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Endothelin enhances lipopolysaccharide-induced expression of inducible nitric oxide synthase in rat glial cells

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Abstract

Lipopolysaccharide is known to stimulate production of nitrite via expression of inducible nitric oxide (NO) synthase in not only macrophages but also glial cells. We found that in glial cell cultures lipopolysaccharide-stimulated inducible NO synthase expression and nitrite accumulation were synergistically enhanced by pretreatment with endothelin, whereas endothelin itself did not induce these responses. Pretreatment with endothelin-1, endothelin-3, and the selective endothelin type B (ET_B) receptor agonist IRL 1620 caused the same effect with similar potencies, suggesting that the synergism was mediated via the endothelin ET_B receptor. A protein kinase C inhibitor, calphostin C, suppressed endothelin-3-enhanced inducible NO synthase expression. Pretreatment with either endothelin-3 or phorbol ester enhanced lipopolysaccharide-induced production of tumor necrosis factor-α (TNF-α). Simultaneous addition of TNF-α increased lipopolysaccharide-stimulated inducible NO synthase expression. These results suggest that the increase in inducible NO synthase expression by endothelin was due to the elevated TNF-α production via protein kinase C. Our findings present the possibility that endothelin is implicated in neurotoxicity via enhancement of inducible NO synthase expression. © 1997 Elsevier Science B.V.

Keywords: Endothelin; Glial cell; Nitric oxide synthase, inducible; TNF-α (tumor necrosis factor-α); Protein kinase C; Endothelin ET_B receptor

1. Introduction

Nitric oxide (NO) is a molecular mediator that has been implicated in many physiological and pathological processes in various tissues including the brain (for review, see Bredt and Snyder, 1994). NO is synthesized by NO synthase, an enzyme for which several isoforms have been identified (Wong and Billiar, 1995). Two distinct types of Ca^{2+}-dependent NO synthase are constitutively expressed, one in some neurons (Bredt and Snyder, 1990) and the other in endothelial cells (Lamas et al., 1992). A Ca^{2+}-independent inducible isoform (inducible NO synthase) can be induced in a wide variety of cell types including glial cells by exposure to endotoxins or cytokines (Wong and Billiar, 1995).

There is increasing evidence that inducible NO synthase is induced after focal and global ischemia primarily in reactive astrocytes (Endoh et al., 1994; Iadecola et al., 1995), and that NO release from glial cells appears to be involved in neuronal cell death (Boje and Arora, 1992; Chao et al., 1992; Hewett et al., 1994). In glial cells, increased expression of endothelin has also been observed following focal and global ischemia (for review, see Patel, 1996). However, the involvement of endothelin in inducible NO synthase expression in the nervous system has never been studied in detail.

The endothelins comprise a family of three isopeptides (endothelin-1, -2, and -3), each with 21 amino acids (Yanagisawa and Masaki, 1989). Endothelin has been implicated in a wide variety of physiological functions in various sites including the central nervous system (Masaki et al., 1992). At least two endothelin receptors, which belong to the superfamily of receptors coupled to the heterotrimeric G protein, have been classified via their different affinities for the three endothelin isopeptides. The order of affinity for the endothelin ET_A receptor is en-

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dothelin-1 ≥ endothelin-2 ≫ endothelin-3, whereas the endothelin ET_B receptor subtype has equal affinity for all three endothelins (Sakurai et al., 1992). In situ hybridization analysis in the rat brain demonstrated that endothelin ET_B receptor mRNA was expressed in glial cells of many brain regions (Hori et al., 1992).

In the present study, we examined the effect of endothelins on lipopolysaccharide-stimulated inducible NO synthase expression in glial cell cultures prepared from neonatal rat brain. We found that pretreatment with endothelin remarkedly potentiated lipopolysaccharide-stimulated inducible NO synthase expression and nitrite accumulation, whereas endothelins by themselves exerted no effect. We suggest the possible involvement of the endothelin ET_B receptor and the protein kinase C system in the regulation of inducible NO synthase expression by endothelins via production of tumor necrosis factor-α (TNF-α).

2. Materials and methods

2.1. Materials

Cyclo (D-Trp-D-Asp–Pro-D-Val–Leu) (BQ-123) was obtained from American Peptide (California), and *N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine (BQ-788), calphostin C, and phorbol 12-myristate 13-acetate (PMA) were from Sigma (Milwaukee). Endothelin-1, -3, and Suc-[Glu^9,$Ala^{11,15}$]-endothelin-1 (8-21) (IRL 1620) were purchased from Peptide Institute (Osaka). Lipopolysaccharide was purchased from Wako Pure Chemicals (Osaka), and tumor necrosis factor-α (TNF-α) was from PEPRO TECHNIC. (New Jersey). The rat TNF-α enzyme-linked immuno solvent assay (ELISA) kits were from BioSource International (California). All other chemicals were of analytical grade.

2.2. Methods

2.2.1. Preparation of glial cell cultures

Glial cell cultures were prepared from the whole brain of neonatal (< 24 h) Wistar rats (Japan SLC, Japan) as described previously (Simmons and Murphy, 1992) with some modifications (Kitamura et al., 1996). The cells were allowed to grow to confluency (12 d) in Dulbecco's modified Eagle medium (DMEM) with 10% (vol/vol) fetal calf serum (heat inactivated), 50 μg/ml penicillin, and 100 μg/ml streptomycin. All cultured cells were kept at 37°C in 5% CO_2/95% air. Subsequently, the cells were shaken (120 rpm for 1 h at room temperature) and the adherent cells were cultured again for 5–6 d and then used in the following experiments. At this point, astrocyte cultures were routinely > 90% positive for glial fibrillary acidic protein, and approximately 7% of the cells were identified as microglia based on ED1 (anti-macrophage/microglia monoclonal antibody) staining. The experiments were carried out in accordance with the NIH guide and were approved by the animal care and use committee of Hokkaido University.

2.2.2. Culture treatment

Glial cells were preincubated with 100 nM endothelins, 100 nM IRL 1620, or 100 nM PMA for 24 h, and then 1–100 ng/ml lipopolysaccharide was added for an additional 8 or 24 h. To examine the effect of TNF-α, the cells were incubated with 200 ng/ml TNF-α and/or 1–10 ng/ml lipopolysaccharide for 8 h. In some experiments, the cells were pretreated with 3 μM BQ-123 or 3 μM BQ-788 for 30 min before the treatment with 100 nM endothelin-3. In another case, the cells were preincubated with 10 or 30 nM calphostin C for 20 min before the treatment with 100 nM endothelin-3.

2.2.3. Determination of nitrite accumulation

Formation of nitric oxide in the culture dishes was determined by the accumulation of nitrite (a stable oxidation breakdown product of nitric oxide) in the incubation medium. After the incubation times indicated, aliquots of the incubation media in 12-well plates were removed for the determination of the nitrite content using the Griess reaction as described previously (Green et al., 1982). Nitrite concentration in the culture medium was determined from a standard curve constructed using known concentrations of $NaNO_2$.

2.2.4. Western blot analysis

The cells were washed once and detached from the dishes with phosphate-buffered saline containing 1 mM EGTA at various incubation times. Detached cells were precipitated by centrifugation ($500 \times g$, 2 min) and suspended in 100 μl of ice-cold buffer A (100 mM potassium phosphate (pH 7.4), 5 mM $MgCl_2$, 0.5 mM benzamidine, 0.5 mM phenylmethanesulphonyl fluoride). Cells were homogenized, at 4°C, by sonication for 30 s at a power output of 20 W (Handy Sonic, UR-20P, Tomy Seiko, Japan) and centrifuged at $40{,}000 \times g$ for 20 min. The cytosol fractions were used for Western blot analysis. Protein samples (10–30 μg protein/lane) were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; 6.5% polyacrylamide) and transferred from the gels to a nitrocellulose membrane by electro–elution at a constant voltage of 100 V for 1 h at 4°C. After the nitrocellulose membrane was incubated with 10 mM Tris–HCl (pH 7.4), 100 mM NaCl, 0.1% Tween 20, and 1% bovine serum albumin to block nonspecific protein binding, the membrane was incubated with the first antibody (anti-mouse inducible NO synthase antibody, diluted to 1:2500, Transduction Laboratories) and second antibody (horseradish peroxidase-linked anti-mouse immunoglobulin antibody, diluted to 1:2000, Amersham). Subsequently, the bands were detected using the enhanced chemiluminescence detection system (ECL kit, Amersham).

2.2.5. TNF-α production

After the incubation times indicated, aliquots of the incubation media in 12-well plates were removed for the determination of the TNF-α content using ELISA kits. ELISA kits from commercial sources were used according to the manufactures' instructions.

2.2.6. Statistical significance

Independent data are expressed as the mean ± S.E. Simple comparisons between two groups were made by Student's *t* test. Multiple comparisons against a single control group were made by one-way analysis of variance (ANOVA) with post hoc test. $p < 0.05$ was considered to be significant.

3. Results

3.1. Effects of endothelins and IRL 1620 on lipopolysaccharide-stimulated nitrite accumulation

Treatment of glial cells with 100 ng/ml lipopolysaccharide for 24 h led to the accumulation of nitrites 3-fold greater than basal levels in the culture medium (Fig. 1A). Interestingly, 100 ng/ml lipopolysaccharide-stimulated nitrite accumulation was significantly enhanced by pretreatment with 100 nM endothelin-1 for 24 h ($p < 0.005$, $n = 3$). Endothelin-1 by itself did not affect the basal level of nitrite production at concentrations between 1 nM and 10 μM (data not shown).

As shown in Fig. 1B, we investigated the effects of endothelin-1, -3, and IRL 1620, a highly selective endothelin ET_B receptor agonist (Takai et al., 1992), on lipopolysaccharide-stimulated nitrite accumulation. Lipopolysaccharide (1–100 ng/ml) induced nitrite accumulation was dose-dependent, and was enhanced by pretreatment with 100 nM endothelin-1, 100 nM endothelin-3, or 100 nM IRL 1620 for 24 h. Endothelin-3 and IRL 1620 by themselves did not affect the basal level of nitrite production at concentrations between 1 nM and 1 μM (data not shown). The level of the synergistic effect of 100 nM endothelin-1 on lipopolysaccharide-induced nitrite accumulation was comparable to that of 100 nM endothelin-3, and that of IRL 1620.

3.2. Effects of endothelins and IRL 1620 on lipopolysaccharide-stimulated inducible NO synthase protein expression

To determine whether or not the synergism between lipopolysaccharide and endothelins might be mediated through enhanced induction of inducible NO synthase protein, expression of the enzyme was determined directly by Western blot analysis using a monoclonal anti-inducible NO synthase antibody. Glial cells stimulated with endothelin-1 alone, IRL 1620 alone, or vehicle (Fig. 2A, lane 3, 5, and 1, respectively) did not express detectable inducible NO synthase proteins. Following incubation with 100 ng/ml lipopolysaccharide for 24 h, a band at the expected inducible NO synthase molecular mass of 130 kDa was revealed (lane 2). Pretreatment with 100 nM endothelin-1 or 100 nM IRL 1620 for 24 h synergistically enhanced the

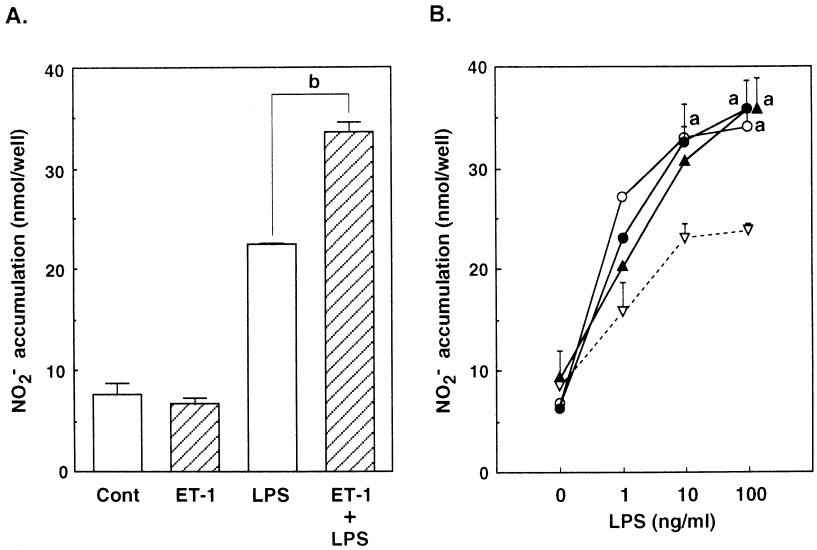

Fig. 1. Synergistic effect of endothelin-1, -3, and IRL 1620 on lipopolysaccharide-induced nitrite accumulation. Glial cells were pretreated with or without 100 nM endothelin-1 for 24 h (A), or pretreated with 100 nM endothelin-1 (○), 100 nM endothelin-3 (●), 100 nM IRL 1620 (▲), or vehicle (▽) for 24 h (B). Then 100 ng/ml lipopolysaccharide (A) or the indicated concentrations of lipopolysaccharide (B) was added for an additional 24 h. Nitrite levels in the culture medium were determined using Griess reagent. ET-1, endothelin-1; LPS, lipopolysaccharide. Data are the means ± S.E. of 3 independent experiments or the means of 2 independent experiments (the results in each experiment agreed within ±10%). [a] $p < 0.05$, significantly different from the value of lipopolysaccharide alone using Student's *t*-test. [b] $p < 0.005$, significantly different from the value of lipopolysaccharide alone using one-way ANOVA with post hoc test.

effect of 100 ng/ml lipopolysaccharide on inducible NO synthase expression 2 to 2.3-fold over the level of lipopolysaccharide alone (Fig. 2A, lanes 4 and 6, respectively and Fig. 2B). And the value of the synergistic effect of 100 nM endothelin-3 on inducible NO synthase induction was comparable to that of endothelin-3 or IRL 1620 (100 nM).

3.3. Effect of endothelin receptor antagonist on endothelin-3-enhanced NO synthase protein expression

Next we examined the effects of subtype-selective antagonists to further investigate the role of the endothelin ET_A and ET_B receptors in mediating inducible NO synthase induction. The synergistic effect of 100 nM endothelin-3 (Fig. 3A, lane 3) was inhibited by pretreatment with 3 μM BQ-788, a selective endothelin ET_B receptor antagonist (Ishikawa et al., 1994), for 30 min (lane 5). In contrast, 3 μM BQ-123, a selective endothelin ET_A receptor antagonist (Ihara et al., 1992) did not have any effect on endothelin-3-enhanced inducible NO synthase expression (lane 7). Neither antagonist alone did stimulate inducible NO synthase expression (data not shown), nor did affect inducible NO synthase expression by 100 ng/ml lipopolysaccharide (lanes 4 and 6). As quantified by laser densitometry, 3 μM BQ-788, but not BQ-123, completely abolished the synergistic effect of 100 nM endothelin-3 (Fig. 3B).

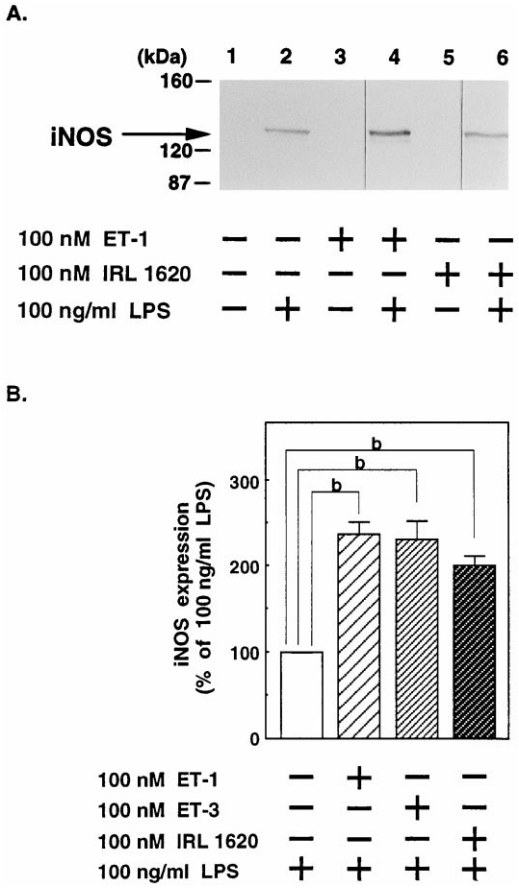

Fig. 2. Enhancement of lipopolysaccharide-stimulated inducible NO synthase expression by endothelin-1, -3, and IRL 1620. Glial cells were pretreated with 100 nM endothelin-1, 100 nM endothelin-3, 100 nM IRL 1620, or vehicle for 24 h. Then 100 ng/ml lipopolysaccharide was added for an additional 24 h. Cytosol fractions of the cells were subjected to SDS–PAGE followed by immunoblot analysis using anti-inducible NO synthase antibody. The data shown in (A) is a representative of 3 independent experiments. Quantitative analysis of the band intensity is represented in (B). iNOS, inducible NO synthase; ET-1, endothelin-1; ET-3, endothelin-3; LPS, lipopolysaccharide. The data are normalized as a percentage of lipopolysaccharide-stimulated inducible NO synthase expression and are the means ± S.E. of 3 independent experiments. $^{b}p < 0.005$, significantly different from the value of LPS alone using one-way ANOVA with post hoc test.

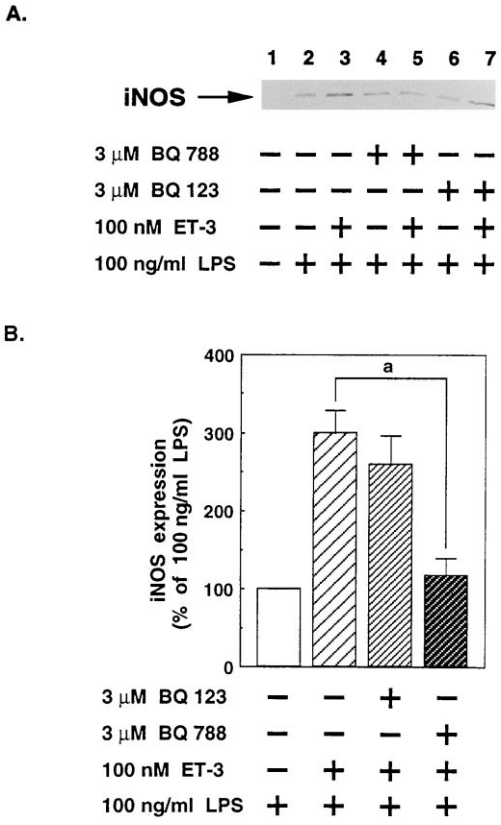

Fig. 3. Effects of BQ-123 and BQ-788 on enhancement of lipopolysaccharide-stimulated inducible NO synthase expression by endothelin-3. Glial cells were preincubated with 3 μM BQ-123 and 3 μM BQ-788 for 30 min. The cells were then treated with 100 nM endothelin-3 or vehicle for 24 h in the presence of the same concentrations of endothelin receptor antagonists. Afterwards, 100 ng/ml lipopolysaccharide was added for an additional 24 h. Cytosol fractions of the cells were subjected to SDS–PAGE followed by immunoblot analysis using anti-inducible NO synthase antibody. The data shown in (A) are representative of 3 independent experiments. Quantitative analysis of the band intensity is shown in (B). iNOS, inducible NO synthase; ET-3, endothelin-3; LPS, lipopolysaccharide. The data are normalized as a percentage of lipopolysaccharide-stimulated inducible NO synthase expression and are the means ± S.E. of 3 independent experiments. $^{a}p < 0.05$, significantly different from the value of lipopolysaccharide alone using one-way ANOVA with post hoc test.

3.4. Involvement of protein kinase C on endothelin-enhanced inducible NO synthase expression

Endothelin receptor activation is coupled to phospholipase C-mediated phosphoinositide hydrolysis and activation of protein kinase C in glial cells (MacCumber et al., 1990; Stanimirovic et al., 1995). To examine the involvement of protein kinase C activity in endothelin-enhanced inducible NO synthase expression, we examined the effect of PMA, an activator of protein kinase C, on lipopolysaccharide-stimulated inducible NO synthase expression. 100 nM PMA by itself did not stimulate inducible NO synthase expression (data not shown). However, pretreatment with 100 nM PMA for 24 h resulted in a synergistic effect on lipopolysaccharide (100 ng/ml)-stimulated inducible NO synthase expression (Fig. 4A, lane 4). The enhancement of inducible NO synthase expression induced by 100 nM PMA was greater than that by 100 nM endothelin-3 (Fig. 4B).

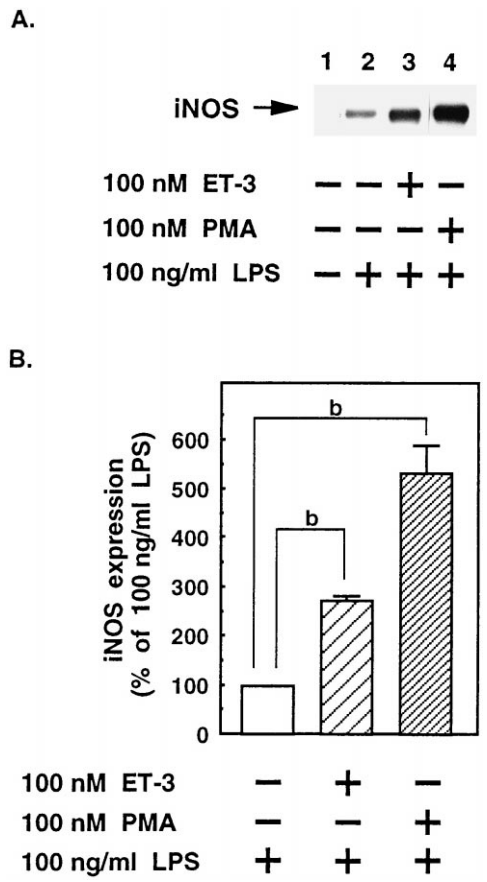

Fig. 4. Effect of PMA on lipopolysaccharide-stimulated inducible NO synthase expression. Glia cells were pretreated with 100 nM endothelin-3, 100 nM PMA, or vehicle for 24 h. Next, 100 ng/ml lipopolysaccharide was added for an additional 24 h. Cytosol fractions of the cells were subjected to SDS–PAGE followed by immunoblot analysis using anti-inducible NO synthase antibody. The data shown in (A) are a representative of 3 independent experiments. Quantitative analysis of the band intensity is represented in (B). iNOS, inducible NO synthase; ET-3, endothelin-3; LPS, lipopolysaccharide. The data are normalized as a percentage of lipopolysaccharide-stimulated inducible NO synthase induction and are the means ± S.E. of 3 independent experiments. [b] $p < 0.005$, significantly different from the value of lipopolysaccharide alone using one-way ANOVA with post hoc test.

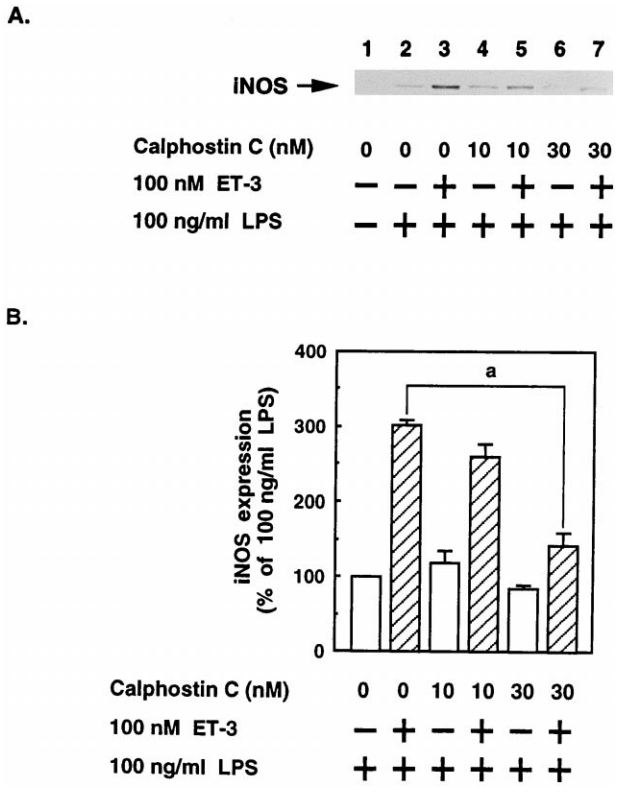

Fig. 5. Effect of calphostin C on endothelin-3-enhanced inducible NO synthase expression. Glial cells were preincubated with 10 or 30 nM calphostin C for 20 min. The cells were then treated with 100 nM endothelin-3 or vehicle in the presence of the same concentrations of calphostin C for 24 h. Then 100 ng/ml lipopolysaccharide was added for an additional 24 h. Cytosol fractions of the cells were subjected to SDS–PAGE followed by immunoblot analysis using anti-inducible NO synthase antibody. The data shown in (A) are representative of 3 independent experiments. Quantitative analysis of the band intensity is represented in (B). iNOS, inducible NO synthase; ET-3, endothelin-3; LPS, lipopolysaccharide. The data are normalized as a percentage of lipopolysaccharide-stimulated inducible NO synthase induction and are the means ± S.E. of 3 independent experiments. [a] $p < 0.05$, significantly different from the value without calphostin C using one-way ANOVA with post hoc test.

Next, we examined the effect of calphostin C, a potent and specific protein kinase C inhibitor (Kobayashi et al., 1989), on endothelin-3-enhanced inducible NO synthase expression. Pretreatment with 30 nM calphostin C for 20 min inhibited the synergistic effect of 100 nM endothelin-3 on lipopolysaccharide-stimulated inducible NO synthase expression (Fig. 5A, lane 7), whereas calphostin C did not stimulate inducible NO synthase expression by itself (data not shown) and did not affect inducible NO synthase expression by 100 ng/ml lipopolysaccharide (lane 6). As summarized in Fig. 5B(b), 30 nM calphostin C significantly inhibited the synergistic effect of 100 nM endothelin-3.

3.5. Effects of endothelin-3 and PMA on lipopolysaccharide-induced TNF-α production

The expression of inducible NO synthase is reported to be induced by lipopolysaccharide and/or various cytokines such as TNF-α (Wang and Marsden, 1995). To investigate whether TNF-α is involved in the synergistic expression of inducible NO synthase by endothelin, we first investigated the effects of endothelin-3 and PMA on lipopolysaccharide-stimulated TNF-α production in glial cells (Table 1). The TNF-α level was examined at two different time points of 8 and 24 h, because kinetics of TNF-α production in response to lipopolysaccharide was known to be variable depending on cell types (Chung and Benevolences, 1990; Lee et al., 1993). Addition of 100 ng/ml lipopolysaccharide for 8 h produced about 220 ng/ml TNF-α proteins, and the pretreatment with 100 nM endothelin-3 for 24 h enhanced the lipopolysaccharide-induced TNF-α expression about 1.4-fold, although this was not significant due to the large standard error. The pretreatment with endothelin-3 significantly enhanced the TNF-α production by lipopolysaccharide stimulation for 24 h 1.7-fold above the level of lipopolysaccharide alone, although the level of TNF-α protein declined to about half of that at 8 h as described previously (Chung and Benevolences, 1990; Chung et al., 1992). Pretreatment with 100 nM PMA for 24 h also enhanced the TNF-α production by lipopolysaccharide. Incubation with 100 nM PMA did not induce TNF-α production by itself.

3.6. Effects of lipopolysaccharide and/or TNF-α on inducible NO synthase induction

We next examined whether or not simultaneous addition of TNF-α enhances lipopolysaccharide-stimulated inducible NO synthase expression. Incubation of glial cells with 200 ng/ml TNF-α alone for 8 h did not stimulate inducible NO synthase expression (Fig. 6A, lane 4). Simultaneous addition of 200 ng/ml TNF-α caused an increase in lipopolysaccharide (1 and 10 ng/ml) stimulated inducible NO synthase expression (Fig. 6A, lanes 5 and 6, and Fig. 6B). Thus, it seems that the increase of TNF-α production induced by endothelin enhanced lipopolysaccharide-stimulated inducible NO synthase expression.

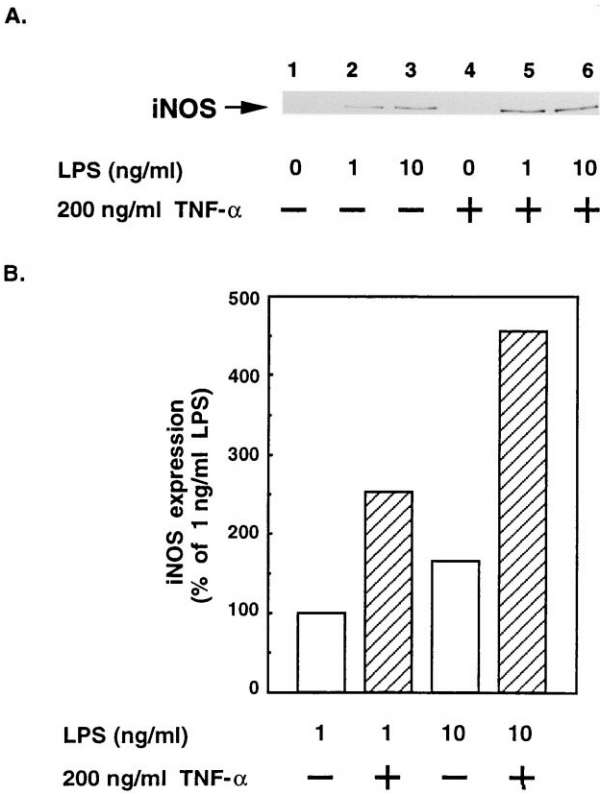

Fig. 6. Effect of TNF-α on lipopolysaccharide-stimulated inducible NO synthase expression. Glial cells were incubated with the indicated concentration of lipopolysaccharide and/or 200 ng/ml TNF-α for 8 h. Cytosol fractions of the cells were subjected to SDS–PAGE followed by immunoblot analysis using anti-inducible NO synthase antibody. The data shown in (A) are a representative of 2 independent experiments. Quantitative analysis of the band intensity is represented in (B). iNOS, inducible NO synthase; LPS, lipopolysaccharide. The data are normalized as a percentage of 1 ng/ml lipopolysaccharide-stimulated inducible NO synthase expression and the mean of 2 independent experiments (the results in each experiment agreed within ±10%).

Table 1
Effects of endothelin-3 and PMA on lipopolysaccharide-induced TNF-α protein expression

	TNF-α (ng/ml) pretreatment for 24 h with		
	none	100 nM ET-3	100 nM PMA
Experiment 1 (8 h)			
None	1.5±0.3	1.1±0.9	N.D.
100 ng/ml LPS	220.8±13.8	323.5±34.9	N.D.
Experiment 2 (24 h)			
None	1.3±0.1	1.4±0.2	1.6±4.4
100 ng/ml LPS	91.9±5.8	157.0±2.7[a]	149.9±5.5[a]

Glial cells were pretreated with 100 nM endothelin-3, 100 nM PMA, or vehicle for 24 h, then 100 ng/ml lipopolysaccharide was added for an additional 8 h (experiment 1), or an additional 24 h (experiment 2). The amount of TNF-α protein in the supernatants was assayed by ELISA. ET-3, endothelin-3; LPS, lipopolysaccharide. Data are the means±S.E. of 3 independent experiments. N.D., not determined.
[a] $p < 0.05$, significantly different from the value of 100 ng/ml lipopolysaccharide using one-way ANOVA with post hoc test.

4. Discussion

In glial cells, 100 ng/ml lipopolysaccharide-stimulated nitrite accumulation and inducible NO synthase expression were enhanced by pretreatment with 100 nM endothelin-1 for 24 h (Fig. 1A, Fig. 2A and B). The synergistic potencies of 100 nM endothelin-1 were similar in cases of both nitrite formation and inducible NO synthase expres-

sion induced by 100 ng/ml lipopolysaccharide, indicating that the stimulatory effect of endothelin-1 on lipopolysaccharide-induced nitrite accumulation is due to the increase in inducible NO synthase expression. In the rat brain, increased levels of endothelin have been demonstrated following focal and global ischemia (Patel, 1996). Additionally there is increasing evidence that the reactive astrocyte expresses inducible NO synthase in response to ischemic insult in an in vivo model of focal and global ischemia (Endoh et al., 1994; Iadecola et al., 1995). These results suggest that endothelin regulates inducible NO synthase expression in the brain in vivo.

The presence of the endothelin ET_B receptor has previously been demonstrated in glial cells throughout the brain (Hori et al., 1992). In addition, microglia with the endothelin ET_B receptor aggregates in rat hippocampus CA1 subfields following transient ischemia (Yamashita et al., 1994). In this study, we demonstrated that endothelin-enhanced inducible NO synthase expression in glial cells is apparently mediated by the endothelin ET_B receptor, because endothelin-1, -3, and the selective endothelin ET_B receptor agonist IRL 1620 increased the effects of lipopolysaccharide on nitrite accumulation and inducible NO synthase expression with similar potencies (Fig. 1B, Fig. 2A and B). Furthermore, the selective endothelin ET_B receptor antagonist BQ-788 abolished the synergistic effect of endothelin-3 on inducible NO synthase expression (Fig. 3).

Activation of protein kinase C signaling pathways plays a modulatory role on inducible NO synthase expression in macrophages and astrocytes (Severn et al., 1992; Simmons and Murphy, 1994). Endothelin is reported to cause increases in phospholipase C activity, concentration of free cytosolic Ca^{2+}, and protein kinase C activity in astrocytes (MacCumber et al., 1990; Stanimirovic et al., 1995). To determine whether protein kinase C is involved in the endothelin-enhanced NO synthase expression, we examined the effect of a specific protein kinase C inhibitor, calphostin C. Calphostin C inhibited the synergistic effect of endothelin-3 on lipopolysaccharide-stimulated inducible NO synthase expression, although 30 nM calphostin C by itself did not stimulate inducible NO synthase expression and did not affect inducible NO synthase expression induced by lipopolysaccharide (Fig. 5). Additionally, lipopolysaccharide (100 ng/ml)-stimulated inducible NO synthase expression was enhanced by pretreatment with 100 nM PMA, the protein kinase C activator, for 24 h (Fig. 4). PMA was more effective on the enhancement of the expression than endothelin-3, probably due to more potent activity of PMA for stimulation of protein kinase C than that of endothelin-3. While protein kinase C does not appear to be necessary for inducible NO synthase expression by lipopolysaccharide alone (Fig. 5; Simmons and Murphy, 1994), our data clearly demonstrated that protein kinase C is involved in the regulation of endothelin-enhanced inducible NO synthase expression.

It is reported that protein kinase C also modulates TNF-α gene expression both in primary rat astrocytes and in the human astroglioma cell lines (Bethea et al., 1992; Chung et al., 1992). In glial cells, we revealed that lipopolysaccharide-stimulated TNF-α production was enhanced by pretreatment with endothelin-3 or PMA (Table 1), suggesting that protein kinase C participates in the enhanced TNF-α-production. Also endothelin-enhanced inducible NO synthase expression seems to be mediated by the increase in TNF-α production. This is because (1) lipopolysaccharide (1 and 10 ng/ml) stimulated inducible NO synthase expression was enhanced by simultaneous addition of 200 ng/ml TNF-α, and (2) the level of TNF-α production (157 ng/ml) enhanced by pretreatment with endothelin-3 was of the same order as that used in Fig. 6. The predominant mechanism underlying the induction of inducible NO synthase is transcriptional regulation. The inducible NO synthase gene is known to be activated by nuclear factor-κB, nuclear factor-interleukin-6, interferon-γ-response element, and a palindromic TNF response element-like site (Wang and Marsden, 1995). It is possible that maximal promoter activation occurs with the synergistic combinations of lipopolysaccharide and TNF-α.

Endothelin is one of the etiological factors linked to the development of ischemic brain injury (Patel, 1996). The expression of TNF-α is elevated after ischemia (Feuerstein et al., 1994). Furthermore, NO produced by inducible NO synthase could play an important role in ischemic damage (Zhang et al., 1995). We showed the synergistic effect of endothelin on lipopolysaccharide-stimulated inducible NO synthase expression in glial cells. It is possible that endothelin modulates inducible NO synthase expression in the brain, and that endothelin plays important pathophysiological roles in the development of delayed neuronal death following ischemia.

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