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PRELIMINARY REPORT

Effect of Insulin on Human Aortic Endothelial Nitric Oxide Synthase

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It has recently been shown that insulin induces vasodilation in human arteries and veins in vivo. This effect of insulin has been shown to be a direct one on the human vein. In view of these observations and the fact that insulin-induced vasodilation is impaired in insulin-resistant states like type 2 diabetes and obesity, we have investigated the hypothesis that insulin may induce the expression of endothelial nitric oxide synthase (e-NOS) in endothelial cells grown from human aortae (HAECs), human lower-limb veins, and human umbilical veins (HUVECs), and microvascular endothelial cells (MVECs) from human adipose tissue. The expression of e-NOS was maximal in HAECs, and therefore, further experiments were performed on these cells. When cells reached 90% confluence, they were induced with different concentrations of insulin (0, 25, 100, and 1,000 μU/mL) for 6 days. The cells were homogenized and e-NOS expression was examined by Western blotting. A dose-dependent induction by insulin of e-NOS in the endothelial cells was clearly demonstrated. There was no detectable level of the inducible NOS isoform (i-NOS), and this effect of insulin was independent of cell proliferation. We conclude that insulin induces a dose-dependent induction of e-NOS in human aortic cells (and possibly arterial/endothelial cells), and this effect may contribute to the overall vasodilatory effect of insulin.

INSULIN has recently been shown to be a vasodilator. This vasodilatory effect has been found in various settings: increased limb blood flow measured either plethysmographically or via a flow probe before and after systemic infusion of insulin[1-4]; vasodilation of dorsal hand veins with the differential transformer technique[5]; and vasodilation of the cephalic vein following infusion of insulin, using an ultrasonograph for imaging.[6] In the demonstrations of increased blood flow following systemic insulin infusion, it has been shown that the coinfusion of N^G-monomethyl-L-arginine, an inhibitor of nitric oxide synthase (NOS), inhibits the increment in limb blood flow.[2,3] Similarly, infusion of methylene blue, an inhibitor of NOS and guanylate cyclase, inhibits insulin-induced vasodilation of the cephalic vein.[6] While the ability of insulin to cause vasodilation in insulin-resistant populations with obesity and type 2 diabetes is impaired,[7-10] the ability of sodium nitroprusside to induce vasodilation in these populations is intact. Since sodium nitroprusside exerts an effect by generating nitric oxide (NO) directly, it would appear that the vasodilatory effect of insulin is exerted by endothelium-mediated NO release, and the basic effect in type 2 diabetes and obesity is an impairment of endothelial NO generation and release.

It has recently been shown that insulin induces an acute release of NO from human umbilical vein endothelial cell (HUVEC) cultures using a NO-specific amperometric electrode,[11] and from cultured rat aortic endothelial cells.[12] In view of these observations and the absence of data on the possible effect of insulin on human arterial endothelial cells, we decided to investigate whether insulin induces an increase in the biosynthesis of NOS in the endothelium in vitro, using cultures of human aortic endothelial cells (HAECs).

MATERIALS AND METHODS

Cell Isolation and Harvesting

Endothelial cells were harvested from human aortae and arterial vessels using the method described by Gospodarowicz et al.[13] The dissected aortae and arteries were placed in phosphate-buffered saline (PBS) supplemented with antibiotics. The vessels were washed several times with PBS and incubated with 0.1% collagenase/dispase solution (Boehringer Mannheim, Indianapolis, IN) in medium 199 ([M199] GIBCO, Grand Island, NY) for 20 minutes. The solution was centrifuged and the cells were collected and harvested in flasks coated with fetal bovine serum (FBS). The cell identity was confirmed by immunohistochemical staining that was positive for factor VIII, *Ulex euro-*

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paeous, EN4, and CD31 and negative for α-actin as described later. HAECs were allowed to reach confluence and then were trypsinized and passaged. All experiments were performed using cultures at passage 5 to 6. HAECs were allowed to reach 90% confluence, washed with PBS, and placed in phenol-free endothelial cell growth medium (Clonetics, Walkersville, MD) containing 2% charcoal/dextran-stripped FBS (Hyclone, Logan, UT) for 24 hours. On the second day, the cells were induced with insulin (0, 25, 100, and 1,000 μU/mL). The medium was changed every 2 days for 6 days. After induction, the cells were collected and homogenized for Western blotting.

HUVECs were isolated from umbilical cords collected aseptically from postdelivery cesarean sections. Cells were isolated in a similar fashion as the HAECs using collagenase/dispase digestion.

The isolation of human microvascular endothelial cells (MVECs) was based on the method of Kern et al.[14] MVECs were isolated from subcutaneous adipose tissue following liposuction. The fat was minced, washed with PBS, and digested with 0.5% collagenase/dispase mixture using M199 as the diluent. The fat was centrifuged at 750× *g* for 10 minutes, and the buoyant fat "tuft" and supernatant were decanted. The pellet was resuspended in growth medium containing M199, 10% FBS, 10 μg/mL endothelial cell growth factor, 5% vitamins, 5% minimum essential amino acids, and antibiotic mixture. The cells were plated on FBS-coated plates, and cells showing different morphological features versus endothelial cells were removed by a sterile stick every 2 days.

Immunohistochemistry

HAECs were grown in monolayers on sterile slides. The slides were then washed twice with PBS-Tween and fixed for 10 minutes at room temperature in an acetic acid:ethanol:water mixture (1:18:1). Slides were stained using BioGenex (San Ramon, CA) supersensitive detection system. Briefly, the slides were blocked with 3% hydrogen peroxide for 10 minutes and incubated with primary antibodies against factor VIII, *Ulex europaeous* (Sigma, St Louis, MO), CD31, EN4, and smooth muscle α-actin (BioGenex). They were then washed and incubated with biotinylated anti-immunoglobulin followed by peroxidase-conjugated streptavidin. Finally, the avidin-biotin complex was detected using 3,3′-diaminobenzidine chromogen.

Western Blot Analysis

Total cell lysates were prepared by washing the adherent cells with PBS followed by 1 mL boiling lysis buffer (1% sodium dodecyl sulfate [SDS], 1 mmol/L sodium orthovanadate, and 10 mmol/L Tris, pH 7.4). The cells were scraped and transferred to a microcentrifuge tube, boiled for an additional 5 minutes, and centrifuged at 14,000× *g* for 5 minutes. Total protein concentrations were determined using a bicinchoninic acid protein assay (Pierce, Rockford, IL). Twenty micrograms of total cell lysate were subjected to 6% SDS–polyacrylamide gel electrophoresis. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked for 1 hour in 5% nonfat dry milk in 0.02% Tween–Tris-buffered saline buffer (TTBS) and then incubated overnight with monoclonal antibodies against endothelial NOS or the inducible NOS isoform ([e-NOS or i-NOS] Transduction Laboratories, Lexington, KY). The membrane was washed 4 times for 15 minutes each with TTBS and then incubated with peroxidase-conjugated goat anti-mouse IgG for 1 hour. Finally, the membrane was washed and developed using super signal chemiluminescence reagent (Pierce). Densitometry was performed with molecular analyst software (Bio-Rad, Hercules, CA).

Cell Proliferation Assay

The CellTiter 96 Aqueous nonradioactive proliferation assay (Promega, Madison, WI) was used to determine the number of viable cells. Briefly, 5,000 cells per well were cultured in 100-μL 96-well plates. Twenty microliters of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfonyl)-2H-tetr azolium was added to each well and incubated for 2 hours at 37°C in a humidified 5% CO_2 atmosphere. Absorbance was recorded at 490 nm using a microplate reader.

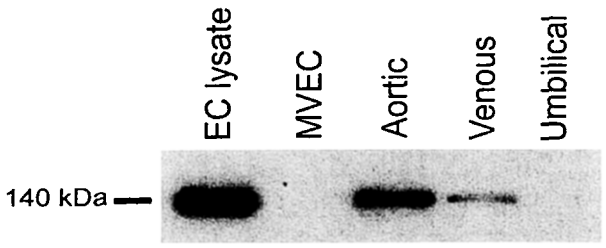

Fig 1. Western blot showing the relative expression of e-NOS in human MVEC, aortic, venous, and umbilical endothelial cells. Note that e-NOS expression is greatest in aortic, medium in venous, and minimal in MVECs and HUVECs (umbilical), in which it appears only at very high x-ray film exposure. EC lysate is an aortic endothelial cell homogenate supplied as a positive control from Transduction Laboratories. These cells were also tested for expression of i-NOS, and no i-NOS bands were observed in any of these cell types (data not shown).

RESULTS

Endothelial cells were grown from human aortae, peripheral veins, and umbilical veins and MVECs were obtained from adipose tissue as already described. Western blots prepared from these cells showed the presence of a 140-kd e-NOS band in the aortic and venous endothelial cell homogenates (Fig 1). The e-NOS band was observed with HUVECs only at very high x-ray film exposure, and there was no detectable band with MVECs. Maximum expression of e-NOS was observed in aortic endothelial cells. Further experiments on e-NOS were therefore performed only in HAECs. There was no detectable

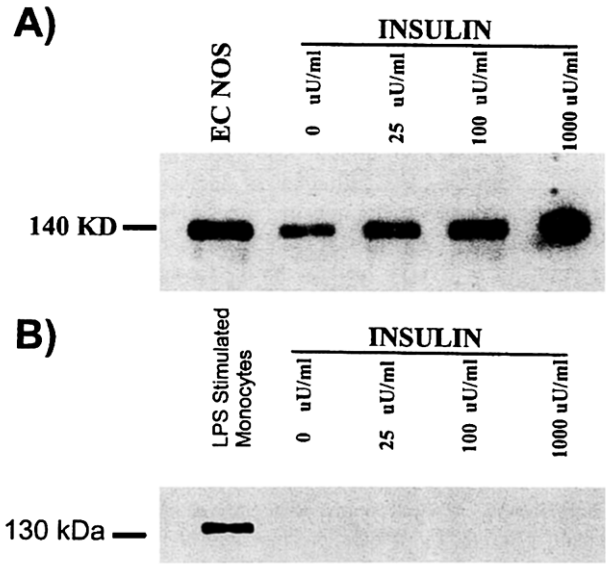

Fig 2. (A) Western blot showing the induction of e-NOS in HAECs. Induction is observed at 25 μU/mL. EC lysate is an aortic endothelial cell homogenate supplied as a positive control from Transduction Laboratories. (B) No i-NOS bands were observed in HAECs induced with different concentrations of insulin, even at very high x-ray film exposure. Macrophage lysate is a macrophage cell homogenate supplied as a positive control from Transduction Laboratories.

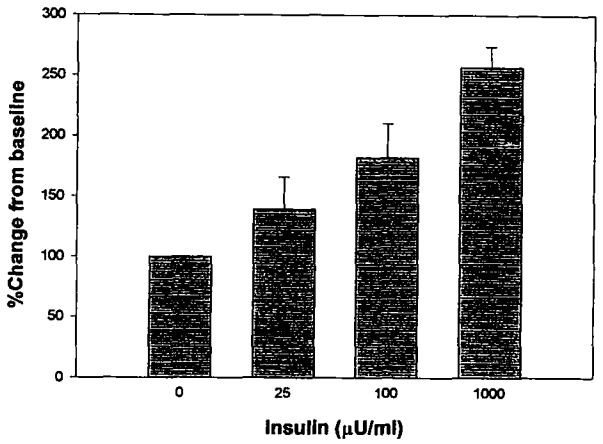

Fig 3. e-NOS induction in HAECs by insulin. Results are the mean ± SD. All values are normalized to a baseline of 100%.

i-NOS in aortic, venous, or microvascular endothelia (data not shown).

HAEC homogenates from cells induced with 0, 25, 100, and 1,000 μU/mL insulin showed a dose-dependent increase in e-NOS following incubation for 6 days (Fig 2A). i-NOS was not detected in these cells (Fig 2B). Densitometric analysis of e-NOS Western blots confirmed the dose-dependent effect of insulin on e-NOS induction in HAECs (Fig 3). This effect of insulin was independent of cell proliferation, since cell counts increased to a similar extent in cultures with and without insulin at various concentrations (Fig 4).

DISCUSSION

Our results demonstrate for the first time that insulin induces an increase in e-NOS in HAECs in culture at physiologically relevant concentrations. This effect is dose-dependent, being the most impressive at 6 days. While insulin has been shown to have an endothelium-dependent NO-mediated acute vasodilatory effect on veins and arteries and to induce the release of NO by HUVECs, there is hitherto no evidence demonstrating that insulin may also have an effect on e-NOS expression in endothelial cells. Our data thus add further to the understanding of insulin-induced NO-mediated vasodilation by extending the previous research showing an acute effect of insulin on NO release by HUVECs.

The mechanism of action of insulin in the increase of e-NOS is not clear. Whether insulin treatment induces de novo NOS synthesis through gene activation or whether it exerts its effect posttranscriptionally is not clear. We are currently investigating whether these effects are mediated through the known cascade of postreceptor events as they occur in classic insulin-responsive cells like adipocytes.

The pathophysiological significance of our findings is obvious. Insulin stimulates the acute release of NO in vivo, as reflected in its acute vasodilatory effect discussed earlier. In addition, Zeng and Quon[11] demonstrated an acute effect of insulin on NO release in vitro following treatment of HUVECs with insulin. This acute NO-releasing effect of insulin on HUVECs was inhibited by N^G-nitro-L-arginine methyl ester (L-NAME), genestein, and wortmannin; thus, this effect is dependent on NOS, tyrosine phosphorylation, and phosphatidylinositol 3-kinase. Insulin-induced NO release in HUVECs had a 50% effective dose of 500 nmol/L and a maximum effective concentration of 1,000 nmol/L. These are clearly pharmacological concentrations and raise the possibility that this effect of insulin may be mediated by insulin-like growth factor-1 (IGF-1) receptors, since insulin binds to the IGF-1 receptor at high concentrations. Indeed, this effect of insulin was inhibited 50% by IGF-1 receptor antibody.[3]

In our experiments, a stimulatory effect of insulin on e-NOS was observed at 25 μU/mL, and there was a dose-dependent

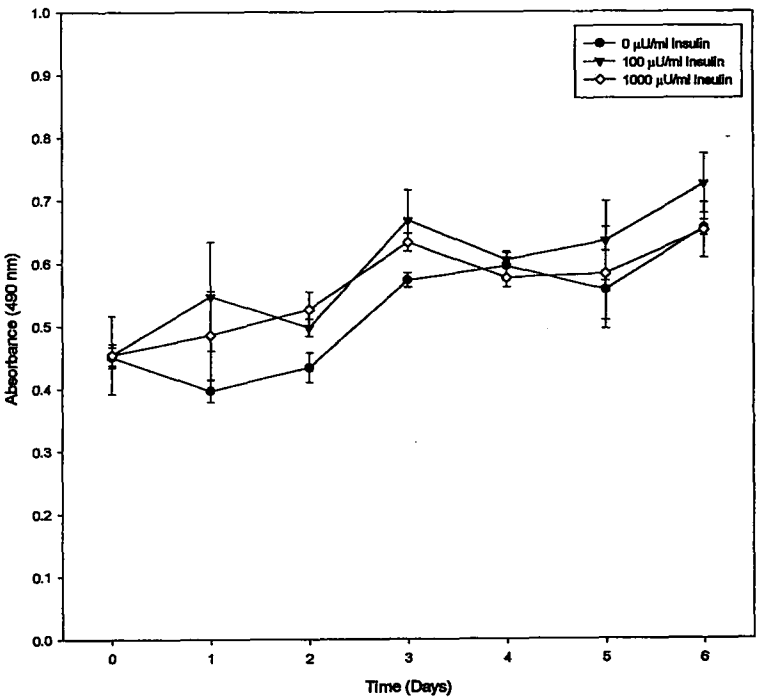

Fig 4. Endothelial cell proliferation over a period of 6 days. Note that there is no significant difference in cell counts in incubates containing 0, 100, or 1,000 μU/mL insulin.

increase at least up to 1,000 μU/mL (6 nmol/L). There was thus a threshold increase in e-NOS at physiological concentrations although the dose-response curve extended into the pharmacological range. It is possible that while the NOS-enhancing effect observed at physiological concentrations is mediated through the insulin receptor, at higher concentrations it is mediated by the IGF-1 receptor. This area requires further elucidation. It should also be noted that the experiments on the acute effect of insulin on endothelial NO release were performed on HUVECs, which, in our experiments, had a markedly lower expression of e-NOS as compared with human aortic cells, which have abundant e-NOS. It is possible that HAECs may respond with an increase in NO release at lower insulin concentrations.

Our data show that insulin also increases the endothelial content of e-NOS, the enzyme responsible for NO synthesis. These observations are consistent with the vasodilatory effect of insulin and extend the previous observation that insulin induces the acute release of NO from endothelial cells harvested from human umbilical veins. Since insulin also exerts a platelet antiaggregatory effect through activation of the platelet NO and guanylate cyclase system,[15] insulin probably helps to maintain the integrity of the vascular lumen. The lack of insulin or resistance to the action of insulin will cause the vascular proconstrictor state with increased platelet aggregability known to occur in diabetes mellitus.[16-19] Furthermore, the fact that endothelial NO may also reduce the expression of adhesion molecules on the endothelial cell surface may prevent the adhesion of monocytes to the endothelium. This adhesion is one of the initial events in atherogenesis.[20] The monocyte-endothelial adhesion leads to monocyte activation, reactive oxygen species (ROS) generation, and protease secretion. ROS generation leads to lipid peroxidation, foam cell formation, and accumulation of foam cells subendothelially to form a fatty streak—this is the first macroscopic lesion of atherosclerosis. Indeed, administration of L-NAME, a NOS inhibitor, in rabbits causes atherosclerosis.[21] Thus, the stimulatory effect of insulin on NOS may be considered antiatherogenic.

These facts are consistent with the concept of diabetes mellitus—both type 1 and type 2—being a proconstrictor and platelet proaggregatory state,[16-19] which is associated with atherosclerosis. Indeed, the vasodilatory effect of insulin has been shown to be markedly impaired in the femoral artery in type 2 diabetes and obesity.[2] We have also demonstrated an impairment in the vasodilatory effect of insulin in our cephalic vein model in type 2 diabetes and in obese subjects with impaired glucose tolerance.[7] It would therefore be of interest to investigate whether endothelial cells harvested from patients with type 2 diabetes and/or obesity have an altered response to insulin.

In conclusion, insulin induces an increase in e-NOS content in HAECs. This effect is consistent with the concept of insulin as a vasodilatory hormone, and provides one mechanism underlying this effect of insulin.

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