CONCISE

PUBLICATIONS

Stimulation of Heme Oxygenase in Macrophages and Liver by Endotoxin

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ABSTRACT In rat peritoneal macrophages, engaged in erythrophagocytosis in vitro, endotoxin stimulated heme oxygenase (HO) activity, which was additive to the substrate-mediated enzyme induction produced by the ingested erythrocyte hemoglobin. Endotoxin neither appeared to injure the erythrocytes, nor did it enhance the rate of erythrophagocytosis. In intact rats, HO activity in both parenchymal and sinusoidal cells of the liver was increased after treatment with endotoxin. It is likely that endotoxin directly stimulates HO activity, a process which may account for the reported rise in bilirubin formation in endotoxin-treated animals. The effect of endotoxin on HO may represent part of the general activation of phagocytic cells by endotoxin.

INTRODUCTION

Leukocytes exposed to endotoxin in serum-containing culture exhibit a variety of metabolic and functional alterations. These include: stimulation of glycolysis, the hexose monophosphate shunt, and RNA synthesis (1, 2); leakage of lysosomal enzymes; (2) and enhanced phagocytosis (2). In the process, the leukocytes appear to inactivate the endotoxin (3, 4). Lymphocytes undergo blastogenesis, (5) and in macrophages, endotoxin produces mitosis (6) and inhibition of migration (7). Moreover, macrophages preexposed to endotoxin become cytotoxic for lymphoma cells (8). In vivo, endotoxin activates the reticuloendothelial system (RES)[1] (9, 10) and exerts an adjuvant-like effect on antibody response (11, 12). Further, in rats, intraperitoneal injection of endotoxin is followed, within 8–24 h, by a doubling of the rate of bilirubin formation (13). The mechanism underlying this increased bilirubin production remained unclear, as it could not be determined whether the endotoxin injured circulating erythrocytes, stimulated erythrophagocytosis, or increased the turnover of cellular hemoproteins.

We now wish to present evidence that endotoxin stimulates heme oxygenase (HO) in macrophages in vitro and in liver in vivo. The activity of this microsomal enzyme system which converts heme to bilirubin IXα (14) is stimulated by heme and hemoproteins, presumably reflecting substrate-mediated enzyme induction (15). Thus, in macrophages engaged in erythrophagocytosis in vitro, the initially low heme oxygenase (HO) activity (16) rises several-fold after ingestion of erythrocytes (17). Similarly, in experimental animals, enzyme activity in hepatic parenchymal (18) or sinusoidal (19) cells increases strikingly after intravenous administration of dissolved hemoglobin or damaged erythrocytes. The stimulation of HO by endotoxin appears

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[1] *Abbreviations used in this paper:* RES, reticuloendothelial system; HO, heme oxygenase; LPS, lipopolysaccharide.

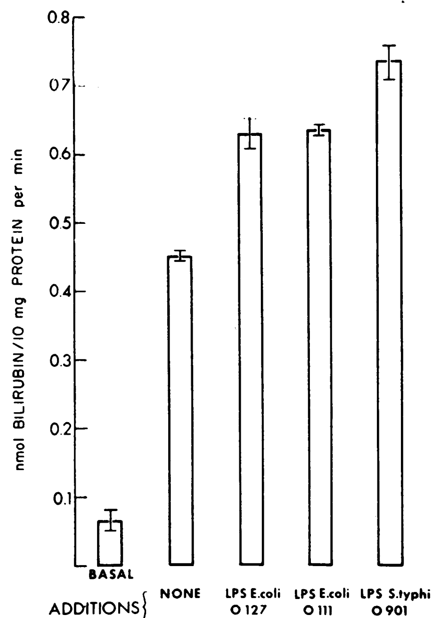

FIGURE 1 The effect of endotoxin on the induction of HO in macrophages engaged in erythrophagocytosis in vitro. HO activity was determined after 4½ h of incubation with the LPS preparation indicated in a final concentration of 50 μg/ml. All incubations were performed in two identical flasks, and in each, enzyme activity was measured in duplicate. Results are given as the mean and range of the four individual measurements. HO activity in resting (basal) macrophages, not engaged in erythrophagocytosis, is shown on the left.

to involve a mechanism which may differ from substrate-mediated enzyme induction.

METHODS

The harvesting and incubation of rat peritoneal macrophages, the preparation of ^{59}Fe-labeled antibody-coated erythrocytes, the quantification of erythrophagocytosis, and the measurement of HO activity have been described previously (17). The conditions of incubation deviated slightly from the system previously used (17), in that the rotating waterbath was set at 50 cycles/min, and the incubation was terminated after 4½ h.

The endotoxin employed in this study was a highly purified lipopolysaccharide (LPS) from *Escherichia coli* 0 111, *E. coli* 0 127, or *Salmonella typhi* 0 901 (Difco Laboratories, Detroit, Mich.), obtained in lyophilized form, dissolved in Medium 199 or in sterile saline, and used interchangeably in these experiments. The two *E. coli* strains are among the most frequent enteropathogenic bacteria isolated from infectious enteritis, and *S. typhi* 0 901 is a pathogenic strain that has been studied extensively. The slightly turbid stock solutions were sonicated before use in order to disperse the larger particles.

The effect of endotoxin on hepatic HO in vivo was studied in adult fed female Sprague-Dawley rats, injected intraperitoneally with a single dose of 0.5 mg LPS from *E. coli* 0 111, *E. coli* 0 127, or *S. typhi* 0 901. Control animals received an intraperitoneal injection of sterile isotonic saline. The rats were sacrificed 18–20 h later, the livers were removed, and HO activity was determined in the 20,000-*g* supernatant fraction of whole liver homogenate, as described previously (20). No apparent differences in liver weight or morphology were observed between treated and control animals. To study the enzyme activity in the sinusoidal (Kupffer) cells of the liver, which functionally represent the organ's sessile macrophage population (21), rat liver was perfused *in situ* with a solution of 0.04% Pronase; sinusoidal cells were separated and isolated by a flotation procedure described in detail elsewhere (19). Contamination of the cell isolate with parenchymal cells did not exceed 4%, as judged by phase microscopy. All enzyme assays were carried out under V_{max} conditions.

RESULTS

Endotoxin (50 μg/ml) showed no cytotoxic effects on macrophages in vitro as judged by glass adherence, morphology by light and electron microscopy, trypan blue exclusion, and phagocytic ability. Nor did endotoxin in this concentration interfere with the rate or magnitude of erythrophagocytosis, since 50±5% of the antibody-coated erythrocytes offered were regularly ingested during the first 2 h of incubation. HO activity in macrophages incubated for 4½ h with endotoxin in the absence of erythrocytes (basal) was comparable to the low activity in native resting macrophages (17) (Fig. 1). Incubation of macrophages with antibody-coated erythrocytes in the absence of endotoxin led to the predictable marked increase in enzyme activity (Fig. 1); we previously showed that this stimulation fulfills the criteria for inductive formation of new enzymes (17). Addition of LPS *E. coli* 0 111, *E. coli* 0 127, or *S. typhi* 0 901 in a final concentration of 50 μg/ml to the erythrophagocytic macrophages resulted in a further stimulation of HO activity (Fig. 1). This augmented stimulation was evident only when the endotoxin was present from the start of the incubation; a 2–3-h delay in adding the endotoxin failed to stimulate enhanced enzyme production. The lower concentration limit for the stimulatory effect of endotoxin on HO was 0.5–1.0 μg/ml, which is similar to the dose response established for the cytotoxic effect for lymphoma cells (8). The observed enzyme stimulation was not due to endotoxin-mediated erythrocyte injury, since the stimulatory effect was lost when the antibody-coated erythrocytes were pretreated for 2 h with high concentrations of endotoxin (2 mg/ml), followed by extensive washing of the cells before incubation. The possibility that the stimulatory effect might be related to endotoxin-induced alteration in the metabolism of the macrophages was also investigated. However, comparison of the following metabolic parameters failed to reveal significant differences between erythrophagocytic macrophages incubated with or without endotoxin: glucose consumption (17), conversion of [1-^{14}C]glucose to $^{14}CO_2$ (17), RNA synthe-

sis from [5-^{3}H]uridine (*22*), overall protein synthesis from [2-^{14}C]leucine (*22*), and incorporation of [2-^{14}C]-leucine into isolated macrophage microsomes (*23*). Finally, phase microscopy of macrophages degrading phagocytized erythrocytes in the presence of endotoxin failed to exhibit an apparent acceleration in the rate of the progressive disintegration of the interiorized erythrocytes. These findings do not exclude the possibility that endotoxin may have a more specific and restricted effect on individual macrophage organelles, detectable only by electron microscopy or cell fractionation.

In intact rats HO activity in whole liver homogenate was increased several-fold after treatment with each of the three endotoxin preparations (Fig. 2). As shown previously (18, 19), the HO activity of whole liver reflects mainly the activity of hepatic parenchymal cells which make up approximately 85% of the total tissue protein. As shown in Fig. 3, basal HO activity was higher in sinusoidal than in parenchymal cells and was doubled in sinusoidal cells obtained from the liver of rats treated with endotoxin. In sinusoidal cells obtained from endotoxin-stimulated liver, the enzyme activity approached the values observed in fully stimulated macrophages in vitro (Fig. 1). It should be noted, however, that because of the relatively small fractional volume of sinusoidal cells in the liver, most of the endotoxin-mediated stimulation of HO activity in the whole liver homogenate (Figs. 2 and 3) was contributed by increased enzyme activity in hepatic parenchymal cells.

DISCUSSION

The reported rise in bilirubin production in rats treated with endotoxin (13) suggested increased heme catabolism. The present demonstration of an endotoxin-related stimulation of HO activity in vivo and in vitro supports this interpretation, and offers a possible explanation for the occurrence of hyperbilirubinemia in gram-negative bacteremia (*24–27*). Among the several possible mechanisms which may account for this finding are: (*a*) endotoxin-produced injury to erythrocytes, causing enhanced susceptibility to erythrophagocytosis and hemolysis, (*b*) accelerated catabolism of cellular heme or heme enzymes, particularly in the liver (28), and (*c*) direct stimulation of the HO system.

Although indirect evidence has been presented that endotoxin alters surface properties of circulating erythrocytes, (*29*) a process which may lead to enhanced erythrophagocytosis and eventually to hemolysis and hemoglobinemia, the present observations in vitro do not support this mechanism as accountable for the endotoxin-mediated stimulation of HO. Specifically, endotoxin did not increase the rate or magnitude of erythrophagocytosis by the macrophages; nor did pretreatment of the erythrocytes with endotoxin, followed by washing of the cells, result in enhanced stimulation of HO activity.

A second possible mechanism, assuming that endotoxin may enhance the degradation or turnover of heme or heme enzymes in the liver (28), could account for our demonstration of elevated enzyme activity in he-

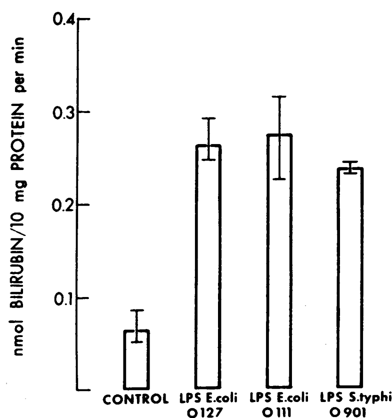

FIGURE 2 The effect of endotoxin on HO in rat liver in vivo. Each animal received an intraperitoneal injection of 0.5 mg LPS in 1 ml sterile saline 18–20 h before the removal of the liver. The bars represent the mean and range of values in three to six identically treated animals.

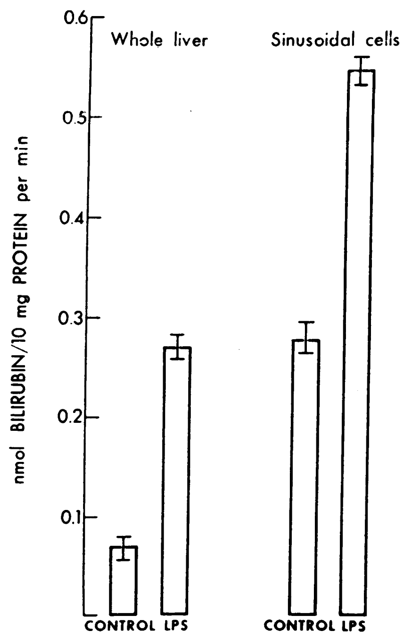

FIGURE 3 Comparison of the effect of endotoxin on HO in whole rat liver and in hepatic sinusoidal cells. Two rats were treated either with 0.5 mg LPS *S. typhi* 0 901 or with sterile saline as indicated in Fig. 2. Bars represent the mean of quadruplicate determinations, brackets the range of values.

patic parenchymal cells. On the other hand, it would fail to explain the endotoxin-induced increase in HO activity in the hepatic sinusoidal cells in vivo and in the erythrophagocytic macrophages in vitro, since both of these cell types contain very low intrinsic hemoprotein concentrations.

The third possibility, that endotoxin directly stimulates HO in hepatic parenchymal and sinusoidal cells in vivo and in macrophages engaged in erythrophagocytosis in vitro, is consistent with all present findings. While no information is available on the optimal time interval required for maximal stimulation of the enzyme in the two hepatic cell populations, in vivo HO activity clearly was increased 18–20 h after a single injection of endotoxin (Figs. 2 and 3). In macrophages in vitro, on the other hand, a stimulatory effect of endotoxin was detectable only in association with erythrophagocytosis (Fig. 1) and only when the endotoxin was present at the time of active erythrophagocytosis; neither pretreatment of the erythrocytes with endotoxin, nor its delayed addition to the incubation mixure, enhanced enzyme simulation. This suggests that, under the experimental conditions in vitro, endotoxin gained access to the macrophages only at the time of, or in association with, the ingestion of antibody-coated erythrocytes.

The mechanism by which endotoxin stimulates HO is not clear. In contrast to previous studies (1, 2), we were unable to detect endotoxin-mediated stimulation of carbohydrate, RNA, or protein metabolism in macrophages engaged in erythrophagocytosis. A possible explanation for this difference may be the deliberate omission of serum from our incubation mixture. It should be noted, moreover, that estimation of overall protein and RNA synthesis, as performed in the present experiments, does not exclude the possibility that endotoxin may have a more specific and restricted stimulatory effect on the synthesis of individual cell organelles or organelle components. Finally, the endotoxin-mediated stimulation of HO in macrophages and in hepatic cells may simply represent one aspect of the general activation of phagocytic cells by endotoxin. Thus, it may be related to the enhanced phagocytosis (1), accelerated killing of ingested bacteria (1), and augmented cytotoxicity for malignant cells (8), which have been noted under the influence of endotoxin.

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