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EFFECTS OF INSULIN ON NITRIC OXIDE PRODUCTION IN CULTURED HUMAN ENDOTHELIAL CELLS

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It has been reported that insulin treatment improves hypertension in the patients with diabetes mellitus. The mechanisms of the antihypertensive effect of insulin, however, remains to be fully elucidated. Nitric oxide (NO) is generated in the endothelial cells and tonically regulates blood pressure. In the present study, we investigated the effect of insulin on NO production in cultured human endothelial cells. Insulin (1 μM, 10 μM and 100 μM) increased NO metabolite (NO_2^-) levels in the medium and NO synthase (NOS) activity in cultured human endothelial cells. Insulin also stimulated proliferation and mitogen-associated protein kinase activity of the endothelial cells. These findings suggest that insulin reduces blood pressure by stimulating NO production in the endothelial cells, possibly via induction of NOS molecule and proliferation of endothelial cells.

Key words: insulin/nitric oxide (NO)/nitric oxide synthase (NOS)/endothelial cell/growth

Hypertension is one of the complications in diabetes mellitus (DM) and a risk factor for such serious vascular episodes as myocardial infarction and cerebral infarction in diabetic patients (1, 2). Previous studies on hyperinsulinemia and blood pressure in the patients with DM suggested that insulin has vasodilative and hypotensive effects (3, 4). The antihypertensive effect of insulin has been explained either by the secondary action via improved glucose metabolism after insulin treatment or by the direct action on peripheral arterial vessels (3, 5, 6).

Nitric oxide (NO) has been identified as an endothelium-derived relaxing factor which acts as a modulator of vascular resistance under physiological conditions (7, 8). NO metabolite levels in plasma and urine were elevated in the diabetic patients treated with insulin (9, 10). These results raise the possibility that the antihypertensive effect of insulin is closely related to endothelial NO generation. To clarify this possibility, we studied the effects of insulin on NO production in human endothelial cells. Our findings suggest that insulin improves hypertension by stimulating NO production in endothelium and proliferation of endothelial cells.

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MATERIALS AND METHODS

Drugs

Recombinant human insulin (Novolin N) was purchased from Novo Nordisk (Bagsvaerd, Denmark). L-[^{3}H] arginine (2.33 GBq/mmol), [γ-^{32}P]ATP (111 GBq/mmol) and mitogen-associated protein (MAP) kinase assay kit were purchased from Amersham Life Science (Buckinghamshire, England). L-[^{14}C]citrulline (2.2 GBq/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Tetrahydrobiopterin was a generous gift from Suntory Biomedical Research Center (Osaka, Japan). All other chemicals were of the purest grade available from regular commercial sources.

Endothelial cell culture

Human endothelial cells were obtained from Clonetics (San Diego, CA, U.S.A.), maintained and subcultured in modified MCDB 131 medium supplemented with 2% fetal calf serum, 12 mg/L bovine brain extract, 1 mg/L hydrocortisone, 10 μg/L

human epidermal growth factor, 50 mg/L gentamycin and 50 μg/L amphotericine, and cultured in the same medium under a humidified atmosphere of 5% CO_2-95% air at 37 ℃. Endothelial cells were plated on 96-well plates (ICN Biomedicals, Aurora, OH, U.S.A.) for 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay, poly-L-lysine coated 24-well plates (Sumitomo Bakelite, Tokyo, Japan) for nitrite (NO_2^-) and nitrate (NO_3^-) assay, and poly-L-lysine coated 6-well plates (Sumitomo Bakelite, Tokyo, Japan) for measurements of NO synthase (NOS) activity and MAP kinase activity.

Nitrite (NO_2^-) *and nitrate* (NO_3^-) *assay*

After 30-min preincubation with Dulbecco's modified Eagle's medium (DMEM) which contains 1 g/L glucose, cells were incubated with DMEM containing various concentrations of insulin for 4 h under a humidified atmosphere of 5% CO_2-95% air at 37℃. Then, the incubation medium was collected and centrifuged at 10,000 rpm for 10 min. The resulting supernatant was stored at -20℃ until assayed.

Nitrite and nitrate concentrations were measured by the NOx analyzing HPLC system (ENO-10, EICOM, Kyoto, Japan) as reported previously (11).

NOS activity assay

Following 30-min preincubation with DMEM which contains 1 g/L glucose, the incubation medium was replaced by DMEM containing various concentrations of insulin (1 μM, 10 μM and 100 μM). After incubation for 4 h under a humidified atmosphere of 5% CO_2-95% air at 37℃, the cells were washed with phosphate buffered saline (PBS, pH 7.4) and collected with 0.5 ml of 50 mM Tris-HCl (pH 7.4) containing 1 mM dithiothreitol, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin and 1 mM phenylmethylsulfonyl fluoride. After sonication, cells were centrifuged at 100,000 x g for 30 min at 4℃. Resulting supernatant was stored at -80℃ until assayed for NOS activity.

NOS activity was measured by production of L-[^{3}H]citrulline from L-[^{3}H]arginine. Aliquot (50 μl) of supernatant was incubated at 37℃ for 20 min in the presence of 50 mM Tris-HCl (pH 7.5), 1mM NADPH, 10 μM flavin adenine dinucleotide, 10 μM tetrahydrobiopterin, 40 μM L-arginine, 3.7 MBq of L-[^{3}H]arginine and 4 mM $CaCl_2$ in a total volume of 100 μl. The reaction was halted by the addition of 1.25 ml of ice-cold 20 mM HEPES (pH 5.5), and the total volume was applied on a Dowex-50Wx 8 (200-400 mesh, Na^+ form) column preequilibrated with 20 mM HEPES (pH 5.5). Flow-through and 2 ml wash of distilled water were collected and the radioactivity was measured by a liquid scintillation counter. The recovery rate of citrulline determined by applying L-[^{14}C]citrulline solution was 80 - 90%. The L-[^{3}H]citrulline concentration produced was corrected by the recovery rate.

MTT assay

After 3-day culture with drugs added to the culture medium which contains 1 g/L glucose every 48 h, viable cell number was measured with MTT assay as previously reported (12).

MAP kinase activity

After 24 h incubation without serum, MAP kinase activity in the cells was measured as reported previously (12).

Protein content

Protein content was measured with Bio-Rad protein assay with bovine serum albumin as the standard (13).

Statistical analysis

All results were expressed by means ± SEM. The statistical differences were evaluated with ANOVA and Fisher's test. A probability level of $P<0.05$ was considered significant.

RESULTS AND DISCUSSION

After the cells were cultured with insulin for 4 h under a humidified atmosphere of 5% CO_2-95% air at 37℃, NOx in the incubation medium was measured. Most of NOx in the cultured medium was detected as NO_2^- (data not shown). NO_2^- levels in the culture medium increased in a dose-

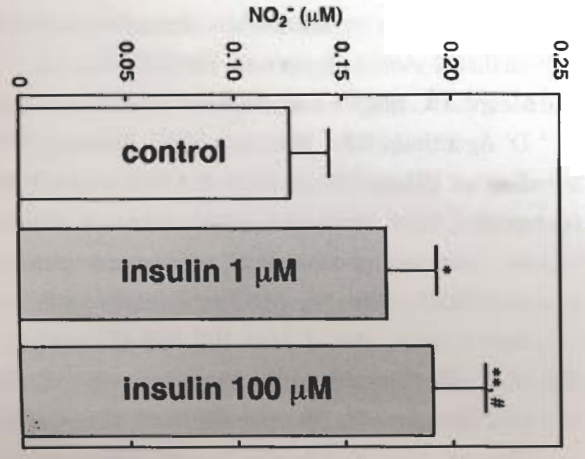

Fig. 1. Effect of insulin on NO_2^- concentration in the incubation medium of human endothelial cells. After human endothelial cells were incubated with insulin (1 μM and 100 μM) for 4 h, the incubation medium was collected and centrifuged at 10,000 rpm for 10 min. The supernatant was applied on the NOx analysis system. Each value shows the mean ± SEM of 5 different experiments. *, $p<0.05$ vs. control group. **, $p<0.01$ vs. control group. #, $p<0.05$ vs. the group added with 1 μM insulin.

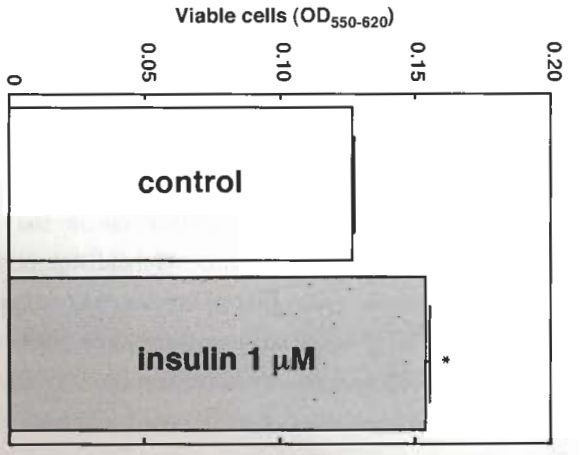

Fig. 3. Effect of insulin on the number of viable human endothelial cells. After human endothelial cells were cultured with 1 μM insulin for 3 days, viable cell number was estimated by MTT assay as described in the text. Each value shows the mean ± SEM of 10 different experiments. *, $p<0.05$ vs. control group.

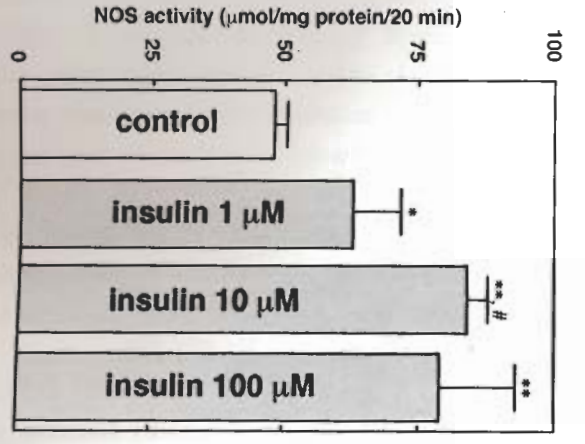

Fig. 2. Effect of insulin on NOS activity in human endothelial cells. After human endothelial cells were incubated with insulin (1 μM, 10 μM and 100 μM) for 4 h, the cells were collected, sonicated and centrifuged. The supernatant was used for the NOS assay as described in the text. Each value shows the mean ± SEM of 5 different experiments. *, $p<0.05$ vs. control group. **, $p<0.01$ vs. control group. #, $p<0.05$ vs. the group added with 1 μM insulin.

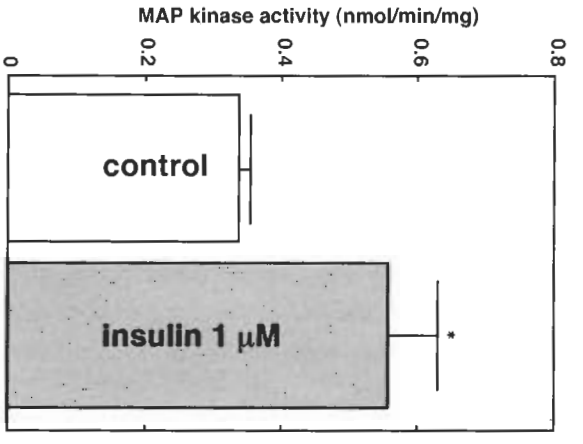

Fig. 4. Effect of insulin on MAP kinase activity in human endothelial cells. After starvation for 24 h, human endothelial cells were incubated with 1 μM insulin for 2.5 min at 37℃. MAP kinase activity of cell lysates was measured with a MAP kinase assay kit and [γ-^{32}P] ATP (Amersham Life Science) according to the manual of the assay kit. Each value shows the mean ± SEM of 6 different experiments. *, $p<0.05$ vs. control group.

related manner by insulin (Fig. 1). These data suggest that insulin stimulates NO generation in the endothelial cells, which is consistent to previous findings (14-16). At the end of the incubation with insulin, cells were collected and NOS activity was measured. NOS activity in the human endothelial cells increased by insulin in a dose-related manner (Fig. 2). Since we measured NOS activity

in the presence of excess arginine and tetrahydrobiopterin, these data suggest that insulin induces NOS molecule in the endothelial cells. NOS activity is dependent on intracellular Ca^{2+} concentrations. The insulin-induced increase in NOS activity can be due to an increase in the intracellular Ca^{2+} concentration. We did not investigate this possibility. Since insulin has trophic actions (17, 18), we finally examined in cultured human endothelial cells whether insulin stimulates proliferation of endothelial cells which produce NO. When the human endothelial cells were cultured with 1 μM insulin for 3 days, viable cell number significantly increased more than contol (Fig. 3). After the endothelial cells were stimulated by 1 μM insulin for 2.5 min, MAP kinase activity increased in the treated cells compared with the control cells (Fig. 4). Similar effects of insulin were observed at the concentrations of 10 μM and 100 μM, and the effects of insulin on cell proliferation and MAP kinase were maximal at the concentration of 1 μM (data not shown). These data taken together suggest that insulin stimulates proliferation of endothelial cells. It is speculated, therefore, that insulin stimulates NO production by both induction of NOS molecule and enhancement of endothelial proliferation. Although the concentrations of insulin used in the present study were much higher than the plasma concentration, it may be possible that the NO-producing action of insulin is exerted on the endothelium in the vicinity of pancreatic β cells.

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