Dokkyo Journal of Medical Sciences
37(1) : 9～14, 2010

Originals

Effect of Insulin on Aortic Rings Isolated from Wistar Rats

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SUMMARY

Background : Insulin increases nitric oxide (NO) production via the phosphatidylinositol 3-kinase (PI3K) pathway and endothelin (ET)-1 secretion via the mitogen-activated protein kinase (MAPK) pathway in vascular endothelial cells. In normal vascular endothelial cells, the insulin effect via the PI3K pathway is thought to occur slightly earlier than that via the MAPK pathway. However, the direct effect of insulin on vascular rings isolated from an animal model has not been fully examined. The main purpose of study was to investigate the potential vasodilation-effect of insulin on the isolated vascular rings of rats.

Material and Methods : Descending aorta rings isolated from Wister rats were used in exo-vivo study. In study using culture cells, bovine aortic endothelial cells were used, and the effect of insulin on the production of nitric oxide (NO) and the secretion of endothelin (ET)-1 was investigated. NO production was evaluating using diaminofluorescein-2, a NO-specific dye.

Results : Insulin (250 nM)-stimulated NO production evaluated using diaminofluorescein-2 (DAF-2) dependency on the NO concentration in BAECs indicated a significant increase in NO compared with controls. Addition of 250 nM insulin also significantly increased ET-1 secretion and phosphorylation of Akt at Ser473 and eNOS at Ser1177 (Ser1179 in bovine eNOS) in BAECs, compared with controls. Insulin treatment showed a slight tendency to increase phosphorylation of extracellular signal-regulated kinase (ERK) 1/2, but this change did not reach statistical significance. A cumulative insulin concentration of 3×10^{-6} M produced only a small change in the diameter of aortic rings from Wister rats (7.3 ± 3.5 % vs. 3.6 ± 3.5 % with 3 $\times 10^{-6}$ M saline ; both n = 3). Addition of acetylcholine to these rings caused dilatation of almost 100 %.

Conclusion : Insulin increases production of NO and secretion of ET-1 in BAECs, but has little effect on aortic rings isolated from Wister rats.

Key Words : insulin, NO, ET-1

INTRODUCTION

Insulin increases the levels of vasodepressors such as nitric oxide (NO) and prostaglandin (PG) I_2 in normal vascular endothelial cells, with NO thought to be the major vasodepressor produced by insulin[1,2)]. NO production by insulin is dependent on the phosphatidylinositol 3-kinase (PI3K)/Akt/endothelial NO synthetase (eNOS) pathway, which is distinct from the classical calcium-dependent pathway activated via G protein-coupled receptors such as the acetylcholine (Ach) receptor[1～9)]. Simultaneously, insulin also stimu-

Received September 25, 2009 ; accepted November 9, 2009
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lates the mitogen-activated protein kinase (MAPK)/endothelin-1 (ET-1) pathway[1,10,11], and ET-1 is a strong vasoconstrictor in endothelial cells. The opposite effects of insulin indicate that the balance of NO production and ET-1 secretion by insulin stimulation in vascular smooth muscle cells has an important role in maintaining normal vascular tonus in healthy subjects[1].

The insulin effect on the PI3KAkt/eNOS pathway is thought to occur slightly earlier than that on the MAPK/ET-1 pathway in normal vascular endothelial cells[1]. Given the apparent priority of insulin for the PI3K/Akt/eNOS pathway over MAPK/ET-1 pathway, we hypothesized that insulin would have a mild or moderate vasodilatatory effect on vascular rings. However, the direct effect of insulin on vascular rings isolated from an animal model has not been fully investigated. Therefore, in the current study we investigated the effect of insulin on aortic rings isolated from Wistar rats. We also confirmed the effects of insulin on NO production and ET-1 secretion in bovine aortic endothelial cells.

MATERIALS AND METHODS

Cell culture

Bovine aortic endothelial cells (BAECs) in primary culture (Cell Systems, Kirkland, WA, USA) were grown to 95% confluence in Dulbecco's modified Eagle's medium (DMEM) including 10% fetal bovine serum (FBS) at 37°C in a humid atmosphere with 5% CO_2. Cells were used between passages 4 and 6.

Measurement of NO in BAECs

Production of NO was assessed using diaminofluorescein-2 (DAF-2 ; Daiichi Pure Chemicals, Tokyo, Japan), an NO-specific fluorescent dye[12]. After overnight starvation, cells were washed in phosphate-buffered saline (PBS) and treated for 5 min with PBS containing 10 μM L-arginine, 10 μM DAF-2, and 0 or 250 nM insulin. An insulin concentration of 250 nM was used based on the significant increase in NO production observed at this concentration in a preliminary study in BAECs (data not shown). Supernatant was collected 5 min after addition of DAF-2 and NO production was measured as fluorescence intensity (FI) using an F-2500 Spectrophotometer (Hitachi, Tokyo, Japan) at wavelengths of 495 nm (excitation maximum) and 515 nm (emission maximum).

Endothelin-1 (ET-1) assay

ET-1 in the supernatant was assayed using an Endothelin-1 IBL Kit (Immune-Biological Laboratories, Gunma, Japan)[13]. Each sample for ET-1 measurement was collected under the same conditions as those used for NO measurement.

Vasodilatatory effects of insulin in Wistar rats

Animal studies were conducted according to the "Guiding principles for the Care and Use of Laboratory Animals" of the Japanese Pharmacological Society. All animal studies were performed by Hamamatsu Pharma Research, Inc. (Hamamatsu, Japan).

Nine-week old male Wistar (WT) rats (non-diabetic controls) were purchased from Japan SLC, Shizuoka, Japan. Rats were anesthetized with an overdose of pentobarbital and sacrificed by phlebotomy from the carotid artery. The chest of each rat was opened and the entire descending aorta was immediately dissected. Rings of the thoracic aorta of 5 mm in length were then prepared under a microscope. Preparations included rings with and without endothelial cells. Each ring was mounted vertically between two hooks in organ chamber myographs filled with 5 ml of Krebs-Henseleit solution of composition 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$ and 11 mM glucose aerated with 95% O_2 and 5% CO_2. Isometric tension was measured using a carrier amplifier (AP-621G, Nihon Kohden, Tokyo, Japan) and recorded with a multiple pen recorder (R-64, Rikadenki, Tokyo, Japan). After equilibration for 60 min, the rings were precontracted with a submaximal concentration of phenylephrine (10^{-6} M). Subsequently, the rings were exposed to cumulative concentrations of acetylcholine (Ach), nitroglycerin (NTG) or insulin. Control rings were treated with same amount of saline.

Western blotting

Cell lysates were prepared using 300 μg of lysis buffer [100 mM NaCl, 20 mM HEPES (pH 7.9), 1% Triton X-100, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, complete protease inhibitor cocktail, and phos-

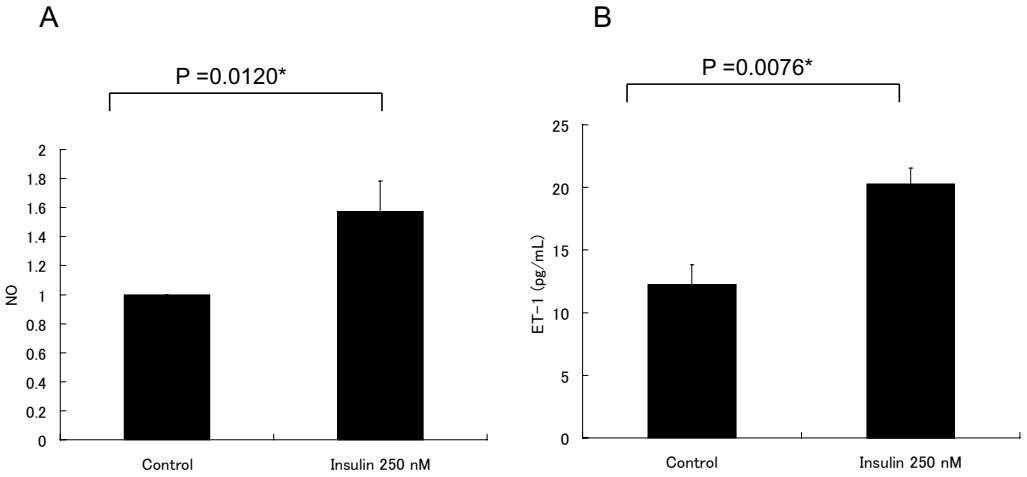

Fig. 1 The effect of insulin on NO production (A) and ET-1 secretion (B) on BAECs. * shows statistical significance ($P < 0.05$). n = 3, respectives

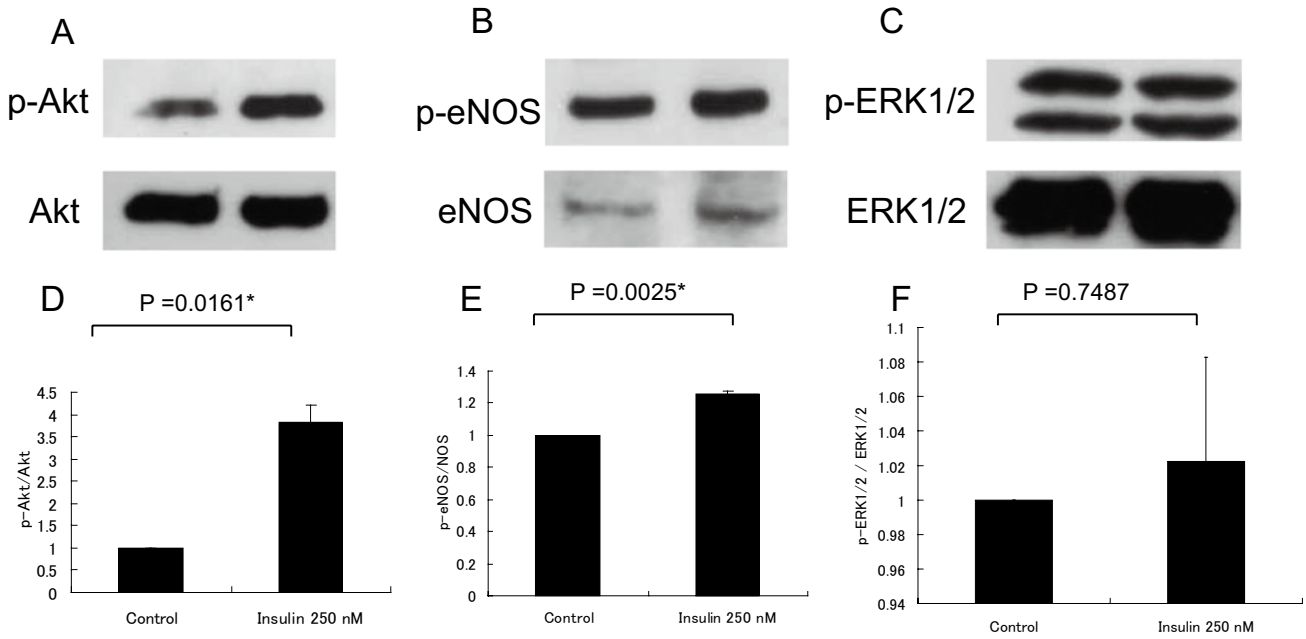

Fig. 2 The effect of insulin on phosphorylation of Akt, eNOS and ERK1/2 on BAECs. Representative samples are shown (A-C). In (D, E), * shows statistical significance ($P < 0.05$). n = 3, respectives

phatase inhibitor cocktail] (Roche Applied Sciences, Indianapolis, IN, USA). Samples (50 μg total protein) were separated by 10% SDS-PAGE and then immunoblotted on polyvinylidene difluoride membranes. The blots were incubated with the primary antibody at 4℃ overnight and probed with a peroxidase-conjugated secondary antibody using standard methods. Immunoreactive proteins were visualized on X-ray films by enhanced chemiluminescence. Blots were quantified by scanning densitometry (GS-800 Calibrated Densitometer, Bio-Rad Laboratories, Hercules, CA, USA). Anti-Akt antibody, anti-phospho-Ser473-Akt antibody, anti-eNOS antibody, anti-phospho-Ser1177 (Ser1179 in bovine) eNOS antibody, anti-extracellular signal-regulated kinase (ERK) 1/2 antibody, and anti-phospho-ERK1/2 antibody were obtained from Cell Signaling Technology (Danvers, MA, USA).

Statistical methods

All data are presented as means ± standard error (SE). Comparison between two variables was performed using an unpaired t test. $P < 0.05$ was considered to indicate statistical significance in all analyses.

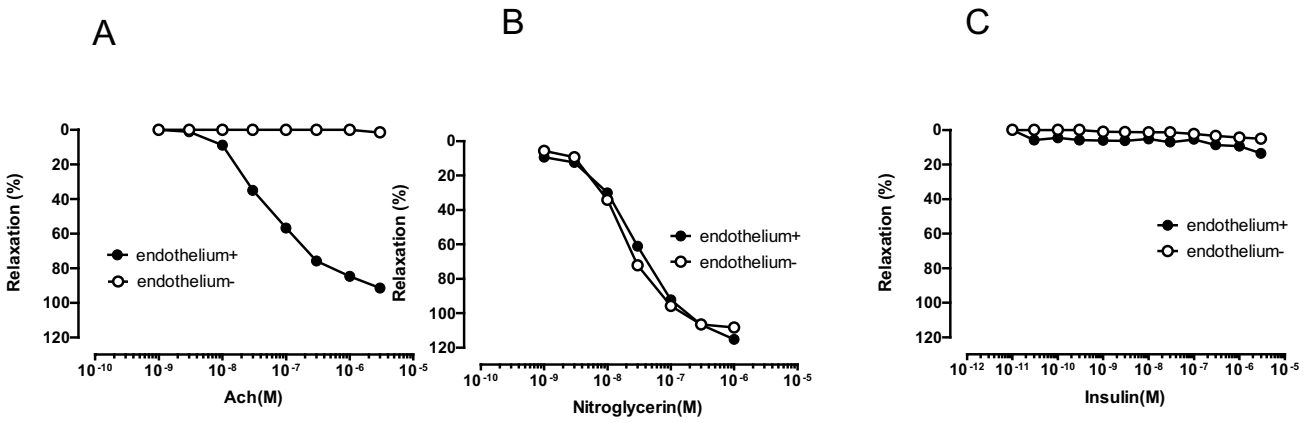

Fig. 3 Effect of Ach (A), nitroglycerin (B), and insulin (C) on dilatation of rat thoracic aorta rings (n = 2 for respective rings).

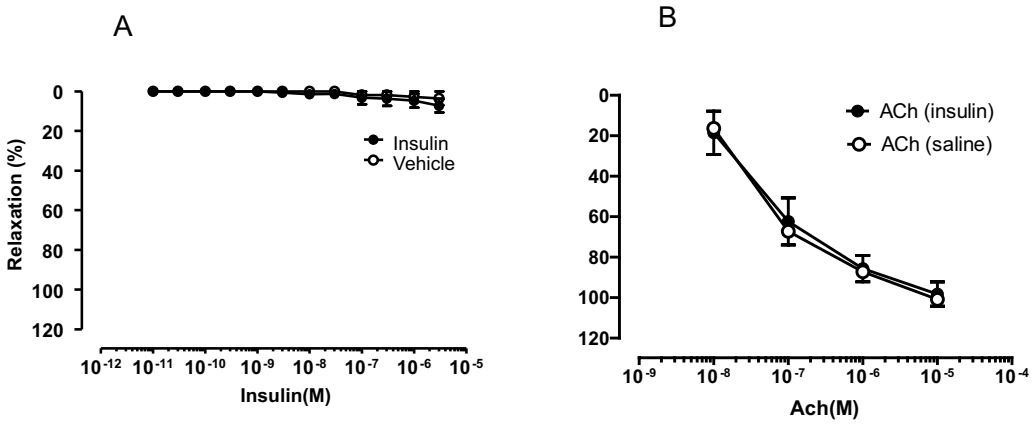

Fig. 4 Effect of insulin on dilatation of rat thoracic aorta rings with endothelium (A), and additional effect of Ach on dilatatin of these rings (n = 3 for respective rings).

RESULTS

Insulin-induced NO production and ET-1 secretion in BAECs

Insulin (250 nM)-stimulated NO production evaluated using the DAF-2 dependency on the NO concentration in BAECs indicated a significant increase of NO compared with controls (Fig. 1A). Addition of 250 nM insulin also significantly increased ET-1 secretion in BAECs (Fig. 1B).

Insulin-stimulated phosphorylation of Akt at Ser473 and eNOS at Ser1177 in BAECs

Addition of insulin (250 nM) to BAECs significantly increased phosphorylation of Akt at Ser473 and eNOS at Ser1177 (Ser1179 in bovine eNOS), compared with controls (Fig. 2A, B, D, E). Insulin treatment also showed a tendency to increase phosphorylation of ERK1/2, but this effect did not reach statistical significance (Fig. 2C, F).

Effect of insulin on dilatation of rat thoracic aorta rings

Rings with and without endothelium were prepared from two WT rats. Cumulative addition of Ach ($1 \times 10^{-9} - 1 \times 10^{-6}$ M) caused 91.5 % relaxation of rings with endothelium (n = 2), but had no effect on rings without endothelium (dilatation of 1.5 % at 3×10^{-6} M Ach ; Fig. 3A). Cumulative addition of NTG ($1 \times 10^{-9} - 1 \times 10^{-6}$ M) produced about 100 % relaxation in rings with and without endothelium (both n = 2 ; Fig. 3B). In contrast, insulin (1×10^{-6} M) had little effect on relaxation of either type of ring, with 13.6 % and 5.1 % relaxation of rings with and without endothelium, respectively (both n = 2 ; Fig. 3C). An additional study was performed in aortic rings with endothelium prepared from three more WT rats. In these rings, in-

sulin (3×10^{-6} M) produced relaxation of 7.3 ± 3.5 %, compared to 3.6 ± 3.5 % with the same volume of saline (both n = 3, not significant ; Fig 4A). Ach caused 100 % dilatation of these rings (Fig. 4B).

DISCUSSION

The results of the study confirm previous findings showing that insulin increases NO production, ET-1 secretion, and the levels of phosphorylated eNOS and phosphorylated ERK1/2 [1,2,5,8~11] in BAECs. The increase in p-ERK stimulated by insulin was slight and the change was not significant in this study, which was probably due to the relatively low insulin concentration. Our data are generally consistent with the established concept that insulin promotes NO production and ET-1 secretion by enhancing the respective PI3K/Akt/eNOS and MAPK/ET-1 pathways.

The balance of insulin-stimulated NO and ET-1 production in vascular smooth muscle cells is important for maintenance of vascular tonus, and the vasodilatatory effect of insulin through an increase in NO is thought to occur slightly before the vasoconstrictive effect produced by an increase in ET-1 in a normal state [1]. Therefore, we expected that insulin would show a clear vasodilatatory effect on vascular aortic rings isolated from Wistar rats. However, we found that insulin had little effect on the diameter of aortic rings with or without endothelial cells : the 7 % relaxation found at a super-physiological insulin concentration (3×10^{-6} M) in rings with endothelial cells was insignificant compared with relaxation of about 3 % in similar saline-treated rings. This result corresponds to that in very recent study in 10 week-old age C57B16 mice fed a normal diet, in which about 7 % relaxation of isolated aortic rings was found at a super-physiological insulin concentration of about 3×10^{-6} M [14]. The small effect of insulin on normal aortic rings suggests that insulin stimulation of NO via the PI3K/Akt/eNOS pathway and ET1 via the MAPK/ET-1 pathway are in balance, without the suggested priority of NO production over that of ET-1 [1].

In the current study, we did not investigate the effect of insulin on aortic rings isolated from a type 2 diabetic rat model such as the Goto Kakizaki (GK) rat. However, in a robust insulin-resistant state, it is likely that ET-1 secretion induced by insulin occurs prior to NO secretion in endothelial cells due to selective insulin resistance, which is generally recognized in type 2 diabetes or metabolic syndrome [1,15]. In fact, a recent study showed reduced insulin-stimulated relaxation of aortic rings (less than 5 % relaxation) in insulin receptor-knockout mice compared with wild type mice [14]. Furthermore, in a previous clinical study, we found that flow-mediated vasodilatation (FMD), which reflects endothelial cell-dependent NO production, in type 2 diabetic patients has a tendency to decrease compared with that in non-diabetic patients [16]. Therefore, we speculate that addition of insulin to precontracted aortic rings from type 2 diabetic rats will have less effect on the ring diameter compared to the effect on rings from normal rats, but that insulin may inhibit aortic dilatation by Ach.

The strongest vasodepressor produced by insulin is thought to be NO [1], but insulin-stimulated PGI_2 may also be an important vasodepressor [1,17~19]. We did not investigate the effect of insulin on PGI_2 production or the effect of PGI_2 on aortic rings in the current study, and it will be of interest to examine the balance of PGI_2 and ET-1 through investigation of the effect of insulin on aortic rings in the presence of an eNOS inhibitor.

In the current study, we missed investigating the effect of insulin for NO production or ET-1 secretion on BAECs or that for vasodilation and vasoconstriction on vascular rings under existence of NO synthetase inhibitor such as N^w-nitro-L-arginine methyl ester (L-NAME), or PI3K inhibitors such as wortamanin and LY294002 ; these drugs inhibit PI3K/eNOS/NO pathway of insulin. Furthermore, regarding MAPK/ET-1 pathway, these effects of insulin under drugs such as p44/pERK inhibitor, PD98059 and U0126 was not investigated. The use of these drugs may have more clarified the effect of insulin on vascular endothelial cells, and this is one of limitations of this study.

Finally, the number of aortic rings investigated in this study was relatively small ; at least, 4-5 aortic rings should have been used.

In conclusion, we found that insulin has little effect on the diameter of aortic rings isolated from Wistar rats. This result suggests that the insulin-stimulated NO production and ET-1 secretion is approximately in balance in normal rats. The potentially harmful effect

of insulin on aortic rings prepared from an animal model of type 2 diabetes requires investigation in a further study.

Disclosure summary : The authors have nothing to disclose.

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