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Cold storage sensitizes hepatocytes to oxidative stress injury

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Abstract Liver cold storage leads to oxygen free radical production and reperfusion injury. Antioxidants are effective in suppressing reperfusion injury in rat livers when used in the reperfusion medium. However, in clinical liver transplantation their effectiveness is not clear, which may be due to the way they are used (in the recipient). In this study we compare the effectiveness of antioxidants when used in the reperfusion medium versus the cold storage solution in isolated hepatocytes and the isolated perfused liver. Hepatocytes were cold stored in UW solution for 24 h. Oxidative stress, induced by t-butyl hydroperoxide (tBHP), was measured in the presence of one of five different antioxidants – deferoxamine (DFO), dithiothreitol (DTT), trolox, tocopherol, dimethylthiourea (DMTU) – in the reperfusion buffer or UW solution. Efficacy was judged by reduction in membrane damage (LDH release) during rewarming. Also, rat livers were cold stored for 48 h in UW solution (± antioxidant) and reperfused (± antioxidants). Efficacy was judged by the effect on enzyme release and bile production. Cold storage of hepatocytes for 24 h sensitized them to oxidative stress. The concentration of tBHP required to induce maximal cell death (80 %–90 % LDH release) was reduced from 1.3 mM (fresh cells) to 0.37 mM (LD-50 values). All antioxidants except DMTU suppressed oxyradical-induced LDH release when used in the reperfusion medium, but only DFO was effective when used in the UW solution. In the isolated perfused liver, DFO, DTT, and trolox were effective and suppressed enzyme release when added to the reperfusion buffer, but none were effective when used in the UW solution. We conclude that cold storage sensitizes liver cells to oxidative stress. The most effective antioxidant was the iron chealator, DFO, which was effective in the reperfusion buffer (isolated perfused liver or hepatocytes) but not in the UW solution when tested in the isolated perfused liver. Suppression of reperfusion injury in liver transplantation could be obtained by antioxidant therapy. However, it is unclear how best to deliver the antioxidants to the site of oxyradical generation.

Key words Hepatocytes, cold storage, oxidative stress injury · Cold storage, hepatocytes, oxidative stress injury · Oxidative stress injury, hepatocytes, cold storage

Introduction

Reperfusion of statically cold-preserved livers results in the formation of oxyradicals that can initiate membrane damage [15] by reacting with proteins, enzymes, and polynucleotides that could ultimately lead to cell death and possible microcirculatory blockage. These events may cause injury to the newly transplanted liver, resulting in primary nonfunction or initial poor function. These complications in clinical liver transplantation lead to increased costs with the possible need for retransplantation, extensive intensive care stays, an increased chance of rejection and/or death of the recipient.

Numerous antioxidants have been shown to suppress reperfusion injury caused by oxyradicals in livers and other organs and under different stress conditions [2, 9, 10, 12, 13]. There has been increasing interest in utilization of antioxidants in organ transplantation with clinical trials of some antioxidants (superoxide dismutase, allopurinol) reported [14]. However, results in the clinics as well as in laboratory models have not given conclusive evidence that antioxidants could play a significant role in improving transplant outcome.

In general, antioxidants have been most often used in the recipient. The lack of efficacy in clinical use may be due to poor distribution of the antioxidant at the site of oxyradical production. Oxyradical generation appears to occur very rapidly upon rewarming and reperfusion of cold-stored organs [3]. Thus, to be effective, the antioxidants need to be present at the site of generation of oxyradicals prior to reperfusion.

One of our goals in liver preservation is to enhance of the efficacy of the University of Wisconsin (UW) solution. One type of additive that could be useful is an antioxidant compound. In this study, we have used isolated cold-stored hepatocyte and isolated perfused liver models to determine if antioxidants can effectively suppress cell death caused by oxidative stress after cold storage and simulated reperfusion (rewarming-reoxygenation). The antioxidants were tested for effectiveness in the reperfusion media and/or in the cold storage solution.

Materials and methods

Hepatocyte preparation

Sprague-Dawley rats (Harlan Sprague Dawley, Madison, Wis.) weighing 300–400 g were used for these studies. Hepatocytes were separated from the liver by collagenase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) digestion according to the method of Seglen [24], as described previously by Marsh et al. [17]. Hepatocyte suspensions were judged suitable for use if greater than 80 % of the cells excluded trypan blue and lactate dehydrogenase (LDH) release was less than 10 %.

Hepatocyte preservation

Hepatocyte suspensions to be cold stored were sedimented from the isolation medium (600 *g* for 2 min) and resuspended in cold (4 °C) UW solution previously equilibrated with nitrogen for 15 min to lower dissolved oxygen. Hepatocytes were stored at a protein concentration of 5 ± 1 mg/ml, as determined by the Biuret method. Hepatocyte suspensions in UW solution were placed in 50-ml polycarbonate tubes and gassed with nitrogen. The tubes were sealed, put in a refrigerator (5 °C), and left undisturbed for 24 h to simulate cold ischemia (anoxia and no perfusion). In some studies, antioxidants were used during preservation and were added directly to the UW solution immediately before use.

Hepatocyte reperfusion

Fresh and 24-h cold-stored hepatocytes were sedimented (600 *g* for 2 min) and resuspended at a protein concentration of 5 ± 1 mg/ml in Krebs-Henseleit bicarbonate (KHB) containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 11.1 mM glucose, 10 mM $NaHCO_3$, and 20 mM HEPES. Five-milliliter aliquots of the resuspended hepatocytes were placed into a 25-ml Erlenmeyer flask and continuously shaken in a Lab-Line orbital shaker (90 rpm) at 37 °C. The gas phase was room air yielding an average partial pressure of oxygen in the solution of 150 mm Hg. Pro-oxidants were added to hepatocyte suspensions just before initiation of normothermic incubation. The pro-oxidants used were tert-butyl hydroperoxide (tBHP), menadione [28], acetaminophen [20], and $HgCl_2$ [8]. All pro-oxidants were obtained from Sigma Chemical (St. Louis, Mo.). In some experiments, antioxidants were added to the hepatocyte suspensions 20 min before addition of the pro-oxidant. The six antioxidants tested were N-acetylcysteine (NAC), vitamin E (α-tocopherol), trolox, dimethylthiourea (DMTU), dithiothreitol (DTT), and deferoxamine (DFO). A concentrated solution of α-tocopherol was prepared using ethanol such that less than 10 µl was added to the incubation medium. After 120 min of normothermic incubation, aliquots of the suspensions were removed for analysis.

LDH measurement

A 0.5-ml aliquot of the hepatocyte suspension was centrifuged (13,000 rpm for 1 min, Eppendorf microcentrifuge) and LDH activity was measured in the cell-free supernate fluid (extracellular LDH) by following the rate at which NADH was converted to NAD at 340 nm in the presence of pyruvate [1]. Total LDH activity was determined in a 0.5-ml aliquot of hepatocytes that had been ultrasonically disrupted. Results are expressed as the percentage of total LDH that was extracellular (percentage of LDH release).

TBA Reactants measurement

Thiobarbituric acid (TBA) reactants were determined by the method described by Stacey et al. [25]. Briefly, 0.5 ml of hepatocyte suspension was added to 1 ml of 10 % trichloroacetic acid, followed by 50 µl of 2 % butylated hydroxytoluene. An additional 2 ml of a 0.67 % solution of 2-thiobarbituric acid was added and the samples were incubated in a boiling water bath for 15 min. Following incubation, the samples were centrifuged at 4800 rpm for 2 min and the absorbance of the supernatant was determined at 532 nm. 1,1,3,3-Tetraethoxyproprane (Sigma Chemical) was used to generate a standard curve.

Isolated perfused liver

Whole livers were harvested from animals anesthetized with 7.2 % chloral hydrate i.p. The bile duct was cannulated and a catheter was placed in the portal vein. The livers were flushed with 10 ml of cold (4 °C) UW solution equilibrated with room air and containing 1000 units heparin. The livers were removed and then flushed with an additional 5 ml of cold UW solution before being stored at 4 °C for 0–48 h. After cold storage, the livers were reperfused at 37 °C with KHB equilibrated with 95 % O_2 / 5 % CO_2 in a recirculating system. The perfusion rate was 3 ml min^{-1} per g liver [19]. The livers were perfused for 90 min and perfusate collected for measuring LDH and aspartate aminotransferase (AST) using spectrophotometric assay kits (LDH kit 340-LD and AST kit DG 158-K, Sigma Chemical). Bile was collected every 15 min and weighed.

Statistics

All data represent at least three experiments and are expressed as means ± SEM. The data were analyzed using the paired *t*-test and a probability of below 0.05 was considered statistically significant.

Results

Cold storage sensitizes hepatocytes to oxidative stress

The pro-oxidant tert-butyl hydroperoxide (tBHP) causes oxidative stress in hepatocytes [23], leading to increased leakage of cytosolic enzymes, including LDH. The concentrations of tBHP that caused nearly complete (80 %–90 %) leakage of LDH from 24-h cold-stored cells (0.6–1.0 mM) had practically no effect on LDH leakage from freshly isolated cells (Fig. 1). The concentration of tBHP to give 50 % release of LDH (LD-50) was estimated from the titration curves shown in Fig. 1. In freshly isolated cells, the LD-50 concentration was 1.23 mM versus only 0.37 mM in cold-stored hepatocytes.

The increased sensitivity of cold-stored hepatocytes to tBHP was not unique to this pro-oxidant. Other pro-oxidants commonly used in studies of oxidative stress in hepatocytes showed an effect similar to tBHP (Table 1). The LD-50 values for menadione, acetaminophen, and $HgCl_2$ were estimated from titration curves obtained as described for tBHP. Cold-stored hepatocytes were 1.8–3.6 times more sensitive to pro-oxidants than freshly isolated hepatocytes, as judged by increased LDH leakage.

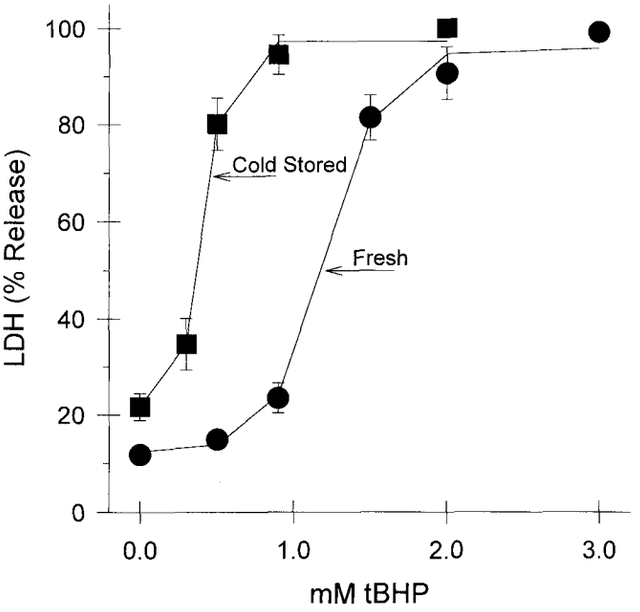

Fig. 1 Percent LDH release in rat hepatocytes exposed to the pro-oxidant tert-butyl hydroperoxide (tBHP). Values are means ± SEM of five to nine hepatocyte preparations

Table 1 Effect of pro-oxidants on hepatocytes. Values are means ± SEM for four to seven hepatocyte preparations. * $P < 0.05$ vs control

Pro-oxidant	LD50	
	Control	24 h CS
tBHP (mM)	1.23 ± 0.03	0.37 ± 0.03*
Menadione (μM)	204 ± 36	56 ± 8*
Acetaminophen (mM)	106 ± 6.1	55.9 ± 6.9*
$HgCl_2$(μM)	27.1 ± 1.1	15.4 ± 1.7*

Inhibition of t-BHP-induced cell death by antioxidants

We tested the effects of six antioxidants for their capability to suppress tBHP-induced release of LDH from 24-h cold-stored hepatocytes. Each antioxidant was present only in the reperfusion medium at the concentrations given in the legend to Fig. 2. The concentration of tBHP used to induce cell death was the LD-50 concentration (0.37 mM) obtained from a previous experiment (Fig. 1). In this set of experiments, tBHP caused an increase in LDH leakage from about 20 % to about 60 %. Five of the six antioxidants studied effectively suppressed (significant at $P < 0.05$) tBHP-induced cell death. Only the hydroxyl radical scavenger, dimethylthiourea (DMTU), was ineffective.

We also measured the effect of antioxidants on tBHP-induced lipid peroxidation as assessed by the concentration of thiobarbiturate reactive substances (TBARS) in the hepatocyte suspension after 24-h cold storage and rewarming (Fig. 3). tBHP at the LD-50 dose increased TBARS from about 0.15 nmol/mg protein to 0.75 nmol/mg protein. The antioxidants that blocked tBHP-induced cell death also blocked tBHP-induced lipid peroxidation. The inhibition of TBAR for-

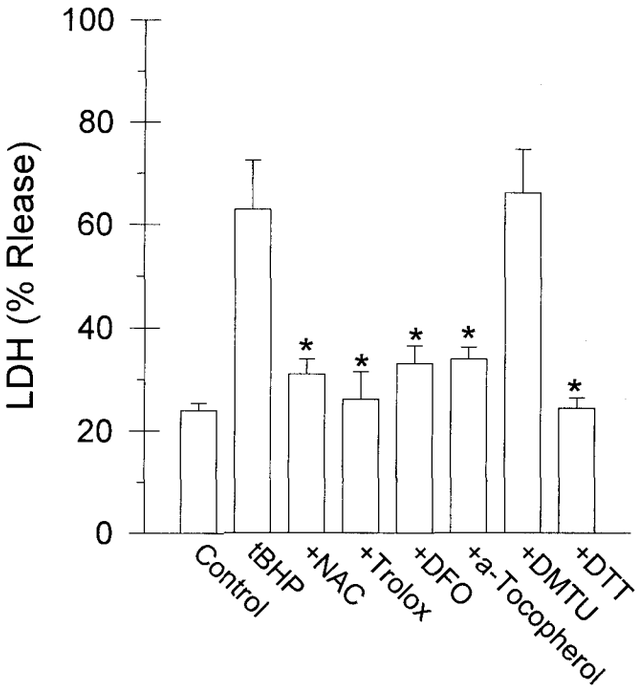

Fig. 2 Effect of antioxidant pretreatment on percent LDH release in 24-h cold-stored rat hepatocytes incubated for 2 h at 37 °C in KHB in the presence of 0.37 mM tBHP. After cold storage, hepatocytes were given a 20-min preincubation with each antioxidant prior to the addition of tBHP. Antioxidants were used in the following concentrations: N-acetylcysteine (NAC) 5 mM, trolox 3 mM, deferoxamine (DFO) 2.5 mM, a-tocopherol 1 mM, dimethylthiourea (DMTU) 3 mM, and dithiothreitol (DTT) 3 mM. Data are expressed as means ± SEM for five to seven hepatocyte preparations. $^*P < 0.05$ vs hepatocytes exposed to tBHP without antioxidant pretreatment

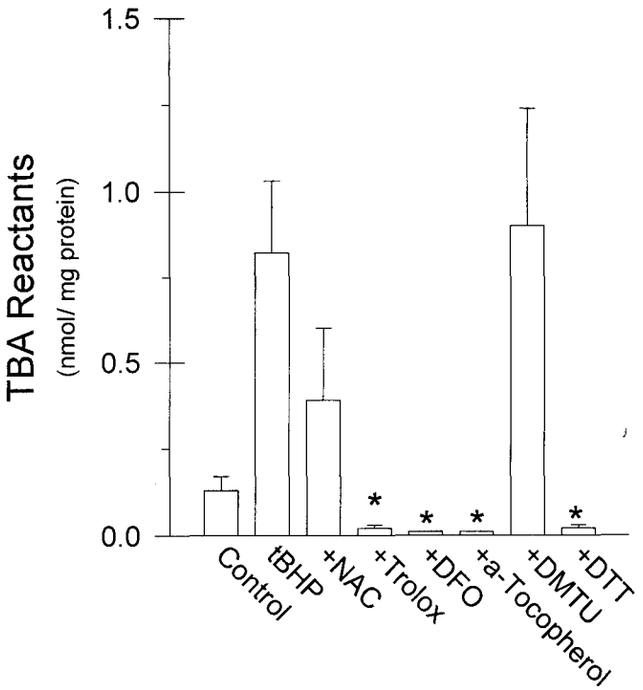

Fig. 3 Effect of antioxidant pretreatment on lipid peroxidation in 24-h cold-stored hepatocytes incubated for 2 h at 37 °C in the presence of 0.37 mM tBHP. Thiobarbituric acid reactive substances (TBARS) were used as an indicator of lipid peroxidation. Values are means ± SEM of three to five hepatocyte preparations. $^*P < 0.05$ vs hepatocytes exposed to tBHP without antioxidant pretreatment

mation by the antioxidants was significant except for NAC. DMTU, which did not prevent tBHP-induced cell death, also did not block tBHP-induced lipid peroxidation.

Effect of antioxidants in the cold storage solution on tBHP-induced cell death

Hepatocytes were cold stored for 24 h in UW solution containing different concentrations of three antioxidants (DFO, trolox, and DTT). We chose these antioxidants because the previous experiment showed them to be the most effective. After cold storage, the hepatocytes were resuspended in KHB without antioxidants, but with tBHP (0.37 mM). In 24 h cold-stored hepatocytes, tBHP increased LDH release from about 20 % to over 90 % (Fig. 4). Only DFO in the UW solution blocked tBHP-induced cell death (LDH 30 %–40 %). Trolox and DTT were ineffective. DFO was effective at each of the concentrations tested (2.5–10 mM).

Effect of DFO on oxidative stress in the isolated perfused rat liver after cold storage

As shown above, DFO effectively blocked oxidative stress induced by tBHP in hepatocytes. DFO was effective when used both in the reperfusion buffer and in the preservative (UW solution). To test the effectiveness of DFO in the whole liver, we used the isolated perfused rat liver model of cold storage/reperfusion. In this model, 48 h rather than 24-h cold storage was used as it induces significantly more injury (enzyme release and reduction in bile production). Furthermore, livers are almost viable after 24 h of cold storage but not after more than 40 h of cold storage, as tested by orthotopic transplantation [26]. DFO was tested either as an additive to the reperfusion buffer (2.5 mM) or in the UW solution (2.5 mM).

After 48 h of cold storage there was a large increase in LDH and AST release from the liver during reperfu-

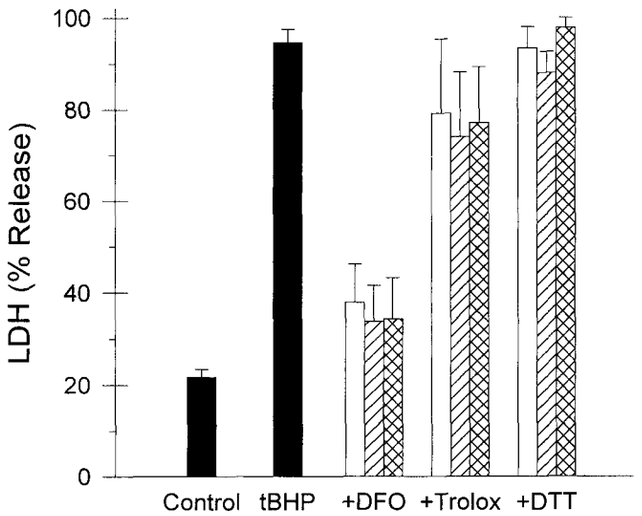

Fig. 4 LDH release in rat hepatocytes cold stored in the presence of antioxidants. Isolated hepatocytes were cold stored in the UW solution with and without three different concentrations of each antioxidant. The concentrations of antioxidants used were: deferoxamine (DFO) 2.5, 5, and 10 mM; Trolox 3, 5, and 10 mM; and Dithiothreitol (DTT) 5, 10, and 20 mM. Of the three antioxidants, only DFO provided significant protection ($P < 0.05$) against the injury caused by tBHP. *Bars* represent means ± SEM of three to five hepatocyte preparations

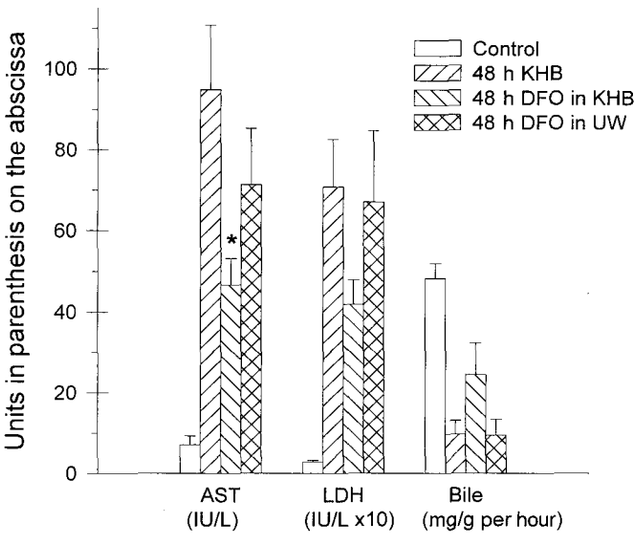

Fig. 5 Effect of the antioxidant deferoxamine (DFO) in whole liver preservation. Rat livers were used fresh or cold stored for 48 h and then reperfused with KHB at 37 °C for 90 min. AST (IU/L) and LDH (IU/L × 10) leakage was determined at the end of reperfusion. Bile production (mg g^{-1} h^{-1}) was determined from samples taken during reperfusion. *$P < 0.05$ vs livers preserved without DFO

sion (Fig. 5). Additionally, bile flow was reduced significantly from control (0-h preservation) values. DFO in the reperfusion buffer suppressed reperfusion-induced enzyme release, which was significantly lower for AST (94.9 ± 15.9 U/l vs 46.7 ± 6.5 U/L, $P < 0.05$) but not for LDH (708.2 ± 117.6 U/L vs 418.6 ± 60.2 U/L, $P = 0.07$). DFO in the reperfusion medium also suppressed, although not significantly, the decrease in bile production caused by cold storage/reperfusion.

We also studied the effect of DFO when used only in the preservative. Rat livers were flushed with cold (4 °C) UW solution containing 2.5 mM DFO. After 48 h of cold storage, the livers were reperfused without DFO. DFO had practically no beneficial effect (Fig. 5) and did not significantly suppress reperfusion-induced enzyme release or the inhibition of bile flow caused by cold storage.

Discussion

In this study we show that cold storage sensitizes isolated hepatocytes to oxidative stress. In our study oxidative stress was induced during rewarming by a pro-oxidant, tBHP. The mechanism of cell death by tBHP involves rapid oxidation of reduced glutathione, peroxidation of cellular lipids, alterations in calcium metabolism, and the formation of oxyradicals [23]. These are events that are thought to occur in transplanted livers that develop primary nonfunction or initial poor function. We show that the concentration of t-BHP (and other pro-oxidants) required to induce cell death is 1.8–3.6 times less than the concentration required to induce the same degree (LD-50) of cell death in freshly prepared hepatocytes. Therefore, cold ischemic storage of hepatocytes reduces their capacity to resist exposure to conditions that result in the generation of oxyradicals.

We developed this model of oxidative stress in cold-stored hepatocytes to determine which antioxidant would best suppress cell death under these conditions. We presumed that the antioxidant that was most efficacious under these conditions would be worthy of testing in a more rigorous model, such as the intact isolated perfused liver. Significant improvements in experimental preservation of livers by antioxidants could lead to clinical applications.

A pro-oxidant was chosen to induce oxidative stress because short-term preservation (24–48 h) of isolated hepatocytes does not induce significant cell death as measured by increased plasma membrane permeability to vital dyes or by leakage of cytosolic enzymes. Consequently, this model is not sensitive enough to allow screening of antioxidants unless cell death by oxidative stress can be reproducibly induced. Also, oxidative stress in the liver is thought to result from pro-oxidants generated upon reperfusion. The pro-oxidants are

thought to originate from activation of endothelial cells, Kupffer cells, or infiltrating macrophages and neutrophils. Use of a pro-oxidant such as tBHP simulates the reperfusion-induced generation of oxidative stress in the liver and allows a test of how preservation sensitizes hepatocytes to this form of injury. Finally, recent studies show that oxidative stress in the hypoxic or ischemic liver not only causes microcirculatory disturbances but also damages hepatocytes [7].

The antioxidants were chosen because they have been shown to be beneficial in some organ preservation models. N-acetylcysteine is a sulfhydral reagent that acts as a cysteine delivery system and stimulates glutathione synthesis in the liver [5]. DTT suppresses oxidation of proteins containing sulfhydral groups and preserves enzyme and membrane integrity by suppression of oxidation of sulfhydral groups [27]. Vitamin E and its water-soluble analogue, trolox, terminate the reactions leading to lipid peroxidation and are thought to help conserve glutathione under conditions of oxidative stress [18]. The iron chelator, DFO, reduces the concentration of free iron in the cell and the formation of hydroxyl radicals, which are thought to play a prominent role in induction of oxidative stress and lipid peroxidation [21]. DMTU is a hydroxyl radical scavenger that is permeable to the plasma membrane [22]. A relatively high concentration of the antioxidants was used (about 3 mM) and suppression of oxidative stress might be due to more than one mechanism.

All of the antioxidants tested, except DMTU, effectively blocked tBHP-induced cell death and lipid peroxidation when added to the reperfusion buffer. However, only DFO was effective when added to the UW solution. If the mechanism of protection by DFO is due to iron chelation, this points to free iron as an initiator of damage in tBHP-induced cell death. Thus, binding free iron may suppress oxidative stress in livers after cold storage and reperfusion. The results suggest that DFO was effective in the UW solution because it entered the cell and was able to bind free iron. When hepatocytes were resuspended in the rewarming buffer and exposed to tBHP, even though DFO may have been washed out of the cells, free iron would have been sufficiently reduced and oxidative stress suppressed. The other antioxidants that were not effective in the UW solution may have been washed out of the cell during the rewarming period and the effective concentration lowered by dilution in the suspending buffer.

Precisely why 24 h of cold storage sensitizes hepatocytes to iron-induced oxidative stress is unclear at the present time. It may be caused by the loss of naturally occurring antioxidants such as glutathione, vitamin E, or coenzyme Q10, stimulation of the rate of production of oxyradicals, or changes in the plasma membrane that may predispose them to lipid peroxidation.

Because DFO was effective in suppressing oxidative stress in rewarmed hepatocytes when included in the UW solution, we selected this antioxidant for testing in the whole liver exposed to preservation/reperfusion. Cold storage of the rat liver leads to membrane injury and leakage of cytosolic enzymes into the reperfusion buffer. The degree of membrane damage increases with time of preservation and is significantly greater after 48 h of preservation than after 24 h [16]. The membrane injury appears to be related partially to oxidative stress since we show that antioxidants in the reperfusion buffer significantly suppressed enzyme leakage. Although DFO, added to the UW solution, suppressed oxidative stress in cold-stored isolated hepatocytes, it was ineffective in the whole liver as tested by the isolated perfused liver. It is unclear why DFO was effective in one model but not the other. It could be due to a difference in distribution of DFO in hepatocytes versus the whole liver. Preservation of the whole rat liver is obtained by flushing the organ with a small volume of UW solution (5–10 ml), whereas isolated hepatocytes are exposed to a much greater volume of UW solution/volume of tissue. Thus, in the whole liver model, an effective concentration of DFO may not have accumulated in the hepatocytes due to either poor distribution of the UW solution throughout the liver or limited diffusion under conditions of static cold storage. This illustrates a difficulty that is encountered when attempting to improve cold storage preservation of livers by simply adding agents to the cold storage solution. The agents may not reach effective concentrations in static cold storage of the liver or may be readily flushed out of the liver upon reperfusion, thus losing their efficacy upon reperfusion.

Antioxidants could play a significant role in reducing the extent of reperfusion injury in the cold-stored liver. However, by themselves, these agents may not be able to suppress primary nonfunction or initial poor function significantly in the clinical arena because the events that cause these problems are most likely multifactorial in origin. Nevertheless, suppression of reperfusion injury could improve the short- and long-term outcome of liver transplantation. Some of the difficulties in using antioxidants are illustrated by this study. Although effective when present during reperfusion, administering antioxidants to the recipient may present complications. For instance, DFO, which is an effective antioxidant, has vasoactive properties that may be a contraindication to its use in a liver transplant recipient [6]. The use in the preservation solution may not be effective because of inadequate distribution throughout the liver. There may also be a rapid flushout of the antioxidant into the systemic circulation upon reperfusion.

The use of antioxidants in cold-stored/reperfused organs could be beneficial as shown in laboratory studies with lazeroids [4, 11], DFO, and other antioxidants [2, 9, 10, 12, 13]. The beneficial effects, however, have

been obtained when the antioxidant was used in donor pretreatment or in the recipient. Beneficial effects specifically related to an antioxidant when used in a preservation solution have not been convincingly documented. A major problem is getting the antioxidant to the site of generation of the oxyradicals, at the proper concentration, and at the appropriate time. This may require the use of liposome-encapsulated antioxidants or hypothermic machine perfusion to equilibrate the tissue with the antioxidants.

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