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Prostanoid Production by Lipopolysaccharide-Stimulated Kupffer Cells

GARY J. BOWERS, M.D.,*‡ THOMAS J. MACVITTIE, PH.D.,* E. F. HIRSCH, M.D.,‡ JAMES C. CONKLIN, M.D.,* RONALD D. NELSON,† RUDOLPH J. ROETHEL, B.S.,† AND MITCHELL P. FINK, M.D.†

*Armed Forces Radiobiology Research Institute, Defense Nuclear Agency, Bethesda, Maryland 20814-5145; †Naval Medical Research Institute, Bethesda, Maryland 20814-5055; ‡Boston University Medical Center, Department of Surgery, Boston, Massachusetts 02118

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Although some data suggest that macrophages in the reticuloendothelial system (RES) are important sources of thromboxane A_2 (TxA_2) and prostacyclin (PGI_2) during endotoxic shock, we are unaware of data documenting the ability of hepatic macrophages (Kupffer cells) to release either TxA_2 or PGI_2 when exposed to lipopolysaccharide (endotoxin, LPS). In this study, Kupffer cells were examined for their ability to release prostaglandin E_2 (PGE_2), TxA_2, and PGI_2 following stimulation with 0, 1.0, 50.0, and 100.0 μg/ml of *Escherichia coli* LPS. Kupffer cells were obtained from rat livers by enzymatic digestion with 0.05% collagenase followed by enrichment of the macrophage population on the basis of differences in density and adherence among the various cell populations isolated. Based on several criteria (phagocytosis of opsonized sheep erythrocytes, positive staining for esterase and peroxidase, failure to replicate), 95% of adherent cells were Kupffer cells. After 4 days of incubation, cells were stimulated with various doses of LPS for 4 and 8 hr. Prostanoid concentrations in culture supernatants were determined by radioimmunoassay. Increasing doses of LPS significantly ($P < 0.001$) increased the concentration of immunoreactive PGE_2 ($iPGE_2$) and $iTxB_2$ (the stable metabolite of TxA_2). The concentration of i6-keto-$PFG_{1\alpha}$ (stable metabolite of PGI_2) increased following stimulation with 1.0 μg/ml of LPS, but declined as the dose of LPS was increased. The results provide evidence that endotoxin-activated Kupffer cells, like other macrophage populations, release several metabolites of arachidonic acid. Kupffer cell-derived prostanoids, particularly TxA_2, may be important mediators of some of the pathophysiologic manifestations of acute endotoxemia.

INTRODUCTION

Thromboxane (Tx) A_2 and prostacyclin (PGI_2) have been implicated as contributing to the pathophysiologic manifestations of endotoxic shock [13]. Thromboxane A_2 is a potent vasoconstrictor [29] and inducer of platelet [18] and leukocyte [33] aggregation. There is evidence that TxA_2 participates in the pathogenesis of endotoxin-induced pulmonary hypertension [8, 16], disseminated intravascular coagulation [1, 35], hepatocellular dysfunction [1, 35], and mortality [1, 35]. Prostacyclin is a vasodilator [2] and platelet antiaggregant [27]. This prostanoid may contribute to delayed hypotension in experimental endotoxic shock [7] and decreased systemic vascular resistance in human sepsis [31].

Several lines of evidence suggest that macrophages within the reticuloendothelial system (RES) are an important source of TxA_2 and PGI_2 in endotoxic shock. First, systemically administered endotoxin is primarily sequestered by fixed macrophages (Kupffer cells) within the liver [26]. Second, endotoxin stimulates TxA_2 and prostacyclin release from cultured peritoneal macrophages [9]. Third,

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stimulation of the RES sensitizes animals to the prostaglandin (PG)-releasing effects of endotoxin whereas RES depression has the opposite effect [10].

Although endotoxin stimulates Kupffer cells to release PGE_2 [4, 11] and a variety of other inflammatory mediators and lytic enzymes [3, 4, 11, 23, 24], we are unaware of data indicating that Kupffer cells synthesize TxA_2 or PGI_2 when challenged with endotoxin. The purpose of the present study was to determine the effects of the endotoxin *Escherichia coli* on *in vitro* TxA_2, PGI_2, and PGE_2 production by rat Kupffer cells.

MATERIALS AND METHODS

Materials. Halothane (Ayerest Laboratories, New York, N. Y.), Hanks' balanced salt solution (HBSS) with (+) and without (−) calcium and magnesium salts (GIBCO, Grand Island, N. Y.), collagenase (Millipore Corp., Freehold, N. J.), trypsin (DIFCO, Detroit, Mich.), *E. coli* lipopolysaccharide (055:B5; DIFCO), TxB_2 and 6-keto-$PGF_{1\alpha}$ (Upjohn, Kalamazoo, Mich.), activated charcoal (Sigma, St. Louis, Mo.), Dextran T-70 (Pharmacia Fine Chemicals AB, Upsala, Sweden), and lymphocyte separation medium (LSM; Litton Bionetics, Kensington, Md.) were purchased from the indicated suppliers. Assay kits for PGE_2, "Atomlight" scintillation fluid, [^{3}H]TxB_2, and [^{3}H]6-keto-$PGF_{1\alpha}$ were purchased from New England Nuclear (Boston, Mass.). Antibodies against TxB_2 (stable metabolite of TxA_2) and 6-keto-$PGF_{1\alpha}$ (stable metabolite of prostacyclin) were kindly provided by Dr. L. Levine (Boston, Mass.). These antibodies demonstrated less than 2% cross-reactivity with other prostanoids. Assay kits ("Bio-Rad") for protein determination were obtained from Bio-Rad Laboratories (Richmond, Calif.). Falcon 24-well plastic tissue culture plates were obtained from Beckton Dickinson Labware (Oxnard, Calif.). Male Sprague–Dawley rats (250–300 g) were purchased from Taconic Farms (Germantown, N. Y.).

In general, Kupffer cells were cultured in medium consisting of minimum essential medium (GIBCO) supplemented with 2% (w/v) glucose (Fisher Scientific, Fair Lawn, N. J.), 15% (v/v) fetal calf serum (FCS), penicillin (50 U/ml), streptomycin (50 μg/ml), and 0.2 m*M* glutamine (all obtained from GIBCO). The medium employed for studies of LPS-stimulated prostanoid release was identical to the above except that FCS was deleted.

The buffer for radioimmunoassay of TxB_2 and 6-keto-$PGF_{1\alpha}$ consisted of Trizma-7.0 (Sigma)-buffered saline containing 0.1% (w/v) gelatin (DIFCO), 2 m*M* $MgSO_4$ and 0.2 m*M* $CaCl_2$.

Cell isolation and maintenance. Under halothane anesthesia, the liver was exposed via a midline laparotomy. The portal vein was cannulated and the liver perfused with approximately 150 ml of warm (37°C) HBSS (−) until the organ blanched. The liver was then perfused with 10 ml of HBSS (+) containing 0.05% (w/v) collagenase, after which the organ was excised, stripped of adherent tissues, minced into small fragments, and digested in 0.05% collagenase in HBSS (+) for 60 min at 37°C with continual stirring. After filtering through nylon mesh to remove nondigested fragments and washing several times with iced HBSS (−), the resulting cell suspension was subjected to LSM density gradient centrifugation (400*g* for 45 min at 4°C). The nonparenchymal cell (NPC) fraction was collected from the interface and washed multiple times using HBSS (−). Viability was determined on the basis of trypan blue dye exclusion. The cells were suspended in enriched Kupffer cell culture medium ($1.0–1.5 \times 10^6$ cells/ml) and transferred to multiwell tissue culture plates (1.0 ml/well). Cultures were maintained at 37°C in humidified air containing 5% CO_2. Following overnight incubation, the cultures were washed briefly once with HBSS (−) containing 0.1% trypsin (w/v) and followed by multiple washes with HBSS (−) to remove debris and loose or nonadherent cells. The cells were incubated

for 4 days with daily washings and media changes prior to use in studies of prostanoid production after endotoxin stimulation.

Cell identification. Adherent cells did not proliferate during the time course of the study. These cells showed positive staining for both esterase [22] and peroxidase [19] and phagocytized opsonized sheep erythrocytes. In general, greater than 95% of adherent cells met these accepted criteria [19] for identification as Kupffer cells.

Endotoxin stimulation. After 4 days of incubation, the cultured cells were washed several times with HBBS (−). The cells were then reincubated for 4 or 8 hr in serum-free medium (1.0 ml/well) containing 0, 1.0, 50.0, or 100.0 μg/ml of LPS. At the end of the specified time, the supernatants from each well were collected and stored individually at −60 to −80°C in polypropylene tubes. Viability of the cells following LPS stimulation was determined by trypan blue staining. The cells were then washed and stored frozen for subsequent protein determination.

Protein determinations. The protein content of the frozen cell monolayer was determined following three freeze–that cycles using the Bio-Rad microassay technique [6].

Radioimmunoassay. Immunoreactive PGE_2 ($iPGE_2$) was determined using the kit supplied by New England Nuclear, according to the procedure specified in the package insert for assays of $iPGE_2$ in urine. The lower limit of sensitivity for this assay was 2.5 pg/ml. With slight modifications, the assay for $iTxB_2$ and i6-keto-$PGF_{1\alpha}$ was as previously described [14]. Briefly, the assay was conducted in 12 × 75-mm plastic tubes, containing 100 μl of tissue culture medium or standard, 100 μl of tritiated antigen dissolved in assay buffer, 100 μl of appropriately diluted antibody in assay buffer, and 300 μl of assay buffer. Standards were prepared in assay buffer. After overnight incubation at 4°C, bound antigen was separated from free by centrifugation after the addition of 900 μl of assay buffer containing 0.03% (w/v) dextran and 0.3% (w/v) charcoal. Supernatants were decanted into scintillation vials containing 5 ml of scintillation fluid and counted for 10 min in a scintillation counter. Concentrations of $iPGE_2$, $iTxB_2$, and i6-keto $PGF_{1\alpha}$ in sample unknowns were determined by comparison with a standard curve after log-logit transformation of the data. Samples were always run in duplicate and the results averaged. Variations between duplicate samples was less than 10%.

Statistical analyses. In each experiment, each condition (defined by LPS dose and incubation time) was run in quadruplicate. All data are expressed as the arithmetic mean ± standard errors. Data for $iTxB_2$ were obtained in two replicate experiments (i.e., eight entries per data point). Other results were obtained in a single experiment. Data were analyzed by two-way analysis of variance, using a fully randomized design, with endotoxin dose and incubation time being the independent sources of variation. Differences with $P \leq 0.05$ were considered significant.

RESULTS

Only 25–30% of the original population added to the cultures remained adherent to the plates after overnight incubation and washing as determined by cell counts of the removed nonadherent cells. This is consistent with estimates of the constituent proportion of Kupffer cells within the liver's nonparenchymal cell population [30]. It was not practical to count the total number of adhered cells within each well nor were we able to accurately estimate the number from representative fields as the cells generally did not assume a uniform distribution within the culture wells. Nonetheless, calculated estimates based on the original number of cells incubated would suggest that each well contained 10^5 cells. Protein content of the individual wells varied from 3 to 5 μg/well, suggesting minor variation in the number of adherent cells present among the wells. Variation in protein content did not correlate with variation in prostanoid production.

Viability was greater than 95% for freshly isolated Kupffer cells as well as those incubated for 4 days in the macrophage medium. Exposure of the cells for 4 to 8 hr to the highest concentration of LPS used in the study (100 μg/ml) decreased viability to 80–85%. Lower LPS concentrations did not affect viability.

The concentrations of $iTxB_2$, $iPGE_2$, and i6-keto-$PGF_{1\alpha}$ detected in the supernatants of Kupffer cells cultures are depicted in Figs. 1, 2, and 3, respectively. Supernatants from unstimulated cultures contained detectable levels of all three prostanoids. Lipopolysaccharide affected production of $iTxB_2$, $iPGE_2$, and i6-keto-$PGF_{1\alpha}$. Depending on the prostanoid, there were marked differences in the effects of increasing LPS dose and duration of incubation on measured prostanoid concentrations.

Resting Kupffer cells released 55.2 ± 6.6 and 72.8 ± 9.9 pg/ml of $iTXB_2$ into the media at 4 and 8 hr, respectively (Fig. 1). In the presence of 1 μg/ml LPS, the detectable concentration of $iTxB_2$ was essentially unchanged from control for both time periods. However, in the presence of 50 μg/ml LPS, 188.5 ± 38.7 pg/ml at 4 hr and 232.8 ± 70 pg/ml at 8 hr were detected in the supernatants. Stimulated macrophages released 235.2 ± 36.6 pg/ml and 256.0 ± 52.5 pg/ml at 4 and 8 hr, respectively, when incubated with 100 μg/ml LPS. Thus, dose of LPS ($F(3,88) = 25.38$; $P < 0.001$) but not incubation time ($F(1,88) = 1.01$) significantly affected TxA2 production.

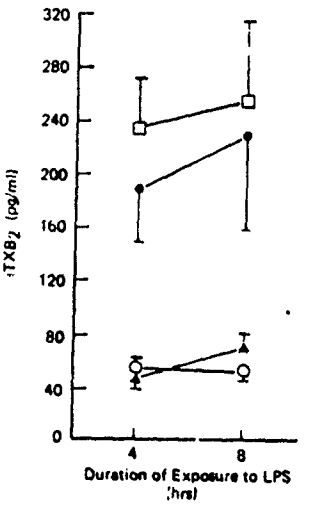

FIG. 1. Concentration of $iTxB_2$ in supernatants from LPS-stimulated Kupffer cells. Each point represents the mean ± SE of eight separate measurements obtained from two experiments. □, 100 μg LPS; ●, 50 μg LPS; ○, 1 μg LPS; ▲, 0 μg LPS.

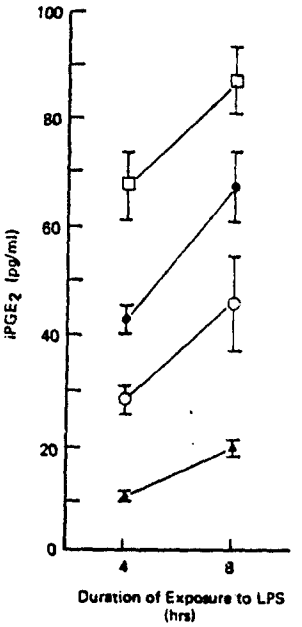

FIG. 2. Concentration of $iPGE_2$ in supernatants from LPS-stimulated Kupffer cells. Each point represents the mean ± SE of four separate measurements (one experiment). □, 100 μg LPS; ●, 50 μg LPS; ○, 1 μg LPS; ▲, 0 μg LPS.

The concentration of $iPGE_2$ also increased as a function of increasing LPS concentration ($F(3,40) = 85.91$; $P < 0.001$) (Fig. 2). At 8 hr a similar rise was noted in the presence of similarly increasing doses of LPS. The effect of incubation time was also significant ($F(1,40) = 34.08$; $P < 0.001$).

When stimulated with the lowest concentration of LPS (1 μg/ml), Kupffer cell production of i6-keto-$PGF_{1\alpha}$ rose from 79.2

± 18.2 pg/ml for nonstimulated cells to 156.0 ± 1.8 pg/ml (4 hr) and 81.2 ± 13.6 pg/ml to 147.5 ± 13.7 pg/ml (8 hr) (Fig. 3). However, unlike $iTxB_2$ and $iPGE_2$, with 50 μg/ml of LPS, the concentration of i6-keto-$PGF_{1\alpha}$ decreased. This decline was further pronounced in the presence of 100 μg/ml LPS. The dose effect was significant ($F(3,40) = 10.18$; $P < 0.001$); the effect of incubation time was not ($F(1,40) = 0.22$).

Table 1 presents, as a function of LPS concentration, the ratio of mean i6-keto $PGF_{1\alpha}$ concentration to mean $iTxB_2$ concentration. The addition of 1 μg/ml of LPS resulted in this ratio increasing from baseline values of 1.4 (4-hr cultures) and 1.1 (8-hr cultures) to 2.8 (both 4- and 8-hr cultures). Adding increasingly larger doses of LPS, however, caused the ratio to progressively and significantly ($F(3,3) = 232.4$, $P < 0.001$) decline to 0.2 at the highest LPS concentration tested (100 μg/ml).

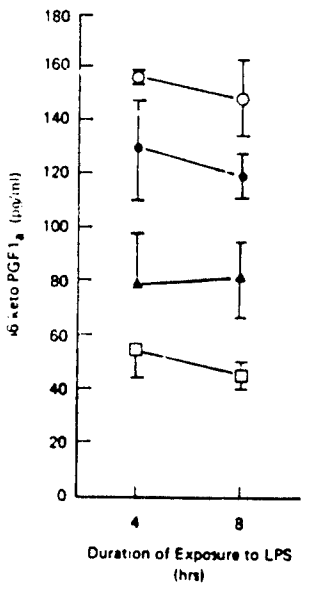

FIG. 3. Concentration of i6-keto-$PGF_{1\alpha}$ in supernatants from LPS-stimulated Kupffer cells. Each point represents the mean ± SE of four separate measurements (one experiment). □, 100 μg LPS; ●, 50 μg LPS; ○, 1 μg LPS; ▲, 0 μg LPS.

TABLE 1

RATIO OF MEAN i6-KETO-$PGF_{1\alpha}$:$iTxB_2$ IN SUPERNATANTS FROM LPS-STIMULATED KUPFFER CELLS

Concentration of LPS (μg/ml)	Duration of exposure to LPS (hr)	
	4	8
0	1.4	1.1
1	2.8	2.8
50	0.7	0.5
100	0.2	0.2

DISCUSSION

Hepatic dysfunction, ranging from mild hyperbilirubunemia and serum transaminase elevations to overt organ failure, occurs in septic patients [5]. The mechanisms underlying this phenomenon are incompletely understood, although it is likely that endotoxins derived from gram-negative bacteria are involved [21,30]. Intravenously administered endotoxin localizes within the liver, being cleared from the circulation by Kupffer cells lining the sinusoids [26,30]. In the presence of endotoxin hepatocellular dysfunction occurs including altered carbohydrate, fat, and protein metabolism [21]. Although hepatocytes have receptors for endotoxins [30], high-resolution audioradiographic studies have failed to demonstrate the ability of these cells to internalize endotoxin [26]. Thus, it is not clear whether endotoxin is capable of causing direct toxicity to hepatocytes. On the other hand, there is convincing evidence that endotoxemia triggers an inflammatory response within the liver characterized by Kupffer cell activation, platelet aggregation, fibrin deposition, intravascular thrombosis, and polymorphonuclear leukocyte infiltration [21]. This inflammatory response may cause injury to hepatocytes, either because of relative ischemia secondary to intravascular thrombosis or because of extracellular release of potent inflammatory mediators by acti-

vated Kupffer cells [3, 4, 23–25] and/or infiltrating leukocytes [34].

It is well established that peritoneal macrophages synthesize a variety of arachidonic acid (AA) metabolites when stimulated with LPS [9, 10, 20]. Production of PGE_2 by endotoxin-stimulated hepatic macrophages (Kupffer cells) *in vitro* has been reported previously [4, 11]. In the present study, we have confirmed this observation and, in addition, have shown that (1) high LPS concentrations (50 and 100 μg/ml) trigger the release of TxA_2, and (2) whereas PGI_2 synthesis is increased by a low LPS dose (1 μg/ml), higher doses of LPS result in less detectable PGI_2.

Thromboxane A_2 induces vasoconstriction (hence low flow) and platelet aggregation [18, 29]. Within the liver, endotoxin-induced venous stasis and platelet aggregation may derive from TxA_2 released by activated Kupffer cells. The procoagulant activity (PCA) of Kupffer cells is stimulated by endotoxin in a dose–response relationship similar to that observed for LPS-stimulated TxA_2 release [23, 25]. This PCA peaks within 6–8 hr following exposure to LPS [25], a time course during which increased amounts of $iTxB_2$ were present in our Kupffer cell cultures (Fig. 1). Thus, LPS-activated Kupffer cells release two agents which can promote the intrahepatic intravascular thrombosis associated with endotoxins. Additionally, TxA_2 causes leukocyte aggregation [33]. Thus, Kupffer cell-derived TxA_2 may also contribute to the accumulation of inflammatory cells within the liver during endotoxemia.

Changes in Kupffer cell production of PGI_2 are more sensitive to LPS concentrations than TxA_2. A dose which had no discernable effect on TxA_2 synthesis (1 μg/ml) augmented PGI_2 release into the media. The physiologic properties of PGI_2 antagonize those of TxA_2 and therefore, PGI_2 released by low concentrations of LPS may minimize the effects of basal TxA_2 production. Conceivably, PGI_2 might also antagonize the thrombotic effects of PCA stimulated by low LPS concentrations [25].

Inhibition of prostacyclin synthetase by cyclo-oxygenase-derived products may explain why increasing doses of LPS decreased PGI_2 production. Fatty acid hydroperoxides inhibit prostacyclin synthetase [17, 32]. Such species are produced following the oxidation of AA [12]. Increased mobilization of AA triggered by LPS may lead to inactivation of prostacyclin synthetase and shunting of intermediates into PGE_2 and/or TxA_2 synthesis. In support of this, selective thromboxane synthetase inhibitors have been shown to increase the production of 6-keto-$PGF_{1\alpha}$ in human peritoneal macrophages [15].

We used doses of LPS that are high relative to presumed circulating concentrations of endotoxin in septic patients. Endotoxins, however, are highly concentrated within the liver [26]. In rabbits, following an intravenous infusion of 250 μg of ^{125}I-LPS, as much as 40–50% of the infused dose is detectable in the liver within 5 min [26]. This corresponds to a tissue concentration ranging from 0.7 to 2.0 μg/g of liver tissue. Within the liver, it is the Kupffer cell which accumulates endotoxin. Therefore, the concentration of endotoxin per gram of macrophage tissue, a small component of the total liver mass, would accordingly be much higher. In rats, a 250-μg dose of LPS is a small dose. In our laboratory, 15 mg/kg is an LD_{50} dose. Thus, in a 0.3-kg rat, 4.5 mg of LPS would result in a liver concentration of 2.25 mg/g liver tissue (assuming 50% uptake by a 10-g liver).

Endotoxin is toxic to Kupffer cells. Following the infusion of endotoxin into mice, swelling of Kupffer cells occurs within a short period of time [21]. In culture, exposure to endotoxin causes Kupffer cells to become vacuolated, rounded, and to leak various intracellular enzymes [24]. Our present observations substantiate the toxicity of endotoxin for Kupffer cells, but only at the highest dose (100 μg/ml) of LPS employed. In general, our data agree with previous reports [4, 24], although the toxicity observed in our study was not as severe, probably reflecting relatively shorter incubation times.

In summary, we showed that endotoxin

stimulates cultured rat Kupffer cells to produce TxA_2, PGI_2, and PGE_2. Dose and time effects appeared to be unique for each prostanoid. Whether Kupffer cell-derived prostanoids are important in the pathophysiology of experimental endotoxic shock or clinical sepsis remains to be established. Unpublished observations from our laboratory and results reported by other [1, 10], however, suggest that this may indeed be the case. Nonetheless, the data presented here substantiate that LPS-activated Kupffer cells release AA metabolites which have properties that can profoundly alter the microenvironment of the liver.

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