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Short communication

Endothelin ET_A receptor antagonist reverses the inhibitory effect of platelet-derived growth factor on cytokine-induced nitric oxide production

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Abstract

Cytokines and cytokine-induced nitric oxide (NO) play important roles in inflammatory glomerular diseases, and both platelet-derived growth factor and transforming growth factor-β inhibit cytokine-induced NO production. In this study, we demonstrated that a selective endothelin ET_A receptor antagonist, BQ-485 (Hexahydro-1*H*-azepinylcarbonyl-Leu-D-Trp-D-Trp-OH), reversed the inhibitory effect of platelet-derived growth factor on cytokine-induced NO production, but not that of transforming growth factor-β. Our findings suggest a difference between the inhibitory mechanisms of platelet-derived growth factor and transforming growth factor-β on cytokine-induced NO production.

Keywords: Nitric oxide (NO); Platelet-derived growth factor; Transforming growth factor-β; Endothelin ET_A receptor; Mesangial cells

1. Introduction

Nitric oxide (NO) has been shown to inhibit mesangial cell proliferation, extracellular matrix accumulation (Craven et al., 1997), and platelet aggregation and adhesion. The release of cytokines by primed macrophages during glomerular inflammation can induce the synthesis of several types of cytokines by mesangial cells. Two such cytokines, interleukin-1β and tumor necrosis factor-α, induce the expression of NO synthase in rat mesangial cells, leading to the production of large amounts of NO (Pfeilschifter et al., 1992). It has been reported that not only do platelet-derived growth factor (Pfeilschifter, 1991) and transforming growth factor (Beck and Sterzel, 1996) inhibit cytokine-induced NO production in rat mesangial cells, but also that endothelin-1 dose (Beck et al., 1995; Hirahashi et al., 1996). However, the mechanism underlying the inhibition remains to be clarified. To investigate the possibility that endothelin is involved in the inhibitory effect of platelet-derived growth factor and transforming growth factor-β on cytokine-induced NO production, we examined the effect of selective endothelin receptor antagonists on their inhibitory effects.

2. Materials and methods

2.1. Chemicals

Endothelin-1, purchased from the Peptide Institute (Osaka, Japan), was dissolved in 0.1% acetic acid solution. Human interleukin-1β was purchased from Genzyme (Boston, MA), human platelet-derived growth factor BB chain, transforming growth factor-β, endothelin-1, and tumor necrosis factor-α were from Boehringer Mannheim (Tokyo, Japan), lipopolysaccharide was from Sigma (St. Louis, MO), and RPMI 1640, Dulbecco's modified essential medium without phenol red, and fetal bovine serum were from Gibco (Grand Island, NY). Concentrated interleukin-1β, tumor necrosis factor-α, and lipopolysaccharide were diluted in Dulbecco's modified essential medium without phenol red but containing insulin-transferrin-sodium selenite medium supplement purchased from Sigma. BQ-485

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was generously provided by Banyu Pharmaceutical (Ibaraki, Japan). BQ-788 (*N-cis*-2,6-Dimethylpiperidinocarbonyl-L-g-MeLeu-D-Trp(MeOCO)-D-Nle-OH) was purchased from Calbiochem (Switzerland). All other chemicals and reagents were obtained from commercial sources and were of analytical grade.

2.2. Cell culture and measurement of nitrite

Rat glomerular mesangial cells were cultured and characterized as described previously (Nakazato et al., 1993). Mesangial cells were plated onto 35-mm dishes at an initial density of approximately 2.0×10^3 cells/cm^2 in RPMI 1640 supplemented with 10% fetal bovine serum. When the cells reached confluence, the medium was replaced with serum-free Dulbecco's modified essential medium containing 5 μg/ml insulin, 5 μg/ml transferrin, and 5 ng/ml selenite for 72 h, and then the cells were incubated with the medium containing interleukin-1β (1 nM), tumor necrosis factor-α (2000 u/ml), and lipopolysaccharide (100 ng/ml) for 48 h. At 48 h, a 100-μl aliquot of the culture medium was collected, and the nitrite level was measured by the Griess reaction (Hishikawa et al., 1995). The nitrite accumulation observed in dishes without cells run in parallel with those containing mesangial cells was then subtracted from each cumulative value.

2.3. Western blot analysis of inducible NO synthase protein

The amount of inducible NO synthase protein was analyzed by Western blot with anti-mouse inducible NO synthase antibody (Transduction Laboratories). The antibody was used at a 500-fold dilution. The confluent cells in 35-mm dishes (5×10^5 cells/dish) were lysed in 100 μl of solution containing 10% glycerol, 2.3% sodium dodecyl sulfate, Tris–HCl (pH 6.8) 62.5 mM, 0.01% bromophenol blue, and 5% mercaptoethanol. The lysate was then heated at 100°C for 5 min. Whole cell lysates containing 10 μg of protein were subjected to 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The separated proteins were electrophoretically transferred to polyvinylidene difluoride membranes. Membranes were blocked in blocking buffer (10 mM Tris–HCl [pH 7.5], 100 mM NaCl, 0.5% Tween-20, 5% nonfat dry milk) for 1 h and incubated with anti-inducible NO synthase antibody for 1 h at room temperature as previously described (Hishikawa et al., 1995). They were then incubated with secondary polyclonal peroxidase-conjugated anti-mouse antibody (Amersham), followed by staining with the enhanced chemiluminescence technique developed by Amer-

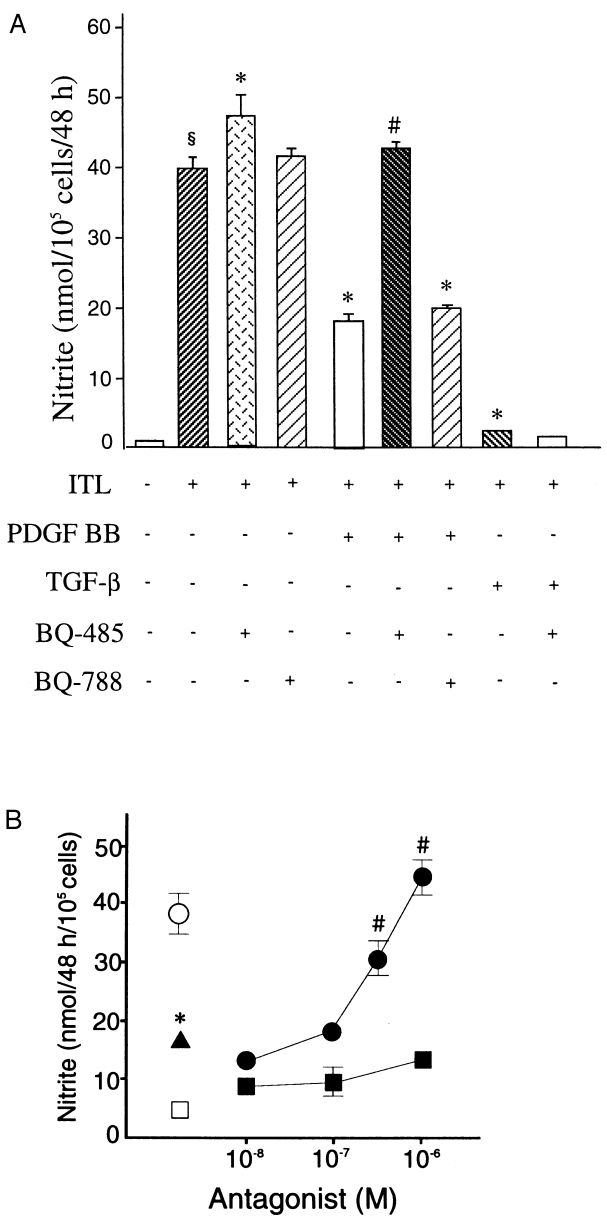

Fig. 1. (A) Effect of the endothelin ET_A receptor antagonist BQ-485 and endothelin ET_B receptor antagonist BQ-788 on inhibition of nitrite accumulation by platelet-derived growth factor BB chain(PDGF BB) and transforming growth factor-β (TGF-β)). Cells were incubated for 48 h under the experimental conditions indicated in the figure. The following concentrations were used: PDGF BB 100 ng/ml; TGF-β 1 ng/ml; BQ-485 1 μM; BQ-788 1 μM. ITL refers to treatment with a combination of interleukin-1β (1 nM), tumor necrosis factor-α (2000 U/ml), and lipopolysaccharide (100 ng/ml). BQ-485 and BQ-788 were added to the medium 30 min before treatment with ITL. Values are means ± S.E.M. ($n = 4$). * $P < 0.05$ vs. ITL only. § $P < 0.05$ vs. no treatment. # $P < 0.05$ vs. ITL plus PDGF BB. (B) Dose-dependent effects of the endothelin ET_A receptor antagonist BQ-485 and the endothelin ET_B receptor antagonist BQ-788 on the inhibitory action of platelet-derived growth factor BB chain (PDGF BB). Cells were preincubated for 30 min with the concentration of BQ-485 or BQ-788 shown and then incubated with medium only (□), with ITL only (○), with ITL and 100 ng/ml PDGF BB (▲), or with ITL, 100 ng/ml PDGF BB, and the concentration of BQ-485 (●) or BQ-788 (■) shown. ITL refers to treatment with a combination of interleukin-1β (1 nM), tumor necrosis factor-α (2000 U/ml), and lipopolysaccharide (100 ng/ml). Values are means ± S.E.M. ($n = 3$). * $P < 0.05$ vs. ITL only. # $P < 0.05$ vs. ITL plus PDGF BB.

sham. The intensities of the inducible NO synthase-specific bands were quantified by densitometry.

2.4. Statistical analysis

Multiple comparisons were evaluated by analysis of variance, followed by Fisher's protected least significant difference test. Student's *t*-test was used for comparisons between two experimental groups. All results are expressed as means ± S.E.M. in the text and figures. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Effects of platelet-derived growth factor BB chain and transforming growth factor-β on cytokine-stimulated nitrite accumulation

We used human interleukin-1β, human tumor necrosis factor-α, and lipopolysaccharide, alone and in combination, to induce NO production in mesangial cells. The largest effect on mesangial cells was seen at 48 h with a combination of interleukin-1β, tumor necrosis factor-α, and lipopolysaccharide at concentrations of 1 nM, 2000 u/ml, and 100 ng/ml, respectively. We therefore used this combination in subsequent experiments. The accumulation of nitrite was accelerated between 24 and 48 h after cytokine treatment, and we therefore measured nitrite accumulation within 48 h of cytokine treatment. The nitrite accumulation was markedly inhibited in a concentration-dependent manner by simultaneous treatment of cells with both platelet-derived growth factor BB chain and transforming growth factor-β.

3.2. Effects of a selective endothelin ET_A antagonist and a selective endothelin ET_B antagonist on nitrite accumulation

To investigate the mechanism of the inhibitory effect of platelet-derived growth factor BB chain on inducible NO synthase, we further investigated the contribution of endothelin ET_A receptors and endothelin ET_B receptors by using receptor antagonists. We used BQ-485, a selective endothelin ET_A receptor antagonist, and BQ-788, a selective endothelin ET_B receptor antagonist. Applied individually, BQ-485 enhanced cytokine-induced nitrite accumulation. The inhibitory effect of platelet-derived growth factor BB chain (100 ng/ml) was abolished by BQ-485 pretreatment in a concentration-dependent manner (Fig. 1B), and the nitrite accumulation was enhanced to some extent, indicating that the endothelin ET_A receptor plays a role in the inhibition of nitrite accumulation. We then used the selective endothelin ET_B antagonist BQ-788 to evaluate the contribution of the endothelin ET_B receptor; however, it did not significantly affect the inhibitory action of platelet-derived growth factor BB chain at concentrations less than 1 μM (Fig. 1B). BQ-788 may act as a nonspecific antagonist of endothelin ET_A and ET_B receptors at concentrations exceeding 10 μM (personal communication, Dr. M. Ihara, Tsukuba Research Institute, Banyu Pharmaceutical, Tsukuba, Japan). We therefore used BQ-788 at concentrations below 1 μM. By contrast, the inhibitory effect of transforming growth factor-β on nitrite accumulation was not influenced by BQ-485 (1 nM ~ 10 μM). These findings indicate that the endothelin ET_A receptor subtype, but not the endothelin ET_B, receptor subtype, plays a major role in the inhibition by platelet-derived growth factor BB chain, of cytokine-stimulated NO production, although endothelin ET_A receptors appeared not to be involved in the inhibitory effect of transforming growth factor-β.

3.3. Effect of BQ-485 on platelet-derived growth factor BB chain and transforming growth factor-β-induced inhibition of inducible NO synthase protein expression

We performed Western blot analysis to further evaluate the effect of BQ-485 at the level of inducible NO synthase

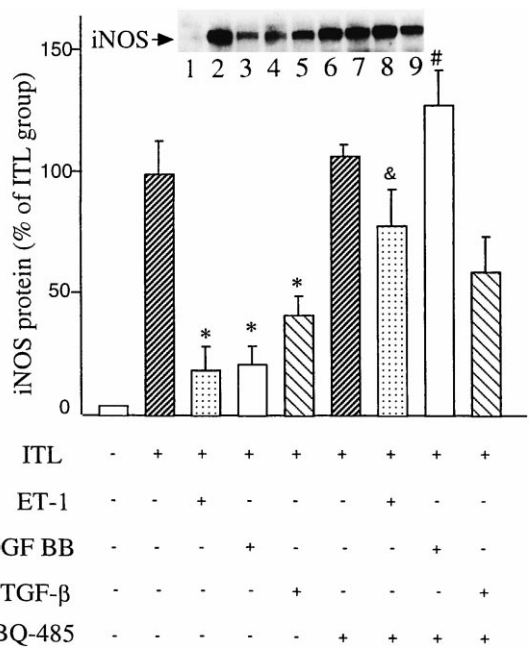

Fig. 2. Effect of the ET_A receptor antagonist BQ-485 on platelet-derived growth factor BB chain (PDGF BB) and transforming growth factor-β (TGF-β)-induced inhibition of cytokine-stimulated inducible NO synthase (iNOS) protein (130 kDa) expression. Western blots of iNOS protein in mesangial cells incubated for 24 h with vehicle (lane 1), ITL (lane 2), ITL plus 100 nM endothelin-1 (ET-1) (lane 3), ITL plus 100 ng/ml platelet-derived growth factor BB chain (PDGF BB) (lane 4), ITL plus 1 ng/ml and transforming growth factor-β (TGF-β) (lane 5), ITL plus 1 μM BQ-485 (lane 6), ITL plus 100 nM ET-1 and 1 μM BQ-485 (lane 7), ITL plus 100 ng/ml PDGF BB and 1 μM BQ-485 (lane 8), and ITL plus 1 ng/ml TGF-β and 1 μM BQ-485 (lane 9) are shown. ITL refers to treatment with a combination of interleukin-1β (1 nM), tumor necrosis factor-α (2000 U/ml), and lipopolysaccharide (100 ng/ml). The signal densities relative to those of lane 2 are shown in the inset. $* P < 0.05$ vs. ITL only. $\# P < 0.05$ vs. ITL plus PDGF BB. $\& P < 0.05$ vs. ITL plus ET-1.

protein expression. Western blotting with anti-inducible NO synthase antibody revealed that cytokine-induced inducible NO synthase protein (~ 130 kDa) was detectable at 24 h but had disappeared by 48 h (data not shown). Platelet-derived growth factor BB chain and transforming growth factor-β inhibited the cytokine-stimulated increase in inducible NO synthase protein to a similar extent as they inhibited nitrite accumulation (Fig. 2). The platelet-derived growth factor BB chain-induced inhibition was completely abolished by BQ-485, and the inducible NO synthase protein expression was enhanced to some extent. The transforming growth factor-β-induced inhibition was not influenced by BQ-485. These Western blotting data were consistent with those for nitrite accumulation.

4. Discussion

Our results demonstrated, for the first time, that a selective endothelin ET_A receptor antagonist, BQ-485, effectively abolished the inhibitory effect of platelet-derived growth factor BB chain on the induction of inducible NO synthase but had no effect on the inhibitory effect of transforming growth factor-β. BQ-485 also reversed the inhibitory effect of endothelin-1 on the induction of inducible NO synthase, but this effect was not as strong as in case of platelet-derived growth factor BB chain. These results suggest that different endothelin receptor subtypes are involved in the inhibition of the cytokine-induced stimulation of inducible NO synthase. The selective endothelin ET_B receptor antagonist, BQ-788, had no significant effect on the inhibitory effect. These findings suggest the possibility that the endothelin ET_A receptor, but not the endothelin ET_B receptor, may play a major role in the inhibitory effects of platelet-derived growth factor BB chain on the induction of inducible NO synthase. However, a nonselective endothelin ET_A and endothelin ET_B receptor antagonist, TAK 044 (cyclo {D-α-aspartyl-3-[(4-phenylpiperazin-1-yl) carbonyl]-L-alanyl-L-α-aspartyl-D-2-(2-thienyl) glycyl-L-leucyl-D-tryptophyl}disodium salt) (kindly provided by Takeda Chemical Industries, Osaka, Japan), showed a more potent effect on the inhibitory actions of platelet-derived growth factor BB chain and endothelin-1 than did BQ-485 (data not shown). This finding suggests that the signaling pathway involving the endothelin ET_B receptor interacts with the signaling pathway involving the endothelin ET_A receptor and potentiates the inhibitory effect on the induction of inducible NO synthase. It was recently reported (Kohno et al., 1994) that endothelin-1 modulates the mitogenic effect of platelet-derived growth factor BB chain via the endothelin ET_A receptor. This finding suggests the possibility that platelet-derived growth factor BB chain inhibited the induction of inducible NO synthase not only by exerting a direct effect via the endothelin ET_A receptor but also by enhancing the production of endothelin-1. In fact, we detected a slight but significant enhancement of endothelin production by platelet-derived growth factor BB chain in the presence of interleukin-1β, tumor necrosis factor-α, and lipopolysaccharide (data not shown). It is also possible that BQ-485 modulates the receptor binding or intracellular signalling of platelet-derived growth factor BB chain, but we should await another study to clarify these points.

As shown in Fig. 2, BQ-485 not only attenuated the inhibitory effect of platelet-derived growth factor BB chain, but caused a significant amplification of inducible NO synthase protein levels. As we detected a significant enhancement of endothelin production in the presence of interleukin-1β, tumor necrosis factor-α, and lipopolysaccharide (data not shown), BQ-485 might reverse the inhibitory effect of endothelin-1 stimulated by these cytokines and stimulate the induction of inducible NO synthase. It is possible that BQ-485 unmasks a stimulatory effect of platelet-derived growth factor BB chain, but there is no report about the stimulatory effect of platelet-derived growth factor BB chain on the induction of inducible NO synthase and this point remains to be elucidated.

Increased platelet-derived growth factor production and platelet-derived growth factor-β receptor expression under inflammatory conditions, such as mesangial proliferative glomerulonephritis, have been demonstrated in vivo. Fukuda et al. (1996) reported that, in vivo, endothelin-1 functions as a potent mitogen in mesangial cells in an autocrine or paracrine fashion, and that endothelin ET_A receptor expression increases rather than that endothelin ET_B receptor expression decreases. These findings may suggest that antagonism of the endothelin ET_A receptor is beneficial in glomerulonephritis because it inhibits the mitogenic function of endothelin-1 and platelet-derived growth factor.

Since the large amounts of NO produced by inducible NO synthase can be expected to inhibit mesangial proliferation and extracellular matrix accumulation, our results suggest that BQ-485 may be useful as a therapeutic agent for mesangial proliferative glomerulonephritis.

Acknowledgements

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