G972R IRS-1 Variant Impairs Insulin Regulation of Endothelial Nitric Oxide Synthase in Cultured Human Endothelial Cells

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Background—Impaired insulin-mediated vasodilation might contribute to vascular damage in insulin-resistant states. Little is known about insulin regulation of nitric oxide (NO) synthesis in insulin-resistant cells. The aim of this work was to investigate insulin regulation of NO synthesis in human umbilical vein endothelial cells (HUVECs) carrying the IRS-1 gene G972R variant, known to be associated with impaired insulin activation of the PI3-kinase (PI3-K) pathway in transfected cells.

Methods and Results—HUVECs were screened for the presence of the G972R-IRS-1 (HUVEC-G972R) variant by restriction fragment length polymorphisms. After 24-hour exposure to 10^{-7} mol/L insulin, endothelial NO synthase (eNOS) mRNA (reverse transcription–polymerase chain reaction), eNOS protein levels (Western blotting), and NOS activity (conversion of [3H]arginine into [3H]citrulline) were increased in wild-type HUVECs (HUVEC-WT), whereas they did not change from baseline in HUVEC-G972R. Compared with HUVEC-WT, in HUVEC-G972R after 2 and 10 minutes of insulin stimulation, IRS-1–associated PI3-K activity was reduced by 47% and 32%, respectively; Akt phosphorylation was decreased by 40% at both time points; and eNOS-Ser1177 phosphorylation was reduced by 38% and 51%, respectively. In HUVEC-WT, eNOS-Thr495 phosphorylation decreased after insulin stimulation. In contrast, in HUVEC-G972R, eNOS-Thr495 phosphorylation increased after insulin stimulation and was 40% greater than in HUVEC-WT.

Conclusions—Our data demonstrate that genetic impairment of the (IRS)-1/PI3-K/PDK-1/Akt insulin signaling cascade determines impaired insulin-stimulated NO release and suggest that the G972R-IRS-1 polymorphism, through a direct impairment of Akt/eNOS activation in endothelial cells, may contribute to the genetic predisposition to develop endothelial dysfunction and cardiovascular disease. (Circulation. 2004;109:399-405.)

Key Words: endothelium • insulin • nitric oxide synthase

Insulin resistance is associated with atherosclerosis and coronary artery disease. Both the metabolic alterations occurring in the insulin-resistant state and the possible detrimental effects of hyperinsulinemia have been proposed to explain this association. However, it has also been hypothesized that vessel wall–selective insulin resistance could contribute to atherosclerosis in humans. Insulin promotes vasodilation and increases blood flow, thus participating in the regulation of hemodynamic homeostasis. Zeng and Quon first demonstrated a role for PI3-kinase (PI3-K) in the induction of NO by insulin in the endothelium, showing that inhibition of insulin-induced activation of PI3-K led to impaired NO availability. Then it was observed that this effect depends primarily on the activation of the insulin receptor (IR)/insulin receptor substrate (IRS)-1/PI3-K/PDK-1/Akt signaling cascade. The IRS-1 phosphorylation that follows IRβ tyrosine kinase activation leads to IRS-1 interaction with the PI3-K p85 regulatory subunit. PI3-K subsequently activates Akt, which in turn phosphorylates endothelial NO synthase (eNOS) on serine 1177 and enhances eNOS transcription. It has been demonstrated that IRS-1 and PDK1 are required for insulin-stimulated production of NO in endothelial cells. Endothelial dysfunction, ie, reduced NO availability, a pivotal step in the pathogenesis of atherosclerosis, is a feature of insulin-resistant states such as type II diabetes, obesity, and hypertension, and it may be dependent on both metabolic and genetic factors. We recently demonstrated that hyper-
glycemia, via hexosamine pathway activation, induces selective insulin resistance in endothelial cells, leading to impaired eNOS activation.\textsuperscript{13} Although data supporting the existence of a “primitive,” genetic, insulin resistance of the endothelium are not at present available, 2 elements suggest the existence of a selective endothelial insulin resistance. First, insulin-resistant first-degree relatives of type II diabetic patients exhibit reduced insulin-dependent endothelial vasodilation compared with control independently of other cardiovascular risk factors.\textsuperscript{14} Moreover, endothelial dysfunction is present in insulin-resistant IRS-1--null mice, an animal model exhibiting features of the insulin-resistance syndrome.\textsuperscript{15} We and others have observed that a common polymorphism in the IRS-1 gene, the G972R-IRS-1 variant, is associated with impaired IRS-1 ability to recruit the p85 regulatory subunit of PI3-K, leading to diminished activation of the PI3K/Akt pathway in several cell types.\textsuperscript{16–20} The purpose of the present study was, therefore, to use human endothelial cells from carriers of this variant as a model to determine whether a polymorphism known to impair insulin action might reduce insulin ability to activate the signaling pathway that regulates eNOS activity and expression.

Methods

Materials

Antibodies and reagents used were anti-phosphotyrosine, anti-IRS-1, anti-p85, anti-Ser1177, anti-Thr945, and anti-eNOS (Upstate Biology); anti-Akt, anti-Ser473-Akt, anti-FOXO1, and anti-Thr24FOXO1 (Cell Signaling Technology); and anti-human eNOS (Transduction Laboratories). All other chemicals were from SIGMA.

Cell Culture

Umbilical cords were obtained from randomly selected healthy mothers delivering at the Chieti University Hospital. Umbilical cord arteries were screened for the presence of the polymorphism of interest as described below. Primary human umbilical vein endothelial cells (HUVECs) were obtained as described previously.\textsuperscript{21} After perfusion of umbilical cords with 0.1% collagenase at 37°C, HUVECs were grown on 0.2% gelatin-coated tissue culture plates in M199 endothelial growth medium (BioWhittaker) supplemented with 20% FBS, 10 μg/mL heparin, and 50 μg/mL ECGF (Sigma). In all experiments, cells were used between the third and sixth passages in vitro.

The G972R-IRS-1 variant was assessed in DNA extracted from both umbilical cord arterial fragments and HUVECs and detected by digestion of polymerase chain reaction (PCR) products with the restriction enzyme Mvul (Roche Applied Science) as previously described.\textsuperscript{22}

RNA Extraction and Reverse Transcription-PCR Analysis

Total RNA was extracted from the HUVECs by use of the SV Total RNA Isolation System (Promega). Total RNA was denatured together with oligo-dT primer for 10 minutes at 70°C, and polyadenosine (poly-A) RNA was reverse-transcribed for 60 minutes at 42°C with StrataScript II (50 U/mL) (Stratagene). PCR was performed with Taq DNA polymerase with the buffer supplied by the manufacturer (Promega). Oligonucleotide primers were constructed from a published sequence of human GAPDH, 5'-CCACCATATGGCAAATTCCATGGCA-3' (sense) and 5'-TCTAGAGCCAGGTCTACGCACACC-3' (antisense) generating a 597-bp fragment (annealing temperature, 60°C) for 25 cycles and human eNOS (sense) 5'-GTGATGGCGAAGCGATGTAAG-3' and 5'-CCGACGGCCGAAA-3 (antisense) (annealing temperature, 58°C) for 35 cycles. PCR products were electrophoresed and analyzed by ethidium bromide staining. The gels were then photographed and scanned to quantify the obtained reverse transcription-PCR products.

Western Blot Analysis

eNOS protein was analyzed by Western blot analysis using an anti-human eNOS antibody as previously described.\textsuperscript{7}

IRS-1, Akt, and eNOS Phosphorylation and Anti-Phosphotyrosine–Associated PI3-K Activity

HUVECs were serum-starved for 16 hours and incubated in the presence or absence of insulin 10−7 mol/L for the indicated times. Cell lysis, immunoprecipitations, Western blot, and PI3-K assays were performed as described.\textsuperscript{13,18–20} eNOS phosphorylation on Ser1177 and Thr945 was assessed after total eNOS immunoprecipitation. Membranes blotted with anti-phosphotyrosine or phosphoactive antibodies were stripped and reprobed with primary antibodies to verify expression levels. Protein phosphorylation levels were normalized to the matching densitometric values of nonphosphorylated proteins. Autoradiographs were quantified with a FluorImager (Biorad).

eNOS Activity

HUVECs were serum-starved for 16 hours and incubated in the presence or in the absence of insulin 10−7 mol/L for 24 hours. NOS activity was assayed by measuring the ability of cell lysates, normalized to cell protein content, to convert L-{[H]}arginine (185×10^5 Bq, 5 μCi, Amersham) into L-{[H]}citrulline, as described by Aldieri et al.\textsuperscript{23}

eNOS Immunofluorescence

HUVECs were plated onto gelatin-coated coverslips, serum-starved for 16 hours, and stimulated with insulin (10−7 mol/L) for 0, 2, and 10 minutes. At the end of treatment, cells were fixed in acetonemethanol 1:1 for 3 minutes at −20°C and rinsed with Dulbecco’s PBS plus 0.1% BSA for 5 minutes at room temperature. Cells were then incubated with 5% goat serum in Dulbecco’s PBS/BSA for 30 minutes at room temperature followed by a 2-hour incubation with polyclonal P-eNOS antibody and monoclonal eNOS antibody for 2 hours at room temperature. Anti-rabbit Texas Red–labeled (diluted 1:100) and anti-mouse fluorescein isothiocyanate–labeled (1:100) secondary antibodies (Jackson Immunoresearch Laboratories) were incubated for 1 hour at room temperature. Slides were mounted with SlowFade (Molecular Probes), and cells were observed with an inverted Nikon microscope fitted with a Nikon confocal imaging system.

Statistical Analysis

Results are reported as mean±SEM in arbitrary units. Statistical analyses were performed by 1-way ANOVA or Student’s t test as indicated. Values of P<0.05 were considered statistically different.

Results

Effect of Insulin on Activity of the Metabolic IRS/PI3-K Pathway and of the Mitogenic MAPK Pathway in WT and HUVEC-G972R Cells

The extent of basal and insulin-stimulated tyrosine phosphorylation of IRβ and IRS-1 was similar in HUVEC–wild-type (WT) and HUVEC-G972R cells (Fig 1A). In HUVEC-WT cells, on insulin stimulation, IRS-1 interaction with the p85 regulatory subunit of PI3-K increased by 5- and 7.5-fold at 2 and 10 minutes, respectively (P<0.01 and P<0.001), and IRS-1–associated PI3-K activity increased by 2.2-fold at 2 minutes and by 64% at 10 minutes (P<0.01 and P<0.05, respectively) (Figure 1A). In HUVEC-G972R, compared with HUVEC-WT, IRS-1 association with the p85 subunit of
PI3-K was reduced by 60% at 2 minutes and by 50% at 10 minutes of insulin stimulation, respectively (P<0.001 and P<0.01; Figure 1A). Compared with HUVEC-WT, HUVEC-G972R cells exhibited a 50% (P<0.001) reduction in basal IRS-1–associated PI3-K activity. When cells were exposed to insulin stimulation, compared with WT, HUVEC-G972R IRS-1–associated PI3-K activity was reduced by 47% and 32% at 2 and 10 minutes of insulin stimulation, respectively (P<0.001 and P<0.01; Figure 1A).

Insulin induced MAPK phosphorylation in both HUVEC-WT and HUVEC-G972R cells. Interestingly, at 10 minutes after insulin stimulation, MAPK phosphorylation was significantly higher in HUVEC-G972R than HUVEC-WT cells (P<0.05) (Figure 1B).

**Effect of Insulin on Akt Phosphorylation in HUVEC-WT and HUVEC-G972R Cells**

Basal Akt expression was comparable in HUVEC-WT and HUVEC-G972R cells (Figure 2A). In HUVEC-WT, insulin induced a 2.1-fold increase in Akt phosphorylation after 2 minutes (P<0.001) and a 148% increase after 10 minutes (P<0.01) (Figure 2A). In contrast, insulin failed to increase Akt phosphorylation significantly in HUVEC-G972R cells. Thus, in these cells, Akt phosphorylation levels after insulin stimulation were 40% lower than those observed in HUVEC-WT at both 2 and 10 minutes (P<0.01) (Figure 2A).

To assess whether reduced Akt phosphorylation in HUVEC-G972R cells was associated with impaired activation of Akt substrates, we measured the transcription factor FOXO-1 phosphorylation. We observed that in HUVEC-G972R cells, reduced activation of Akt was associated with impaired FOXO-1 phosphorylation at 10 minutes (Figure 2B) (P<0.05).

**Effect of Insulin on eNOS Phosphorylation in WT and HUVEC-G972R Cells**

Next, we assessed the ability of insulin to modulate eNOS phosphorylation on both the Akt consensus site at Ser1177 and at Thr495. On insulin stimulation, in HUVEC-WT, eNOS Ser1177 phosphorylation increased by 35% at 2 minutes (P<0.01) and by 2.1-fold at 10 minutes (P<0.001) (Figure 3A). Compared with HUVEC-WT, HUVEC-G972R cells exhibited a 38% reduction of basal Ser1177 eNOS phosphorylation; on insulin stimulation, Ser1177 eNOS phosphoryla-
tion was similarly reduced by 38% at 2 minutes and by 2.2-fold at 10 minutes compared with HUVEC-WT (Figure 3A). As a complementary approach, we measured eNOS Ser1177 phosphorylation by an immunofluorescence assay. By this assay, we confirmed that insulin-stimulated HUVEC-G972R displayed reduced Ser1177 phosphorylation compared with HUVEC-WT (P<0.01) (Figure 3B).

In basal conditions, eNOS Thr495 phosphorylation was 27% less in HUVEC-G972R than in HUVEC-WT cells. However, in HUVEC-WT, eNOS Thr495 phosphorylation decreased after insulin stimulation, by 59% at 2 minutes and by 32% at 10 minutes (P<0.01 versus basal for both time points (Figure 2A)). In contrast, in HUVEC-G972R cells, insulin enhanced eNOS Thr495 phosphorylation by ~2-fold.

Figure 2. Activation of Akt pathway in WT and HUVEC-G972R. Effects of insulin stimulation in WT and HUVEC-G972R cells on (A) Akt Ser473-phosphorylation and (B) Thr24-FOXO-1 phosphorylation (n=3; **P<0.01, *P<0.05; ANOVA).

Figure 3. eNOS phosphorylation in WT and HUVEC-G972R. Effects of insulin stimulation in WT and HUVEC-G972R cells on (A) eNOS Ser1177 and Thr495 phosphorylation and (B) eNOS Ser1177 phosphorylation by an immunofluorescence assay (n=3; *P<0.05, **P<0.01, ***P<0.001; ANOVA).
after 10 minutes, giving a 40% increment in comparison with HUVEC-WT at this time point (Figure 3A).

Effect of Insulin on eNOS mRNA and Protein Levels in WT and HUVEC-G972R Cells

We also evaluated the ability of insulin to modulate eNOS mRNA and protein levels in HUVEC-WT and HUVEC-G972R cells (Figure 4, A and B, respectively). Compared with cells exposed to vehicle, in HUVEC-WT, insulin increased both eNOS mRNA and protein levels significantly, by 2.5- and 2.3-fold, respectively (both \( P < 0.001 \)). In HUVEC-G972R, on the contrary, no difference between cells exposed to insulin or vehicle could be detected in either eNOS gene expression or protein levels.

Effect of Insulin on eNOS Activity in WT and HUVEC-G972R Cells

The impact of the G972R substitution on NOS activity was also examined. To this end, we monitored the conversion of L-\(^{3}H\)arginine into L-\(^{3}H\)citrulline. As shown in Figure 5, in HUVEC-WT, L-\(^{3}H\)citrulline synthesis from L-\(^{3}H\)arginine was 0.32 ± 0.04 pmol · min \(^{-1} \) · mg total protein \(^{-1} \) after insulin stimulation, which was significantly greater than the L-\(^{3}H\)citrulline synthesis rate observed in the unstimulated cells (0.11 ± 0.01 pmol · min \(^{-1} \) · mg total protein \(^{-1} \), \( P < 0.01 \)). This insulin effect was significantly greater (\( P < 0.01 \)) than the stimulated conversion rate observed in HUVEC-G972R, which was 0.13 ± 0.02 versus 0.11 ± 0.01 pmol · min \(^{-1} \) · mg total protein \(^{-1} \) in unstimulated cells. When exposed to LPA (a phospholipid growth factor that induces NO production via mobilization of intracellular Ca\(^{2+} \)) to assess the integrity of eNOS activation system, both WT and G972R-HUVECs exhibited a comparable increase in NO production.

NOS basal activity in HUVEC-WT and HUVEC-G972R was identical. Preincubation with L-NAME induced a significant inhibition in insulin-stimulated NOS activity in HUVEC-WT (\( P < 0.01 \)).

Discussion

Insulin resistance is the hallmark of the metabolic syndrome (a cluster of cardiovascular risk factors including hypertension, glucose intolerance, visceral obesity, hypertriglyceridemia, and low plasma HDL cholesterol), and it is associated with accelerated atherosclerosis.\(^1\) As to the mechanism(s) linking insulin resistance and accelerated atherosclerosis, it is thought that compensatory hyperinsulinemia, which is generally associated with insulin resistance, may be involved, although the impact of hyperinsulinemia on vessel wall pathophysiology remains controversial. In fact, on one site, hyperinsulinemia might exert potentially dangerous mitogenic effects on smooth muscle cells through spillover activation of the insulin-like growth factor-1 receptor,\(^1\) whereas on

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**Figure 4.** eNOS mRNA and protein expression in insulin-stimulated WT and HUVEC-G972R. Effects of insulin stimulation in WT and HUVEC-G972R cells on (A) eNOS mRNA and (B) eNOS protein levels (n=3; **\( P < 0.01 \); ANOVA).

**Figure 5.** Insulin-stimulated eNOS activity in WT and HUVEC-G972R. Effects of insulin stimulation in WT and HUVEC-G972R on eNOS activity. Stimulation with 5 \( \mu \)mol/L of LPA was shown for comparison (n=3; **\( P < 0.01 \); ANOVA).
another site, it might be protective (it is known that insulin induces vasodilation by modulating eNOS activity and expression through activation of the IR/IRS-1/PI3-K/PDK-1/Akt signaling cascade4–7). Thus, if the signaling pathways leading to the theoretically protective insulin actions on the vessel wall were to be impaired in insulin-resistant states, this insulin resistance at the vessel site might be among the culprits for the accelerated atherosclerosis associated with impaired insulin sensitivity. Furthermore, we and others have recently observed that inhibition of the effects of insulin on the PI3-K pathway by activation of the hexosamine pathway or by pharmacological inhibitors, such as wortmannin, enhances insulin-induced mitogenic effects depending on the MAPK pathway. Interestingly, in classic insulin target tissues, such as skeletal muscle, insulin resistance is selectively restricted to the PI3-K pathway.26 Therefore, it is possible that a selective and genetic impairment of the PI3-K pathway could induce a “resistance” to the vasodilatory action of insulin on endothelium. To gain insight into this possibility, we addressed the question of whether primary endothelial cells naturally carrying the G972R-IRS-1 variant exhibited impaired eNOS activation, an early event in the developmental process leading to atherosclerosis.9,10 The functional effect of the G972R-IRS-1 polymorphism on insulin signaling has been extensively characterized.16–20 Thus, it has been demonstrated that the presence of the Gly→Arg change at codon 972-IRS-1 causes a specific defect in binding of the p85 regulatory subunit of PI3-K to the IRS-1 variant.16,20 This results in a decrease in IRS-1-associated PI3-K activity and in the subsequent reduced activation of the Ser/Thr kinase-Akt, a key enzyme linking PI3-K activation to multiple biological functions of insulin, including glucose transport, glycogen synthesis, and eNOS activation.17–20

In HUVECs naturally expressing the G972R-IRS-1 variant, we observed cell-specific impairment of insulin action, as revealed by defective insulin-stimulated eNOS activation and expression. Indeed, we observed that in the cells carrying the G972R-IRS-1 variant, the IRS-1/PI3-K/PDK-1/protein kinase B/Akt insulin-signaling cascade was impaired (as documented by reduced IRS-1-associated PI3K activity and reduced insulin-stimulated Akt phosphorylation), whereas MAPK pathway activity was increased. This resulted in both impaired insulin-stimulated eNOS expression (as documented by insulin failure in increasing eNOS mRNA and protein levels in HUVEC-G972R) and impaired eNOS activation (as documented by reduced eNOS-Ser1177 phosphorylation and increased Thr495 phosphorylation in the cells carrying the variant). It is conceivable that both reduced protein expression and reduced activation contributed to the reduced NO production in HUVEC-G972R after 24 hours of insulin stimulation. In this regard, the choice of measuring NO production after 24 hours of insulin stimulation was made so as to detect the activity of the eNOS newly synthesized in response to insulin. Phosphorylation on serine/threonine residues has been shown to regulate eNOS activity.27 Among the numerous potential phosphorylation sites, Ser1177 in the reductase domain and Thr495 within the calmodulin-binding domain have a functional role. Ser1177 is rapidly phosphorylated on cell activation by shear stress, growth factors, and hormones. When Ser1177 is phosphorylated, the flux of electrons through the reductase domain and, as a consequence, also NO production are increased 2- to 3-fold above basal levels.28 Several potential proinflammatory/atherogenic stimuli have been shown to inhibit Ser1177 phosphorylation.13,29,30 By contrast, Thr495 is constitutively phosphorylated by PKCs in the endothelial cells investigated to date, and it is a negative regulatory site, ie, phosphorylation causes a decrease in enzyme activity through interference with the binding of calmodulin to the calmodulin-binding domain.27 Protein phosphatase 1 dephosphorylates Thr495, and inhibition of protein phosphatase 1 results in the hyperphosphorylation of Thr495, which inhibits eNOS activity.31 Our study supports the concept that genetic impairment in the upstream activators of Akt could reduce its ability to phosphorylate eNOS on Ser1177. Interestingly, we also observed that reduced insulin-dependent PI3-K activity could lead to impaired Thr495 dephosphorylation. As to the mechanism(s) for this, insulin-induced phosphatases leading to eNOS Thr495 dephosphorylation have not, so far, been clearly identified, but protein phosphatase 2A and protein phosphatase 1 could be plausible candidates.27,28 Our data also support the hypothesis that to fully activate NO release, Thr495 dephosphorylation acts in concert with serine 1177 phosphorylation; however, the role of Thr495 phosphorylation in eNOS activation remains controversial.29

In summary, our data offer experimental evidence, in a cellular model of insulin resistance, that genetic impairment of the IRS-1/PI3-K/PDK-1/Akt insulin-signaling cascade determines impaired insulin capacity of stimulating NO release and demonstrate a potential mechanism by which insulin resistance can be involved in vascular dysfunction and hence abnormalities.

Our data, however, might also have relevant clinical implications. Previous studies have shown that the frequency of the G972R-IRS-1 polymorphism is significantly higher in patients with angiographic evidence of coronary artery disease compared with control individuals.30 When adjusted for other risk factors, the relative risk of coronary artery disease associated with the G972R-IRS-1 polymorphism was 2.93-fold higher than in wild-type individuals, and it increased to 6.97-fold in obese subjects and to 27.3-fold in subjects with clinical features of insulin resistance syndrome.30 Furthermore, a recent genome-wide linkage analysis of the acute coronary syndrome has mapped a susceptibility locus on chromosome 2q33-q37.3, which harbors the gene encoding IRS-1.31

Our results provide evidence that the G972R-IRS-1 variant has a direct negative effect on endothelial function; this could contribute to the increase risk for coronary artery disease observed in individuals carrying the polymorphism. Interestingly, in a population of untreated hypertensive subjects, we have recently observed that subjects carrying the G972R-IRS-1 polymorphism are characterized by endothelial dysfunction, as determined in vivo by strain-gauge plethysmography–measured blood flow changes in response to acetylcholine infusion (F. Perticone, M. Federici, and G. Sesti, unpublished observations, 2003).
In conclusion, the present results, together with the evidence discussed above, strongly suggest that the G972R-IRS-1 polymorphism, through a direct impairment of Akt/eNOS activation in vascular endothelial cells, may contribute to the genetic predisposition to develop endothelial dysfunction and hence cardiovascular disease.

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