Roles for Insulin Receptor, PI3-Kinase, and Akt in Insulin-Signaling Pathways Related to Production of Nitric Oxide in Human Vascular Endothelial Cells

Guangyuan Zeng, MD, PhD; Frederick H. Nystrom, MD, PhD; Lingamanaidu V. Ravichandran, PhD; Li-Na Cong, MD; Martha Kirby, BS; Howard Mostowski, BS; Michael J. Quon, MD, PhD

Background—Previously, we demonstrated that insulin stimulates production of nitric oxide (NO) in endothelial cells. However, specific insulin-signaling pathways mediating production of NO have not been elucidated.

Methods and Results—We developed methods for transfection of human umbilical vein endothelial cells (HUVECs) and direct measurement of NO to begin defining insulin-signaling pathways related to NO production. HUVECs were cotransfected with enhanced Green Fluorescent Protein (eGFP) and another gene of interest. Transfection efficiencies >95% were obtained by selecting cells expressing eGFP. Overexpression of insulin receptors in HUVECs resulted in an ∼3-fold increase in production of NO in response to insulin. In contrast, HUVECs overexpressing a tyrosine kinase–deficient mutant insulin receptor had a dose-response curve similar to that of control cells. Overexpression of inhibitory mutants of either phosphatidylinositol 3-kinase (PI3K) or Akt resulted in nearly complete inhibition of insulin-stimulated production of NO. Overexpression of an inhibitory mutant of Ras had a much smaller effect.

Conclusions—Receptor kinase activity is necessary to mediate production of NO through the insulin receptor. Both PI3K and Akt contribute importantly to this process, whereas the contribution of Ras is small. (Circulation. 2000;101:1539-1545.)

Key Words: insulin ■ endothelium ■ signal transduction ■ nitric oxide ■ receptors

Insulin has vasodilator actions in vivo that depend on endothelium-derived nitric oxide (NO). Sensitivity to vasodilator actions of insulin is positively correlated with insulin sensitivity with respect to glucose metabolism in some studies. Insulin-resistant insulin receptor substrate-1 knockout mice are hypertensive, whereas insulin sensitizers such as troglitazone lower blood pressure in humans. These in vivo data suggest that insulin signaling related to production of NO in vascular endothelium may play a role in coupling metabolic and hemodynamic homeostasis.

We previously reported that insulin stimulates production of NO in human umbilical vein endothelial cells (HUVECs). Specific actions of insulin are frequently mediated by distinct pathways. For example, in adipose cells, phosphatidylinositol 3-kinase 3-kinase (PI3K) and Akt participate in insulin-stimulated translocation of the glucose transporter GLUT4, whereas Ras pathways mediate mitogenic effects of insulin. Here, we begin to elucidate specific insulin-signaling pathways involved with production of NO by directly measuring NO in HUVECs overexpressing wild-type or inhibitory forms of signaling molecules previously examined in relation to the metabolic actions of insulin.

Methods

Vectors
pCIS2 is an expression vector (from C. Gorman: Choi et al) generating high levels of expression in HUVECs. pCIS-eGFP is cDNA for enhanced green fluorescent protein from pEGFP-1 (Clontech Laboratories, Inc, Palo Alto, Calif) ligated into pCIS2. pCIS-HOOK is cDNA for HOOK from pHOOK-1 (Invitrogen Corp, San Diego, Calif) ligated into pCIS2. HOOK has an extracellular fragment that binds to 4-ethoxymethylene-2-phenyl-2-oxazoline-5-one (phOx), hIR-WT is an expression vector for human insulin receptor. hIR-K1030A is an expression vector for kinase-inactive insulin receptor (from S.I. Taylor). Δp85 is a bovine p85 regulatory subunit of PI3K lacking inter-SH2 domain required for binding and activation of p110, Akt-K179A is a kinase-inactive Akt, pCIS-N17-ras is a dominant inhibitory mutant of ras, SRE-luc is a serum response element of c-fos driving expression of luciferase reporter (obtained from J.E. Pessin: Yamauchi et al).

Culture and Transfection
HUVECs (Clonetics Corp, San Diego, Calif) were maintained in primary culture and serum starved overnight in endothelial basal media (an endothelial growth media [EGM] from Clonetics Corp) without additives before NO measurements. HUVECs (∼70% confluent) were transfected with Transit-100 (Pan Vera Corp). Eight hundred microliters of Opti-Mem (GIBCO BRL) was mixed with 40

Received August 3, 1999; revision received October 8, 1999; accepted October 21, 1999.
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µL Transit-100 and incubated for 5 minutes in a polystyrene tube. Eight micrograms of plasmid DNA was added to the mixture and incubated for another 5 minutes. Cells in each 80-cm² flask were washed with Opti-Mem, and 17 mL of Opti-Mem plus the Transit-100/DNA mixture was added. After incubation at 37°C for 4 hours, media were replaced with EGM. Transfected cells were incubated overnight before further studies.

Cell Selection
Cells were cotransfected with pCIS-eGFP and another vector. After overnight incubation, cells were trypsinized, pelleted (400 g, 5 minutes), and resuspended in 1 mL ice-cold DPBS (without Ca²⁺ and Mg²⁺) containing 2% fetal bovine serum and 0.1% NaN₃. Cells were passed through a sterile 53-µm nylon mesh and kept on ice before sorting. We used a Becton-Dickinson FACStar Plus cell sorter (5-W argon laser, 488 nm) with FL-1 photomultiplier tube and 100-µm sorting nozzle (~2200 events/s) to select cells expressing eGFP. Cells were collected in 0.5 mL of EGM containing 20% FBS, 1000 U/mL penicillin, and 1 mg/mL streptomycin. After sorting, cells were transferred to 6-well dishes (containing 2 mL EGM) and incubated at 37°C.

Cotransfection Efficiency
Cells were cotransfected with pCIS-eGFP and pCIS-HOOK (4 µg of each plasmid/flask). The following day, cells were incubated with ~3×10⁴ Capture-Tec beads (coated with phOx) in 10 mL EGM for 30 minutes at 24°C on a shaking platform. After being washed twice with EGM (10 mL), cells expressing HOOK were identified by visualization of beads bound to cells. Cotransfection was assessed with the use of a microscope with illumination at 488 nm.

Cell Surface Binding of [¹²⁵I]-Insulin
Specific binding of [¹²⁵I]-insulin was measured in cells overexpressing hIR-WT or hIR-K1030A as described.⁷

Immunodetection of Insulin Receptors and Akt
HUVECs were transfected in 100-mm dishes. After 1 day, cells were serum-starved overnight and then treated with or without 100 nmol/L insulin for 3 minutes. Medium was aspirated, and the flask of cells was frozen on liquid nitrogen. Cells were lysed in 0.5 mL RIPA buffer (20 mmol/L Tris, pH 7.4, 2.5 mmol/L EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50 mmol/L NaF, 1 mmol/L Na₂VO₄, and a complete protease inhibitor cocktail; Boehringer Mannheim) and centrifuged at 10 000g for 10 minutes. Samples were separated by SDS-PAGE (7.5% gel) and immunoblotted with antibody against the α-subunit of the insulin receptor (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif), an antiphosphotyrosine antibody (4G10, Upstate Biotechnology Inc, Lake Placid, NY), or an anti-Akt antibody.¹⁰

Cotransfection of N17-ras and SRE-luc
HUVECs were cotransfected with SRE-luc (0.5 µg) and either pCIS2 or pCIS-N17-ras (0.5 µg) in 6-well plates. Cells were then serum starved overnight followed by incubation in the absence or presence of 10% serum at 37°C for 4 hours. Luciferase activity was determined in cell lysates.¹⁴

Measurement of NO in Transfected HUVECs
After FACS selection of transfected HUVECs, ~200 000 cells were seeded in 1 well of a 6-well dish and serum starved overnight. The next day, cells were washed and equilibrated in 2 mL DMEM (containing 25 mmol/L glucose, 20 mmol/L HEPES, 0.1% BSA, pH 7.4). An NO-selective amperometric electrode was used to directly measure insulin-stimulated production of NO at 24°C, pH 7.4, as described.⁷

Results
Transfection/Selection of Endothelial Cells
We achieved ≈20% transfection efficiencies (Figure 1E). Our method had higher efficiencies than those reported previously for human endothelial cells.¹⁵–²¹ Nevertheless, our ability to assess effects of transgenes on production of NO under these conditions is limited because of the large proportion of untransfected cells. Therefore, we used a fluorescently activated cell sorter (FACS) to rapidly select cells cotransfected with enhanced Green Fluorescent Protein (eGFP) and our gene of interest (enrichment >95%) (Figure 1). To assess cotransfection efficiency, we transfected HUVECs with pCIS-eGFP and pCIS-HOOK and directly visualized cells (data not shown). Nearly all cells
expressing eGFP also expressed HOOK (cotransfection efficiency \( \approx 99\% \)).

### Role of Receptor Tyrosine Kinase

Overexpression of insulin receptors was demonstrated by binding of \([^{125}\text{I}]\)-insulin to HUVECs before transfection, after cotransfection with eGFP and receptors, and after FACS selection (Table). Before selection, we observed an \( \approx 10\)-fold increase in insulin binding in cells transfected with hIR-WT or hIR-K1030A. After selection, insulin binding in these cells increased to \( \approx 50\)-fold over the control group. This additional 5-fold increase is consistent with a 20\% transfection efficiency and subsequent enrichment to \( \approx 100\% \) (compare with Figure 1).

We assessed receptor autophosphorylation by immunoblotting lysates of transfected (but not selected) cells that were treated without or with insulin (Figure 2). Overexpression of hIR-WT and hIR-K1030A was observed by immunoblotting (Figure 2A). Antiphosphotyrosine immunoblots showed a small increase in phosphorylation of endogenous insulin receptors on insulin stimulation of control cells. For cells overexpressing hIR-WT, there was a large increase in tyrosine phosphorylation of recombinant receptors in response to insulin, whereas cells transfected with hIR-K1030A were similar to the control (Figure 2B). Furthermore, stimulation of transfected cells with supraphysiological insulin levels (1 \( \mu \text{mol/L} \)) resulted in phosphorylation of hIR-WT that was greater than that observed with insulin stimulation at 100 nmol/L (Figure 2C).

We next examined effects of insulin receptor constructs on production of NO in transfected/selected HUVECs (Figure 3). In control cells, insulin stimulated a dose-dependent increase in NO (ED\(_{50} = 700 \text{ nmol/L} \)). The response to maximal insulin stimulation (\( \approx 110 \text{ nmol/L} \) NO from 2\( \times 10^5 \) cells in 2 mL media) and the ED\(_{50} \) were similar to untransfected HUVECs.\(^7\) Overexpression of hIR-WT caused a 3-fold increase in NO production in response to insulin without significantly affecting insulin sensitivity (Figure 3A). In contrast, the insulin dose-response of cells overexpressing hIR-K1030A was similar to that of control cells (Figure 3B). These results suggest that intact kinase activity is essential for the insulin receptor to mediate effects on production of NO in HUVECs.

### Role of PI3K and Akt

In HUVECs overexpressing Dp85, insulin-stimulated production of NO was almost completely inhibited except at the highest dose of insulin tested (Figure 4). Thus, PI3K may play an important role in the insulin-signaling pathways related to production of NO.

We inquired whether Akt (serine-threonine kinase downstream from PI3K) also plays a role in this pathway by overexpressing the inhibitory mutant \( \text{Akt}-\text{K179A} \).\(^{10,22,23}\) Overexpression of \( \text{Akt}-\text{K179A} \) was confirmed by immunoblotting (data not shown). We also performed in vitro kinase assays to demonstrate that \( \text{Akt}-\text{K179A} \) did not generate detectable kinase activity in response to insulin stimulation and that phosphorylation of a cotransfected wild-type Akt was inhibited (data not shown). In cells overexpressing Akt-

<table>
<thead>
<tr>
<th>Construct</th>
<th>Increase in Binding After Transfection</th>
<th>Increase in Binding After FACS Selection</th>
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<tbody>
<tr>
<td>pCIS2</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>hIR-WT</td>
<td>11( \times )</td>
<td>61( \times )</td>
</tr>
<tr>
<td>hIR-K1030A</td>
<td>13( \times )</td>
<td>48( \times )</td>
</tr>
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Bound/free ratios were used to determine the fold increase in insulin binding. Results shown are average of 2 independent experiments.

![Figure 2](http://circ.ahajournals.org/)

**Figure 2.** Overexpression of insulin receptors in HUVECs. A, Cell lysates from HUVECs transfected with pCIS2 (lanes 1 and 2), hIR-WT (lanes 3 and 4), or hIR-K1030A (lanes 5 and 6) and treated without or with insulin (100 nmol/L) were immunoblotted with insulin receptor \( \alpha \)-subunit antibody. B, Antiphosphotyrosine immunoblot of duplicate samples. C, Antiphosphotyrosine immunoblot of cell lysates from HUVECs transfected with hIR-WT and stimulated with insulin (100 nmol/L, 1 \( \mu \text{mol/L} \)) for 4 minutes. Representative blots are shown from experiments repeated independently 3 times.
K179A, we observed nearly complete inhibition of production of NO in response to insulin except at the highest dose of insulin tested (Figure 5).

Role of Ras
To test the role of Ras, we overexpressed a dominant inhibitory mutant (N17-Ras) in HUVECs. We first assessed the ability of N17-Ras to inhibit serum stimulation of an SRE-luc construct. In control cells, serum stimulation caused a 4-fold increase in luciferase activity (Figure 6). In contrast, in cells cotransfected with pCIS-N17-ras and SRE-luc, luciferase activity was significantly inhibited in both basal and serum-stimulated states. Thus, N17-Ras inhibited Ras-dependent signaling in HUVECs. In contrast to Dp85 and Akt-K179A, overexpression of N17-Ras did not significantly affect insulin-stimulated production of NO except for a slight inhibition at the highest insulin doses tested (Figure 7).

Discussion
Transfection/Selection of HUVECs
We studied HUVECs because tissue culture models do not reproduce all the physiological characteristics of bona fide endothelial cells. Establishing stable transfections in primary culture is not feasible because cells have a limited ability to divide. Moreover, a variety of transfection techniques in human endothelial cells have had limited success.15–21 We developed a novel strategy to achieve transient transfection efficiencies in HUVECs that approach 100%. The insulin response of HUVECs that undergo this transfection/selection procedure with an empty vector is similar to that of untransfected cells with respect to production of NO. This suggests
that transfected/selected cells respond appropriately to insulin and effects of overexpressing signaling molecules can be evaluated in a physiologically relevant cell.

Role of Insulin Receptor
Using insulin binding and immunoblotting, we demonstrated overexpression of recombinant insulin receptors in HUVECs and verified their expected behavior with respect to autophosphorylation. Overexpression of hIR-WT caused a large increase in insulin responsiveness without any increase in sensitivity. This may be due to biological characteristics of HUVECs and technical limitations of our measurement system. In HUVECs, pharmacological concentrations of insulin are required to detect production of NO. As discussed previously, this may be due to limitations in electrode sensitivity, the necessity to perform measurements at 24°C, and the fact that HUVECs may not be as sensitive to insulin as endothelial cells in small vessels. Another limitation of our system is an inability to compare basal levels of NO production between experimental groups (ie, in the absence of insulin) because the electrode must be recalibrated each time it moves. We assume NO production is similar for all groups in the absence of insulin and compare only relative differences we elicit in response to insulin. It is possible that overexpression of wild-type insulin receptors per se is sufficient to elicit biological activity. For example, we demonstrated that overexpression of insulin receptors in adipose cells is sufficient to recruit GLUT4 to the cell surface in the absence of insulin. Thus, assumptions that basal levels of NO production are the same in all groups (if incorrect) may impair our ability to detect increases in insulin sensitivity caused by overexpression of the receptor. Finally, limitations in our electrode may make it difficult to detect shifts in insulin sensitivity.

Our results imply that there are few “spare receptors” in HUVECs with respect to production of NO. That is, maximal receptor occupancy is required to achieve a maximal effect. Under these conditions, effects of overexpression of insulin receptors on basal production of NO would be minimized. Without “spare receptors,” overexpression of insulin receptors would tend to effect larger increases in insulin responsiveness rather than sensitivity (as we observed). Although the insulin dose required to elicit a detectable effect on NO production in our in vitro system is much larger than the physiological levels of insulin that stimulate NO-dependent increases in limb blood flow, we demonstrated that the insulin receptor can increase autophosphorylation in response to these large doses of insulin under our conditions. Nevertheless, one should be cautious about extrapolating from our results to effects on whole-body physiology. Properties of endothelial cells overexpressing insulin receptors may not necessarily reflect insulin action in vascular endothelium in the intact organism.

Since overexpression of the kinase-inactive insulin receptor did not alter the dose-response curve, we conclude that the receptor kinase is necessary to mediate insulin-stimulated production of NO in endothelial cells under our experimental conditions. We did not observe an inhibitory effect of this mutant that has been described in other contexts. However, this is not unprecedented because we previously reported that a kinase-inactive insulin receptor was “dead” with respect to metabolic signaling in adipose cells but was not inhibitory. Differences in cell type and transfection methods may influence formation of mutant/wild-type receptor hybrids and explain why inhibitory interactions between recombinant kinase-inactive receptors and endogenous receptors are not always observed. Indeed, hybrid recombinant/endogenous receptors may be limited because insulin receptor dimers form before release from the endoplasmic reticulum.

Role of PI3K
We hypothesize that parallels exist between metabolic insulin signaling pathways and pathways related to production of NO. We previously demonstrated that PI3K is necessary for insulin-stimulated translocation of GLUT4 in rat adipose cells. We used an inhibitory mutant of PI3K because compounds such as wortmannin often have other nonspecific effects. These nonspecific effects may account for why we observed a more complete inhibition with Δp85 in this study than we did with wortmannin in a previous study. Since overexpression of Δp85 causes nearly complete inhibition of insulin-stimulated production of NO in HUVECs, we conclude that PI3K also may play a role in vasodilator actions of insulin. The role of PI3K in the production of NO is probably not unique to insulin signaling because several studies reported that wortmannin blocks production of NO in endothelial cells in response to stimulation by vascular endothelial growth factor and insulin-like growth factor-1, which also signal through receptor tyrosine kinases. Nevertheless, PI3K activity per se is unlikely to be sufficient to stimulate eNOS activity because we have previously shown that platelet-derived growth factor (PDGF) stimulation of HUVECs (known to stimulate PI3K activity in endothelial cells) did not increase production of NO. This highlights another parallel between metabolic insulin-signaling pathways and insulin signaling related to production of NO in that PDGF-stimulated activation of PI3K in adipose cells is not sufficient to cause translocation of GLUT4 in the absence of overexpression of PDGF receptors.

There are several possible explanations for why overexpression of Δp85 did not cause complete inhibition of insulin-stimulated production of NO at high insulin concen-
trations. Overexpression of Δp85 might not be sufficient to completely inhibit endogenous PI3K activity. If inhibitory effects of Δp85 depend on competition for limiting factors, expression levels and compartmentalization of these factors may influence the extent of inhibition of PI3K activity. In addition, PI3K may not be the only mediator of this action of insulin, and parallel pathways may contribute to production of NO. Finally, if a small fraction of sorted cells were not expressing Δp85, the production of NO in response to insulin in the untransfected cells might be detectable at high insulin doses.

**Role of Akt**

Akt is a downstream effector of PI3K involved with antiapoptotic effects of growth factors and metabolic effects of insulin. The kinase-inactive Akt mutant used in this study has been extensively characterized and has dominant inhibitory actions in other contexts. We have shown that this Akt mutant has no detectable kinase activity in response to insulin and is able to inhibit kinase activity of a cotransfected wild-type Akt. The significant inhibition of insulin-stimulated production of NO caused by overexpression of Akt-K179A strongly suggests that Akt plays an important role in mediating vasodilator actions of insulin in endothelium. The incomplete inhibition observed at the highest insulin doses may be due to the factors discussed above for PI3K. The magnitude of inhibition with respect to production of NO caused by Akt-K179A in HUVECs is larger than the inhibitory effect we previously reported on translocation of GLUT4 in adipose cells. Thus, it is possible that Akt may play a more important role in insulin-stimulated production of NO than in insulin-stimulated glucose transport.

**Role of Ras**

Ras-dependent insulin signaling pathways play a major role in mitogenic actions of insulin but are less important for metabolic actions of insulin. We demonstrated that N17-Ras was capable of blocking ras-dependent signaling in HUVECs. Nevertheless, in contrast to inhibitory mutants of PI3K and Akt, overexpression of N17-Ras in HUVECs caused a very small inhibition of insulin-stimulated production of NO only at the highest insulin doses used. Thus, relative to PI3K and Akt, the role of Ras in insulin-stimulated production of NO is small. This result is similar to what we previously observed with respect to insulin-stimulated translocation of GLUT4 in adipose cells and highlights another parallel between metabolic insulin-signaling pathways and pathways related to production of NO.

**Conclusions**

We present a novel transfection system for HUVECs that allows us to dissect insulin-signaling pathways related to production of NO in a physiologically relevant cell. The insulin receptor tyrosine kinase, PI3K, and Akt all play significant roles in insulin-stimulated production of NO, whereas Ras is less important. Striking parallels between metabolic insulin-signaling pathways and pathways related to vasodilator actions of insulin provide additional support for the hypothesis that the vascular endothelium is a physiological target of insulin that couples regulation of glucose metabolism with hemodynamics.

**Acknowledgments**

This work was supported in part by a Research Award from the American Diabetes Association to Dr Quon.

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_Circulation._ 2000;101:1539-1545
doi: 10.1161/01.CIR.101.13.1539

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7322. Online ISSN: 1524-4539

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