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The Effect of Cilostazol, a Cyclic Nucleotide Phosphodiesterase III Inhibitor, on Heparin-Binding EGF-like Growth Factor Expression in Macrophages and Vascular Smooth Muscle Cells

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Heparin-binding EGF-like growth factor (HB-EGF) is a mitogen for smooth muscle cells (SMC) and is detected in SMC and macrophages in atherosclerotic plaques, suggesting that HB-EGF may be associated with the pathogenesis of atherosclerosis. The present study indicates that cilostazol, a phosphodiesterase III inhibitor, suppresses the expression of HB-EGF in rat aortic SMC and in U-937 cells, a macrophage-like cell line, stimulated by lipopolysaccharide. Further, cilostazol diminished the induction of HB-EGF mRNA by methylglyoxsal, which is a reactive dicarbonyl metabolite produced as the result of a glycation reaction and which might be associated with macroangiopathy caused by hyperglycemia. Cilostazol suppressed the production of HB-EGF protein in the conditioned medium of SMC. These data suggest that cilostazol might act by suppressing the progression of atherogenesis by means of suppressing the expression of HB-EGF in SMC and macrophages.

Heparin-binding EGF-like growth factor (HB-EGF) is a member of the EGF family and shares 35-40% protein sequence homology in its EGF domain (1,2) with other members such as EGF (3), transforming growth factor-α (TGF-α) (4), amphiregulin (5), betacellulin (6), epiregulin (7), neuregulin (8), and NTAK/neuregulin 2 (9-11). Although HB-EGF as well as EGF and TGF-α activate the EGF receptor, HB-EGF, in contrast to EGF and TGF-α, has been characterized as a much more potent mitogen for SMC. The heparin-binding properties of HB-EGF appear to play a role in facilitating its interaction with cell surface proteoglycans and the modulation of its biological properties (12). It has been suggested that HB-EGF is involved in atherogenesis, since HB-EGF is produced by SMC (13,14), macrophages (1), endothelial cells (15), T-lymphocytes (16), and platelets (Kayanoki et al. unpublised observation) in culture and can be detected in SMC and macrophages of atherosclerotic plaques (17).

Cyclic nucleotide phosphodiesterase (PDE) regulates a wide variety of physiological responses in many type of cells and tissues, including platelet aggregation (18), vascular relaxation (19), and cardiac muscle contraction (20). Agents which affect cyclic nucleotide metabolism have received considerable attention for use as therapeutic agents (21), since cyclic nucleotides are known to be key substances, not only in the regulation of cellular function, but also for modulating cellular responces to extracellular events. Cilostazol {6-[4-(1-cyclohexyl-1*H*-tetrazol-5-yl)-butoxy]-3,4-dihydro-2-(1*H*)-quinolinone, MW 369.47} constitutes one such PDE-III inhibitor and has been used clinically as an anti-platelet agent (22). Recent reports indicate that cilostazol suppresses the proliferation of rat aortic smooth muscle cells (23) and rat mesangial cells (24). Moreover, dibutyl cyclic AMP decreased the induction of platelet-derived growth factor-A (PDGF-A) chain mRNA levels and the secretion of PDGF-AA protein by thrombin in human saphenous vein smooth muscle cells (25). The present study reports an investigation of the effect of cilostazol on the expression of HB-EGF in rat aortic smooth muscle cells and U-937 cells. The study is undertaken to determin if the suppression of HB-EGF expression is associated with the inhibition of SMC growth by PDE inhibitors.

Abbreviations used: EGF, epidermal growth factor; HB-EGF, heparin-binding EGF-like growth factor; SMC, smooth muscle cells; TGF-α, transforming growth factor-α; PDGF, platelet derived growth factor; PDE, phosphodiesterase; LPS, lipopolysaccharide; DMEM, Dulbecco's modified Eagle's medium.

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MATERIALS AND METHODS

Materials. Cilostazol was obtained from Otsuka Pharmaceutical Co. (Tokyo, Japan). Lipopolysaccaride (LPS) was obtained from Wako Chemical Co. Ltd. (Kyoto). Methylglyoxal was obtained from Sigma (St. Louis, MO) and further purified by distillation under reduced pressure (b.p. 26 °C, 20 mmHg) (26).

Cell culture. U-937 cells purchased from ATCC were cultured with RPMI 1640 (Nikken Biomedical Laboratories) containing 10% fetal calf serum (FCS), 100 units/ml penicillin G and 100 μg/ml streptomycin sulfate in a humidified atmosphere of 5% CO_2 at 37 °C. Subconfluent cells were stimulated with LPS in fresh media and were incubated for an additional 4 h for mRNA preparation. Rat aortic vascular SMC were isolated from the thoracic aortae of male Wistar rats (body weight 250 g) by enzymatic dissociation according to the method of Gunther et al. (27), and cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FCS, penicillin G (100 units/ml), and streptomycin (100 μg/ml). SMC from passages 4-8 were used for the experiments.

RNA isolation and Northern blot analysis. After incubation with the agents indicated above, cells were washed twice with PBS, scraped off the plates in the presence of 5 mM EDTA, and isolated by centrifugation. Total RNA was isolated by extraction with acid guanidinium thiocyanate-phenol-chloroform (28) and quantitated by measuring the absorbance at 260 nm. Twenty mg of total RNA were heat-denatured at 65°C for 15 min in the presence of 50% formamide and gel running buffer [40 mM morpholinopropanesulphonic acid (Mops), 10 mM sodium acetate, and 1 mM EDTA, pH 7.0] and electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde. The size-fractionated RNAs were transferred onto a Zeta-Probe membrane (Bio Rad) overnight by capillary action, and incubated at 80°C for 60 min. After hybridization with a ^{32}P-labeled human HB-EGF cDNA (1) or rat HB-EGF cDNA probe at 42°C in the presence of 50% formamide, the membranes were washed twice for 80 min at 55 °C with 30 mM sodium citrate, 300 mM NaCl, pH 7.5, and 0.1% sodium dodecyl sulfate. Kodak XAR films were exposed for 1-3 days with an intensifying screen at −80°C. In order to normalize mRNA content, gels were stained with ethidium bromide. All other DNA and RNA manipulations were conducted according to methodology described Maniatis *et al.* (29).

Measurement of HB-EGF activity. Rat aortic vascular SMC were cultured with or without 10 μM cilostazol for 2 days and the conditioned medium was collected. HB-EGF activity was estimated by EP170.7 cell DNA synthesis assay (16) and anti-rat HB-EGF neutralizing antibodies (Horikawa et al., submitted).

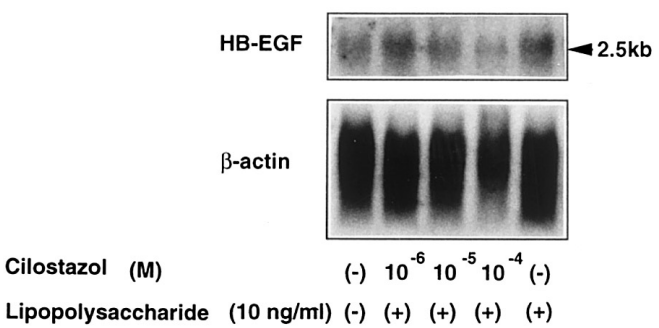

FIG. 1. Suppression of LPS-induced HB-EGF expression by cilostazol in U-937 cells. U-937 cells were stimulated with 10 ng/ml LPS with or without cilostazol for 3 h. Total RNA was isolated and Northern blots were performed using ^{32}P-labeled human HB-EGF cDNA (upper panel) and ^{32}P-labeled human β-actin (lower panel).

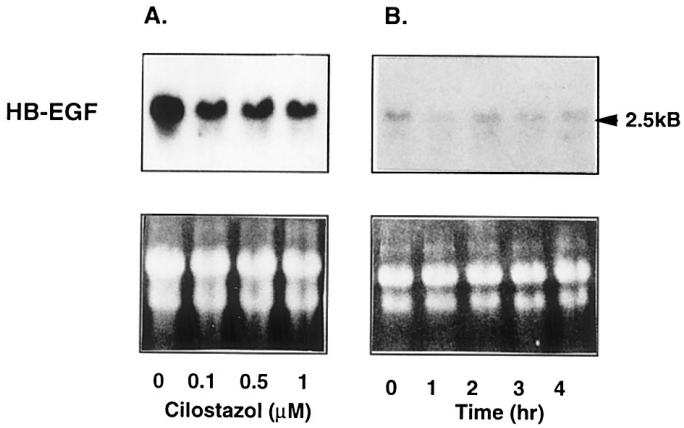

FIG. 2. Suppression of HB-EGF mRNA expression by cilostazol in SMC. (A) SMC were incubated with cilostazol for 3 h. Total RNA was isolated and analyzed by Northern blotting using ^{32}P-labeled human HB-EGF cDNA (upper panel) and ribosomal RNA was indicated in the lower panel. (B) SMC were incubated with 1 μM cilostazol for appropriate intervals. At the time points indicated, total RNA was isolated and analyzed by Northern blotting.

RESULTS

Suppression of LPS-induced HB-EGF expression by cilostazol in U937 cells. It has been reported that macrophages play an important role in atherogenesis via their production of several types of growth factors and cytokines (30). U-937 cells, a macrophage-like cell line, expressed HB-EGF mRNA, even in an unstimulated condition while LPS up-regulated its mRNA level by approximately three fold (Fig. 1). Cilostazol suppressed HB-EGF mRNA expression up-regulated by LPS in a dose dependent manner (Fig. 1).

Suppression of HB-EGF mRNA expression by cilostazol in SMC. The proliferation of SMC is a major event in the progression of atherosclerosis. It has clearly been shown that SMC have been characterized to produce HB-EGF both *in vivo* and *in vitro* (13,14,17), which may stimulate SMC proliferation in an autocrine manner. The addition of 100 nM cilostazol in cultured SMC suppressed the expression level of HB-EGF mRNA by approximately 50% (Fig.2A). In addition, cilostazol constantly suppressed the expression of HB-EGF mRNA during the entire four hour incubation (Fig.2B).

Suppression of methylglyoxal-induced HB-EGF mRNA expression by cilostazol in SMC. Recently, methylglyoxal, a physiological substrate for the glyoxalase system, has been reported to induce HB-EGF mRNA by increasing intracellular peroxide levels, suggesting that these effects might be involved in the development of diabetic complications, including atherosclerosis. In this study, we examined whether cilostazol suppressed methylglyoxal-induced HB-EGF mRNA ex-

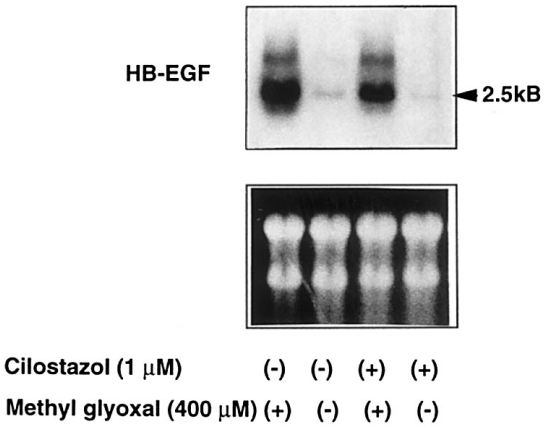

FIG. 3. Suppression of methylglyoxal-induced HB-EGF mRNA expression by cilostazol in SMC. SMC were incubated with a combination of 1 μM cilostazol and 400 μM methylglyoxal for 3 h. Total RNA was isolated and analyzed by Northern blotting using ^{32}P-labeled human HB-EGF cDNA (upper panel) and ribosomal RNA was indicated in the lower panel.

pression in SMC. As shown in Figure 3, the addition of 1 μM cilostazol to cultured SMC suppressed HB-EGF mRNA expression up-regulated by methylglyoxal by approximately 40%.

Cilostazol suppressed production of HB-EGF protein in the conditioned medium of SMC. To measure the HB-EGF released into the conditioned medium of SMC treated with or without cilostazol, an EP170.7 cell assay was employed (16). A ^{3}H-thymidine incorporation assay of SMC showed that the conditioned medium of SMC, which had been treated with 10 μM cilostazol stimulated HB-EGF activity in the conditioned medium of non-stimulated SMC by approximately 45% (Fig.4).

DISCUSSION

The production of several growth factors which cause the proliferation of proliferate SMC are thought to be a major event in the progression of atherosclerosis (30). It has been shown that various types of growth factors stimulate SMC proliferation (30). These findings suggest that agents which interfere with growth factor signaling to proliferate SMC may be of therapeutic value in the prevention and retardation of atherosclerosis. Among these types of growth factors, HB-EGF is a highly potent factor in addition to PDGF (1). HB-EGF is produced by most types of cells which are associated with atherosclerosis (1,13-17). In the present study, we investigated whether cilostazol, a cyclic AMP phosphodiesterase III inhibitor, was capable of supressing the expression of HB-EGF in vascular SMC and a monocyte-like cell line, U-937 cells.

Cilostazol has been reported to suppress the proliferation of several types of cells, such as rat SMC (23) and rat mesangial cells (24). Moreover, some investigaters reported that increased cAMP levels decresed the induction of PDGF-A chain mRNA levels by thrombin. In thrombin-stimulated SMC, increased cAMP levels significantly decreased the secretion of PDGF-AA protein (25). The data herein show that non-stimulated, as well as stimulated expression, of HB-EGF mRNA was suppressed by cilostazol. Taken together, the proliferation of rat SMC could conceivebly be suppressed by cilostazol, as the result of the suppression of both HB-EGF and PDGF by the agent.

Cilostazol has been clinically characterized as an anti-platelet agent. Its anti-aggregatory effect is generally thought to be a contributor in the therapy of atherosclerotic disorders. The present study indicates that the inhibition of growth factors, especially HB-EGF, by the agent might also be valuable in anti-atherosclerotic therapy. Furthermore, in diabetic conditions, the *in vivo* Maillard reaction is accelerated, as a result of continuous hyperglycemia. The serum concentration of methylglyoxal and 3-deoxyglucosone, reactive dicarbonyl metabolites, produced as the result of glycation reaction, are increased in patients with diabetes mellitus. Recently, we reported that methylglyoxal, an intermediate produced in the Maillard reaction, dramatically increased the expression of HB-EGF in SMC (26). In the present study, cilostazol suppressed the induction of HB-EGF by methylglyoxal in SMC. This suggested that cilostazol might also be a useful therapeutic agent for treatment of diabetic angiopathy.

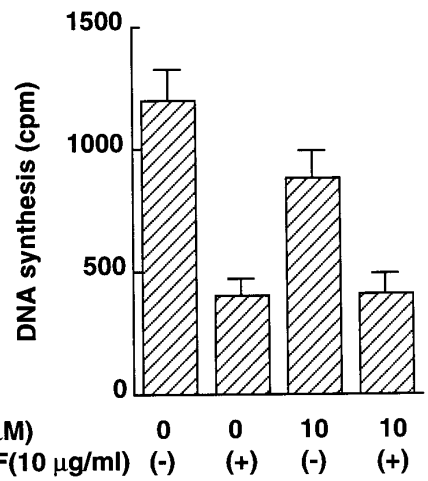

FIG. 4. Diminished ability to proliferate EP170.7 cells of cilostazol-treated conditioned medium of SMC. SMC were cultured with or without 10 μM cilostazol for 2 days and the conditioned media were collected. HB-EGF activities of the conditioned media were measured by EP170.7 cell assay. HB-EGF activity was neutralized by anti-rat HB-EGF neutralizing antibody #19.

Cilostazol inhibits cAMP-phosphodiesterase isozyme PDE-III and subsequently increases intracellular cAMP. Elsewhere, dibutyryl cAMP increases intracellular cAMP so that the induction of the PDGF-A chain mRNA by thrombin is suppressed (25). Collectively, this latter finding and our data, suggest that cAMP itself suppresses the induction of several growth factors, such as HB-EGF and PDGF. Recently, we found that the elevation of intracellular calcium is essential for the induction of HB-EGF (Kayanoki et al., unpublished observation). Since an increase of cAMP levels is thought to decrease intracellular calcium concentration, this decrease might be responsible for the suppressed expression of HB-EGF by cilostazol.

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