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Modulation by endothelin-1 of lipopolysaccharide-induced cyclooxygenase 2 expression in mouse peritoneal macrophages

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Abstract

We investigated the modulation by endothelin-1 of lipopolysaccharide-induced cyclooxygenase 2 expression and prostaglandin E_2 production by mouse peritoneal macrophages. Our previous report showed that endothelin-1 at concentrations above 10^{-11} M induced cyclooxygenase 2 expression through mainly endothelin ET_B receptors and that an endothelin ET_B receptor-mediated process was not involved in cyclooxygenase 2 activation in macrophages stimulated by lipopolysaccharide for 4 h. In the present study, when macrophages were stimulated by lipopolysaccharide for 12 h in the presence of endothelin-1 (10^{-15} to 10^{-8} M), cyclooxygenase 2 expression and prostaglandin E_2 production were enhanced by 1.2- to 1.6-fold. The endothelin ET_B receptor selective antagonist, BQ788 (*N-cis*-2,6-dimethylpiperidino-carbonyl-L-γ-methyl-leucyl-D-L-methoxycarbonyl-tryptophanyl-D-norleucine), significantly inhibited this synergistic effect of endothelin-1. In addition, the cyclooxygenase 2-selective inhibitor, NS398 (*N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide), also suppressed this effect. Western blot analysis showed that the endothelin ET_B receptor was up-regulated by lipopolysaccharide in a time- and concentration-dependent manner, and that this up-regulation was inhibited by NS398. From these results, we conclude that endothelin-1 promotes lipopolysaccharide-induced cyclooxygenase 2 activation in the delayed phase through endothelin ET_B receptors up-regulated by lipopolysaccharide.

Keywords: Endothelin-1; Macrophage; Cyclooxygenase 2; Lipopolysaccharide; Endothelin ET_B receptor

1. Introduction

Endothelin-1 is a highly potent vasoconstrictor (Yanagisawa et al., 1988) and has a variety of biological effects on various types of cells, including monocytes/macrophages. The endothelin-1 signaling cascade involves several prostanoids, which may contribute to endothelin-induced contractility and mitogenesis (Filep et al., 1991; Simonson and Dunn, 1993; Kester et al., 1994). Recently we showed that a high concentration of endothelin-1 (10^{-11} to 10^{-9} M) induces cyclooxygenase 2 expression and prostaglandin E_2 production by mouse peritoneal macrophages through mainly endothelin ET_B receptors and partially ET_A receptors (Shimada et al., 1998). It has been demonstrated that macrophages produce prostaglandin E_2 via the cyclooxygenase 2-dependent pathway in response to proinflammatory cytokines or bacterial lipopolysaccharide (Morham et al., 1995; Matsumoto et al., 1996; Naraba et al., 1998). In addition, human macrophages can also produce endothelin-1 by lipopolysaccharide stimulation (Ehrenreich et al., 1990; Chanez et al., 1996). These reports raise the possibility that endothelin-1 may play an important role in the pathogenesis of lipopolysaccharide-induced inflammatory reactions; however, it remains unclear what the effect of endothelin-1 is on lipopolysaccharide-stimulated inflammatory cells. In the present study, we demonstrated the synergistic modulation by endothelin-1 of cyclooxygenase 2 expression and prostaglandin E_2 production in mouse peritoneal macrophages stimulated by lipopolysaccharide for 12 h. We found that the endothelin ET_B receptor was up-regulated by lipopolysaccharide, which may contribute to this modulation by endothelin-1.

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2. Materials and methods

2.1. Materials

The following commercial preparations were used: lipopolysaccharide (*E. coli*, 055: B5) and the cyclooxygenase 2 selective inhibitor, NS398 (*N*-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide) from Nacalai Tesque (Kyoto, Japan), endothelin-1 from Peptide Institute (Kyoto, Japan), BQ788 (*N*-*cis*-2,6-dimethylpiperidino-carbonyl-L-γ-methyl-leucyl-D-L-methoxycarbonyl-tryptophanyl-D-norleucine) from Banyu (Tokyo, Japan), antibody against cyclooxygenase 2 from Cayman Chemicals (MI, USA) and antibody against human endothelin ET_B receptor from Immunobiological Laboratories (Gunma, Japan).

2.2. Preparation and activation of mouse peritoneal macrophages

This study was performed in accordance with The Japanese Pharmacological Society Guide for the Care and Use of Laboratory Animals. The local Animal Care Committee approved all procedures at Nara Medical University.

Brewer's thioglycolate medium (4.05%, W/V) (Nacalai Tesque, Kyoto, Japan) was injected i.p. into 10-week-old C57 Black/6 mice (Kiwa Experimental Laboratory Animal, Wakayama, Japan). Peritoneal exudate cells were collected on day 4 by washing the cavity with 10 ml of ice-cold sterile heparinized calcium- and magnesium-free phosphate buffer saline (PBS). The collected cells were immediately centrifuged, the supernatants were discarded and peritoneal cells were immediately seeded in 12-well plates in 1 ml of Dulbecco's Modified Eagle's Medium (DMEM; Gibco-BRL, France) containing 10% fetal bovine serum (Nacalai Tesque). After a 90-min incubation at 37°C with 5% CO_2, about 90% of adherent cells were macrophages, as assessed by measurement of their esterase activity. Then the adherent cells were collected with a rubber policeman and were suspended at 1.0×10^6 cells/ml in DMEM and seeded in 12-well plates (1 ml/well). Cell viability throughout the experiment exceeded 95% as determined by trypan blue dye exclusion and measurement of lactate dehydrogenase (LDH) in cell supernatants with an LDH-UV test kit (Wako, Tokyo, Japan). Adherent macrophages were washed with sterile calcium and magnesium-free PBS warmed to 37°C and

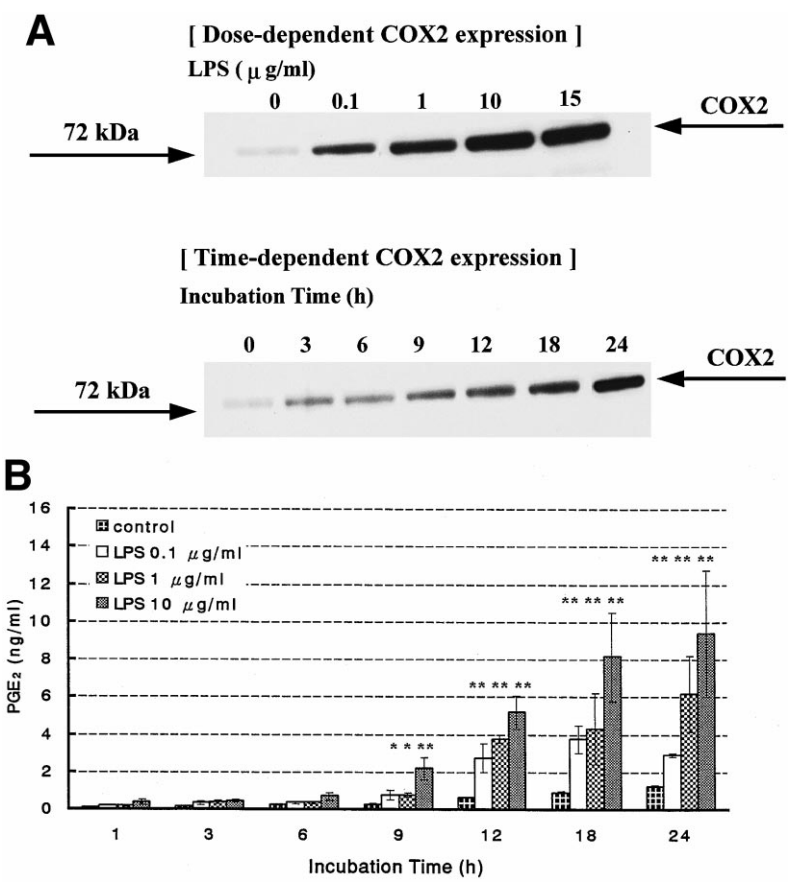

Fig. 1. Cyclooxygenase 2 protein expression and prostaglandin E_2 production by lipopolysaccharide. (A) shows representative experiments of time- and concentration-dependent cyclooxygenase 2 protein expression, which was determined by Western blot as described in Section 2. (B) shows prostaglandin E_2 production, which was measured as described in Section 2. Values are expressed as means ± S.D. of five independent experiments. ** or * indicates a statistically significant difference between cells stimulated by lipopolysaccharide and control at $P < 0.01$ or $P < 0.05$. Abbreviation: COX2; cyclooxygenase 2, LPS; lipopolysaccharide, PGE_2; prostaglandin E_2.

incubated in the medium with or without lipopolysaccharide at 10 μg/ml for up to 24 h.

2.3. Western blot

Macrophage protein (40 μg) was separated by electrophoresis on 7.5% sodium dodecyl sulfate-polyacrylamide gels and transferred to hybond polyvinylidene difluoride membranes (Amersham). The specificity of the antiserum used for immunodetection of cyclooxygenase 2 was determined in the presence of two purified forms of prostaglandin synthetase (sheep placenta). Rabbit polyclonal antiserum against human and murine cyclooxygenase 2 (72–74 kDa) did not cross-react with murine cyclooxygenase 1 (68 kDa). Endothelin ET_B receptors were detected by using rabbit polyclonal antibody against the human endothelin ET_B receptor (51 kDa). The protein bands were visualized by the enhanced chemiluminescence detection system (Amersham) using Kodak X-AR film. Determination of cyclo-oxygenase 2 and endothelin ET_B receptors was performed by densitometric analysis (Bio-Rad Laboratories, Tokyo, Japan).

2.4. Prostaglandin E_2 and LDH assay

The prostaglandin E_2 concentration in supernatants was measured by enzyme-linked immunoassay kit (Cayman). We measured whether the prostaglandin E_2 concentration in DMEM containing 10% fetal bovine serum without cultured macrophages changed within 12 h and verified that it was not degraded and was stable in the present study. The cytotoxicity of reagents was assessed by measurement of LDH in cell supernatants, using the LDH-UV test kit.

2.5. Statistical analysis

Data are expressed as means ± standard deviation (S.D.). One-way Analysis of Variance was used to determine group differences. If the group values were statistically significant ($P < 0.05$), post hoc analyses were conducted using the Fisher's Protected Least-Significant Difference test.

3. Results

3.1. Cyclooxygenase 2 expression and prostaglandin E_2 production by lipopolysaccharide-stimulated macrophages

Western blot analysis showed a time- and concentration-dependent increase of cyclooxygenase 2 protein expression in mouse peritoneal macrophages stimulated by

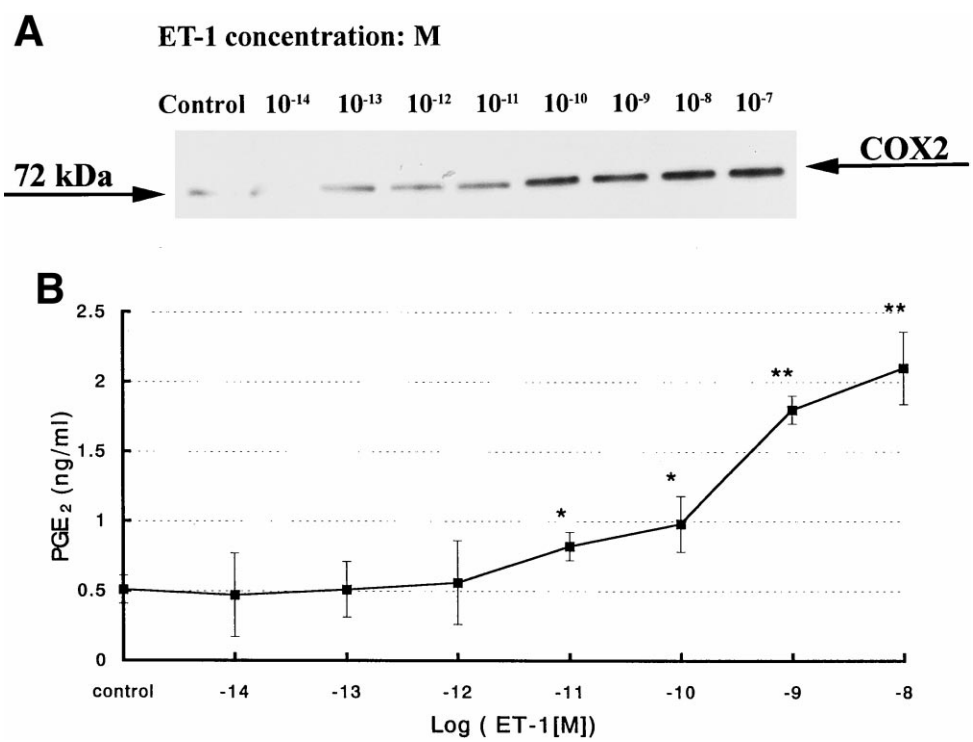

Fig. 2. Cyclooxygenase 2 expression and prostaglandin E_2 production by endothelin-1. (A) shows representative experiments of cyclooxygenase 2 protein expression, which was determined by Western blot as described in Section 2. (B) shows prostaglandin E_2 production, which was measured as described in Section 2. Values are expressed as means ± S.D. of five independent experiments. ** or * indicates a statistically significant difference between cells stimulated with endothelin-1 and control cells at $P < 0.01$ or $P < 0.05$. Abbreviation: COX2; cyclooxygenase 2, ET; endothelin, PGE_2; prostaglandin E_2.

lipopolysaccharide (Fig. 1A). Prostaglandin E_2 production was also increased by lipopolysaccharide in a time- and concentration-dependent manner (Fig. 1B). The significant increase of cyclooxygenase 2 expression was first noted at 6 h and then developed further.

3.2. Effects of endothelin-1 on lipopolysaccharide-induced cyclooxygenase 2 expression and prostaglandin E_2 production

Endothelin-1 at concentrations above 10^{-15} M promoted lipopolysaccharide-induced cyclooxygenase 2 protein expression in 12 h. When the concentration was above 10^{-11} M, endothelin-1 increased cyclooxygenase 2 protein expression and prostaglandin E_2 production by itself in a concentration-dependent manner (Fig. 2). Therefore, low concentrations of endothelin-1 (below 10^{-11} M) synergistically increased cyclooxygenase 2 protein expression and prostaglandin E_2 production in lipopolysaccharide-stimulated macrophages. High concentrations of endothelin-1 (above 10^{-11} M) showed additive effects on lipopolysaccharide-induced responses, which appeared to be independent of the concentration (Fig. 3).

The selective endothelin ET_B receptor antagonist, BQ788, at 10^{-7} M (Mihara et al., 1994) failed to inhibit lipopolysaccharide-induced cyclooxygenase 2 protein expression and prostaglandin E_2 production in 12 h. However, it suppressed the synergistic increase induced by endothelin-1 (10^{-14} M) of cyclooxygenase 2 protein expression and prostaglandin E_2 production (Fig. 4).

3.3. Up-regulation of endothelin ET_B receptor by lipopolysaccharide

Western blot analysis using anti-human endothelin ET_B receptor antibody showed broad bands at 51 kDa in molecular weight, which was consistent with a previous report (Hiraki et al., 1997). The amount of this protein was minimal and did not change during culture without any stimulation, but its level was increased by lipopolysaccharide (10 μg/ml) in 6 to 12 h (Fig. 5A). Endothelin ET_B receptor up-regulation by lipopolysaccharide was detected at 0.01 μg/ml, and the level at 12 h was 1.2- to 1.8-fold that measured without stimulation (control) (Fig. 5B).

3.4. Effects of cyclooxygenase 2 inhibitor, NS398 on cyclooxygenase 2 expression, prostaglandin E_2 production and endothelin ET_B receptor regulation

Macrophages were pretreated with the cyclooxygenase 2-selective inhibitor, NS398, at 10^{-5} M for 30 min (Futaki

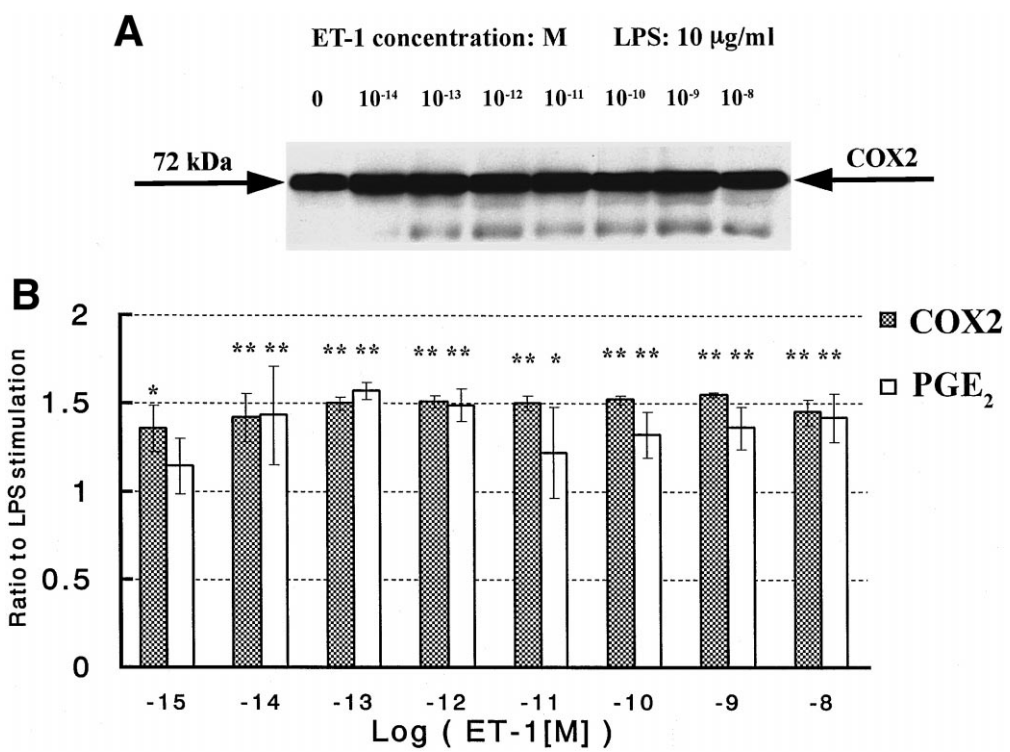

Fig. 3. The effects of endothelin-1 on lipopolysaccharide-induced cyclooxygenase 2 protein expression and prostaglandin E_2 production. (A) shows representative experiments of cyclooxygenase 2 protein expression after 12 h of lipopolysaccharide (10 μg/ml) stimulation with or without endothelin-1. Cyclooxygenase 2 protein expression was determined by Western blot as described in Section 2. (B) shows the densitometry of the cyclooxygenase 2 protein band and prostaglandin E_2 production, which are expressed as ratios to those measured after 12 h of lipopolysaccharide (10 μg/ml) stimulation. Values are expressed as means ± S.D. of the five independent experiments. ** or * indicates a statistically significant difference between cells stimulated by lipopolysaccharide with and without endothelin-1 at $P < 0.01$ or $P < 0.05$. Abbreviation: COX2; cyclooxygenase 2, LPS; lipopolysaccharide, PGE_2; prostaglandin E_2, ET; endothelin.

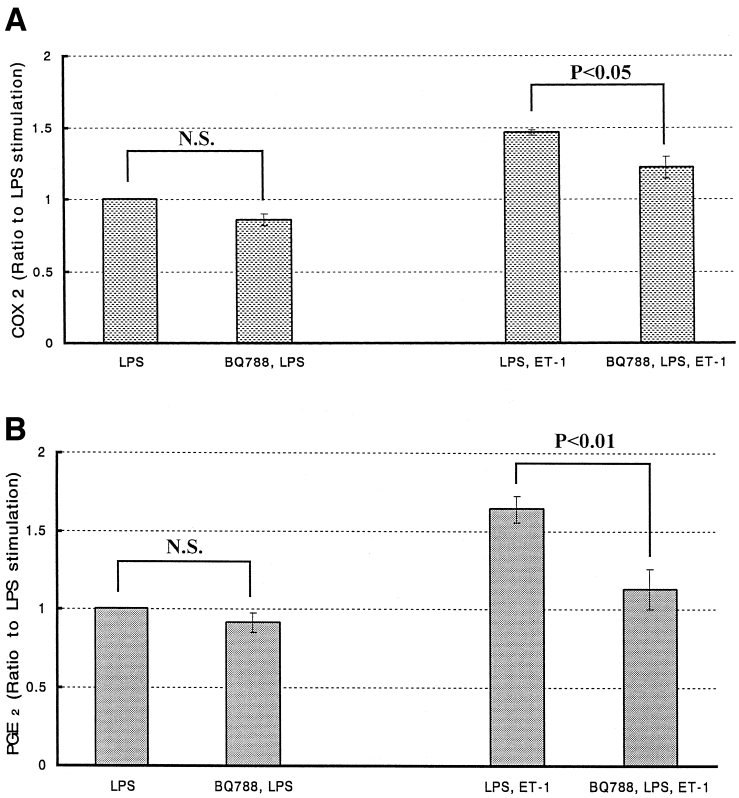

Fig. 4. Effect of BQ788 (10^{-7} M) on the stimulation by endothelin-1 (10^{-14} M) of lipopolysaccharide-induced cyclooxygenase 2 expression and prostaglandin E_2 production. (A) and (B) show the effects of BQ788 on cyclooxygenase 2 protein expression and prostaglandin E_2 production stimulated by lipopolysaccharide with or without endothelin-1. Cyclooxygenase 2 protein or prostaglandin E_2 production is expressed as the ratio to that in response to 12 h of lipopolysaccharide (10 μg/ml) stimulation. Abbreviation: COX2; cyclooxygenase 2, LPS; lipopolysaccharide, ET; endothelin, PGE_2; prostaglandin E_2, N.S.; no statistically significant difference.

et al., 1994). NS398 suppressed lipopolysaccharide-induced prostaglandin E_2 production and its synergistic increase by endothelin-1 (data not shown). In contrast, NS398 had no effects on lipopolysaccharide-induced cyclooxygenase 2 protein expression; however it clearly suppressed the synergistic increase elicited by endothelin-1 (Fig. 6).

Moreover, NS398 significantly inhibited endothelin ET_B receptor up-regulation by lipopolysaccharide (Fig. 7).

4. Discussion

Previous reports showed that lipopolysaccharide induced cyclooxygenase 2 expression, resulting in prostaglandin E_2 production by macrophages in the delayed phase (Naraba et al., 1998). In addition, we showed that high concentrations of endothelin-1 (10^{-11}–10^{-8} M) also induced cyclooxygenase 2 expression and then prostaglandin E_2 production by macrophages in a time- and concentration-dependent manner (Shimada et al., 1998). The present study demonstrated that a low concentration of endothelin-1 (below 10^{-11} M) synergistically promoted cyclooxygenase 2 expression and prostaglandin E_2 production by lipopolysaccharide-stimulated macrophages in 12 h. Such a synergistic effect of endothelin-1 on lipopolysaccharide-induced cyclooxygenase 2 expression was not concentration dependent. The reason for this is that the expression of cyclooxygenase 2 protein was already maximal after stimulation with lipopolysaccharide and endothelin-1. In contrast, the synergistic modulation by endothelin-1 of lipopolysaccharide-induced prostaglandin E_2 production appeared to be dependent on the concentration. Therefore, endothelin-1 may influence the enzymatic activity of cyclooxygenase 2 in a different fashion from its protein expression.

The mechanism of synergistic modulation by endothelin-1 involves an endothelin ET_B receptor-mediated process as indicated by the effect of BQ788 pretreatment. In addition, lipopolysaccharide up-regulated endothelin ET_B receptors on macrophages. The latency of this regulation almost coincided with the progressive curve of synergistic modulation by endothelin-1. From these results, it is concluded that endothelin-1 promotes lipopolysaccharide-induced cyclooxygenase 2 protein expression and prostaglandin E_2 production in 12 h through endothelin ET_B receptors up-regulated by lipopolysaccharide.

Oda et al. (1997a,b) showed that endothelins enhanced the expression of lipopolysaccharide-induced inducible form of nitric oxide synthase through endothelin ET_B

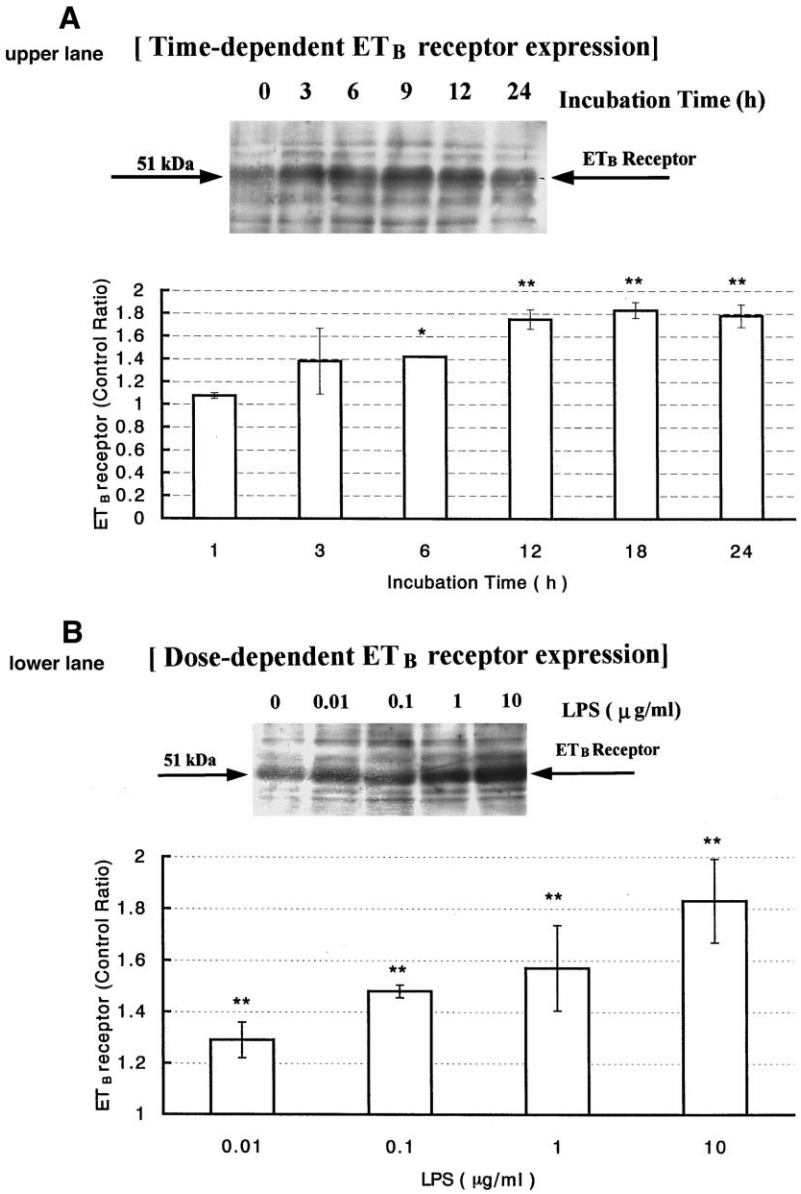

Fig. 5. Changes of endothelin ET_B receptor protein expression by lipopolysaccharide. (A) shows time-dependent endothelin ET_B receptor protein expression stimulated by lipopolysaccharide (10 μg/ml) [upper lane] and the densitometric results [lower lane], as determined by Western blot and densitometric analysis as described in Section 2. (B) shows concentration-dependent endothelin ET_B receptor protein expression measured after 12 h of lipopolysaccharide stimulation [upper lane] and the densitometric result [lower lane]. Values are expressed as means ± S.D. of the five independent experiments. ** or * indicates a statistically significant difference between cells stimulated by lipopolysaccharide and control cells at $P < 0.01$ or $P < 0.05$. Abbreviation: ET; endothelin, LPS; lipopolysaccharide.

receptors in rat glial cells. They also found that endothelin-1 inhibited the expression of inducible nitric oxide synthase in rat glial cells prepared from neonatal rat brain (1997). King et al. (1997) showed that endothelin-1 stimulated human monocytes to produce nitric oxide predominantly via endothelin ET_B receptors. These reports and the present study indicate that endothelin and endothelin ET_B receptor related mechanisms may contribute to the production of important biological factors such as nitric oxide and prostanoids under lipopolysaccharide stimulation.

Pretreatment with the cyclooxygenase 2-selective inhibitor, NS398, inhibited prostaglandin E_2 production by lipopolysaccharide alone, while this inhibitor had no effect on lipopolysaccharide-induced cyclooxygenase 2 expression. In contrast, NS398 inhibited the synergistic increase by endothelin-1 (10^{-14} M) of lipopolysaccharide-induced cyclooxygenase 2 expression. From these results synergistic modulation by endothelin-1 may be dependent on lipopolysaccharide-induced cyclooxygenase 2 activation and its reaction products including prostaglandin E_2. In addi-

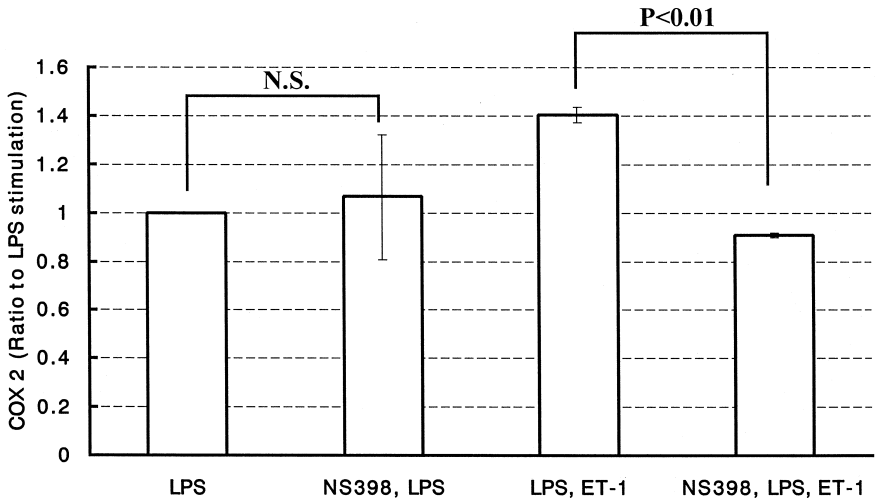

Fig. 6. Effect of NS398 (10^{-5} M) on the stimulation by endothelin-1 (10^{-14} M) of lipopolysaccharide-induced cyclooxygenase 2 protein expression. The density of the cyclooxygenase 2 protein band is expressed as a ratio to that measured after 12 h of lipopolysaccharide (10 μg/ml) stimulation. Values are expressed as means ± S.D. of the five independent experiments. Abbreviation: COX2; cyclooxygenase 2, LPS; lipopolysaccharide, ET; endothelin, N.S.; no statistically significant difference.

tion, NS398 suppressed lipopolysaccharide-induced endothelin ET_B receptor up-regulation. Mallat et al. (1996) showed that the endothelin ET_B receptor is selectively up-regulated by agents that elevate cyclic adenosine monophosphate (c-AMP) levels, such as prostaglandin E_2 in human hepatic stellate cells. Therefore, it is considered that prostanoid, especially prostaglandin E_2, may play a pivotal role in the mechanism of endothelin ET_B receptor up-regulation by lipopolysaccharide.

It has already been demonstrated that endothelin-1 stimulates or inhibits c-AMP accumulation (Takigawa et al., 1995) as well as receptor-operated and voltage-gated calcium channels (Takuwa et al., 1989; Wright and Malik, 1995). The present study did not show whether the endothelin ET_B receptor, particularly lipopolysaccharide-induced newborn endothelin ET_B receptor, was involved in c-AMP accumulation in macrophages, or whether it influenced other signal cascades. And we have no data about whether endothelin-1 regulates the endothelin ET_B receptor by itself. Thus, the present report cannot demonstrate the detailed mechanism of the synergistic modulation by endothelin-1 of the lipopolysaccharide-induced inflammatory response, and further work up will be required. However, this is the first report indicating the possibility that endothelin-1 potentiates endotoxin-mediated inflammatory reactions through endothelin ET_B receptors, and may help explain the pathogenesis of various inflammatory diseases.

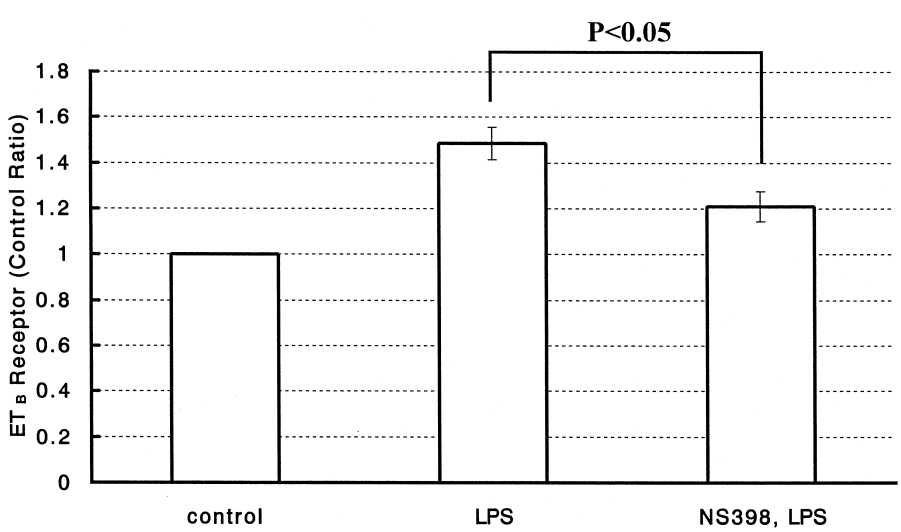

Fig. 7. Effect of NS398 (10^{-5} M) on lipopolysaccharide-induced up-regulation of endothelin ET_B receptor protein expression. The density of the endothelin ET_B receptor protein band is expressed as a ratio to that measured when there was no stimulation (control). Values are expressed as means ± S.D. of five independent experiments. Abbreviation: ET; endothelin, LPS; lipopolysaccharide.

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