Decreased cardiac expression of vascular endothelial growth factor and redox imbalance in murine diabetic cardiomyopathy

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Han B, Baliga R, Huang H, Giannone PJ, Bauer JA. Decreased cardiac expression of vascular endothelial growth factor and redox imbalance in murine diabetic cardiomyopathy. Am J Physiol Heart Circ Physiol 297: H829–H835, 2009. First published June 26, 2009; doi:10.1152/ajpheart.00222.2009.—Type 1 diabetes is associated with a unique form of cardiomyopathy that is present without atherosclerosis. Redox imbalance and/or changes in vascular endothelial growth factor (VEGF) expression have been associated with diabetes-related cardiomyopathy. However, the mechanisms of these changes and their interrelationships remain unclear. Using a murine type 1 diabetes model, we tested the hypothesis that alterations in cardiac performance are associated with decreased cardiac microvascular prevalence, as well as downregulation of VEGF isoforms. We also investigated oxidative stress as a contributor to regulate individual VEGF isoforms and microvascular rarefaction. Significant and rapid hyperglycemia was observed at 1 wk post-streptozotocin (STZ) and persisted throughout the 5-wk study. Left ventricular (LV) fractional shortening was reduced at week 1 and 5 post-STZ insulin relative to age-matched controls. We also observed the early reduction in E/A ratio at 1 wk. Immunostaining for CD31 and digital image analysis demonstrated a 35% reduction in microvessels/myocardial area, indicative of rarefaction, which was highly correlated with fractional shortening. Furthermore, a significant increase in the prevalence of protein 3-nitrotyrosine was observed in the diabetic cardiac tissue, shortening. Furthermore, a significant increase in the prevalence of protein 3-nitrotyrosine was observed in the diabetic cardiac tissue, which was inversely associated with microvascular rarefaction. The expressions of three VEGF isoforms were significantly reduced to different extents. The reduction of VEGF164 was associated with GSSG accumulation. These data demonstrate that the mouse model of STZ-induced diabetes has hallmark features observed in humans with respect to nonischemic systolic and diastolic performance and microvascular rarefaction, which are associated with changes in VEGF isoform expression and redox imbalance in the myocardium.

microvascular density; vascular endothelial growth factor; cardiomyopathy; oxidants

Materials and Methods

Type 1 diabetes mellitus is complicated by severe progressive cardiovascular diseases, including hypertension, congestive heart failure, and coronary artery disease, with over 75% of all diabetic patients dying from cardiovascular events (15, 16, 21). Many of these complications may be secondary to errors in lipid metabolism and attendant atherosclerosis (14). However, a nonatherogenic cardiomyopathy independent of vascular complications in type 1 diabetes has been recognized for over 20 yr (39). This unique form of cardiac disease occurs in roughly 30% of all type 1 patients and presents as early diastolic abnormalities followed by later decline in systolic function, overt failure, and death (12). These issues are particularly relevant in pediatric populations of type 1 diabetic patients, wherein early and progressive cardiomyopathy can precede large-vessel occlusive crises. While this syndrome is well recognized, the mechanisms involved are poorly understood, and specific therapeutic strategies in this patient population currently remain undefined (45).

Diabetic cardiomyopathy is characterized by microvascular pathology, independent of coronary disease, leading to progressive heart failure (10, 11, 22). Microvascular changes may lead to reduced perfusion and mismatch of myocardial supply and demand. These microvascular abnormalities may lead to ischemia in the absence of coronary atherosclerosis and contribute to adverse cardiovascular events in the diabetic patient (26, 32). VEGF is the major angiogenic factor (25) that induces angiogenesis under reduced tissue oxygen tension, leading to the development of new blood vessels to meet the metabolic demands of the tissues. Diabetic microvascular complications are considered to be influenced by angiogenic factors, including VEGF, as a response to both ischemia and hyperglycemia (7). Impairments in VEGF expression and action have been found to occur in diabetes (6, 48, 53). Several isoforms of VEGF have been identified, which not only differ in their amino acid sequence, but also induce specific biological responses (13). VEGF164 is the most abundant and biologically active form (33). Here, we test whether these VEGF isoforms were regulated distinctly in STZ-induced diabetic model.

Oxidative stress is associated with diabetic complications in both humans and animals (4, 9, 17, 35, 38, 49–51). Hyperglycemia can stimulate reactive oxygen species (ROS) production from a variety of sources (34). Besides ROS, recycling of GSH with its disulfide product (GSSG) is a major component of intracellular redox regulation. The balance between reduced and oxidized GSH is considered as an index of the redox state. Thus, the ratio of GSH to GSSG is well recognized as an important index of cellular protection and redox regulation (8). However, their regulations in the heart are poorly understood, and the relationship to VEGF isoform expression, microvessel density, and cardiac functional impairment are unclear. The present investigation was designed to address this issue.

The central hypotheses of these studies are that the mouse model of streptozotocin (STZ)-induced type 1 diabetes mimics the cardiovascular abnormalities observed in the clinical settings, and that these changes are related to reductions in microvessel densities, which are associated with distinct myocardial VEGF isoform regulation and oxidative stress.

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Animals. All aspects of animal use in this study were performed in accordance with the guidelines of the National Institutes of Health and approved by the Institutional Animal Care and Use Committee.
Hyperglycemia was induced in pathogen-free male CF-1 mice (age 6 wk, n = 18, Harlan Laboratories) weighing 16–18 g, with a single dose of STZ (150 mg/kg ip, prepared daily in citrate buffer, pH 4.5, for maximal stability) or vehicle control. Animals were housed in a sterile cage rack system with HEPA-filtered air circulation (~50 air changes/h, Allentown Caging).

Animals were studied longitudinally at 0, 1, and 5 wk post-STZ (n = 18 each time point). Eighteen age-matched mice were treated with vehicle and studied at 0, 1, and 5 wk, to evaluate control parameters for this time-dependent study. Statistical differences within these control groups (0 vs. 1 vs. 5 wk) were not observed for any parameter evaluated. These observations were pooled and served as control values. In all figures shown, these control values are represented at time 0. Blood glucose collected in the morning was determined at each time point (0 vs. 1 vs. 5 wk) with a Glucometer Encore (Ames) clinical blood glucose monitor. Mice were killed at 5 wk with 100 mg/kg ip pentobarbital sodium (Abbott Laboratories).

Assessment of cardiac function. At 0, 1, and 5 wk post-STZ, in vivo cardiovascular function was determined using a Sonos 1000 echocardiography unit (Hewlett-Packard, Andover, MA), as previously described (28). Mice were anesthetized by isoflurane inhalation (0.25–1.5% isoflurane in 95% O2/5% CO2), and normothermia was maintained by a heating pad. In separate studies, systolic blood pressure was determined using a tail cuff monitor (model 289Z, IITC Life Sciences). LV diastolic blood pressure was determined using a tail cuff monitor (model 289Z, IITC Life Sciences). A 7.5-MHz pediatric probe (optimized and dedicated to rodent studies) placed in the parasternal, short-axis orientation recorded LV systolic (LVd) and diastolic internal dimensions (LVIDd). Three loops of M-mode data were captured for each animal, and data were averaged from at least 5 beat cycles/loop. Parameters were determined using the American Society for Echocardiography leading-edge technique in a blinded fashion. These parameters allowed the determination of left ventricular (LV) fractional shortening (FS) by the equation: FS = [(LVIDd − LVIDs)/LVIDd] × 100%

Ascending aortic flow waveforms were recorded using a continuous wave Doppler flow probe oriented in a short-axis, suprasternal manner. Peak aortic flow and velocity-time integrals (VTI) were calculated from these waveforms. After death, aortic root cross-sectional area was measured, and cardiac output (CO) was calculated by the equation: CO = heart rate × VTI × aortic cross-sectional area. Autopsy measurements of aortic root cross-sectional area were conducted. We observed no significant age- or diabetes-related changes in LV outflow dimensions at any time point studied (day 0 and 5-wk controls and 1- and 5-wk diabetic mice). LV diastolic function was evaluated by the ratio of the E/A wave from the transmitral valve flow waveform.

Cardiac histology and immunohistochemistry. The apical portion of the heart was equatorially bisected just distal to the mitral valve and immersed in 10% Formalin. Tissues were paraffin embedded and blocked according to standard procedures.

Following fixation and paraffin embedding, cardiac tissues were prepared as 5-μm cross sections and mounted on slides. Tissue morphology and fibrosis were evaluated by using Masson’s trichrome. In addition, cardiac cross sections were immunostained using specific antibodies directed against protein 3-nitrotyrosine (protein-3NT; 1:400 dilution, Upstate Biotechnology) and platelet endothelial cell adhesion molecule (PECAM; 1:100 dilution, Santa Cruz). Exposure of the tissue sections to 0.06% wt/vol diamobenzidine, followed by methanamine counterstaining, provided visualization of immunoreactivity. Staining (isotypic) of control tissues exposed for the same duration to nonimmune rabbit IgG in place of the primary antibody provided demonstration of antibody specificity.

Image capture and digital image analysis. Cross-sectional areas of each heart were visualized with an Olympus BX-40 microscope (×800 magnifications, Olympus) and captured using an Insight QE digital camera (Diagnostic Instruments). Six images were captured per tissue section in the designated area encompassing the entire cross section of L.V. This provided a straightforward method by which to obtain images representative of the anterior, posterior, lateral, and septal walls of the LV. Images were then analyzed for extent of diamobenzidine signal in each tissue using research-based digital image analysis software (Image Pro Plus 4.0; Media Cybernetics), as previously described (5, 27, 29).

In addition to quantification of immunostaining by integrated optical density analysis for total content of PECAM, we also assessed the number of microvessels by a digital imaging approach, as previously published (44). Briefly, color segmentation was used to delineate positive endothelial cell (microvessel) staining from the rest of the tissue. Positive objects were filtered based on size and aspect ratios to separate microvessels from single cells and/or inappropriate staining. Using Image Pro Plus software and a custom-designed analyzing program, images were segmented in such a fashion to count all vessels with an area of ≤100 μm. Vessels were identified by a circular or elliptical structure with a centralized clear lumen. The final count was then checked by the user to ensure that vessels were counted appropriately. Since capillary density may be related to myofiber atrophy, the myofiber per unit area was evaluated. In our experiment, no changes in myofiber area were detected between the diabetic and control groups. Results of microvascular density are expressed as microvessels per unit area of tissue. Intraobserver variability (one blinded observer, 50 myocytes, 3 trials) and interobserver variability (two blinded observers, 50 myocytes each) for this approach were less than 3 and 5%, respectively.

Western immunoblotting. Ten percent tissue homogenates were prepared in 50 mM NaPO4, 50 mM serine borate, and 17.5 mM EDTA (pH 7.4). Protein concentrations were determined by the Bradford assay. Fifteen micrograms of protein from each sample were heated at 95°C for 10 min and separated on NuPAGE Novex 12% bis-Tris gels (Invitrogen). After electrophoresis, proteins were transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat milk in Tween 20 (0.1%)-Tris-buffered saline (10 mM Tris, pH 7.5; 150 mM NaCl), and incubated with a VEGF antibody (Santa Cruz) for 1 h at room temperature. Horseradish peroxidase-conjugated secondary antibody (Vector Laboratories), enhanced chemiluminescence reaction (PerkinElmer Life Sciences), image capture using UVP EpiChemi dark room system, and image analysis (LabWorks, Media cybernetics) were used to assess the relative amount of target at the appropriate molecular weight. Three VEGF isoforms (VEGF164, VEGF184, and VEGF206) were recognized as reference proteins (19). The process was repeated three times from the same sample, and six samples in each group were examined.

GSH and GSSG measurement. Tissue content of both GSH and GSSG was measured by the enzyme recycling method described by Adams et al. (1). Cardiac levels of GSH and GSSG, which are expressed in nanomoles per milligram protein, were calculated from the concentrations measured in the respective acid supernatants.

Statistical analysis. Parameters between diabetic and control groups were evaluated for significance by one-way ANOVA for all functional data and Student t-tests for all histological data. Significant correlations were assessed using Pearson’s correlation analysis. A total of 30–35 data points were used for each regression analysis. In all cases, results are expressed as means ± SE, and significance was defined as P < 0.05.

RESULTS

Mouse model of hyperglycemia. Blood glucose concentrations were determined as described above. As expected, significant and rapid hyperglycemia was observed at 1 wk post-STZ and persisted throughout the 5-wk study (Table 1). Mice whose blood glucose was ≤200 mg/dl at 1 wk were excluded from further study. STZ-treated mice demonstrated significant cachexia during the progression of diabetes, as body weights
were 12% lower than age-matched controls at 5 wk of diabetes (Table 1).

Cardiac function and morphology. For the duration of the study, systolic blood pressures and heart rates remained unchanged (shown in supplemental material; the online version of this article contains supplemental data). High-quality cardiac ultrasound imaging was achieved under light inhalation anesthesia, as described in MATERIALS AND METHODS. Cardiac diastolic function was assessed by the A/E ratio (Fig. 1). An early (1 wk) decrease in the E peak velocity, which translated to a significant decrease in the E/A ratio, was observed, suggesting that passive ventricular filling due to ventricular dilation in these mice is impaired early in disease progression. However, this impairment was not evident at 5 wk. Statistically significant impairment in LVFS was observed at both 1 and 5 wk. An increase in LVIDs, indicative of LV dilation, was also observed at 1 and 5 wk post-STZ.

Aortic Doppler flow velocity analysis revealed a trend toward decreased CO and ventricular stroke volume in the STZ mouse model. However, they did not reach statistical significance (shown in supplementary material).

No significant increase in heart weight (hypertrophy) was observed in 5-wk diabetic animals. LV fibrosis was detected by Masson’s trichrome stain, assessed by digital imaging. No significant elevations in fibrosis were observed in 5-wk diabetic hearts compared with control. However, morphological analysis showed a significant increase in LV lumen area, with no evidence of increase in LV wall area, suggestive of ventricular dilated cardiomyopathy (Fig. 2).

Cardiac microvessel prevalence. Anti-PECAM (also called CD31, a specific endothelial cell marker) antibody was used to demarcate capillaries. Figure 3A is a representative photomicrograph from LV cross sections stained for PECAM staining. Total PECAM staining (indicative of total endothelial cells) was not different between the 5-wk diabetic and control groups (shown in supplementary material). However, upon further investigation, using an image analysis technique to count the number of microvessels (based in size and aspect ratios) showed a significant (26%, P < 0.05) decrease in the number of microvessels in the 5-wk diabetic myocardium compared with age-matched control mice (Fig. 3B). This decrease showed a significant correlation to the contractile dysfunction seen in the diabetic mice (P < 0.05, Fig. 3C).

Cardiac VEGF protein expression. VEGF isoform expression was quantified by Western blot. Figure 4A shows representative VEGF isoform bands by Western blot in the 5-wk diabetic and control groups. The expression of two VEGF isoforms (VEGF188 and VEGF164) in the heart was decreased significantly in STZ-treated mice (P < 0.05, Fig. 4B).

Cardiac redox state. Cardiac GSH and GSSG concentrations were determined by the enzyme recycling methods, in cardiac homogenates from control and 5-wk diabetic animals. Figure 5, A and B, shows the quantification of GSH and GSSG levels, respectively, expressed as nanomoles per milligram protein. Results indicate that 5-wk post-STZ injection caused significantly enhanced GSSG levels without affecting the levels of GSH. As a result, the GSH-to-GSSG ratio was significantly reduced in the STZ-induced diabetic heart, indicative of enhanced oxidative stress (Fig. 5C). A significant correlation between the GSSG content and VEGF164 prevalence was observed (Fig. 5D).

The prevalence of protein-3NT, a stable biomarker of ROS and nitrogen species, was determined by immunohistochemistry. A significant increase in the total protein-3NT content in the 5-wk diabetic tissues was observed (Fig. 6A). Furthermore, the degree of myocardial protein-3NT content was inversely associated with microvessel density measurements (P < 0.05, Fig. 6B).

DISCUSSION

Although diabetes has traditionally been defined as a metabolic disorder, a majority of the morbidity and mortality associated with this disease is directly attributable to cardiovascular causes and is strongly predicted by hyperglycemia (14). Diabetes mellitus is an independent risk factor for a variety of cardiovascular diseases, including hypertension, atrial fibrillation and arrhythmia, cardiomyopathy, and heart failure (21). The cause of accelerated cardiovascular disease in diabetic patients is multifactorial, although atherosclerotic events (secondary to the loss of metabolic control) (16) have a major influence on morbidity and mortality in diabetes. Interestingly, a subset of diabetic patients, particularly younger patients, develops a specific cardiomyopathy in the absence of
clinically detectable atherosclerosis and/or coronary artery disease (12, 30, 37, 40). The mechanisms involved in this non-ischemic cardiomyopathy are not defined, but this form of heart disease is often considered “microvascular”, since there is little or no evidence of large-vessel lesions. Herein, we hypothesized that alterations in cardiac performance in a mouse model of diabetes and hyperglycemia are associated with decreased cardiac microvascular prevalence, as well as downregulation of VEGF isoforms. We also investigated glutathione as a contributor to regulate individual VEGF isoforms. Before exploring the underlying mechanisms, we tested if the STZ mouse model established in our lab mimics type 1 diabetes-related cardiomyopathy seen in patients.

First recognized in 1963, the STZ rodent model has historically been valuable for basic insights of the consequences of hyperglycemia in vivo (46). Since this mouse model is devoid of severe hyperlipidemia and atherosclerotic events (typical in type 2 cardiac etiologies), it may be most appropriate in modeling aspects of this nonatherogenic type 1 cardiomyopathy (47). We observed an early reduction in the E wave and E/A ratio, indicative of diastolic dysfunction, and changes in multiple indicators of systolic function late in the progression of the disease. These included increases in LV inner dimensions, as measured by echocardiography (LVIDs), and a significant reduction in percent LVFS. These impairments are highly consistent with the clinical presentation of type 1 cardiomyopathy and suggest the value of the STZ model in this setting.

While the cardiac macrovascular effects of diabetes are well recognized, diabetic microvascular studies have generally focused on diabetic retinopathy. However, few studies have shown that there are cardiac microvascular functional abnormalities (deficits, unlike overproliferation in the retina) in diabetes, both clinically and in animal models (31, 36, 45, 52). It has been suggested that these microvascular abnormalities might contribute to the myocardial functional deficits through

![Representative images (A) and morphological analysis of CTRL and 5-wk STZ-induced diabetic mice. B: heart weight (WT); C: fibrosis; D: LV tissue area; E: LV lumen area. Values are means ± SE (n = 6). *P < 0.05 compared with CTRL.](image1)

![Representative images (A) and quantitation of cardiac microvessel density and association to cardiac function in CTRL and 5-wk STZ-induced diabetic mice. B: microvessel density; C: fractional shortening. Values are means ± SE (n = 6). *P < 0.05 compared with CTRL.](image2)
episodes of silent local myocardial ischemia, in times of increased myocardial demand. The cardiac microvasculature consists of vessels with diameters of $\leq\frac{500}{9262}$ m in humans, a heterogeneous mix of small arterioles, capillaries, and postcapillary venules, responsible for the distribution of blood, water, and solutes to the working cells of the heart. This network cannot be visualized angiographically and normally requires the use of intracoronary Doppler catheters, contrast-enhanced MRI, or other advanced imaging in vivo (18). Conversely, postsacrifice imaging is far less complicated. We developed an imaging method using the specific endothelial cell marker PECAM to delineate endothelial cells. Staining was then sorted based on shape and aspect ratios to filter out individual cells, large vessels, and other inappropriately stained components. This resulted in counting only microvessels, which we defined as staining with an aspect ratio of 1:1 to 3:1 and a size $\leq\frac{100}{9262}$ m (as determined on an initial control set). Although there was no change in total endothelial cell staining (defined by CD31 detection in situ), there was a significant decrease in the number of microvessels found in the diabetic myocardium compared with the control myocardium, and this microvessel depletion was also highly correlated to systolic dysfunction.

VEGF is implicated in diabetic microvascular complications (6, 7, 25, 41, 48) and is a major mediator of angiogenesis and neovascularization under both physiological and pathophysiological conditions, as well as important for the maintenance of the existing endothelium (2, 23, 24). Through alternative splicing of mRNA, a single VEGF gene gives rise to multiple distinct protein isoforms (20). These isoforms apparently differ in their expression patterns, and several recent reports suggest that they may also differ in their tissue kinetics and cellular effects (33). In our laboratory, different VEGF isoforms have been found to execute distinct actions on endothelial cells in vitro (20). Therefore, to investigate three most common VEGF isoform changes in our model might provide more information regarding microvessel rarefaction in diabetes. We observed decreased prevalence of VEGF$_{188}$ (40% decrease) and VEGF$_{164}$ (45% decrease) in this model, whereas VEGF$_{120}$ prevalence was unchanged. Recent studies by others have also shown that VEGF$_{164}$ and its primary receptor are decreased by

![Figure 4](http://ajpheart.physiology.org/)

A. Band intensities were normalized to actin in each lane. B: VEGF/actin. Values are means $\pm$ SE ($n = 6$). *$P < 0.05$ for VEGF$_{188}$ and VEGF$_{164}$ compared with CTRL.

![Figure 5](http://ajpheart.physiology.org/)

Fig. 5. GSH (A), GSSG levels (B), GSH/GSSG ratio (C), and association to VEGF prevalence (D) in CTRL and 5-wk STZ-induced diabetic mice. Values are means $\pm$ SE ($n = 6$). *$P < 0.05$ compared with CTRL.

![Figure 6](http://ajpheart.physiology.org/)

Fig. 6. The content of protein-3-nitrotyrosine (3NT; A) and association to microvessel density (B) in CTRL and 5-wk STZ-induced diabetic mice. Values are means $\pm$ SE ($n = 6$). *$P < 0.05$ compared with CTRL.
as much as 40–70% in diabetic myocardium (6). Our observations support the concept that the VEGF isoforms may be discretely regulated in myocardium, and that downregulation of VEGF isoforms may mediate the loss of microvessels during diabetes. Further study of the unique and specific properties of VEGF isoforms, especially as they relate to reduced myocardial microvessel density, may help explain cardiovascular dysfunction in diabetes and may lead to improved therapeutics.

In addition to reduced microvessel densities and companion reductions in VEGF isoforms, results of the present study indicated that 5 wk of STZ-induced diabetes caused significant oxidative stress (as detected by increased protein-3NT prevalence and reduced GSH-to-GSSG ratio). These results corroborate previous findings (4). It has been shown that the diabetic heart is exposed to increased oxidative stress due to elevated glucose levels, diabetic neuropathy, increased sympathetic activity, amplified renin-angiotensin system activity, and myocardial ischemia/function hypoxia (42, 43). The resulting increase in ROS and nitrogen species can, in turn, lead to altered signal transduction, abnormal gene expression, and activation of apoptotic pathways, possibly causing myocardial cell death (3). Enhanced oxidative stress in diabetes was also associated with irreversible damage of protein by the production of advanced glycosylation end products (51). In our studies, we also observed an inverse correlation of myocardial GSSG and the most prevalent VEGF isoform (VEGF164), suggesting that there is association of redox status and VEGF expression during diabetes. Given that VEGF is critical for vascular maintenance in normal myocardium, this association may be a critical component of diabetic cardiomyopathy. Furthermore, the degree of myocardial protein-3NT content was inversely associated with microvessel density measurements ($P < 0.05$). These data suggest that oxidation may be contributing to the observed rarefaction, but clearly more investigations are needed to define the interplay between these two variables (e.g., does oxidation cause rarefaction, or does rarefaction promote oxidation?).

In summary, using a well-recognized mouse model of diabetes, we observed a significant reduction in microvessel density, reduced expression of selected VEGF isoforms, and an increase in oxidative stress in myocardium. These changes were apparently linked and were statistically associated with measures of LV performance. Collectively, our observations suggest that microvascular rarefaction contributes to diabetes-related cardiomyopathy in the absence of atherosclerosis. Further studies to determine the mechanisms of this rarefaction in this animal model and in humans may lead to better treatments for this rather unique form of heart dysfunction.

REFERENCES


DECREASED CARDIAC VEGF AND REDOX IMBALANCE IN DIABETES


