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In Vitro and In Vivo Expression of Inducible Nitric Oxide Synthase During Experimental Endotoxemia: Involvement of Other Cytokines

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Abstract In this study, we investigated the expression of genes for inducible nitric oxide synthase (iNOS), tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), interleukin 6 (IL-6) of Kupffer cells in the presence of lipopolysaccharide (LPS), and the tissue expression of iNOS in a rat liver after LPS injection at various time intervals. The effects of L-N^{G}-nitroarginine-methyl-esther HCl (L-NAME), a NO inhibitor, also were examined. The mRNA transcripts of TNF-α, IL-1β, and IL-6 were rapidly detected no more than at 1 h after LPS stimulation, whereas the iNOS transcript was detectable from 3 h after LPS stimulation and maximally increased at 12 h. This fact suggested that these early induced cytokines were related to expression of iNOS. Using an anti-iNOS antiserum raised against recombinant iNOS protein, immunohistochemical analysis was made to reveal kinetics of NO producing cells. The cells immunoreactive for iNOS appeared at 6 h post-LPS injection in the sinusoids of the liver. By structural and immunohistochemical studies, almost all iNOS positive cells were identified as Kupffer cells and endothelial cells. The number of cells immunoreactive for iNOS increased until 12 h post-LPS injection. At 24 h after LPS injection, iNOS positive cells were restricted to the foci of spotty necrosis. Hepatic injury measured by released enzymes was increased by pretreatment of L-NAME before LPS injection. J. Cell. Biochem. 65:349–358. © 1997 Wiley-Liss, Inc.

Key words: inducible NOS; TNF-α; IL1-β; IL-6; endotoxemia

The effects of infection with gram-negative bacteria are thought to be caused by endotoxins, particularly LPS present in the wall of these bacteria. Experiments using the rats injected with LPS have been performed since 1978, allowing the investigation of various biological changes induced by LPS administration [Morrison et al., 1978]. The LPS portion of bacterial endotoxins plays a major role in the activation of serum complement by both alternative and classical pathways. In addition to the activation of complements, the cytokines are released by LPS administration. TNF-α and IL-1 play major roles in the pathogenesis of endotoxemia [Morrison et al., 1978, 1987]. A number of cell types (monocytes, tissue macrophages, neutrophils, and platelets) can interact with LPS and are activated to produce cytokines and other inflammatory mediators [Luster et al., 1994; Lynn et al., 1992]. NO is one of the factors released from these inflammatory cells [Billiar et al., 1992]. It has been shown that macrophages [Ding et al., 1988], nerve cells, hepatocytes [Curran et al., 1989, 1990], and endothelial cells produce NO. Its functions include tumoricidal activity, vasodilation [Stuehr et al., 1989], neurotransmission [Garthwaite et al., 1988], and suppression of T cell proliferation [Isobe et al., 1992; Kawabe et al., 1992]. NO production can occur by two types of enzymes. Constitutive NOS (cNOS) are expressed in various cell types, such as endothelial cells, neurons, and neutrophils. These cNOS produce low amounts of NO. On the other hand

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macrophages, Kupffer cells, hepatocytes, and glia cells express iNOS and can produce high amounts of NO. The expression of iNOS is stimulated by the exposure to cytokines and bacterial endotoxins. Previous studies have revealed that, in vitro, IFN-γ which is the dominant iNOS-inducing agent in macrophages does not require the presence of additional host-derived factors or microbial products [Stenger et al., 1994].

Recently, some reports have demonstrated the tissue expression and distribution of iNOS in various tissue during the course of infectious diseases [Afework et al., 1994; Thomsen et al., 1994]. Although many in vitro studies, which have documented cytotoxicity against hepatocytes related to NO, little is known about the tissue expression of iNOS and the kinetics of NO secreting cells during endotoxemia. In this study, we investigated the gene expressions of cytokines in Kupffer cells by LPS stimulation, and the relationship with iNOS promotion. We also examined, using antiserum raised against recombinant iNOS protein, the tissue expression of iNOS and the effect of an NO inhibitor, L-NAME, in rats injected with LPS.

MATERIALS AND METHODS

Animals

Male Wistar rats weighing from 200 to 250 g obtained from Chubu Science Company (Nagoya, Japan) were provided with food and water ad libitum and kept on a 12-h light-dark cycle in a temperature- and humidity-controlled environment at the Institute for Laboratory Animal Research (Nagoya University School of Medicine, Nagoya, Japan).

Media and Reagents

Murine recombinant interferon gamma (IFN-γ) was kindly provided by Shionogi Pharma. Corp. (Tokyo). RPMI 1640 medium was purchased from GIBCO (Grand Island, NY), fetal calf serum (FCS) from Irvine Scientific (Santa Ana, CA), Eagle's minimum essential medium from Nissui Pharmaceutical Co. (Tokyo), and L-N^G-nitroarginine-methyl-esther HCl (L-NAME) from BIOMOL Researh Laboratories, Inc (Philadelphia, PA). Lipopolysaccharide (LPS); *Esherichia coli* 0111 B4 (Difco) also was obtained. Isolated cells were maintained in RPMI medium containing 10% FCS, 100 U of penicilline/ml, 100 mg of streptomycin/ml and 25 mM HEPES (N-2-hydroxyethylpiperazin -N'-2-ethanesulfic acid, Sigma), as a complete medium.

Cell Line

RAW264.7, a mouse macrophage cell line, was obtained from the American Type Culture Collection (ATCC). Cells were grown in complete medium. at 37°C in 5% CO_2.

Isolation of Kupffer Cells

Kupffer cells were obtained using a perfusion technique described by Emeis and Planque [1976]. Briefly, the liver was perfused for 2 min in situ via the portal vein with Gey's balanced salt solution containing 0.2% Pronase E (Sigma). The liver was minced and incubated in 80 to 100 ml of the solution for 60 min at 37°C (pH 7.3) with continuous stirring. During this incubation period, 0.5 mg of DNase (Sigma) in 1 ml of Hanks' balanced salt solution was added at 20- and 40-min time points to aid in digestion of extracellular debris. The cells were filtered through a sterile stainless wire screen, pelleted at 250 g three times, and washed three times in MEM. Approximately 1.0 x 10^8 non-parenchymal cells were, thus, obtained from the liver. This suspension often included erythrocytes, which were lysed by osmolar shock. Kupffer cells were isolated from this suspension by its adherence property. Nonparenchymal cells were allowed to adhere overnight in a glass dish. This population was considered to be Kupffer cells, on the basis of results from immunohistochemical staining performed with ED1 (macrophage and its subpopulation positive monoclonal antibody) [Heuff et al., 1993a,b]. More than 95% of these adherent cells were ED1 positive. Ultrastructural appearence by electron microscopy also showed that this population had the characteristics of Kupffer cells. Cell viability was determined to be greater than 90% by the trypan blue exclusion method.

Detection of iNOS, TNF-α, IL-1β, and IL-6 mRNA in Cultured Kupffer Cells

Kupffer cells were cultured in the presence of LPS (5 mg/ml), or in medium alone. Total RNA was isolated from these cells using the guanidium thiocyanate-phenol-chroloform extraction method by Chomczynski and Sacchi [1987]. For reverse transcriptase PCR (RT-PCR) proce-

dure, single-stranded cDNA for use in PCR reactions was synthethized in the manner as follows; RT reaction mixture, which consisted of 1 μg of each RNA preparation, 1 μl of 50 pmol/μl oligo$(dT)_{18}$ primer, 2 μl of2.5 mM dNTP mixture, 4 μl of 5 x first strand buffer (100 mM Tris-HCl, 500 mM KCl, 15 mM $MgCl_2$), 0.4 μl of 0.1 M DTT, and 10 μl of RNase free distilled water, were overlaid incubated at 70°C for 5 min, then at 37°C for 10 min and add 200 U of reverse transcriptase (MMLVRT) (GenHunter, Co., Ltd., USA), and again at 37°C for 50 min. After the procedure the enzyme was inactivaed at 95°C for 3 min.

The PCR mixture contained 5 ml of 10x PCR buffer (containing 500 mM KCl, 100 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$), 1 U of Taq DNA polymerase (Nippon Gene, Tokyo), 4 μl of 2.5 mM dNTP mixture, 10 pmol/μl of the primers, 2 μl of this RT production, and 37 μl of distilled water. The primers for the cytokines and β-actin were as follows: β-actin sense 5'AAG TGT GAC GTT GAC ATC CGT3' antisense 5'CTC ATC GTA CTC CTG CTT GCT3' (243 bp); iNOS sense 5'TTG GTG TTT GGG TGC AGG C3' antisense 5'AGG AAA AGA CCG CAC CGA A3' (367 bp); TNF-α sense 5'TCT CAT CAG TTC CAT GGC C3' antisense 5'TCT TGA TGG CAG AGA GGA GG3' (328 bp); IL-1β sense 5'CTC ATT GTG GCT GTG GAG AAG3' antisense 5'GGA ATA GTG CAG CCA TCT TTA G3' (632 bp); and IL-6 sense 5'ATA CCA CCC ACA ACA GAC CAG T3' antisense 5'GAT GAG TTG GAT GGT CTT GGT C3' (469 bp).

The tubes were incubated in a thermal cycler (PJ2000, Takara Shuzo Co., Ltd., Tokyo) at 95°C for 30 s, 54°C for 1 min, 72°C for 1 min and 30 s (22 to 30 cycles) and 72°C for 5 min (once). The appropriate cycles for the cytokines and iNOS were determined by several trials to detect clear bands. The numbers of cycles were 25 for β-actin, iNOS, and TNF-α. IL-1b was amplified for 22 cycles, IL-6 for 30 cycles. After the reaction, 5 μl of this PCR product was analyzed on a 0.8% agarose gel stained with ethidium bromide in Tris borate-EDTA buffer (90 mM Tris, 90 mM boric acid, 1.0 mM EDTA). RT-PCR products were analysed quantiatively using a densitometer (NIH Image 1.59).

Anti-iNOS Antibody and Western Blot

The polyclonal antibody against the murine iNOS was obtained from the sera of rabbit immunized with purified recombinant iNOS protein. Sense 5'GTC GTC GAC GTA GAC GAT GAC AAA ATG GCT TGC CCC TGG AAGT3' and antisense 5'CCT AAG CTT ATG TTT GCC GTC ACT CCG3' were used for the construction of the iNOS vector using the QIA expression system (QIAGEN, USA). The purified protein from bacteria containing the iNOS expression vector was injected into the rabbits subcutaneously. The sera from these immunized rabbits were used for the experiments.

Cultured RAW cells (1.0 x 10^6) and isolated Kupffer cells were collected and lysed. Ten microgram of proteins were subjected to SDS-Page followed by immunoblotting with the rabbit anti-iNOS antibody (described above) and ^{125}I-protein A.

In Vivo Administration of LPS

All experiments were performed under sterile conditions with ether anesthesia. The LPS was injected in a single intravenous dose of 5 mg/kg via the tail veins of Wistar rats. A control rat was injected with saline only. At 3, 6, 12, and 24 h intervals following LPS injection, the rats were sacrificed and their livers were fixed by perfusion via the portal vein, with 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), gradually the tissue was immersed in graded 10%, 15%, 20%, and 25% sucrose PBS solutions. To examine the effect of L-NAME, 50 mg of L-NAME, diluted in 500 μl of physiological saline, was injected intraperitoneally for 3 days prior to LPS injection.

Immunohistochemistry of iNOS

For immunohistochemical staining, 6 μm sections were cut and air-dried. iNOS staining was performed as follows: Sections were rinsed in PBS, immersed in 1% normal goat serum, then incubated with anti-iNOS antiserum diluted 1:100 at room temperature. Next, they were washed with PBS and blocked intrinsic peroxidase with immersing the sections in 0.3% H2O2 in methanol for 20 min. After washing with PBS, sections were incubated with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody at a dilution of 1:100 for 30 min. Peroxidase activity was visualized by DAB (WAKO, Japan) applied for approximately 3 min. Sections were counterstained with hematoxylin.

RESULTS

Expression of iNOS and Other Cytokines mRNA

Kinetics of iNOS and other cytokines transcripts in cultured Kupffer cells stimulated with LPS was examined. mRNA was isolated from stimulated Kupffer cells at various time intervals and cytokines and iNOS mRNA transcripts were amplified with RT-PCR. The constitutive expression of β-actin was used to adjust each cDNA concentration. The expression of iNOS mRNA started at 3 h after LPS exposure and maximally increased at 12 h, whereas TNF-α, IL-1β, and IL-6 mRNA transcripts expressed at 1 h after LPS exposure. TNF-α and IL-1β mRNA transcripts was maximally increased at 1 h, and decreased gradually until 12 h. IL-6 mRNA was detected at 1 h and peaked at 3 h (Fig. 1).

Western Blot Analysis of iNOS

Isolated Kupffer cells and RAW cells were analyzed by immunoblotting. They were cultured in medium ,alone, or stimulated with various concentrations of IFN-γ (300 U/ml), LPS (5 μg/ml), or both. After 12 h, total cell lysates from unstimulated or stimulated cells were processed for determination of the iNOS activity. The anti-iNOS antiserum reacted with proteins present in RAW cells stimulated with IFN-γ alone, or IFN-γ plus LPS. Kupffer cells, however, expressed the iNOS protein by the stimulation of LPS alone or IFN-γ plus LPS. This finding revealed that the induction of iNOS differed between cell types, as stimuli causing the expression of iNOS varied. Kupffer cells, in vitro, can be stimulated only with LPS and express iNOS (Fig. 2A). The accumulation of NO in the various cultures support this fact (data not shown).

Figure 2B illustrates the time course of iNOS expression in Kupffer cells cultured in the presence of LPS. The iNOS expression was seen as a band which was faintly present 6 h following LPS stimulation. At 12 h after LPS stimulation, the band had become strong. At 1 and 3 h after stimulation, the induction of iNOS was not observed.

Immunohistochemistry of Isolated Kupffer Cells

To examine the availability of our anti-iNOS antiserum for immunohistochemistry, we prepared Kupffer cells as described in the Materials and Methods and cultured them on Lab Tek chamber slides (Nunc, Inc. USA). These cells were reacted with the anti-iNOS antibody. Nonactivated Kupffer cells demonstrated no immunoreactivity for the anti-iNOS antiserum (Fig. 3A), whereas Kupffer cells stimulated with LPS reacted with the anti-iNOS antiserum (Fig. 3B).

Immunohistochemistry Performed on Tissue Sections of Liver at Various Time Intervals

Three h following LPS injection, cells immunoreactive for iNOS were seldom detected (Fig. 4A). Six h following LPS injection, iNOS-positive cells (black) appeared in the sinusoids (Fig. 4B). Twelve h following LPS injection, iNOS-positive cells peaked. There were small clusters of immunoreactive infiltrating cells and small foci of necrosis throughout the liver. Morphologically the iNOS-positive cells appeared to be Kupffer cells or endothelial cells (Fig. 4C). Twenty-four h following LPS injection, foci of spotty necrosis were resolving. The number of iNOS-positive cells in the sinusoids was decreasing except for foci of spotty necrosis (Fig. 4D).

The Effects of L-NAME Treatment

Hepatic damage was assessed by the release of the hepatocellular enzymes that escaped into the serum (Table I). Serum concentration of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were measured at the various times of liver extraction, namely at 3, 6, 12, and 24 h after LPS injection. As time passed, both the AST and ALT levels became elevated. Surprisingly, pretreatment with L-NAME significantly increased the levels of AST and ALT at 3 h post-LPS injection. L-NAME treated rats could not survive more than 5 h, so that we could measure only at 3 h intervals.

We also demonstrated immunohistochemical staining of liver sections 3 h post-LPS injection that revealed a large range of necrosis and small vessels obstructed with clot on tissue sections from L-NAME treated rats (data not shown).

DISCUSSION

There have been many reports about various gram negative endotoxemia models which have been stimulated by the administration of LPS to experimental subjects. In peripherally injected animals, an increase in the number of Kupffer cells has been observed to start at 6 h post-LPS injection [Van et al., 1988]. LPS is

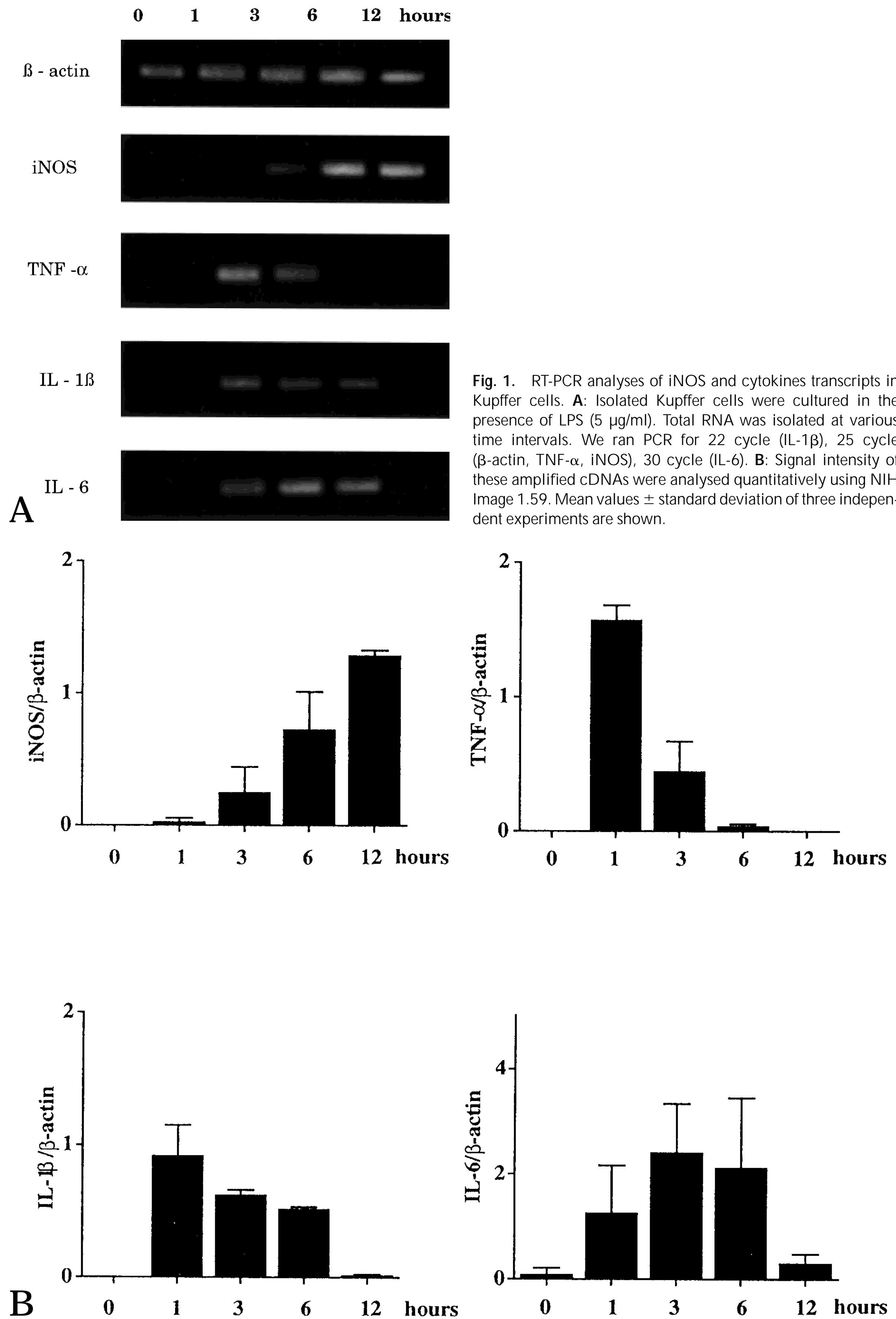


Fig. 1. RT-PCR analyses of iNOS and cytokines transcripts in Kupffer cells. **A**: Isolated Kupffer cells were cultured in the presence of LPS (5 μg/ml). Total RNA was isolated at various time intervals. We ran PCR for 22 cycle (IL-1β), 25 cycle (β-actin, TNF-α, iNOS), 30 cycle (IL-6). **B**: Signal intensity of these amplified cDNAs were analysed quantitatively using NIH Image 1.59. Mean values ± standard deviation of three independent experiments are shown.

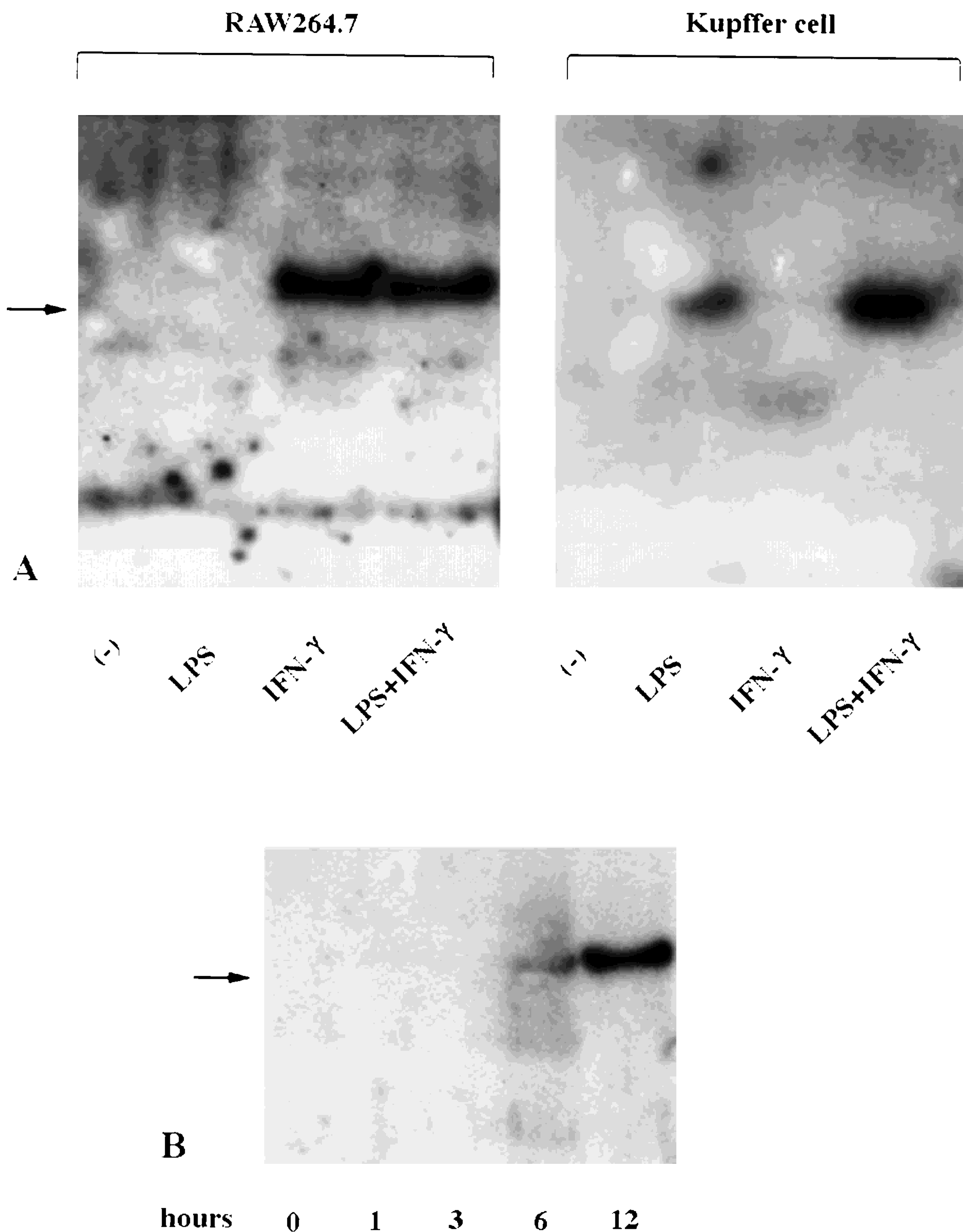
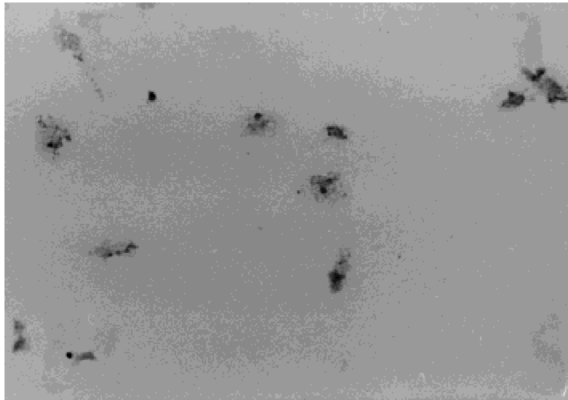


Fig. 2. Western blot of iNOS. Induction of iNOS in a macrophage cell line (RAW264.7) and Kupffer cells. **A**: Both types of cells were cultured in medium, alone, or stimulated by LPS (5 μg/ml), IFN-γ (300 U/ml), or LPS plus IFN-γ for 12 h. Total cell lysates from unstimulated or stimulated preparations were immunoblotted with anti-iNOS antiserum. Arrow shows a 130 kDa band, the typical molecular weight of iNOS. Time course study of iNOS expression in isolated Kupffer cells. **B**: Isolated Kupffer cells were cultured in the presence of LPS (5 μg/ml), and at various time intervals cells were prepared for immunoblotting. A 130 kDa band appeared at 6 h post-injection and strong expression was noted at 12 h.

A



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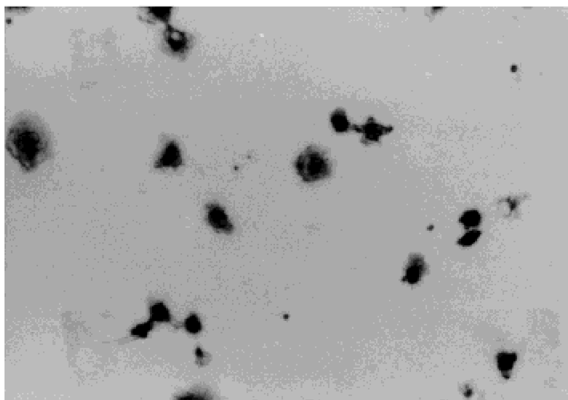
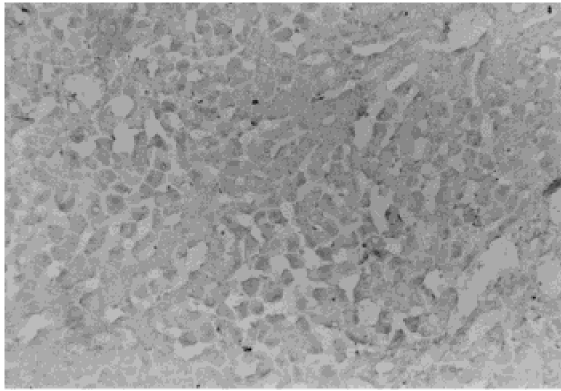


Fig. 3. Isolated Kupffer cells, cultured in medium alone, or stimulated with LPS (5 μg/ml), were reacted with anti-iNOS antiserum. Kupffer cells in medium alone did not express iNOS (**A**); those stimulated with LPS for 12 h reacted with the anti-iNOS antiserum (**B**) (magnification x 400).

thought to be taken up by the liver, especially by Kupffer cells, immediately following injection, but not by the hepatocytes [Mathison et al., 1979; Uragoh et al., 1988]. The cytokines, such as TNF-α, that are secreted from Kupffer cells may be implicated as mediators of the profound pathologic changes produced by LPS. Kupffer cells are thought to play a pivotal role in LPS-induced liver injury. We investigated the expression of TNF-α, IL-1β, and IL-6 in rat Kupffer cells in the presense of LPS. These mRNA transcripts started to express no more than at 1 h following LPS stimulation. TNF-α mRNA transcript maximally expressed at 1 h, and decreased soon. These results are consistent with those of previous report [Luster et al., 1994]. IL-1, TNF-α, and IL-6 are rapidly secreted, and at the mRNA transcriptional level, IL-1α, IL-1β, TNF-α, and IL-6 mRNA are maximally increased within 40 min of LPS administration. IL-1α and TNF-α secretion is mainly due to the Kupffer cells. Moreover, the results of in vivo challenges in the mouse liver are similar to those of in vitro studies [Luster et al., 1994]. We revealed that iNOS as well as IL-6 mRNA expressed later than TNF-α and IL-1β. Earlier studies reported that TNF-α and IL-1β induce NO production of Kupffer cells [Spitzer, 1994]. Therefore our present results well correspond with the view that the TNF-α and IL-1β produced at early time casually relate to the induction of iNOS and IL-6. Although the kinetics of the production and regulation of cytokines in the liver have been elucidated, cytotoxicity against hepatic parenchyma and the function of those cytokines remain unknown.

The localization and distribution of iNOS and cNOS have recently been investigated in various tissues including that from the nervous system [Afework et al., 1994], tumors [Thomsen et al., 1994], and the vascular system [Tsujino et al., 1994]. Although studies in vitro have demonstrated that both Kupffer cells and hepatocytes can express iNOS following stimulation with various cytokines and endotoxins [Geller et al., 1993, 1994], little is known about in vivo responses of hepatic parenchymal and nonparenchymal cells to endotoxins. According to our immunohistochemical results, the iNOS expression was detectable in hepatic nonparenchymal cells (i.e., Kupffer cells and endothelial cells), but not in parenchymal cells. Inducible NOS expression was documented 6 h following LPS administration by both Western blot analysis and immunohistochemistry. At 12 h post-LPS injection, the expression became maximal, and iNOS positive cells were found throughout the sinusoids. Morphological differentiation between macrophage-like cells (Kupffer cells, monocyte) and endothelial cells was not possible using these immunohistochemical methods since both types of cells exist within the sinusoids. The exact identification of Kupffer cells and endothelial cells can only be achieved using ultrastructural analysis or a specific biochemical marker. Our results suggest that the in vivo administration of LPS does not induce



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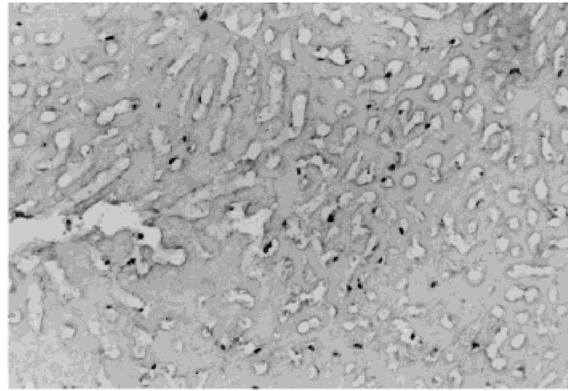

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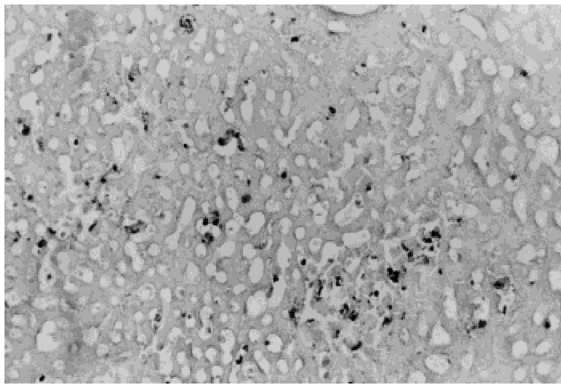

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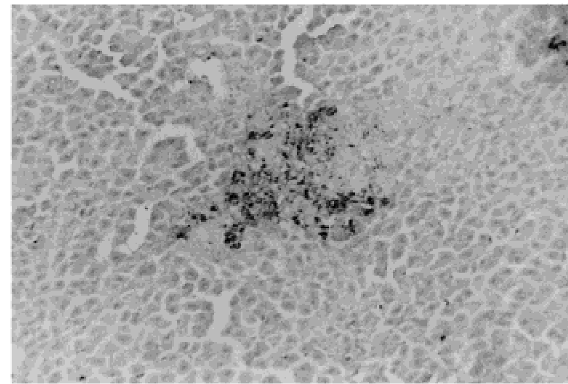

D

Fig. 4. Immunohistochemistry of iNOS in liver section following LPS injection. **A**: Three h following LPS injection, immunoreactive cells were not identified on the sections. **B**: However, 6 h after LPS injection, immunoreactive cells were scattered within the sinusoids. **C**: At 12 h post-injection, the number of immunoreactive cells had increased, and spotty necrosis of the sinusoids and clusters of inflammatory cells were observed. **D**: At 24 h post-injection, foci of spotty necrosis were still present, but less immunoreactive cells were observed in the sinusoids (magnification x 200).

the expression of iNOS in hepatocytes. This finding obviously conflicts with those of previous reports [Geller et al., 1993; Curran et al., 1989] stating hepatocytes contain iNOS and produce large amounts of NO following stimulation by cytokines and endotoxins in vitro. However, Spolarics et al. [1993] have demonstrated that the in vivo LPS administration does not elicit the expression of iNOS mRNA in hepatic parenchymal cells. Thus our immunohistochemical results are consistent with those of Spolarics et al. Geller et al. [1993] reported that iNOS mRNA in hepatocyte was detected following chronic immflammation triggered by *Corynebacterium parvum* injection in vivo. But the expression of iNOS in hepatocyte in vivo was quite weak as compared with in vitro stimulated by multiple cytokines. Why the cytiokines produced by LPS administration induced iNOS in Kupffer cells but not in hepatocytes? Earlier reports showed that hepatocytes require four types of signals; IFN-γ, TNF-α, IL-1β, and LPS,

TABLE I. Hepatic Damage Measured by Serum AST and ALT*

	AST (IU/l)	ALT (IU/l)
Normal	148.5 ± 0.7	48 ± 2
L-NAME(−)		
3 h	357 ± 4.6	65 ± 9.6
6 h	671 ± 188	262 ± 112
12 h	550 ± 131	184 ± 26
24 h	1,678 ± 540	740 ± 253
L-NAME(+)		
3 h	720 ± 180	132 ± 35

*Rats were treated with or without L-NAME before LPS injection. Plasma was obtained when sacrificed at various time intervals following LPS injection. Normal represents neither LPS nor L-NAME injection. Values are the mean ± standard error of three different experiments.

to express iNOS strongly. We showed that Kupffer cells expressed TNF-α and IL-1β, but not IFN-γ after stimulation with LPS [Aono et al., unpublished observation]. We therefore speculate that lack of IFN-γ production after LPS administration resulted in poor expression of iNOS in hepatocytes.

NO is said to have both protective and destructive effects in biological feature. Some reports have shown that NO inhibitors improve the symptoms due to arthritis, ulcerative colitis, Crohn's disease, and autoimmune diseases [Schmidt et al., 1994]. On the other hand, NO mediates anti-inflammatory effects such as the inhibition of neutrophil adhesion [Kubes et al., 1991], T-cell proliferation [Isobe et al., 1992], and platelet aggregation [Shultz et al., 1992]. In vitro studies have documented the relationship between stimuli (cytokines and endotoxins) and NO-producing cells, it is likely that all of the physiological effects of NO in the liver have not yet been elucidated.

Liver injury caused by in vivo LPS administration is thought partly because extremely swollen Kupffer cells, platelet aggregation, and granulocytes obstruct the sinusoids, and result in liver tissue damage [Shibayama., 1987; Van et al., 1988]. By the light microscopy and electron microscopy findings, the hypothesis has been established on the basis of these structural analysis. Sinusoidal blood flow might become retarded in sinusoids where plugging occurs. This may result in local ischemia, causing ultrastructural changes in the surrounding cells [Van et al., 1988]. One of the most important effects of NO is vasodilation, so NO keeps sinusoidal blood flow. The experiments using NO inhibitors support this hypothesis. Treatment with the NO inhibitor, L-NAME, increases the susceptibility of a subject to LPS changes. As in previous reports [Harbrecht et al., 1994], our data regarding escaped hepatocellular enzymes demonstrates the protective effect of NO on liver injury, since pretreatment with L-NAME increased the levels of these enzymes at the same time point. According to these data, NO make protective effects in the liver during endotoxemia. Other reports have documented, however, that iNOS mutant mice are resistant to LPS-induced mortality in contrast to wild-type [Wei et al., 1995]. The result of L-NAME treatment is quite different from that of iNOS mutant mice. As L-NAME inhibits both iNOS and cNOS. These contrary results lead to the speculation that a large amount of NO produced by iNOS is cytotoxic, but a little NO from cNOS regulates the sinusoidal tone and maintains hepatic blood flow, resulting in the prevention of parenchymal cell damage. In addition, Wink et al. [1993, 1995] reported that NO protected against cellular damage and cytotoxicity from reactive oxygen species. NO production decreased by pretreatment of L-NAME, so that oxygen radicals were not scavenged. By other biological effects of NO, platelet aggregation [Shultz et al., 1992]. and Tcell proliferation [Isobe et al., 1992] were suppressed. These functions can keep the microcirculation in the liver.

In conclusion, TNF-α, IL-1β, and IL-6 are secrated immediatedly after LPS stimulation, whereas iNOS expresses from 3 h after LPS stimulation and increased up to 12 h. TNF-α, IL-1β were thought to enhance iNOS expression by autocrine or paracrine stimulation. In vivo LPS administration revealed that iNOS was mainly expressed in Kupffer cells and endothelial cells not in hepatocytes.

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