Inhibition of Phosphatidylinositol 3-Kinase Enhances Mitogenic Actions of Insulin in Endothelial Cells*

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The concept of “selective insulin resistance” has emerged as a unifying hypothesis in attempts to reconcile the influence of insulin resistance with that of hyperinsulinemia in the pathogenesis of macrovascular complications of diabetes. To explore this hypothesis in endothelial cells, we designed a set of experiments to mimic the “typical metabolic insulin resistance” by blocking the phosphatidylinositol 3-kinase pathway and exposing the cells to increasing concentrations of insulin (“compensatory hyperinsulinemia”). Inhibition of phosphatidylinositol 3-kinase with wortmannin blocked the ability of insulin to stimulate increased expression of endothelial nitric-oxide synthase, did not affect insulin-induced activation of MAP kinase, and increased the effects of insulin on prenylation of Ras and Rho proteins. At the same time, this experimental paradigm resulted in increased expression of vascular cellular adhesion molecules-1 and E-selectin, as well as increased rolling interactions of monocytes with endothelial cells. We conclude that inhibition of the metabolic branch of insulin signaling leads to an enhanced mitogenic action of insulin in endothelial cells.

Insulin profoundly influences the function of the vascular endothelium (1–5). In humans, physiological levels of insulin stimulate increased production of nitric oxide (NO)1 in the vasculature resulting in vasodilation and increased blood flow (1, 5). Intriguingly, vasodilator actions of insulin are impaired in individuals who are also resistant to metabolic actions of insulin (6). Although associations between vascular disease and insulin-resistant states such as diabetes, obesity, and hypertension have been firmly established, the mechanisms linking endothelial dysfunction and accelerated atherosclerosis with insulin resistance (typically defined as decreased sensitivity or responsiveness to metabolic actions of insulin) have not been fully elucidated. With in vivo studies, it is particularly challenging to differentiate potentially distinct influences of insulin resistance per se from effects of compensatory hyperinsulinemia. In vitro studies in vascular endothelial cells demonstrate that insulin may stimulate production of NO by increasing both the expression and the activity of endothelial nitric-oxide synthase (eNOS) (7–9). Activation of phosphatidylinositol 3-kinase (PI 3-kinase) is necessary to promote both increased expression and activity of eNOS in response to insulin (7–9). Interestingly, PI 3-kinase is also a key signaling molecule mediating metabolic actions of insulin in adipose tissue and skeletal muscle (reviewed in Ref. 10). Thus, abnormalities in PI 3-kinase-dependent pathways that are shared among different tissues may provide one molecular explanation for the frequent associations of vascular disease and insulin-resistant states (4).

Recent studies (11, 12) in both humans and animals demonstrate that regulation of the insulin receptor substrate-1 (IRS-1)/PI 3-kinase-dependent branch of insulin signaling may be distinct from regulation of the Ras/mitogen-activated protein kinase (MAP kinase)-dependent insulin signaling pathway. In fact, in many models of metabolic insulin resistance, insulin signaling via the IRS-1/PI 3-kinase pathway is impaired, whereas the MAP kinase pathway is unaffected (11, 12). Compensatory hyperinsulinemia resulting from insulin resistance may stimulate increased production of plasminogen activator inhibitor-1, endothelin, and various proliferative events in vascular smooth muscle cells via MAP kinase-dependent pathways (13–17). We have demonstrated recently that the ability of insulin to increase the prenylation of Ras and Rho proteins is mediated via the Shc/MAP kinase pathway and is completely independent of PI 3-kinase activity (18, 19). Enhanced prenylation of these small molecular weight GTPases increases the mitogenic responsiveness of cells to a variety of growth factors.

One might envision that hyperinsulinemia resulting from insulin resistance would drive intact Shc/MAP kinase-dependent pathways to increase prenylation of Ras and Rho leading to enhanced mitogenic responsiveness of endothelial cells. In the present study, we designed experiments to mimic the typical “insulin-resistant” state by blocking PI 3-kinase-dependent signaling and exposing cells to increased concentrations of insulin (“compensatory hyperinsulinemia”). The effects of insulin to stimulate prenylation of Ras and Rho proteins, expression of eNOS and adhesion molecules (VCAM-1 and E-selectin), and the interaction of leukocytes with the endothelium were exam-
ned in human umbilical vein endothelial cells (HUVEC). We found that inhibition of PI 3-kinase lead to enhanced mitogenic actions of insulin in endothelial cells. Thus, we have identified an additional plausible mechanism for insulin resistance and hyperinsulinemia to contribute to vascular complications of diabetes.

**EXPERIMENTAL PROCEDURES**

**Materials**—All standard chemicals were from Sigma. Anti-Ras monoclonal antibody and eNOS antibody were purchased from Transduction Laboratories (Lexington, KY). Anti-Rho-A antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). p44/42 MAP kinase and phospho-p44/42 MAP kinase antibodies were purchased from Cell Signaling, New England Biolabs (Beverly, MA). Supplies and reagents for SDS-PAGE were purchased from Bio-Rad, and the enhanced chemiluminescence kit was obtained from Amersham Biosciences. Recombinant human VEGF-165 was purchased from R & D Systems (Minneapolis, MN). HUVEC in primary culture and endothelial basal media (EBM) were obtained from Clonetics Corp. (San Diego, CA). For all experiments involving HUVEC, cells were seeded in 60-mm dishes, grown in EGM-2 as described (7, 9), and used between passages 3 and 4.

Assessment of eNOS Expression in Endothelial Cells—HUVEC were serum-starved for 14 h in EBM-B (endothelial basal media supplemented with 1% platelet-deprived horse serum) and then treated with or without insulin (100 nM) for 24 h in the absence or presence of wortmannin (50 nM). Cell lysates were prepared using lysis buffer (150 mM NaCl, 5 mM MgCl₂, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM NaVO₃, 1 mM sodium pyrophosphatase, 1% Triton X-100, 0.05% SDS, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 50 mM Hepes, pH 7.5), and samples were subjected to immunoprecipitation using an antibody against eNOS, according to standard methods. Samples were then separated by 8% SDS-PAGE followed by immunoblotting with anti-eNOS antibody.

Measurement of MAP Kinase Phosphorylation in Endothelial Cells—HUVEC were serum-starved for 24 h in EBM-B and then treated with insulin (100 nM) in the absence or presence of wortmannin (50 nM) for 24 h. Cells lysates were prepared with lysis buffer, and samples were immunoprecipitated with p44/42 MAP kinase antibodies. Samples were analyzed by SDS-PAGE, determined by Western blotting using phospho-p44/42 MAP kinase antibodies, and visualized by chemiluminescence.

Measurement of FTase and GGTase-II Activity and Amounts of Far-nylated p21Ras and Geranylgeranylated Rho-A—HUVEC at 95% confluence were serum-starved overnight in EBM-A (15 mM Hepes, pH 7.4), Rho-A were immunoprecipitated from equal volumes from each phase. Labeled antisense RNA probes were synthesized by in vitro transcription using T7 polymerase (Ambion Maxiscript kit) and gel-purified. RPA was performed on total RNAs using the Ambion RPA kit. An RNA probe of human cyclinophilin (nucleotides 135–209, GenBank™ accession number X52956) was used as an internal control for RNA quantification. All signals were visualized and analyzed by densitometric scanning (LAS-1000, Fuji Film, Tokyo). Data are expressed as mean ± S.D. Statistical significance was tested using one-way analysis of variance followed by the Student-Newman-Keuls test. Statistical significance was set at p < 0.05.

**RESULTS**

We have shown previously that wortmannin completely inhibits the acute effect of insulin to stimulate production of NO in HUVEC (7). In the present study we demonstrate that treatment of HUVEC with 50 nM wortmannin also fully inhibits the effect of insulin on the expression of eNOS in these cells (Fig. 1). These experiments are consistent with previous results demonstrating that the effect of insulin to increase eNOS expression and activity in endothelial cells is mediated by the PI 3-kinase pathway (7, 9). In contrast, under similar conditions, the ability of insulin to stimulate the phosphorylation of MAP kinase remained unaffected (Fig. 2), that is insulin (10 nM) promoted comparable phosphorylation of MAP kinase both in control and the wortmannin-treated cells.

We then examined the effect of insulin on the activity of far-nylated p21Ras (FTase) and geranylgeranylated Rho-A (GGTase I) in wortmannin-treated cells. In control HUVEC, insulin (10 nM) stimulated both prenyltransferases at 1 h of incubation with a return toward basal activity at 24 h (Fig. 3, A and B). In the wortmannin-treated cells, the effect of insulin on FTase was significantly increased at 24 h (Fig. 3A). A pattern of activation of GGTase I was somewhat different in

![Fig. 1. Effect of insulin and wortmannin on the expression of eNOS in HUVEC. Insulin-stimulated induction of eNOS expression is blocked by wortmannin (Wort) in HUVEC. Cells starved in EBM-B for 14 h were treated without or with insulin (100 nM, 20 h) in the absence or presence of wortmannin (50 nM). One group was treated with wortmannin alone. Cell lysates (125 μg of total protein) were subjected to immunoprecipitation with anti-eNOS antibody. The samples were then separated by 8% SDS-PAGE followed by immunoblotting with anti-eNOS antibody. A representative blot from experiments that were repeated independently three times is shown. The relative levels of expression were quantified by scanning densitometry of the immunoblots. Results are means ± S.E. of three independent experiments expressed in arbitrary density units.](https://www.jbc.org/content/273/13/1795/F1.large.jpg)
the wortmannin-treated HUVEC. The effect of insulin was significantly greater both at 1 and 24 h of incubation (Fig. 3).

As expected, increased activity of the prenyltransferases was followed by the corresponding increases in the amounts of farnesylated p21Ras and geranylgeranylated Rho-A in wortmannin-treated cells exposed to either 10 or 100 nM insulin for 24 h (Fig. 4).

We have shown previously that increased availability of farnesylated p21Ras and geranylgeranylated Rho-A in wortmannin-treated cells exposed to either 10 or 100 nM insulin for 24 h enhanced the mitogenic effectiveness of other growth factors, such as platelet-derived growth factor, epidermal growth factor, and insulin-like growth factor-1 in various cells (20, 21). Similarly, cells with increased availability of geranylgeranylated Rho-A also displayed increased mitogenic responsiveness to agents working via the Rho pathway (22, 23). Therefore, we hypothesized that increased availability of farnesylated p21Ras and geranylgeranylated Rho-A in HUVEC might increase the responsiveness of these cells to other growth-promoting agents. To investigate this point, we examined the effect of insulin, in the presence of wortmannin, on the vascular endothelial growth factor (VEGF)-induced expression of the adhesion molecules, VCAM-1 and E-selectin. As expected, VEGF significantly increased the expression of VCAM-1 and E-selectin in control cells (Fig. 5). Insulin alone did not exert any significant effect on the expression of mRNA of these adhesion molecules. Nevertheless, in concert with previously published observations (25, 26), it significantly attenuated the effect of VEGF (Fig. 5). However, in the presence of wortmannin, insulin lost its attenuating effect and potentiated the effect of VEGF on the expression of VCAM-1 and E-selectin (Fig. 5).

We then addressed the functional implications of an increased expression of the adhesion molecules. Because the process of monocyte adhesion to endothelium begins with rolling interactions and progresses to a complete stop, we assessed these two phases of monocyte interaction with the insulin-treated endothelial cells in the presence of wortmannin (Fig. 6). The experiments were performed in a flow chamber under a low shear stress (0.3 dynes/cm²). We found that rolling interactions of monocytes with the endothelial cells in response to insulin were significantly increased by the blockade of PI 3-kinase (Fig. 6A). In addition, under these conditions, leukocytes were more likely to come to complete stops than in control experiments or in the presence of either insulin or wortmannin alone (Fig. 6B). Thus, the two essential early steps required for monocyte adhesion to endothelium (i.e. initial rolling and arrest of motion) were significantly increased when the cells were treated with insulin in the presence of wortmannin.

**DISCUSSION**

Investigators exploring contributions of insulin resistance and the resulting compensatory hyperinsulinemia to the pathogenesis of vascular diseases that accompany the metabolic...
syndrome X must reconcile an apparent paradox. On the one hand, insulin resistance signifies impaired insulin action. On the other hand, hyperinsulinemia raises the possibility of increased insulin action in certain contexts. For example, in endothelial cells, diminished insulin action secondary to acquired or inherited insulin resistance readily explains the impaired ability of insulin to stimulate production of NO and normal vasodilation (4–6). At the same time, hyperinsulinemia may cause overproduction of endothelin-1 and plasminogen activator inhibitor-1 by endothelial cells that contribute to vascular disease (13–17). An unresolved question is how physiological responses related to either insulin resistance or hyperinsulinemia can occur simultaneously in the same cell. One potential explanation for this important pathophysiological conundrum was suggested by the demonstration that insulin resistance along the IRS-1/PI 3-kinase pathway of insulin signaling does not necessarily coincide with resistance in other signaling pathways. In fact, the Ras-MAP kinase signaling pathway retains normal sensitivity to insulin in both humans and animals who are resistant to the metabolic actions of insulin (11, 12).

We have demonstrated recently (27, 28) that the effect of insulin to stimulate prenylation of Ras and Rho proteins is actually increased in tissues of insulin-resistant humans and animals. Insulin promotes the phosphorylation of the α-subunit of FTase and GGTase I and stimulates the activities of both prenyltransferases (18–23). Even though stimulatory effects of insulin on prenyltransferases and the amounts of prenylated Ras and Rho proteins are relatively small (15–30%, see Figs. 3 and 4), they are of high physiological significance, for they result in a 2-fold potentiation of action of other growth-promoting agents working via the Ras- and Rho-dependent pathways (19–23).

Although insulin at high concentrations may act through the receptors for insulin-like growth factor-1 (29), we have demonstrated that the effect of insulin on the prenyltransferases is not mimicked by other growth factors, requires the presence of an intact insulin receptor, and is mediated via the Shc-MAP kinase pathway (19, 21). Analogous to congenital adrenal hyperplasia, where the influence of ACTH is directed along an unaffected branch of steroidogenesis, we postulate that insulin signaling via the MAP kinase pathway is unaffected (and its signaling to the prenyltransferases is actually increased) in the presence of insulin resistance along the PI 3-kinase pathway. Thus, metabolic insulin resistance caused by impaired PI 3-kinase pathways may result in compensatory hyperinsulinemia that increases the activity of the prenyltransferases through unopposed Shc-MAP kinase pathways (Fig. 7).

Our current experiments strongly support our hypothesis and demonstrate an increased responsiveness of HUVEC to VEGF in the presence of an experimental paradigm mimicking the state of metabolic insulin resistance and compensatory hyperinsulinemia. In vascular endothelial cells, blockade of the PI 3-kinase signaling pathway with wortmannin increased the ability of insulin to augment the activities of FTase and GGTase I as well as the amounts of prenylated p21Ras and Rho-A. Concomitantly, the effects of VEGF on the production of VCAM-1 and E-selectin in HUVEC were increased, and the endothelial cells attracted significantly greater numbers of mononuclear cells (Fig. 6). Because insulin-stimulated production of NO is a PI 3-kinase-mediated event (7, 9), our results are also consistent with a recent study showing that the effect of insulin to inhibit expression of ICAM-1 in endothelial cells may be dependent on production of NO (25).

Increased expression of cellular adhesion molecules, including ICAM-1, VCAM-1, and E-selectin, is believed to be among the earliest steps in the process of atherogenesis (30). These adhesion molecules are not only expressed in the endothelial cells but are also released into the circulation via either shedding or an alternative pathway (31). Increased levels of circulating adhesion molecules are correlated with future coronary events (32–34). Similarly, increased levels of circulating adhesion molecules are consistently found in patients with type 2 diabetes and other conditions associated with insulin resistance (reviewed in Refs. 35 and 36). Furthermore, in healthy volunteers, Chen et al. (35) have found a significant correlation between the degree of insulin resistance and circulating concentrations of E-selectin, ICAM-1, and VCAM-1, that is the most insulin-resistant individuals displayed the highest concentrations of all three soluble adhesion molecules (35). Our findings that insulin (with or without VEGF) can potentiate the expression of these adhesion molecules when the metabolic (PI 3-kinase-dependent) branch of insulin signaling is inhibited may provide a molecular explanation for the increased cellular
PI 3-Kinase Inhibition Enhances Mitogenic Actions of Insulin

Fig. 6. Effect of insulin- and wortmannin-treated endothelial cells on monocyte adhesion and arrest. Insulin/wortmannin treatment of endothelial cells increases adhesion of Wehi 274.1 monocytes under flow. A, rolling cells/min. Wehi 274.1 monocytes were injected into a laminar flow chamber at 10^6 cells/ml at the shear stress of 0.3 dynes/cm². Images were collected at 30 frames/s and analyzed to count the number of cells/min that formed rolling adhesions to localized endothelial cells. Endothelial cells were either untreated or treated with 100 nM wortmannin, 10 nM insulin, and a combination of both for 24 h.

In the presence of insulin resistance, the PI 3-kinase-dependent (metabolic) branch of insulin signaling is blocked, the mitogenic branch is normal and insulin signaling to the prenyltransferases is augmented. Insulin signaling to prenyltransferases flows via activation of Shc and MAP kinase (19). Greater availability of prenylated Ras and Rho proteins and decreased production of NO in endothelial cells potentiate the effects of other growth factors leading to increased expression of adhesion molecules. f-p21Ras, farnesylated p21Ras; ggRho-A, geranylgeranylated Rho-A.

In summary, we show that in an in vitro model of metabolic insulin resistance with hyperinsulinemia, insulin is unable to stimulate eNOS expression but is able to increase production of adhesion molecules in the same endothelial cells (possibly via enhanced prenylation). We postulate that insulin resistance in vivo simultaneously results in decreased NO production and compensatory hyperinsulinemia that augments prenylation of Ras and Rho proteins. This may contribute to the increased responsiveness of endothelial cells to atherogenic actions of VEGF and other growth factors.

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