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Full paper

DPP-4 inhibition protects human umbilical vein endothelial cells from hypoxia-induced vascular barrier impairment

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ABSTRACT

Dipeptidyl peptidase-4 (DPP-4) inhibitors are relatively new class of anti-diabetic drugs. Some protective effects of DPP-4 on cardiovascular disease have been described independently from glucose-lowering effect. However, the detailed mechanisms by which DPP-4 inhibitors exert on endothelial cells remain elusive. The purpose of this research was to determine the effects of DPP-4 inhibitor on endothelial barrier function. Human umbilical vein endothelial cells (HUVECs) were cultured and exposed to hypoxia in the presence or absence of Diprotin A, a DPP-4 inhibitor. Immunocytochemistry of vascular endothelial (VE-) cadherin showed that jagged VE-cadherin staining pattern induced by hypoxia was restored by treatment with Diprotin A. The increased level of cleaved β-catenin in response to hypoxia was significantly attenuated by Diprotin A, suggesting that DPP-4 inhibition protects endothelial adherens junctions from hypoxia. Subsequently, we found that Diprotin A inhibited hypoxia-induced translocation of NF-κB from cytoplasm to nucleus through decreasing TNF-α expression level. Furthermore, the tube formation assay showed that Diprotin A significantly restored hypoxia-induced decrease in number of tubes by HUVECs. These results suggest that DPP-4 inhibitior protects HUVECs from hypoxia-induced barrier impairment.

1. Introduction

As shown in the clinical trials such as Framingham study and Finnish population-based study, diabetes mellitus is associated with a significant risk of coronary heart disease.[1,2] Patients with diabetes have higher mortality from cardiovascular disease than people without diabetes.[1,2] Although there is controversy regarding intensive glucose lowering therapy to patients with type 2 diabetes who has cardiovascular risk,[3] improvement in glycemic control is associated with reduction in the risk of complications including myocardial infarction and microvascular complications.[4]

Dipeptidyl peptidase-4 (DPP-4) inhibitors are oral anti-hyperglycemic agents which prevent degradation of incretins such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP), consequently increase insulin secretion.[5] Neuropeptides, cytokines, growth factors, and chemokines as well as incretins were identified as DPP-4 substrates.[6] Although an increasing number of clinical and basic studies have shown the pleiotropic effects of antidiabetic agents, the effects of DPP-4 inhibitors on occurrence of macrovascular complications in patients with diabetes were not recognized in large randomized controlled trials.[7–9] As for microvascular complications, recent studies have shown that DPP-4 inhibitors have beneficial effects. It was reported that linagliptin improved flow-mediated vasodilatation after 28 days of treatment.[10] Saxagliptin reduced the development and progression of microalbuminuria.[7] Several lines of evidence indicated that DPP-4 inhibitors exert antiinflammatory effects, and therefore contribute to prevention of atherosclerosis.[11,12] However, further evidence is necessary to confirm the effects of DPP-4 inhibitors on vascular complications.

We reported that the inhibition of DPP-4 activity leads to reduction in infarct size after myocardial infarction and hypoxia-induced apoptosis in cardiomyocytes and human umbilical vein

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endothelial cells (HUVECs).[13,14] Hyperglycemia leads to endothelial barrier dysfunction and it precedes the development of type 2 diabetes.[15–17] Endothelial barrier dysfunction is a key element of arteriosclerosis. Therefore, we investigated the effect of DPP-4 inhibitor on endothelial barrier. Hypoxia exacerbates endothelial barrier dysfunction, leading to the disrupted vascular homeostasis and development of cardiovascular disease.[18–20] Hypoxia induces the expression levels of DPP4 mRNA and protein.[21] Endothelial cells form monolayers that cover the internal surface of blood vessels and they are joined together by intercellular junction proteins.[22,23] Although adherens junctions in endothelial cells regulate vascular integrity, the effect of DPP-4 inhibitors on adherens junctions remains undetermined. Here, we focused on adherens junctions, the barrier function of the endothelial cells and investigated the effects and mechanisms of DPP-4 inhibitor on hypoxia-induced barrier impairment in HUVECs.

2. Materials and methods

2.1. Cell culture

HUVECs were seeded at 4.0×10^5 cells/well in collagen coated 12-well plate and maintained in endothelial growth medium-2 (EGM-2; Lonza, Walkersville, MD) in normoxic chamber (a mixture of 95% air and 5% CO_2) at 37 °C until the cells were confluent. Subsequently, after washing by PBS (Sigma), the cultured media were exchanged to Endothelial Basal Medium-2 (EBM-2; Lonza, Walkersville, MD) and pre-incubated for 1 h in normoxic chamber. Then, the cells were divided into three groups; Nx group; incubated in normoxic chamber, Hx group; incubated in hypoxic chamber (1% O_2, 5% CO_2, and 94% N_2 at 37 °C) (WAKEN 9000EX, Waken B Tech Co., Ltd, Kyoto, Japan), and Hx + DipA (incubated in EBM-2 containing 100 μM of DPP-4 inhibitor, Diprotin A) (Peptide Institute, Osaka, Japan) in hypoxic chamber at 37 °C as described previously.[14] After 24 h of the incubation, each group of cells were washed with PBS and harvested. Diprotin A was added in the groups to be assigned to Hx + DipA 24 h before the media were changed to EBM-2 (Fig. 1). The concentration of 100 μM Diprotin A was followed by our previous findings that DPP-4 activity was inhibited over 80% by 100 μM Diprotin A in HUVECs.[14]

2.2. Immunofluorescence microscopy

For immunostaining of vascular endothelial (VE)-cadherin, HUVECs were seeded and cultured on cover glasses in 12-well plates for 24 h. Subsequently, cells were fixed with 4% paraformaldehyde. After removed fixative solution, cells were washed with PBS and blocked with 0.2% Triton X-100 and 2% Bovine Serum albumin (BSA). After blocking, the cells were incubated with anti-VE-cadherin primary antibody (C-19, Santa Cruz, CA), followed by detection using Donkey anti-Goat IgG (H + L) secondary antibody with conjugated Alexa Flour 488 (Invitrogen, Carlsbad, CA). Nuclei were stained with Hoechst 33258 (Life Technologies, Grand Island, NY). For detecting nuclear translocation of nuclear factor -κB (NF-κB), the cells were seeded and cultured on cover glasses in 12-well plates for 24 h. After fixed with methanol for 10 min, cells were washed with PBS and blocked with 0.2% Triton X-100 and 2% BSA. After blocking, cells were incubated with anti- NF-κB p65 (C-20, Santa Cruz, CA) and washed with PBS, followed by detection using Goat Anti-rabbit Cy3 IgG (H + L) secondary antibody (Abcam). Nuclei were stained with Hoechst 33258 (Life Technologies, Grand Island, NY). Stained cells were determined using a Zeiss LSM780 confocal microscope (Carl Zeiss Inc, Jena, Germany).

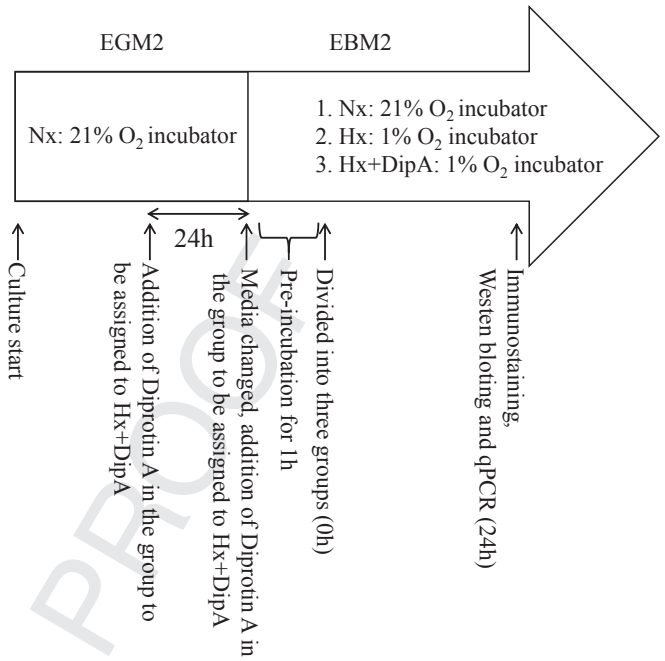

Fig. 1. The scheme of the cell culture protocol.

2.3. Quantitative real time polymerase chain reaction

HUVEC total RNA of each group was isolated using RNA iso Plus (Takara). Reverse transcription was performed using ReverTra Ace qPCR RT Master Mix (TOYOBO, Japan) according to the manufacturer's manual. Quantitative realtime polymerase chain reaction (qPCR) was performed by THUNDERBIRD Probe qPCR Mix (TOYOBO) using Light cycler 480 (Roche, Rotkreuz, Switzerland). The following primer sets were used; tumor necrosis factor -α (TNF-α) (left- cagcctcttctccttcctgat, right- gccagagggctgattagaga), and 18S (left-gcaattattccccatgaacg, right-gggacttaatcaacgcaagc).

2.4. Western blotting analysis

The cells were lysed in RIPA Buffer (10 mM Tris–HCl pH 8.0, 140 mM NaCl, 10 mM EDTA, 0.1% SDS, 1% Triton X-100, 1% Sodium Deoxycholate, 50 mM NAF, 1 mM Na_3VO_4, 5 μg/mL Leupeptin, 1 mM PMSF). After centrifuged at 12,000 rpm for 30 min, the supernatant was transferred to a fresh tube and the lysates were subjected to SDS-PAGE, and the protein bands were then transferred to a polyvinylidene difluoride membrane. After blocking with 5% Skim milk (WAKO), the membranes were incubated with the primary antibody against β-catenin (BD biosciences) overnight, and then incubated with peroxidase-linked anti-mouse IgG antibody (GE Healthcare UK, Ltd., Buckinghamshire, UK) for 1 h. The immunoblots were developed by enhanced chemilumino- fluorescence method with ECL prime (GE Healthcare UK, Ltd., Buckinghamshire, UK). Finally, the signals were measured and analyzed with EZ capture MG (Atto, Tokyo, Japan). Quantification of the images was performed using the ImageJ software followed by the calculation of the ratio of cleaved β-catenin relative either to the overall protein expression or β-tubulin expression.

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