

Full Paper

Insulin Induces Internalization of the Plasma Membrane 5-Hydroxytryptamine_{2A} (5-HT_{2A}) Receptor in the Isolated Human Endothelium-Denuded Saphenous Vein via the Phosphatidylinositol 3-Kinase Pathway

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Received September 22, 2011; Accepted December 5, 2011

Abstract. The aim of this study was to investigate the relaxant effect of insulin on the 5-hydroxytryptamine (5-HT)-induced constriction of the human endothelium-denuded saphenous vein (SV) and its signal transduction pathway. During the 5-HT-induced sustained constriction of vessels, insulin induced vasorelaxation in a concentration-dependent manner. This insulin-induced vasorelaxation was partially attenuated by L-NAME, a nitric oxide synthase (NOS) inhibitor, and was abolished by wortmannin, a phosphatidylinositol 3-kinase (PI3-K) inhibitor. Insulin increased the Ser⁴⁷³ phosphorylation of Akt. Endothelial NOS and inducible NOS protein expressions were observed in SV smooth muscle when insulin induced relaxation of SV vessels precontracted with 5-HT. Although insulin did not affect the total protein level of 5-HT_{2A} receptors, it decreased the particulate protein level and reciprocally increased the soluble protein level of 5-HT_{2A} receptors in a concentration-dependent manner. These results demonstrate that insulin can induce the internalization of 5-HT_{2A} receptors from the plasma membrane to the cytoplasm. The insulin-induced internalization of 5-HT_{2A} receptors was abolished by wortmannin but was not affected by L-NAME. These results suggest that the relaxant effect of insulin on 5-HT-induced vasoconstriction is mediated in part by the internalization of plasma membrane 5-HT_{2A} receptors and the production of nitric oxide via the PI3-K/Akt pathway.

Keywords: insulin-induced vasorelaxation, saphenous vein (SV), internalization of 5-HT_{2A} receptor, diabetes mellitus (DM), nitric oxide (NO)

Introduction

The saphenous vein (SV) continues to be the most commonly used conduit vessel for coronary artery bypass grafting (CABG) because of its availability and suppleness (1). One of the most serious problems in CABG is that the bypass grafts often go into spasm after implantation into coronary arterial circulation, which leads to

premature occlusion and increased perioperative morbidity (2, 3). The release of 5-hydroxytryptamine (5-HT) from activated platelets plays a crucial role in the constriction of bypass grafts when surgical handling removes the endothelium (4, 5). Furthermore, diabetes mellitus (DM) is a well-established risk factor for perioperative complications in CABG (6, 7).

We recently reported that in isolated human endothelium-denuded SVs, the constrictions caused by 5-HT were significantly greater (hyperreactivity) in the DM patients than in the non-DM patients (8). In the study, we demonstrated that the hyperreactivity to 5-HT in the SV

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Published online in J-STAGE on January 27, 2012 (in advance)

doi: 10.1254/jphs.11172FP

smooth muscle of DM patients resulted from not only the enhanced phosphorylation of myosin light chain phosphatase (MLCP) but also the defective protein level of MLCP (8). Thus, we discovered that the defective protein levels of MLCP in the DM group may partially explain the poor patency of SV grafts harvested from DM patients.

Because DM is associated with defects in insulin action, one important mechanism responsible for the vascular hyperreactivity in DM patients is the impaired insulin-mediated relaxation of their vasculature. A large number of studies have reported that insulin induces vasorelaxation by mechanisms that include the stimulation of nitric oxide (NO) production and the reduction in vascular smooth muscle cell intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) (9–18). Moreover, we previously reported that insulin induces the internalization of the plasma membrane 5-HT_{2A} receptor, which was fused to yellow fluorescent protein for visualization and was stably expressed in HEK293 cells. These findings suggest a possible mechanism for the lower vascular reactivity to 5-HT in non-DM patients (19).

The aim of this study was to investigate the relaxant effect of insulin on the 5-HT-induced constriction of human endothelium-denuded SVs and its signal transduction pathway. We hypothesized that insulin exerts a relaxant effect on 5-HT-induced vasoconstriction that results from not only the NO production but also the internalization of the plasma membrane 5-HT_{2A} receptor. To test this hypothesis, vascular rings of human endothelium-denuded SV were used for functional and biochemical experiments.

Materials and Methods

Preparation of blood vessels and contractile studies

Human SVs were obtained from 16 patients who were undergoing CABG at Miyazaki Prefectural Nobeoka Hospital (Nobeoka) or surgical varicose-vein treatment at Kuwabara Clinic (Miyazaki). At Miyazaki Prefectural Nobeoka Hospital, portions of each SV were sectioned into the desired lengths for bypassing the occluded coronary arteries, and the remainder was used for the experiments. At Kuwabara Clinic, portions of the SV with varicose-veins were sectioned, as a surgical treatment, from each patient with the varix of the lower extremity. We used only tiny portions of each SV that were not extended. After each vein was obtained, it was immediately placed in modified Krebs buffer, which had been previously aerated with 95% O₂ – 5% CO₂, and transported to our laboratory. The composition of the modified Krebs buffer solution was as follows: 118.0 mM NaCl, 4.7 mM KCl, 25.0 mM NaHCO₃, 1.2 mM MgSO₄, 1.1

mM KH₂PO₄, 2.5 mM CaCl₂, 0.01 mM EDTA, and 11.0 mM glucose. The final pH of the modified Krebs buffer was adjusted to 7.4. The vessel was carefully cut into 2-mm rings, which were denuded of endothelium by inserting a syringe needle into the lumen and then gently rolling each ring back and forth. Each ring was suspended between stainless steel hooks in a 5-ml organ bath containing modified Krebs buffer maintained at 37°C and continuously aerated with 95% O₂ – 5% CO₂. One hook was connected to a force transducer (Nihon-Kohden, Tokyo) to record the isometric tension in a computer system (PowerLab8/30; Bio Research Center Co., Ltd., Nagoya). The SV segment grafted into the coronary arterial circulation is exposed to higher pressure than when it is located originally in the venous circulation. We therefore applied a resting tension of 2.0 g to the SV ring preparation as applied to the human internal thoracic artery ring preparation in our previous study (20). The SV rings were stretched progressively to the optimal resting tension (2.0 g) and were allowed to equilibrate for 1 h. The buffer was changed every 30 min. Thereafter, the presence of functional endothelium was assessed. The rings were preconstricted with noradrenaline (Sankyo, Tokyo) (1 μM) and acetylcholine (Daiichi Pharmaceutical Co., Ltd., Tokyo) (30 nM) was added to the organ bath. If a relaxant effect was not observed, we considered functional endothelium to be absent. The preparation was washed twice with fresh modified Krebs buffer solution, and it gradually relaxed from the noradrenaline-induced vasoconstriction. After the tension of the rings had completely returned to basal levels, each ring was preconstricted with 5-HT (Sigma Chemical Co., St. Louis, MO, USA) (30 nM). After 7 min, a single concentration of insulin (Humulin R; Eli Lilly Japan, Kobe) (ranging from 0.1 nM to 1 μM) was applied to each organ bath. The vasorelaxant effect of insulin in the presence of 5-HT was evaluated using the percentage of the 5-HT-induced vasoconstriction just before addition of insulin as 100% in each ring, and the values were compared with those obtained from time-control experiments in which the vehicle for insulin was applied. In another series of experiments, *N*^G-nitro-L-arginine methyl ester hydrochloride (L-NAME; Nacalai Tesque, Kyoto) (300 μM), wortmannin (Sigma) (100 nM), L-NAME plus wortmannin, or L-NAME plus PD98059 (Sigma) (10 μM) was added to each organ bath; 15 min later, each ring was preconstricted with 5-HT (30 nM); and then insulin (300 nM) was added to each organ bath. The vasorelaxant effect of 300 nM insulin in the presence of 5-HT was evaluated using the percentage of the 5-HT-induced vasoconstriction just before the addition of insulin as 100% in each ring, and the values were compared with values obtained from time-control experiments in which the

vehicle for insulin was applied.

Western blot analysis of Ser⁴⁷³phosphorylated Akt, Akt endothelial NOS (eNOS), inducible NOS (iNOS), and β -actin

Following the contractile studies, the SV rings were flash frozen in liquid nitrogen and stored at -80°C . Frozen SV rings were homogenized in ice-cold cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) containing 1 mM phenylmethylsulfonyl fluoride and centrifuged at $12,000 \times g$ for 15 min at 4°C . An aliquot (100 μl) of the resulting supernatant fluid was mixed with 100 μl of $2 \times$ sodium dodecyl sulphate (SDS) electrophoresis sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 10% 2-mercaptoethanol, and 4% SDS] at 98°C for 4 min. Total amounts of protein were measured using a bicinchoninate protein assay kit (Nacalai Tesque). Equal amounts of protein (5.5 μg per lane) were separated by SDS-10% or SDS-12.5% polyacrylamide gel electrophoresis and then transferred onto a polyvinylidene difluoride membrane (Hybond-P). The membrane was preincubated with 3% bovine serum albumin in Tween-Tris-buffered saline [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.1% Tween-20] and reacted with mouse or rabbit antibodies (1:2000) against Ser⁴⁷³ phosphorylated Akt, Akt, iNOS, β -actin (Cell Signaling Technology), or eNOS (BD Transduction Laboratories, Lexington, KY, USA) overnight at 4°C in Immuno-enhancer Reagent A (Wako, Osaka). After repeated washing, the immunoreactive bands were reacted with horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies in Immuno-enhancer Reagent B (Wako), visualized using the Immuno-Star enhanced chemiluminescent detection system (Wako), and quantified using a luminoimage LAS-4000 analyzer (GE Healthcare Japan, Tokyo). The amount of Akt phosphorylation was normalized by the amount of Akt protein. The amounts of eNOS and iNOS were normalized by the amount of β -actin. These normalized protein levels were expressed as ratios: a value of 1 represents each protein level obtained from the SV vessels 7 min after the addition of 30 nM 5-HT.

Western blot analysis of the 5-HT_{2A} receptor in soluble and particulate fractions

Following the contractile studies, the SV rings were flash frozen in liquid nitrogen and stored at -80°C for western blot analysis. Western blot analysis of the 5-HT_{2A} receptor in soluble and particulate fractions was performed by using an established protein extraction method that was recently reported (21, 22). Briefly, frozen SV rings were homogenized in transmembrane protein extraction buffer 1 (ProteoExtract Transmembrane Protein Extraction Kit; Calbiochem-Novabiochem, San Diego,

CA, USA) and centrifuged at $1,000 \times g$ for 5 min at 4°C . An aliquot (100 μl) of the resulting supernatant was mixed with $2 \times$ SDS electrophoresis sample buffer (100 μl) and used as the soluble fraction. Collected pellets were solubilized in extraction buffer 2A and centrifuged at $16,000 \times g$ for 15 min at 4°C . An aliquot (100 μl) of the resulting supernatant was mixed with $2 \times$ SDS electrophoresis sample buffer (100 μl) and used as the particulate fraction. Equal amounts of protein (5.5 μg per lane) were separated by SDS-10% polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Hybond-P). The membrane was reacted with rabbit antibody (1:2000) against the 5-HT_{2A} receptor (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C in Immuno-enhancer Reagent A. The amounts of particulate and soluble 5-HT_{2A} receptors were normalized by the total amount of the 5-HT_{2A} receptors. These normalized protein levels were expressed as ratios: a value of 1 represents each protein level obtained from the SV vessels 7 min after the addition of 30 nM 5-HT.

Ethics

The Ethics Committees at both Miyazaki Prefectural Nobeoka Hospital and Kyushu University of Health and Welfare approved this study. All patients provided written consent to participate in the study. These experiments were conducted in accordance with *The Code of Ethics of the World Medical Association (Declaration of Helsinki) for Experiments Involving Humans*.

Statistical analysis

The data are presented as mean \pm standard error of the mean (S.E.M.). Statistical comparisons were made using one-way analysis of variance (ANOVA) and then by the Newman-Keuls multiple range test. Significance was assumed at $P < 0.05$. Statistical analyses were performed using SPSS (Statistical Package for Social Science 16.0J for Windows).

Results

Effect of insulin on 5-HT-induced vasoconstriction

We first investigated the effect of insulin on 5-HT-induced vasoconstriction. The addition of 30 nM 5-HT significantly induced vasoconstriction, which peaked at 3 min after adding 5-HT and sustained for at least 30 min. Insulin induced the relaxation of SV vessels precontracted with 5-HT in a concentration-dependent manner (Fig. 1A). Insulin alone did not affect the resting tension (data not shown). As shown in Fig. 1A, concentrations above 30 nM insulin significantly reduced 5-HT-induced vasoconstriction. Treatments of 300 nM and 1 μM insulin

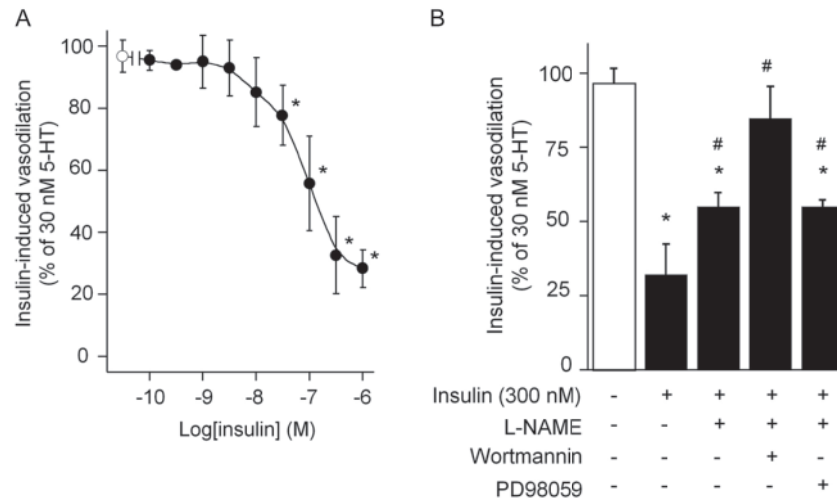


Fig. 1. The relaxant effect of insulin on isolated human endothelium-denuded SV vessels precontracted with 5-HT and the effects of inhibitors on insulin-induced vasorelaxation. **A:** The vasorelaxant effect of insulin in the presence of 5-HT. Each vascular ring was precontracted with 30 nM 5-HT, and then vehicle (open circle) or a single concentration of insulin (closed circle) ranging from 0.1 nM to 1 μ M was added to the organ baths. The maximal relaxant effect of insulin at each concentration was obtained approximately 15 min after insulin treatment. The vasorelaxant effect of insulin in the presence of 5-HT was evaluated as a percentage of the 5-HT-induced vasoconstriction just before the addition of insulin as 100% in each ring. Data are expressed as the mean \pm S.E.M. ($n = 5 - 6$). * $P < 0.05$, compared with values obtained from time-control experiments applying the vehicle for insulin (open circle). **B:** Effects of inhibitors on the insulin-induced vasorelaxation. In another series of experiments, 300 μ M L-NAME, L-NAME plus 100 nM wortmannin, or L-NAME plus 10 μ M PD98059 were added to the organ baths; and 15 min later, each ring was precontracted with 30 nM 5-HT, and then the vehicle (open column) or 300 nM insulin (closed column) was added to the organ baths. The vasorelaxant effect of insulin in the presence of 5-HT was evaluated using the percentage of the 5-HT-induced vasoconstriction just before the addition of insulin as 100% in each ring. Data are expressed as the mean \pm S.E.M. ($n = 4$). * $P < 0.05$, compared with values obtained from time-control experiments applying the vehicle for insulin (open column), # $P < 0.05$ compared with insulin alone.

exerted the maximal relaxation to $32.7\% \pm 5.0\%$ and $28.3\% \pm 6.1\%$, respectively.

Effects of inhibitors of NOS, PI3-K, and MEK on insulin-induced relaxation in vessels precontracted with 5-HT

Next, to determine the signal transduction pathway for the relaxant effect of insulin on the 5-HT-induced vasoconstriction, we examined the effects of L-NAME, wortmannin, L-NAME plus wortmannin, or L-NAME plus PD98059 on the relaxation induced by 300 nM insulin in the vessels precontracted with 30 nM 5-HT. These inhibitors did not significantly affect the vasoconstriction induced by 5-HT (data not shown). As shown in Fig. 1B, insulin significantly decreased 5-HT-induced vasoconstriction by $68.0\% \pm 10.8\%$. The insulin-induced vasorelaxation was partially attenuated by L-NAME by $45.1\% \pm 4.8\%$ and was abolished by L-NAME plus wortmannin. In addition, the insulin-induced vasorelaxation was abolished by wortmannin alone (data not shown). PD98059 did not affect the insulin-induced vasorelaxation after treatment with L-NAME alone (Fig. 1B).

Effects of inhibitors of NOS and PI3-K on phosphorylation of Akt in SV smooth muscle during insulin-induced relaxation of SV vessels precontracted with 5-HT

To determine whether the insulin-induced phosphorylation of Akt is mediated via the PI3-K pathway in SV vessels precontracted with 5-HT, we investigated the effects of inhibitors of NOS and PI3-K on the insulin-induced phosphorylation of Akt during 30 nM 5-HT-induced vasoconstriction. Insulin increased the Ser⁴⁷³ phosphorylation of Akt in a concentration-dependent manner (Fig. 2A). Concentrations above 1 nM insulin significantly increased the phosphorylation of Akt. Treatment with 300 nM insulin increased the phosphorylation of Akt by 9.4 ± 0.7 -fold. As shown in Fig. 2B, the insulin-induced phosphorylation of Akt was abolished by L-NAME plus wortmannin but was not affected by L-NAME. In addition, wortmannin alone abolished the insulin-induced phosphorylation of Akt (data not shown).

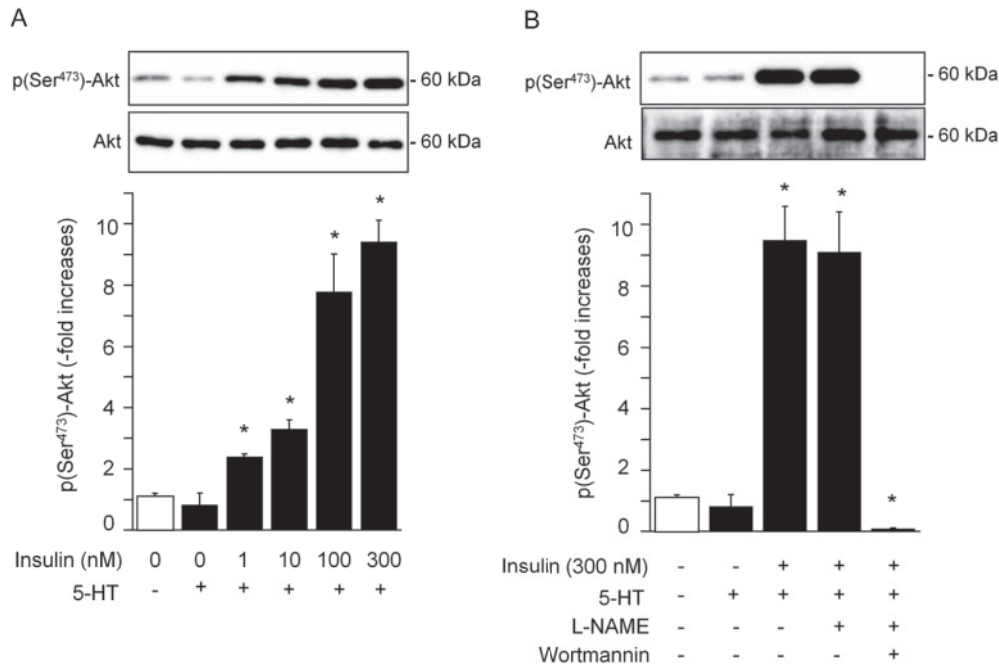


Fig. 2. Insulin-induced Ser⁴⁷³ phosphorylation of Akt in isolated human endothelium-denuded SV vessels precontracted with 5-HT and the effects of inhibitors on insulin-induced Ser⁴⁷³ phosphorylation of Akt. Each vascular ring was precontracted with 30 nM 5-HT, and then, the vehicle or a single concentration of insulin ranging from 1.0 to 300 nM was added to the organ baths. Approximately 15 min after treatment with vehicle or insulin, the SV rings were flash frozen in liquid nitrogen and stored at -80°C until western blot analysis. The amount of Akt phosphorylation was normalized by the amount of Akt protein and expressed as ratios: a value of 1 represents the phosphorylated protein level obtained from the SV vessels 7 min after the addition of 30 nM 5-HT (data not shown). **A:** Insulin-induced Ser⁴⁷³ phosphorylation of Akt in isolated human endothelium-denuded SV vessels precontracted with 5-HT. Data are expressed as the mean \pm S.E.M. ($n = 4$). $*P < 0.05$, compared with values obtained from time-control experiments applying vehicle for insulin (2nd column from left). **B:** Effects of inhibitors on insulin-induced Ser⁴⁷³ phosphorylation of Akt. The rings were treated with 300 μM L-NAME or L-NAME plus 100 nM wortmannin 15 min before the addition of 5-HT. Then, the same experiments as described in panel A were carried out. Data are expressed as the mean \pm S.E.M. ($n = 4$). $*P < 0.05$, compared with values obtained from time-control experiments applying the vehicle for insulin (2nd column from the left).

Expressions of eNOS and iNOS in SV smooth muscle during the insulin-induced relaxation of SV vessels precontracted with 5-HT and insulin-induced iNOS expression

To clarify which NOS isoforms are involved in insulin-induced NO production in SV smooth muscle, we conducted a western blot analysis for eNOS and iNOS after 300 nM insulin treatment of SV vessels precontracted with 30 nM 5-HT. The expressions of eNOS and iNOS were observed in SV smooth muscle (Fig. 3A). As shown in Fig. 3B, insulin increased the protein levels of iNOS in SV smooth muscle in a concentration-dependent manner. Concentrations of insulin above 10 nM significantly increased the protein level of iNOS. The addition of 300 nM insulin significantly increased protein level of iNOS by 2.1 ± 0.2 -fold.

Insulin-induced internalization of 5-HT_{2A} receptors from plasma membrane to cytoplasm during insulin-induced relaxation of SV vessels precontracted with 5-HT and effects of inhibitors of NOS and PI3-K on the insulin-induced internalization of 5-HT_{2A} receptors

We examined whether insulin induces the internalization of 5-HT_{2A} receptors from the plasma membrane to the cytoplasm in SV smooth muscle. Insulin did not affect the total protein level of 5-HT_{2A} receptors at any concentration (Fig. 4A). Insulin, but not 30 nM 5-HT, decreased the particulate protein level of 5-HT_{2A} receptors. Concentrations of insulin above 100 nM significantly decreased the particulate protein level of the 5-HT_{2A} receptor in a concentration-dependent manner (Fig. 4A). On the other hand, insulin, but not 30 nM 5-HT, increased the soluble protein level of the 5-HT_{2A} receptor. Concentrations of insulin above 100 nM significantly increased the soluble protein level of 5-HT_{2A} receptors (Fig. 4B). As shown in Fig. 4, A and B, treat-

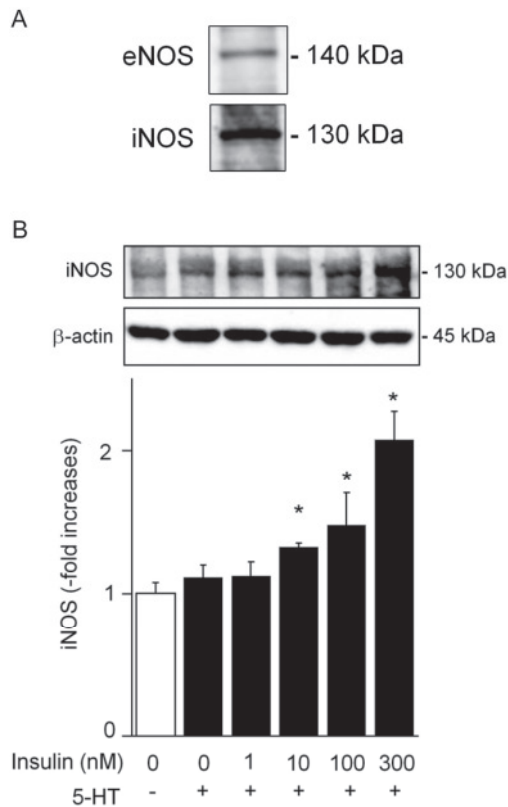


Fig. 3. Expressions of eNOS and iNOS and insulin-induced iNOS inductions in isolated human endothelium-denuded SV vessels pre-constricted with 5-HT. Each vascular ring was pre-constricted with 30 nM 5-HT, and then, vehicle or a single concentration of insulin ranging from 1.0 to 300 nM was added to the organ baths. Approximately 15 min after treatment with vehicle or insulin, the SV rings were flash frozen in liquid nitrogen and stored at -80°C until western blot analysis. A: Expressions of eNOS and iNOS during 300 nM insulin-induced relaxation of SV vessel pre-constricted with 5-HT. B: Insulin-induced iNOS inductions in SV vessels pre-constricted with 5-HT. The amount of iNOS was normalized by the amount of β -actin and expressed as ratios: a value of 1 represents the protein level of iNOS obtained from the SV vessel 7 min after the addition of 30 nM 5-HT. Data are expressed as the mean \pm S.E.M. ($n = 4$). * $P < 0.05$, compared with values obtained from time-control experiments applying vehicle for insulin (2nd column from left).

ment with 300 nM insulin significantly decreased the particulate protein level of the 5-HT_{2A} receptor by 0.6 ± 0.1 -fold and reciprocally increased the soluble protein level of the 5-HT_{2A} receptor by 2.8 ± 0.5 -fold, indicating that insulin induces the internalization of the 5-HT_{2A} receptor from the plasma membrane to the cytoplasm in SV smooth muscle.

Next, to clarify the signal transduction pathway for the insulin-induced internalization of the 5-HT_{2A} receptor, we examined the effects of inhibitors of NOS and PI3-K on the insulin-induced internalization of the 5-HT_{2A} receptor. As shown in Fig. 4C, the insulin-induced inter-

nalization of the plasma membrane 5-HT_{2A} receptors was abolished by wortmannin but was not affected by L-NAME.

Discussion

In this study, we found that insulin induces the vasorelaxation of endothelium-denuded SVs pre-constricted with 5-HT in a concentration-dependent manner, and this relaxation is mediated in part by NO production and by the internalization of plasma membrane 5-HT_{2A} receptors via the PI3-K/Akt pathway.

The underlying mechanisms of insulin-induced vasorelaxation have generally been considered to be related to NO production. A considerable number of studies have reported that insulin induces NO-dependent vasorelaxation via endothelial-dependent (12, 13) or endothelial-independent (9, 14) pathways. However, several studies have reported that insulin induces endothelial-independent and NO-independent vasorelaxation through a mechanism involving the activation of potassium channels (15, 16). To elucidate the role of NO in insulin-induced vasorelaxation, we examined the effects of L-NAME on this process. Indeed, insulin-induced vasorelaxation was partially attenuated by L-NAME, suggesting that the insulin-induced vasorelaxation is mediated in part by NO production. We observed eNOS and iNOS expressions in SV smooth muscle during the insulin-induced relaxation of SV vessels pre-constricted with 5-HT. In addition, insulin increased the expression of iNOS in a concentration-dependent manner. These results suggest that the insulin-induced NO production is mediated by both eNOS and iNOS in SV smooth muscle. Our finding that insulin stimulates NO production in endothelium-denuded vasculature is consistent with previous reports (17, 18).

Furthermore, we observed that approximately half of the maximal vasorelaxation induced by insulin still occurred in the presence of L-NAME, while the relaxant effect was abolished by wortmannin but not by the MEK inhibitor PD98059. We also observed that insulin increased the Ser⁴⁷³ phosphorylation of Akt in a concentration-dependent manner, which was abolished by wortmannin. Collectively, these results demonstrate that insulin activates the PI3-K/Akt pathway and consequently induces vasorelaxation involving NO-dependent and NO-independent components.

We previously reported that insulin induces the internalization of the plasma membrane 5-HT_{2A} receptor, which was fused with yellow fluorescent protein for visualization and stably expressed in HEK293 cells (19). To determine whether insulin could induce the internalization of the plasma membrane 5-HT_{2A} receptor in en-

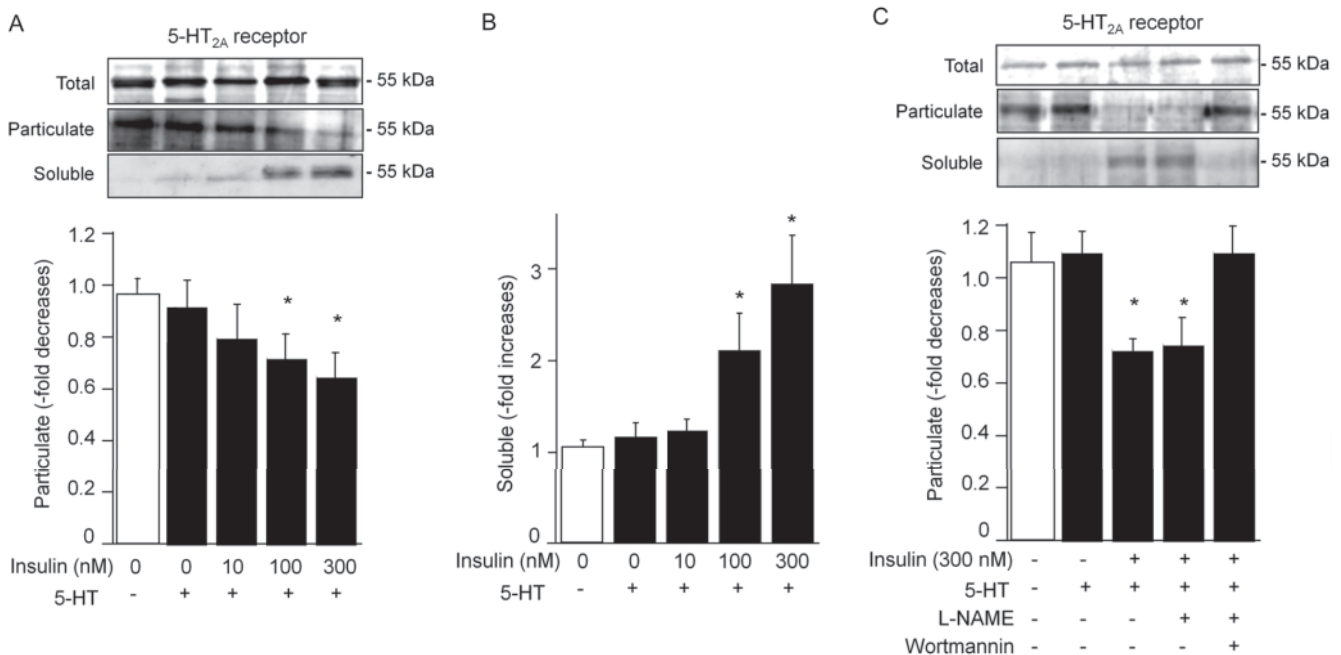


Fig. 4. Insulin-induced internalization of the 5-HT_{2A} receptors from the plasma membrane to the cytoplasm in isolated human endothelium-denuded SV vessels preconstricted with 5-HT, and the effects of inhibitors on the insulin-induced internalization of the 5-HT_{2A} receptors. Each vascular ring was preconstricted with 30 nM 5-HT, and then, vehicle or a single concentration of insulin ranging from 10 to 300 nM was added to the organ baths. Approximately 15 min after treatment with vehicle or insulin, the SV rings were flash frozen in liquid nitrogen and stored at -80°C until western blot analysis. The amounts of particulate and soluble 5-HT_{2A} receptors were normalized by the total amount of 5-HT_{2A} receptor and expressed as ratios: a value of 1 represents each protein level obtained from the SV vessels 7 min after the addition of 30 nM 5-HT. A: Effect of insulin on the protein level of particulate 5-HT_{2A} receptor. Data are expressed as the mean \pm S.E.M. (n = 4). **P* < 0.05, compared with values obtained from time-control experiments applying vehicle for insulin (2nd column from left). B: Effect of insulin on the protein level of soluble 5-HT_{2A} receptor. Data are expressed as the mean \pm S.E.M. (n = 4). **P* < 0.05, compared with values obtained from time-control experiments applying vehicle for insulin (2nd column from left). C: Effect of inhibitors on the insulin-induced decrease in the protein level of particulate 5-HT_{2A} receptor. The rings were treated with 300 μ M L-NAME or L-NAME plus 100 nM wortmannin 15 min before the addition of 5-HT. Then, the same experiments as described above were carried out. Data are expressed as the mean \pm S.E.M. (n = 4). **P* < 0.05, compared with values obtained from time-control experiments applying the vehicle for insulin (2nd column from the left).

endothelium-denuded SV vessels, we conducted western blot analysis of 5-HT_{2A} receptors in the soluble and particulate fractions as well as the total protein after our contractile studies. In our experiments, insulin did not affect the total protein level of 5-HT_{2A} receptors, but we observed a decrease in the particulate protein level and a reciprocal increase in the soluble protein level of 5-HT_{2A} receptors in a concentration-dependent manner. These results demonstrate that insulin can induce the internalization of 5-HT_{2A} receptors from the plasma membrane to the cytoplasm. To investigate which signaling pathways were activated during insulin-induced NO-independent vasorelaxation, we focused on the PI3-K/Akt pathway because Doronin et al. reported that the phosphorylation of Akt mediated the internalization of G protein-coupled receptors, such as the β_2 -adrenergic receptor, in response to insulin (23). In this experiment, the

insulin-induced internalization of 5-HT_{2A} receptors was abolished by wortmannin but was not affected by L-NAME. These results suggest that the internalization of 5-HT_{2A} receptors was dependent on the PI3-K/Akt pathway, but was not due to NO production.

In conclusion, this study shows that the relaxant effect of insulin on the 5-HT-induced constriction of endothelium-denuded SV vessels is mediated in part by the internalization of the plasma membrane 5-HT_{2A} receptor as well as the production of NO via the PI3-K/Akt pathway. Our results reveal for the first time that defects in the insulin-induced sequestration of the 5-HT_{2A} receptor may partially explain the poor patency of SV grafts in DM patients when 5-HT is released from activated platelets.

We recently reported that in isolated human endothelium-denuded SVs, 5-HT_{2A} and 5-HT_{1B} receptors similarly contribute to 5-HT-induced vasoconstriction (24).

Thus, further investigation will be required to clarify whether insulin could induce the internalization of not only the 5-HT_{2A} receptor but also the 5-HT_{1B} receptor.

Acknowledgments

This study was supported in part by Japan Society for the Promotion of Science; Grants-in-Aid for Scientific Research (C) 22591556 to RY and for the Co-Research Project at Kyushu University of Health and Welfare.

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