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Inhibition of LPS activation of Kupffer cells by transition metals

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Abstract

Background—Bacterial endotoxins are the principal agents causing sepsis and septic shock. Cytokine cascades produced by cellular interactions to endotoxins can cause cardiovascular failure followed by multi-organ failure and death. Endotoxin intravenously administered to mice can have fatal consequences. Previous studies have shown that the transition metals Mn^{++}, Cr^{+++} can be protective.

Methods—The effects of Mn^{++}, Cr^{+++}, Zn^{++} and Cu^{++} on LPS binding to rat Kupffer cell extracts were analyzed using dot blots, SDS-PAGE, and western transfer. Kupffer cells were isolated from rat livers by collagenase perfusion, differential centrifugation and adhesion to plastic.

Results—5mM Mn^{++}, Zn^{++}, Cr^{+++} and Cu^{++} completely inhibited LPS binding. Isolated Kupffer cells were also exposed to Mn^{++} and to LPS and TNF-α release measured. The presence of Mn^{++} significantly ($p<0.05$) reduced TNF-α production by Kupffer cells in response to LPS. Experiments to determine if these effects were mediated by binding to LPS-binding proteins showed this was not the case. More likely a complex occurs between the metal and LPS. We also showed significantly enhanced uptake of LPS into Kupffer cells in the presence of Mn++.

Conclusions—The data is consistent with the metals binding to LPS via its two phosphate groups and neutralizing their charge. These data also support the hypothesis that there is enhanced cellular up-take by non-receptor mediated methods such as absorptive pinocytocis. At the same time receptor binding and activation of the cells is inhibited. This can explain the effects of transition metals on LPS toxicity.

Introduction

Bacterial endotoxins are principal players in the often fatal systemic reactions associated with sepsis and septic shock, the most dangerous of which is refractory hypotension. This causes inadequate organ perfusion with cardiovascular collapse multi-organ failure and death [1]. Interaction of endotoxins with cells of the macrophage/monocyte lineage result in many events including production of reactive oxygen species, TNF-α and other cytokines [2]. The key cell involved in the detoxification of endotoxins is the liver fixed macrophage or Kupffer cell [3]. Kupffer cells are the largest population of fixed macrophages in the body and serve to cleanse

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the portal blood of antigens and toxins before it is sent to the systemic circulation [4]. A number of endotoxin binding proteins have been described including CD14, CD11/18, an 80kD protein and the scavenger receptor. For many years there was no clear evidence that Kupffer cells express a specific endotoxin receptor that signals the cytokine response[5]] however, it is generally accepted now that CD14 in the presence of LPS binding protein (LBP) will act as a true endotoxin receptor in Kupffer cells. Nevertheless Kupffer cells can respond to LPS in the absence of serum, thus without binding with LBP, and this suggests that non CD14/Toll receptor pathways can also operate in these cells. The mechanics of these non-CD14 pathways are not known.

Endotoxin intravenously administered to mice can have fatal consequences [6]. Previous studies have shown that the transition metals manganese and chromium can be protective [7, 8]. Other studies have also shown the protective effects of Zn^{++} (0.4-2mg/Kg) in rats given lethal quantities of S. typhimurium endotoxin [9]. Evidence that zinc can inhibit acute ethanol induced liver injury has also implicated these metals. It has been suggested that Zinc can prevent endotoxin translocation through the gut wall [10] and thus serves as a mechanism to reduce the effects of alcohol on liver function. Alcohol induced hepatic changes and TNF elevations were inhibited in animals pre-treated with zinc [10]. Here we present an alternative hypothesis and suggest that zinc and other transition metals can react directly with endotoxin not only inhibiting gut permeability to endotoxin but also directly preventing receptor interaction and signaling in the liver while retaining the ability to endocytose and detoxifiy LPS.

Methods

Sources of lipopolysaccharide

Salmonella minnesota LPS was obtained from Sigma Chemical Co (St. Louis, Mo.). Covalent modification of LPS with methyl benzimidate (Sigma Chemical Co., St. Louis, MO.) to allow iodination has been described previously [11]. Modified LPS was radio iodinated by the Chloramine T procedure [12]. The average specific activities for ^{125}I-LPS (wild and rough chemotypes) range from 0.27 to 0.75 μCi/μg.

Ligand blotting with ^{125}I-labelled wild type LPS

Isolated rat Kupffer cells were extracted with 1% SDS, 2mM PMSF, 1 μg/ml leupeptin and aprotinin in TBS. After removing the debris by centrifugation, the extracts were run on 10% SDS-polyacrylamide gels and the separated proteins were electrophoretically transferred to nitrocellulose membranes. The membranes were blocked with 10% skim milk in TBS-T (0.05% Tween-20 in TBS), washed, and exposed to ^{125}I-labelled wild type LPS in both the presence and absence of excess (100 μg/ml) unlabelled wild-type LPS at room temperature. After incubation the membranes were extensively washed in TBS-T and autoradiographed at -70 °C. For the dot-blot assay, 2μl of protein matched partially purified Kupffer cell protein was blotted onto nitrocellulose membrane and allowed to dry. The membrane was blocked with 10% skim milk in TBS-T at room temperature for 45 minutes while being shaken. The membrane was then washed three times with TBS, and incubated with 4 ml TBS containing ^{125}I-labeled LPS in presence or absence of 5-10 mM Mn++, Mg++, Ca++, Zn++, Cu++, Cr+++ and EDTA in a pouch for 45 minutes at room temperature with shaking. The membrane was washed for 1 hour with several changes in TBS-T, air dried and autoradiographed. All blots were carried out in triplicate using two separate samples of partially purified Kupffer cell LPS binding proteins. Typical blots are seen in Figs 1 and 3.

Isolation of Rat Kupffer Cells

Kupffer cells were isolated according to our standard laboratory protocol [13] from the livers of male Sprague Dawley rats. The rats were fasted overnight and the livers perfused through the portal vein with collagenase. Suspended cells were separated into parenchymal and non-parenchymal fractions by differential centrifugation. Kupffer cells were purified from the non-parenchymal cell fraction by using 17.5 % solution of metrizamide (Accurate Chemicals & Scientific Corpn., Westbury, NY) in Gey's balanced salt solution (Life Technologies Inc., Grand Island, NY) for final separation. The interface layer containing Kupffer cells was isolated and washed in phosphate buffer. Further purification of the cells was achieved by attachment to the plastic plates for 2 h. Generally 1×10^8 cells were routinely isolated from one liver. This is a standard procedure in our laboratory and has produced consistently similar results. Kupffer cells were identified by their ability to phagocytose 1μm latex particles, and their staining for endogenous peroxidase activity [13]. Cell isolated in this way were 85% Kupffer cells and >95% viable (Trypan Blue exclusion).

Bio-gel chromatography

The LPS-binding proteins were partially purified as described by us elsewhere [14]. Bio-gel A-1.5m beads (Bio-Rad Laboratories, Hercules, CA) were loaded into a glass column (2 cm wide, 34 cm long) and equilibrated with 0.3% SDS in TBS. Void volume was approximately 35 ml. Chromatography was run with a Pharmacia pump (1.5 ml/min) and Gilson fraction collector, and (0.75 ml/fractions were collected). A 1% SDS protein lysate (1.75 ml) was diluted to 3.5 ml final volume with 0.3% SDS in TBS and then loaded into the column. Absorbance of each fraction was determined using a spectrometer at 280nm. Fractions were then selected and dot blotted onto nitrocellulose to perform a dot-blot assay with ^{125}I-LPS as the ligand. Densitometric values from the dot-blots were then obtained for each selected fraction. Relative densitometric values of the dots were presented as binding activity.

Cytokine determination

Cytokines were measured in the supernatants from the cultured cells 6 hours after exposure to 1μg/ml LPS or to LPS and 10mM Mn++ for 1 hour. Incubations were carried out at 37°C. Controls were untreated cells and cells exposed to 10mM Mn++ only unless otherwise indicated. Cytokines were measured using the Cytoscreen ELISA kits (Biosource, CA) according to the manufacturer's instructions. Incubations were done in the presence and absence of 5% fetal bovine serum to distinguish between CD14/Toll receptor activation and other mechanisms of LPS binding. All measurements were carried out in triplicate.

^{125}I-Labeled LPS uptake by rat Kupffer cells

Isolated Kupffer cells were suspended in GBSS with 1% BSA, with a final concentration of 3.0×10^7 cells/ml. The cells were then divided into three experimental groups: group A had ^{125}I-labeled LPS (1 μg/ml final concentration), the group B had ^{125}I-labeled LPS (1 μg/ml) and an excess of unlabeled LPS, and group C had ^{125}I-labeled LPS and 10mM Mn++. Cells were incubated at 37°C for 5, 30, and 60 minutes while being shaken. At each time point, 100 μl (3.0×10^6 cells) in triplicate were removed from each experimental group and added to a tube containing 200 μl of oil (dibutyl phthalate and dioctyl phthalate, 3:1). The tubes were centrifuged at 3000 rpm for 2 minutes. The bottom of the tubes, containing the cell pellet, were then cut into blank tubes and counted in a gamma counter [13].

Statistical analysis

Data was analyzed using Student's t test. The significance of the difference was determined using the generalized Wilcoxon test, a p value < 0.05 was considered significant.

Results

The identification of two rat Kupffer cell LPS-binding proteins, $p31^{LPB}$ and $p34^{LPB}$, and their specificity of binding to LPS are reported by us elsewhere [14]. In this report the effects of Ca ++, Mg++, Mn++, Zn++, Cu++, and EDTA on LPS binding to LPS- binding rat Kupffer cell proteins were analyzed using dot blots, SDS-PAGE, and western transfer to nitrocellulose. Mn ++, Zn++ and Cu++, (10mM) all totally inhibited the binding of ^{125}I-LPS. Mg++ and Ca++ showed much weaker effects (Figs. 1a, 1b and 1c), Cr+++ was also highly inhibitory (data not shown). Concentrations of the metals down to 1mM were equally effective. This suggests that the system is saturated at these concentrations. Lower concentrations were not used in this study. Addition of EDTA prevented the inhibitory effects due to chelation of metal ions (Fig. 1a), demonstrating specificity of metal ion interaction with LPS or the LPS-binding Kupffer cell proteins. The effects of Mn++ were investigated at greater depth as this metal has been shown to prevent death in mice treated with lethal concentrations of LPS (7). Isolated rat Kupffer cells were exposed to Mn++ (5mM, 10mM, and 20mM) and to LPS (1 μg/ml) and TNF-α release was measured, to determine if Mn++ could influence LPS- induced cytokine production in Kupffer cells. The presence of Mn++ significantly reduced the TNF-α production of Kupffer cells in response to LPS treatment (Fig. 2). Similar data was obtained for IL-6 and IL-10 (Tables 1 & 2). Cells were tested for viability before and after treatment using Trypan Blue exclusion. The cells remained viable throughout the course of the experiments. From these initial experiments it was not clear if the effects of transition metals were mediated by their binding to the LPS binding proteins or whether a complex between the metal and LPS was being formed. Figure 3A shows the results obtained when the LPS binding proteins (partially purified by chromatography on Bio Gel A1.5m columns [14]), were pre-incubated with Mn++, dot blotted onto nitrocellulose and incubated with labeled LPS (column A). There was no inhibition of LPS binding; however, when the blot was carried out using labeled LPS previously incubated with Mn++ complete inhibition of binding was seen (column B). A second set of experiments shown in Figure 3B shows that incubation of the binding proteins on the membrane followed by washing and incubation with labeled LPS did not result in inhibition. These data suggest that Mn++ is binding to LPS and not directly to the binding proteins. Since the LPS Mn++ complex is not capable of binding to the Kupffer cell LPS binding proteins, it is possible that the metal ion binds to one or both of the two phosphate groups on LPS and neutralize their charge. This may affect receptor binding and activation of the Kupffer cells. Experiments were also conducted to determine if the LPS complex with Mn ++ affected LPS uptake by isolated rat Kupffer cells. Figure 4 shows reduced uptake of ^{125}I-LPS in the presence of unlabeled LPS. Because there is a non-specific uptake component for LPS with Kupffer cells [15] it is difficult to achieve complete inhibition of uptake with cold LPS as shown in Fig. 4. However, inhibition of LPS uptake was achieved using a 50 fold excess of unlabeled ligand. Conversely, in the presence of Mn++ an increased rate of uptake of LPS was observed.

Conclusions

These data suggest that the binding proteins are not involved in receptor mediated endocytosis of LPS as inhibition of uptake in the presence of Mn++ would be expected. However, the increased uptake in the presence of Mn++ suggests that direct insertion of LPS and internalization by absorptive pinocytosis may be occurring [15]. Neutralization of negative charges (phosphate groups) on endotoxin by the transition metal could account for a more effective insertion of LPS into the plasma membrane. Interfering with these phosphate groups causes abolition of the TNF-α response. Monophosphoryl Lipid A for example is inactive in eliciting a cytokine response [14].

It is likely that transition metals can not only inhibit gut permeability to endotoxins [10] but can directly inhibit endotoxin receptor interactions in the liver while still allowing pinocytosis and detoxification by the Kupffer cells and hepatocytes [16]. There are, therefore, possible clinical ramifications of transition metal treatment of septic patients. The studies showing pre-treatment with Mn++ increased survival of rats treated with endotoxin supports this idea [7]. Manganese is neurotoxic and studies have shown that it affects microgilia by increasing the production of pro-inflammatory cytokines and nitric oxide [17]. However, another example showed that Zinc pretreatment of mice attenuates TNF-α production and reduces liver injury caused by alcohol [18]. Aban-Mohamed *et al* showed in rats that zinc can also inhibit nitric oxide formation in response to LPS [19]. Interestingly this study also showed that LPS treatment reduced the amount of serum zinc Ievels in the rats [19]. This would suggest that a complex between endotoxin and zinc is being formed that is more rapidly cleared from the circulation as we have suggested may be happening with manganese. A Study has also shown a binding between Shigella dysenteriae S and R strain endotoxins and copper [20]. Mandali *et al* showed that following an oral dose, serum chromium in early weaned pigs was reduced in endotoxin treated animals [21] and Kaplanski showed that both plasma zinc and iron levels were reduced in rats treated with LPS, [22] data which is also supportive of our study. However, not all studies have shown beneficial effects of transition metals during sepsis. Kraus *et al* showed in pigs that zinc treatment during acute endotoxemia was detrimental and caused increased serum levels of TNF-α and IL-6 [23]. Thus further examination of the biological effects of transition metals on the biological properties of endotoxins is needed to determine if they may be beneficial to patients with gram negative bacterial infections.

Acknowledgements

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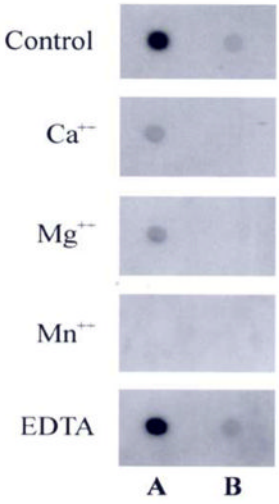

Fig 1A

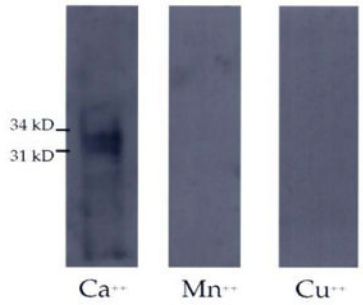

Fig 1C

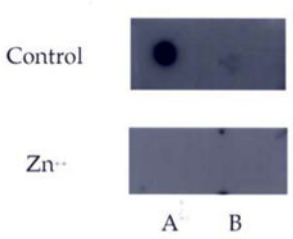

Fig 1B

Figure 1.

(A) ^{125}I LPS binding to partially purified rat Kupffer cell protein in the presence and absence of divalent cations Mn ++, Mg ++ and Ca ++ (10mM). Kupffer cell protein; A. (2μl) were dot blotted onto nitrocellulose. BSA; B. (2μl, 2mg/ml) was used as a negative control. The effects of a metal chelator (EDTA) were also examined.

(B) ^{125}I LPS binding to partially purified rat Kupffer cell protein in the presence and absence of the cation Zn ++ (10mM). Kupffer cell protein; A. (2μl) were dot blotted onto nitrocellulose. BSA; B. (2μl, 2mg/ml) was used as a negative control.

(C) ^{125}I LPS binding to LPS-binding rat Kupffer cell proteins run on 10% SDS/PAGE and Western transferred to nitrocellulose. This shows the effect of Ca ++, Mn ++ and Cu++ ions (5mM) on specific LPS binding proteins.

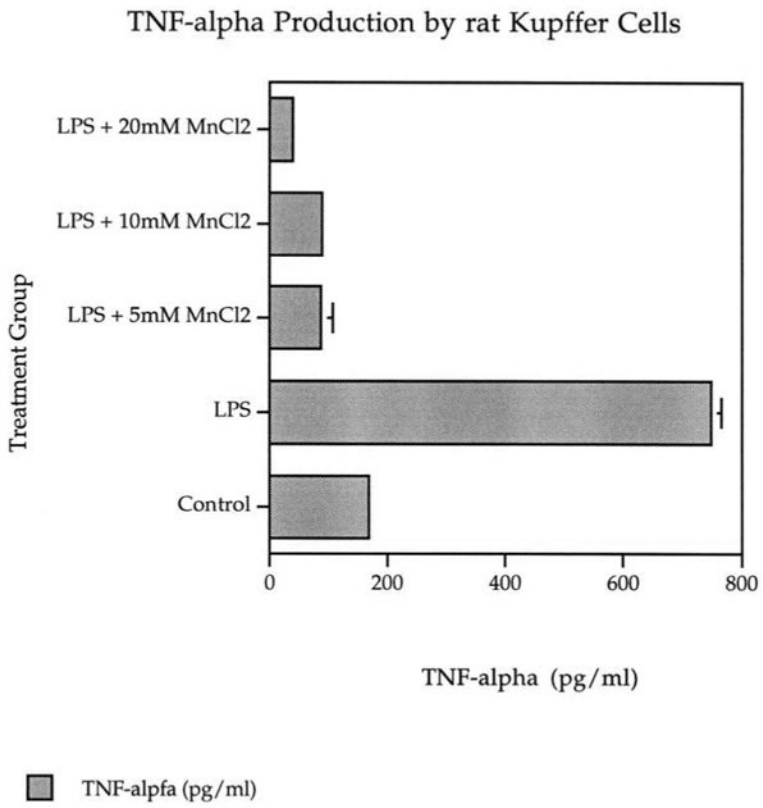

Figure 2.

TNF-α production by Kupffer cells in response to LPS (0.5μg/ml), in the presence and absence of Mn ++ is compared to the untreated control. Differences between the LPS treated cells without Mn++ compared with the Mn++ treated cells were significant ($p<0.001$).

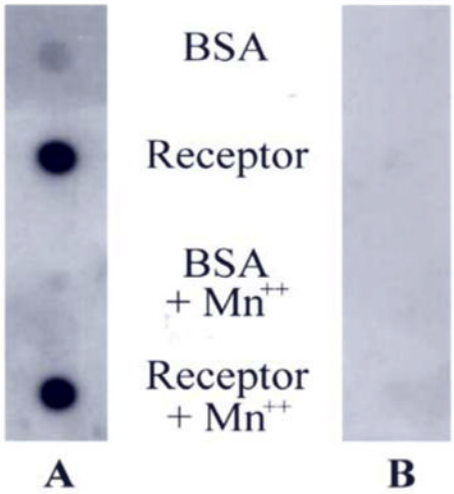

Fig 3A

Fig. 3B

Figure 3.

(A) Binding of ^{125}I-LPS to partially purified rat Kupffer cell protein (Receptor) pre-incubated with 5mM Mn ++. Control and Mn ++ incubated extracts were spotted onto membranes with BSA controls. Membrane A was incubated with ^{125}I-LPS and membrane B with ^{125}I-LPS plus 5mM Mn ++.

(B) Partially purified rat Kupffer cell protein (Receptor) and BSA as a control were spotted onto membranes. Membrane A was incubated with 5mM Mn++ and Membrane B with TBS only. After drying both membranes were incubated with ^{125}I-LPS at room temperature for 30 mins followed by washing drying and autoradiography.

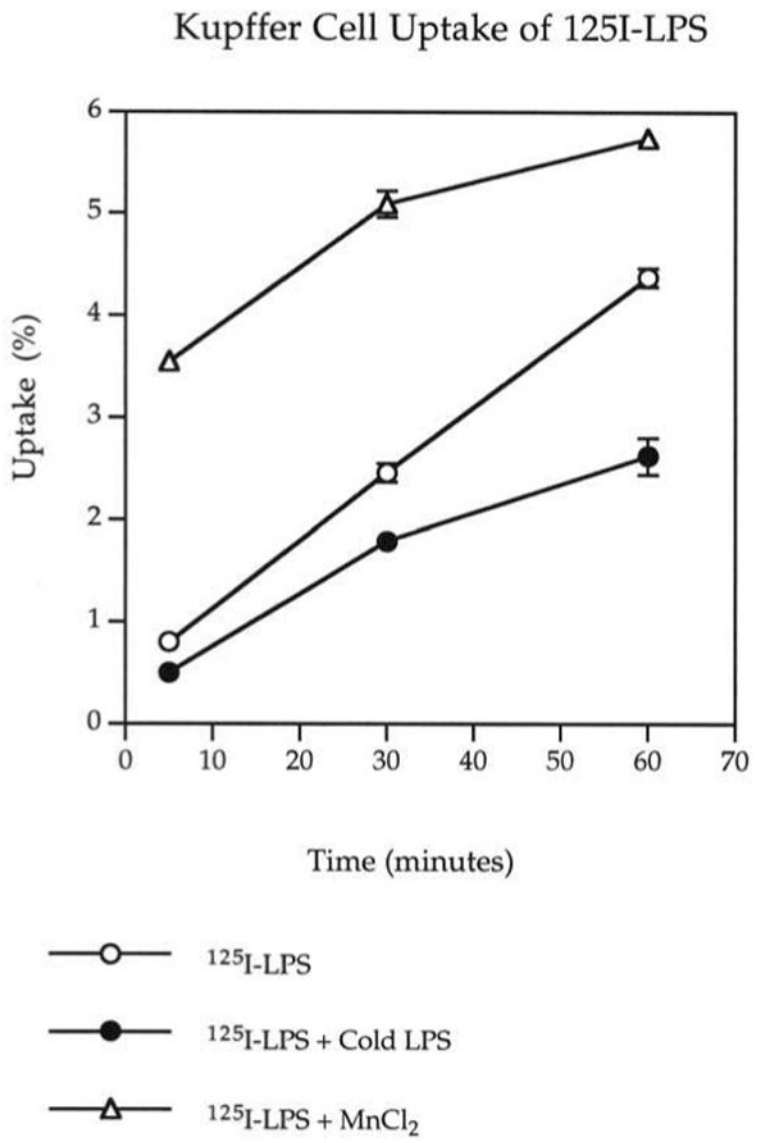

Figure 4.

Binding and internalization of ^{125}I-LPS by isolated rat Kupffer cells. Uptake of ^{125}I-LPS was inhibited in the presence of excess unlabeled LPS. In the presence of Mn++ (10mM) uptake was significantly increased ($p<0.01$ at 30mins),