Attenuation of glucose-augmented expression of TGF-β1 and oncofetal fibronectin in human umbilical vein endothelial cells by 2-aminophenoxazine-3-one

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Abstract

The aim of this study was to investigate whether 2-aminophenoxazine-3-one (Phx-3) may attenuate the increased mRNA level of TGF-β1 and extra domain oncofetal fibronectin (EDB-FN) in human umbilical vein endothelial cells (HUVECs) treated with higher concentrations of glucose, because these proteins are considered to play critical roles in diabetic retinopathy. The mRNA level of TGF-β1 was significantly increased in HUVECs treated with high concentration of glucose (15 mM), compared with that in the cells with a normal concentration of glucose (5 mM). The levels of mRNA and protein of TGF-β1 returned to the normal levels, when high glucose-treated HUVECs were administered with 10 μM Phx-3, at which concentration the proliferation of HUVECs was not affected. The level of EDB-FN mRNA was also augmented in HUVECs treated with higher concentration of glucose, and was reversed by 10 μM Phx-3 administration. The present results suggest that Phx-3 has the ability to attenuate the levels of mRNA of TGF-β1 and EDB-FN which are inducible in HUVECs treated with higher concentration of glucose, and thereby may prevent or limit angiogenesis in diabetic retinopathy. The present results also support the concept that the TGF-β1 level regulates the levels of EDB-FN in endothelial cells. Phx-3 may prevent high glucose-associated endothelial damage, vascular angiogenesis and diabetic retinopathy in diabetic patients, by downregulating the expression of TGF-β1 and EDB-FN.

Introduction

It has been recognized that the thickening of the capillary basement membrane, associated with increased deposition of extracellular matrix protein, is the main event causing blindness in adults with diabetic retinopathy as well as mortality due to diabetic macroangiopathy. Fibronectin (FN) is an extracellular matrix protein, which plays key roles in various cellular events including proliferation and migration of endothelial cells and fibroblasts, and therefore may be respon-
may help prevent the development of diabetic retinopathy.

On the other hand, Khan et al. who reported that glucose-induced expression of EDB-FN in endothelial cells is mediated by transforming growth factor-β1 (TGF-β1) and endothelin-1 (ET-1)⁶, suggesting that the changes in the levels of these cytokines may affect those of EDB-FN. We previously demonstrated that glucose-induced expression of ET-1 in HUVECs was reversed to normal levels by Phx-3, which was produced by the reactions of o-aminophenol with bovine hemoglobin solution⁹. This result suggests that the levels of EDB-FN may be reduced by this phenoxazine in HUVECs treated with higher concentration of glucose.

It is also interesting to investigate whether Phx-3 may reduce the glucose-induced expression of TGF-β1 in HUVECs or not, because the reduction of TGF-β1 level causes the level of EDB-FN. These facts prompted us to investigate the effects of Phx-3 on the mRNA expression of TGF-β1 and in HUVECs.

Materials and methods

Phx-3

Phx-3 was prepared by the reactions of bovine hemoglobin solution and o-aminophenol hydrochloride (Wako Pure Chemicals, Tokyo), and was purified using a column of Sephadex LH20 (4 cm × 50 cm), previously equilibrated with 50% ethanol, and eluted with 50% ethanol. Purified Phx-3 was identified by measuring UV and visible spectra, and by thin layer chromatography⁹. The chemical structure is shown in Fig. 1.

Cell culture methods and cell proliferation assay

HUVECs were seeded on a 96-well microplate (Asahi Technoglass, Tokyo) at a density of 5,000 cells per well in EGM-2 culture medium CAMBREX MD, USA) supplemented with 2% fetal bovine serum (FBS, CAMBREX) and 10 μg/1 human recombinant epidermal growth factor, and were cultured at 37°C in 5% CO₂/air for 24 hours. HUVECs were used at the 7th passage. Appropriate concentrations of glucose (final concentrations: 5 mM, as normal glucose concentration or 15 mM, as high glucose concentration) were added to the medium, when the cells were 80% confluent. 10 μM Phx-3 dissolved in dimethyl sulfoxide (DMSO) was diluted with EGM-2 and added to the culture medium containing HUVECs to make a final concentration of 10 μM. Cell proliferation was evaluated by the microculture tetrazolium WST-8 assay using a Cell Counting Kit-8® (Dojindo Molecular Technologies, Kumamoto, Japan)⁹.

Isolation of RNA and synthesis of cDNA

HUVECs (1 × 10⁶) were grown on poly-L-lysine coated dishes at 37°C for 24 hours. Then, the cells were treated with 5 mM glucose alone, 5 mM glucose plus 10 μM Phx-3, 15 mM glucose alone or 15 mM glucose plus 10 μM Phx-3, and were incubated at 37°C for 24 hours. Then, total RNA was isolated from HUVECs using TRizol® reagent (Invitrogen, Japan)¹⁰. After addition of chloroform, samples were centrifuged to separate aqueous and organic phases. RNA was recovered from the aqueous phase by isopropyl alcohol precipitation and suspended in DEPC-treated water. RNA was quantified by measuring UV absorbance at 260 nm. The purity of samples was determined by calculating the ratio of absorbance at 260 nm and 280 nm.

Four μg of total RNA was used for cDNA synthesis using the SuperScript I® system (Invitrogen). Briefly, RNA was added to Oligo (dT) primers (Invitrogen) and denatured at 70°C for 10 min. Reverse transcription was carried out by addition of MMLV reverse transcriptase and incubation of samples at 42°C for 55 min and 70°C for 15 min. The resulting cDNA produced were stored at −20°C.

Quantification of Real Time PCR

Real time polymerase chain reaction was carried out in the Opticon2™ (Bio Rad Japan, Tokyo) using SYBR Green 1®. PCR reactions were performed in 96-well multiplate™ (Bio Rad) with a final volume of 20 μl. The reaction mixture consisted of 10 μl of 2× DYNAmo™ SYBR Green qPCR kit (Bio Rad), 0.5 μl of each forward and reverse 10 μmol/l primers, 8 μl H₂O and 1 μl cDNA template. Primers for TGF-β1, total FN, and EDB-FN were designed by use of the Primer 3 software (Whitehead Institute, Cambridge, MA, USA). The gene-specific forward and reverse primer sequences are 5’-GCCCAGTGGTGGCAACATG-3’ and 5’-CGTTGCTCCCTGACAGAAG-3’ for TGF-β1 with the expected 63 bp PCR product, and 5’-CATGAGCCAGTGCAAGCC-3’ and 5’-TCCTGCAAGCTGTTGTTGA-3’ for total FN with the expected 184 bp PCR product, 5’-GGTTGGTGGGAACC-3’ and 5’-ATGTTGGTGGGCAAGAAG-3’ for EDB-FN with the expected 130 bp PCR product, 5’-GAAGTGGTGGCAAC-3’ and 5’-CTACCAGAGATGTATGG-3’ for 18S rRNA with the expected 153 bp PCR product.

The following PCR programs was performed on the Opticon 2: an initial denaturation at 95°C for 15 min to activate DNA polymerase, followed by a 39 cycle pro-
gram consisting of heating at 20°C/s to 95°C with 10s hold, cooling at 51°C for 20s for annealing and heating at 20°C/s to 72°C with 30s hold for extension. The fluorescent level of products was measured at 80°C after the extension phase. PCR was immediately followed by a melting curve analysis to determine the melting point of the double stranded PCR product produced. Each mRNA level was quantified using the standard curve method. Standard curves were constructed using serially diluted standard template. Ct value was used to compute mRNA levels from the standard curve. Each mRNA level was normalized by using the ratio to ribosomal RNA, to account for differences in reverse transcription efficiencies and amount of cDNA in reaction mixtures.

**Immunoblotting analysis of TGF-β1 protein**

HUVECs (1×10⁶ cells) were grown on poly-L-lysine coated dishes, at 37°C, for 24 hours. Then, the cells were treated with 5 mM glucose alone, 5 mM glucose plus 10 μM Phx-3, 15 mM glucose alone or 15 mM glucose plus 10 μM Phx-3, and were incubated at 37°C for 24 hours. The cells in each dish were rinsed twice with chilled PBS and lysed with the solution composed of RIPA Lysis buffer Kit (Santa Cruz Biotechnology, CA, USA), for 30 min at 4°C. The whole cell extracts were sedimented at 15,000 rpm for 5 min to remove the insoluble material. Total protein concentration in the supernatants was determined using the Bio-Rad Protein Assay Reagent (Life Science, Tokyo, Japan). Namely, the extracts containing 20 μg proteins were heated to 100°C for 5 min, and subjected to SDS-PAGE electrophoresis. The proteins were transferred to PVDF membranes using XCell II SureLock Mini-Cell system (Invitrogen). The membranes were blocked with 5% non-fat milk prior to immunoblot analysis reacted with anti-human TGF-β1 mouse IgG (Sigma, St Louis, MO, USA) diluted to 5,000 times by CanGet Signal kit (Toyobo Co Ltd, Osaka, Japan) for primary antibody and anti-mouse IgG horseradish peroxidase linked whole antibody from sheep (GE Healthcare, UK) diluted to 20,000 times for secondary antibody. Monoclonal anti-α-tubulin (Sigma) diluted to 5,000 times was used as an internal control. Finally, the blots were visualized using the enhanced chemiluminescence system (ECL advanced, GE Healthcare).

**Statistics**

The data were expressed as means ± S.E.M. and were analyzed by ANOVA followed by Tukey’s post-hoc test. A p-value less than 0.05 was considered to indicate a statistically significant difference.

**Results**

**Effects of Phx-3 on endothelial proliferation**

Proliferation of HUVECs in the presence of normal (5 mM) glucose or high (15 mM) glucose, and the effects of various concentrations of Phx-3 (10 to 100 μM) for 24 hours was estimated by WST-3 assay. It was found that the cell counts were increased gradually during 24 hours incubation of HUVECs in the presence of 5 mM or 15 mM glucose, 5 mM glucose plus 10 μM Phx-3 or 15 mM glucose plus 10 μM Phx-3. Since the results agreed well with those described in our previous report, the data were not presented. The curves of these groups increased at the similar rates after 24 hours. These results indicated that proliferation of HUVECs were not affected by Phx-3 at concentration of 10 μM. Thus, we used 10 μM Phx-3 for the following experiments.

**High glucose augmented expression of TGF-β1 mRNA**

We investigated the level of TGF-β1 mRNA in HUVECs treated with either 5 mM glucose alone, 5 mM glucose plus 10 μM Phx-3, 15 mM glucose alone or 15 mM glucose plus 10 μM Phx-3 for 24 hours. As shown in Fig. 2, the level of TGF-β1 mRNA in the HUVECs treated with 5 mM glucose was not altered by addition of 10 μM Phx-3 after 24 hours. On the other hand, the levels of TGF-β1 mRNA in the HUVECs treated with 15 mM glucose alone increased up to approximately 200% of 5 mM glucose alone after 24 hours (third column from the left in Fig. 2). Such augmented expression of TGF-β1 mRNA was reversed to the levels of normal control by 10 μM Phx-3 (fourth column from the left in Fig. 2).

Fig. 3 shows the representative immunoblotting patterns of TGF-β1 in cells treated with 5 mM glucose alone, 5 mM glucose plus 10 μM Phx-3, 15 mM glucose alone or 15 mM glucose plus 10 μM Phx-3. The relative ratio of TGF-β1 protein to α-tubulin was not changed in HUVECs treated with 5 mM glucose plus 10 μM Phx-3 (second column from the left in Fig. 4), compared with the cells treated with 5 mM glucose alone.

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**Fig. 2** Changes in expressed amounts of mRNA of TGF-β1 in HUVECs. Level of mRNA are expressed as the ratio of TGF-β mRNA to 18S rRNA. Cells were treated with 5 mM glucose alone, 5 mM glucose plus 10 μM Phx-3, 15 mM glucose alone, and 15 mM glucose plus 10 μM Phx-3 for 24 hours (n=3) (*P<0.05).
We investigated the expression of EDB-FN mRNA in HUVECs by calculating the ratio of EDB-FN mRNA to total FN mRNA. Cells were treated with either 5 mM glucose alone, 5 mM glucose plus 10 μM Phx-3, 15 mM glucose alone or 15 mM glucose plus 10 μM Phx-3 for 24 hours shown in Fig. 5. The level of EDB-FN mRNA in the HUVECs with 5 mM glucose was not affected by addition of 10 μM Phx-3 (second column from the left in Fig. 5) (P > 0.05). The levels of EDB-FN mRNA in the HUVECs treated with 15 mM glucose increased up to approximately 250% of 5 mM glucose alone (third column from the left in Fig. 5). However, this was reversed to the levels of 5 mM glucose alone by the addition of 10 μM Phx-3 (fourth column from the left in Fig. 5).

**Discussion**

The vascular damage caused by hyperglycemia in patients with diabetes may induce dysfunction of endothelial cells and the activation of smooth muscle cells in the vascular wall, initiating the events in angiogenesis and atherosclerosis. These events are promoted by various cytokines and proteins such as TGF-β1, ET-1, plasminogen activator inhibitor-1 (PAI-1), intercellular adhesion molecule 1, vascular cell adhesion molecule 1 and fibronectin, which are produced and released from endothelial cells and smooth muscle cells in the vascular wall. Khan et al. reported that the production of both TGF-β1 and EDB-FN was enhanced in HUVECs treated with higher concentrations of glucose.

We demonstrated that the level of TGF-β1 mRNA in HUVECs was promoted by the addition of high concent-...
trations of glucose (Fig. 2) which is consistent with the report of Khan et al., and that high concentrations of glucose induced TGF-β1 mRNA level was attenuated by addition of 10 μM Phx-3 (Fig. 2). This result was confirmed by the changes in TGF-β1 in HUVECs treated with high glucose and Phx-3 (Fig. 3 and 4).

The behavior of EDB-FN mRNA level was consistent with that of TGF-β1 mRNA. The level of EDB-FN mRNA increased in HUVECs treated with high concentrations of glucose, and was reversed to normal values by addition of Phx-3 (Fig. 5). We have previously showed that expression of endothelin-1, which is known to stimulate the gene expression of EDB-FN in endothelial cells, in HUVECs is augmented by high concentrations of glucose, and this augmentation was attenuated by Phx-3. These results support the view that TGF-β1 and ET-1 regulate the expression of EDB-FN in endothelial cells as indicated by Khan et al.

Phx-3 is a phenoxyazine compound that was originally identified as Questiomycin B, but its biological activities have been unclear until recently. Later, it was found to be produced by the reactions of o-aminophenol with human or bovine hemoglobin solution. Recently, Phx-3 has been shown to have various biological activities such as antimicrobial effects on mycobacteria and Chlamydia pneumoniae and anticancer effects on malignant melanoma cell line and human pancreas cancer cells. We previously demonstrated that Phx-3 downregulates the high concentrations of glucose augmented-embryonic form of smooth muscle myosin heavy chain, and PAI-1, ET-1 in HUVECs. In this study, we showed that TGF-β1 and EDB-FN, which play a critical role in the angiogenesis in diabetic retinopathy, were upregulated in HUVECs treated with high concentrations of glucose, and were downregulated by Phx-3 (Fig. 2–5). The mechanism for Phx-3 downregulation of TGF-β1, endothelin-1 and EDB-FN remains obscure. Since Phx-3 shows little adverse effects on mice. Phx-3 may be beneficial for preventing diabetic angiogenesis and thereby delaying or preventing the development of diabetic retinopathy, where various angiogenic events are provoked by high concentrations of glucose.

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References


脱落静脈血管内皮細胞における高濃度ブドウ糖によって惹起されるTGF-β1と癌胎児性ファイブロネクチンの増加に対する
2-Aminophenozone-3-oneの作用

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【要旨】糖尿病網膜症は成人の失明の主たる要因であり、大血管障害は生命予後を悪化させる。その病態は細胞外マトリックスの増加による毛細血管基底膜の肥厚であり、中でもファイブロネクチンが血管内皮細胞、線維芽細胞の増殖、遊走に関与している。Khanらは、選択的プロモーションによって生成される多様なファイブロネクチンのうちEBFの癌胎児性ファイブロネクチン（EDF-FN）が糖尿病網膜症で多く発現することを報告し、血管新生のEDF-FNが重要な役割を演じることを示唆した。また、高濃度ブドウ糖により増加するTGF-β1、エンドセリン1とEDF-FNとの関連も明らかとなった。一方、水溶性フェノキサシン(Phx-3)は血液成分をもと抗生物質として発見されたが、私共は血管内皮細胞培養系にて高濃度ブドウ糖条件下で増加する胎児性平滑筋ミオシン重鎖、PAI-1、エンドセリン1などの因子がPhx-3で抑制されることを明らかにし、Phx-3が高濃度ブドウ糖関連血管内皮障害を抑制する可能性を示唆した。今回は、Phx-3濃度が20μM以下でヒト脱落静脈血管内皮細胞（HUVEC）の増殖を阻害しないことを確認した。さらに、HUVECを正常濃度ブドウ糖条件下（5 mM）および高濃度ブドウ糖条件下（15 mM）で、それぞれにPhx-3（10μM）を添加した4群のHUVECを24時間培養し、TGF-β1およびEDF-FNにおけるmRNAの発現量をreal time RT-PCR法を用いて比較検討した。その結果、高濃度ブドウ糖条件下ではTGF-β1は約2倍に、EDF-FNでは約2.5倍に増加したが、Phx-3添加群ではその増加はほぼ完全に抑制された。TGF-β1の蛋白発現量においても同様の結果であった。以上の結果より、Phx-3は高濃度ブドウ糖によって惹起されるHUVEC内的血管新生因子の増加に対して抑制的に作用する可能性を有することが明らかになった。このような血管網膜症の進展阻止につながる可能性が示唆された。

（キーワード）ヒト脱落静脈血管内皮細胞、糖尿病、2-アミノフェノキサシン-3-オン、TGF-β1、癌胎児性ファイブロネクチン