptosis in Chang human hepatocytes. Accordingly, the inhibition of ROS and, in particular, mitochondrial ROS had a greater effect on reducing hepatocyte apoptosis. As previously noted, hepatocytes undergoing necrosis will release intracellular ROS into the liver parenchyma and induce both hepatocyte and endothelial cell activation.[36] Therefore, our observations have implications not only for hepatic IRI but also for liver diseases in which chronic hypoxia leads to continued ROS production and ongoing liver damage. In separate experiments, we exposed human hepatic sinusoidal endothelial cells (HSECs) to the same in vitro model of warm IRI. HSECs did not increase intracellular ROS during hypoxia and H-R and did not undergo any significant level of cell death during hypoxia and H-R (R.H.B., unpublished data, 2010). These observations show that liver epithelial and endothelial cells have different responses to hypoxia and H-R, and these differences are likely to shape the hepatic inflammatory microenvironment. On the basis of our data, we speculate that in warm IRI, hepatocyte ROS (not HSEC-derived ROS) may be important regulators of hepatic injury. The precise role of HSECs during warm IRI is beyond the scope of this particular study.

In rat livers, treatment with antioxidants can prevent IRI.[37] In limited human studies, ischemia has induced the expression of ROS scavengers within the liver.[38] Despite the presence of induced antioxidant mechanisms, human hepatocytes isolated from normal livers, the livers of patients with ALD, and the livers of patients with biliary cirrhosis do not appear to be protected against cell death during hypoxia and H-R. Hepatocytes isolated from normal resected liver tissue were, however, surprisingly resistant to ROS-mediated apoptosis and necrosis. This finding has important implications for research involving human hepatocytes and suggests that studies should be interpreted in the context of the hepatocyte source; hepatocytes isolated from resected hepatic tissues of patients with liver tumors are likely to respond differently to physiological stress. As mentioned earlier, the reason for this difference remains unknown. Furthermore, which hepatocyte response reflects the true physiological response remains to be determined. In separate experiments, NAC, Rot, and DPI were used to treat HepG2, Huh7, and PLC/PRF/5 human hepatoma cell lines during hypoxia and H-R. These inhibitors induced overwhelming cell death in these particular cell lines, and this indicated vastly different responses to hypoxia and H-R in comparison with primary hepatocytes (data not shown). Therefore, although previous studies have shown cytoprotective effects of antioxidants and ROS inhibitors, we report here for the first time that the inhibition of mitochondrial and NADPH oxidase–derived ROS reduces primary human hepatocyte apoptosis and necrosis.

A single strategy aimed at the amelioration of the harmful effects produced by IRI has not yet been adopted into general clinical practice. Experimental interventions to reduce ROS have shown potential for minimizing liver injury in various models. ROS scavengers,[39] thioredoxin mimetics,[40] and the delivery of antioxidant genes[41] have been shown to partially suppress the effects of IRI. However, the clinical application of such compounds has been limited for toxicological and technical reasons. NAC, however, has clinical potential because it is known to be well tolerated at doses that should be clinically effective. Indeed, NAC is used clinically in several settings; for example, it is used as a hepatoprotective agent for acute liver failure and acetaminophen toxicity. Furthermore, although NAC administration has been shown to improve hepatic microcirculation and bile flow after hepatic IRI,[7] we now show that NAC can also reduce all ROS production in human hepatocytes with a concomitant decrease in apoptosis and necrosis during normoxia, hypoxia, and H-R. Recent studies have suggested that pleiotropic compounds are required to treat IRI because of the diverse nature of the problem.[42] We suggest that exogenous NAC could be a straightforward, practical, and beneficial strategy for ameliorating human hepatocyte cell death during IRI. Indeed, a recent randomized study showed that the systemic infusion of NAC before liver procurement reduced graft dysfunction and early graft loss after liver transplantation.[43]

Although some authors have challenged the pathophysiological relevance of intracellular oxidant stress during reperfusion,[44] we have clearly shown that hepatocyte ROS generated by mitochondria and NADPH oxidase can lead directly to significant hepatocyte cell death; we suggest that although other sources of ROS, such as neutrophils and KCs, are capable of contributing to tissue ROS accumulation in IRI, they may not be the only pathways leading to ROS-

Figure 4. (A) ROS accumulation in human hepatocytes during H-R mediates apoptosis. Human hepatocytes isolated from normal tissue, diseased tissue, and normal resected tissue were treated with 20 mM NAC, 2 μM Rot, or 10 μM DPI during H-R, and the percentages of cells that were stained with both the ROS probe DCF and the apoptotic marker annexin V were assessed by flow cytometry. The cross-plots show ROS on the *x* axis and apoptosis on the *y* axis. The percentages of human hepatocytes that were stained with both DCF and annexin V are shown in parentheses. The data are representative of 3 normal hepatocyte preparations, 3 biliary disease preparations, 3 ALD preparations, and 5 normal resected liver preparations. $^*P < 0.001$. (B) ROS accumulation in human hepatocytes during H-R mediates necrosis. Human hepatocytes isolated from normal tissue, diseased tissue, and normal resected tissue were treated with 20 mM NAC, 2 μM Rot, or 10 μM DPI during H-R, and the percentages of cells that were stained with both the ROS probe DCF and the necrotic marker 7-AAD were assessed by flow cytometry. The cross-plots show ROS on the *x* axis and necrosis on the *y* axis. The percentages of human hepatocytes that were stained with both DCF and 7-AAD are shown in parentheses. The data are representative of 3 normal hepatocyte preparations, 3 biliary disease preparations, 3 ALD preparations, and 5 normal resected liver preparations. $^*P < 0.01$.

mediated hepatocyte damage. An important caveat to our data is that oxidative stress in human hepatocytes after hypoxia and H-R may differ between hypoxia at 4°C and hypoxia at 37°C.[45] Therefore, although these data can be applied to the transplant setting, they are not wholly reflective of the in vivo situation.

In summary, our data show that human hepatocyte responses to hypoxia and H-R are determined by their particular microenvironment. Both apoptosis and necrosis are regulated by endogenous human hepatocyte ROS, and inhibitors of ROS generation significantly improve hepatocyte viability by reducing ROS generation. The use of NAC offers an opportunity to modulate hepatic IRI and improve patient outcomes after OLT, possibly through its addition to preservation fluids. In addition, our studies demonstrate for the first time that hepatocytes taken from normal tissue, normal resected tissue, and diseased tissue may vary considerably in their functional and metabolic responses to hypoxic stress.

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