Testicular lactate dehydrogenase-X, which is involved in sperm maturation and metabolism, also was not affected *in vivo* [6, 15] despite the demonstration of inhibition *in vitro*, by a number of workers [14, 16, 17], thus reaffirming the fact that gossypol has different effects *in vitro* and *in vivo*.

The significant feature of the present investigation is that the effect of oral gossypol on drug-metabolising enzymes was examined at the dosage regimens with demonstrated anti-fertility effect [6]. One of the possible explanations for the inconsistency between the *in vitro* and *in vivo* observations could be due to the occurrence of gossypol in a protein bound form, leading to inadequate levels of free gossypol to bring about inhibitory action. Working with isolated rabbit heart, Qian *et al.** observed the inhibition of the ventricular contractibility by gossypol in Lacke's solution but not in blood. Similarly, incubation with human serum albumin *in vitro* has been found to protect human and hamster lactate dehydrogenase-X from gossypol inhibition [18].

Based on these studies it can be safely concluded that gossypol fed orally over a period of 5–7 weeks at the dosage which produces infertility is unlikely to affect or inhibit xenobiotic detoxification.

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The effect of hyperthermia on conversion of rat hepatic xanthine dehydrogenase to xanthine oxidase*

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Interest in the potential of hyperthermia in the treatment of cancer stems from research demonstrating an increased sensitivity of cancerous cells to hyperthermia, compared to that of normal tissue [1, 2]. Hyperthermic liver perfusion has been utilized in attempts to treat patients with liver metastases arising from resectable colorectal cancer [3, 4]. One problem that appears to limit application of this technique, however, is the significant hepatotoxic effects of hyperthermic perfusion [4, 5].

Hepatotoxicity caused by hyperthermic perfusion is manifested by elevations in SGOT and LDH enzyme levels, and pathologically characterized by a centrolobular necrosis [4, 5]. We have suggested that this heat-induced toxicity is a consequence of oxidative stress, resulting in lipid peroxidative damage [5]. The process of lipid peroxidation is thought to be initiated by the reaction of an activated oxygen species with polyunsaturated fatty acids of cellular phospholipids, resulting in a chain-reaction formation of lipid hydroperoxides and aldehyde derivatives [6]. Lipid peroxidative processes and their biological consequences have been the subject of extensive research and numerous reviews [6–8].

Previous reports support the contention that hyperthermia results in oxidative stress within biological systems. Depletion of glutathione, an important cellular antioxidant, has been shown to increase the thermal sensitivity of cells in culture at 42–43° [9–13]. The redox state of hepatic cytoplasm, as measured by the lactate/pyruvate ratio, has been shown to be reduced markedly in both dogs and humans during hyperthermic perfusion, again with significant changes observed at 42–43° [14–16].

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One mechanism suggested to result in the intracellular formation of either active oxygen species or oxygen-derived free radicals is the activity of the cytosolic enzyme, xanthine oxidase (XO, EC 1.2.3.2) [17, 18]. XO is capable of catalyzing the one electron reduction of molecular oxygen to yield the radical anion, superoxide ($O_2^{\cdot}$) [17, 19]. While there remains some debate in the literature about the actual species initiating the lipid peroxidation process, it has been clearly established that superoxide may serve as an important precursor for hydrogen peroxide (H_2O_2) and the highly reactive hydroxyl radical (OH·) [17, 20, 21]. Further, superoxide may either cause or facilitate the release of iron from ferritin, a step clearly implicated in the processes of lipid peroxidation [20–22].

XO exists in two structurally distinct forms, either the NAD^+-dependent dehydrogenase (type D) or oxidase (type O) [18, 23–25]. It is the latter form which reduces molecular oxygen to the superoxide radical. Under normal physiological conditions, XO is present predominantly in the type D [25–27]. Several different treatments have been shown by Della Corte and Stirpe [24, 26, 27] to cause conversion of the type D to the type O form, including (i) proteolysis, (ii) incubation at 37°, (iii) storage at −20°, (iv) exposure to certain organic solvents or (v) sulfhydryl reagents.

We have hypothesized that hyperthermic treatment of liver would result in a significant conversion of XO from the D to the O form and, further, that such conversion would result in elevated rates of superoxide formation. We have examined this hypothesis by evaluating the effect of time and temperature on the conversion of rat liver XO from type D to type O *in vitro* and also *in vivo* following hyperthermic perfusion. We have also attempted to characterize the nature of the temperature-induced conversion by examination of the reversibility of such conversion and determination of the activation energy of the type O enzyme-catalyzed reaction.

Methods

Male Sprague–Dawley rats, 275–325 g (King Animal Laboratories, Inc., Oregon, WI), were given standard lab rodent chow (Rat–Mouse Chow, Purina, St. Louis, MO) and water *ad lib.* and acclimatized for 1 week after receipt.

For *in vitro* experiments, rats were anesthesized with ether, and livers were flushed *in situ* with ice-cold phosphate buffer (KPE; 0.01 M KPO_4, 0.15 M EDTA, pH 7.4) and immediately excised and stored in KPE buffer prior to homogenization. Livers were rinsed and homogenized in 4 vol. of KPE buffer.

For *in vivo* experiments, livers were perfused by a recirculation technique with precise temperature control as previously described [28], with 175 ml Krebs–Henseleit bicarbonate solution, pH 7.4. A simple silastic tubing membrane oxygenator was employed to mix the perfusate with humidified $O_2 + CO_2$ (95:5). Flow rate through the liver was maintained at 3–4 ml/min/g liver by adjusting the height of the inflow reservoir [29]. Three livers were each perfused at 37, 42, and 42.5° for 3 hr. After perfusion, livers were flushed with ice-cold KPE buffer.

The livers were homogenized in 4 vol. of ice-cold KPE buffer in a Potter–Elvehjem homogenizer. The homogenate was centrifuged at 12,000 *g* for 10 min, and the resulting supernatant fraction was further centrifuged at 105,000 *g* for 60 min. The supernatant fraction derived from this ultracentrifugation step was utilized as the XO preparation, as previously described [23, 24].

Total XO activity was determined as described by Stirpe and Della Corte [24]. The reaction mixture contained 0.2 ml of cytosolic XO preparation, 60 μM xanthine and 0.67 mM NAD^+ in 50 mM potassium phosphate buffer (pH 7.8 containing 0.1 mM EDTA) in a final volume of 3 ml. Appearance of uric acid was monitored for 10 min at 292 nm, using a molar extinction coefficient of 9750 [30]. XO oxidase activity was determined in an identical system, but without NAD^+.

The effect of temperature on the rate of uric acid formation by XO was determined by performing the assay for XO total and oxidase activity at 35, 37, 39, 41, 42, 43 and 45°. The effect of temperature on the conversion of XO from type D to type O was determined by incubation of aliquots of the cytosolic XO preparation for 1 and 2 hr at 25, 35, 40 and 45° followed by assay for XO total and type O activity at 20°. The effect of hyperthermic perfusion on the conversion of XO to type O was determined by assay of XO total and oxidase activity in the 105,000 *g* supernatant fraction at 20° after each perfusion.

The effect of temperature on the rate of XO type O superoxide formation was tested as described by Fridovich [31]. A preparation of 100% type O enzyme was obtained by incubation of a cytosolic XO preparation at 37° for 5 hr as previously described [24]. The 1.0-ml reaction mixture contained xanthine oxidase (10–20 μl), xanthine (0.2 mM) and cytochrome *c* (30 μM) in 0.05 mM potassium phosphate buffer, pH 7.8, with 0.1 mM EDTA. Superoxide production was calculated from the absorption at 550 nm by using a molar extinction coefficient of 21,000 for cytochrome *c* [31]. Initial reaction velocities were determined in a Gilford Response spectrophotometer and were used to determine activation energy for this reaction by Arrhenius plot.

Where possible, data were tested by analysis of variance. Comparison between means was by least significant difference test.

Results

The percentage of XO activity of rat hepatic cytosol represented by the type O form was increased markedly as a function of increasing assay temperature, as shown in Fig. 1. Total activity, however, remained relatively constant in this experiment. This may reflect a progressive inactivation of the type D form of activity with elevated temperature. The activation energy for the generation of superoxide by type O enzyme was determined over the temperature range utilized in this experiment and was found by Arrhenius plot to be 9.2 kcal/mol (Fig. 2).

Incubation of the cytosolic XO preparation at hyperthermic temperatures for time periods similar to those utilized in hyperthermic perfusion resulted in a substantial temperature-dependent increase in the percentage of total XO activity represented by the type O form (Fig. 3). The change in type O form activity in cytosol incubated at 25° was entirely reversible by incubation with dithioerythritol (DTE), suggesting that no structural changes had occurred. However, DTE treatment had no discernible effect on type O activity in cytosol incubated at or above 35°.

Hyperthermic liver perfusion for a 3-hr period resulted in a significant increase in the percentage of total XO activity represented by the type O form (Fig. 4). Incubation of the hepatic homogenates prepared from hyperthermic perfused liver with DTE substantially reversed the D to O form conversion.

Discussion

We have postulated that the hepatotoxicity which occurs following hyperthermic liver perfusion is a consequence of oxidative stress, manifested in the processes of lipid peroxidation [5]. In this research, we have investigated the role that conversion of xanthine oxidase (XO) from an NAD^+-dependent dehydrogenase (type D) to the superoxide ($O_2^{\cdot}$)-generating type O form may play in causing this oxidative stress. We have hypothesized that conversion of XO from type D to type O, as a consequence of hyperthermia, would result in elevated rates of production of superoxide which may then serve as a source for oxygen-derived free radical species.

The exact role the formation of superoxide may play in the initiation or propagation of lipid peroxidation has not been clearly established. The potential of a direct superoxide–lipid interaction appears to be limited due to the

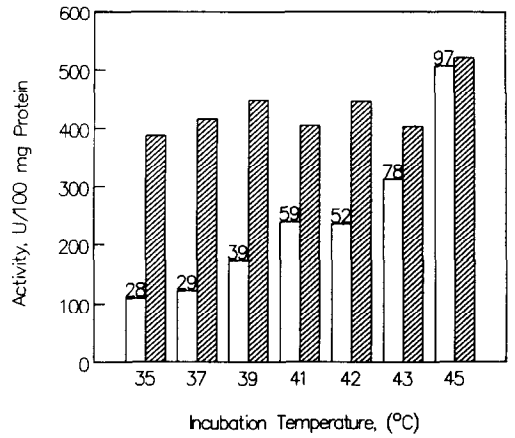

Fig. 1. Effect of a 20-min hyperthermic incubation on D form and total XO activity of rat hepatic cytosol. Bars represent the mean for O form (□) or total XO activity (▨). The values above the open bars are the percentage of total activity represented by the O form.

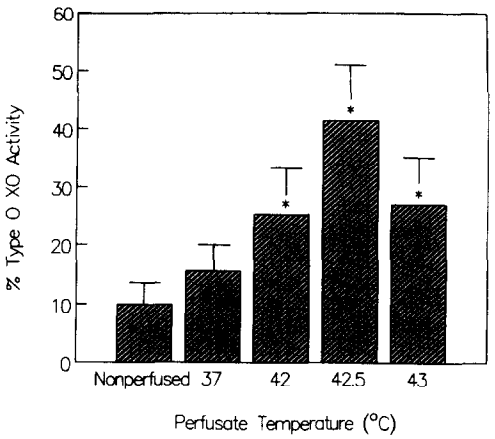

Fig. 4. Effect of hyperthermic perfusion on conversion of XO from type D to type O form in rat liver. Values are means ± SD for groups of N = 4. Means marked with an asterisk were significantly different from nonperfused mean value, $P < 0.05$.

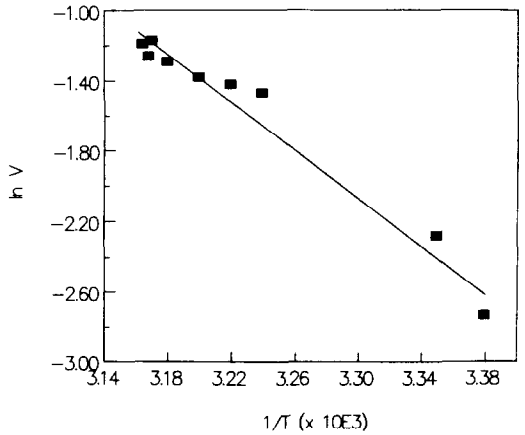

Fig. 2. Temperature dependence of the XO generation of superoxide. Values are in initial velocity vs 1/incubation temperature. $r^2 = 0.964$.

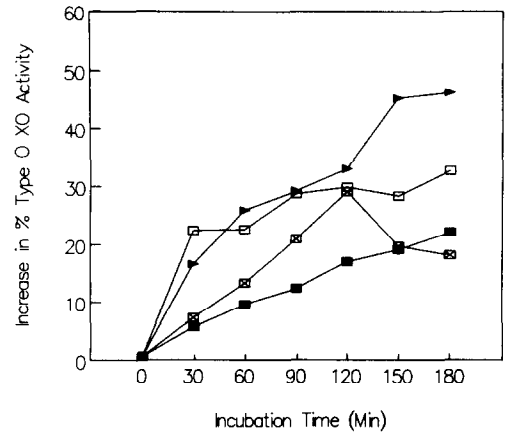

Fig. 3. Effect of incubation time and temperature on the hyperthermic-induced conversion of XO D to O form in rat hepatic cytosol. Values are means for groups of N = 3 determinations/time and temperature. Key: (■) 25°; (□) 35°; (▶) 40°; and (⊠) 45°.

relatively low reactivity of superoxide in aqueous medium [32]. However, superoxide may lead to the formation of the hydroxyl radical, via an iron-catalyzed Haber–Weiss reaction [33]. It has also been suggested that superoxide, as well as XO itself, may play a role in the release of iron from the iron storage molecule ferritin [22, 34]. Such "free" or, more precisely, low molecular weight chelated iron has been implicated in the initiation of lipid peroxidation [35].

Our initial experiment demonstrated that incubation of rat hepatic cytosol for even short periods of time at elevated temperatures resulted in a significant increase in the percentage of XO in the type O form. These results are in agreement with those of Della Corte and Stirpe [27] and emphasize the relative ease by which XO can be converted to the superoxide-generating type O oxidase *in vitro* [36]. Similar results were obtained following incubation for a time period analogues to that of our hyperthermic liver perfusion protocol. However, the magnitude of the D to O conversion was significantly less in this experiment.

Results from the *in vivo* experiment, a 3-hr hyperthermic perfusion of rat liver, were consistent with the *in vitro* experiments. We observed a significant increase in the percentage of total XO activity represented by type O form as a function of perfusate temperature. The percent O form was maximally increased in liver perfused at 42.5°, to approximately four times the nonperfused control levels, suggesting that the potential for the formation of superoxide in these tissues had been increased markedly.

There appears, however, to be a basic difference between the *in vitro* and *in vivo* experiments, beyond simply the magnitude of the D to O conversion. Della Corte and Stirpe have shown that the type D to O conversion may be reversed significantly with DTE treatment, except when the conversion had been accomplished by proteolysis [24, 27]. In our *in vitro* experiment, we were unable to reverse the D to O conversion with such treatment following incubation at 35° or higher. This suggests that at least some aspects of the D to O form conversion in rat hepatic cytosol were proteolytic in nature. In contrast, in the *in vivo* experiment, XO O form could be readily reconverted to the D form by treatment with DTE and, hence, is not reflective of proteolytic changes in the enzyme. It is interesting to note that an enzyme system for the conversion of XO O to D has been identified [37]. It is tempting to postulate that, in the case of our *in vivo* experiment, the cellular machinery responsible for the

reconversion of O to D form remained intact, in contrast to the *in vitro* experiments.

The experiments described here were intended to suggest one possible mechanism for increased oxidative stress consequential to hyperthermic liver perfusion. The data described are consistent with the hypothesis that hyperthermia results in the conversion of XO from type D to type O, with the concomitant increased production of superoxide. The role this may play in the development of peroxidative damage in a hyperthermic situation will be the subject of future research.

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