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Effect of cilostazol, a cAMP phosphodiesterase inhibitor, on nitric oxide production by vascular smooth muscle cells

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Abstract

We investigated the effects of cilostazol, a cAMP phosphodiesterase inhibitor, on nitric oxide (NO) synthesis in cultured rat vascular smooth muscle cells. Incubation of the cultures with interleukin-1β (10 ng/ml) for 24 h caused a significant increase in the accumulation of nitrite, a stable metabolite of NO. Although cilostazol by itself showed no effect on nitrite accumulation, it stimulated interleukin-1β-induced nitrite accumulation in a concentration-dependent manner (10^{-8}–10^{-5} M). This effect of cilostazol was completely abolished in the presence of N^G-monomethyl-L-arginine, actinomycin D or dexamethasone. The cilostazol-induced nitrite production was accompanied by increased inducible NO synthase protein expression. In the presence of dibutyryl-cAMP, interleukin-1β-induced nitrite accumulation was further increased, but the stimulatory effect of cilostazol on nitrite accumulation was blunted. The effect of cilostazol was also abolished in the presence of Rp-8-bromoadenosine-3′,5′-cyclic monophosphorothioate, a competitive inhibitor of protein kinase A. Addition of cilostazol to the cultures significantly increased intracellular cAMP levels of vascular smooth muscle cells. These results indicate that cilostazol increases NO synthesis in interleukin-1β-stimulated vascular smooth muscle cells, at least partially through a cAMP-dependent pathway.

Keywords: Interleukin-1; Nitrite; Nitric oxide (NO) synthase

1. Introduction

Nitric oxide (NO) is synthesized from L-arginine by two enzymes. The generation of NO by constitutive, Ca^{2+}-dependent NO synthase (cNOS) from the vascular endothelium plays an important role in homeostasis of the vascular system. Recently, two isoforms of constitutive NO synthase have been cloned from rat brain (nNOS, or type I NOS) and vascular endothelium (eNOS, or type III NOS) (Moncada et al., 1991; Nathan and Xie, 1994). The inducible, Ca^{2+}-independent enzyme (iNOS, or type II NOS) is expressed in target cells such as macrophages, vascular smooth muscle cells and cardiac myocytes after stimulation with endotoxin and cytokines (Dinerman et al., 1993; Ikeda et al., 1995). NO inhibits platelet aggregation (Radomski et al., 1987), proliferation of vascular smooth muscle cells (Garg and Hassid, 1989), and leukocyte adhesion to endothelial cells (Niu et al., 1994). Therefore, NO synthase induction in vascular smooth muscle cells may play a role in local vascular injury associated with atherosclerosis.

The new synthetic platelet-aggregation inhibitor cilostazol (6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)-butoxy]-3,4-dihydro-2-(1*H*)-quinolinone), a specific inhibitor of cAMP phosphodiesterase (Umekawa et al., 1984), has been used clinically for the treatment of arteriosclerosis obliterans as an anti-platelet agent. Recently, it has been shown that cilostazol inhibits proliferation of vascular smooth muscle cells (Takahashi et al., 1992), increases peripheral blood flow in arteriosclerosis obliterans (Yasuda et al., 1985), and prevents reocclusion after coronary arterial thrombolysis (Saitoh et al., 1993). However, there have been no reports concerning the effects of cilostazol on the generation of NO, another modulator of vascular contraction and

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proliferation. In this study, we investigated the effects of cilostazol on inducible NO synthase activity in cultured rat vascular smooth muscle cells.

2. Materials and methods

2.1. Culture of cells

Primary cultures of vascular smooth muscle cells were obtained from the media of thoracic aortae of Sprague-Dawley rats (200–250 g) as described previously (Ikeda et al., 1991). The cells were grown in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The cultures were harvested twice a week by treatment with 0.125% trypsin and passaged at a 1:3 ratio in 100-mm culture dishes. A typical growth experiment was performed using cultured cells at passage 5–10. Cells (3×10^4/ml) were plated in 24-well or 100-mm culture dishes in DMEM, supplemented as described above, and allowed to grow to subconfluence, after which they were pre-incubated in DMEM containing 0.5% fetal bovine serum and supplemented with insulin (5 μg/ml) and transferrin (5 μg/ml) for 24 h, and used for the experiments described below.

2.2. Measurement of nitrite

NO production by the cultured cells was determined by measuring the nitrite contents of the culture media. Vascular smooth muscle cells plated in 24-well dishes were incubated in DMEM containing 0.5% fetal bovine serum at 37°C. The nitrite contents of culture media were determined by mixing 500 μl of medium with an equal volume of Griess reagent (1 part 0.1% naphthylethylenediamine dihydrochloride to 1 part 1% sulfanilamide in 5% phosphoric acid) (Green et al., 1982). The absorbance at 550 nm was measured and the nitrite concentration was determined by interpolation of a calibration curve of standard sodium nitrite concentrations against absorbance. After washing, cells were dissolved in 0.2 ml of 1% sodium dodecyl sulfate and used for protein assay (BCA protein assay kit); bovine serum was used as a standard. Nitrite levels were corrected by those of protein, and data are shown as nmol per mg protein.

2.3. Assay for inducible NO synthase protein

The expression of inducible NO synthase protein was analyzed by immunoblotting with an anti-inducible NO synthase antibody as described previously (Koide et al., 1993). Briefly, vascular smooth muscle cells were lysed in a buffer containing 50 mM Tris/Cl, pH 7.5, 1 mM EDTA, 1 μM leupeptin, 1 μM pepstatin A, 0.1 mM phenylmethylsulfonyl fluoride and 1 M dithiothreitol, and sonicated. The homogenates were then centrifuged at $100\,000 \times g$ for 20 min, and the supernatants (60 μg protein) were subjected to 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were electrophoretically transferred onto nitrocellulose membranes, and the resultant blots were incubated with the anti-inducible NO synthase antibody for 2 h followed by peroxidase-labeled donkey anti-rabbit IgG for 1 h. Peroxidase-labeled proteins were visualized by incubation with peroxidase color development reagents containing the enzyme substrate 3,3′-diaminobenzidine using $NiCl_2$ as an enhancer.

2.4. Measurement of cAMP

After incubation in 24-well dishes with cilostazol for 1 h, vascular smooth muscle cells were immediately immersed in 0.2 ml of 0.1 N HCl to stop the reaction. Cells were then collected into glass tubes with a rubber policeman, boiled for 5 min, and then centrifuged at $2500 \times g$ for 15 min at room temperature. The supernatants were decanted, and after 0.05 ml of 50 mM sodium acetate was added to each tube, cells were kept at −70°C until they were assayed for cAMP content. The pellets were dissolved in 0.2 ml of 1% sodium dodecyl sulfate and kept at 4°C until protein assay. Intracellular cAMP contents were measured with a commercial enzyme immunoassay kit using the manufacturer's high sensitivity acetylation protocol (Amersham, UK) and were corrected by those of protein, and data are shown as nmol per mg protein.

2.5. Statistical analysis

Data are expressed as means ± S.E.M. For comparisons between multiple groups, we determined the significance of differences between group means by analysis of variance using the least significant difference for multiple comparisons. Differences at P values of < 0.05 were considered to be statistically significant.

2.6. Materials

Cilostazol and human recombinant interleukin-1β were obtained from Otsuka Pharmaceutical (Tokyo, Japan). Rp-8-bromoadenosine-3′,5′-cyclic monophosphorothioate (Rp-8-Br-cAMPS) was purchased from Biolog Life Science Institute (Bremen, Germany). N^G-monomethyl-L-arginine (L-NMMA), actinomycin D, dexamethasone and N^2,2′-*O*-dibutyryl cAMP (db-cAMP) were from Sigma (St. Louis, MO, USA). A polyclonal anti-inducible NO synthase antibody, which was raised against rat liver inducible NO synthase, was kindly provided by Dr. H. Esumi (National Cancer Institute, Kashiwa, Japan). All other chemicals used were of the highest grade commercially available.

3. Results

3.1. Effects of cilostazol on nitrite accumulation

Accumulation of nitrite in the medium represents the summation of NO synthase activity during the time period studied, since NO secreted by cells is rapidly decomposed to the more stable products nitrite and nitrate. As reported previously (Ikeda and Shimada, 1994), interleukin-1β (10 ng/ml) stimulated the accumulation of nitrite in a time-dependent manner. The levels of nitrite increased significantly within 6 h and continued to increase for at least 24 h after exposure to interleukin-1β. Nitrite accumulation stimulated by interleukin-1β was significantly increased by simultaneous treatment of the cells with cilostazol (10^{-5} M). After a 24-h incubation, the level of interleukin-1β-induced nitrite accumulation in the presence of cilostazol was about 1.5-fold that in the absence of cilostazol.

Fig. 1 shows the concentration-response effect of cilostazol. Although cilostazol by itself did not affect the basal level of nitrite accumulation, it increased interleukin-1β-induced nitrite accumulation in vascular smooth muscle cells in a concentration-dependent manner (10^{-8}–10^{-5} M).

As shown in Fig. 2, simultaneous incubation with the NO synthase inhibitor L-NMMA (10^{-3} M), the RNA synthesis inhibitor actinomycin D (5 μg/ml) or dexamethasone (10^{-6} M) for 24 h completely inhibited cilostazol- as well as interleukin-1β-induced nitrite accumulation.

3.2. Effects of cilostazol on inducible NO synthase protein expression

We next examined whether cilostazol increased inducible NO synthase protein expression in vascular smooth

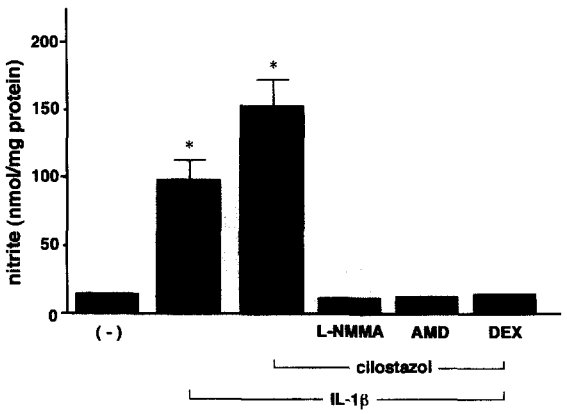

Fig. 2. Effects of L-NMMA, actinomycin D and dexamethasone on cilostazol-induced nitrite accumulation in vascular smooth muscle cells. Cells were exposed to 10^{-5} M cilostazol for 24 h with 10^{-3} M L-NMMA, 5 μg/ml actinomycin D (AMD) or 10^{-6} M dexamethasone (DEX) in the presence of 10 ng/ml interleukin-1β (IL-1β). Data are mean ± S.E.M. of four samples. * $P < 0.01$ compared with control samples indicated as (−).

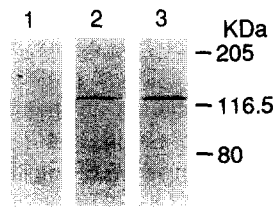

Fig. 3. Expression of inducible NO synthase protein in vascular smooth muscle cells. Cells were incubated with 10 ng/ml interleukin-1β for 24 h with or without 10^{-5} M cilostazol. Cell extracts were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblot analysis using the anti-inducible NO synthase antibody. The positions of the molecular mass markers are indicated on the right. The inducible NO synthase protein band with a molecular mass of about 125 kDa is the band above the 116.5 kDa marker. Lane 1, control; lane 2, interleukin-1β; lane 3, interleukin-1β plus cilostazol. Two independent experiments yielded identical results.

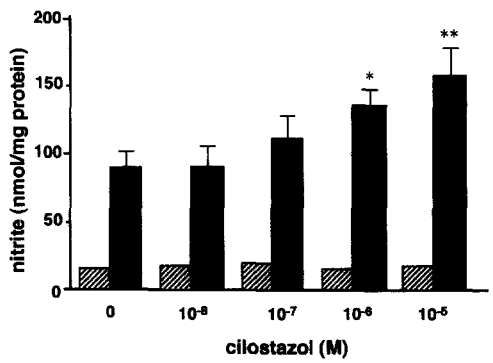

Fig. 1. Concentration-dependence of the stimulatory effect of cilostazol on nitrite accumulation. Vascular smooth muscle cells were incubated for 24 h with (dotted bars) or without (hatched bars) 10 ng/ml interleukin-1β in the presence of various concentrations of cilostazol (10^{-8}–10^{-5} M) as indicated. Nitrite accumulation in the culture medium was measured, and the values were normalized to the protein content per dish. Points and bars represent mean ± S.E.M. ($n = 4$). * $P < 0.05$, ** $P < 0.01$ compared with cells not exposed to cilostazol.

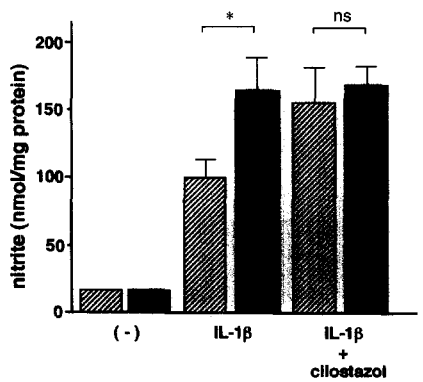

Fig. 4. Effects of db-cAMP on interleukin-1β- and cilostazol-induced nitrite accumulation in vascular smooth muscle cells. Cells were incubated for 24 h with 10 ng/ml interleukin-1β (IL-1β) or interleukin-1β plus 10^{-5} M cilostazol in the presence (dotted bars) or absence (hatched bars) of 1 mM db-cAMP. Nitrite accumulation in the culture medium was measured, and the values were normalized to the protein content per well. Data represent mean ± S.E.M. ($n = 4$). * $P < 0.05$, ns; not significant.

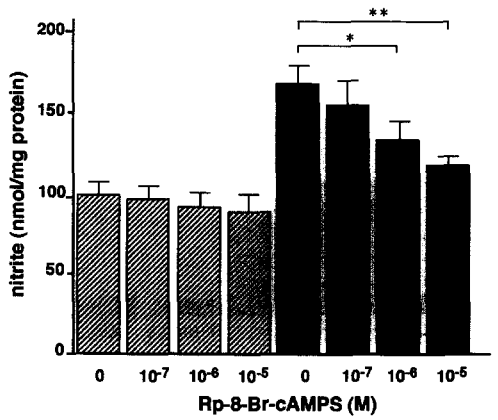

Fig. 5. Effects of Rp-8-Br-cAMPS, a competitive inhibitor of protein kinase A, on nitrite accumulation in vascular smooth muscle cells. Cells were incubated for 24 h with 10 ng/ml interleukin-1β and various concentrations of Rp-8-Br-cAMPS (10^{-7}–10^{-5} M) in the presence (dotted bars) or absence (hatched bars) of 10^{-5} M cilostazol. Nitrite accumulation in the culture medium was measured, and the values were normalized to the protein content per well. Data represent mean ± S.E.M. ($n = 4$). * $P < 0.05$, ** $P < 0.01$.

muscle cells. As shown in Fig. 3, unstimulated cells did not express inducible NO synthase protein. Incubation with interleukin-1β for 24 h caused an induction of inducible NO synthase protein expression, and its expression was further increased in the presence of cilostazol.

3.3. Involvement of cAMP in the action of cilostazol

We then investigated the mechanism of the stimulatory effect of cilostazol on NO synthesis. It has been shown that cilostazol stimulates cAMP formation (Takahashi et al., 1992) and cAMP up-regulates inducible NO synthase expression in rat vascular smooth muscle cells (Koide et al., 1993). As shown in Fig. 4, in the presence of db-cAMP (1 mM), the basal level of nitrite accumulation was not affected, while interleukin-1β-induced nitrite accumulation was increased. Addition of cilostazol to the cultures significantly increased interleukin-1β-induced nitrite accumulation, but its stimulatory effect was blunted in the presence of db-cAMP.

The effect of cilostazol was also significantly inhibited by Rp-8-Br-cAMPS, a competitive inhibitor of protein kinase A, in a dose-dependent manner (Fig. 5), also supporting the notion that the stimulatory effect of cilostazol is mediated through a cAMP-dependent pathway.

We then measured intracellular cAMP levels of vascular smooth muscle cells treated with cilostazol. As shown in Fig. 6, addition of 10^{-5} M cilostazol for 1 h significantly increased intracellular cAMP levels of vascular smooth muscle cells.

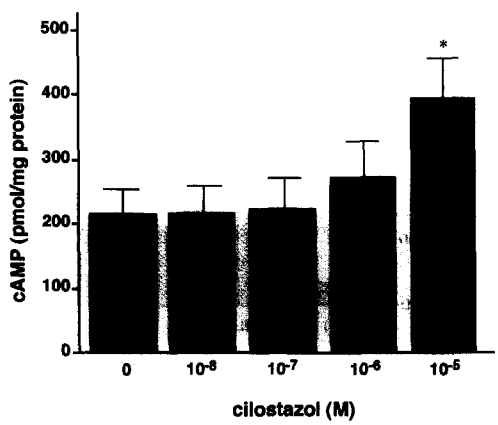

Fig. 6. Effects of cilostazol on intracellular cAMP levels of vascular smooth muscle cells. Cells in 24-well dishes were incubated for 1 h with 10^{-8}–10^{-5} M cilostazol. Intracellular cAMP contents were measured as described in Section 2. Data represent mean ± S.E.M. ($n = 4$). * $P < 0.05$ compared with control cells not exposed to cilostazol.

4. Discussion

In this study, we investigated whether cilostazol affected NO synthesis in vascular smooth muscle cells. Although cilostazol by itself showed no effect on NO production, it stimulated interleukin-1β-induced NO production in a dose-dependent manner. We obtained three pieces of evidence for a causal link between cAMP production and augmentation of NO synthesis by cilostazol in vascular smooth muscle cells. First, cilostazol caused an increase in the cellular levels of cAMP (Fig. 6). Second, cAMP increased interleukin-1β-induced NO production (Fig. 4). Third, the stimulatory effect of cilostazol was blunted in the presence of db-cAMP (Fig. 4) or Rp-8-Br-cAMPS (Fig. 5). These results suggest that cilostazol augments NO production by vascular smooth muscle cells, at least partially through a cAMP-dependent process.

It is generally accepted that cAMP mediates the induction of numerous genes through a specific protein kinase A which phosphorylates a 43-kDa nuclear phosphoprotein termed 'cAMP response element (CRE) binding protein,' a transcription factor which binds to the consensus palindromic motif 'TGACGTCA' termed the cAMP response element, to initiate gene transcription (Montminy and Bilezikjian, 1987; Roesler et al., 1988; Yamamoto et al., 1988; Lowenstein et al., 1992). Evidence for the existence of CRE domains in association with the inducible NO synthase gene is currently lacking, and there is no CRE sequence present in the 5′-flanking region of mouse inducible NO synthase (Xie et al., 1993). The near absence of effects of cilostazol (Fig. 1) and db-cAMP (Fig. 4) on basal NO production suggests, however, that, if CRE-dependent transcription is operating in our system, then this must be conditional on the activation of the numerous cytokine-response elements which have been identified in the inducible NO synthase promoter region (Xie et al., 1993). It has been shown that cAMP up-regulates inducible NO synthase expression in cytokine-stimulated vascular smooth muscle cells (Koide et al., 1993; Hi-

rokawa et al., 1994), mesangial cells (Mühl et al., 1994), and cardiac myocytes (Ikeda et al., 1996). In rat cardiac myocytes, Oddis et al. (1995) recently revealed that cAMP enhances inducible NO synthase mRNA stability following cytokine exposure.

Inducible NO synthase activity is induced in blood vessel wall and cultured vascular smooth muscle cells by endotoxins and cytokines (Dinerman et al., 1993; Ikeda and Shimada, 1994). Joly et al. (1992) demonstrated that in vivo balloon injury induced NO synthase activity in rat carotid arteries even in the absence of endothelium. Hansson et al. (1994) also reported that arterial smooth muscle cells in the neointima formed after deendothelializing balloon injury in the rat carotid artery expressed the cytokine-inducible isoform of NO synthase, and Groves et al. (1992) showed that molsidomine, a NO donor, inhibited platelet adhesion and aggregation following angioplasty of the carotid artery in pigs. Several lines of evidence from both in vitro and in vivo studies have recently suggested a role of endogenous NO as an anti-atherogenic autacoid (Cooke and Tsao, 1994); NO inhibits aggregation of platelets (Radomski et al., 1987), proliferation of vascular smooth muscle cells (Garg and Hassid, 1989), production of various cytokines by endothelial cells (Marcinkiewicz and Chain, 1993), adhesion of leukocytes to endothelial cells (Niu et al., 1994), expression of endothelial adhesion molecules (De Caterina et al., 1995), oxidation of low density lipoprotein (Jessup et al., 1992), and causes vasorelaxation (Karaki et al., 1988). Thus, NO might be produced by vascular tissue under various pathological conditions and inhibits de novo formation of intimal lesions (Cooke and Tsao, 1994).

In the present study, we revealed that cilostazol increases NO synthesis in interleukin-1β-stimulated vascular smooth muscle cells, at least partially through a cAMP-dependent pathway. These findings suggest that cilostazol may inhibit initiation and progression of atherosclerosis by augmenting endogenous NO production by vascular smooth muscle cells. However, to conclude, further investigations should be devised to elucidate the mechanisms and conditions under which NO inhibits atherogenesis.

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