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Liver hyperthermia and oxidative stress: role of iron and aldehyde production

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Hyperthermia has been used to treat cancer in the liver. However, significant hepatotoxicity occurs at a therapeutic temperature of 42–43°C. We have proposed that heat toxicity is the result of oxidative stress from superoxide generation with resultant lipid peroxidation. Further, iron release from liver iron stores (ferritin) appears to play a central role in hyperthermic toxicity. In this study, rat livers were perfused *in situ* at 37 or 42·5°C with and without deferoxamine for 1 h with an asanguinous perfusate. Oxidative stress was assessed by the efflux of glutathione (GSH) into the perfusage. Prior studies by Skibba *et al.* (1989a, 1991) showed that perfusage equivalents of GSH were primarily present as oxidized glutathione (GSSG). Lipid peroxidation was assessed by the measurement of aldehydes appearing in the perfusate and formation of hydrocarbon gases (ethane and pentane) in the perfusion chamber head space. Liver injury was assessed by the leakage of cytosolic enzymes, AST and LDH, into the perfusate. Livers perfused at 42·5°C showed significant rises ($p < 0.05$) in AST and LDH after 60 min of perfusion but perfusion at 42·5°C with deferoxamine added, was not significantly different from perfusion at 37°C. Perfusion at 42·5°C caused an increase in GSH into the perfusate at a level significantly ($p < 0.05$) greater than at 37°C. GSH levels in the liver after 60 min of perfusion decreased from 4.82 ± 0.76 μM/gm at 37°C to 1.48 ± 0.54 μM/gm at 42·5°C ($p < 0.05$) but only fell to 3.42 ± 1.23 μM/gm at 42·5°C with deferoxamine added. Efflux of iron into the perfusate increase significantly with time and temperature. Low molecular weight chelated iron within the liver after perfusion increased from 5.88 ± 1.46 nM/gm at 37°C to 25·8 nM/gm at 42·5°C ($p < 0.05$). Perfusate total aldehyde levels increased from 0.085 ± 0.056 to 0.32 ± 0.09 μM/ml after 60 min at 37°C and 0.87 ± 0.45 to 2.01 ± 0.90 μM/ml at 42·5°C ($n = 8$). There was a significant decrease in total aldehyde levels at 42·5°C with the addition of deferoxamine to the perfusate, 0.36 ± 0.14 to 0.86 ± 0.27 μM/ml, when compared to 42·5°C levels ($p < 0.05$). Levels of ethane and pentane in the perfusion chamber head space showed no significnt changes with time or temperature of perfusion. The data suggest that lipid peroxidation may play a causal role in hyperthermia induced liver toxicity and that iron plays a major role in this injury. Failure of hydrocarbon analysis to support this conclusion appears related to the use of membrane oxygenators.

Keywords: Hyperthermia, lipid peroxidation, iron, glutathione, aldehyde

1. Introduction

Hyperthermia (42–42·5°C) induced by isolation-perfusion in human livers with cancer has been shown to have definite tumoricidal effects on colon cancer meta-

static to the liver (Skibba and Quebbeman 1986). However, significant hepatic toxicity which accompanies such treatment can be attributed directly to the effects of heat on the liver (Skibba and Quebbeman 1987, Skibba *et al.* 1986 a,b). The hepatic injury caused by hyperthermia is similar to and characteristic of numerous forms of insult such as xenobiotic poisoning, ischemic injury, reperfusion injury, organ preservation and heatstroke (Comporti 1985, Weis 1986, Dix and Aikens 1993, Farber 1994, Poli 1993, Kedderis 1996). This same pattern of cellular injury has been shown to be caused by oxidative stress which can initiate lipid peroxidation (Comporti 1985, Six and Aikens 1993, Farber 1994, Kedderis 1996, Poli 1994, Weis 1986). Oxidative stress can be defined as an alteration in the intracellular proxidant-to-antioxidant ratio in favor of proxidants (Sies 1985a).

Toxic cellular damage from lipid peroxidation is strongly linked to a pathological alteration in membrane structure and function (Comporti 1985, Skibba and Quebbeman 1987, Poli 1993, Farber 1994). Similar changes in structure and function have been observed in membranes exposed to hyperthermia in the range of 41–45°C (Lepock 1982). Thus, we proposed that the liver toxicity induced by hyperthermia (41–43°C) was due to oxidative stress (Skibba *et al.* 1986b, 1988, 1989 a,b). This hypothesis was supported by findings of a marked decrease in the redox state of the dog and human liver during hyperthermic perfusion (Skibba and Condon 1983, Skibba *et al.* 1986). Further, we observed a decrease in cytochrome p-450 and microsomal glucose-6-phosphatase activity, along with an increase in lipid conjugated dienes after hyperthermic perfusion (Skibba *et al.* 1990). These membrane changes are markers for lipid peroxidation (Tappel 1980). Hepatocellular membrane damage was also evident by the release of cytosolic enzymes, leakage of cellular potassium, and release of lysosomal enzymes during and after hyperthermic (41–43°C) perfusion of human, dog and rat livers (Bowers *et al.* 1981, Skibba and Condon 1983, Skibba and Quebbeman 1986, 1987, Skibba *et al.* 1986 a,b, 1987, 1989 a,b).

Other markers for lipid peroxidation include formation of aldehydes and the hydrocarbons, ethane and pentane (Wendel and Dumelin 1981, Esterbauer and Cheeseman 1990, Esterbauer *et al.* 1991, Esterbauer 1993). Their formation occurs when lipid hydroperoxides break down in biological systems. They are end-products of lipid peroxidation, the formation of which is dependent on the presence of transition-metal ions to decompose the hydroperoxides (Wendel and Dumelin 1981). These studies show that there is an increase in the efflux of total soluble aldehydes from the perfused rat liver at hyperthermic temperatures (41–43°C) and that the process is in part related to the release of 'free' iron. Further, formation of ethane and pentane did not give clear results.

2. Experimental

Male Sprague-Dawley rats, 250–300 gm (King Animal Laboratories, Inc., Oregon, WI) were provided standard laboratory chow and water *ad libitum* and were acclimatized for 1 week after receipt. Enzymes and cofactors necessary for analysis of perfusate and liver samples were obtained from Sigma Chemical (St Louis, MO, USA).

2.1. *Perfusion techniques*

Livers from rats starved 16 h were perfused *in situ* by a recirculation technique described by Hems *et al.* (1966), but modified for control of liver temperature

(Collins and Skibba 1980). The perfusate consisted of Krebs-Henseleit bicarbonate buffer (pH 7·4) with 2% bovine fraction V albumin in a total volume of 175 ml. A silastic tubing membrane oxygenator was employed to mix the perfusate with humidified oxygen-carbon dioxide. A gas mixing valve was used to control the partial pressures of O_2, CO_2 and pH. Inflow pO_2s were maintained between 250 and 300 mmHg and pCO_2s between 30 and 40 mmHg. Perfusate pH and partial pressure of O_2 and CO_2 were determined every 15 min (IBL1 blood gas analyser, Radiometer, Copenhagen). Perfusion flow rates were 3–3·5 ml/min/gm liver to maintain viability (Sugano *et al.* 1978). A 15 min perfusion period was allowed for equilibration of the preparation before the start of each experiment. Rat livers were perfused for 60 min at 37°C, and 42·5°C with and without deferoxamine added to the perfusate.

2.2. *Analytical methods*

2.2.1. *Total aldehyde assay.* The method described by Lappin and Clark (1951) as taken from Pryor *et al.* (1991) was used to determine the total aldehyde content of perfusate samples. A 0·5 ml sample of perfusate was mixed with 0·5 ml methanol. To this mixture was added 1 ml of a saturated solution of 2,4-dinitrophenylhydrazine (DNPH) in methanol. After mixing the solution, 1 drop concentrated HCl was added and the solution incubated at 50° for 30 min. Tubes containing the reaction mixture were covered with parafilm to minimize loss of methanol due to evaporation during incubation. The contents were cooled in ice water, mixed with 5 ml of 10% KOH in 80% methanol, and analysed at 480 nm within 5 min after the addition of alkali against a reference containing unused perfusate treated similarly. The concentration of aldehydes in the samples was calculated from a calibration curve created by using known amounts of nonanal.

2.2.2. *Total iron assay and measurement of LMWC-Fe (low molecular weight chelated iron).* Perfusate total iron levels were determined by quantitation of the bathophenanthroline-ferrous complex formed after denaturation of protein with acetic acid, reduction of iron by thioglycolic acid, and reaction with bathophenanthroline sulfate as described by Thomas *et al.* (1985). Absorbance of the bathophenanthroline-ferrous complex was determined at 535 nm. LMWC-Fe was determined on liver homogenate after addition of 5 volumes of distilled water containing 1 mM EDTA. An aliquot was then filtered through an Amicon Centroflo CF25 apparatus to preclude ferritin from the filtrate. The filtrates are analysed for total iron as described (Thomas *et al.* 1985, Powers *et al.* 1992).

2.2.3. *Glutathione assay.* Total GSH was determined by the enzymatic technique described by Tietze (1969). A standard curve was prepared using a standard solution of GSH.

2.2.4. *Analysis of hydrocarbons.* A Hewlett-Packard Model 5890 gas chromatograph was used. The method was that described by Zarling and Clapper (1987). Briefly, 50 ml aliquots of gas were collected in airtight Teflon syringes at the specified intervals from the head space of the perfusion chamber. The gas chromatograph was fitted with a gas-sampling valve, which included a 10 ml sampling loop and a flame ionization detector. The injector temperature was 150° and the detector temperature was 225°. The sampling loop was flushed and filled with half of the 50 ml gas sample. The sample was then eluted from a 2 m stainless steel col-

umn (Chromosorb 102) by using nitrogen carrier gas at a flow rate of 30 ml/min. After injection of the sample onto the column, the column temperature was held at 50° for 1 min, increased to 100° by 50°/min, and then allowed a gradual temperature increase of 15°/min over the next 6 min to produce the final temperature of 190°, which was held for 9 min (Zarling and Clapper 1987). The peaks were integrated and plotted by the Hewlett-Packard 5890 integrator. Calibration was with a known gas mixture (Alltech, Deerfield, IL) (Zarling and Clapper 1987).

2.3. *Statistical analysis*

At each temperature and treatment in each experimental group, values of each parameter were summarized by the mean and standard deviation. The comparison of means was performed with the analysis of variance (ANOVA) followed by the least significant difference test (SAS^R). Two means at the same temperature were compared with the t-test. A probability of 0·05 or smaller was used to indicate statistical significance.

3. Results

Hepatotoxicity as a consequence of hyperthermic perfusion was indicated by the temperature-dependent leakage of the cytosolic enzymes AST and LDH into the perfusate as described previously by Skibba *et al.* 1991. The data are shown in Figure 1. Significant ($p < 0·05$) rises related to temperature occurred only after 60 min of perfusion, in the livers perfused at 42·5°C. Enzyme leakage after 60 min of perfusion with deferoxamine at 42·5°C was not significantly different than that of 37°C. The hyperthermic temperature, 42·5°C was selected for these experiments

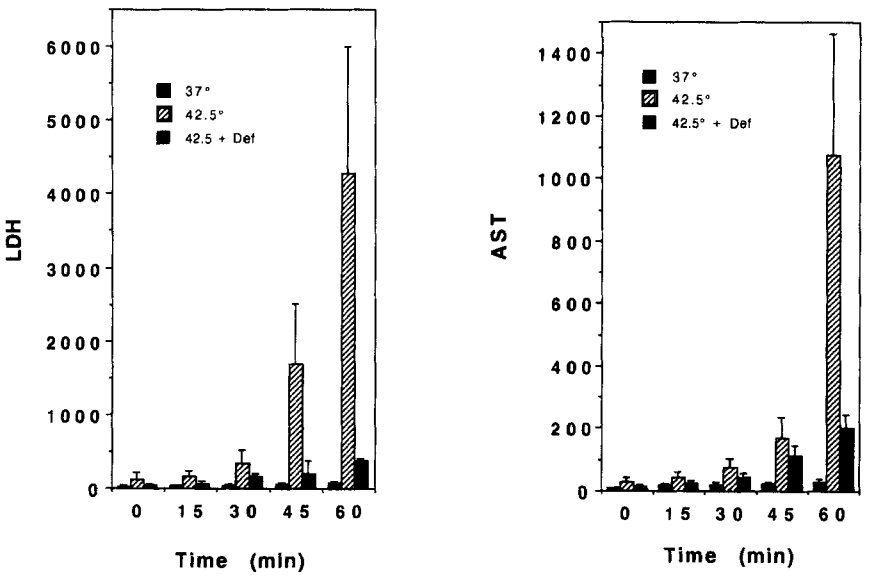

Figure 1. Effect of temperature on leakage of cytosolic enzymes, LDH and AST, into the perfusate. Increases with time at each temperature were significant ($p < 0·05$) for 42·5°C and 42·5°C + Def. Differences due to temperature occurred between 37°C versus 42·5°C and 42·5°C versus 42·5°C + Def ($p < 0·05$). Zero-time values are taken after the 15 min equilibration period of each perfusion. N = 8 for each perfusion group.

because it causes heat-induced oxidative stress with minimal hepatocellular necrosis (Skibba *et al.* 1991).

Oxidative stress was assessed by the efflux of GSH into the perfusate. Prior studies have shown that perfusate equivalents in our experiments were primarily present at GSSG, consistent with the liver being in a state of oxidative stress (Skibba *et al.* 1989, 1991). There was a significant temperature-dependent increased efflux of GSH equivalents into the perfusate (Figure 2). The GSH efflux was accompanied by a decrease in liver GSH content 42·5°C (Figure 2). When perfusion at 42·5°C was carried out with deferoxamine added to the perfusate, the decrease in liver GSH content was significantly ($p < 0{\cdot}05$) less than perfusion at 42·5°C without deferoxamine. The liver GSH content after perfusion at 42·5°C with deferoxamine remained near the level found after perfusion at 37°C (Figure 3).

Lipid peroxidation was assessed by measurement of total aldehydes appearing in the perfusate and release of ethane and pentane into the perfusion chamber. Perfusate aldehydes levels increased at the hyperthermic temperature but this effect was partially blocked with inclusion of deferoxamine in the perfusate (Figure 4). Levels of ethane and pentane in the perfusion chamber head space showed no significant changes with time or temperature of perfusion.

Efflux of iron from the liver was increased at 42·5°C (Figure 5) on comparison to that at 37° but the greatest efflux occurred at 42·5°C when deferoxamine was added to the perfusate. There was a temperature related increase in LMWC-Fe within the liver after perfusion at 42·5°C and a greater increase in LMWC-Fe after addition of deferoxamine (Figure 6).

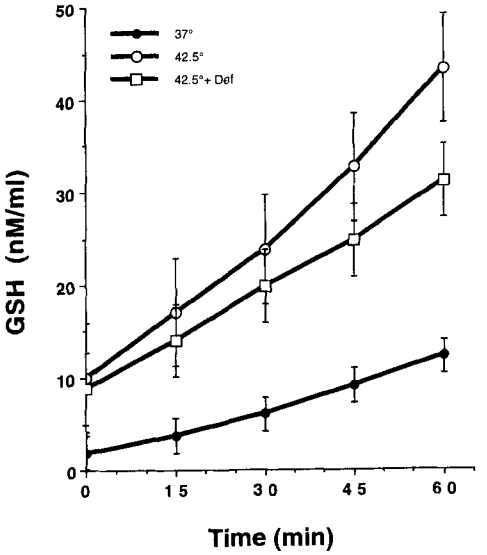

Figure 2. Effect of temperature and deferoxamine on the efflux of total GSH into the perfusate. Perfusate GSH increased with time of perfusion at each temperature (37°C, 42·5°C and 42·5°C + Def). Increases with time at each temperature were significant ($p < 0{\cdot}05$). Differences due to temperature occurred between all three perfusion groups, 37 versus 42·5 and 42·5°C + def, and 42·5 versus 42·5°C + Def ($p < 0{\cdot}05$, LSD). Zero-time values are taken after the 15 min equilibration period of each perfusion. N = 8 for each perfusion group.

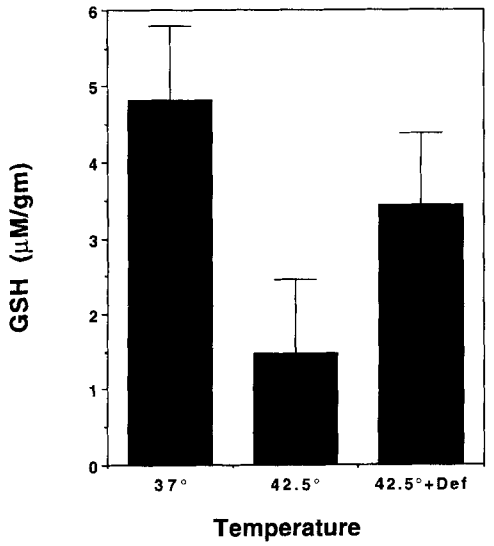

Figure 3. Total GSH content of liver after a 60 min perfusion. Differences occurred between 37 versus 42·5°C (*) and 42·5°C + Def, and 42·5 versus 42·5°C + Def ($p < 0.05$). N = 8 for each perfusion group.

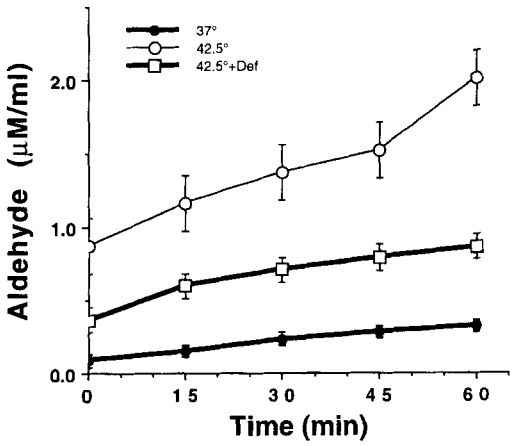

Figure 4. Effect of temperature and deferoxamine on efflux of total aldehyde into the perfusate. Increases with time within each perfusion group were significant ($p < 0.05$). Differences due to temperature occurred between all three perfusion groups, 37 versus 42·5 and 42·5°C + Def, and 42·5 versus 42·5°C + Def ($p < 0.05$, LSD). Zero-time values are taken after the 15 min equilibration period of each perfusion. N = 8 for each perfusion group.

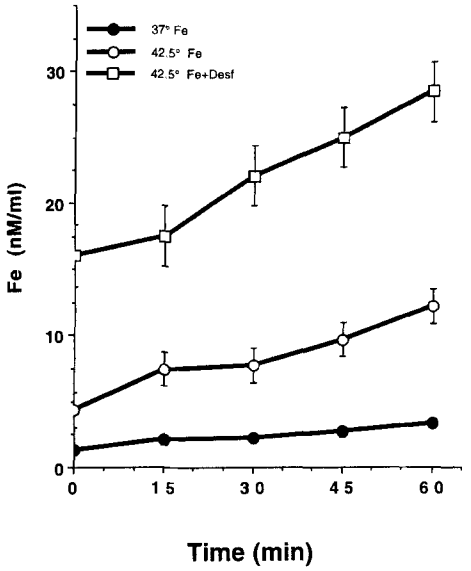

Figure 5. Effect of temperature and deferoxamine on efflux of total iron into the perfusate. Increases with time within each perfusion group were significant ($p < 0.05$). Differences due to temperature occurred between all three perfusion groups, 37 versus 42·5 and 42·5°C + Def, and 42·5 verusus 42·5°C + Def ($p < 0.05$, LSD). Zero-time values are taken after the 15 min equilibration period of each perfusion. N = 8 for each perfusion group.

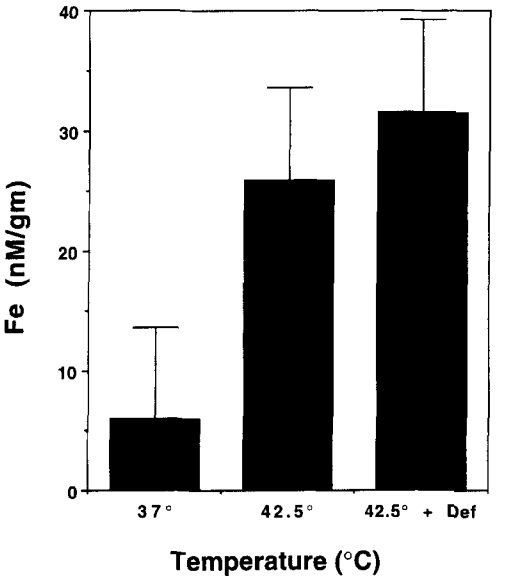

Figure 6. LMWC-Fe content of liver after a 60 min perfusion. Differences occurred between 37 versus 42·5 (*) and 42·5°C + Def (**), and 42·5 versus 42·5°C + Def ($p < 0.05$). N = 8 for each perfusion group.

4. Discussion

The long-term goal of these and prior studies has been to determine the underlying cause(s) of hyperthermic toxicity to the liver and determine if the heat toxicity can be prevented or reversed. Previously, it had been proposed that heat-induced hepatotoxicity in the therapeutic range of 42–43°C was due to oxidative stress, resulting in lipid peroxidation processes, with concomitant cellular injury (Skibba *et al.* 1986a). Oxidative stress can be defined as an alteration in the intracellular proxidant-to antioxidant ratio in favor of proxidants (Sies 1985a). Oxidative stress was manifested by the marked reduction in total liver GSH content (Figure 3) and increased efflux of total GSH (Figure 2) and oxidized glutathione (GSSG) from the liver at hyperthermic temperatures (41–43°C) (Skibba *et al.* 1989 a,b). Cell death which occurs with the use of GSH depleting agents appears to be predominantly related to lipid peroxidation (Comporti 1987, DeLeve and Kaplowitz 1991).

The efflux of GSH from the liver perfused at the hyperthermic temperature, 42·5°C indicates ongoing oxidative stress. The hepatocellular loss of GSH equivalents represent one of the first measurable indicators of intracellular oxidative stress (Sies and Akerboom 1984, Sies 1985b, Skibba *et al.* 1989 b). One source of reactive oxygen species during liver hyperthermia has been identified as that mediated by xanthine oxidase (XO). In previous experiments it has been shown that XO is converted to the oxidase form (type O) during hyperthermic perfusion (Skibba *et al.* 1989, a,b). Oxidase activity is necessary for the generation of superoxide (O_2^-) and hydrogen peroxide (H_2O_2) and the initiation of lipid peroxidation.

O_2^- and H_2O_2 are insufficiently reactive to initiate lipid peroxidation (Weis 1986, Farber 1994). But, the highly reactive hydroxyl radical ($\cdot OH$) can be formed from these reactants, O_2 and H_2O_2, via the iron catalysed Haber-Wiess reaction (Farber 1994, Weis 1986). The availability of ferric iron in combination with active oxygen species such as superoxide, has been shown to be essential to the process of lipid peroxidation and it is central to the metal catalysed oxidation of amino acid residues of proteins (Farber 1984, Minotti and Aust 1989, Schaich 1992, Aust *et al.* 1993, Stadtman 1993). Superoxide, produced by XO, has been shown to reductively release iron from ferritin and promote peroxidation of phospholipid liposomes (Thomas *et al.* 1985, Biemond *et al.* 1986, Boyer and McCleary 1987, Reif *et al.* 1988). We reported that during hyperthermic liver perfusion, O_2^-, generated by the action of XO in the oxidase form, causes the release of iron from ferritin, thereby facilitating lipid peroxidation (Powers *et al.* 1992). Our present data shows that there was an increased efflux of iron from the livers perfused at 42·5°C as well as an increased amount of 'free' iron (LMWC-Fe) in the cytosol at the end of perfusion at 42·5°C (Figures 5 and 6). Further, oxidative stress demonstrated by the efflux of GSH and GSSG from the liver at hyperthermic temperatures was partially inhibited by iron chelation and allopurinol (Powers *et al.* 1992). These data confirm that in the presence of the iron chelator, deferoxamine, there was less GSH efflux from the liver perfused at 42·5°C than perfused at 42·5°C without deferoxamine. Chelation of iron at 42·5°C also maintained liver GSH levels close to those from livers perfused at 37°C (Figure 4).

Oxidative damage, in addition to causing lipid peroxidation, can lead to modification of proteins through the introduction of carbonyl groups (Davies 1987, Dean *et al.* 1991, Stadtman 1993). Oxidative modification of proteins 'marks' them for degradation by most common proteases (Stadtman 1993). This concept is consistent

with the proteolysis observed during hyperthermic perfusion (Skibba and Collins 1978).

Protein carbonyl groups are formed by direct metal-catalysed oxidation of certain amino acid residues of proteins (Stadtman 1993) or by other reactions occurring during membrane lipid peroxidation (Esterbauer and Cheeseman 1990, Dean *et al.* 1991). Cytotoxic chemicals such as 4-hydroxynonenal and other aldehydes released during peroxidation of lipids can react with protein thiol groups and form adducts, thereby creating protein carbonyl groups (Comporti 1985, Esterbauer and Cheeseman 1990, Dean *et al.* 1991, Esterbauer *et al.* 1991, Esterbauer 1993). We have found an increase in the efflux of total soluble aldehydes from the perfused rat liver at the hyperthermic temperature of 42·5°C (Figure 4). This efflux of aldehydes was partially ameliorated by the inclusion of deferoxamine in the perfusate. Thus, aldehydes are generated from metal catalyzed dismutation of lipid hydroperoxides (Dix and Aikens 1993). However, the aldehyde production during liver perfusion at 42·5°C was not completely abrogated by iron chelation. Therefore, there are other reactions which produce these toxic aldehydes during hyperthermic liver perfusion (Esterbauer and Cheeseman 1990, Dix and Aikens 1993, Esterbauer 1993).

Pompella *et al.* (1991) suggested that membrane protein thiols can be attacked by lipid radicals and/or reactive lipid soluble aldehydes. GSH inhibited both lipid peroxidation and protein carbonyl formation in rat liver microsomes (Palamanda and Kehrer 1992). The protection against peroxidation afforded by GSH appears to be related to the maintenance of protein thiols and not to a direct antioxidative effect (Palamanda and Kehrer 1992). Administration of hepatotoxic doses of diquat (a redox cycling compound) to rats increased the carbonyl content of the protein excreted in the bile which probably reflected iron-catalyzed oxidation of the proteins (Gupta *et al.* 1994). In the case of haloalkanes, the formation of carbonyl functions in phospholipid-bound fatty acyl residues and the release of toxic aldehydes capable of reacting with protein and enzymes are important events in their pathogenic mechanisms of cellular damage (Comporti 1985, Dix and Aikens 1993). A main reaction for the formation of carbonyl from unsaturated fatty acids is the dismutation of monohydroperoxides (Dix and Aikens 1993). Peroxidation of phospholipids was shown to precede the cytotoxicity of rat hepatocytes exposed to 1,3-dichloropropane (Suzuki *et al.* 1994).

These data and previous studies by Skibba *et al.* indicate that the response of the liver to hyperthermia (41–43°C) is that of oxidative stress which results from the generation of reactive oxygen species. It is now known that these radicals can function as signal transduction messengers to activate transcription factors (Pahl and Baeuerle 1994 (1986b, 1989a,b, 1990, 1991). Numerous mammalian genes and regulatory proteins involved in transcription are sensitive to oxidative stress. Most notably, oxidative stress is known to induce heat shock proteins (HSPs) (Moseley 1994, Schoeniger *et al.* 1994, Li *et al.* 1995, Tacchini *et al.* 1995). But, GSH depletion and oxidation of protein thiols has also been shown to be linked to the increased transcription of HSP 70 (Liu *et al.* 1996). Further, biologically active aldehydes, in particular, (E)-4-hydroxyalk-2-enals, produced during lipid peroxidation from oxidative stress induce heat shock genes in HeLa cells (Allevi *et al.* 1995).

In conclusion, the initial response to liver hyperthermia appears to be induction of oxidative stress. Both free-radical generation and resulting thiol modification activate a variety of molecular responses, some of which can lead to cell death. From these studies, it is suggested that the iron and production of aldehydes further

modify this response through alteration in the thiol-disulfide redox status in the liver (Slater *et al.* 1995). Iron chelation with deferoxamine can partially ameliorate the heat-induced hepatotoxicity by preserving liver GSH levels and presumably thiol-disulfide redox status. This effect could be related to the decrease in aldehyde production and presumably less protein carbonyl formation.

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