Decreased Cardiac Expression of Vascular Endothelial Growth Factor and Its Receptors in Insulin-Resistant and Diabetic States

A Possible Explanation for Impaired Collateral Formation in Cardiac Tissue

Eva Chou, BA; Izumi Suzuma, MD; Kerrie J. Way, PhD; Darren Opland, BA; Allen C. Clermont, MA; Kelko Naruse, MD, PhD; Kiyoshi Suzuma, MD, PhD; Nancy L. Bowling, PhD; Chris J. Vlahos, PhD; Lloyd Paul Aiello, MD, PhD; George L. King, MD

Background—Inadequate angiogenic response to ischemia in the myocardium of diabetic patients could result in poor collateral formation. Yet, excessive neovascularization in the retina causes proliferative diabetic retinopathy. Since vascular endothelial growth factor (VEGF) is the major angiogenic factor expressed in response to hypoxia, we have characterized expression of VEGF and its receptors in retina, renal glomeruli, aorta, and myocardium in insulin-resistant and diabetic states.

Methods and Results—The expression of mRNA and protein for VEGF and its receptors, VEGF-R1 and VEGF-R2, in the myocardium was decreased significantly by 40% to 70% in both diabetic and insulin-resistant nondiabetic rats. Twofold reductions in VEGF and VEGF-R2 were observed in ventricles from diabetic patients compared with nondiabetic donors. In contrast, expression of VEGF and its receptors were increased 2-fold in retina and glomeruli from diabetic or insulin-resistant rats. Insulin treatment of diabetic rats normalized changes in both cardiac and microvascular tissues.

Insulin increased VEGF mRNA expression in cultured rat neonatal cardiac myocytes.

Conclusions—The results documented for the first time that differential regulation of VEGF and its receptors exist between microvascular and cardiac tissues, which can be regulated by insulin. These results provide a potential explanation for concomitant capillary leakage and neovascularization in the retina and inadequate collateral formation in the myocardium of insulin-resistant and diabetic patients. (Circulation. 2002;105:373-379.)

Key Words: growth substances ■ diabetes mellitus ■ myocardium ■ collateral circulation ■ insulin

Retinal neovascularization is a hallmark of proliferative diabetic retinopathy (PDR), yet hypoxia-induced collateral vessel formation is decreased in myocardium, lower extremities, and chronic wounds.1–3 Hyperglycemia and insulin resistance are risk factors for the development of microvascular and cardiovascular pathologies in diabetes.4,5 Since vascular endothelial growth factor (VEGF) has an essential role in hypoxia-induced angiogenesis and levels are elevated in retina of diabetic patients with PDR, we have characterized vascular expression of VEGF and its receptors in diabetic and insulin-resistant states.6–9

VEGF is a major mediator of neovascularization in physiological and pathophysiological conditions; with crucial roles in developmental blood vessel formation and regulation of hypoxia-induced tissue angiogenesis.6–13 Five isoforms, differing in secretion and heparin-binding properties, are derived from alternative splicing of a single gene. Two VEGF receptor tyrosine kinases have been identified: Flt-1 or VEGF-R1, and KDR/Flk-1 or VEGF-R2.6,10–13 VEGF also has a variety of other endothelial cell actions relating to permeability, vasodilation, and antiapoptosis, and expression may be regulated by cytokines such as transforming growth factor-β, cellular differentiation, and transformation.6,10,14,15 Patients with myocardial ischemia and infarction have elevated levels of VEGF mRNA and hypoxia-inducible factor-1 in myocardial tissues, indicating this to be an important cardiac response to blood and oxygen deprivation.6,7,16,17

Uncontrolled VEGF-induced angiogenesis may cause pathologic neovascularization in retinal microvascular tissue.8,9 Elevated VEGF levels in aqueous and vitreous fluids is reported in patients with active PDR and with nonproliferative or quiescent PDR compared with patients without diabetes.8,9 Similarly, in streptozotocin (STZ)-induced diabetic rats, VEGF expression is reported to be upregulated in retina.
and glomeruli. In contrast, the effect of diabetes and insulin-resistant states on cardiac and cardiovascular expression of VEGF is unknown. Documentation of expression of VEGF and its receptors in cardiac tissue could be valuable because inadequate collateral vascular formation in response to ischemia increases cardiovascular morbidity and mortality rates in diabetic patients. In this study, we characterize the effect of diabetes, insulin resistance, and insulin treatment on expression of VEGF and its receptors in microvascular and cardiac tissues.

Methods

Experimental Animals
Male Sprague-Dawley rats (weight, 200 to 250g; Taconic, Germantown, NY) were used. After an overnight fast, animals were administered citrate saline vehicle or STZ (55 mg/kg IP). Diabetes was confirmed by hyperglycemia (Glucometer Elite, Bayer Corporation). After 2 weeks of diabetes, animals were separated into those receiving the protein kinase C (PKC)-β selective inhibitor, LY333531 (0.012% wt/wt) chow, or control chow (Lilly Research Laboratories). Another group of animals was given insulin pellet implants (Linplant) after sodium amoborabitol anesthesia (10% solution, 1 mL/kg; amylal sodium, Lilly). After 4 weeks of diabetes, animals were killed with CO2, and the tissues were harvested. Zucker lean and insulin-resistant obese fa/fa rats (Harlan, Indianapolis, Ind) and Zucker diabetic fatty rats (Genetic Models Inc, Indianapolis, Ind) were used at 14 weeks of age. Blood glucose measurements were taken, and plasma insulin levels were assayed with double-antibody radioimmunoassay. The Joslin Animal Care Committee approved all procedures.

Human Heart Samples
Human heart samples were obtained from the National Disease Research Interchange (NDRI, Philadelphia, Pa).

RNA Isolation
Isolated ventricle, aorta, and retina were frozen in liquid nitrogen and stored at −80°C. Kidneys were removed intact and cleaned of adipose tissue, and glomeruli were isolated by the sieving method as previously described. Frozen tissue was crushed and homogenized with TRIzol Reagent (Molecular Research Center Inc); total mRNA was isolated by means of guanidium thiocyanate methods.

Northern Blot Analysis
Northern blot analysis was performed with 20 to 25 μg of total RNA as described. Rat VEGF cDNA (kindly provided by Dr Eric Pierce) and KDR cDNA were labeled with 32P-dCTP (NEN Life Sciences) with use of the Multiprime labeling kit (Amersham Pharmacia Biotech). Signals were analyzed with a PhosphorImager (Molecular Dynamics). Densitometric quantification was performed with the use of ImageQuant software (Molecular dynamics). Results are plotted as mean±SD, and n equals the number of groups tested.

Semi quantitative Multiplex Reverse Transcription–Polymerase Chain Reaction
Multiplex polymerase chain reactions were performed for VEGF and VEGF-R1 and -R2, as previously described. Loading differences were normalized to the RRRPPO control gene.

VEGF ELISA
Rat plasma VEGF was determined with the use of Quantikine M Mouse VEGF Immunoassay (R&D Systems).

Results

Expression of VEGF and Its Receptors in Diabetic Rats
Glucose levels in diabetic rats with or without LY333531 treatment significantly increased (528±55 and 495±68 mg/dL, respectively) compared with nondiabetic rats (102±11 mg/dL). Insulin treatment decreased glucose levels significantly below nondiabetic levels (49±15 mg/dL). Body weight in all groups increased significantly during the study. The final weight of diabetic rats with (299±29 g) and without (312±25 g) LY333531 treatment was significantly lower than nondiabetic (393±23 g) or diabetic rats treated with insulin (389±25 g). Plasma VEGF levels were shown to be significantly reduced in diabetic rats (71.3±13 pg/mL) compared with nondiabetic rats (86±13 pg/mL).

VEGF mRNA levels in the myocardium, per Northern blot analysis, decreased 40% after 4 weeks of diabetes compared with nondiabetic controls (P<0.001; data not shown). With the use of quantitative multiplex reverse transcription–polymerase chain reaction (RT-PCR), mRNA expression levels of VEGF isoforms 164 and 188 was detected in rat ventricle (Figure 1A). Significant reductions (40%; P<0.001) in mRNA expression of both isoforms were observed in the ventricle of diabetic rats with or without LY333531 treatment and were partially normalized after 2 weeks of insulin treatment. VEGF isoforms 164 and 188 were expressed in aorta and did not change with diabetes (data not shown).

The retina expressed predominantly VEGF 120 and 164 isoforms, which increased 2-fold in diabetic animals (P<0.001, Figure 1B). Insulin reversed the diabetes-induced increase in VEGF expression, whereas treatment with LY333531 was ineffective. Similar findings were observed in the renal glomeruli, in which mRNA of VEGF isoforms 120, 164, and 188 were detected (Figure 1C). Only VEGF 164 and 188 increased in the diabetic state, and insulin treatment reversed these changes. In contrast to the retina and ventricle, treatment with LY333531 was only partially effective in normalizing VEGF 164 and 188 expression in glomeruli from diabetic rats.

Expression of VEGF-R1 (Flt-1) and VEGF-R2 (KDR) mRNA levels were reduced by 50% and 70%, respectively, in
diabetic rat heart ($P<0.001$, Figure 2A). Similarly, immunoblot analysis of VEGF-R2 in diabetic rat heart showed a significant reduction (33%; $P=0.039$), which was normalized by insulin treatment (Figure 2B). No difference in the protein expression of PECAM-1, an endothelial cell marker, was observed in the ventricles between diabetic and nondiabetic rats (Figure 3). In contrast, VEGF-R1 and VEGF-R2 mRNA expression in retina increased $2.1\pm0.9$- and $2.1\pm0.8$-fold, respectively ($P<0.003$, Figure 2C) in diabetic rats, compared with control animals. Similarly, VEGF-R1 and VEGF-R2 mRNA expression in glomeruli increased 1.7±0.4- and 1.8±0.4-fold, respectively ($P<0.002$, Figure 2D), in diabetic versus control animals. Insulin treatment normalized these changes in retina and glomeruli, whereas LY333531 treatment was ineffective. Expression of VEGF receptors in aorta did not differ between diabetic and nondiabetic animals (data not shown).

**Effect of Insulin Resistance and Obesity-Related Diabetes**

Zucker lean (Lean), insulin-resistant Zucker fa/fa (Fatty), and insulin-resistant spontaneous-diabetic Zucker fa/fa (DM-Fatty) rats were used as models of insulin resistance and type 2 diabetes.22 Plasma glucose levels were 80±16, 121±27, and 364±131 mg/dL for Lean, Fatty, and DMFatty rats, respectively, in the nonfasting state. Final weights of Lean rats were significantly less (333±37 g) than Fatty or DM-Fatty rats (423±70 g and 405±29 g, respectively). Plasma insulin levels were 1.0, 19.4, and 13.8 ng/mL in Lean, Fatty, and DMFatty rats, respectively.

Quantitative RT-PCR analysis of Zucker rat ventricle showed mRNA expression for VEGF isoforms 164 and 188. Expression for VEGF 164 decreased in Fatty (20%) compared with Lean rats ($P=0.05$, Figure 4A). In DMFatty rats, both VEGF 164 and 188 expression decreased (25%) compared with Lean controls ($P=0.05$, Figure 4A). Interestingly, expression of VEGF 164 and 188 in Fatty and DMFatty rats did not differ from each other.

Quantitative RT-PCR analysis of VEGF in retina of Lean rats showed the predominant isoforms to be VEGF 120 and 164 (Figure 4B), both isoforms increased in retina of Fatty rats (30%, $P<0.05$) and in DMFatty rats (40% and 65%, respectively, $P<0.05$), compared with Lean controls. In Fatty rats, VEGF 120, 164, and 188 isoforms in glomeruli modestly increased by 1.3-, 1.2-, and 1.6-fold, respectively, compared with Lean rats (data not shown). Significant increases of 2.0-, 2.5-, and 2.8-fold were observed in DMFatty rats compared with Lean rats.

VEGF-R2 expression in the cardiac tissue of Fatty rats decreased (20%, $P<0.05$), whereas VEGF-R1 expression was unchanged compared with Lean controls (Figure 5A). Protein expression of PECAM-1 was also studied and found to be decreased in Fatty rats (25%) compared with Lean rats (Figure 3). In DMFatty rats, VEGF-R1 and R2 expression in the heart was decreased 30% and 25%, respectively ($P<0.01$). In contrast, retinal expression of VEGF-R1 and R2 slightly increased in Fatty rats, and VEGF-R2 expression significantly increased in DMFatty rats (30%, $P<0.05$) compared with Lean controls (Figure
Glomerular expression of VEGF-R1 and R2 both increased only in DMFatty rats by 1.7-fold compared with Lean rats (data not shown).

Expression of VEGF and VEGF-R2 in Heart From Diabetic Patients

Preliminary evaluation of VEGF and VEGF-2 expression was performed in cardiac samples from 3 patient groups. Nondiabetic subjects were divided into those with cardiac disease history (CtrlHD; age, 48±19 years; 2 women, 1 man) and those without (Ctrl; age, 59±8.5 years; 4 women, 2 men). Causes of Ctrl death were car accidents, intracranial bleed, renal cancer, and liver disease. Causes of CtrlHD death were coronary artery disease, heart failure, and aortic stenosis. Samples were obtained from 3 diabetic subjects (DM; age range, 56 to 65 years; 1 woman, 2 men), 2 with type 2 diabetes and 1 with type 1 diabetes. All diabetic patients had history of hypertension, and 2 had history of myocardial infarction. Causes of death were head trauma, cardiovascular arrest, and myocardial infarction. As shown in Figure 6, in DM ventricle VEGF mRNA levels decreased 60% (P=0.039) and VEGF-R2 mRNA expression decreased 45% (P<0.049), compared with Ctrl. VEGF and VEGF-R2 mRNA levels decreased 500% (P=0.001) and 300% (P=0.005), respectively, in DM ventricle compared with the CtrlHD group. In contrast, VEGF mRNA levels from CtrlHD donors were significantly higher (200%) than Ctrl patients (P=0.004).

Effect of Insulin on VEGF mRNA Expression in Cardiac Myocytes

Since insulin treatment normalized VEGF mRNA expression in the myocardium, the direct effect of insulin on VEGF mRNA expression in cultured rat neonatal cardiac myocytes was characterized. Insulin (100 nmol/L) increased mRNA expression of VEGF 164 and 188 significantly by 2- to 3-fold by 12 hours (Figure 7; P<0.05).

Discussion

These data provide the first comprehensive analysis of the expression of VEGF and its receptors, VEGF-R1 and R2, in both microvascular and cardiac tissue from diabetic and insulin-resistant animals. In addition, we have preliminary
results showing similar decreases in VEGF and VEGF-R2 mRNA expression in ventricle from humans with diabetes. The finding that VEGF mRNA expression decreased in the myocardium but increased in the microvessels demonstrates a major difference in VEGF regulation between microvessels and cardiac tissues. More surprising was the finding that VEGF mRNA levels were decreased in insulin resistance without the presence of clinical diabetes, suggesting other metabolic factors besides hyperglycemia can also regulate VEGF expression in retina and myocardium.

Enhanced expression of VEGF mRNA in the retina is consistent with reports of elevated VEGF protein levels in ocular fluid from patients with PDR.8,9 Our studies also confirm previous reports demonstrating upregulation of VEGF and VEGF receptor mRNA in retina from diabetic rats and supports the concept that VEGF actions are enhanced by diabetes to cause retinal pathologies.6,17,18,24 Similarly, this study is consistent with reports of elevated mRNA expression of VEGF and its receptors in renal glomeruli19; however, a causal role for VEGF in kidney pathology remains unclear. VEGF could contribute to increased plasma flow, microalbuminuria, and mesangial expansion, and possible mechanisms include VEGF-induced vasodilation through activation of nitric oxide synthase or induction of connective tissue growth factor, which is a potent fibrotic factor.25–27

The decrease in cardiac mRNA expression of VEGF and its receptors is also consistent with pathological reports that collateral vascular formation after myocardial ischemia is blunted in diabetic patients and animals.7 Measurements of
cardiac VEGF expression were at the mRNA level, which will need to be confirmed by analysis of VEGF protein in the future. Nevertheless, our results suggest a potential molecular explanation for this clinical observation since collateral vascular formation reportedly correlates with hypoxia-induced increases in VEGF levels in the myocardium in the nondiabetic state.8,28 The paradoxical changes in the expression of VEGF and its receptors suggest that local regulatory factors differ between myocardium and microvessels. Hypoxia is unlikely to be the main cause of the changes because there is no evidence of hypoxia in these tissues from animals with short diabetes duration.7,19 In the myocardium, VEGF and its receptors decreased, indicating that factors other than hypoxia are involved. Hyperglycemia has been reported to induce VEGF expression through increases in oxidants, glycation products, or activation of the PKC-β isoform.16,21,29 However, it is unlikely that activation of the PKC-β isoform plays a major role because treatment with the PKC-β specific inhibitor LY333531 was only effective in the glomeruli.20,29 These results indicate the main effect of PKC-β isoform inhibition is to normalize the actions of VEGF in retinal and renal tissues rather than reducing its expression, which we have published previously.30

The results with Zucker rats provide a novel finding that insulin-resistant and glucose-intolerant states may also decrease VEGF expression in myocardium. This is consistent with clinical observations that increased cardiac mortality is present in both insulin-resistant and diabetic patients, whereas retinal and glomerular lesions are generally not observed until frank hyperglycemia has been established for several years.1,5 The modest increases in retinal VEGF levels exhibited by Zucker fa/fa insulin-resistant rats suggest mild hyperglycemia, as observed in undiagnosed mild type 2 diabetes, could be a risk for the development of diabetic retinopathy. With the onset of severe hyperglycemia, increases in VEGF and its receptors became more pronounced, which is consistent with clinical findings that glycemic control is the major risk factor for diabetic retinopathy and nephropathy.1 This is in contrast with cardiovascular mortality and morbidity, which is increased in people with insulin resistance without diabetes, and is consistent with our finding that cardiac expression of VEGF decreased similarly in insulin-resistant and diabetic states. However, diabetes and insulin-resistant states appear to regulate VEGF receptors differently, since PECAM expression is not changed in the diabetic state but decreased to an equal extent as VEGF-R2 in the myocardium of insulin-resistant rats. These findings also indicate that the decline in VEGF-R2 in insulin resistance could reflect a decreased capillary network in the myocardium. Moreover, reduced cardiac PECAM expression suggests that the vasculature may be reduced before the onset of diabetes if insulin resistance is present.

One potential mechanism for decreased VEGF expression in myocardium in diabetic and insulin-resistant states is loss of insulin-induced VEGF expression. Studies have shown in multiple cells types that insulin can increase VEGF mRNA expression by activating the PI3-kinase/Akt pathway.31 Previously we have reported that insulin-induced activation of the PI3-kinase/Akt pathway is reduced in the vasculature from insulin-resistant rats.22 The major cell types in cardiac tissue include cardiac myocytes, fibroblasts, and endothelial cells, all of which have been shown to produce VEGF. In this study, we isolated rat neonatal cardiac myocytes and demonstrated insulin to induce an increase in VEGF expression. Whether VEGF receptors could be similarly altered, and which signaling pathways may be involved, has yet to be assessed. Nevertheless, the findings are suggestive that the ability of insulin to induce VEGF expression may be inhibited in myocardium exposed to metabolically abnormal states such as insulin resistance, which is often present in poorly controlled diabetic and insulin-resistant patients.

Together, the results from this study with diabetic and insulin resistant rodents, and from the preliminary findings in ventricle from humans with diabetes, offer a potential molecular explanation for the increased risk of cardiovascular morbidity and mortality in patients with insulin resistance and diabetes. Therapeutic approaches targeted to inhibit systemic VEGF activity to treat PDR may have benefit for diabetic nephropathy but may be detrimental to VEGF-induced cardiac neovascularization.

Acknowledgments
This study was supported by grants R01-EY5110, DK-597201, and DK-59725-01, Joslin DERC. Dr Way is a JDFI Postdoctoral Fellow and Dr Suzuma is a Mary K. Iacocca Fellow at Joslin.
References


Decreased Cardiac Expression of Vascular Endothelial Growth Factor and Its Receptors in Insulin-Resistant and Diabetic States: A Possible Explanation for Impaired Collateral Formation in Cardiac Tissue

Eva Chou, Izumi Suzuma, Kerrie J. Way, Darren Opland, Allen C. Clermont, Keiko Naruse, Kiyoshi Suzuma, Nancy L. Bowling, Chris J. Vlahos, Lloyd Paul Aiello and George L. King

_Circulation_. 2002;105:373-379
doi: 10.1161/hc0302.102143

_Circulation_ is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2002 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circ.ahajournals.org/content/105/3/373

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in _Circulation_ can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to _Circulation_ is online at:
http://circ.ahajournals.org//subscriptions/