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Beraprost Sodium, a Prostaglandin I_2 Analogue, Protects Against Advanced Glycation End Products-induced Injury in Cultured Retinal Pericytes

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Abstract

Background: Beraprost sodium, a prostaglandin I2 analogue, has been recently reported to exhibit beneficial effects on atherosclerosis in patients with diabetes. However, effects of beraprost sodium on microvascular injury in diabetes remain to be elucidated. We have previously shown that advanced glycation end products (AGE), senescent macroproteins formed at an accelerated rate in diabetes, caused pericyte apoptosis, thus being involved in the pathogenesis of the early phase of diabetic retinopathy. In this study, we examined whether beraprost sodium can protect against AGE-induced cytotoxicity in cultured retinal pericytes.
Materials and Methods: Intracellular formation of reactive oxygen species (ROS) was detected using a fluorescent probe. DNA synthesis was determined by measuring [3H]thymidine incorporation into cells. Apoptosis was determined by DNA fragmentations, which were quantitatively measured in an enzyme-linked immunosorbent assay.
Results: Beraprost sodium or forskolin, a stimulator of adenylate cyclase, was found to significantly inhibit AGE-induced ROS generation and the subsequent decrease in DNA synthesis in pericytes. Both treatments significantly prevented AGE-induced apoptotic cell death in pericytes. Furthermore, beraprost sodium was found to down-regulate AGE receptor mRNA levels in pericytes.
Conclusion: The results demonstrated that cyclic AMP-elevating agents such as beraprost sodium and forskolin protected retinal pericytes from AGE-induced cytotoxicity through its anti-oxidative properties. Our present study suggests that beraprost sodium may have therapeutic potentials in treatment of patients with early diabetic retinopathy.

Introduction

Beraprost sodium is a newly developed orally active prostaglandin I2 analogue with antiplatelet and vasodilating properties (1). Recently, beraprost sodium has been reported to exhibit beneficial effects on atherosclerosis in both human and animal models (2,3); indeed, it improved ankle pressure index and symptoms in the lower extremities in diabetic patients with arteriosclerosis obliterans (4). However, effects of beraprost sodium on microvascular injury in diabetes remain to be elucidated.

Diabetic retinopathy is one of the most important microvascular complications in diabetes and is a leading cause of acquired blindness among the people of occupational age (5). Development of diabetic retinopathy is characterized by loss of pericytes and increased vascular permeability, followed by neovascularization in the retinas (6,7).

We have recently found that advanced glycation end products (AGE), senescent macroproteins formed at an accelerated rate in diabetes, caused apoptotic cell death in cultured retinal pericytes, thus being involved in the pathogenesis of the early phase of diabetic retinopathy (8,9). In this study, we examined effects of beraprost sodium on reactive oxygen species (ROS) generation, DNA synthesis and apoptotic cell death in retinal pericytes exposed to AGE.

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Materials and Methods

Materials

Bovine serum albumin (BSA) (fraction V), 2,4,6-trinitrobenzenesulfonic acid, forskolin and N-acetylcysteine (NAC) were purchased from Sigma (St. Louis, MO, USA). D-glyceraldehyde was from Nakalai Tesque (Kyoto, Japan). Beraprost sodium was a gift from Kaken Pharmaceutical Co. Ltd. (Tokyo, Japan). [3H]Thymidine, [γ-^{32}P]ATP, and Hybond-N$^+$ nylon membrane were obtained from Amersham Pharmacia

Biotech (Buckinghamshire, United Kingdom). Reverse transcriptase and T4 polynucleotide kinase were purchased from Takara (Kyoto, Japan).

Cells

Pericytes were isolated from bovine retina and maintained in Dulbecco's modified Eagle's medium (Gibco BRL, Rockville, MD, USA) supplemented with 20% of fetal bovine serum (FBS) (ICN Biomedicals Inc., Aurora, Ohio, USA). AGE treatments were carried out in a medium containing 2% FBS. Medium was changed every two days.

Preparation of AGE-Proteins

AGE-BSA was prepared as described previously (10). Briefly, BSA was incubated under sterile conditions with D-glyceraldehyde for 7 days. Then unincorporated sugars were removed by dialysis against phosphate-buffered saline. Control non-glycated BSA was incubated in the same conditions except for the absence of reducing sugars. Preparations were tested for endotoxin using Endospecy ES-20S system (Seikagaku Co., Tokyo, Japan); no endotoxin was detectable. The extent of chemical modification was determined as described with 2,4,6-trinitrobenzenesulfonic acid as a difference in lysine residues of modified and unmodified protein preparations (11). The extent of lysine modification (%) of modified BSA preparations was 65% for AGE-BSA.

Intracellular ROS

Pericytes were treated with 100 μg/ml of AGE-BSA or non-glycated BSA in the presence or absence of 10 nM beraprost sodium or 1 μM forskolin for 24 h. Then the intracellular formation of ROS was detected by using the fluorescent probe CM-H2DCFDA (Molecular Probes Inc., Eugene, OR) as described previously (12,13).

Measurements of [3H]Thymidine Incorporation in Pericytes

Pericytes were treated with 100 μg/ml of AGE-BSA or non-glycated BSA in the presence or absence of various concentrations of beraprost sodium or forskolin for 2 days. Then [3H]thymidine incorporation was determined as described previously (14).

Measurement of Apoptotic Cell Death in Pericytes

Pericytes were treated with 100 μg/ml of AGE-BSA or non-glycated BSA in the presence or absence of 10 nM beraprost sodium or 1 μM forskolin for 5 days. Then cells were lysed and the supernatant was analyzed in an enzyme-linked immunosorbent assay (ELISA) for DNA fragments (Cell Death Detection ELISA, Roche Molecular Biochemicals, Mannheim, Germany).

Primers and Probes

Sequences of the upstream and down stream primers and the internal probe used in the quantitative reverse transcription-polymerase chain reactions (RT-PCR) for detecting bovine receptor for AGE (RAGE) and β-actin mRNAs were the same as described previously (8).

Poly(A)+RNA Isolation and Quantitative RT-PCR

Poly(A)+RNAs were isolated (15) from pericytes treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 10 nM beraprost sodium for 2 days, and then analysed by RT-PCR as described previously (16). Ten-μl aliquots of each RT-PCR reaction mixture were electrophoresed on a 1.2% agarose gel, transferred to a Hybond-N^+ nylon membrane, and the membrane was then hybridized with the respective 32P-end labeled probes (17). The amounts of the poly(A)+RNA templates (30 ng) and the cycle numbers (37 cycles) for amplification were chosen in quantitative ranges where the reactions proceeded linearly, which had been determined by plotting signal intensities as functions of the template amounts and cycle numbers (8). Signal intensities of hybridized bands were measured by microcomputer-assisted NIH Image (Version 1.56).

Statistical Analysis

All values were presented as means ± S. E. Statistical significance was evaluated using the Studentís t test for paired comparison; $p < 0.05$ was considered significant.

Results

Effects of Beraprost Sodium or Forskolin on AGE-Induced Intracellular ROS Generation in Pericytes

As shown in Fig. 1, AGE significantly increased intracellular ROS generation in cultured retinal pericytes. Beraprost sodium or forskolin was found to completely inhibit the AGE-induced increase in ROS generation in pericytes.

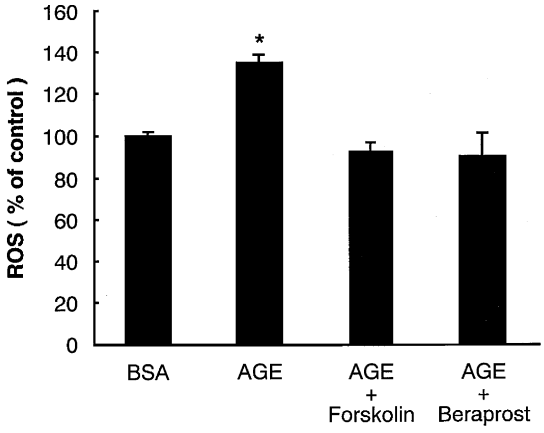

Fig. 1. Effects of beraprost sodium or forskolin on intracellular ROS generation in retinal pericytes. Pericytes were treated with 100 μg/ml of AGE-BSA or non-glycated BSA in the presence or absence of 10 nM beraprost sodium or 1 mM forskolin for 24 h, and then ROS were quantitatively analysed. *, $P < 0.01$ compared to the value of the control with non-glycated BSA alone.

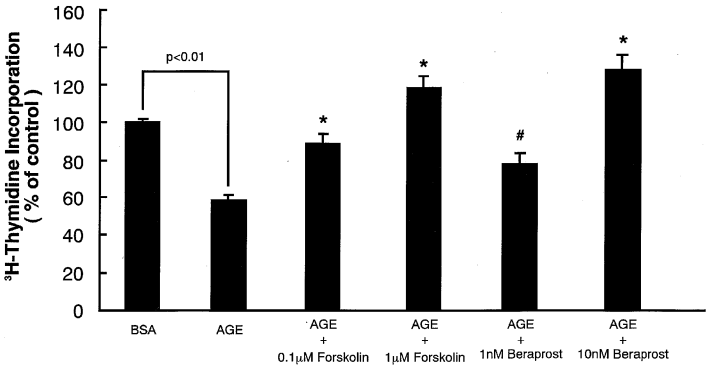

Fig. 2. Effects of beraprost sodium or forskolin on [3H]thymidine incorporation into retinal pericytes. Pericytes were treated with 100 μg/ml of AGE-BSA or non-glycated BSA in the presence or absence of the indicated concentrations of beraprost sodium or forskolin 48 h. For the last 4 h of culture, the cells were pulsed with 2 mCi [3H]thymidine. After cells were washed with ice-cold phosphate-buffered saline, [3H]thymidine incorporation into the cells was assayed. The percentage of [3H]thymidine incorporation is indicated on the ordinate and related to the value with non-glycated BSA alone. #, $P < 0.05$; *, $P < 0.01$ compared to the value of the control with AGE-BSA alone.

Effects of Beraprost Sodium or Forskolin on AGE-Induced Inhibition in DNA Synthesis in Pericytes

We have recently found that an anti-oxidant, NAC, completely prevented the AGE-induced inhibition in DNA synthesis in pericytes (unpublished data). Therefore, we next investigated whether inhibition of ROS generation induced by beraprost sodium or forskolin could block the cytopathic effects of AGE in cultured pericytes. As shown in Fig. 2, beraprost sodium or forskolin prevented the AGE-induced inhibition in DNA synthesis in pericytes in a dose-dependent manner; 10 nM beraprost sodium or 1 μM forskolin completely prevented the deleterious effects of AGE.

Effects of Beraprost Sodium or Forskolin on AGE-Induced Apoptotic Cell Death in Pericytes

We next investigated whether beraprost sodium or forskolin could prevent the AGE-induced apoptotic cell death in cultured pericytes. Apoptosis is characterized by DNA fragmentations due to endogenous endonuclease activation (18). Therefore, we quantitatively measured DNA fragments in the cytoplasm of cultured pericytes. As shown in Fig. 3, beraprost sodium or forskolin significantly inhibited the AGE-induced apoptotic cell death in cultured pericytes.

Effects of Beraprost Sodium on RAGE Gene Expression in Retinal Pericytes

We have recently found that AGE induced apoptotic cell death in bovine retinal pericytes through interaction with RAGE (8,9). Therefore, poly(A)+ RNAs were isolated from pericytes, and analysed by a quantitative RT-PCR technique to determine effect of beraprost sodium on the expression of RAGE genes. As shown in Fig. 4, beraprost sodium down-regulated RAGE mRNA levels in pericytes.

Discussion

In the present study, we demonstrated for the first time that beraprost sodium or forskolin inhibited AGE-induced ROS generation and the subsequent decrease in DNA synthesis and apoptotic cell death in cultured pericytes. Since we have recently found that an antioxidant, NAC, also prevented the AGE-induced cytotoxicity to pericytes, it is probable that beraprost sodium or forskolin could attenuate the deleterious effects of AGE through its anti-oxidative properties. Recently, Shimura et al. reported that ROS-induced endothelial cell injury was significantly blocked by treatments with other cyclic AMP elevating agents such as dibutyryl cyclic AMP and isobutylmethyl xanthine (19). Furthermore, prostacyclin analogue suppressed ROS-induced hepatocyte injury by elevating intracellular cyclic AMP levels (20). These observations suggest that cyclic AMP might mediate protection against oxidative stress in various types of cells.

We were not able to completely clarify here the molecular mechanism for ROS suppression by beraprost sodium in pericytes. However, diphenylene iodonium, an inhibitor NADPH oxidase, did not

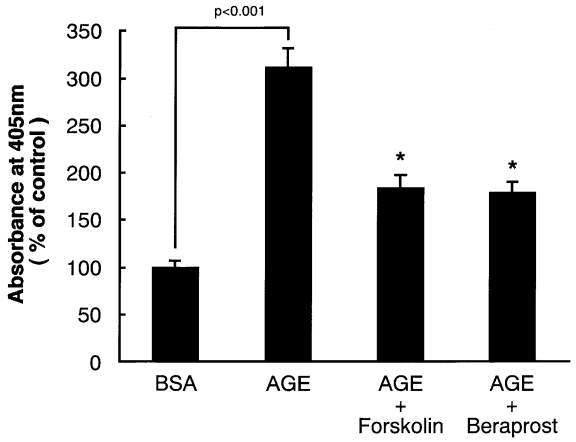

Fig. 3. Effects of beraprost sodium or forskolin on apoptotic cell death in retinal pericytes. Pericytes were treated with 100 μg/ml of AGE-BSA or non-glycated BSA in the presence or absence of 10 nM beraprost sodium or 1 mM forskolin for 5 days. Then cells were lysed and the supernatant analyzed in an enzyme-linked immunosorbent assay for DNA fragments. The percentage of absorbance at 405 nm is indicated on the ordinate and related to the value of the control with non-glycated BSA alone. *, $P < 0.01$ compared to the value of the control of with AGE-BSA alone.

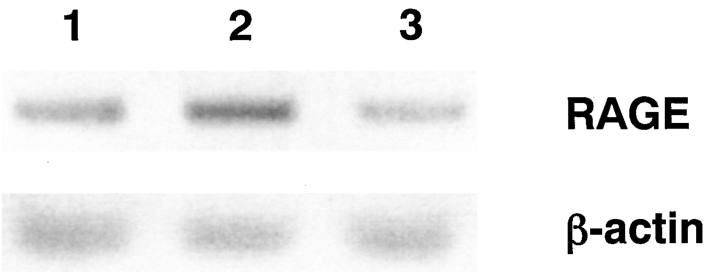

Fig. 4. Effects of sodium beraprost on RAGE gene expression in retinal pericytes. Pericytes were treated with 100 μg/ml AGE-BSA or non-glycated BSA in the presence or absence of 10 nM beraprost sodium for 2 days, and then 30 ng poly(A)+ RNAs were transcribed and amplified by PCR. Each lower panel shows the expression of β-actin genes. PCR amplification for β-actin mRNA was performed for 22 cycles. Lane 1; cells treated with BSA alone, lane 2; cells treated with AGE-BSA alone, lane 3; cells treated with AGE-BSA plus 10 nM beraprost sodium.

prevent the AGE-induced ROS generation and the subsequent inhibition in DNA synthesis in pericytes (data not shown). Therefore, although cyclic AMP elevating agents were known to block ROS generation in neutrophils by suppressing NADPH oxidase (21), it is unlikely that inhibition of NADPH oxidase might be an initial mechanism for beraprost sodium-elicited inhibition of ROS generation in pericytes.

In the present study, beraprost sodium down-regulated RAGE mRNA levels in pericytes exposed to AGE. Since we have previously shown that AGE used in these experiments induced apoptotic cell death in pericytes through interaction with RAGE proteins (8,9), anti-apoptotic effects of beraprost sodium observed here could be, at least in part, ascribed to inhibition of RAGE protein expression in cultured pericytes.

There is a growing body of evidence that beraprost sodium exhibited beneficial effects on atherosclerosis in animal models (3,22). Recently, beraprost sodium was found to be an effective treatment of symptomatic patients with intermittent claudication (2), and it also improved ankle pressure index in the lower extremities in diabetic patients with arteriosclerosis obliterans (4). Since pericytes play an important role in the maintenance of microvascular homeostasis (23,24), our present study provided another beneficial aspects of beraprost sodium; beraprost sodium could halt the progression of diabetic retinopathy by preventing AGE-induced apoptotic cell death in pericytes.

In conclusion, we demonstrated in the present study that beraprost sodium protected cultured pericytes against AGE injury through its anti-oxidative properties. Cyclic AMP elevating agents such as beraprost sodium may theoretically help circumvent the development and progression of diabetic retinopathy by blocking the AGE signaling pathway in pericytes.

Acknowledgments

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