Regulation of Vascular Endothelial Growth Factor Expression and Vascularization in the Myocardium by Insulin Receptor and PI3K/Akt Pathways in Insulin Resistance and Ischemia

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Objective—This study characterized the role of insulin receptors and resistance on vascular endothelial growth factor (VEGF) expression and myocardial vascularization in physiological conditions and after ischemia.

Methods and Results—Cardiac microvascular density was reduced by 30% in insulin-resistant Zucker fatty rats versus lean controls. This was associated with a parallel 40% inhibition of insulin-stimulated activation of both Akt and VEGF expression in the myocardium and cardiomyocytes. In contrast, the activation of Erk1/2 by insulin remained unchanged. In cultured cardiomyocytes, insulin or insulin-like growth factor (IGF)-1 increased VEGF mRNA and protein expression by 2-fold. Inhibition of PI3K/Akt, especially Akt2-mediated cascades but not the Ras/MEK/Erk pathway, using chemical inhibitors, dominant negative adenoviral constructs, or siRNA approaches suppressed VEGF mRNA expression by insulin. Ventricular tissues from muscle insulin receptor knockout (MIRKO) mice, which lack insulin receptors in the myocardium, have significant reductions in insulin but not IGF-1 signaling, VEGF expression, and vascular density before and after ischemia versus controls.

Conclusions—Insulin regulates VEGF gene expression and vascularization in the myocardium specifically via insulin receptors and the activation of PI3K/Akt pathway. Selective inhibition of this pathway may lead to the decreases in VEGF expression and capillary density in the myocardium of patients with insulin resistance. (Arterioscler Thromb Vase Biol. 2006;26:787-793.)

Key Words: cardiomyocyte ■ collateral circulation ■ diabetes ■ insulin resistance ■ vascularization ■ VEGF

Patients with type 2 diabetes and insulin resistance have high morbidity and mortality rates, caused mainly by ischemic heart disease (IHD), partly caused by reduced collateral vessel formation in response to ischemia in the myocardium. Patients with concomitant IHD and diabetes have reduced microvessel density in the myocardium as compared with those with IHD alone or healthy subjects. Microvessel homeostasis is determined by multiple factors, including the expression and actions of vascular endothelial growth factor (VEGF), a potent angiogenic factor. Ventricular cardiomyocytes are a rich source of VEGF production that significantly affects the development and function of cardiac vasculature. We and others have previously shown that diabetes and insulin resistance are associated with attenuated cardiac VEGF expression, which may decrease capillary density in the myocardium in diabetic and insulin resistant states, leading to cardiac dysfunction.

Induction of VEGF expression by insulin has been reported in several cell types including vascular smooth muscle cells, epithelial cells, and fibroblasts, but not in cardiomyocytes or in vivo. We have reported that there is a selective loss of insulin’s signaling via PI3K/Akt pathway in the vascular endothelium in insulin-resistant states, which can cause abnormalities in gene expression such as the decreased expression of endothelial nitric oxide synthase. However, insulin has not been reported to regulate the vascularization of myocardium directly. We propose that insulin’s signaling and actions may also be decreased in the myocardium and contribute to the reduced expression of VEGF in insulin resistance. Furthermore, we have assessed the importance of insulin receptor and signaling on VEGF expression and vascularization in the myocardium using both insulin resistant and insulin receptor-deficient animals in basal and ischemic states.
Materials and Methods

Isolation and Culture of Rat Cardiomyocyte

Neonatal rat cardiomyocytes (NRCM) and adult rat cardiomyocytes (ARCm) were harvested and cultured as previously described.1,3

Insulin and Insulin-Like Growth Factor-1 Stimulation

Cells were serum-deprived for 16 hours and incubated with 100 nmol/L insulin (Humulin R; Lilly Laboratories, Indianapolis, Ind) or 100 ng/mL (13.5 nmol/L) insulin-like growth factor (IGF)-1 (Calbiochem, La Jolla, Calif) with or without a 30-minute pretreatment with P98059 (20 μmol/L) or wortmannin (100 nmol/L) dissolved in 0.01% DMSO (Calbiochem).

Infection of NRCM With Recombinant Adenovirus

Adenoviruses containing cDNA of the LacZ gene or dominant-negative isoforms of Akt, K-Ras, and p85 were used to infect cardiomyocytes at 30 to 300 multiplicity of infection as described.14

Transfection of siRNA

Reduction of Akt1 and 2 isoform expressions were achieved by using siRNA approaches developed by Jiang et al.15 Briefly, NRCM was transfected with 20 nmol scrambled siRNA (5′-CGUGCAUACC-GAGCAGCCCA-3′), with the siRNA targeting to either rat Akt1 (5′-AACCAGGACCAGAAGACUG-3′) or Akt2 (5′-AACCGAGCCAGAAGCGCCUC-3′) using lipofectamin2000 (Invitrogen). Cells were assayed 48 hours after transfection.

Animals

Male Zucker fatty rats and age-matched lean controls (Harlan, Indianapolis, Ind) at 14 to 22 weeks of age were used. Insulin resistance was manifested by increases in body weight (567.0 ± 8.2 g in fatty versus 368.3 ± 4.2 g in lean, P < 0.0001) and hyperinsulinemia (1.21 ± 0.24 ng/mL in fatty versus 0.12 ± 0.07 ng/mL in lean, P < 0.0001) but without diabetes (random blood glucose < 250 mg/dL). Characterization of muscle-specific insulin receptor knockout (MIRKO) mice has been described previously.16 Animals were housed in the Joslin animal facility. The Joslin Institutional Animal Care and Use Committee approved all procedures involving animals.

Euglycemic Hyperinsulinemic Clamp

Both Zucker fatty rats and their lean controls were randomly divided into control and insulin infusion (10 μU/kg per minute) groups that were subjected to 1-hour euglycemic insulin clamp17 and then euthanized with CO2. The ventricular tissue was snap-frozen with liquid N2 and immediately stored at −80°C until analysis.

Myocardium Infarction and Ischemia-Reperfusion in Mice and Rats

Mice at 3 months of age were anesthetized by intraperitoneal injection of pentobarbital (25 to 30 μg/g, intraperitoneal) and the coronary artery was ligated after thoracotomy.18 Mice were kept in animal facility for 7 days after surgery and had free access to food and water. Ischemia-reperfusion was performed in lean and fatty rats at 12 weeks of age.19 Left descending coronary artery was blocked (MIRKO) mice has been described previously. Animals were housed in the Joslin animal facility. The Joslin Institutional Animal Care and Use Committee approved all procedures involving animals.

TaQMan RT-PCR

Quantitative real-time polymerase chain reaction (PCR) was performed using the ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, Calif). Primers for VEGF (Forward: 5′-CGCAAGAATCCCGGTTTAA-3′ and Reverse: 5′-GAAATGCTTTCGCCGTCTGA-3′) were used at a concentration of 1 μmol/L, and probes (6-FAM/TCCGGACGTTCACGTGAG-CCTT(TAMRA) were used at a concentration of 250 nmol/L.

Insulin Stimulates VEGF Expression in Cardiomyocytes Via a PI3K/Akt-Dependent Pathway

Insulin-induced VEGF mRNA expression in cultured NRCM after 12 hours of incubation in a concentration-dependent manner (Figure 1A). VEGF mRNA levels were elevated at all concentrations but were significantly higher at 50 (2-fold) and 100 (2.3-fold) nmol/L of insulin above control (both P < 0.001). At concentrations >100 nmol/L, insulin did not further increase VEGF expression. A 2-fold increase (P < 0.05) in VEGF protein was detected by ELISA assay in conditioned media from NRCM stimulated with 100 nmol/L insulin as compared with controls (Figure 1B). Because insulin stimulated phosphorylation of Akt and Erk-1/2 in both adult and neonatal cardiomyocytes (data not shown), we further characterized the signaling pathway mediating insulin’s effects on VEGF expression. In cultured
NRCM, insulin-increased VEGF expression by 2.3-fold, which was inhibited by PI3K inhibitor, wortmannin, but was not changed by MEK inhibitor PD98059 (Figure 2A). Similarly, IGF-1 induced a 1.8-fold increase in VEGF mRNA expression at 100 ng/mL (≈13.5 nmol/L) that was also blocked by wortmannin but not by PD98059 (Figure 2A).

Infection with a control adenovirus containing the LacZ gene did not affect VEGF expression in NRCM at either basal or insulin-stimulated states (Figure 2B). Consistent with results from the pharmacological inhibitor studies, suppression of PI3K/Akt pathway using either dominant negative p85 (DN-p85) or Akt (DN-Akt)14,20 completely blocked insulin-stimulated VEGF mRNA expression (Figure 2B). Inhibition of Ras/MEK/Erk-1/2 pathway by dominant-negative Ras (DN-Ras)14,20 had no effect on insulin-stimulated VEGF expression (Figure 2B). To further characterize the isoform-specific action of Akt, we decreased the expression of different Akt isoforms selectively using siRNA gene silencing approaches.15 Because cardiomyocytes do not express significant levels of Akt3 isoform,21 we focused on Akt1 and 2 isoforms. As shown in Figure 2C, transfection of siRNA targeting Akt1 or Akt2 selectively decreased the protein expression of Akt1 and 2 isoforms by 40% and 60%, respectively. Reduction of Akt2 isoform expression clearly and selectively inhibited insulin-induced VEGF expression by 79% (Figure 2D). Inhibition of Akt1, however, increased basal VEGF expression by 5-fold, and the addition of insulin failed to increase VEGF mRNA levels further (Figure 2D).

Activation of Akt, Erk, and JNK-1 by Insulin in Cardiomyocytes from Zucker Lean and Fatty Rats

In Vitro and In Vivo

To characterize insulin’s actions in control and insulin-resistant states, cardiomyocytes were isolated from adult Zucker lean and fatty rats and evaluated for insulin-mediated activation of PI3K/Akt and MEK/Erk pathways using phosphorylation of Akt serine473 and Erk-1/2 threonine202/tyrosine204 as surrogate markers for their activation, respectively. Insulin (100 nmol/L) increased mRNA expression of VEGF by 2.3-fold (P<0.05) in cultured cardiomyocytes from Zucker lean control rats, which was not observed in cells from insulin resistant Zucker fatty rats (Figure 3A). In addition, basal VEGF expression was decreased by 40% in cardiomyocytes cultured from fatty rats as compared with the lean controls (Figure 3A). This finding is similar to previous results in ventricular tissues,2 suggesting that cultured cardiomyocytes retained their in vivo phenotype. Stimulation by insulin (100 nmol/L) for 10 minutes significantly increased the phosphorylation of Akt (4.3 fold, P<0.05) in cardiomyocytes from lean control rats but failed to activate Akt significantly in cells from the fatty rats (Figure 3B). In contrast, insulin stimulated phosphorylation of Erk-1/2 to a similar extent in cardiomyocytes from both lean (2.8 fold, P<0.001) and fatty (2.4 fold, P<0.05) rats (Figure 3C). The potential role of JNK-1 activation in the selective inhibition of Akt phosphorylation by insulin was evaluated by the expression of phospho-JNK-1, p-jun and c-fun, which were the same in Zucker lean and fatty rats. (Figure 3D).

In vivo stimulation with a bolus of insulin (10 U) for 10 minutes increased Akt phosphorylation by 3.5-fold (P<0.01) in the ventricular tissues of Zucker lean rats but not in the Zucker fatty rats (Figure 4A). Euglycemic insulin clamp was performed at an insulin infusion rate of 10 mU/kg per minute for 1 hour and induced a 2.3-fold increase in Akt phosphorylation (P<0.05) in the myocardium of healthy Zucker lean rats (Figure 4B). In contrast, insulin’s effect was significantly inhibited in the ventricles of insulin resistant Zucker fatty rats (Figure 4B).

Blood Vessel Density in the Myocardium of Lean and Obese Rats at Basal State and After Ischemia-Reperfusion

The effect of myocardial insulin resistance on blood vessel density was examined in the ventricles of Zucker obese and...
lean rats by immunohistochemical staining using endothelial cell-specific marker *Griffonia Simplicifolia Lectin I* (GSL-I)\(^1^8\). Insulin resistant fatty rats demonstrated a 30% reduction (\(P<0.01\)) in vessel density compared with their age matched lean controls (Figure IA and IB, available online at http://atvb.ahajournals.org). To assess reactive vessel formation, the left coronary descending artery (LCD) was occluded for 1 hour before the removal of the suture and the rats were allowed to recover for 7 more days. Obvious tissue damages were noted in the regions irrigated by LCD (data not shown). Blood vessel density was quantified by measuring the signal of GSL-I over the peri-infarction. This revealed a 40% decrease in capillary density in the fatty rats versus the lean rats, although both groups had increased vessel density in the peri-infarcted zone when compared with basal states (data not shown). Morphologically, lean rats displayed a substantial number of dilated vessels in the peri-infarcted zone and this phenotype was absent in the insulin resistant fatty rats (Figure IC and ID).

**Effect of Insulin Receptors on Cardiac VEGF Expression and Vascularization**

The physiological significance of insulin receptors and their signaling pathways in cardiac VEGF expression and vascularization were directly investigated using hearts from muscle-specific insulin receptor knockout (MIRKO) mice.\(^1^6\) Insulin injection (5 U/kg) into LoxP controls via the inferior vena cava increased the phosphorylation of Akt in the myocardium. This effect of insulin on Akt activation however was decreased by 68% in MIRKO mice (Figure 5). In contrast, infusion of IGF-1 in vivo increased p-Akt expression in both LoxP and MIRKO mice to a similar extent (Figure 5). MIRKO mice at the age of 6 months showed a 45% reduction of VEGF mRNA expression (\(P<0.05\)) in the myocardium compared with LoxP controls (Figure 6A). Protein expression of VE-Cadherin (VE-Cad), a marker of endothelial cells, indicated a reduction of 20% to 25% in the ventricular tissue from MIRKO mice versus LoxP mice.
which was not statistically significant (Figure 6B). To support the finding that capillary density may also be decreased in parallel with changes in VEGF mRNA and VE-Cad, we measured blood vessel density by GSL-I staining (Figure IIA, IIB, and IIC, available online at http://atvb.ahajournals.org), which was decreased by 24% ± 12% (P < 0.05) at basal state.

Characterization of Vascularization in MIRKO Mice After Ischemia

We further evaluated vascularization in the peri-infarcted zone in ventricular tissues from MIRKO and LoxP mice 7 days after coronary artery ligation. Mortality due to cardiac rupture is similar in MIRKO mice (57%; 4/7) and LoxP controls (50%; 2/4). These hearts were processed for GSL-I staining and revealed a 41% reduction (P < 0.05) of blood vessels in the peri-infarcted zone in MIRKO mice as compared with LoxP control mice (Figure IIB, IIE, and IIF). Costaining of the sections for Ki67 expression, a nuclear marker of active cellular proliferation, and GSL-I showed that the proliferation rate of the endothelial cells from LoxP and MIRKO mice was not significantly different (Figure IIIA to IIG, available online at http://atvb.ahajournals.org).

Discussion

We have reported previously that in insulin resistant or diabetic states, the expression of VEGF and its receptors in the myocardium is decreased, although it can be normalized by insulin treatment. Our current findings show that insulin can regulate VEGF expression in the myocardium, which is inhibited in insulin resistance. We have demonstrated that insulin at physiological levels induced significant increases in VEGF expression in cardiomyocytes. The results from MIRKO mice provide strong evidence that insulin receptors are critical in the maintenance of VEGF expression at basal state.

This study showed for the first time that insulin signaling and its effects on VEGF mRNA expression are similar in neonatal and adult rat cardiomyocytes. Insulin binds to insulin receptors and activates IRS proteins to recruit multiple signaling molecules including PI3K, Nck, Grb2, and others, resulting in the activation of Akt and Erk1/2 pathways. Although both the PI3K/Akt and MEK/Erk cascades are critical in the mediation of insulin’s action, insulin’s effect on VEGF expression in the myocardium is mediated mainly through the Akt pathway and not mediated by MAPK activation. This finding differs from our previous report that insulin regulates VEGF production via both PI3K/Akt and Erk pathways in aortic smooth muscle cells. However, our current findings are similar to those in skeletal muscle, which exhibits increased VEGF expression and capillary density when the constitutive active form of Akt is overexpressed.

These results demonstrate that insulin signaling via PI3K/Akt pathway in the myocardium, especially in cardiomyocytes, is selectively inhibited in obesity-induced insulin resistant states, whereas the Ras/MEK/Erk-1/2 signaling cascade remains fully responsive. Similar findings have been reported in the microvasculature and skeletal muscles. We have proposed that the selective inhibition of insulin’s action via the PI3K/Akt pathway in vascular tissues may induce a pro-atherogenic state in insulin resistance, because this pathway mediates insulin’s effects on endothelial nitric oxide synthase expression and activation in the endothelium.

These findings provide the first direct evidence that insulin signaling is selectively inhibited in cardiomyocytes of Zucker rats. At the biochemical level, selective insulin resistance could be caused by metabolic derangements such as hyperglycemia, dyslipidemia, or elevation of free fatty acids that alter intracellular signaling cascades. In this study, cardiomyocytes from Zucker obese rats retain insulin resistance even in culture, suggesting that the inhibition of insulin’s action can persist for days even when the metabolic derangements have been removed. The mechanisms underlying persistent insulin resistance could be multiple, for example, the activation of PKC and JNK-1 pathways. In Zucker fatty rats, it is unlikely that JNK-1 activation is involved because no changes in either expression or activation were observed in cardiomyocytes from lean and fatty rats (Figure 3D). Another possibility is the activation of JAK2 by angiotensin II in cardiac tissue which may be responsible for the selective inhibition of the insulin-mediated activation of the PI3K/Akt pathway.

Further studies are in progress to determine whether persistent activation of PKC, especially the β isoforms, is mediating the selective inhibition of PI3K/Akt pathways in the myocardium of animals with obesity-induced insulin resistance, because activation of PKCβ isoforms has been suggested to mediate angiotensin II-induced JAK phosphorylation in vascular smooth muscle cells. This is possible because PKC activation induced by diabetes has been shown to persist for many years and could be caused by enhanced PKCβ activity caused by diabetes or glucose.

Selectively inhibiting PI3K/Akt pathway could impede myocardial function in several ways. Suppression of insulin-induced Akt activation in the myocardium can affect cardiac
function by decreasing glucose transport and utilization, which will increase the myocardium’s dependence on free fatty acids for fuel consumption, as has been reported in both diabetic and insulin resistant states. Our results from MIRKO mice and insulin resistant Zucker rats demonstrate that insulin receptor and its action via the PI3K/Akt pathway play an important role in the expression of VEGF and vascularization of the myocardium at basal and ischemic states. This is most likely not caused by systemic metabolic disorders in the MIRKO mice, given that the activation of PI3K/Akt pathway is normal in the myocardium of MIRKO mice in response to IGF-1 infusion (Figure 5). The results from the MIRKO mice and Zucker obese rats clearly showed that selective inhibition of the PI3K/Akt cascade in insulin-resistant states is likely responsible for the decrease in VEGF expression and vascularization in the myocardium. It is possible that the inhibition of the PI3K/Akt pathway, which mediates the anti-apoptotic actions of many growth factors and the decrease in angiogenic factors such as VEGF in the insulin-resistant state, may increase endothelial cell apoptosis and decrease angiogenic response to ischemia. These alterations would result in lowering of capillary density at basal level and postischemia in insulin resistance. Further, it appears that insulin-induced VEGF expression in cardiomyocytes is mediated mainly by the Akt2 isoform activation-dependent pathway because siRNA inhibition of Akt2 expression significantly reduced VEGF expression. It is very surprising that IGF-1 was not able to compensate for the loss of insulin’s actions in the MIRKO mice, even though it can also activate the PI3K/Akt pathway and induced VEGF expression. In MIRKO mice, cardiac VEGF expression is decreased although IGF-1/PI3K/Akt axis is intact, suggesting that insulin receptor-mediated cascade plays a unique role that cannot be substituted by IGF-1 axis. Further studies using direct overexpression of VEGF in cardiac tissues of Zucker fatty rats and MIRKO mice are required to define the role of insulin resistance as the cause of the decrease of VEGF expression and impairment of reactive myocardial vascularization after ischemia.

The role of Akt1, however, is still ambiguous because of the significant increase of VEGF expression at basal states induced by reduction of Akt1. Thus, it is unlikely that Akt1 has a major effect on insulin’s stimulatory action on VEGF expression in cardiomyocytes. We cannot determine from these studies which of the stages in the insulin receptor/IRS/PI3K/Akt cascade are specifically inhibited in the insulin-resistant state. However, we and others have suggested that the elevation of endothelin-1 or angiotensin II, or the activation of PKCβ isofrom can increase the Thr/ser phosphorylation of insulin receptors, IRS, and p85/PI3K, which will all decrease the phosphorylation of Akt, and inhibit insulin’s actions. Further studies are needed to pinpoint the sites of the inhibition in the myocardium.

The results from the ischemia studies indicated that the lack of insulin’s specific actions via the PI3K/Akt pathway results in decreases in capillary density. However, DNA synthesis of the capillary cells in the myocardium after ischemia did not differ between control and insulin-resistant rats. These results suggest that proliferative responses, in concordance with Erk1/2 activation, are unchanged. The decrease may be related to an increase in the level of apoptosis, which is consistent with the findings of reduction in Akt activation and lowering of VEGF expression, known regulators of cell survival. Further studies are needed to determine whether the decrease in VEGF expression and capillary density exhibited by the Zucker rats and MIRKO mice will result in decreases in survival or increases in myocardial infarction after ischemia.

In summary, this study has provided a comprehensive analysis of the insulin signaling pathway both in cultured cardiomyocytes and in the myocardium in vivo. A specific and direct role for insulin receptors has been demonstrated on the expression of VEGF and vascularization in the myocardium in vivo. In addition, we found that selective inhibition of PI3K/Akt pathway in the resistant state provides a potential biochemical explanation for the decreased utilization of glucose as a source of fuel and the reduced vascularization of myocardium observed in diabetic and insulin-resistant states but paradoxically preserves the mitogenic actions of insulin. We speculate that the loss of insulin’s action could blunt the upregulation of VEGF expression and vascularization in the myocardium in response to ischemia, contributing to the poor outcome after myocardium infarction observed in diabetic and insulin resistant patients.

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Figure I

**A**

[Images of tissue sections labeled Lean and Fatty]

**B**

Bar graph showing blood vessel density per field (X400) with a p-value of p<0.01.

**C**

[Images of green-stained tissue sections labeled Lean and Fatty]

**D**

Bar graph showing blood vessel density (Fold Change, X200) with a comparison between Lean and Fatty conditions.
Figure II

Blood vessel density/field (X400)

Lox-P  MIRKO

Blood vessel density/field

LoxP  MIRKO

p<0.05

p<0.05
Figure III