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SHORT COMMUNICATIONS

Hepatic microsomal drug metabolism after administration of endotoxin in rats

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Endotoxemia accompanying gram negative bacterial infections is a serious, life-threatening condition that frequently occurs in children and adults with altered immune mechanisms and in older patients after instrumentation of the urinary tract.

A key aspect in the therapeutic management of patients with endotoxin shock is the administration of pharmacologic agents. Since most drugs are partially or completely eliminated by hepatic metabolism prior to their excretion from the body, assessment of the capability of the liver *in vivo* and *in vitro* to metabolize drugs in the course of endotoxemia is essential for the rational formulation of drug dosage regimens in this condition. The purpose of the present investigation was to explore the ability of the liver to metabolize drugs after the administration of endotoxin in rats.

Adult female rats obtained from Holtzman and weighing between 190 and 220 g were used. The animals were fed standard laboratory diet and water *ad lib.* until the time of the intraperitoneal injection of endotoxin or saline, when food and water were removed for the duration of the experiment. Lyophilized, purified lypopolysaccharide from *Escherichia coli* 026 B6 (obtained from Difco Laboratories, Detroit) was freshly reconstituted in sterile pyrogen-free isotonic saline just prior to use and employed as the endotoxin preparation; the dose given was 1 mg in 1 ml of saline. Endotoxin was administered intraperitoneally to six rats between 8:00 and 9:00 a.m.; simultaneously six control animals received an equal volume of isotonic saline by intraperitoneal injection. Twenty-four hr later, under sodium pentobarbital anesthesia, all animals were sacrificed and the livers removed *in toto*. A small piece of each liver was resected for histologic examination and the remainder of the liver was prepared for enzymic studies.

In an additional experiment, a test group of seven rats (four experimental and three control) were treated as outlined but only bilirubin UDPG transferase activity was determined in liver homogenates.

Rat livers were removed, rinsed with cold saline, blotted, weighed and homogenized in the cold (4°) with 2 vol. (2 ml/g of liver) of cold isotonic 1·15% (w/v) KCl for approximately 2 min in a Potter homogenizer with a Teflon pestle. Microsomes were prepared by differential centrifugation at 104,000 *g* for 60 min in a Beckman L2-65B ultracentrifuge.

Microsomes and 9000 *g* supernatant fractions were used immediately or, when assay conditions allowed, maintained at −90° for no longer than 14 days. Simultaneous controls which had been stored under identical conditions were employed in each experiment.

The suspended microsomal pellet obtained from the 104,000 *g* centrifugation was used for the following assays: bilirubin uridine diphosphoglucuronic acid (UDPGA) transferase [1], benzpyrene hydroxylase [2–4], aniline hydroxylase [5] and cytochrome P-450 content [6]. For nitro- and azoreductase activities [7, 8] the 9000 *g* supernatant fraction was used. Protein concentrations were determined by the method of Lowry *et al.* [9], using bovine serum albumin as the reference protein.

Liver material for histology was fixed in 10% formalin and stained with hematoxylin and eosin. The sections were graded as to the type and extent of necrosis, the amount of fat present and the inflammatory response on a 0–4+ basis.

In other experiments with Holtzman rats of the same sex and weight, the LD_{50} to intraperitoneal *E. coli* endotoxin 026 B6 had been determined to be between 2·0 and 2·5 mg. One endotoxin-treated animal of the six injected died in the first 24 hr. The other five and all six controls tolerated the intraperitoneal injection and appeared normal at the time of sacrifice.

The livers of the five saline-treated rats showed no histologic evidence of necrosis, fat or inflammation. On the other hand, the livers of the endotoxin-treated animals demonstrated pathologic abnormalities, but the extent of the lesion was surprisingly limited considering the rather large dose of endotoxin administered. Two rats showed hemorrhagic zonal liver infarcts judged to be 2+ in extent, one rat showed a few scattered mid-zonal infarcts graded as 1+ and the remaining two animals failed to show any infarctions. The degree of pericentral necrosis was 1+ in all endotoxin-treated rats with mild pericentral fat present in all rats. The inflammatory response with polymorphonuclear leucocytes predominating in areas of necrosis was 1–2+ in all animals.

The mean values for the activities *in vitro* of the liver microsomal drug-metabolizing enzymes investigated and of microsomal cytochrome P-450 content were lower in the endotoxin-treated rats. However, statistical significance ($P < 0{\cdot}1$ or smaller) could be established only for bilirubin UDPGA transferase, aniline hydroxylase, cytochrome P-450 and benzpyrene hydroxylase (Table 1).

The extent of hepatocyte damage as documented by light microscopy and the degree of alteration in enzyme activities did not demonstrate any correlation.

Endotoxin causes liver damage by a number of mechanisms. The liver is the main organ for the detoxification of these bacterial products and the integrity of this organ is crucial in the defense against states of endotoxemia [11]. Endotoxemia has been shown to have numerous effects on mitochondrial membrane function [12] and uncouples phosphate esterification in mitochondrial respiration [13]. In addition, endotoxin has been shown to activate adenyl cyclase [14]. While endotoxin directly injures liver cells, it also causes profound circulatory derangements that contribute to the liver injury. Some of these circulatory changes are mediated by the release of vasoactive substances [15], although endotoxin has been shown to cause direct vasoconstrictive effects in the isolated liver preparations [16].

Extensive studies have shown that the damaged liver of carbon tetrachloride-treated rats has decreased activities of microsomal enzymes which catalyze the oxidation and reduction of drugs [17–21] accompanied by a decrease in liver microsomal P-450 [22–24]. These and other biochemical as well as structural disturbances caused by carbon tetrachloride are the result of peroxidative destruction [25] and decreased hydrofobicity [26] of microsomal

Table 1. Hepatic microsomal drug metabolism *in vitro* in endotoxin- and saline-treated rats

Enzyme	Rat treatment	Mean activity*	S. D.	P
Bilirubin UDPGA transferase	Endotoxin	0·64	0·59	< 0·02
	Saline	1·23	0·62	
Aniline hydroxylase	Endotoxin	11·4	2·75	< 0·01
	Saline	48·0	9·30	
Benzpyrene hydroxylase	Endotoxin	0·14	0·06	< 0·1
	Saline	0·29	0·13	
Nitroreductase	Endotoxin	0·78	0·69	< 0·5
	Saline	1·83	1·20	
Azoreductase	Endotoxin	16·6	1·05	< 0·5
	Saline	17·6	1·30	
Cytochrome P-450	Endotoxin	0·31	0·06	< 0·01
	Saline	0·56	0·09	

* Enzyme activities are expressed as ng of the product formed/mg of protein/incubation period. Incubation times were 30 min for bilirubin UDPGA transferase, 10 min for benzpyrene hydroxylase, and 60 min for aniline hydroxylase, nitroreductase and azoreductase. Cytochrome P-450 content was calculated on the basis of an extinction coefficient of 91 cm-mM [10].

membranes; CCl_4 binds irreversibly to ribosomal proteins and lipids [27]. Also, experimental liver disease in mice infected with murine hepatitis virus [28] and in rabbits with surgical obstructive jaundice [29, 30] produced alterations in microsomal drug-metabolizing enzyme activity.

Our findings of decreased microsomal cytochrome P-450 content and low microsomal drug-metabolizing enzyme activities in the liver of rats treated with endotoxin are in general agreement with the results obtained utilizing liver damaged from other etiologies. Our data are insufficient to indicate whether or not a similar mechanism (i.e. damage to the endoplasmic reticulum) is operative. The mechanism by which endotoxin affects microsomal drug enzyme systems remains to be elucidated.

In animals as well as in humans [31, 32] instrinsic liver disease alters drug metabolism. Of interest, in view of the present study, is the possible role of absorbed bacterial endotoxins in initiating or perpetuating liver injury. Rats with a fatty liver due to choline deficiency are no longer able to detoxify endotoxins arising from the gastrointestinal tract. These endotoxins lead to tissue necrosis at doses normally rendered innocuous, and this injury may, in turn, play a key role in the development of cirrhosis in these animals [33, 34]. It is conceivable, therefore, that part of the alterations in drug metabolism in human liver disease may be related to endotoxin effects on the hepatocytes. Drug metabolism in patients with endotoxin shock may be complicated by drug pretreatment as well as other environmental and genetic factors.

In summary, we have demonstrated that endotoxin administered to rats alters hepatic microsomal drug-metabolizing enzyme activity. Endotoxin treatment results in a statistically significant decrease in activities of bilirubin UDPGA transferase, aniline hydroxylase, benzpyrene hydroxylase and in cytochrome P-450 content. Further work is necessary to elucidate the mechanism involved and to determine to what extent our findings are relevant to the therapeutic management of patients with endotoxemia.

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Effect of oximes upon inhibition of bone marrow acetylcholinesterase by neostigmine

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The anti-cholinesterase agent, neostigmine, has been used clinically and experimentally for many years [1]. *In vivo*, in the mouse, neostigmine inhibits femoral marrow acetylcholinesterase (AChE) activity. It also triggers marrow haemopoietic stem cells into DNA synthesis [2]. The demonstration that, *in vitro*, cholinergic agents initiate DNA synthesis in the bone marrow stem cell [3], suggests that the *in vivo* effect of neostigmine may be secondary to a build up of acetylcholine. The oximes 2-PAM (1-methyl pyridinium-2-aldoxime) and toxogonin (*bis*[4-hydroxy iminomethyl pyridinium-1-methyl] ether dichloride) are used clinically to reverse the effects of organophosphorus cholinesterase inhibitors [4, 5]. There is also evidence that, in some systems, the effects of neostigmine can be reversed by oximes [6, 7]. It was thought that the oximes may provide a useful tool in the elucidation of the mechanisms of stem cell triggering by neostigmine, and may also provide a means of reversing the stem cell effect of the anticholinesterase agent. The present study therefore investigated the effects of toxogonin and the chloride of 2-PAM (2-PAM-Cl), *in vitro*, upon inhibition of bone marrow AChE activity by neostigmine.

B_6AF_1/J female mice aged $2\frac{1}{2}$–$4\frac{1}{2}$ months were used as donors of femoral bone marrow. Suspension cultures of marrow cells were set up in Fischer's medium (approx $5–6 \times 10^6$ cells per ml), as previously described [8]. The cells were allowed to equilibrate in a shaking water bath at 37° for 15 min, then the drugs were added and the incubations were carried out, as indicated in the results. At the end of incubation AChE activity was measured by the spectrophotometric method of Ellman *et al.* [9]. It has been shown [10] that oximes accelerate non-enzymic hydrolysis of acetylthiocholine, the substrate for AChE in the Ellman assay. In the present experiments dilution of the oximes for AChE assay was not sufficient to prevent significant non-enzymic hydrolysis. A filtration method was therefore devised to remove the oximes at the end of incubation. Suspensions from all cultures were filtered in those experiments where oximes were used. Aliquots of suspension containing the required cell numbers were passed through Millipore filters (0·45 μm pore size) in Swinnex filter units. Air (12 ml) was passed through each filter and the filters were then dropped into assay bottles containing phosphate buffer. The AChE activity of the cells on the filters was then measured [9]. Throughout the assay the bottles containing the filters were shaken at room temperature to ensure mixing of the solutions above the filters. Blanks containing no cells were also shaken. For the spectrophotometer readings the solutions were poured off the filters, the readings were made, and the solutions immediately poured back onto the filters. Readings were taken every 3–4 min for periods of up to 1 h. In all experiments a marrow sample that had been cultured with oxime only was included, to check that the drug had been removed by the filtration procedure.

Readings taken during the first 30 min of assay were used in the calculations. Using the method of least squares, the slope of the line of best fit was calculated. This gave the change in absorbance units per min. Values obtained by subtraction of blank values from sample values were used to calculate the percentage protection or reversal.

$$\%\ \text{Protection or reversal*} = [(z - y)/(x - y)] \times 100$$

x = control (no treatment) value
y = value from sample treated with neostigmine only
z = value from sample treated with neostigmine plus oxime

As 10^{-6} M neostigmine completely inhibited AChE activity, but 10^{-10} M neostigmine had no effect upon the enzyme activity, 10^{-8} M neostigmine was used in most of the experiments.

Table 1 shows the effect of adding 10^{-3} M or 10^{-4} M 2-PAM-Cl or toxogonin to the cultures 10 min before addition of neostigmine. A high level of protection is seen when 10^{-3} M 2-PAM-Cl is added before 10^{-8} M neostigmine, and the level of protection is similar whether the cultures are incubated for 30, 90 or 120 min. 10^{-3} M 2-PAM-Cl is much less effective when 10^{-7} M or 10^{-6} M neostigmine is added. 10^{-4} M 2-PAM-Cl provides some degree of protection against 10^{-8} M neostigmine when incubated for 30 or 60 min, but when the incubation is extended to 90 or 120 min the protective effect disappears. 10^{-3} M toxogonin partially protects against 10^{-8} M neostigmine and to a lesser extent against 10^{-7} M neostigmine, but 10^{-4} M toxogonin has no protective effect against 10^{-8} M neostigmine.

* Protection when oxime added before neostigmine, reversal when added afterwards.