

## 2-Amino-phenoxazine-3-one Attenuates Glucose-Induced Augmentation of Embryonic Form of Myosin Heavy Chain, Endothelin-1 and Plasminogen Activator Inhibitor-1 in Human Umbilical Vein Endothelial Cells

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The aim of this study was to investigate the changes in mRNA level of embryonic form of myosin heavy chain (SMemb), endothelin-1 (ET-1) and plasminogen activator inhibitor-1 (PAI-1), which are considered to be involved in the angiogenesis and atherosclerosis in diabetic blood vessels, in human umbilical vein endothelial cells (HUVECs) caused by high ambient glucose, and the effects of 2-aminophenoxazine-3-one (Phx-3), which was produced by the reaction of bovine hemoglobin with *o*-aminophenol, on them. The mRNA level of SMemb, ET-1 and PAI-1 and the level of SMemb protein were extensively upregulated in HUVECs treated with high concentration of glucose (15 mM), compared with those in the cells with normal concentration of glucose (5 mM). The migration activity of HUVECs evaluated by the cell migration assay was accelerated by 15 mM glucose. When 10  $\mu$ M Phx-3, at the concentration of which the proliferation of HUVECs was not affected, was administered to HUVECs with 15 mM glucose, the mRNA level of SMemb, ET-1 and PAI-1 and the level of SMemb protein were significantly downregulated to the normal levels in the cells. However, when 10  $\mu$ M Phx-3 was administered to HUVECs with 5 mM of glucose, the mRNA level of SMemb, ET-1 and PAI-1 and the level of SMemb protein were not affected. The migration activity of HUVECs, which was accelerated by high glucose, was reversed by 10  $\mu$ M Phx-3. The present results suggest that Phx-3 may be a drug to prevent the high glucose-associated endothelial damage, vascular angiogenesis in diabetic patients, by inhibiting the expression of angiogenic factors, such as SMemb, ET-1 and PAI-1, in the endothelial cells.

**Key words** human umbilical vein endothelial cell; high glucose; 2-aminophenoxazine-3-one

Endothelial migration, proliferation and differentiation have been recognized to be associated with diabetes induced angiogenesis and atherosclerosis,<sup>1–3</sup> inflammation,<sup>2,4,5</sup> and angiogenesis in malignancies,<sup>2,6</sup> where a number of cytokines and growth factors released by the endothelial cells and the smooth muscle cells are involved. In diabetes, the vascular damage caused by hyperglycemia may induce dysfunction of endothelial cells and the activation of smooth muscle cells in the vascular wall, initiating the events in angiogenesis and atherosclerosis.<sup>1–3</sup> Therefore, it may be important to study the expression of the factors in these cells under hyperglycemic conditions. Especially, expression of endothelin-1 (ET-1), a vasoconstrictive peptide, plasminogen activator inhibitor-1 (PAI-1), a prothrombic protein, and ICAM-1, VCAM-1 and ECAM-1, a group of adhesion proteins, are of significant interest in this context.<sup>7–12</sup> Chen *et al.*<sup>7,8</sup> reported that the upregulated expression of endothelin-1 was shown in human umbilical vein endothelial cells (HUVECs) under hyperglycemic conditions, however, the behavior of PAI-1 in the cells was not studied under such conditions, hitherto.

On the other hand, the phenotypic switch of smooth muscle cells from contractile to synthetic type manifested by expression of an embryonic form of myosin heavy chain (SMemb) was demonstrated to be relevant to the atherosclerosis in diabetes.<sup>1,13</sup> However, it was not sufficiently investigated whether SMemb may be expressed in the HUVECs

and whether the expression of SMemb may be changed under hyperglycemic conditions.

The development of drugs to attenuate the expression of endothelial factors in the vascular endothelial cells are critical for preventing the angiogenesis and atherosclerosis in the diabetic patients. Lohray *et al.*<sup>14</sup> indicated that a phenoxazine analogue of phenyl propanoic acid activated peroxisome proliferator-activator inhibitor gamma (PPAR $\gamma$ ), which is highly expressed in adipocytes,<sup>15</sup> and is involved in the improvement of type 2 diabetes through insulin sensitizing mechanism.<sup>16</sup> Chemically synthesized phenoxazines are generally insoluble in water, and have been shown to exert limited biological effects including anticancer activity.<sup>17,18</sup> Hence, phenoxazine compounds have not been studied in terms of biological activities, except for actinomycin D having strong anticancer effects but causing adverse effects.<sup>19</sup> In the 1990s, Tomoda *et al.*<sup>20–22</sup> reported that novel and relatively water-soluble phenoxazines such as 2-amino-4,4 $\alpha$ -dihydro-4 $\alpha$ ,7-dimethyl-3*H*-phenoxazine-3-one (Phx-1), 3-amino-1,4 $\alpha$ -dihydro-4 $\alpha$ ,8-dimethyl-2*H*-phenoxazine-2-one (Phx-2), and 2-amino-phenoxazine-3-one (Phx-3) were produced, when *o*-aminophenol and its derivatives (2-amino-5-methylphenol, 2-amino-4-methylphenol) were reacted with human or bovine hemoglobin. Among these phenoxazines, the biological effects of Phx-1 have been exclusively studied, in terms of antitumor effects *in vivo* and *in vitro*,<sup>23,24</sup> immunosuppressive effects,<sup>25,26</sup> anti-proliferating effects on

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polio virus transfected in Vero cells.<sup>27)</sup> However, the biological activity of Phx-3 remained obscure, though only anti-mycobacterial activity of Phx-3 was indicated by Anzai *et al.*<sup>28)</sup> and Shimizu *et al.*<sup>22)</sup> Therefore, it will be tempting to study the effects of novel phenoxazines on high glucose-induced changes in the endothelial factors that may be involved in the diabetes induced angiogenic and atherogenic processes.

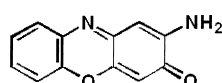
Present manuscript deals with the augmentation of SMemb, ET-1 and PAI-1 in human umbilical endothelial cells (HUVECs) under hyperglycemic conditions, and the attenuating effects of Phx-3 on high glucose augmented expression of these endothelial factors in HUVECs. The possibility of Phx-3 to treat the atherosclerotic lesions in type 2 diabetes was suggested.

## 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 by using a column of Sephadex LH20 (4 cm×50 cm), previously equilibrated with 50% ethanol, and eluted with 50% ethanol.<sup>22)</sup> Purified Phx-3 was identified by measuring UV and visible spectra, and by a thin layer chromatography.<sup>22)</sup> 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 5000 cells per well in EGM-2 culture medium (Takara Bio Japan, Tokyo) supplemented with 2% fetal bovine serum (FBS, Invitrogen Japan, Tokyo) and 10 μg/l human recombinant epidermal growth factor, and were cultured at 37 °C, in 5% CO<sub>2</sub>/air, for 24 h. 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. Ten mM 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 assay using WST-8 (Dojindo Molecular Technologies, Kumamoto, Japan).<sup>29)</sup>

**Isolation of RNA and Synthesis of cDNA** HUVECs (1×10<sup>6</sup> cells) were grown on poly-L-lysine coated dishes, at 37 °C, for 24 h. 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 8 h. Then, total RNA was isolated from HUVECs using TRIzol<sup>TM</sup> reagent (Invitrogen Japan).<sup>8)</sup> 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. Purity of samples was de-



Phx-3

Fig. 1. Chemical Structure of Phx-3

termined by calculating the ratio of absorbance at 260 nm and 280 nm.

Four micrograms of total RNA was used for cDNA synthesis using the Superscript II<sup>TM</sup> system (Invitrogen Japan). Briefly, RNA was added to Oligo (dT) primers 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. The resulting cDNA products were stored at -20 °C.

**Real Time PCR** Real time polymerase chain reaction (RT-PCR) was carried out in the Opticon2<sup>TM</sup> (MJ Japan, Tokyo) using SYBR·Green 1.<sup>30)</sup> PCR reactions were performed in 96-well multiplate<sup>TM</sup> (MJ Japan) with a final volume of 20 μl. The reaction mixture consisted of 10 μl of 2×DyNAmo<sup>TM</sup> SYBR·Green qPCR kit (MJ Japan), 0.5 μl of each forward and reverse 10 μmol/l primers, 8 μl H<sub>2</sub>O and 1 μl cDNA template. Primers for SMemb, ET-1, PAI-1 and β-actin were designed by use of the Primer 3 software (Whitehead Institute, Cambridge, MA, U.S.A.). The gene specific forward and reverse primer sequences are 5'-AGAGGAGGCAATACAGTGG-3', 5'-TGGAGAGCCTTAAGACACAG-3' for SMemb mRNA with the expected 117 bp PCR product, 5'-TCCTCTGCTGGTTCCTGACT-3', 5'-CAGAACTCCACCCCTGTGT-3' for ET-1 mRNA with the expected 243 bp PCR product, 5'-CCCACCTTCTCAGGCTGTTTC-3', 5'-CCGTTGAAGTAGAGGGCATT-3' for PAI-1 mRNA with the expected 187 bp PCR product.

The following PCR program was performed on the Opticon2: an initial denaturation at 95 °C for 10 min to activate DNA polymerase, followed by a 39 cycle program consisting of heating at 20 °C/s to 95 °C with 10 s hold, cooling at 59 °C with a 30 s hold for annealing and heating at 20 °C/s to 72 °C with 30 s hold for extension. PCR was immediately followed by a melting curve analysis to determine the melting point of the double stranded PCR product produced. mRNA levels were quantified using standard curve method. Standard curves were constructed using serially diluted standard template. Ct value was used to compute mRNA levels from the standard curve. mRNA levels were normalized to β-actin, whose primers were 5'-CCTCTATGCCAACACAGTGC-3' (forward), and 5'-CATCGTACTCCTGCTTGCTG-3' (reverse), to account for differences in reverse transcription efficiencies and amount of cDNA in reaction mixtures.

**Immunoblotting Analysis of SMemb Protein** HUVECs (1×10<sup>6</sup> cells) were grown on poly-L-lysine coated dishes, at 37 °C, for 24 h. 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 8 h. The cells in each dish were rinsed twice with chilled PBS and lysed with the solution composed of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 1% Triton X-100, 1% deoxycholate), and a complete tablet protease inhibitor cocktail (Roche, Indianapolis, IN), for 30 min at 4 °C. The whole cell extracts were sedimented at 15000 rpm for 15 min to remove the insoluble material. Total protein concentration in the supernatants was determined using the Bio-Rad Protein Assay<sup>®</sup> reagent (Life Science, Tokyo). Namely, the extracts containing 50 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 (Invitro-

gen Japan, Tokyo). The membranes were blocked with 5% non-fat milk prior to immunoblot analysis reacted with a mouse anti-human SMemb IgG antibody (dilution 1:3000, Yamasa Co. Ltd., Tokyo) and a horseradish peroxidase-conjugated goat anti-mouse secondary antibody (dilution 1:2000, Amersham Biosciences, Tokyo). Finally, the blots were visualized using the enhanced chemiluminescence system (ECL Plus western Blotting Detection System, Amersham Biosciences).

**Cell Migration Assay of HUVECs** Measurement of migrating ability of HUVECs was performed according to the method of Yamakawa *et al.*<sup>31</sup> HUVECs were cultured in endothelial basal medium (EBM, Sigma-Aldrich Japan, Tokyo, Japan) supplemented with 2% FBS under a humidified atmosphere of 5% CO<sub>2</sub> in air. The cells (1×10<sup>6</sup> cells/dish) in EBM were fluorescence-labeled by incubation with 3 μM 5-carboxyfluorescein, diacetoxymethyl ester (DCECF-AM, Dojindo Laboratories, Kumamoto, Japan) for 30 min at 37°C. After the labeled cells had been washed with EBM, 5×10<sup>4</sup> of them in 300 μl medium were added into culture insert with 8 μm-pore HTS FluoroBlok™ Inserts membrane (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) precoated with Matrigel™ matrix (Becton Dickinson) with a density of 25 μg/insert. Then, 3 μl of 1 mM Phx-2 was added to this medium. The culture insert was set into the well of a 24-well plate which contained 700 μl of EBM with 10% FBS as a chemoattractant. The serum was used without heat inactivation. The appropriate amount of recombinant human vascular endothelial growth factor protein (rhVEGF, Becton Dickinson) was added to the both side of medium and incubated at 37°C for 8 h in a CO<sub>2</sub> incubator. Following incubation, FluoroBlok™ Inserts membrane was examined under a fluorescence microscope and fluorescence of invaded cells was read directly in a fluorescence plate reader with bottom reading capabilities at excitation/emission wavelengths of 530/590 nm every 1 h without further manipulation. Only those labeled cells that have invaded the Matrigel™ matrix and passed through the pores of the FluoroBlok™ membrane will be detected.

**Statistics** The data were expressed as mean±S.E.M. and were analyzed by ANOVA followed by *post-hoc* test. Differences were considered significant at values of *p*<0.05.

## RESULTS

**Effect 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 300 μM) for 24 h was studied by WST-8 assay (Fig. 2). The cell counts were increased gradually during 8 h 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. The proliferation curve of these groups increased at the similar rates after 8 h. However, the cell counts were much suppressed in the presence of 5 mM glucose plus Phx-3 over 30 μM, or 15 mM glucose plus Phx-3 over 30 μM, dependent on the dose of Phx-3 added, showing that Phx-3 suppresses the proliferation of HUVECs at higher concentrations over 30 μM, but does not affect it at the concentration of 10 μM.

### Glucose Augmented Expression of the Vasoactive Fac-

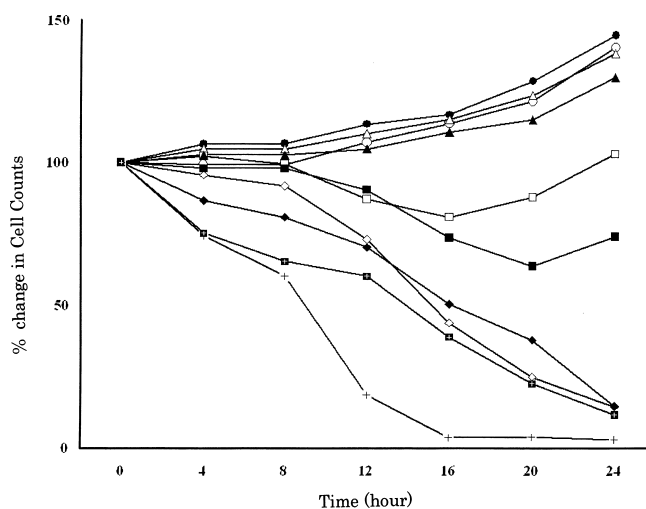


Fig. 2. Effects of Phx-3 on the Proliferation of HUVECs Treated with Normal Concentrations or High Concentrations of Glucose

(○): normal glucose (5 mM); (△): normal glucose+10 μM Phx-3; (□): normal glucose+30 μM Phx-3; (◇): normal glucose+100 μM Phx-3; (+): normal glucose+300 μM Phx-3; (●): high glucose (15 mM); (▲): high glucose+10 μM Phx-3; (■): high glucose+30 μM Phx-3; (◆): high glucose+100 μM Phx-3; (■): high glucose+300 μM Phx-3. Each point shows the mean values (*n*=5).

**tors and the Effects of Phx-3 on the Expression** We studied the mRNA level of SMemb, ET-1 and PAI-1 in HUVECs treated with either normal concentrations of glucose alone (5 mM), normal concentrations of glucose (5 mM) plus 10 μM Phx-3, high concentrations of glucose alone (15 mM) or high concentrations of glucose (15 mM) plus 10 μM Phx-3 for 8 h. As shown in Fig. 3A, the level of SMemb mRNA of the HUVECs with normal concentrations of glucose (5 mM) was not affected by the addition of 10 μM Phx-3. The levels of SMemb mRNA of the HUVECs treated with high concentrations of glucose increased to 200% of the control (5 mM glucose alone), while those of HUVECs treated with high concentrations of glucose and 10 μM Phx-3 decreased extensively (by 50% of HUVECs with high concentrations of glucose alone). The mRNA level of ET-1 (Fig. 3B) and PAI-1 (Fig. 3C) in HUVECs was not altered in the HUVECs treated with 5 mM glucose and 10 μM Phx-3, compared with the cells treated with 5 mM glucose alone. The mRNA level of ET-1 and PAI-1 was upregulated by 15 mM glucose (by 210% and 220%, respectively), but these were reversed to the levels of normal control (5 mM glucose alone) by the addition of 10 μM Phx-3.

Figure 4 shows the representative immunoblotting patterns of SMemb protein in each cells treated with 5 mM glucose alone, 15 mM glucose alone, 5 mM glucose plus 10 μM Phx-3 or 15 mM glucose plus 10 μM Phx-3. Consequently, it was found that the amounts of SMemb protein were not altered in the HUVECs treated with 5 mM glucose and 10 μM Phx-3, compared with the cells treated with 5 mM glucose alone. These were extensively increased in HUVECs treated with high glucose (15 mM) alone for 8 h, compared with those treated with 5 mM glucose alone. Furthermore, the additional treatment of 10 μM Phx-3 reversed the high glucose effect. These results are consistent with those for the mRNA level of SMemb as shown in Fig. 3.

**Effect of Phx-3 on Glucose Augmented Endothelial Cell Migration** Since the migration of endothelial cells to

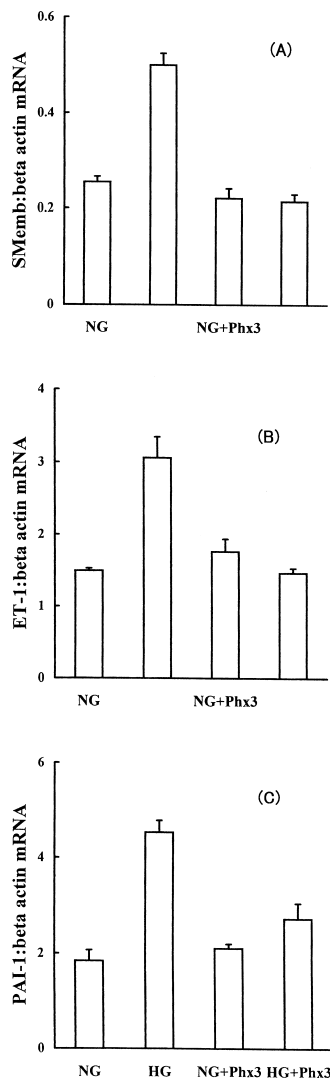


Fig. 3. Changes in Expressed Amounts of mRNA of SMemb, ET-1 and PAI-1 in HUVECs Treated with  $10 \mu\text{M}$  Phx-3 ( $n=4$ )

(A) The amounts of SMemb mRNA; (B) the amounts of ET-1 mRNA; (C) the amounts of PAI-1 mRNA. NG, HUVECs with normal glucose concentrations (5 mM); HG, HUVECs with high glucose concentrations (15 mM); NG+Phx3, HUVECs with normal glucose concentrations (5 mM) and  $10 \mu\text{M}$  Phx-3; HG+Phx3, HUVECs with high glucose concentrations (15 mM) and  $10 \mu\text{M}$  Phx-3. The amounts of each mRNA were based on the mRNA amounts of  $\beta$ -actin, as a house-keeping gene transcript.

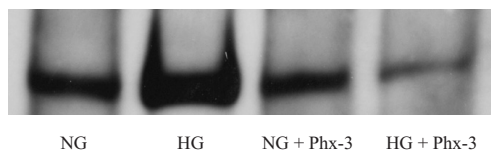


Fig. 4. Immunoblotting Patterns of SMemb in HUVECs Treated with  $10 \mu\text{M}$  Phx-3

NG, HUVECs with normal glucose concentrations (5 mM); HG, HUVECs with high glucose concentrations (15 mM); NG+Phx-3, HUVECs with normal glucose concentrations (5 mM) and  $10 \mu\text{M}$  Phx-3; HG+Phx-3, HUVECs with high glucose concentrations (15 mM) and  $10 \mu\text{M}$  Phx-3.

the extracellular matrix has been considered as a marker of atherosclerotic process in diabetes, we studied the migration ability of HUVECs which were treated with normal concentration of glucose (5 mM) or high concentration of glucose (15 mM) in the presence or absence of  $10 \mu\text{M}$  Phx-3 for 8 h

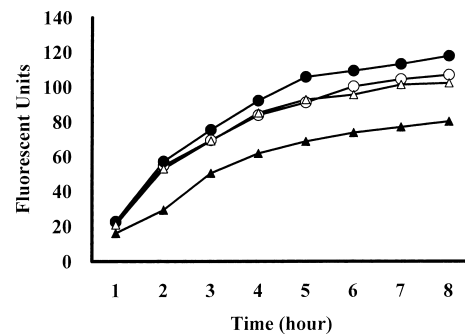


Fig. 5. Changes in Counts of Migrating Cells after Treatment of HUVECs with Normal Glucose, Normal Glucose plus  $10 \mu\text{M}$  Phx-3, High Concentrations of Glucose or High Concentrations of Glucose plus  $10 \mu\text{M}$  Phx-3

Data was expressed as the mean percent migration derived from the mean fluorescent units of cell migration through the Matrigel coated FluoroBlok inserts. (○): normal concentrations of glucose (5 mM); (△) normal glucose (5 mM) +  $10 \mu\text{M}$  Phx-3; (●): high concentrations of glucose (15 mM); (▲) high concentrations of glucose (15 mM) +  $10 \mu\text{M}$  Phx-3. Each point shows the mean values ( $n=5$ ).

(Fig. 5). Consequently, it was found that the counts of migrating HUVECs were significantly increased in the cells treated with high glucose, compared with the cells treated with normal concentration of glucose at each point examined for 8 h. On the contrary, when HUVECs were treated with both high glucose and  $10 \mu\text{M}$  Phx-3, the counts of migrating HUVECs were reduced more markedly than those of the cells with high glucose alone, at each time examined for 8 h. At 8 h, the counts of migrating HUVECs treated with 15 mM glucose and  $10 \mu\text{M}$  Phx-3 were about 67% of those treated with 15 mM glucose alone. However, the counts of migrating HUVECs were not affected when HUVECs with 5 mM glucose was treated with  $10 \mu\text{M}$  Phx-3. These results show that the migration activity of HUVECs was accelerated under hyperglycemic conditions, but was reduced by the treatment with  $10 \mu\text{M}$  Phx-3.

## DISCUSSION

Since diabetes mellitus leads to endothelial dysfunction and development of atherosclerotic vascular diseases,<sup>1,2,7</sup> it is critical to identify alteration of vasoactive factors involved in such events in the endothelial cells to identify potential targets of newer therapy as well as novel disease biomarker. It has recently been shown that various intracellular, extracellular and intercellular factors of the endothelial cells and smooth muscle cells, such as SMemb, ET-1, PAI-1, ICAM-1, VCAM-1, ECAM-1, and fibronectin *etc.* are involved in the atherosclerotic process in diabetes.<sup>1,9-12</sup>

SMemb was originally found in embryonic smooth muscle cells,<sup>32</sup> and shown to be increased at the mRNA and protein levels in diabetic meningeal cells.<sup>13</sup> SMemb has been characterized to cause vascular smooth muscle cells to switch from contractile to synthetic type, possibly leading to atherosclerotic lesions.<sup>13,32</sup> However, SMemb was not quantified at the mRNA level in HUVECs. ET-1, a potent vasoconstrictor, is implicated as a cause of the micro- and macrovascular complications of diabetes mellitus,<sup>7,8</sup> and is detected in large quantities in HUVECs treated with high glucose.<sup>33</sup> PAI-1, on the other hand accelerates the proliferation of HUVECs through VEGF activation, promoting the angiogenesis<sup>34</sup> and is detectable in HUVECs.<sup>10</sup>

Present results demonstrated that the mRNA level of these factors tightly relevant to angiogenesis and atherosclerosis in diabetes were upregulated in HUVECs treated with high ambient glucose (Fig. 3). Namely, we found that SMemb was expressed at the mRNA level in HUVECs, and that high glucose resulted in the increased mRNA level of SMemb (Fig. 3) and the level of SMemb protein (Fig. 4) in the cells, for the first time. It remains unclarified whether such increased SMemb in HUVECs may be directly linked to the intensified invasion activity of the cells as shown in Fig. 4 or not. We found that mRNA of PAI-1 was also expressed in HUVECs and was upregulated in the cells with high ambient glucose (Fig. 3). We confirmed the increased expression of ET-1 at the mRNA level in HUVECs with high ambient glucose (Fig. 3), as has been shown by Keynan *et al.*<sup>33</sup>) These results strongly support the views that the changes in the factors such as SMemb, ET-1 and PAI-1 in the endothelial cells may be associated with the angiogenesis and atherosclerosis under hyperglycemic conditions.

Furthermore, we found that Phx-3, which is equivalent to questionycin A, whose actions have not been clarified except for the anti-mycobacterial activity,<sup>22,28</sup>) attenuated the increased expression of SMemb, ET-1 and PAI-1 in HUVECs caused by high ambient glucose (Fig. 3), and inhibited the increased invasion activity of the cells with high ambient glucose (Fig. 5). These attenuating effects of Phx-3 on HUVECs with high ambient glucose may be beneficial for inhibiting the progress of diabetic angiogenesis and atherosclerosis accompanying the migration of the activated endothelial cells into the extracellular matrix. However, the mechanism for attenuating action of Phx-3 on the expression of intracellular factors in HUVECs with high ambient glucose is currently obscure. Present results suggest Phx-3, which has been shown to have little adverse effects on mice (our unpublished data) may be a useful drug to treat diabetes, atherosclerosis and cancer accompanying blood vessel proliferations, which awaits further investigation.

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