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ROLE OF NITRIC OXIDE IN IMMUNOLOGICAL LIVER DAMAGE IN MICE

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Abstract—The role of nitric oxide (NO) in immunological liver injury in mice was studied. Moderate increases in plasma NO levels and liver damage were seen after the injection of either *Bacillus* Calmette-Guérin (BCG) or lipopolysaccharide (LPS) alone in mice. Administration of LPS following BCG injection resulted in a remarkable elevation of the plasma NO level and severe liver damage. The elevation of the NO level and the liver damage induced by BCG or BCG + LPS were not affected by the administration of L-arginine. The BCG-induced increase of plasma NO was inhibited by N^G-monomethyl-L-arginine (NMA) treatment without effect on the elevated plasma glutamic pyruvic transaminase (GPT) and glutamic oxaloacetic transaminase (GOT) levels. The BCG + LPS-induced elevation of plasma GPT and GOT levels was more pronounced after NO production was inhibited by NMA treatment. The action of NMA mentioned above was partially reversed by the simultaneous administration of L-arginine. These findings suggest that NO plays a protective role against liver injury induced by BCG + LPS in mice.

Key words: nitric oxide; liver injury; lipopolysaccharide

The synthesis of NO† from L-arginine is known to exist in a number of animal cells including endothelial cells [1], macrophages [2–4], cerebellar neurons [5], neutrophils [6], Kupffer cells [7] and hepatocytes [8, 9]. Common to all of the cell types is the use of L-arginine as a substrate for NO synthesis. However, both the triggering signals for NO release and the amount of NO production vary greatly with different cell types. Endothelial cells, neurons and neutrophils instantaneously produce a small amount of NO, which appears to have a role as an intercellular or intracellular messenger by activating soluble guanylate cyclase [1, 5, 10]. Macrophages and hepatocytes produce much greater quantities of NO, which is not detectable until several hours after exposure to specific inflammatory or septic stimuli [8–10]. It has been proposed that the accumulation of macrophages in the liver after the injection of a primer is involved in the pathogenesis of liver injury [11, 12]. Although a role for macrophage NO in tumor cells [3, 13, 14], fungal cytostasis [15] and killing of the intracellular parasite *Leishmania* [16] has been reported, the function of NO from either macrophages or hepatocytes in liver damage has not been ascertained. This study was undertaken to determine if an alteration in NO production would influence the degree of hepatic damage in mice.

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† Abbreviations: NO, nitric oxide; BCG, *Bacillus* Calmette-Guérin; LPS, lipopolysaccharide; NMA, N^G-monomethyl-L-arginine; GPT, glutamic pyruvic transaminase; and GOT, glutamic oxaloacetic transaminase.

MATERIALS AND METHODS

Animals

Female Kunming strain mice, weighing 22–24 g, were obtained from the Animal Center of the Chinese Academy of Medical Sciences, Beijing. They were allowed food and tap water *ad lib.* until 12 or 16 hr of fasting before they were killed.

Reagents

NMA, a potent inhibitor of NO synthase, was donated by Dr. T. R. Billiar, Department of Surgery, University of Pittsburgh (U.S.A.). *N*-1-Naphthylethylenediamine dihydrochloride and D-galactosamine were purchased from the Sigma Chemical Co. (St. Louis, MO). Salmonella enteritidis-derived LPS was obtained from Difco Laboratories, Detroit, MI. BCG, grown for 14 days in a glycerol-free culture medium, was bought from the Institute of Biological Products, Chinese Ministry of Public Health.

Experimental liver injury

Immunologic liver injury. Mice were injected via the tail vein with 0.2 mL of the BCG culture (approximately 5×10^7 viable units per mouse) or with saline alone; 12 days later, the mice were injected intravenously with 7.5 μg of LPS in 0.2 mL saline or with saline alone. Ten hours after the injection of LPS, the mice were decapitated. Blood and liver samples were collected for biochemical and pathological examinations, respectively.

Chemical liver injury. Carbon tetrachloride was prepared as a 0.1% solution in peanut oil prior to its use. Acetaminophen and D-galactosamine were dissolved in saline and neutralized with 1 N NaOH to pH 6.8 just before use. Carbon tetrachloride

(10 μL/kg), acetaminophen (230 mg/kg), and D-galactosamine (800 mg/kg) were administered to mice intraperitoneally. The mice were decapitated after 16 hr of fasting. Blood and liver samples were collected for biochemical and pathological examinations.

Administration of NMA and L-arginine

NMA and L-arginine in 0.2 mL of saline with LPS were simultaneously injected i.v. into mice.

Determination of plasma transaminases

Plasma GPT and GOT were determined using kits produced by the Beijing Chemical Factory.

Measurement of plasma NO

Heparinized blood samples that had been preserved on ice were analyzed within the shortest possible time. The method described by Shechter [17] was used for measuring NO_2^-, an oxidized product of NO. Briefly, 0.1 mL of plasma was deproteinized in a zinc sulfate–sodium hydroxide deproteinizing system, and then a suitable volume of the supernatant was diazotized on ice with 0.1 mL of 3% sulfanilic acid in 20% glacial acetic acid; 15–20 min later, 0.1 mL of 6 mM *N*-1-naphthylethylenediamine dihydrochloride was added and the samples were allowed to stand for 60 min at room temperature for the development of a red–violet color. Absorbance was read at 545 nm on a spectrophotometer. Sodium nitrite was used as a standard.

Pathological examination of the liver

A piece of the same lobe of liver from each mouse was fixed in 10% neutral formalin and embedded in paraffin wax. The sections were stained with hematoxylin and eosin. The necrosis of the liver was graded and scored as follows: 0, no necrosis; 1, mild necrosis; 2, moderate necrosis and 3, severe necrosis. The index of necrosis in each group was deduced by dividing the sum of the scores by the number of mice in each group.

Statistical analysis

All values are expressed as means ± SD. The values were statistically analyzed, using Student's *t*-test.

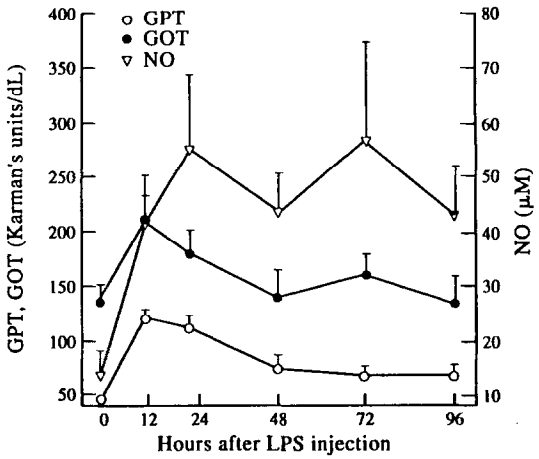

Fig. 1. Dynamic changes of plasma NO_2^-, GPT, and GOT levels in mice challenged with BCG + LPS. Mice were injected intravenously with 5×10^7 units of BCG per mouse and 12 days later with 7.5 μg of LPS in saline or saline alone. Plasma GPT, GOT and NO were determined at different times after LPS injection. Each point is the mean ± SD for 8–10 mice.

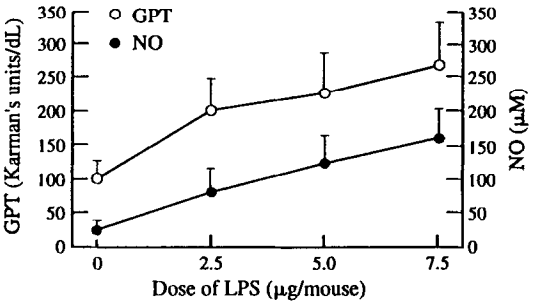

Fig. 2. Correlation between plasma NO_2^- level and GPT activity in mice treated with BCG + LPS. Mice received injections of various doses of LPS 12 days after i.v. injections of BCG (5×10^7 units/mouse). Plasma GPT and NO were determined 10 hr after LPS injection. Each point is the mean ± SD for 10–12 mice.

RESULTS

Alteration of plasma NO and transaminase levels in immunologic liver injury in mice

When the mice were first injected with BCG and then challenged with LPS, significant elevations of plasma NO, GPT and GOT levels were observed. In the preliminary experiment, plasma NO_2^-/NO_3^- levels were determined separately; the data showed that the plasma level of NO_2^- was parallel to that of NO_3^-. Therefore, in the regular experiments, only plasma NO_2^- was measured to indicate nitric oxide levels. The transaminase level reached its peak 12 hr after LPS injection and then decreased with the passage of time, whereas the NO_2^- level increased later and fluctuated during the 4 days of the experiment (Fig. 1). The degree of elevation of plasma NO_2^- and GPT levels varied with the dose of LPS injected (Fig. 2).

Changes of plasma GPT and GOT levels in BCG + LPS-treated mice after modulation of NO synthesis by NMA and L-*arginine*

As shown in Fig. 3, administration of BCG alone resulted in a mild elevation of plasma transaminases and NO levels in mice. Treatment of mice with NMA depressed the elevation of NO but had no effect on the elevated transaminases. L-Arginine showed no effect on the rise of the NO level or on the changes of plasma GPT and GOT levels. Mice given only LPS demonstrated a minimal elevation of plasma GPT and GOT levels as well as plasma NO level. Injection of LPS in BCG-primed mice,

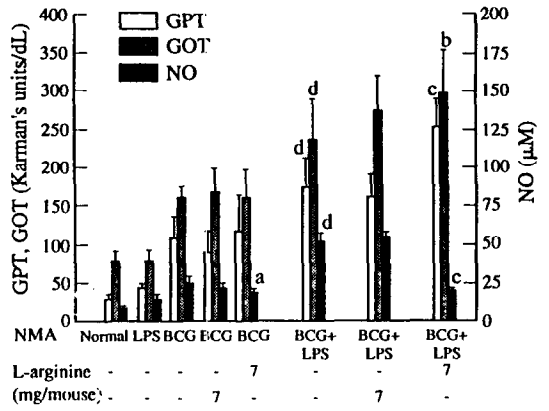

Fig. 3. Influence of NMA and L-arginine on the increases of plasma NO_2^-, GPT, and GOT levels in mice treated with BCG + LPS. Mice were injected i.v. with 5×10^7 units of BCG and 12 days later with 7.5 μg of LPS. NMA or L-arginine (7 mg/mouse) was injected simultaneously with or without LPS. Mice were killed 10 hr after LPS injection, and plasma NO_2^-, GPT, and GOT levels were determined. Each value is the mean ± SD for 5–12 mice. Key: (a) $P < 0.05$ vs the BCG group, (b) $P < 0.05$ vs the BCG + LPS group, (c) $P < 0.01$ vs the BCG + LPS group, and (d) $P < 0.01$ vs the LPS group.

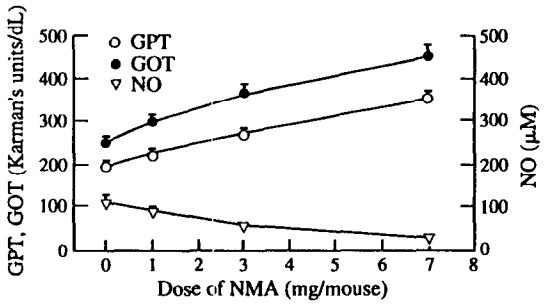

Fig. 4. Dose effect of NMA on plasma NO_2^-, GPT, and GOT levels in mice challenged with BCG + LPS. Each mouse was injected i.v. with 5×10^7 units of BCG and 12 days later with 7.5 μg of LPS. Various doses of NMA were administered to mice simultaneously with LPS. Blood was collected 10 hr after the LPS injection for determination of GPT, GOT, and NO_2^-. Each point is the mean ± SD for 11–12 mice.

however, induced a remarkable rise in plasma NO, GPT and GOT levels ($P < 0.01$ vs the LPS group). The plasma NO level was 2-fold higher than that of mice given BCG alone and 5-fold greater than that of normal mice.

In mice pretreated with BCG, simultaneous injection of NMA with LPS significantly blocked the elevation of the NO level while dramatically increasing plasma GPT and GOT levels (Fig. 3). The degree of suppression of NO production and the elevation of plasma GPT and GOT were proportional to the dose of NMA administered (from 1 to 7 mg/mouse) (Fig. 4). In contrast, the injection of L-arginine combined with LPS had no effect on the elevation of plasma NO and transaminase levels (Fig. 3).

From Fig. 5, it can be seen that a dose of 14 mg of L-arginine almost completely reversed the suppression of NO production and the increase of plasma GPT and GOT levels induced by NMA administration (7 mg/mouse). A dose of 7 mg of L-arginine showed a similar but weaker effect.

Changes of plasma transaminases and NO levels in chemical liver injury in mice

Administration of carbon tetrachloride, acetaminophen, or D-galactosamine to mice resulted in a remarkable rise in plasma GPT activity. However, no significant change of NO levels was observed in any of the three models of chemical liver injury (Table 1).

Findings of the pathological examination

The livers from animals injected with BCG alone contained a number of monocyte infiltrations in the portal region and a large number of macrophages in the form of small granulomas in the sinusoids. Marked hypertrophy of the Kupffer cells was also seen. Administration of LPS to the BCG-pretreated mice induced necrosis and thrombi within the small vessels and sinusoids, in addition to the formation of granulomas. The injection of NMA resulted in even greater necrosis and formation of small peripheral infarcts. Table 2 shows that injection of LPS in mice pretreated with BCG induced notable necrosis in the liver. The necrosis was slightly ameliorated or remarkably worsened by simultaneous injection of L-arginine or NMA with LPS, respectively.

DISCUSSION

It has been reported that severe hepatitis can be induced by injecting a small dose of bacterial LPS into BCG or *Corynebacterium parvum*-pretreated mice [18, 19]. A hypothesis was proposed recently that monocytes or macrophages migrated into the mouse liver after priming with *C. parvum* and released soluble factors toxic to hepatocytes when they were challenged with LPS [11].

NO is a reactive nitrogen intermediate, synthesized from L-arginine by NO synthase. It has been reported that NO is involved in the cytotoxicity of activated Kupffer cells to hepatocytes *in vitro* [7, 20]. However, the role of NO in liver injury induced by BCG + LPS in mice was not determined. As described in this paper, a remarkable elevation of both plasma NO and transaminase levels was found after injection of LPS into BCG-pretreated mice and a good correlation between NO and transaminases was also seen, indicating that the increase of plasma NO is closely related to liver injury. However, no remarkable change of plasma NO was found in carbon tetrachloride, acetaminophen- or D-galactosamine-induced liver injury models, implying that the role of NO in the pathogenesis of these models is minor.

Surprisingly, liver damage induced by BCG + LPS

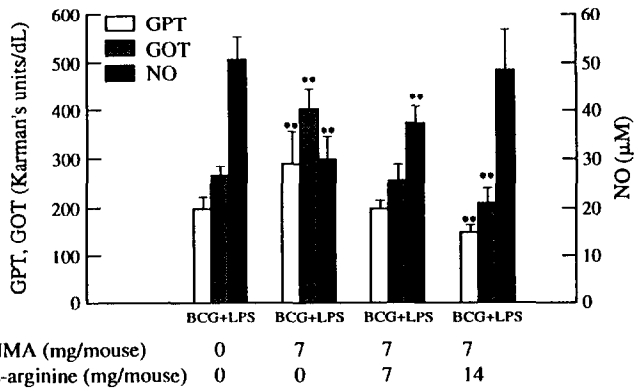

Fig. 5. Antagonistic effect between L-arginine and NMA on plasma NO_2^-, GPT, and GOT levels in mice treated with BCG + LPS. Mice were injected i.v. with 5×10^7 units of BCG and 12 days later received 7.5 μg of LPS intravenously. NMA or NMA + L-arginine at the indicated doses was administered simultaneously to mice with LPS. Ten hours later, blood was collected for determination of plasma NO_2^-, GPT and GOT levels. Each bar is the mean ± SD of 10–12 mice. Key: (**) $P < 0.01$ vs the BCG + LPS group.

Table 1. Plasma NO_2^- and GPT levels in mice treated with carbon tetrachloride, acetaminophen, or D-galactosamine*

Group	N	NO_2^- (μM)	GPT (U/dL)
Normal	8	20.37 ± 7.10	33 ± 9
CCl_4	10	21.52 ± 8.56*	363 ± 113†
Acetaminophen	10	22.91 ± 7.40*	447 ± 39†
D-Galactosamine	10	22.87 ± 8.11*	185 ± 105†

* Mice were injected i.p. with CCl_4 (10 μL/kg), acetaminophen (230 mg/kg), or D-galactosamine (800 mg/kg). Blood was collected 16 hr later for determination of plasma NO_2^- and GPT. Values are means ± SD.
* Not significantly different from normal ($P > 0.05$).
† Significantly different from normal ($P < 0.01$).

Table 2. Influence of NMA and L-arginine on liver necrosis induced by BCG or BCG + LPS in mice*

Group	N	Index of liver necrosis
Normal	10	0
LPS	10	0
BCG	10	0
BCG + NMA	8	0
BCG + L-arginine	10	0
BCG + LPS	10	0.55
BCG + LPS + L-arginine	12	0.42
BCG + LPS + NMA	11	1.27

* Mice were injected i.v. with BCG (5×10^7 units/mouse) and 12 days later with LPS (7.5 μg/mouse). NMA (5 mg/mouse) and L-arginine (7 mg/mouse) were simultaneously injected i.v. with LPS in saline (pH 7.4). Ten hours after LPS challenge, the mice were killed, and liver samples were collected for pathological examinations.

was increased instead of reduced after NO production was inhibited by NMA, a competitive inhibitor of NO synthase. Further study revealed that the inhibiting effect of NMA on NO production could be reversed by the simultaneous injection of L-arginine, the precursor of NO synthesis, with NMA. The pathological examination supported the biochemical findings. Since the inhibition of NO synthesis was associated with greater hepatic damage, it appears that NO does not induce acute hepatocellular damage in this model but instead may function to protect against liver injury mediated by activated macrophages.

The action of NO includes vasodilation [1, 2, 21], inhibition of platelet function [22] such as aggregation [23] and adhesion [24], cytotoxicity [12] and cell-to-cell communication in the central nervous system [5]. The former two actions of NO are beneficial to improving necrosis of the liver cells. There is also the possibility that blocking NO production by systemic injection of a relatively large dose of NMA may lead to significant derangements in systematic hemodynamics, and may alter significantly either hepatic artery or splanchnic blood flow and potentially contribute to the changes seen in hepatocellular enzyme levels. Therefore, it is conceivable that blocking NO production by NMA leads to large vessel occlusion by promoting the vasoconstriction and/or by facilitating the accumulation of platelets. The findings of the pathological examination confirmed the possibility that the action of NO includes inhibition of vascular thrombosis. It is possible that NO exhibits its protective action against liver injury through vasodilation and inhibiting platelet aggregation and adhesion.

The present finding is contrary to the predicted effects of NO in liver damage proposed by other authors [7, 20], implying that the role of NO in immunological liver injury is very intricate. Further study on the role of NO in this immunological liver injury model is in progress in our laboratory.

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