Involvement of tumor necrosis factor α, rather than interleukin-1α/β or nitric oxides in the heme oxygenase-1 gene expression by lipopolysaccharide in the mouse liver

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Abstract Heme oxygenase-1 (HO-1) is induced under various oxidative stress conditions, such as lipopolysaccharide (LPS) insult. Induction of HO-1 by LPS is reported to be mediated through interleukin-1β (IL-1β), rather than other inflammatory cytokines in the mouse liver. However, we found that IL-1α/β knockout (KO) mice responded well to LPS insult, as did wild-type mice with respect to HO-1 mRNA induction (about 30-fold increase). In contrast, tumor necrosis factor α KO (TNFαKO) mice responded very weakly to LPS in the HO-1 mRNA expression, but not metallothionein mRNA. Recent studies reveal that nitric oxide from Kupffer cells is involved in HO-1 induction in the liver produced by LPS. Therefore, nitrite and nitrate concentrations in the liver were also measured and these parameters did not increase in either IL-1KO or TNFαKO. In addition, the phosphorylation of c-JUN N-terminal kinase (JNK) and p38, but not extracellular signal-regulated kinase, was very low in TNFαKO mice due to LPS administration. All of these findings indicate that TNFα is a major candidate to trigger HO-1 induction in response to LPS stimulation, and that its message is likely transduced through JNK and p38 pathways.

Key words: Heme oxygenase-1; Lipopolysaccharide; Cytokine; Interleukin-1; Tumor necrosis factor α; Liver; Gene deficient mouse; Nitric oxide; Signal transduction; c-JUN N-terminal kinase; p38; Extracellular signal-regulated kinase; Metallothionein

1. Introduction

Heme oxygenase-1 (HO-1) is a rate-limiting enzyme for heme degradation and is known to be a stress marker because of its rapid inductive response to various oxidative stresses [1–4]. Lipopolysaccharide (LPS) is one of the well known inducers of HO-1 in many different tissues or cell lines [5–8]. A mechanism to induce HO-1 by LPS has been shown to be mediated by DNA binding activation of activated protein-1 (AP-1) [5,9], and interleukin-1β (IL-1β) elevated after LPS administration is suggestive of being one of the major intermediary cytokines in mouse liver [10,11]. Recently, nitric oxide (NO) produced by LPS stimulation has been shown to lead to HO-1 gene expression in the Kupffer cell [11,12].

Gene-modified animals are a good tool to understand biological mechanisms in a whole body. However, no study on the possible involvement of cytokines on HO-1 gene expression by LPS using cytokine deficient mice has previously been carried out. We assumed that using these gene-modified animals would be useful for studying the mechanism of HO-1 mRNA induction by LPS since LPS produces various cytokines that work as a network. Therefore, we used IL-1α/β deficient (IL-1 knockout (KO) mouse) and tumor necrosis factor α (TNFα) deficient mice (TNFαKO mouse) to compare the effect of LPS on HO-1 gene expression. In this study, we also examined a possible role of NO to induce HO-1 mRNA expression. For this purpose, we determined nitrite and nitrate (NOx) concentrations, the stable oxidation products of NO in the liver, after LPS administration. Additionally, we investigated the changes in the mitogen-activated protein (MAP) kinase to the induction of HO-1 gene expression evoked by LPS, which associates with AP-1 DNA binding activation [13].

2. Materials and methods

2.1. Reagents

LPS from *Escherichia coli* serotype (O111:B4) was purchased from Sigma Aldrich Japan (Tokyo, Japan). Deoxycytidine-5′-[α-^{32}P]-triphosphate (3000 Ci/mmol) and [γ-^{32}P]adenosine-5′-triphosphate (5000 Ci/mmol) were from Japan Isotope Association (Tokyo, Japan). Complete™ protease inhibitor cocktail was from Roche Diagnostics GmbH (Mannheim, Germany). Polyclonal antibodies against p44/42 MAP kinase, SAPK/c-JUN N-terminal kinase (JNK) or p38 MAP kinase, phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAP kinase (Thr180/Tyr182) and monoclonal antibody against phospho-p44/42 MAP kinase (Thr202/Tyr204) were from Cell Signaling Technology, Inc. (Beverly, MA, USA). All other reagents used were of the highest grade commercially available.

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2.2. Animals and treatment

All animal experiments were carried out under the control of the Regulation of the Committee of Animal Care and Welfare of Showa University. The IL-1KO mouse was established by Horai et al. [14] and the TNFαKO mouse was established by Tagawa et al. [15]. Balb/c mice were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan) and mated with either IL-1KO or TNFαKO. Wild-type, homozygous and heterozygous progenies were selected by their respective gene expressions and the lines for wild-type or homozygous animals were established. LPS was dissolved in saline and injected intraperitoneally at the dose of 0.1 mg/kg.

2.3. RNA extraction and Northern blot

Total RNA was isolated from the liver of each mouse using the acid GTC–phenol–chloroform extraction method [16] as previously described [2]. Total RNA (20 μg) was fractionated by electrophoresis on 1% agarose gel followed by transfer onto a nylon membrane. The RNA blot was hybridized with ^{32}P-labeled cDNA for HO-1 (1.1 kb, *Bam*HI fragment of pMp32cod) and metallothionein (MT)-1 (0.4 kb, *Hin*dIII/*Eco*RI fragment of pmMT-1), and oligonucleotide for mouse 18S ribosomal RNA (5′-ACGGTATCTGATCGTCTTCGAACC-3′; this sequence corresponds to the complement of nucleotides 1044–1067 of the mouse gene for 18S rRNA). The hybridization levels were quantitated with a bio-imaging analyzer (BAS3000, Fuji Photo Film Co., Tokyo, Japan).

2.4. Cytokine concentrations

IL-1β, IL-6 and TNFα concentrations in mouse serum were determined using enzyme-linked immunosorbent assay kits (IL-1β and IL-6, Endogen, Woburn, MA, USA; TNFα, Biosource, Camarillo, CA, USA).

2.5. NOx concentration

NOx in the liver was determined by the method of Yamada et al. [17] using the NO detector–high-performance liquid chromatography system (ENO-10, Eicom, Kyoto, Japan). Livers were perfused with saline containing 1 mM EDTA and 0.1% aprotinin and immediately stored in 20% glycerol solution. The tissue was homogenized with at least four volumes of 20% glycerol solution, and the supernatant after centrifugation of the homogenate was used as a sample to measure NOx. NOx in the supernatant was separated by a reverse-phase separation column packed with polystyrene polymer (NO-PAK, 4.6×50 mm, Eicom, Kyoto, Japan), and nitrate was reduced to nitrite in a reduction column packed with copper-plated cadmium fillings (NO-RED, Eicom, Kyoto, Japan). The nitrite concentration was measured by the reaction with Griess reagent to form a purple azodye in a reaction coil.

2.6. Phosphorylation of p38, JNK and extracellular signal-regulated kinase (ERK)

Liver was perfused with 10 mM Tris–HCl (pH 7.4) containing 1 mM EDTA, 250 mM sucrose, 1 mM APMSF, 2 μg/ml aprotinin, 10 mM *p*-nitrophenyl phosphate, 20 mM β-glycerophosphate, 50 μM sodium orthovanadate and 10 mM sodium molybdate and homogenized with 10 volumes of the homogenizing buffer (20 mM Tris–HCl (pH 7.4) containing 150 mM NaCl, 1% NP-40, 100 μg/ml DNase I, 50 μg/ml RNase A, 1 mM APMSF, 10 mM *p*-nitrophenyl phosphate, 20 mM β-glycerophosphate, 50 μM sodium orthovanadate and 10 mM sodium molybdate and Complete™ protease inhibitor cocktail). The homogenized solution was centrifuged for 20 min at 15 000 rpm and the supernatant was used for the immunoblot analysis. Protein concentration was determined by the method of Bradford [18], using a protein reagent (Bio-Rad Labs., Hercules, CA, USA).

The supernatant fraction (25 μg protein) was solubilized in 2% SDS and the proteins were separated by polyacrylamide gel electrophoresis (3% stacking gel, 10% separating gel) according to the method of Laemmli [19]. After electrophoresis, the proteins were transferred to polyvinylidene difluoride membranes (Japan Genetics Co., Tokyo, Japan) at 80 mA for 50 min. Western blots were performed using polyclonal antibodies against p44/42 MAP kinase, SAPK/JNK or p38 MAP kinase, phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 MAP kinase (Thr180/Tyr182) and monoclonal antibody against phospho-p44/42 MAP kinase (Thr202/Tyr204). Signal was detected using chemiluminescence (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK). Molecular weight was calculated with prestained SDS–PAGE standards (Bio-Rad Labs., Hercules, CA, USA) that was applied to the same gel run samples.

2.7. Statistical analysis

Data were subjected to statistical analysis by the Kruskal–Wallis non-parametric analysis of variance test followed by Dunn's multiple comparison test. The accepted level of significance was set at $P < 0.05$.

3. Results

We first examined the dose-dependent effects of LPS on HO-1 gene expression in wild-type mice livers and found that there were no significant differences in HO-1 mRNA among the doses of 0.1, 0.5 and 1 mg/kg at 2 h after the administration (data not shown). Therefore, we chose 0.1 mg/kg of LPS in this study. Fig. 1 represents the effect of LPS on HO-1 gene expression in IL-1KO, TNFαKO and wild-type mice livers. We also examined the response of MT mRNA, another stress responsive protein, under the same experimental conditions. Unexpectedly, HO-1 mRNA expression in response to LPS in the IL-1KO liver showed almost the same level as that seen in the wild-type mice. In contrast, TNFαKO mice showed a very weak HO-1 gene expression, but not MT gene, in response to LPS. Namely, LPS did not produce the increase of HO-1 gene expression, but rather enhanced MT mRNA expression in the TNFαKO mice in a manner similar to that of the wild-type mice livers. In IL-1KO mice, LPS tended to increase MT response to a greater degree than that observed in the wild-type mice.

Under the same experimental conditions, we measured IL-1β, IL-6 and TNFα concentrations in the mice sera (Fig. 2). As shown in Fig. 2, TNFαKO mice showed very limited ability to produce IL-1β in response to LPS and responded to

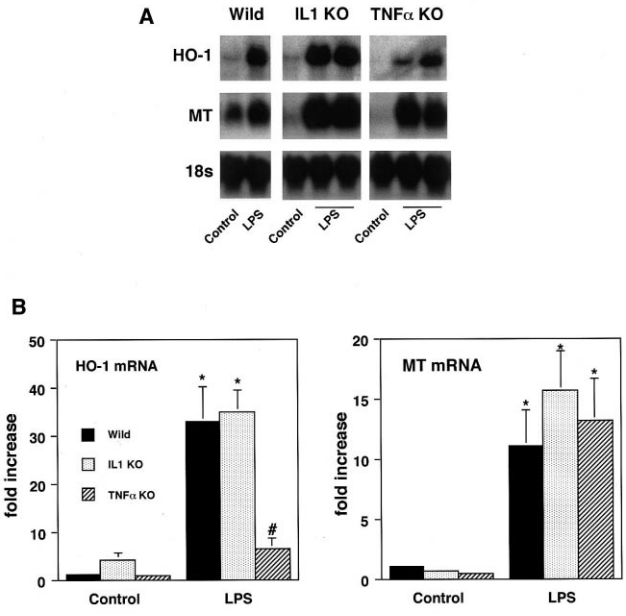

Fig. 1. Effect of LPS on HO-1 and MT gene expression in wild-type, IL-1KO and TNFαKO mouse livers. LPS (0.1 mg/kg) was injected intraperitoneally and livers were collected at 2 h after treatment. A: Northern blot analysis was performed. B: Blots for HO-1 and MT mRNA were semi-quantified and normalized with that for 18S ribosomal RNA. Symbols represent the mean ± S.E.M. for seven or 10 mice. *Significantly different from controls at $P < 0.05$. #Significantly different from wild-type mice treated with LPS at $P < 0.05$.

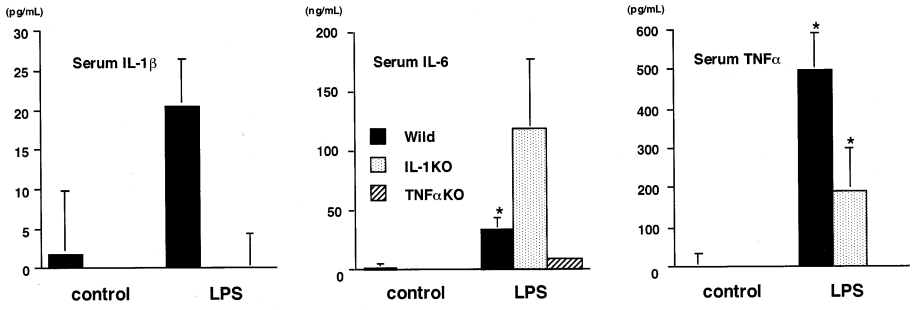

Fig. 2. Effect of LPS on various cytokines in mouse serum. Experimental conditions were the same as in Fig. 1. Serum cytokine concentrations were determined as described in Section 2. Symbols represent the mean ± S.E.M. for three or four mice. *Significantly different from controls at $P < 0.05$. #Significantly different from wild-type mice treated with LPS at $P < 0.05$.

about 1/10 of IL-6 production when compared to those produced in the wild-type mice. In contrast, LPS was able to increase the IL-6 content three-fold, but it decreased TNFα to 40% of the control value, in IL-1KO mice compared to that seen in the wild-type mice.

In order to clarify the possible role of TNFα in the HO-1 gene expression induced by LPS, we examined the effects LPS on heterozygous and homozygous animals (Fig. 3). As shown in Fig. 3, we found that the magnitude of HO-1 mRNA expression in TNFα (+/−) mice treated with LPS was reduced to about 80% of the wild-type mice when examined at 2 h after the administration.

Recently, it has been reported that NO produced in the Kupffer cells after LPS stimulation is a strong mediator for the induction of HO-1 mRNA [11,12]. Then, nitrite and nitrate concentrations were measured in the mouse liver of the stimulation with LPS. As shown in Fig. 4, nitrate was the major NO product and was 100 times more abundant than nitrite in the liver of wild-type, IL-1KO as well as TNFαKO mice. In wild-type mice livers, nitrate level was increased to about two-fold the control levels after stimulation with LPS. However, neither IL-1KO nor TNFαKO mouse livers showed any increase of nitrate levels in the liver. On the other hand, nitrite levels in the wild-type, IL-1KO and TNFαKO mouse livers were not changed appreciably after LPS stimulation. Nitrite concentrations in the liver of IL-1KO and TNFαKO controls were higher than that of the wild-type mice; however, again the nitrite concentration was only about 1/100 of the nitrate concentration in the liver. Under the same experimental conditions, we also measured plasma NOx concentrations and found that there was no alteration of the product in any mice (data not shown).

Since LPS lacked the induction of HO-1 mRNA in TNFαKO mice, we finally examined the changes in MAP kinase resulting in AP-1 DNA binding activation, which have been shown to promote an increase in HO-1 gene transcription. As shown in Fig. 5, we found that TNFαKO mice exhibited lower levels of the phosphorylated JNK and p38. In contrast, LPS increased ERK phosphorylation in TNFαKO mice, as well as in wild-type mice.

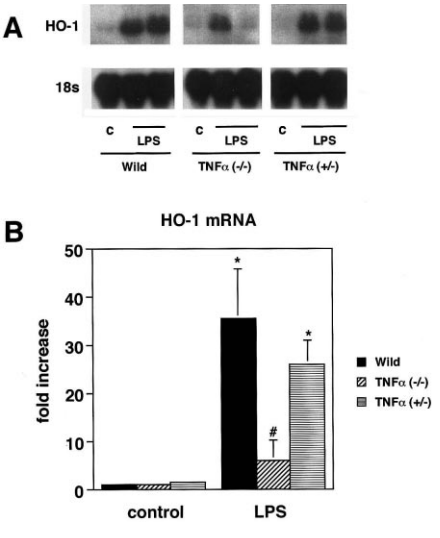

Fig. 3. Effect of LPS on HO-1 mRNA in TNFα(−/−) and TNFα(+/−) mouse livers. A: Northern blot analysis was performed. B: Blots for HO-1 were semi-quantified and normalized with that for 18S ribosomal RNA. Symbols represent the mean ± S.E.M. for five or six mice. *Significantly different from controls at $P < 0.05$. #Significantly different from wild-type mice treated with LPS at $P < 0.05$.

4. Discussion

It is difficult to identify the factor that plays the most prominent role in HO-1 gene expression during LPS stimulation because LPS stimulates various factors, including inflammatory cytokines and NO, which have been shown to induce HO-1, individually [20]. The present study has shown that

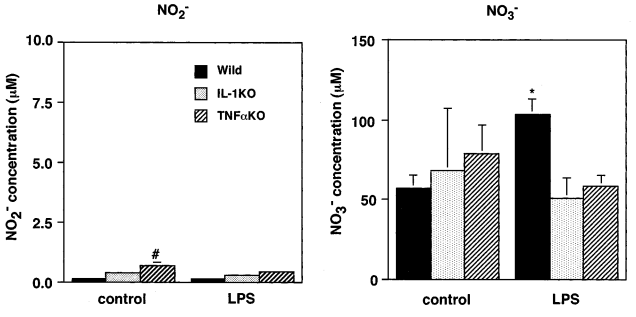

Fig. 4. Effect of LPS on nitrate and nitrite concentrations in wild-type, IL-1KO and TNFαKO mouse livers. Nitrate and nitrite concentrations in mouse livers were detected as described in Section 2. Bars represent the mean ± S.E.M. for four or six mice. *Significantly different from controls at $P < 0.05$.

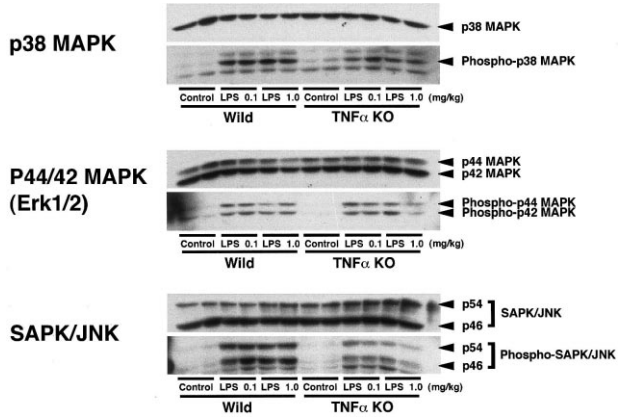

Fig. 5. Effect of LPS on MAP kinases in wild-type and TNFαKO mouse livers. Mice were treated with LPS (0.1 mg/kg or 1.0 mg/kg) and the livers were collected at 1 h after the treatment. Immunoblotting analysis was investigated as described in Section 2.

TNFα is one of the key cytokines to produce HO-1 gene expression under LPS stimulation in mouse livers. Previous studies revealed the involvement of IL-1β and/or NO, released from Kupffer cells, not TNFα or IL-6 in HO-1 mRNA accumulation after LPS stimulation [10–12]. However, the TNFαKO mouse has only a weak response to IL-1β and NO production after LPS administration. Therefore, IL-1β and/or NOs might work only marginally to induce HO-1 mRNA.

We also studied effects of neutralizing antibodies against either IL-1β or TNFα to examine which cytokine is involved in HO-1 gene expression in response to LPS (data not shown). Neutralizing TNFα antibody suppressed HO-1 mRNA induction about 30% and IL-1β antibody by about 10% in the wild-type mice. This indicates that the results obtained by employing cytokine KO mice are basically consistent with what has occurred in wild-type mice although neutralizing antibodies do not work as effectively as what we expected.

It has been reported that MT induction by LPS is regulated through IL-6 [21]. LPS-mediated elevation of IL-6 content in IL-1KO mice was higher than the wild-type animals, which possibly leads to the enhanced MT expression in the former mice. However, TNFαKO mice showed a similar response of MT mRNA to that in wild-type mice with a lower amount of IL-6 in the serum. Therefore, the present study indicates that there are other factors that cause MT induction in addition to IL-6. In this respect, however, further study will be needed.

The increase of nitrate in the wild-type mouse evoked by LPS was not to as great an extent as compared to that of HO-1 mRNA induction. Furthermore, IL-1KO mice that expressed high HO-1 mRNA by LPS administration did not produce a high NOx concentration. Taken together, NO may have a limited role in the induction of HO-1 mRNA by LPS when compared to TNFα in the murine liver, although previous studies have shown the importance of NO in vivo and in vitro [11,22].

LPS initially binds to the CD14 and toll-like receptor at the cell surface and activates MAP kinase pathways [13,23]. It is also reported that HO-1 gene expression by LPS stimulation is mediated by the activation of a transcription factor, AP-1 [5,9,20]. The present study suggests that p38 and JNK are involved in the signal transduction of LPS, which activates AP-1 followed by HO-1 gene expression.

The present findings employed with cytokine KO mice support the hypothesis that TNFα plays a more critical role in HO-1 mRNA induction by LPS in mouse livers than IL-1β and NOx, and this process is mediated by p38 and JNK.

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