Nitrogen Metabolism and Lipid Peroxidation during Hyperthermic Perfusion of Human Livers with Cancer[1]

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ABSTRACT

Isolation-perfusion was used as a means of heating human livers with cancer. Perfusion was at 42–42.5°C for 4 h. Perfusate constituents were analyzed in an attempt to identify factors contributing to the hepatotoxic effects of hyperthermia. During perfusion the perfusate constituents analyzed were: urea; total amino acids; uric acid; malonaldehyde; and lysosomal enzymes. Hepatic ammonia for urea synthesis is derived from degradation of amino acids, amines, and nucleic acids. An increase in proteolysis was reflected in the increase in urea from 0.6 ± 0.2 mM to 1.9 ± 8 mM and total amino acids from 1.0 ± 0.6 mM to 4.4 ± 1.7 mM during the 4 h of perfusion at 42–42.5°C. An increase in purine catabolism occurred as evidenced by an increase in perfusate uric acid from 1.7 ± 1.0 mg/100 ml to 6.1 ± 2.7 mg/100 ml. Free oxygen radicals, which can lead to lipid peroxidation, are generated by the action of xanthine oxidase on xanthine. Lipid peroxidation occurring during perfusion was assessed by an increase in malonaldehyde from 2.3 ± 1.3 μM to 10.4 ± 10.0 μM. An increase in acid phosphatase in the perfusate from 38 ± 15 units/liter to 78 ± 45 units/liter occurred, suggesting labilization of lysosomes, perhaps through lipid peroxidation. Proteolysis and lipid peroxidation are suggested to be two interrelated factors contributing to heat toxicity in the perfused human liver with cancer.

INTRODUCTION

Hyperthermia, 42–43°C, was shown to be an effective tumoricidal agent (1–5). Hyperthermia, 42°C, can be induced in the liver by isolation-perfusion (6, 7). This therapy was used to treat patients with cancer metastatic to the liver (8). Results indicate that hyperthermia was tumoricidal for metastatic colon cancer, but there remained an identifiable rim of tumor cells at the periphery of metastatic tumor nodules. However, there was marked variation in the hepatotoxic effects of heat on the liver. This varied from mild elevations in serum enzymes to hepatic necrosis in one patient which appeared directly attributable to the thermotherapy.

Heat-induced hepatotoxicity varies with the method(s) or condition(s) under which the liver is heated and is manifest through changes in hepatic functional integrity (9). The functional integrity of the isolated perfused liver can be assessed by analysis of perfusate constituents. Several investigators studied the effects of heat on liver tissue using the isolated perfused liver and described changes with temperature and time on bile flow and glucose, lactate, and fatty acid metabolism (6, 7, 9–16). The liver remained functional in a range of 42–43°C.

Perfusate constituents were analyzed during hyperthermic perfusion of human livers with cancer in an attempt to identify factors contributing to the hepatotoxic effects of heat. Data from these studies suggested that nitrogen metabolism and lipid peroxidation appeared to be two such factors. The potential contribution of these factors to heat-induced hepatotoxicity is the subject of this paper.

MATERIALS AND METHODS

Six patients were selected who had primary or metastatic cancer in the liver, but they were without detectable tumor elsewhere in the body. If the tumor was metastatic to the liver, the primary tumor had been removed. One patient had a melanoma metastatic to the liver and had refused chemotherapy. The second patient had a primary cholangiolar carcinoma of the liver and had been treated for 5 mo with 5-fluoro-deoxyuridine via the implantable pump. The other four patients had metastic colon cancer to the liver. Two had refused chemotherapy, and the other two had been treated with systemic 5-fluorouracil therapy. Informed consent was obtained. The protocol was approved by the Human Research Review Committee of the Medical College of Wisconsin. The details of the operative technique were as described by these investigators (8).

Urea, uric acid, creatinine, calcium, and phosphorus were determined by automated chemistry panels in the clinical laboratories. Total α-amino acids were measured by the ninhydrin assay (17). Acid phosphatase (p-nitrophenylphosphatase) and β-glucuronidase were assayed as described (18, 19). The TBA[3] test was used to measure lipid peroxidation. TBA reacts with malonaldehyde to give a red species absorbing at 532 nm (20). Measurement of TBA-reactive material in the perfusate was similar to that described (21). A 0.5-ml aliquot of perfusate plasma was mixed with 2.0 ml of 20% trichloroacetic acid and set on ice for 10 min. One ml of a 0.67% TBA solution and 0.1 ml of a 0.2% butylated hydroxytoluene in ethanol were added. The solution was heated for 15 min in a boiling water bath. After cooling and centrifugation, the absorbance of the supernatant was determined a 535 nM. The TBA value was determined by a standard curve of 1,1,3,3-tetraethoxy-propane and compared to the values calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1}.

Continuous variates were summarized by the sample size, mean, and standard deviation. A two-way analysis of variance and *post hoc* multiple comparison methods (least significant difference and Dunnett's test) were used to test for changes in mean level across time points of data capture (22). Multiple comparison of each time point mean was made with every other time point mean by the least significant difference test. The 0 time mean was compared to each mean obtained at 5, 30, 60, 90, 120, 150, 180, 210, and 240 min via Dunnett's test. A P value of < 0.05. was considered statistically significant.

RESULTS

The levels of the perfusate constituents urea, total amino acids, uric acid, acid phosphatase, and malonaldehyde were analyzed and identified as factors related to the hepatotoxic effects of heat on the human liver. There was an increase in perfusate urea, phosphate, creatinine, total amino acids, uric acid, acid phosphatase, and malonaldehyde. Analysis of variance indicated that these changes were significant. Calcium decreased significantly during perfusion.

Urea concentrations increased with time throughout the perfusion (Fig. 1). The changes were significant ($P < 0.05$) after 30 min in comparison to the concentration at the start of perfusion. After 60 min, the further increases in urea concentrations were not significantly different from the 60-min value. Total amino acids increased significantly in the perfusate during perfusion. At 30 min, the changes were significant ($P < 0.05$)
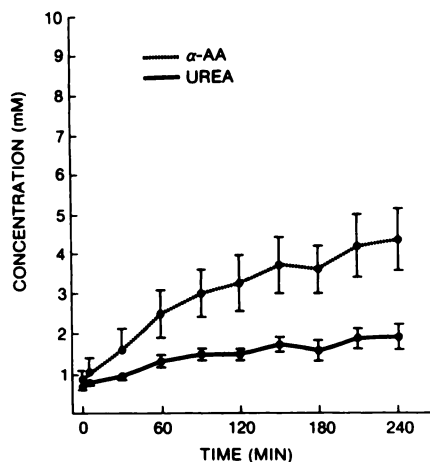
Fig. 1. Time course of changes in perfusate levels of urea and total amino acids (α-AA) during hyperthermic liver perfusion. *Points*, pooled data from six perfusions; *bars*, SE.
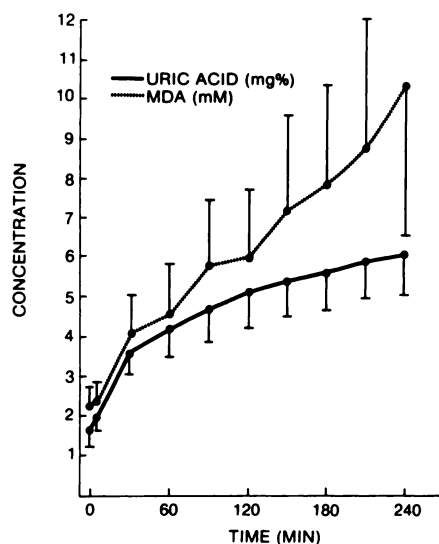

Fig. 2. Time course of changes in perfusate uric acid and MDA during hyperthermic liver perfusion. *Points*, pooled data from six perfusions; *bars*, SE.

Fig. 3. Time course of changes in perfusate lysosomal enzymes during hyperthermic liver perfusion. *Points*, pooled data from six perfusions; *bars*, SE.

Table 1 *Effect of hyperthermic perfusion on perfusate levels of creatinine, calcium, and phosphorus*

Time (min)	Creatinine (mg/100 ml)	Calcium (meq/liter)	Phosphorus (mg/100 ml)
0	0.47 ± 0.11[a]	3.91 ± 0.86	5.69 ± 1.58
5	0.58 ± 0.09	3.56 ± 0.68	5.74 ± 1.24
30	0.91 ± 0.25	3.27 ± 0.53	6.64 ± 2.45
60	1.18 ± 0.27	3.38 ± 0.44	6.69 ± 1.42
120	1.31 ± 0.28	3.41 ± 0.30	6.82 ± 1.67
180	1.41 ± 0.34	3.35 ± 0.32	5.81 ± 2.48
240	1.45 ± 0.38	3.35 ± 0.34	5.67 ± 2.97

[a] Mean ± SD from perfusion of six human livers with cancer at 42–42.5°C.

min level to subsequent time points (Table I). Phosphorus showed an increase which was significant at 30 min.

DISCUSSION

Hyperthermia caused hepatic injury similar to that seen after exposure to hepatotoxic chemicals (9). Hepatocellular injury was manifested by elevations in serum enzymes, serum glutamic oxaloacetic transaminase, serum glutamic pyruvic transaminase, and lactate dehydrogenase. Pathological changes consisted of a centrolobular lesion varying from fine granular degeneration to necrosis, dilatation of portal veins, and congestion of centrolobular sinusoids (9). The underlying mechanism(s) for heat-induced hepatotoxicity remains speculative. Lipid peroxidation and proteolysis occurred during hyperthermic perfusion of human livers with cancer. The results cannot be compared to perfusion at 37°C since no normothermic, nontherapeutic perfusions were carried out. Some comparisons can be made to data obtained from rat and dog liver perfusion experiments.

Perfusate urea increased with time during these perfusions (Fig. 1). Hepatic ammonia is derived for urea synthesis from degradation of amino acids, amines, and nucleic acids. Urea increased with time during perfusion of rat and dog livers, but it failed to show a significant difference between 37, 42, and 43°C (7, 9, 16). Rates of ureogenesis from endogenous substrates were unchanged or increased with temperatures up to 42°C in the perfused rat liver (11, 12, 23).

The increase in total amino acids in the perfusate during perfusion at 42.5°C indicated that there was ongoing proteolysis during perfusion at that temperature (Fig. 1). Amino acids were released into the perfusate during perfusion of rat livers until a constant level was established in the perfusion medium (24,

in comparison to the 0-time concentration. Significant increases continued to occur during each 30-min period of perfusion (Fig. 1). Thus, ongoing proteolysis is indicated by the increase in urea and total amino acids.

Perfusate uric acid increased during perfusion (Fig. 2). The increase was significant ($P < 0.05$) at 30 min in comparison to the level at the start of perfusion. Significant increases continued to occur in the fourth h of perfusion in comparison to the level at 90 min.

Malonaldehyde levels increased significantly ($P < 0.05$) during perfusion in comparison to the 0-time concentration (Fig. 2). After 120 min, further increases in MDA were not significant in comparison to the level at 120 min.

An increase in perfusate acid phosphatase (p-nitrophenylphosphatase) activity occurred during perfusion (Fig. 3). The increases were significant ($P < 0.05$). There was no increase in the level of β-glucuronidase during the perfusion.

Perfusate constituents creatinine, calcium, and phosphorus showed significant changes during perfusion (Table 1). Creatinine, which is related to nitrogen metabolism, increased significantly ($P < 0.05$) over base line. Calcium levels decreased significantly ($P < 0.05$) in comparison with the 0-time and 5-
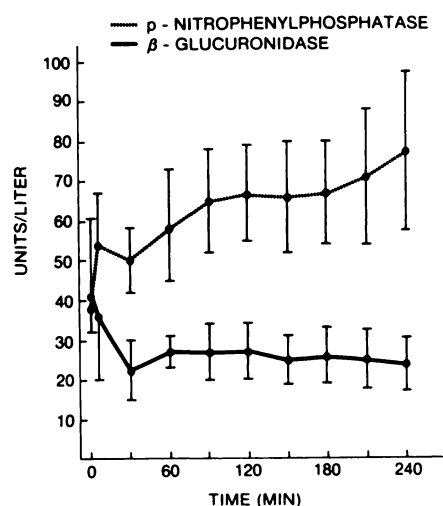
25). The accumulation of amino nitrogen or endogenous protein degradation was inhibited by amino acids with time of perfusion of the rat or dog liver at 37°C when a mixture of amino acids was added to the perfusate (6, 7, 9, 16). There was a significant ($P < 0.05$) increase in total amino acids with time and temperature during perfusion of the dog liver at 42 and 43°C (7, 16). Thus, proteolysis appeared to be increased during hyperthermic perfusion.

Another source of urea nitrogen could have been from the degradation of nucleic acids and purine nucleotides. Evidence for this degradation was the increase in uric acid during perfusion (Fig. 2). One origin of the purines for uric acid synthesis could come from the tumoricidal effects occurring during hyperthermic perfusion. A second source would be from adenine nucleotides which were not resynthesized to ATP. The synthesis of 1 mol of urea requires 4 ATPs. Heat treatment of isolated mouse liver mitochondria caused uncoupling of oxidative phosphorylation in a temperature-dependent fashion which becomes significant between 41 and 45°C (26). Futile cycling occurred during hyperthermic perfusion of rat livers with cancer (27). During these perfusions total phosphorus increased in the perfusate (Table 1). This also may be related to consumption of high energy intermediates.

An increased uric acid synthesis resulted from the increased purines. Uric acid synthesis required xanthine oxidase. XO acting on xanthine was the presumed source of superoxide which led to the appearance of the lipid peroxidation product MDA. MDA increased during the perfusion (Fig. 2). The xanthine oxidase activity of rat and human liver was reported to be predominantly a dehydrogenase (type D) which could be readily converted into an oxidase (type O) by proteolytic enzymes (28, 29). In order for XO to be the source of superoxide, it must be converted to the type O form at hyperthermic temperatures. In vitro, rat liver XO increased to greater than 50% O form at cytotoxic temperatures (30).

One lysosomal enzyme, p-nitrophenyl phosphatase, increased during the perfusions (Fig. 3). This could indicate labilization of the lysosomes which would be a source of proteolytic enzymes for conversion of xanthine oxidase type D to type O and enhanced proteolysis. There was no increase in β-glucuronidase activity in the perfusate. This enzyme is a glycoprotein which is cleared by the liver (31, 32). Since the enzyme level in the perfusate appeared to remain at a constant level, it could be inferred that there was a continued release of the enzyme into the perfusate during perfusion (Fig. 3). Prior studies showed that there was no inhibition of receptor-mediated endocytosis of glycoprotein by the perfused rat liver at hyperthermic temperatures (33).

However, lysosomal labilization could be the result of lipid peroxidation (34–37). Exposure of lysosomes to systems which generate superoxide, including xanthine oxidase, caused an increase in free nonsedimentable lysosomal enzymes (35–37).

Another effect which would be related to lipid peroxidation was the decrease in perfusate calcium which occurred during perfusion (Table 1). This could be referred to as calcium influx, a final event in cytotoxicity (38).

In summary, these data suggest a hypothesis to explain the hepatotoxic effects of hyperthermia. Hyperthermia causes tumor cell death along with ATP catabolism. This produces an increase in hypoxanthine and production of uric acid through the action of xanthine oxidase. Superoxide production, from the action of xanthine oxidase, leads to lipid peroxidation of cellular membranes. Labilization of the lysosomes and calcium influx follow. Further proof of this hypothesis is needed.

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