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Short communication

Ethanol potentiates lipopolysaccharide- or interleukin-1β-induced nitric oxide generation in RBE4 cells

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Abstract

Our present study investigated the effects of ethanol treatment on inducible nitric oxide (NO) synthase pathway from lipopolysaccharide- or interleukin-1β-treated cultured rat blood-brain barrier cell line (rat brain endothelial 4 cells: RBE4 cells). Cells were lipopolysaccharide- or interleukin-1β-treated with or without ethanol (50, 100 or 200 mM) for 16 or 24 h. Inducible NO synthase activity and mRNA expression were measured using Griess reaction and reverse transcription-polymerase chain reaction (RT-PCR) respectively. In the absence of lipopolysaccharide or interleukin-1β, ethanol treatments failed to stimulate inducible NO synthase gene expression. Lipopolysaccharide or interleukin-1β increased nitrite production and inducible NO synthase mRNA levels, and ethanol potentiated this effect. We concluded that ethanol could aggravate the consequences of NO generation by RBE4 cells after inducible NO synthase induction following inflammation or sepsis. This ethanol action on NO generation could contribute to circulatory failure associated with shock due to sepsis or hemorrhage, and alter blood-brain barrier permeability.

Keywords: Nitric oxide (NO); Nitric oxide (NO) synthase, inducible; Ethanol; Blood-brain barrier; RBE4 cell; Inflammation; Endotoxin

1. Introduction

Nitric oxide (NO) is an important messenger and effector molecule involved in several biological functions including blood flow regulation and native immunity. NO is synthesized by oxidation of the terminal guanidino nitrogen atom(s) of L-arginine by a family of enzymes designated nitric oxide synthases (NO synthases) (see Nathan, 1992). Two types of NO synthases have been identified in numerous cell types: constitutive and inducible. Two types of NO synthases have been identified in endothelial cells of macrovessels. The present study was conducted with a recently described immortalized rat brain microvessel endothelial cell line, rat brain endothelial 4 cells (RBE4 cells). These cells were immortalized by transfection with a plasmid containing the E1 A adenovirus gene; they exhibit in culture a nontransformed phenotype and express typical endothelial markers (Durieu-Trautmann et al., 1991, 1993a; Roux et al., 1993). Only the inducible isoform of NO synthase has been detected in RBE4 cells (Durieu-Trautmann et al., 1993b). A large excess of NO is generated during inflammation or sepsis and can alter blood-brain barrier permeability (Shukla et al., 1995). On the other hand, several studies indicate that ethanol can influence NO synthase activity in both peripheral and central tissues, but the mechanisms have not been clearly elucidated. Some reports have shown that ethanol modulates NO generation. Ethanol has been reported to suppress lipopolysaccharide-induced NO generation in C_6 glioma cells (Syapin, 1995), in the lung in vivo and in vitro (Greenberg et al., 1994) and in Kupffer and hepatic endothelial cells (Spolarics et al., 1993). However, ethanol has also been shown to up-regulate inducible NO synthase expression in interleukin-1β-treated cultured vascular smooth muscle cells (Durante et al., 1995). Ethanol may have differential

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effects according to cell type on the inducible NO synthase pathway after exposure to cytokines or microbial products.

The aim of this study was to characterize the effect of ethanol on the inducible NO synthase pathway after induction by lipopolysaccharide or interleukin-1β in RBE4 cells.

2. Materials and methods

2.1. Materials

Alpha medium/Ham's F10 without phenol red, geneticin sulfate, Moloney-murine leukemia virus reverse transcriptase were purchased from Gibco BRL; bacterial lipopolysaccharide (*E. coli*, serotype no. 0127:B8) and N^G-methyl-L-arginine monoacetate were obtained from Sigma; fetal calf serum, trypsin/EDTA and recombinant human basic fibroblast growth factor were obtained from Boehringer; collagen type I was obtained from Institut Jacques Boy (France); Taq DNA polymerase was obtained from Eurobio; recombinant human interleukin-1β (specific activity $3 \cdot 10^8$ U/mg) was obtained from Genzyme; *N*-(1-naphthyl)ethylenediamine dihydrochloride and sulfanilamide were obtained from ICN.

2.2. Cell culture

RBE4 cells were cultured as previously described (Durieu-Trautmann et al., 1993b) in Alpha Medium/Ham's F10 (1 : 1) without phenol red supplemented with 10% heat inactivated fetal calf serum, 2 mM L-glutamine, 300 μg/ml geneticin sulfate, 2 μg/ml ciprofloxacin, 1 ng/ml basic fibroblast growth factor, under a 5% CO_2 atmosphere at 37°C. RBE4 cells were passaged twice a week by harvesting with trypsin/EDTA and spread at a density of 10^4 cells/cm^2 onto collagen-coated dishes; in 75 cm^2 flasks (for RNA isolation), and into 12-well dishes (12 mm in diameter, for nitrite assay), and used between passages 30 and 50.

2.3. Cell stimulation

When cells reached 80% confluence, they were then exposed to interleukin-1β (5 ng/ml) or lipopolysaccharide (10 μg/ml) with or without different concentrations of ethanol. For ethanol treatment, ethanol was added at final concentrations of 50, 100 and 200 mM using absolute ethanol. Cells were treated for 16 h (RNA preparation) or 24 h (nitrite determination).

2.4. Nitrite assay

NO generation was determined by measuring the release of nitrite in the supernatant as previously described (Southan et al., 1995). Aliquots (500 μl) of culture medium were mixed with an equal volume of Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% phosphoric acid), the mixture was incubated at room temperature for 10 min and the absorbance at 550 nm was then measured. Nitrite concentrations were determined relative to a standard curve by using culture medium containing various concentrations of sodium nitrite.

2.5. Reverse transcription and amplification of mRNA by the polymerase chain reaction assay

Total cellular RNA was extracted using the RNA Insta-Pure System (Eurogentec Belgium) according to the manufacturer's procedure, then briefly dried under vacuum, resuspended in ribonuclease-free water and quantified by measuring its absorbance at 260 nm. The first strand of cDNA was synthesized by reverse transcription. A 30 μl reverse transcription reaction mixture containing 2 μg total RNA (heated at 65°C for 10 min and then quenched on ice for 5 min), reverse transcription buffer (50 mM Tris/HCl pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 5 mM dithiothreitol, 1 mM deoxynucleosides triphosphate, 33 U RNasin inhibitor, 250 pmol $pd(N)_6$ and 400 U Moloney-murine leukemia virus reverse transcriptase was incubated at 37°C for 1 h, heated to 95°C for 5 min and then quick-chilled on ice. Primers for polymerase chain reaction (PCR) of inducible NO synthase were obtained from Genosys, and were selected from the cDNA encoding for inducible NO synthase from rat liver (Adachi et al., 1993; EMBL/Gen bank: D12520): 5′-GACTGAGACTCTGGC-CCCACG-3′ (nucleotides 8–28) and reverse 5′-CCTT-TGTTACGGCTTCCAGCC-3′ (nucleotides 675–695). Glyceraldehyde-3-phosphate dehydrogenase was used as standard and primers obtained from Clontech. PCR was performed in a Perkins-Elmer/Cetus thermal cycler on 4 μl of the cDNA reaction mixture at final concentrations of PCR buffer, 2.5 mM $MgCl_2$, 200 μM deoxynucleosides triphosphate, 40 pmol of each of the downstream (5′) and upstream (3′) primers and 2.5 U Taq DNA polymerase in a total volume of 50 μl. The amplification profile involved four linked files as follows: 5 min at 95°C for 1 cycle; 1 min at 95°C, 1 min at 65°C and 1 min at 72°C for 30 cycles for inducible NO synthase or 23 cycles for standard; and finally 8 min at 72°C for 1 cycle. The PCR was carried out at different cycle numbers to obtain results higher than the limit of detection, but below the PCR plateau. A 5 μl aliquot of each amplified PCR sample (inducible NO synthase and standard) was electrophoresed on a 2% agarose gel. The gel was stained with ethidium bromide and photographed for densitometry analysis. The expected size of the amplified fragments was evaluated using the 1 kb marker (700 base pairs for inducible NO synthase and 1000 base pairs for standard).

2.6. Densitometry analysis

Densitometry analysis was performed as follows: a black and white CCD camera (IVC 500 BC) allowed image acquisition using a Matrox (IDS 542) card placed in an IBM AT computer. Grey levels were scored using PC SCOPE 2:0 image analysis software (I2S, Bordeaux, France).

2.7. Statistical analysis

For nitrite determination, means ± S.E.M. of 3–6 wells from two independent experimentations were used. Student's *t*-test was used to compare different treatments and controls.

For mRNA determination, densitometry values (arbitrary units) were recorded for both inducible NO synthase and standard signals. The inducible NO synthase mRNA/standard mRNA ratios were calculated for each treatment. Three independent experiments were performed for each treatment. Means ± S.E.M. of each ratio were calculated and compared using Student's *t*-test.

3. Results

Treatment of RBE4 cells with interleukin-1β (5 ng/ml) or lipopolysaccharide (10 μg/ml) for 24 h significantly stimulated the release of nitrite (1.7- and 2.5-fold respectively) (Fig. 1). The simultaneous addition of ethanol 200 mM to the RBE4 cells significantly increased both the interleukin-1β- and the lipopolysaccharide-induced release of nitrite (approximately 60%) (Fig. 1). This enhancement is more pronounced for the higher doses of ethanol ($P < 0.001$ for 200 mM compared to inducer). Addition of ethanol alone (100 or 200 mM) failed to increase nitrite production. Both interleukin-1β- and lipopolysaccharide-induced release of nitrite, and the ethanol-mediated enhancement of this effect, were significantly blocked by the concomitant addition of the specific NO synthase inhibitor N^G-methyl-L-arginine (L-NMMA) (100 μM) to RBE4 cells (Fig. 1). Interestingly, the L-NMMA inhibition of treated (lipopolysaccharide or interleukin-1β)-alcoholized cells was significantly more pronounced in lipopolysaccharide- than in interleukin-1β-treated cells (67% versus 36%).

The exposure of RBE4 cells to interleukin-1β (5 ng/ml) or lipopolysaccharide (10 μg/ml) for 16 h resulted in significant enhancement of inducible NO synthase mRNA levels compared to untreated cells (Fig. 2, lane 1 for lipopolysaccharide, lane 5 for interleukin-1β and lane 9 for untreated cells). No signal was detected in untreated cells and in cells exposed to ethanol (50, 100 or 200 mM) alone (Fig. 2, lane 10 for ethanol 200 mM; data not shown for ethanol 50 and 100 mM). The mRNA levels of interleukin-1β- or lipopolysaccharide-stimulated inducible NO synthase were significantly increased (23%, $P < 0.05$ and 10%, $P < 0.05$, respectively) by the simultaneous addition

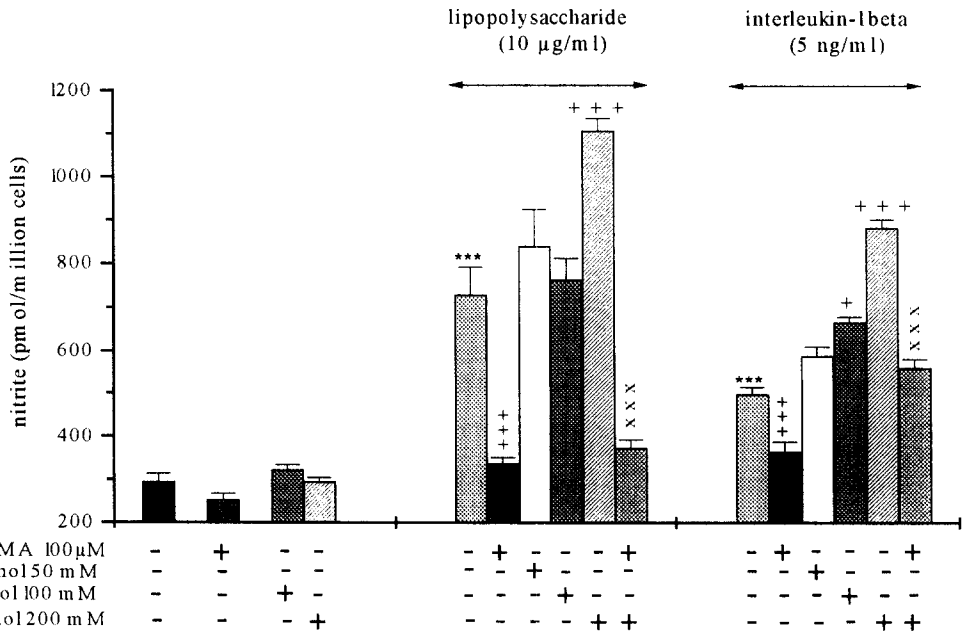

Fig. 1. Effect of lipopolysaccharide, interleukin-1β and ethanol on nitrite accumulation in the supernatant of cultured RBE4 cells. Cells were stimulated for 24 h. Columns represent nitrite production with (+) or without (−) different treatments. Results are expressed as means ± S.E.M. of 3–6 wells from two independent experiments. ***, $P < 0.001$ compared to untreated; +, $P < 0.05$, and +++, $P < 0.001$ compared to lipopolysaccharide- or interleukin-1β-treated cells; ×××, $P < 0.001$ compared to lipopolysaccharide- or interleukin-1β-treated alcoholized cells (200 mM).

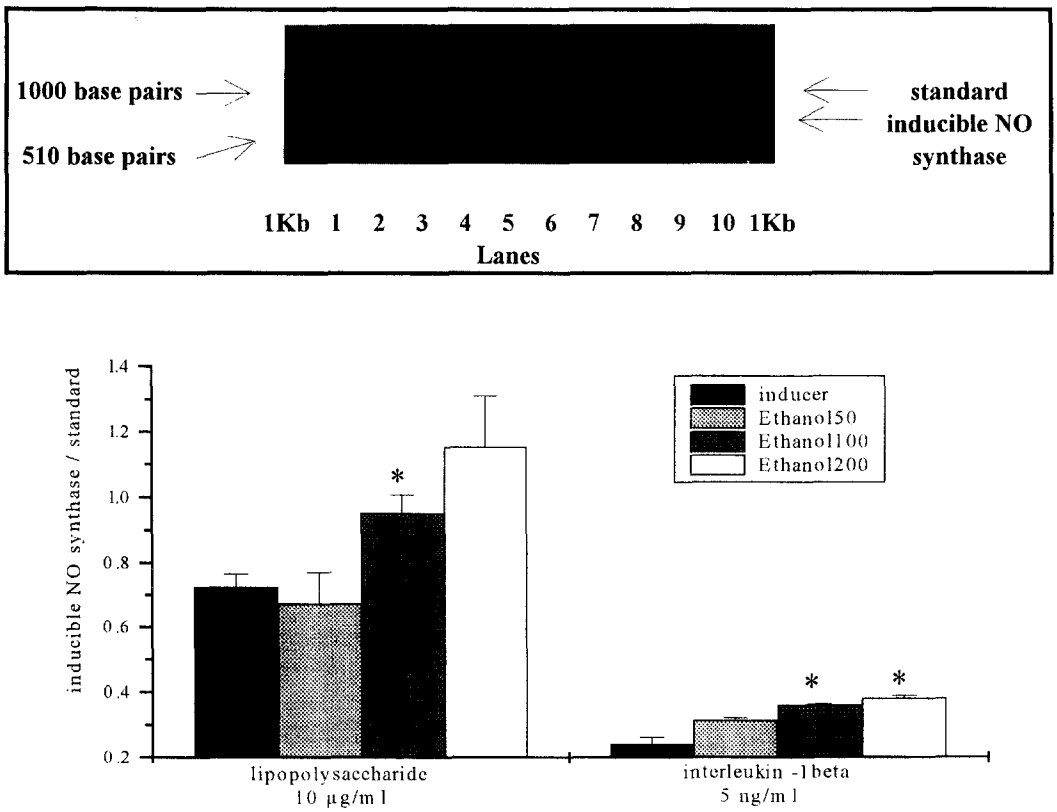

Fig. 2. Effect of ethanol (50, 100 or 200 mM) on inducible NO synthase mRNA levels in lipopolysaccharide- or interleukin-1β-treated RBE4 cells. Cells were stimulated for 16 h. Results are expressed as means ± S.E.M. of three independent experiments. The photo represents one typical experiment of three independent experiments. Molecular weight standards were the 1 kb ladder. *Lane 1:* lipopolysaccharide (10 μg/ml); *lanes 2–4:* lipopolysaccharide (10 μg/ml) with ethanol 50, 100 and 200 mM respectively. *Lane 5:* interleukin-1β (5 ng/ml); *lanes 6–8:* interleukin-1β (5 ng/ml) with ethanol 50, 100 and 200 mM respectively. *Lane 9:* untreated cells; *lane 10:* ethanol 200 mM. *, $P < 0.05$ compared to inducer.

of ethanol (100 mM) (Fig. 2, lane 3 for lipopolysaccharide and lane 7 for interleukin-1β).

4. Discussion

The present study demonstrated that ethanol potentiates the lipopolysaccharide- or interleukin-1β-induced expression of the inducible NO synthase gene in RBE4 cells, and the subsequent generation of NO. We also showed that the regulatory effect of ethanol on inducible NO synthase expression was not stimulus-dependent, since an induction has been obtained with both lipopolysaccharide and interleukin-1β.

The subsequent generation of NO, and its potentiation by ethanol, are blocked by the specific NO synthase inhibitor L-NMMA (Nathan, 1992), indicating that NO synthase activity is responsible of nitrite generation. These results agree with those of Durante et al. (1995) using vascular smooth muscle cells. However, lipopolysaccharide induction of nitrite production is higher (1.5-fold) than that observed using interleukin-1β (Fig. 1). The difference between the two treatments is more pronounced when mRNA levels are compared (about 2.8-fold). We suggest that the concentration of interleukin-1β used (5 ng/ml) was less inducer than that of lipopolysaccharide (10 μg/ml). We tested (data not shown) different concentrations of the two inducers according other data (Durante et al., 1995; Kanno et al., 1994). We chose 5 ng/ml for interleukin-1β and 10 μg/ml for lipopolysaccharide as a balance between low concentrations resulting in an undetectable signal (< 1 ng/ml for interleukin-1β and 0.1 μg/ml for lipopolysaccharide) and high concentrations (> 10 ng/ml for interleukin-1β and 20 μg/ml for lipopolysaccharide) resulting in a too strong signal for analyzing ethanol effect.

A short half-life (4 h) of interleukin-1β-induced inducible NO synthase mRNA has been demonstrated (Kanno et al., 1994) in different types of endothelial cells. It seems that the persistent accumulation of nitrite is not exactly correlated with mRNA levels, and could explain the inconsistency between nitrite production and mRNA levels. Moreover, Durante et al. (1995) suggested that ethanol action on cytokine-induced inducible NO synthase expression could involve a stabilizing effect on the transcript.

The molecular mechanism by which ethanol potentiates the expression of inducible NO synthase from lipopolysaccharide- or interleukin-1β-treated RBE4 cells is not known, but some hypotheses can be argued. Inducible NO synthase gene expression is regulated by different transcription factors, including NF-kB (Lowenstein et al., 1993) and we can speculate an ethanol action on these transduc-

tion pathways, as reported in recent presentations (Greenberg et al., 1996; Mandrekar et al., 1996). On the other hand, many data have shown an ethanol action on different membrane sites, altering membrane protein function, affecting G proteins and their related systems, changing ion channel function, and regulating receptor subunit expression (see review of Moring and Shoemaker, 1995). Consequently, an action of ethanol on membrane associated transduction pathways during lipopolysaccharide or interleukin-1β treatments could potentiate inducers' effects as shown in the present data.

Similar observations have been reported for other proteins. For instance, chronic ethanol treatment alters $GABA_A$ receptor gene expression, increasing the $\alpha 6$ and decreasing the $\alpha 1$ and $\alpha 2$ subunits of this brain receptor (Mhatre and Ticku, 1992).

Other authors have reported an increase (Durante et al., 1995) or decrease (Kolls et al., 1995; Greenberg et al., 1994, 1995; Syapin, 1995) in inducible NO synthase mRNA levels by alcohol after different inducers' stimulation in different cell types. In these different data, the effect of ethanol on inducible NO synthase differed according to cell types, ways of administration of ethanol (in vivo or in vitro) and inducer used (lipopolysaccharide, interleukin-1β, tumor necrosis factor-α for instance). Taken together, all these data, including our present work, show that ethanol by itself fails to regulate inducible NO synthase gene expression, but modulates the effects of different proinflammatory agents and cytokines.

Nevertheless, one previous study demonstrated that in vivo ethanol alone induced NO generation in hepatocytes, and could contribute to ethanol hepatotoxicity (Wang et al., 1995).

The ethanol potentiation observed in our study appears to be an aggravating factor during sepsis or inflammation, associated with circulatory failure. This ethanol target in blood-brain barrier cells may participate in ethanol brain toxicity following pathological disorders, specially when blood-brain barrier permeability is increased after excessive NO generation (Shukla et al., 1995).

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