Insulin-stimulated Production of Nitric Oxide Is Inhibited by Wortmannin

Direct Measurement in Vascular Endothelial Cells

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Abstract

Hypertension is associated with insulin-resistant states such as diabetes and obesity. Nitric oxide (NO) contributes to regulation of blood pressure. To gain insight into potential mechanisms linking hypertension with insulin resistance we directly measured and characterized NO production from human umbilical vein endothelial cells (HUVEC) in response to insulin using an amperometric NO-selective electrode. Insulin stimulation of HUVEC resulted in rapid, dose-dependent production of NO with a maximal response of ~ 100 nM NO (200,000 cells in 2 ml media; ED_{50} ~ 500 nM insulin). Although HUVEC have many more IGF-1 receptors than insulin receptors (~ 400,000, and ~ 40,000 per cell respectively), a maximally stimulating dose of IGF-1 generated a smaller response than insulin (40 nM NO; ED_{50} ~ 100 nM IGF-1). Stimulation of HUVEC with PDGF did not result in measurable NO production. The effects of insulin and IGF-1 were completely blocked by inhibitors of either tyrosine kinase (genestein) or nitric oxide synthase (L-NAME). Wortmannin (an inhibitor of phosphatidylinositol 3-kinase [PI 3-kinase]) inhibited insulin-stimulated production of NO by ~ 50%. Since PI 3-kinase activity is required for insulin-stimulated glucose transport, our data suggest that NO is a novel effector of insulin signaling pathways that are also involved with glucose metabolism. (*J. Clin. Invest.* 1996. 98:894–898.) Key words: hypertension • insulin resistance • diabetes • obesity

Introduction

Hypertension is frequently associated with insulin resistant states such as diabetes and obesity (1, 2). However, mechanisms linking hypertension with insulin resistance (decreased sensitivity to insulin with respect to glucose uptake and metabolism) are not well understood (3, 4). Since insulin resistance usually leads to compensatory hyperinsulinemia, some investigators have hypothesized a direct role for insulin in hypertension (for reviews see references 1, 5). The effects of insulin to promote renal tubular reabsorption of sodium, sympathetic nervous system activity, and proliferation of vascular smooth muscle cells tend to increase plasma volume, cardiac output, and peripheral vascular resistance. However, these effects are opposed by direct vasodilatory actions of insulin in some vascular beds. Thus, the net hemodynamic effect of insulin, if any, is a tendency to lower blood pressure. For example, patients with insulinomas who have high insulin levels without insulin resistance are usually normotensive. Interestingly, drugs that improve insulin sensitivity also lower blood pressure in hypertensive humans and rats (6–9). This suggests that it may be abnormalities underlying insulin resistance rather than insulin per se that are causally related to hypertension.

An elegant series of experiments by Baron and co-workers showed that sensitivity to the vasodilatory action of insulin is positively correlated with insulin sensitivity with respect to glucose uptake in normal, obese, and diabetic individuals (10–13). Furthermore, using inhibitors of nitric oxide synthase (NOS),[1] they have shown that the vasodilatory action of insulin is most likely mediated by nitric oxide (NO) (14). Thus, it is possible that defects in insulin signaling leading to insulin resistance with respect to glucose metabolism may also lead to defects in insulin-stimulated production of NO. This would be predicted to cause an impaired vasodilatory response to insulin resulting in a relative elevation in peripheral vascular resistance that may contribute to hypertension.

To gain insight into potential mechanisms linking insulin signal transduction with hypertension we have directly measured and characterized NO production in response to insulin in primary cultures of human umbilical vein endothelial cells (HUVEC) using an NO-specific amperometric electrode. We report, for the first time, direct measurement of a rapid, dose-dependent production of NO from HUVEC in response to insulin. The effects of insulin on NO production appear to be mediated, in part, through the insulin receptor utilizing a wortmannin-dependent pathway. Our data suggest that NO is a novel effector of insulin signaling pathways that are also involved with glucose metabolism.

Methods

Human umbilical vein endothelial cells. Primary cultures of HUVEC pooled from multiple donors were obtained from Clonetics Corp. (San Diego, CA) and cultured in 6-well plates at 37°C, 5% CO_2 with Endothelial Cell Growth Media (EGM, Modified MCDB 131 from Clonetics) containing bovine brain extract with heparin (12 μg/ml),

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1. *Abbreviations used in this paper:* HUVEC, human umbilical vein endothelial cells; NO, nitric oxide; NOS, nitric oxide synthase.

hEGF (10 ng/ml), hydrocortisone (1 μg/ml), 2% fetal bovine serum, gentamicin (50 μg/ml), and amphotericin-B (50 ng/ml). All studies were conducted on cells that underwent fewer than four passages. HUVEC were serum starved overnight in Endothelial Basal Media (EGM without the additives) prior to NO measurements.

Direct measurement of NO. All NO measurements were done at 24°C, pH 7.4 using the NO-501 Nitric Oxide Monitoring Device (Inter-Medical Ltd, Salisbury, UK) inside a shielded Faraday cage. This amperometric device utilizes a platinum/iridium measuring electrode covered with a semi-permeable NO-selective membrane in conjunction with a carbon reference electrode. When a voltage is applied across these electrodes in solution, NO generates a small current in the measuring electrode that is directly proportional to the NO concentration. Electrodes were allowed to equilibrate for at least 30 min in PBS before use. We calibrated the device by generating a standard curve using the NO donor S-nitroso-N-acetyl-DL-penicillamine (SNAP, obtained from Research Biochemicals International, Natick, MA) in PBS according to the manufacturers instructions. It was assumed that a 1 μM solution of SNAP will generate an NO concentration of 1.3 nM and that there is a linear relationship between the amount of SNAP and the amount of NO produced over the range we are measuring. Because the sensitivity of the electrode varies from day to day, a new standard curve was generated on the same day of each experiment. A linear least-squares fit was obtained for each standard curve and used to calculate the NO concentrations for each experiment. On the day of the experiment, after overnight incubation in serum free Endothelial Basal Media, cells were washed with DMEM (containing 25 mM glucose, 20 mM Hepes, pH 7.4, 24°C) and allowed to equilibrate in 2 ml of media. The electrodes were placed in the well so that the tips of the electrodes were 5 mm apart and ~ 1 mm above the surface of the cells. Various concentrations of agonists were added and the signal generated from the NO response processed through a 16 bit A/D converter and recorded on a desktop computer. After NO measurements were complete, cells were trypsinized and counted in a Coulter counter so that data could be normalized for cell number.

Ligand binding studies. To generate data for Scatchard plots, HUVEC were grown to confluence in 6-well plates and incubated overnight at 4°C with tracer concentrations of either [^{125}I]-insulin, [^{125}I]-IGF-1, or [^{125}I]-PDGF-BB (Amersham Life Sciences, Inc., Cleveland, OH) in the presence of varying concentrations of unlabeled ligand as described previously (15).

Results

Insulin stimulation of HUVEC. We used the NO donor SNAP to calibrate the electrode and generate a standard curve prior to each experiment (Fig. 1 *A*). We were able to reliably generate linear standard curves over the range of NO concentrations measured in our experiments. After addition of insulin to cells that had been washed and incubated overnight in serum free media, a sharp transient signal related to mechanical disturbance of the electrode was followed by a sustained signal within a minute or two that was indicative of NO production by HUVEC (Fig. 1 *B*). Addition of buffer (without insulin) to the cells, or addition of insulin to media in the absence of cells, did not result in the generation of a sustained signal by the NO electrode (data not shown). The dose-dependent insulin effect on NO production was quite reproducible (Fig. 2 *B*). When data were normalized for cell number (using a standard of 200, 000 cells per well of a six-well plate in a volume of 2 ml media, 24°C, pH 7.4), HUVEC produced an NO concentration of ~ 100 nM in response to a maximally stimulating dose of insulin (5 μM). The ED_{50} for this insulin effect, estimated from a best fit curve, was ~ 500 nM.

To gain additional information about the mechanisms whereby insulin stimulates NO production in HUVEC, we pretreated cells with either L-NAME (an arginine analog that is a competitive inhibitor of NOS), genestein (a tyrosine kinase inhibitor), or αIR-3 (an antibody against the IGF-1 receptor) before stimulating cells with insulin. L-NAME or genestein completely blocked the production of NO in response to insulin suggesting that activation of both the insulin receptor tyrosine kinase and NOS are involved with insulin-stimulated NO. αIR-3 inhibited the maximal insulin response by ~ 50% without affecting the ED_{50} significantly suggesting that some of the insulin signal may be mediated through the related IGF-1 receptor. To determine if insulin signaling pathways related to production of NO share elements in common with insulin signaling of glucose transport, we pre-treated cells with 500 nM wortmannin (an inhibitor of phosphatidylinositol 3-kinase

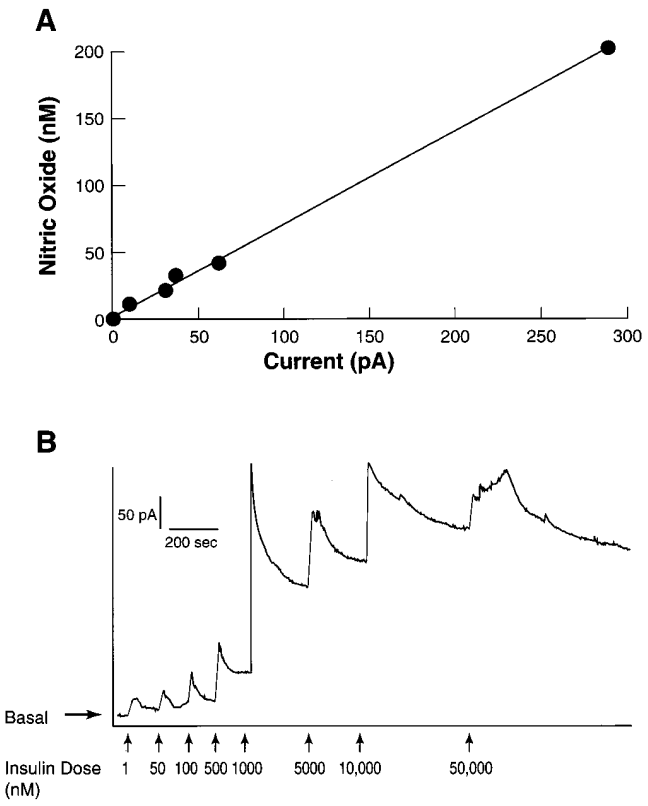

Figure 1. Direct measurement of NO from HUVEC. (*A*) A representative standard curve is shown ($r^2 = 0.99$). A new standard curve was generated on the day of each experiment. The concentration of NO (nM) is plotted as a function of the current generated by the NO electrode (pA). Our NO electrode has a linear response over the range of NO concentrations measured. (*B*) Representative output generated from an experiment where NO production was measured in response to increasing concentrations of insulin. Current generated by the NO electrode is recorded as a function of time. HUVEC were cultured in six-well plates and serum-starved overnight prior to NO measurements. After a stable baseline signal was achieved (shown as Basal), increasing concentrations of insulin were added to the media at the indicated times. In this figure, one can see a brief transient signal caused by mechanical disturbance of the electrode followed by a sustained signal in response to insulin that increases in an insulin dose-dependent fashion. The concentrations of insulin shown represent cumulative concentrations.

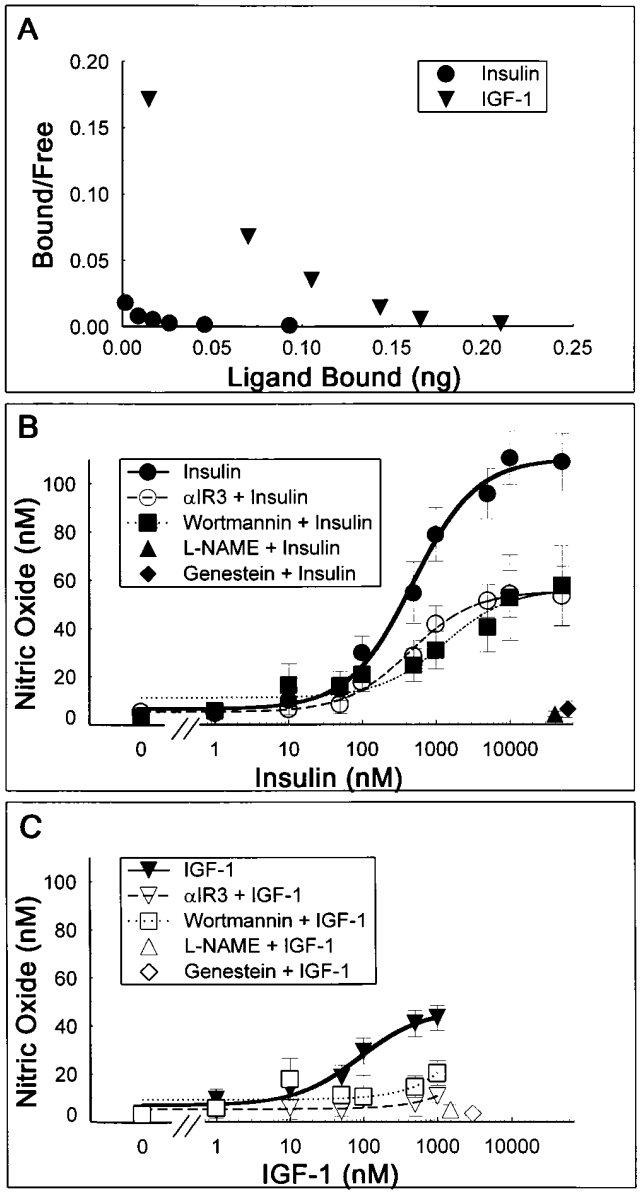

Figure 2. Differential effects of insulin and IGF-1 on production of NO in HUVEC. (*A*) Scatchard analysis of insulin and IGF-1 binding studies. The data shown are the average of triplicate determinations from a representative experiment that was repeated independently twice. From this experiment we estimated that there are approximately 10 times as many IGF-1 receptors as insulin receptors on HUVEC. (*B*) Production of NO from HUVEC in response to insulin in the presence or absence of various inhibitors. Data shown are the mean±SEM of n independent experiments. (●) insulin alone, $n = 12$; (▲) pre-treatment with 1 mM L-NAME for 1 h, $n = 7$; (◆) pretreatment with 25 μM genestein for 15 min, $n = 3$; (■) pre-treatment with 500 nM wortmannin for 40 min, $n = 4$; (○) pretreatment with 25 nM αIR-3 for 5 min, $n = 5$. To estimate ED_{50} values, the insulin dose-response curves were fit to the equation $y = a + b\,[x / (x + k)]$ using a Marquardt-Levenberg nonlinear least squares algorithm. When plotted on linear-log axes, this equation gives a sigmoidal curve where the parameters are associated with the following properties: a = basal response, a + b = maximal response, k = half-maximal dose (ED_{50}), and x = concentration of insulin. (*C*) Production of NO from HUVEC in response to IGF-1 in the presence or absence of various inhibitors. (▼) IGF-1 alone, $n = 13$; (△) pre-treatment with 1 mM L-NAME for 1 h, $n = 3$; (◇) pretreatment with 25 μM genestein for 15 min, $n = 4$; (□) pre-treatment with 500 nM wortmannin for 40 min, $n = 4$; (▽) pre-treatment with 25 nM αIR-3 for 5 min, $n = 4$.

(PI 3-kinase). Wortmannin caused an ~ 50% inhibition of the maximal insulin effect suggesting that PI 3-kinase is involved with the signaling of NO production by insulin. Because high concentrations of wortmannin may inhibit other lipid kinases such as PI 4-kinase, we also performed experiments using a lower dose of wortmannin (100 nM) and obtained similar results (data not shown).

IGF-1 stimulation of HUVEC. Although the affinity of insulin for the IGF-1 receptor is ~ 100-fold less than for the insulin receptor, it is possible that insulin is signaling NO production, in part, through the IGF-1 receptor. Therefore, we used ligand binding studies to estimate the number of insulin and IGF-1 receptors on HUVEC and directly measured IGF-1–stimulated production of NO from HUVEC. Scatchard analysis demonstrated the presence of ~ 10 times as many IGF-1 receptors as insulin receptors on HUVEC (~ 400,000 and ~ 40,000 receptors per cell respectively) (Fig. 2 *A*). Similar to our results with insulin, we observed that L-NAME, genestein, and wortmannin inhibited production of NO in response to IGF-1 (Fig. 2 *C*). However, despite the fact that there are 10 times as many IGF-1 receptors as insulin receptors on HUVEC, maximal IGF-1 stimulation resulted in an NO concentration that was only ~ 40% of that observed with maximal insulin stimulation. The ED_{50} for this IGF-1 effect, estimated from a best fit curve, was ~ 100 nM.

As another approach to investigating signaling of NO production by insulin and IGF-1 receptors, we examined the effect of the order of addition of each agonist. Interestingly, addition of insulin to cells that had been maximally stimulated with high concentrations of IGF-1 (where IGF-1 is presumably also occupying insulin receptors) did not result in a further significant increase in NO production (Fig. 3). Similarly, addition of IGF-1 to cells maximally stimulated with insulin did not result in a further significant increase in NO production. That is,

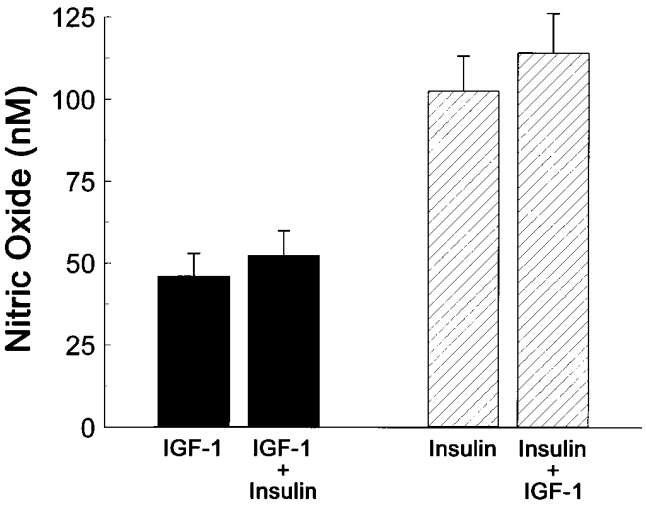

Figure 3. Effect of agonist order on production of NO from HUVEC. Data shown are mean±SEM of 9 independent experiments. NO production in response to 1 μM IGF-1 stimulation was not significantly increased following subsequent addition of 50 μM insulin (solid bars, $P = 0.22$). NO production in response to 50 μM insulin was not significantly increased following subsequent addition of 1 μM IGF-1 (hatched bars, $P = 0.15$). However, NO production from insulin treatment alone was significantly greater than that from IGF-1 treatment alone ($P < 0.001$).

the NO level produced by stimulation with IGF-1 followed by insulin is only half of that produced by stimulation with insulin followed by IGF-1. If cells are treated first with a half-maximal dose of insulin (500 nM) followed by increasing conentrations of IGF-1, there is an additive effect of IGF-1 on the NO response. If cells are treated first with a half-maximal dose of IGF-1 (100 nM) followed by increasing concentrations of insulin, there is also a small additive effect of insulin on the NO response. However, the maximal NO response to insulin in cells pre-treated with 100 nM IGF-1 is intermediate between the insulin response seen without IGF-1 pre-treatment and the insulin response seen after pre-treatment with a maximally stimulating dose of IGF-1 (data not shown).

PDGF stimulation of HUVEC. To further investigate the issue of receptor specificity, we attempted to measure production of NO in HUVEC in response to PDGF since it is known that PDGF stimulation of endothelial cells results in activation of PI 3-kinase (16). Although we were able to detect the presence of PDGF receptors on HUVEC using tracer-labeled ligand binding studies, we were unable to detect production of NO in response to PDGF at concentrations of up to 100 ng/ml of the BB isoform (data not shown).

Discussion

One of the major physiological roles of endothelium is to mediate the vasodilatory response to various agonists by regulated production of NO (17–19). The synthesis of NO is catalyzed by isoforms of NOS that are expressed in a tissue specific manner. eNOS, the predominant isoform in endothelial cells is essential for regulation of basal blood pressure (20). Unlike iNOS which undergoes transcriptional regulation in response to glucocorticoids and cytokines (mediated by tyrosine kinase dependent mechanisms), eNOS is thought to be acutely regulated by a calcium and calmodulin dependent mechanism (21). However, it has recently been reported that IGF-1 can stimulate production of NO from HUVEC in a tyrosine-kinase dependent manner that is likely mediated through activation of eNOS (22). Results from our present study showing that insulin-stimulated production of NO in HUVEC is completely blocked by genestein or L-NAME are consistent with the possibility that eNOS may be acutely regulated through ligand activated tyrosine kinase receptors such as the insulin and IGF-1 receptors.

Although the presence of insulin receptors on endothelial cells has been well documented, the physiological functions of these receptors in vascular endothelium are not well understood (23). Unlike classical insulin targets such as muscle and adipose tissue, endothelial cells are not generally very responsive to metabolic effects of insulin such as increased glucose transport because the insulin responsive glucose transporter GLUT4 is not expressed in these cells. However, increased blood flow to muscle as a result of the vasodilatory action of insulin contributes to insulin's effect on glucose disposal in vivo (24–26). One established function of insulin receptors in vascular endothelial cells is related to transport of insulin across the endothelium to target tissues (27). Our data suggest another important function of the insulin receptor on endothelial cells may be to mediate vasodilation through the production of NO. The results we obtained in HUVEC are not necessarily generalizable to all endothelium. It is possible that different types of endothelial cells may have different responses to insulin in terms of NO production. For example, the endothelium of small arterial vessels in skeletal muscle seem particularly sensitive to the vasodilatory actions of insulin (14). Perhaps some heterogeneity among endothelial cell types might be explained by different distributions of insulin or IGF-1 receptors.

It is possible that some of insulin's effects on NO production in HUVEC are being mediated through IGF-1 receptors. High concentrations of insulin are known to activate IGF-1 receptors as well as insulin receptors even though the binding affinity of insulin for the IGF-1 receptor is $\sim$ 100 times less than for the insulin receptor (23). Our finding that there are approximately 10 times as many IGF-1 receptors as insulin receptors on HUVEC is consistent with previous reports (28, 29). Other evidence consistent with the possibility of insulin signaling through the IGF-1 receptor include our observation that the ED_{50} for insulin-stimulated production of NO is higher than that reported for other actions of insulin such as glucose transport (30). Furthermore, the maximal effect of insulin is not achieved until concentrations exceed 5 μM (a concentration capable of fully activating IGF-1 receptors). Nevertheless, insulin stimulated NO levels that were twice that seen with IGF-1 stimulation. The magnitude of the IGF-1 effect we observed was similar to what has been reported previously (22). Furthermore, the IGF-1 receptor blocking antibody αIR-3 was able to completely inhibit NO production in response to IGF-1 but only blocked 50% of the insulin signal. Therefore, while some of insulin's effects on NO production may be mediated through the IGF-1 receptor, there is a significant effect that is specifically mediated by the insulin receptor.

Interestingly, the order in which insulin and IGF-1 were added to the cells affected the level of NO produced. These results are consistent with the possibility that the signal generated by the insulin receptor depends, in part, on which ligand is bound. In other words, cells treated first with high concentrations of IGF-1 that occupy both IGF-1 receptors and insulin receptors generate less NO than cells treated first with high concentrations of insulin. Subsequent addition of the other ligand does not generate additional signal because the receptors are already occupied. One explanation for this differential signaling from the insulin receptor might be ligand-specific differences in conformational changes that occur in the receptor as a result of binding insulin or IGF-1 (31). When we stimulated the cells with submaximal concentrations of insulin or IGF-1 where receptor occupancy is lower, we were able to see additive effects of subsequent addition of the other ligand. However, cells treated first with submaximal concentrations of IGF-1 had a somewhat blunted response to insulin consistent with the hypothesis that insulin and IGF-1 are competing for receptors that can distinguish between related ligands and mediate different biological effects.

PI 3-kinase is a necessary effector of insulin signaling related to glucose transport (32). Since pre-treatment of HUVEC with wortmannin (an inhibitor of PI 3-kinase) inhibited the production of NO by insulin, our data suggest that insulin signal transduction pathways for NO share common elements with insulin signal transduction pathways related to glucose transport. To determine if PI 3-kinase activity is sufficient for NO release, we stimulated HUVEC with PDGF. Like insulin, PDGF is a growth factor that signals through a ligand activated tyrosine kinase receptor. Many downstream effectors of the insulin receptor are also downstream effectors of the PDGF re-

ceptor (33, 34). In particular, PDGF is known to stimulate PI 3-kinase activity in endothelial cells (35). The fact that we were unable to detect production of NO in response to PDGF suggests that neither tyrosine kinase activity nor PI 3-kinase activity per se is sufficient for the production of NO in HUVEC. This result is similar to what is known about signaling of glucose transport in insulin sensitive tissues. That is, PDGF stimulation of adipocytes does not increase glucose transport even though the resultant increase in PI 3-kinase activity is comparable to that generated by insulin stimulation (and PI 3-kinase is a necessary effector for insulin-stimulated glucose transport) (32, 36, 37, Quon et al., unpublished data).

In summary, we provide direct evidence for specific signaling of NO production through the insulin receptor. In addition, our data suggest that differential signaling from the insulin receptor depends on the particular ligand that is occupying the receptor. This ligand specificity may be important for generating appropriate signaling for the diverse effects on growth, differentiation and metabolism mediated by the insulin receptor in various tissues. We conclude that NO is a novel effector of insulin action in vascular endothelium. Our study does not directly address abnormalities seen in hypertension-associated insulin resistant states such as diabetes and obesity. However, our finding that insulin stimulates production of NO from endothelium together with the observation that this insulin-signaling pathway may share common signaling elements with insulin-stimulated glucose transport raises the possibility that insulin resistance with respect to production of nitric oxide may contribute to hypertension in patients who are also insulin resistant with respect to glucose metabolism.

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