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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 suggest that 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.

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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 liver injury during IRI are complex and include extracellular matrix accumulation, growth factor signaling, and apoptosis. The ability to modulate IRI may therefore offer several therapeutic benefits.

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. 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. 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). 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. 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. 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, whereas others have shown that the absence or elimination of KCs does not prevent tissue damage in IRI. 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. 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. Furthermore, the inhibition of mitochondrial complexes I and III can ameliorate ROS production in human hepatoma cell lines and rat hepatocytes. Other enzymes, such as the flavoenzyme nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, can also produce ROS in rat hepatocytes. The accumulation of intracellular ROS induces cell death, and during hepatic IRI, hepatocytes undergo both apoptosis and necrosis. Some studies have supported apoptosis as the primary mode of death, and others have supported necrosis. 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 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. 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 hepatic IRI, we showed 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 50g 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 μg/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% CO₂ 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% CO₂ and 95% N₂ 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. 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. 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 Kingdom). 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.
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
diseases. The result of cholestasis, a common feature of these cytosolic antioxidant defenses, which is possibly a reflection of increased engagement of hepatocyte ROS accumulation during H-R. This may increase in ROS production during hypoxia and H-R. We have found that human hepatocyte apoptosis and necrosis during hypoxia and H-R mirrored the level of intracellular ROS accumulation. Hepatocytes isolated from normal liver tissue and diseased 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. 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 lines26 and murine27 and rodent hepatocytes28 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

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

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Biliary Cirrhosis</th>
<th>ALD</th>
<th>Resected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normoxia</td>
<td>30.3 (13.3-53.5)</td>
<td>11.9 (6.2-21.1)</td>
<td>226.5 (217.1-247.1)</td>
<td>352.8 (256.4-450.5)</td>
</tr>
<tr>
<td>Hypoxia</td>
<td>121.9 (39.6-166.2) *</td>
<td>255.2 (139.9-444.0) *</td>
<td>295.9 (229.2-281.2)</td>
<td>377.1 (284.3-547.2)</td>
</tr>
<tr>
<td>H-R</td>
<td>271.1 (217.7-370.0) *</td>
<td>102.6 (35.2-151.0) *</td>
<td>294.7 (278.2-311.1)</td>
<td>506.1 (332.2-874.0)</td>
</tr>
</tbody>
</table>

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.
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.
resected liver preparations. *disease preparations, 3 ALD preparations, and 5 normal represent of 3 normal hepatocyte preparations, 3 biliary data are expressed as means and standard errors. The data are described in the Materials and Methods section. ROS production ROS accumulation was determined by flow cytometry as within the comparatively hypoxic hepatic environment. ROS is emphasized by our finding that increased 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 Lemasters²⁰ 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 hepatocyte apoptosis and only partially inhib-
number of factors, including calcium overload, cal-
pain activation, and lysosome rupture, force the cell
to undergo necrosis.\textsuperscript{34} In contrast, Wang et al.\textsuperscript{35} de-
amonstrated that ROS derived from mitochondria
directly activated apoptosis in Chang human hepato-
cytes. Accordingly, the inhibition of ROS and, in par-
ticular, mitochondrial ROS had a greater effect on
reducing hepatocyte apoptosis. As previously noted,
hepatocytes undergoing necrosis will release intracel-
lular ROS into the liver parenchyma and induce both
hepatocyte and endothelial cell activation.\textsuperscript{36} There-
fore, 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 hy-
poxia 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 microen-
vironment. 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 pre-
vent IRI.\textsuperscript{37} In limited human studies, ischemia has
induced the expression of ROS scavengers within the
liver.\textsuperscript{38} Despite the presence of induced antioxidant
mechanisms, human hepatocytes isolated from nor-
mal 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 men-
tioned 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 dif-
ferent 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 mitochon-
drial and NADPH oxidase–derived ROS reduces pri-
mary 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 scav-
engers,\textsuperscript{39} thioredoxin mimetics,\textsuperscript{40} and the delivery of
antioxidant genes\textsuperscript{41} have been shown to partially sup-
press the effects of IRI. However, the clinical applica-
tion of such compounds has been limited for toxico-
logical and technical reasons. NAC, however, has
clinical potential because it is known to be well toler-
ated 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. Fur-
thermore, although NAC administration has been
shown to improve hepatic microcirculation and bile
flow after hepatic IRI,\textsuperscript{7} we now show that NAC can also
reduce all ROS production in human hepatocytes
with a concomitant decrease in apoptosis and necro-
sis during normoxia, hypoxia, and H-R. Recent stud-
ies have suggested that pleiotropic compounds are
required to treat IRI because of the diverse nature of
the problem.\textsuperscript{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.\textsuperscript{43}

Although some authors have challenged the patho-
physiological relevance of intracellular oxidant stress
during reperfusion,\textsuperscript{44} we have clearly shown that he-
patocyte 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-
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.\textsuperscript{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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**REFERENCES**


