Insulin Signaling in Arteries Prevents Smooth Muscle Apoptosis


Objective—Insulin is an antiapoptotic factor of cultured vascular cells, but it is not clear whether it also exerts antiapoptotic effects on vascular cells in vivo. We studied insulin receptor signaling in the arteries of normal and diabetic rats to establish whether insulin exhibits antiapoptotic activity toward vascular smooth muscle cells in vivo as well as in vitro.

Methods and Results—Western blot analysis and real-time polymerase chain reaction revealed α- and β-subunits of the insulin receptor in association with insulin receptor substrate-1 and phosphatidylinositol 3-kinase in the media of the aorta and carotid artery. The insulin receptor signaling pathway was partially activated under physiological conditions, further activated by intravenous insulin injection, and was attenuated in streptozotocin-induced diabetic rats. Lipopolysaccharide injection induced more apoptosis of vascular smooth muscle cells in diabetic rats than in control rats, whereas insulin prevented apoptosis in the aortic wall. An in vitro study suggested that the antiapoptotic effect of insulin was mediated by phosphatidylinositol 3-kinase.


Key Words: diabetic macroangiopathy ■ antiapoptotic effect of insulin ■ vascular smooth muscle cells ■ Akt/protein kinase B ■ phosphatidylinositol 3-kinase

The artery is a target organ of insulin, where the insulin receptor (IR) and downstream signaling molecules are present.1-3 Insulin induces DNA synthesis,4,5 chemotaxis,6 and uptake of glucose and amino acids in cultured vascular smooth muscle cells (VSMCs),7 and increases intimal thickening after balloon injury in the rat carotid artery.8 In addition, insulin infusion into the forearm increases production of NO by endothelial cells and induces vasodilation.9 Insulin was found recently to exert antiapoptotic effects on cultured VSMCs,10 vascular endothelial cells,11 and monocytes.12 This effect is mediated by phosphatidylinositol 3-kinase (PI3K) and the downstream signal Akt/protein kinase B (PKB) but does not require activation of mitogen-activated protein kinases.13 VSMC apoptosis occurs in atherosclerotic lesions in humans, rabbits, and pigs, and in the thickened intima formed after percutaneous transluminal coronary angioplasty (PTCA) or balloon injury in rat carotid arteries.14 Induction of smooth muscle apoptosis decreases intimal thickening and potentially decreases restenosis after PTCA. At the same time, smooth muscle apoptosis decreases the number of smooth muscle cells in the fibrous cap, resulting in plaque instability.15 Diabetes is a major risk factor for unstable plaque formation,16 and it has not been elucidated whether vascular cell apoptosis increases as a result of diabetes or whether insulin exerts an antiapoptotic effect on vascular cells in vivo.

In this study, we report that insulin signaling is diminished and that lipopolysaccharide (LPS)-induced apoptosis of smooth muscle cells increases in the aorta of streptozotocin (STZ)-induced diabetic rats. Furthermore, insulin prevents apoptosis of VSMCs.

Materials and Methods

Antiphosphotyrosine monoclonal antibodies (PY-20) were purchased from Signal Transduction Laboratories, and polyclonal antibodies against α- and β-subunits of the p110 subunit of PI3K, IR-α subunit, IR substrate-1 (IRS-1), and caspase3 were obtained from Santa Cruz Biotechnology, and polyclonal antibody against poly-(ADP-ribose)-polymerase (PARP) was purchased from Roche Diagnostics. Recombinant human insulin was obtained from Eli Lilly or Becton Dickinson Labware. Recombinant rat interferon-γ (IFN-γ) was kindly provided by TNO Primate Center (Rijswijk, Netherlands), recombinant human interleukin-1β (IL-1β) by Otsuka Pharmaceutical Co., and recombinant tumor necrosis factor-α (TNF-α) by Dainippon Pharmaceutical Co. All other reagents were purchased from Sigma.

Animals

Seventy-five male Sprague-Dawley rats (6 to 8 weeks of age; Charles River Japan Inc, Osaka, Japan) were fed standard rodent
chow and handled in accordance with the National Cardiovascular Center Research Institute animal care and use committee protocol. Rats were intraperitoneally anesthetized with 30 mg/kg body weight sodium pentobarbital and perfused with ice-cold sodium vanadate solution (0.5 mmol/L Na$_2$VO$_4$, 10 mmol/L phosphate buffer, pH 7.4, 150 mmol/L NaCl, and 20 mmol/L H$_2$O$_2$) before being euthanized. Diabetic rats were prepared by injecting 50 mg/kg body weight STZ. Recombinant human insulin (60 µg in 1 mL saline; Becton Dickinson Labware) was injected intravenously into control or diabetic rats 4 weeks after STZ administration. Insulin (10 IU/kg; Eli Lilly) was subcutaneously administered once per day for 3 days to study the signaling pathway. Regular insulin (10 IU/kg; Eli Lilly) was subcutaneously injected intravenously into control or diabetic rats 4 weeks after STZ injection. The aorta was then perfused–fixed with 10% buffered formalin after 72 hours. Paraffin-embedded sections were stained by immunoperoxidase (Trevigen, Inc.) and in aortic tissue by terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) method, and apoptotic stromal cells in the prostate of castrated rats were used as a positive control. The number of TUNEL-positive cells and the total number of cells in the aortic sections were counted under a light microscope. Serial sections were stained with goat polyclonal anti-CPP32 antibody (Santa Cruz Biotechnology) and DAPI.

**DNA Ladder**

DNA fragmentation in cultured cells was detected using a TACS apoptotic DNA laddering kit (Trevigen, Inc.) and in aortic tissue by the method of Cohen and Duck.  

**Statistical Analysis**

The data are presented as mean±SEM. The statistical significance of differences between the 2 groups was determined by Student’s t test, and multiple comparisons were analyzed by 1-way ANOVA with Dunnett test when a difference was indicated. $P<0.05$ was considered statistically significant.

**Results**

**Functional IR Signaling Pathway in the Artery**

Western blot analysis of the immunoprecipitate of anti–IR-β showed the presence of the β-subunit of IR (95 kDa) in the tissue lysate of the normal aorta and carotid artery of rats fed ad libitum (Figure 1A). Reprobing of the same membrane with anti–IR-α revealed that IR-α is associated with IR-β in the arterial wall (Figure 1A). Also, reprobing with antiphosphotyrosine showed that IR-β was tyrosine-phosphorylated (Figure 1A), indicating that IR was partially activated in the artery under physiological conditions. The insulin-like growth factor-I (IGF-I) receptor was also detected in the same tissue lysate, and it too was tyrosine-phosphorylated (data not shown).

IRS-1 was also detected in the tissue lysate of the artery and was partially tyrosine-phosphorylated (Figure 1B). Sequential reprobing of the same membrane with antibodies against IR-β, the IGF-I receptor, and PI3K revealed that IRS-1 was associated with IR-β and PI3K (Figure 1B) but not with the IGF-I receptor (data not shown). The level of IRS-1 protein was lower in the artery than in the liver or in hepatoma G2 cells when normalized against the total protein content (data not shown). In addition, real-time PCR analysis revealed the presence of mRNA of these components of the IR signaling pathway in the artery (Figure 1C).

The removal of endothelial cells did not significantly decrease the amount of IR (data not shown), indicating that most of the IR signaling molecules detected originated from medial smooth muscle cells.

**Exogenous Insulin Further Activates IR Signaling in the Artery**

To investigate whether the IR signaling pathway in the artery responds to changes in the plasma insulin level, a therapeutic dose of insulin was injected into the rats. Intravenous injection increased the tyrosine phosphorylation of IR within 5
minutes (Figure 2A). Tyrosine phosphorylation of IRS-1 and the association between IRS-1 and 2 subunits of PI3K, the P85 regulatory and P110 catalytic subunits, were also increased, indicating that PI3K in the arterial wall was activated by the injection (Figure 2B). An insulin injection at the same dose did not change the level of tyrosine phosphorylation of the IGF-I receptor (Figure 2C). PI3K activation was confirmed by measuring PI3K activity in the IRS-1 immunoprecipitate; its activity increased 2-fold 5 minutes after injection (Figure 2D). In addition, Akt phosphorylation also increased (Figure 2E). These findings show that the insulin signaling pathway in the arterial wall responds to changes in plasma insulin in a manner similar to that of the classical target organs of insulin.

IR Signaling Is Attenuated in Arteries of STZ-Induced Diabetic Rats

To further confirm that the insulin signaling pathway in the arterial wall is affected by the plasma insulin level, aortas of STZ-induced diabetic rats and control rats were examined. The plasma insulin level was significantly reduced in the diabetic rats, in which it was 1.26\pm 0.2 ng/mL compared with 18.2\pm 2 ng/mL in control rats (n=5; P<0.01), and the tyrosine phosphorylation of IRS-1 in the aorta was significantly lower in diabetic rats than in nondiabetic rats when normalized against the same amount of IRS-1 (Figure 3A). PI3K associated with IRS-1 was also decreased in STZ-induced diabetic rats (Figure 3A). There was no significant difference in the protein levels of IR, IRS-1, PI3K, or Akt.
Cytokines induced the translocation of Bax from the cytosol to the mitochondrial fraction and the release of cytochrome c from mitochondria. These changes were prevented by insulin (data not shown).

Insulin Exhibits Antiapoptotic Activity Toward VSMCs In Vitro

Because insulin prevents apoptosis in different cell types and PI3K plays a role in the antiapoptotic effect of various growth factors, we examined whether insulin prevents the cytokine-induced apoptosis of rat VSMCs in a PI3K-dependent manner. As reported previously, the combination of IFN-γ, TNF-α, and IL-1β induced apoptosis in VSMCs as early as 24 hours after incubation, and the number of apoptotic cells increased after 72 hours to a maximum value of 25% of the total number of cells (Figure 4A). Insulin partially inhibited in a concentration-dependent manner apoptosis induced by the combination of cytokines (Figure 4B).

Two PI3K inhibitors, wortmannin and LY294002, abolished the antiapoptotic effect of insulin (Figure 4B).

The antiapoptotic effect of insulin and the inhibition of this effect by PI3K inhibitors were confirmed by DNA ladder formation (Figure 4C) and activation of caspase-3 (Figure 4D). Insulin inhibited DNA fragmentation induced by cytokines, and wortmannin inhibited the antiapoptotic effect of insulin. Similarly, insulin inhibited the activation of caspase-3 in smooth muscle cells treated with cytokines, and wortmannin and LY294002 blocked the effect of insulin.

Insulin Prevents LPS-Induced Apoptosis of VSMCs in Diabetic Rats

To study whether insulin has antiapoptotic activity in vivo, we injected LPS and insulin into STZ-induced diabetic rats. The intravenous injection of 0.1 mg/kg LPS induced significant amounts of apoptosis in aortic medial smooth muscle cells after 72 hours (Figure 5A through 5D). The nuclei of apoptotic VSMCs had numerous small vesicles (Figure 5B and 5C). They were different from the hypertrophic nuclei of activated muscle cells after 72 hours (Figure 5A through 5D). The number of apoptotic cells was counted after 48 hours. Each column represents mean ± SEM of 4 to 5 samples and significant differences (P < 0.01; ANOVA) between the cytokine-treatment groups. C, The antiapoptotic effect of insulin was also tested by DNA ladder formation. DNA was extracted from VSMCs cultured in DMEM supplemented with 10% FCS (lane 1), 0.1% BSA (lane 2), a combination of cytokines (lane 3) for 48 hours, a combination of cytokines with 10 nmol/L insulin (lane 4), or a combination of cytokines with 10 nmol/L insulin plus 1 μmol/L wortmannin (lane 5). M indicates DNA size markers. D, Apoptosis was induced in VSMCs in the presence or absence of insulin, 1 μmol/L wortmannin, or 10 μmol/L LY294002. The number of apoptotic cells was counted after 48 hours.

Insulin prevented LPS-induced apoptosis of the medial smooth muscle cells in diabetic rats comparable to that in control rats at 19.1 ± 1.1, 15.3 ± 1.9, and 2.5 ± 0.4 ng/mL, 1, 2, and 8 hours after injection, respectively (P < 0.01). Insulin administered just before LPS injection prevented LPS-induced apoptosis of the medial smooth muscle cells in diabetic rats. Insulin inhibited DNA fragmentation (Figure 5F) and proteolytic cleavage of PARP, which is a sensitive marker indicative of apoptosis onset (Figure 5G).
IR signaling is diminished in the vascular wall in STZ diabetic rats, and this decrease is associated with the increased susceptibility to apoptosis of smooth muscle cells. Exogenous insulin activates IR, but not the IGF-I receptor, and prevents smooth muscle apoptosis.

In agreement with previous reports, we found in the arterial wall a functional IR signaling pathway similar to that in cultured VSMCs. The arterial IR signaling pathway is partially activated in rats fed ad libitum and further activated by exogenous insulin injection. Although vascular endothelial and smooth muscle cells possess IR signaling molecules, endothelial denudation revealed that most of the signaling originates from medial smooth muscle cells. The IGF-I receptor, which was partially tyrosine-phosphorylated, was not associated with IRS-1 and was not activated by pharmacological doses of exogenous insulin. These findings suggest the limited involvement of IGF-I receptor signaling in the antiapoptotic effect of insulin.

In STZ-induced diabetes rats, the activation of IR and the downstream signaling pathway was diminished in the arteries, whereas the protein levels of these molecules were not changed. Decreased insulin signaling was accompanied by the increased susceptibility of the VSMCs to LPS-induced apoptosis. Insulin injections prevented apoptosis, reflecting the antiapoptotic activity of insulin signaling in the vascular wall. Having diabetes did not increase vascular cell apoptosis for up to 3 months (data not shown), suggesting that insulin signaling is required for survival only when vascular cells are exposed to apoptosis-inducing factors.

The antiapoptotic effect of insulin was confirmed by in vitro experiments. Insulin partially prevented smooth muscle apoptosis induced with a mixture of cytokines in vitro. It prevented cytokine-induced translocation of Bax and cytochrome c release from mitochondria, and the activation of caspase-3. As reported previously, PI3K and Akt/PKB are involved in the antiapoptotic action of insulin, and inhibitors of PI3K abolished the effects of insulin. It is not clear why apoptosis is only partially prevented by insulin in vitro but completely prevented in vivo. It could be attributable to a lack of sensitivity of the apoptosis assay in vivo resulting from different stimuli or different phenotypes of the VSMCs.

Two potential implications exist based on our observations for accelerated atherosclerosis in diabetes. First, decreased insulin activity in diabetes potentially accelerates the apoptosis of VSMCs in the fibrous cap, leading to weakness and rupture of the plaque. Clinically, diabetes is a major risk factor for plaque instability. Gene transfer of P53 to the lesion induces smooth muscle apoptosis and plaque rupture in apolipoprotein E–deficient mice. Our findings indicate that smooth muscle cells are more susceptible under diabetic conditions than under nondiabetic conditions to cell death.
induced by apoptosis-inducing agents found in the body, such as circulating LPS, infectious agents, and cytokines locally produced by vascular cells.\textsuperscript{32} Furthermore, diabetes results in production of additional apoptosis-inducing factors such as advanced glycation end products and radicals.\textsuperscript{33}

Second, increased smooth muscle apoptosis may account for medial calcification found in the arteries of diabetic patients. Medial calcification or Monkeborg sclerosis is frequently found in diabetes and end-stage renal failure and is positively correlated with the prognosis of these diseases.\textsuperscript{34} Apoptosis was found recently to potentially regulate vascular calcification in vitro\textsuperscript{35} and in vivo.\textsuperscript{36} Apoptotic bodies, such as matrix vesicles, bind calcium phosphate, and thus act as nucleation sites for calcification. VSMC apoptosis is accompanied by bone-related molecules such as matrix Gla protein in vitro and in vivo.\textsuperscript{37}

Our study was limited to a model of type 1 diabetes. However, a previous study found that insulin signaling in the arteries is diminished in hyperinsulinemic obese animals with insulin resistance,\textsuperscript{3} and therefore, VSMCs may also be susceptible to apoptosis in insulin-resistant type 2 diabetes.

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