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SHORT COMMUNICATIONS

Lipid peroxidation caused by hyperthermic perfusion of rat liver*

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Isolation-perfusion has been used as a means of providing controlled hyperthermia to human livers with cancer [1, 2]. Hyperthermia applied in this manner has shown significant tumoricidal effects for colon cancer metastatic to the liver [3]. However, the significant hepatotoxicity which accompanies such treatment can be attributed directly to the effects of heat on the liver [2, 4, 5]. Hepatocellular injury caused by hyperthermia was demonstrated by the release of cytosolic enzymes, lactate dehydrogenase (LDH†) and aspartate aminotransferase (AST), and lysosomal enzymes, *p*-nitrophenyl phosphatase and β-glucuronidase into the perfusate during perfusion and into the blood after perfusion [1–5]. Similar leakage of hepatic enzymes has been reported with hyperthermic perfusion of dog and rat liver [2, 6, 7]. While the mechanism of the heat-induced injury has not been determined yet, the leakage of hepatocellular enzymes, as well as cytosolic potassium, clearly suggests that membrane damage or structural alteration is involved [8].

We previously suggested lipid peroxidation as the toxic mechanism underlying hypthermia-induced hepatocellular injury [4]. The peroxidative deterioration of membranes has been postulated to be the primary cause for cellular damage associated with the formation of free radicals in biological systems [9–11]. In the case of hyperthermia, the source of free radicals must be generated within the cell. While such a mechanism has yet to be identified, one source of radical oxygen species during hyperthermia could be the enhanced production of O_2^- (superoxide) and H_2O_2 (hydrogen peroxide) normally generated during aerobic metabolism as intermediates in redox processes leading from oxygen to water [9, 12].

Another source for the formation of radical oxygen species during hyperthermia could be the action of xanthine oxidase (XO) on xanthine during purine catabolism [4, 13]. An increase in purine catabolism occurred during hyperthermic perfusion of human and rat livers as evidenced by an increase in perfusate uric acid and allantoin respectively [4, 13, 14]. In order for XO to be the source of oxygen free radicals, it must be converted from its normal dehydrogenase form to an oxidase, type O [15–17]. This transition can be accomplished by heat [16, 18], and, indeed, we have noted a significant increase in the amount of XO in the type O form after hyperthermic perfusion of rat liver [13, 14, 18].

An additional biochemical change associated with hyperthermic perfusion of rat liver has been the marked reduction in the liver glutathione (GSH) content, and the efflux of both GSH and oxidized glutathione (GSSG) from the liver at hyperthermic temperatures [13, 14]. Similar changes in liver GSH have been attributed by Sies [19] to oxidative stress. Continued oxidative stress may lead to lipid peroxidation [20].

Evidence for lipid peroxidation following hyperthermic liver perfusion is lacking, and methods for detecting lipid peroxidation in biological systems are indirect. Examples include measurements of malonaldehyde, conjugated lipid dienes, lipid peroxides, alkanes, loss of cytochrome P450 activity and inhibition of lipid-dependent glucose-6-phosphatase [21–24]. Our goal in this report is to present evidence in support of the hypothesis that lipid peroxidation is the cause for the hepatotoxity accompanying hyperthermic liver perfusion. In these experiments, lipid peroxidation was assessed by a decrease in microsomal cytochrome P450 and glucose-6-phosphatase activity and the formation of diene conjugates during hypthermic liver perfusion.

Methods

Male Fisher 344 rats, 250–300 g (King Animal Laboratories, Inc., Oregon, WI), were provided standard laboratory rodent chow (Rat–Mouse Chow, Purina, St. Louis, MO) and water *ad lib.* and were acclimatized for 1 week after recept. Livers from fed rats were perfused *in situ* by a recirculation technique with accurate temperature control as previously described by Collins and Skibba [25]. The perfusate consisted of Krebs–Henseleit bicarbonate buffer (pH 7.4) in a total volume of 175 mL. A silastic tubing membrane oxygenator was employed to mix the perfusate with humidified oxygen–carbon dioxide (95:5). Perfusate pH and partial pressure of O_2 and CO_2 were determined every 15 min (IBL1 blood gas analyzer, Radiometer, Copenhagen). A flow rate greater than 3 mL/min/g was employed during all perfusions in order to maintain viability [26]. The bile duct was cannulated with polyethylene tubing (PE 20), and bile flow was measured. A 10-min perfusion period was allowed for equilibration of the preparation before the start of each experiment. Livers were perfused for 1, 2 and 3 hr at 37, 42, 42.5 and 43°.

During perfusion, perfusate samples were obtained at 0, 15, 30, 45 and 60 min for determination of AST, LDH, allantoin, total GSH, GSSG and electrolytes. At the end of perfusion the liver was flushed with 50 mL of ice-cold potassium–phosphate buffer (150 mM KCl, 10 mM potassium-phosphate and 0.1 mM EDTA, pH 7.4) and homogenized with a Potter–Elvehjem homogenizer in 4 vol. of potassium–phosphate buffer. This 20% liver homogenate was processed for the isolation of microsomes as described [27]. The P450 was measured by the method of Omura and Sato [28], conjugated dienes by the method described by Recknagel and Glende [29] and the glucose-6-phosphatase activity as described [30]. XO activity (total and type O) was determined as described by Stirpe and Della Corte [16]. AST, LDH and electrolytes were determined by automated chemistry panels. Allantoin, total GSH and GSSG were measured as referenced in prior papers [13, 14].

Data were collected from four liver perfusions at each temperature and time of perfusion. A total of forty-eight rat liver perfusions was carried out for these experiments. Where possible, data were tested by analysis of variance (ANOVA). Comparison between means was by the least significant difference test (LSD).

Results

We observed a significant increase in the amount of diene conjugates as a function of perfusion temperature (Fig. 1).

* A preliminary report of this work has been published [Skibba J, Powers R, Stadnicka A and Kalbfleisch J, Lipid peroxidation after hyperthermic perfusion of rat liver *Fed Proc* **2**(4): A376, 1988].

† Abbreviations: LDH, lactate dehydrogenase; AST, aspartate aminotransferase; XO, xanthine oxidase; GSH, glutathione; GSSG, oxidized glutathione; ANOVA, analysis of variance; LSD, least significant difference test; and PUFA, polyunsaturated fatty acid.

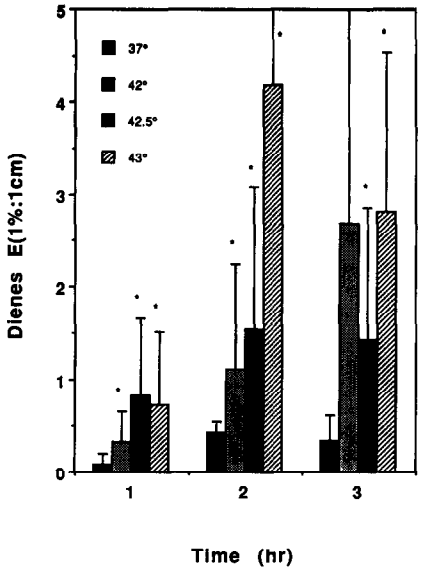

Fig. 1. Effects of hyperthermic perfusion on hepatic conjugated diene levels. All absorbance measurements have been corrected to a uniform base of 1 mg lipid/mL. This value was then multiplied by 10 to obtain the absorbance at a concentration of 1%. This yields the so-called E (1%:1 cm) value. Values are means ± SD, N = 4. Key: (*) mean response was significantly different from 37° ($P < 0.05$).

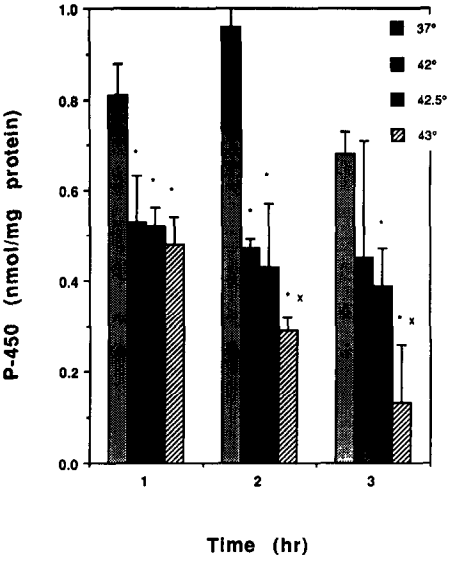

Fig. 3. Effect of hyperthermic perfusion on microsomal cytochrome P450 content. Values are means ± SD, N = 4. Key: (*) mean response was significantly different from 37° ($P < 0.05$), and (×) mean response was significantly different from 42° ($P < 0.05$).

At 1, 2 and 3 hr, the mean responses at 37° were different from those at 42, 42.5 and 43° ($P < 0.05$). There were no significant changes with duration of perfusion at each temperature.

Time- and temperature-dependent depletion of glucose-6-phosphatase and cytochrome P450 activity was observed in rat livers perfused for 1–3 hr at hyperthermic temperatures. Microsomal glucose-6-phosphatase activity decreased significantly ($P < 0.05$) from 37° only after 2 hr of perfusion at 42, 42.5 and 43° (Fig. 2). However, significant changes in cytochrome P450 content induced with hyperthermic perfusion occurred within 1 hr (Fig. 3). When compared to normothermic perfusion, there was a significant decrease ($P < 0.05$) in cytochrome P450 content after perfusion at 42, 42.5 and 43° for 1, 2 and 3 hr. There were no changes with time at each temperature except at 43°.

XO showed a significant conversion from the dehydrogenase to the oxidase (type O) after hyperthermic liver perfusion. The increase in type O XO at 42–43° was significantly ($P < 0.05$) greater than after perfusion at 37°. Also, there was a marked reduction in liver glutathione content after perfusion at hyperthermic temperatures (42–43°) when compared to perfusion at 37°. These data are not presented in detail since we have reported previously similar data from previous experiments [13, 14, 18]. The measurements were carried out during these experiments to assure us that these changes occurred prior to or simultaneously with the indicators of lipid peroxidation.

Liver perfusion at 42–43° was accompanied by a temperature-dependent increase in leakage into the perfusate of the cytosolic enzymes AST and LDH. Further, potassium leakage into the perfusate also occurred and was increased significantly ($P < 0.05$) at 42–43° on comparison to 37°. Lastly, there were temperature-dependent increases in the efflux of total glutathione and allantoin from the liver into the perfusate. These observations were consistent with prior reports by us and others and the data are not represented [2, 6–8, 13, 14].

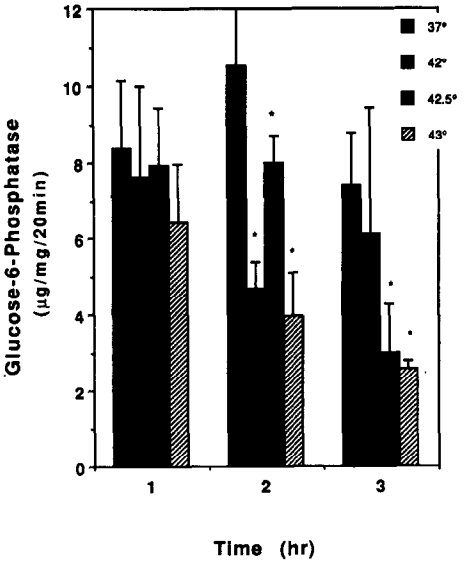

Fig. 2. Effect of hyperthermic perfusion on liver microsomal glucose-6-phosphatase activity. Significant changes with time occurred after perfusion at 37, 42.5 and 43° ($P < 0.05$). Values are means ± SD, N = 4. Key: (*) mean response was significantly different from 37° ($P < 0.05$).

Discussion

The peroxidative breakdown of polyunsaturated fatty acid (PUFA) chains of membrane lipids has been implicated in the pathogenesis of many types of liver injury and especially in the hepatic damage induced by toxic chemicals [9–11]. The pathologic similarity between such injury and the hepatotoxicity accompanying hyperthermic liver per-

fusion has led us to hypothesize that oxidative stress and lipid peroxidation are the mechanistic basis for hyperthermia-induced liver injury. Lipid peroxidation is comprised of three events: (1) initiation, (2) propagation and (3) termination. Initiation is the reaction of the PUFA with a reactive free radical resulting in the abstraction of a hydrogen to form a lipid radical (diene conjugate), which then reacts with molecular oxygen, forming a peroxylipid radical. Propagation is the reaction of a peroxylipid radical with another PUFA side chain of a lipid molecule to yield a lipid hydroperoxide and lipid radical, thus conserving the number of radicals in the reaction sequence. Termination is the removal of free radicals, where two radicals combine to yield a nonradical product, thus ending the chain reaction [31].

Formation of diene conjugated fatty acids has been considered an indicator of damage caused by free radical reactions [31, 32]. While the assay of diene conjugates as a quantitative measure of free radical formation and lipid peroxidation has been criticized because of potential errors [31], this parameter provides a useful relative indicator of the extent of free radical reactions and damage. Several groups have shown that the hepatotoxic damage caused by free radical generating haloalkanes is reflected by an increase in diene conjugation [32]. Moreover, their results correlated well with other indirect evidence of free radical activity and lipid peroxidation. In our experiment, perfusion of rat livers at 42–43° for 1–3 hr resulted in formation of diene conjugates in an amount significantly greater than that formed during perfusion at 37° (Fig. 1).

Inactivation of glucose-6-phosphatase and/or cytochrome P450 has been used as an indirect measure of lipid peroxidation [10, 11, 23]. Haloalkane-induced and iron-stimulated lipid peroxidation were found to be correlated with both decreased glucose-6-phosphatase and cytochrome P450 [10, 32–34]. Loss of cytochrome P450 and glucose-6-phosphatase activity was observed after hyperthermic liver perfusion at 42–43° for 1–3 hr. No exogenous hepatotoxins were added to the perfusate. Lipid peroxidation would thus have to be the consequence of endogenously produced free radicals generated under hyperthermic conditions.

One mechanism suggested to result in the intracellular formation of oxygen-derived free radicals is the activity of the cytosolic enzyme XO (EC 1.2.3.2) [9, 15]. XO exists in two distinct forms, either as an NAD^+-dependent dehydrogenase (type D) or oxidase (type O) [15–17]. It is the latter form which generates O_2^- and H_2O_2 by the reduction of O_2 while oxidizing xanthine or hypoxanthine [9, 15]. Under normal physiologic conditions, XO is present predominantly in the type D form [16, 17]. We have shown previously that hyperthermic liver perfusion results in the conversion of XO from type D to type O, with concomitant increased production of superoxide [13, 14, 18]. A similar conversion was observed during these perfusions at 42–43°.

These data support the premise that the toxic liver injury of hyperthermia is induced by oxidative stress with subsequent lipid peroxidation. The peroxidative breakdown of membrane lipids results in a complex series of biochemical and biophysical events which cause perturbations in the functions of several subcellular organelles, e.g. microsomes, lysosomes, mitochondria and plasma membranes [10, 11]. Indeed, hyperthermic liver perfusion has been shown to inhibit microsomal drug metabolism [35, 36], a finding which can be accounted for by the inactivation of cytochrome P450. Destruction of cytochrome P450 by lipid peroxidation has been shown not to be confined to special isoenzymes of cytochrome P450 [37].

Damage to lysosomal membranes has been observed after liver perfusion at 42–43° as evidenced by labilization of the lysosomes and release of lysosomal enzymes into the perfusate [38]. Similar damage of lysosomes as a consequence of peroxidative events has been reported [39, 40].

Lastly, peroxidative alterations of plasma membranes have resulted in an increase in K^+ permeability and release of cytosolic enzymes, AST and LDH [10, 11], which are similar to alterations accompanying hyperthermia of the liver [2, 6–8].

In summary, the data presented support the premise that hyperthermia-induced hepatocellular injury is the end result of lipid peroxidation. Evidence for lipid peroxidation is the formation of diene conjugates and the decrease in microsomal P450 and glucose-6-phosphatase activity during hyperthermic liver perfusion.

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The stereospecific inhibition of endogenous triacylglycerol synthesis by fenoprofen in rat isolated adipocytes and hepatocytes

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Most of the 2-arylpropionate non-steroidal anti-inflammatory agents such as fenoprofen, ibuprofen and ketoprofen are used clinically as racemates, although anti-inflammatory activity is thought to reside primarily with the *S*-enantiomers [1]. The *R*-enantiomers were, until recently, thought to be inactive, or at best, to act as prodrugs for the active *S*-enantiomer formed via stereospecific chiral inversion [2, 3], involving formation of a highly reactive *R*-2-arylpropionyl–CoA thioester intermediate [4–6]. Fatty acyl–CoA thioesters are the endogenous substrates for triacylglycerol synthesis and, consistent with the stereospecific formation of *R*-2-arylpropionyl–CoA thioesters, fenoprofen [7] and ibuprofen [8] undergo stereospecific incorporation into rat triacylglycerols, forming hybrid fenoprofen– and ibuprofen–triacylglycerols.

Racemic mixtures of various 2-arylpropionates have been shown to inhibit cholesterogenesis and fatty acid synthesis *in vitro*, and also to decrease serum triacylglycerol and cholesterol levels *in vivo* in rats [9]. The hypolipidaemic activity of the 2-arylpropionates appears to be correlated with their capacity to form hybrid triacylglycerols [9], and in this paper we now report the effects of *R*- and *S*-fen-