Regulation of Endothelial Constitutive Nitric Oxide Synthase Gene Expression in Endothelial Cells and In Vivo
A Specific Vascular Action of Insulin

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Background—The vasodilatory effect of insulin can be acute or increase with time from 1 to 7 hours, suggesting that insulin may enhance the expression of endothelial nitric oxide synthase (eNOS) in endothelial cells. The objective of the present study was to characterize the extent and signaling pathways by which insulin regulates the expression of eNOS in endothelial cells and vascular tissues.

Methods and Results—Physiological concentrations of insulin (10^{-10} to 10^{-7} mmol/L) increased the levels of eNOS mRNA, protein, and activity by 2-fold after 2 to 8 hours of incubation in cultured bovine aortic endothelial cells. Insulin enhanced eNOS gene expression in microvessels isolated from Zucker lean rats but not from insulin-resistant Zucker fatty rats. Inhibitors of phosphatidylinositol-3 kinase (PI-3 kinase) decreased the effect of insulin on eNOS gene expression, but a general protein kinase C (PKC) inhibitor, GF109203X or PKC\(\beta\) isoform inhibitor, LY333531 enhanced eNOS expression. In contrast, PKC activators inhibited both the activation by insulin of PI-3 kinase and eNOS mRNA levels. Overexpression of PKC\(\beta\) isoform in endothelial cells inhibited the stimulation by insulin of eNOS expression and PI-3 kinase activities in parallel.

Conclusions—Insulin can regulate the expression of eNOS gene, mediated by the activation of PI-3 kinase, in endothelial cells and microvessels. Thus, insulin may chronically modulate vascular tone. The activation of PKC in the vascular tissues as in insulin resistance and diabetes may inhibit PI-3 kinase activity and eNOS expression and may lead to endothelial dysfunctions in these pathological states. (Circulation. 2000;101:676-681.)

Key Words: insulin ■ nitric oxide ■ RNA ■ endothelium ■ cells ■ diabetes mellitus ■ proteins

Insulin has multiple physiological effects on the vascular tissues such as vasodilation,1–4 which may be endothelial cell dependent and can be inhibited by inhibitors of nitric oxide synthase (NOS).5–6 Zeng and Quon7 suggested that insulin can increase the production of NO in cultured endothelial cells within a few minutes, indicating an activation of NOS via the insulin receptors. However, the vasodilatory effect of insulin in vivo may have both acute and prolonged actions; Utriainen et al8 and other researchers2–5 showed that the effect of insulin on forearm blood flow continued to increase even after 6 to 7 hours of infusion. In addition, the concentrations of insulin required to rapidly activate NO in cultured endothelial cells were much higher than physiological levels.7 These results suggest that insulin could also be increasing the production of NO in vivo by inducing both the expression and the activation of NOS in the endothelial cells.

We investigated the possibility that one mechanism of the vasodilatory effect of insulin is to increase the expression of endothelial NOS (eNOS) in endothelial cells. Although all 3 types of NOS have been reported to be expressed by endothelial cells, eNOS is the most abundant form8 and can be regulated by acetylcholine, hypoxia, sheer stress, lysophosphatidylcholine (LPC), and cytokines.8–11

In the present study, we characterized the effect of insulin on the expression of eNOS both in cultured endothelial cells and in microvessels from lean and insulin-resistant rats. In addition, the signaling pathway and regulation of the action of insulin on eNOS gene expression were studied.

Methods

Cell Culture

Bovine aortic endothelial cells (BAECs) from passages 4 to 10 were isolated and used as described previously.12 Confluent cells were placed in DMEM containing 1% platelet-deprived horse serum (PDHS) for 24 hours before being studied and pretreated with the following inhibitors: phosphatidylinositol-3 (PI-3) kinase–selective inhibitors wortmannin (Sigma Chemical Co)13 and LY294002 (BIOMOL Research Laboratories),14 protein kinase C (PKC) activator phorbol-12-myristate-13-acetate (PMA) (Sigma Chemical Co), general PKC inhibitor GF109203X (GFX) (Calbiochem-Novabiochem Corp),15 and PKC\(\beta\) isoform–selective inhibitor

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LY335351 (Lilly Inc). Cells were then stimulated with insulin (Sigma Chemical Co), recombinant insulin-like growth factor-1 (IGF-1) (Upstate Biotechnology), and LPC (Avanti Polar Lipid) or α-IR3 antibodies (Sigma).

Construction of Replication-Deficient Recombinant Adenovirus Containing PKCβI cDNA
The construction of a replication-deficient recombinant adenovirus for PKCβI expression was performed as described previously. Adenovirus-mediated gene transfer to confluent BAECs was performed through a 1-hour adenoviral infection of 10^9 pfu/mL at 37°C in DMEM containing 10% PDHS as described previously. The infected BAECs were then incubated in DMEM containing 1% PDHS for 24 hours, incubated with or without insulin (100 nmol/L) for an additional 6 hours, and harvested. AdV-CMV-PKCβI- or β-galactosidase (β-Gal)-infected BAECs were assessed for PKC activity and protein expression as previously described.

Isolation of Vascular Stroma From Epididymal Fat Pads of Zucker Rats
Vascular stromas were obtained from the epididymal fat pads of 12-week-old Zucker lean and fatty rats (Harlan Sprague Dawley, Inc). Epididymal fat pads were isolated, minced, and incubated with 0.2% collagenase I for 30 minutes at 37°C. Then, they were fractionated with the use of a Dounce homogenizer and centrifuged at 3000g for 20 minutes to isolate vessels from adipocytes. Vascular stroma were washed with DMEM containing 0.2% BSA and incubated with DMEM containing 0.2% BSA with or without insulin for 6 hours at 37°C. The purity of the isolated vascular stroma was quantified through immunohistochemical staining with anti–factor VIII antibody and through immunoblotting of the stroma with antibodies to smooth muscle cell α-actin. Only preparations that were stained positively in ≥90% of the vessels were used.

RNA Isolation and Northern Blot Analysis
Total RNA from cultured BAECs, PKCβI-overexpressed BAECs, and vascular stroma from the epididymal fat pads of Zucker rats were isolated according to the guanidinium thiocyanate-phenol-chloroform method with TRI Reagent (Molecular Research Center) and solution D containing 4 mol/L guanidinium thiocyanate, 25 mmol/L sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 mol/L 2-mercaptoethanol. Total RNA (20 μg) was fractionated and hybridized to 650-bp cDNA fragments of rat eNOS (kindly provided by Dr Morris F. White, Joslin Diabetes Center, Boston, Mass), which were labeled with the use of a DNA labeling system (Multiprime; Amersham Corp). The quantification of eNOS mRNA levels was performed with a PhosphorImager (Molecular Dynamics) and normalized to 36B4 mRNA.

Immunoblot Analysis of eNOS
Cells were washed 3 times with ice-cold PBS, pH 7.4, lysed in 50 mmol/L Tris, pH 7.5, 2 mmol/L EDTA, 0.5 mmol/L EGTA, 2 mmol/L PMSF, 25 μg/mL leupeptin, 0.1 mg/mL aprotinin, 1 mmol/L dithiothreitol, 50 mmol/L NaF, and 1% Triton X-100 (Sigma Chemical Co); scraped from the dish; rotated for 1 hour at 4°C; and centrifuged for 15 minutes at 14 000g. Protein concentrations of the supernatant were measured according to the method of Bradford and separated with the use of 6% SDS-PAGE as described previously. The membrane was incubated for 1 hour with polyclonal anti-human eNOS antibody (Transduction Laboratories) diluted in PBS containing 0.1% Tween-20 and 1% BSA, washed 3 times for 10 minutes with PBS containing 0.1% Tween-20, and incubated with 0.1 μCi/mL [3H]l-protein A (Amersham Life Science, Inc). Protein levels of eNOS were quantified with a PhosphorImager.

Assay of PI-3 Kinase Activity
After preincubation with or without 100 nmol/L PMA for 30 minutes, BAECs were stimulated with insulin (100 nmol/L) for 5 minutes. Cells were processed as described previously for this assay. Aliquots of proteins from the supernatant were immunoprecipitated with 10 μL/ml anti-α-insulin receptor substrates (IRS)-2 antibodies (kindly provided by Dr Morris F. White, Joslin Diabetes Center, Boston, Mass) for 2 hours and bound to protein A–Sepharose beads at 4°C as described previously. The lipids were quantified with a PhosphorImager.

Assay of NOS Activity
The amount of NOS activity produced by BAECs was measured by using an NOS Detect assay kit (Transduction Laboratories) according to the manufacturer’s instructions. Briefly, BAECs were harvested in PBS containing 1 mmol/L EDTA and centrifuged at 12 000g. The pellets were lysed in homogenization buffer containing 25 mmol/L Tris, pH 7.4, 1 mmol/L EDTA, and 1 mmol/L EGTA and centrifuged at 12 000g. Aliquots from the supernatant were used for the measurement of NOS activity through the conversion of [3H]l-arginine to [3H]l-citrulline. Data were normalized by the amount of protein and reaction time.

PKC Activity Assay and Immunoblotting Studies
After adenoviral infection, confluent BAECs were harvested and PKC activity were measured as previously described. Briefly, PKC activities were measured according to 32P labeling of 100 μmol/L PKC-specific peptide substrate RRKTLRRL. For immunoblotting studies, total cell lysate (75 μg/lane) was fractionated with the use of PAGE and detected with the use of antibodies to the PKCβisoform (Santa Cruz Biotechnology, Inc). A detailed description of the method was reported previously.

Statistical Analysis
Data are expressed as mean±SEM and were analyzed with the use of the Newman-Keuls test for ANOVA for multiple comparisons. A value of P<0.05 was considered statistically significant.

Results
Effect of Insulin on mRNA and Protein Levels of eNOS
Insulin (100 nmol/L) significantly augmented the eNOS mRNA expression of eNOS at 1 hour by 33±9%, reached 71±21% at 6 hours, and attained a maximum of 2-fold at 12 hours, which was maintained for 24 hours (Figure 1A). Expression of eNOS mRNA responded to insulin (Figure 1B) with a significant increase even at 0.1 nmol/L. At 10 nmol/L insulin, eNOS mRNA level was significantly increased by 50±12%, and a maximum effect of 2-fold was attained at 100 nmol/L. Therefore, stimulation with 100 mmol/L insulin with an incubation time of 6 hours IGF-1 (25 nmol/L) also increased the eNOS mRNA level by 47±10% in human umbilical endothelial cells. The addition of αIR3, an IGF-1 receptor–specific antibody (1 μg/mL), inhibited the effect of IGF-1 by 60% but did not decrease the effect of insulin. Insulin also increased eNOS protein levels at 6 hours by 43±16% and reached a maximum of 2-fold at 24 hours, which was maintained for 36 hours.

Effect of PI-3 Kinase Inhibitors Wortmannin and LY294002 on Expression of eNOS
The acute effect of insulin on NO production in endothelial cells was reported to be inhibited by wortmannin, a PI-3 kinase inhibitor. To determine whether PI-3 kinase activation could be increasing mRNA expression and protein levels of eNOS, 2 structurally different PI-3 kinase inhibitors, wortmannin (100 nmol/L) and LY294002 (50 nmol/L), were preincubated with BAECs before the addition of insulin (100 nmol/L).
Insulin increased the mRNA level of eNOS by 58±20% compared with control, but the effect of insulin was inhibited by preincubation with wortmannin (Figure 2A). Similar to eNOS mRNA levels, insulin significantly increased the eNOS protein level by 74±9%, which was completely inhibited by the addition of wortmannin (Figure 2B).

The pretreatment of BAECs with another PI-3 kinase inhibitor, LY294002 (50 nmol/L), completely inhibited the induction of eNOS mRNA expression by insulin. Unlike wortmannin, LY294002 significantly decreased the basal mRNA expression of eNOS without insulin treatment by 30±4%. Correspondingly, LY294002 inhibited the increases in eNOS protein levels stimulated by insulin and decreased the basal eNOS protein level by 72±5%.

Insulin (100 nmol/L) significantly increased NOS activity from 115±9 to 176±7 pmol·mg protein⁻¹·min⁻¹ after 24 hours (P=0.01, n=6). Preincubation with wortmannin (100 nmol/L) for 15 minutes significantly decreased insulin-induced NOS activity to 123±13 pmol·mg protein⁻¹·min⁻¹, but the basal levels of NOS activity were unchanged.

**Figure 3.** Effect of PMA on eNOS mRNA expression stimulated with or without insulin in BAECs. A, Time course of PMA-induced eNOS mRNA expression. After BAECs were starved for 24 hours, cells were treated with 100 nmol/L PMA for indicated times (P<0.05 vs 0 hours). B, Effect of PMA on insulin-induced eNOS mRNA expression. Cultured BAECs were starved for 24 hours and treated with 100 nmol/L insulin for 6 hours after pretreatment with or without 100 nmol/L PMA for 30 minutes. Then, 20 µg of total RNA extracted from BAECs was used for Northern blot analysis with 32P-labeled eNOS probes as described in Methods (P<0.01 vs control [Con.], #P<0.01 vs insulin). Results are expressed as a percentage of control and shown as mean±SEM of 3 different experiments (A) and of 4 different experiments (B).

**Effect of PMA on Insulin-Induced eNOS mRNA Expression and PI-3 Kinase Activities**

Because PKC activation is observed in the vascular tissue in diabetes and may regulate eNOS in BAECs,12,16,21 the actions of PMA, a PKC agonist, on eNOS expression were studied (Figure 3). In time course experiments, PMA (100 nmol/L) did not change the eNOS mRNA level for the initial 6 hours but significantly increased the expression of eNOS mRNA after 12 and 24 hours of incubation by 66±11% and 105±14%, respectively (Figure 3A). In contrast, when BAECs were preincubated with PMA for 30 minutes, the effect of insulin on eNOS mRNA levels was inhibited (14±13%) (Figure 3B).

Because insulin may increase NO production via activation of PI-3 kinase through the tyrosine phosphorylation of its receptors and IRS,22 the effects of PKC activation on the insulin induction of eNOS expression and PI-3 kinase activity were examined in parallel. Insulin significantly increased
PKCβ1 isoform–specific inhibitor, LY333531, on insulin-induced eNOS mRNA expression in BAECs. Cultured BAECs starved for 24 hours were pretreated with 5 μmol/L GFX or 20 nmol/L LY333531 for 30 minutes and stimulated with or without 100 nmol/L insulin for 6 hours. RNA was extracted from cells, and Northern blot analysis was performed with 20 μg of total RNA per lane. Results are normalized by levels of 36B4 mRNA expression and expressed as a percentage of control and as mean±SEM of 4 different experiments (*P<0.05 vs control, **P<0.01 vs control [Con.]).

IRS-2–associated PI-3 kinase activity by 5.4±0.4-fold. When BAECs were preincubated with PMA (100 nmol/L) for 30 minutes, insulin-induced IRS-2–associated PI-3 kinase activity was mostly inhibited. However, the basal PI-3 kinase activity was not changed with PMA treatment.

**Effect of PKC Inhibitors on eNOS mRNA Expression**

The exposure of BAECs to the PKC inhibitor GFX (5 μmol/L) without insulin for 6 hours increased the expression of eNOS mRNA by 38±10% (Figure 4). The expression of eNOS mRNA was greater in cells exposed to both insulin and GFX (by 76±20% compared with control cells or those incubated with either insulin or GFX alone). We have reported that hyperglycemia may preferentially activate PKCβ isoforms in the vascular cells.16 To explore the possibility that the PKCβ isoform could also have a role in regulation of the activation by insulin of PI-3 kinase and eNOS expression, the effect of LY333531 (20 nmol/L), a PKCβ isoform inhibitor, was characterized.16 The addition of LY333531 also increased eNOS mRNA expression by 60±14%, which is similar to insulin or GFX alone. LY333531 and insulin together did not have a significant additive effect.

**Effect of Overexpression of PKCβ Isoform on Insulin-Induced eNOS mRNA Level**

To determine directly whether the PKCβ isoform can regulate the effect of insulin on eNOS expression, we overexpressed the PKCβ1 isoform in BAECs through the use of replication-deficient adenovirus containing cDNA of the PKCβ1 isoform. Compared with control cells infected with adenovirus containing the β-Gal gene, cells infected with adenovirus containing the PKCβ1 gene had a 50-fold increase in the protein for the PKCβ1 isoform. Total PKC activities were also increased by 11- and 7-fold in the cytosol and membrane fractions, respectively.

Insulin (100 nmol/L) enhanced eNOS mRNA expression in BAECs with or without infection with adenovirus containing only β-Gal by as much as 2-fold (Figure 5). In contrast, insulin did not increase eNOS mRNA levels in cells infected with adenovirus containing the PKCβ1 isoform. The expression of eNOS was not changed by overexpression of the PKCβ1 isoform (Figure 5) at the basal level. In contrast, LPC (100 μmol/L) increased eNOS mRNA levels by 5- and 4.5-fold in control and adenovirus-containing β-Gal cells, respectively. In BAECs infected with the adenoviral-PKCβ1 isoform, LPC increased eNOS mRNA by 4-fold, which was not significantly different from controls.

**Effect of Insulin on eNOS mRNA Level in Vascular Stroma Isolated From Epididymal Fat Pads of Zucker Fatty and Lean Rats**

To determine whether insulin can also change eNOS expression in vascular tissue, we characterized eNOS mRNA levels in vascular stroma isolated from Zucker lean and fatty insulin-resistant rats, a model of insulin resistance.23 The expression of eNOS mRNA with or without insulin (100 nmol/L) for 6 hours in the vascular stroma isolated from insulin-resistant models (Zucker fatty rats) showed that the basal levels of eNOS mRNA expression were significantly decreased to 29±5% of vascular stroma derived from Zucker lean rats (Figure 6). The contents of vascular stroma in both preparations were found to be similar through the use of immunostaining with factor VIII antibodies and immunoblotting with antibodies to smooth muscle cell α-actin. Moreover, insulin increased eNOS mRNA levels by 50±16% in the vascular stroma from the Zucker lean rats but was ineffective in vascular stroma isolated from the insulin-resistant rats.

**Discussion**

One of the important vascular actions of insulin is its vasodilatory effect, which is associated with NO production, either from endothelial cells or from perivascular neuronal cells.1–6 The possibility that insulin can enhance the production of NO is supported by the findings that insulin and IGF-1 increased NO production in endothelial cells in <1...
Figure 6. Effect of insulin on eNOS mRNA expression in vascular stroma from epididymal fat pads of Zucker fatty rats. Vascular stroma was isolated from epididymal fat pads of Zucker fatty and lean rats as described and incubated with or without 100 nmol/L insulin for 6 hours. Total RNA (20 μg) was used for Northern blot analysis. Results are expressed as a percentage of control and shown as mean±SEM of 5 different experiments (*P<0.05 vs control [Con.] in Zucker lean rats, **P<0.01 vs control in Zucker lean rats).

This suggested that insulin can directly activate eNOS because protein and mRNA levels of eNOS could not increase so rapidly. However, the acute effect of insulin on NO production cannot account for all of the vasodilatory effects of insulin in vivo, because some physiological studies have reported that the vasodilatory effect of insulin continues to increase even after 7 hours of infusion. These results suggest that the vasodilatory effect of insulin has a sustained component that requires several hours of stimulation when near-physiological concentrations of insulin are used. Our findings that physiological concentrations of insulin enhanced the expression of eNOS in the endothelial cells only after 4 hours of incubation, with maximum effects observed at 8 hours, are consistent with the sustained component of the vasodilatory effects of insulin.

The effect of insulin on eNOS mRNA levels was observed between 0.1 and 100 nmol/L, which corresponded closely to the range of binding and activation of insulin receptors in the endothelial cells and to the physiological levels of insulin in the plasma. It is interesting that the rapid effect of insulin on NO production in cultured human umbilical endothelial cells required pharmacological insulin concentrations of 10 to 10,000 nmol/L. This suggests that the acute effect of insulin on NO production may not be significant under physiological conditions and may be mediated in part by IGF-1 receptors.

The parallel increases in the mRNA, protein, and enzymatic activities of eNOS indicate that the effect of insulin is primarily due to the elevation of steady state levels of eNOS mRNA. It is not possible from these results to determine whether the effect of insulin on eNOS mRNA levels was due to increases in the transcription rate or the half-life of eNOS mRNA. However, it is likely that the changes are due to increases in the transcription rate, which is the most common mechanism via which insulin regulates the mRNA levels of many genes in cells.

The signaling pathways of insulin action on eNOS mRNA appear to involve mainly the insulin receptors, because the maximal effect on eNOS mRNA level was attained with <100 nmol/L, a concentration of insulin shown to bind minimally to IGF-1 receptors in endothelial cells. In addition, the effect of insulin on eNOS mRNA was not prevented by inhibitory antibodies to IGF-1 receptors. The effects of the PI-3 kinase inhibitors wortmannin and LY294002 suggest that the activation of PI-3 kinase is involved. Interestingly, the rapid effect of insulin on NO production from endothelial cells was also inhibited by wortmannin, although the direct effect of insulin on PI-3 kinase was not measured in the previous study.

PKC activation appears to modulate the effect of insulin on eNOS mRNA expression; rapid PKC activation induced by phorbol esters caused inhibition of insulin-stimulated PI-3 kinase activity and eNOS mRNA expression. The findings that eNOS expression was increased by the long-term incubation of PMA and by PKC inhibitors are consistent because both maneuvers will reduce PKC activities in endothelial cells. These findings confirmed previous reports that PKC inhibition increased eNOS mRNA in BAECs.

The findings that both general PKC inhibitor GFX and specific PKCβ isoform inhibitor LY333531 increased basal eNOS levels suggest that PKC activities may regulate eNOS mRNA levels in endothelial cells. The use of the PKCβ isoform inhibitor LY333531 (20 nmol/L, a concentration that selectively inhibited the PKCβ isoform) indicated that the activation of PKCβ isoform may have a selective effect on eNOS expression. This finding was surprising in that the PKCβ isoform is expressed to a lesser extent than other PKC isoforms in the endothelial cells. The inhibitory effect of the PKCβ isoform on eNOS mRNA level is directly confirmed through the overexpression of the PKCβ isoform in endothelial cells with the use of adenoviral vectors containing full-length DNA of the PKCβ isoform. The inhibitory effect of PKC activation on eNOS expression may be specific to insulin because the stimulating effect of LPC was not affected. The finding that the activation of the PKCβ isoform can inhibit eNOS expression in endothelial cells is of particular interest in the area of diabetic vascular complications because the activation of PKCβ isoforms has been associated with various vascular dysfunctions in the retina, renal glomeruli, and cardiovascular systems of diabetic animals and with cardiac hypertrophy in transgenic mice.

The results obtained for the microvessels isolated from the Zucker fatty and lean rats support the likelihood that our findings in cultured endothelial cells have physiological meaning and that this action of insulin is blunted in insulin-resistant states. These in vivo findings are consistent with previous reports that showed the total NOS activities were decreased in the skeletal muscle and neuronal tissues of Zucker fatty rats. The basal expression of eNOS was also much lower in insulin-resistant Zucker fatty rats than in lean animals, suggesting that insulin may also modulate eNOS levels in the vessels at the basal state. However, specific measurements of the signaling steps of insulin must be determined to document the extent of insulin resistance in the vascular tissues in insulin-resistant animals or humans.

In summary, insulin can modulate eNOS expression chronically both in vitro and in vivo, which may enhance the NO production induced by other agonists such as acetylcholine.
Because the enhancement of NO production causes vasodilation and inhibits smooth muscle growth, it is possible that at physiological levels in an insulin-sensitive state, insulin can indirectly have antiatherosclerotic effects. In the presence of hyperglycemia and insulin resistance, which are known to activate PKC and induce the inhibition of PI-3 kinase activities in the vasculatures, the effect of insulin on eNOS expression is blunted, resulting in the loss of its vasodilatory effects. Further studies are necessary to determine whether PKC activities are increased in the insulin-resistant state and whether the use of the inhibitor of the PKC isoform or of specific insulin sensitizers can improve the vascular actions of insulin and endothelial cell dysfunctions.

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