Overexpression of Vascular Endothelial Growth Factor-B in Mouse Heart Alters Cardiac Lipid Metabolism and Induces Myocardial Hypertrophy


Abstract—Vascular endothelial growth factor (VEGF)-B is poorly angiogenic but prominently expressed in metabolically highly active tissues, including the heart. We produced mice expressing a cardiac-specific VEGF-B transgene via the α-myosin heavy chain promoter. Surprisingly, the hearts of the VEGF-B transgenic mice showed concentric cardiac hypertrophy without significant changes in heart function. The cardiac hypertrophy was attributable to an increased size of the cardiomyocytes. Blood capillary size was increased, whereas the number of blood vessels per cell nucleus remained unchanged. Despite the cardiac hypertrophy, the transgenic mice had lower heart rate and blood pressure than their littermates, and they responded similarly to angiotensin II–induced hypertension, confirming that the hypertrophy does not compromise heart function. Interestingly, the isolated transgenic hearts had less cardiomyocyte damage after ischemia. Significantly increased ceramide and decreased triglyceride levels were found in the transgenic hearts. This was associated with structural changes and eventual lysis of mitochondria, resulting in accumulation of intracellular vacuoles in cardiomyocytes and increased death of the transgenic mice, apparently because of mitochondrial lipotoxicity in the heart. These results suggest that VEGF-B regulates lipid metabolism, an unexpected function for an angiogenic growth factor. (Circ Res. 2008;103:1018-1026.)

Key Words: VEGF-B • cardiac hypertrophy • cardiac metabolism • fatty acids • mitochondria

Members of the vascular endothelial growth factor (VEGF) family, currently comprising 5 mammalian proteins, are major regulators of blood and lymphatic vessel development and growth. VEGF is essential for vasculogenesis and angiogenesis, whereas VEGF-C is necessary for lymphangiogenesis. Although not required for embryonic development, placenta growth factor and VEGF-D are likely to play more subtle roles in the control of angiogenesis and lymphangiogenesis, or function under pathological conditions.1,2

VEGF-B exists as 2 isoforms generated by alternative splicing. VEGF-B167 has a heparin-binding carboxyl terminus, whereas VEGF-B166 contains a hydrophobic carboxyl terminus, is O-glycosylated, and proteolytically processed.3 Both isoforms bind to VEGF receptor (VEGFR)-1 and neuropilin-1 but not to the major mitogenic endothelial cell receptors VEGFR-2 or VEGFR-3.4,5 VEGF-B has a wide tissue distribution, being most abundant in the myocardium, skeletal and vascular smooth muscle, as well as in brown adipose tissue.6 Mice lacking VEGF-B are viable and fertile and display only a mild phenotype in the heart. This is manifested as an atrial conduction abnormality characterized by a prolonged PQ interval in 1 strain (C57Bl background),7 or as a smaller heart size with impaired recovery after myocardial ischemia in another (129/SvJ or 129/SvJ × C57Bl/6J background).8 Furthermore, VEGF-B has also been implicated in pathological vascular changes in inflammatory arthritis9 and in protecting the brain from ischemic injury.10 However, the ability of VEGF-B to stimulate angiogenesis directly is poor in many tissues. VEGF-B did not stimulate vessel growth when delivered into muscle or periventricular tissue via adenoviral vectors.11,12 On the contrary, VEGF-B overexpressed in endothelial cells of transgenic mice was able to potentiate...
rather than initiate, angiogenesis.13 And unlike VEGF, VEGF-B did not increase vascular permeability.8,13

Here we have studied the effects of VEGF-B in the skin and heart, by overexpression via the keratin-14 and α-myosin heavy chain (αMyHC) promoters, respectively. VEGF-B promoted very little angiogenesis. However, mice overexpressing VEGF-B in the heart gradually developed cardiac hypertrophy with increased cardiomyocyte size and, surprisingly, had lower blood pressure, lower heart rate and larger blood vessels in the myocardium than their wild-type (WT) littermates. The hypertrophy was associated with changes in mitochondrial morphology and eventual lysis of mitochondria. We found marked changes in the lipidic profiles of the transgenic hearts, including increased amounts of ceramides. These results suggest that VEGF-B is capable of altering cardiac lipid metabolism, with overexpression ultimately leading to cardiomyopathy.

Materials and Methods
An expanded Materials and Methods section is available in the online data supplement at http://circres.ahajournals.org.

Generation of Transgenic Mice
To generate keratin-14–VEGF-B transgenic mice, DNA from the human VEGF-B gene was cloned into the keratin-14 expression vector (provided by Dr Elaine Fuchs, The Rockefeller University, New York, NY). To generate the heart-specific transgene, cDNA encoding human VEGF-B167 was ligated into the αMyHC promoter expression vector (a gift from Dr Jeffrey Robbins, Children’s Hospital, Cincinnati, Ohio). Expression cassettes were injected into fertilized mouse oocytes of FVB background. Mice were PCR-genotyped using tail DNA. All mouse experiments were approved by the Provincial State Office of Southern Finland and carried out in accordance with institutional guidelines.

Immunohistochemistry
Sections of paraformaldehyde-fixed, paraffin-embedded mouse hearts were stained with rat antibodies against mouse platelet endothelial cell adhesion molecule (Pecam)-1 (BD Pharmingen) using biotinylated anti-rat IgG (Vector Laboratories) and TSA-kit (NEN Life Sciences) for detection. The number and size of Pecam-1–stained vessels were quantified using ImageJ program (NIH).

Results are presented as averages ± SD.

Acetone-fixed cryosections were stained with antibodies against collagen IV (CosmoBio), laminin-1 (a gift from Päivi Liesi, University of Helsinki, Helsinki, Finland), VEGFR-1 (a gift from Dr Bronke Pytowski, ImClone Systems), neuropilin-1 or VEGF-B (R&D Systems). AlexaFluor488-conjugated anti-rabbit or AlexaFluor594-conjugated anti-goat (Molecular Probes) antibodies were used for detection. Cardiomyocyte sizes were quantified from laminin-1-stained photomicrographs using Axiovision program (Zeiss).

Echocardiography
Transtracheal echocardiography was performed using Acuson Ultrasound System (Sequoia512) and a 15-MHz linear transducer. Mice were anesthetized with fentanyl citrate, fluanisone, and midazolam, and normal body temperature was maintained.

Telemetric Analysis
Heart rate and mean arterial pressure were recorded from left carotid artery using telemetric implants as previously described,13 except xylazine was used for anesthesia. Data collection was started on the fifth day when the normal circadian rhythm of the mice had returned. Data were gathered day and night for 2 weeks, every 5 minutes for 10 seconds.

Measurements of Mitochondrial Redox State and Tolerance of Short-Term Ischemia in Isolated, Perfused Hearts
Aorta was cannulated immediately after cervical dislocation and perfusion commenced. The heart was enclosed in a thermostatic, light-tight chamber connected to a spectrophotometer–fluorometer. Left ventricular pressure was monitored using a saline-filled cannula connected to a Statham P231D pressure transducer and SP1400 pressure monitor. Parameters were calculated with custom-designed software. Venous effluent from the heart was collected in 1-minute aliquots and lactate dehydrogenase (LDH) washout measured. The values are presented as means ± SE.

Angiotensin II–Induced Pressure Overload
Angiotensin (Ang) II was administered via subcutaneous Alzet-minipumps (Scanbur AB) for 1 week (0.1 mg/kg/h). Echocardiography was performed using VEVO 779 Ultrasound System (VisualSonic).

Electron Microscopy
Left ventricular samples were fixed with glutaraldehyde, postosmicated, and embedded in epon. Regions of interest were examined with JEOL 1400 EX Transmission Electron Microscope.

Lipidomic Analysis of Heart Tissue
Lipid extracts of heart tissue, mixed with a labeled lipid standard mixture, were analyzed on a Waters Q-Tof Premier mass spectrometer combined with Acquity Ultra Performance liquid chromatography. Results are presented as means ± SEM.

Results
VEGF-B is Minimally Angiogenic in the Skin
We generated transgenic mice overexpressing VEGF-B in the epidermis under the keratin-14 promoter (Figure 1A and B in the online data supplement). Dermal vessel density and branching were only slightly increased (≈20%; supplemental Figure IC), thus VEGF-B only minimally promoted angiogenesis in the skin.

VEGF-B Encoded by a Heart-Specific Transgene is Associated With Pericellular Matrix
As the heart is one of the major sites of VEGF-B expression and the primary organ affected on deletion of the VEGF-B gene, we studied the effects of VEGF-B in this tissue. For this, we overexpressed the heparin-binding VEGF-B isoform, VEGF-B167, under the α-myosin heavy chain (αMyHC) promoter (Figure 1A). The VEGF-B167 transgene was expressed at 6- and 8-fold compared to the endogenous VEGF-B gene in the myocardium of the 2 transgenic mouse lines studied, as analyzed by Northern and Western blotting (supplemental Figure ID and data not shown), and by immunohistochemistry (Figure 1B). Much of the extracellular VEGF-B167 protein colocalized with the pericellular matrix component collagen IV (Figure 1B). Intracellular staining was also observed in denatured samples (Figure 1B, bottom row, middle). VEGFR-1 and neuropilin-1 distribution partially overlapped with that of collagen IV and laminin (Figure 1B and 1C and data not shown), suggesting that extracellular VEGF-B was bound to heparan sulfates in the basal laminae of myocardial blood vessels and cardiomyocytes.
The Hearts of the αMyHC-VEGF-B<sub>167</sub> Mice Have Larger Blood Vessels

Immunohistochemical staining for Pecam-1 revealed a lower blood vessel density in the hearts of αMyHC-VEGF-B<sub>167</sub> mice than in WT littermates (236±33 versus 297±38 vessels per microscopic field, n=13, P<0.001; Figure 4A through 4C). However, when calculated per number of cardiomyocyte nuclei in the analyzed area, the number of vessels in transgenic hearts was comparable to that of WT hearts (1.68±0.19, n=7, versus 1.95±0.24, n=5, P>0.05, Figure 4D). The blood vessels in the myocardium of the αMyHC-VEGF-B<sub>167</sub> mice were larger than those of their littermates (average vessel area 1223±244 versus 944±130 pixels, P<0.002, Figure 4E). This was associated with an increase in the number of endothelial cells per vessel cross-section (0.64±0.11, n=6, versus 0.51±0.09, n=4, P<0.004). Thus, the total area covered by blood vessels in the myocardium of the transgenic mice was similar to that of controls (277236±44831 versus 274278±37929 pixels per microscopic field, P>0.5; Figure 4F). This indicates that although VEGF-B<sub>167</sub> overexpression did not affect the vessel number per cardiomyocyte, the vessel area per cardiomyocyte was increased.

Mitochondrial Redox State

Central for energy metabolism and heart function is fatty acid oxidation coupled to ATP production in mitochondria. The myocardial content of cytochrome c oxidase (cytochrome aa<sub>3</sub>), a marker of the mitochondrial respiratory chain, was similar in transgenic and control hearts. The steady state oxidation–reduction level of cytochrome aa<sub>3</sub> tended to be slightly more reduced in transgenic hearts than in controls (Figure 5A and 5B), which was also the case for the fluorescent flavoproteins. These mainly reflect the activity of mitochondrial lipoamide dehydrogenase, which is in equilibrium with the NADH/NAD<sup>+</sup> pool<sup>16</sup> and can therefore be used as a redox indicator for NADH/NAD<sup>+</sup> in the mitochondrial matrix. When the cytochrome aa<sub>3</sub> reduction percentage was plotted against mechanical work output during electric pacing to varying heart rates, the plots were linear without a
significant difference between the slopes of transgenic and control hearts. Cytochrome aa3 reduction at the intercept (representing 0 work output) was similar for both transgenic and control hearts, indicating no difference in the mitochondrial coupling efficiency (data not shown).

Ischemia and Pressure Overload Tolerance

LDH washout from the transgenic hearts after a 20-minute period of ischemia was significantly less than from WT controls (Figure 5C), suggesting less cellular damage on ischemia-reperfusion. Despite this indication of myocardial protection, the relative work output (pressure development/heart rate) during the first 10 minutes of reperfusion tended to be lower in the transgenic hearts (64 ±2% versus 90±18%, for TG, n=3, and WT, n=6, hearts, respectively; Figure 5D). After 20 minutes of reperfusion this difference disappeared, the work output being 82±7% (n=3) in the αMyHC-VEGF-B167 hearts and 85±18 (n=3) in the controls (Figure 5D).

Both transgenic and WT mice responded to Ang II–induced pressure overload by decreasing left ventricular internal diameter and stroke volume (WT untreated 34.26 ±4.47 µL, WT Ang II–treated 28.01±4.48 µL, n=6, P<0.05; TG untreated 39.13±5.17 µL, TG Ang II–treated 28.12±4.47 µL, n=6, P<0.01). Furthermore, the Ang II–treated transgenic mice did not show any functional impairment when compared to Ang II–treated WT littermates in echocardiographic analysis, further confirming that the VEGF-B induced hypertrophy does not compromise heart function.

Mitochondrial Changes in the Transgenic Hearts Are Associated With Abnormal Lipid Accumulation

Despite normal cytochrome concentrations in the transgenic hearts, electron microscopic analysis revealed progressive changes in the structure of cardiomyocyte mitochondria in 2- and 6-month-old transgenic mice (Figure 6A through 6D and supplemental Figure III), including swelling and altered morphology of the cristae (white arrows, Figure 6C), apparently leading to mitochondrial lysis (black arrows).
changes accumulated in older transgenic mice, and intracellular vacuoles were visible in the transgenic hearts in light microscopy at 1 year of age (arrows in Figure 6E). Mitochondrial changes suggested altered energy metabolism in the cardiomyocytes, which use mainly fatty acids as an energy source.

Previous studies have indicated that increased myocardial fatty acid uptake or decreased fatty acid utilization leads to cardiac hypertrophy followed by the accumulation of toxic lipid species.17,18 We therefore carried out lipidomic profiling of the hearts. This analysis revealed a significant increase in de novo synthesized ceramide levels in the transgenic hearts, as measured by the ceramide to sphingomyelin ratio (Figure 7A and supplemental Figure IV), whereas the triacylglycerols were decreased, as normalized to total phospholipid concentrations (Figure 7B) or to tissue weight (data not shown). Ceramides are known for their toxicity and accumulate in pathological conditions such as diabetes mellitus or adipose tissue inflammation.19,20 Thus, ceramide accumulation was the probable reason for the observed mitochondrial damage and increased mortality of the αMyHC-VEGF-B167 transgenic mice.

**Discussion**

In the present study, we show that elevated expression of VEGF-B induces very little angiogenesis in the skin or heart.

![Figure 3](http://circres.ahajournals.org/doi/abs/10.1161/CIRCRESAHA.108.186786)

**Figure 3.** Heart rate, blood pressure, and survival of the transgenic and WT mice. Telemetric measurements of the heart rate (A), as well as systolic and diastolic blood pressure (B), of 5-month-old homozygous αMyHC-VEGF-B167 male mice and WT controls. The values are presented as averages ± SD. **P<0.001.

**Figure 4.** Altered pattern of myocardial capillaries in the αMyHC-VEGF-B167 hearts. Sections of the hearts from male αMyHC-VEGF-B167 (A) and WT littermate (B) mice were stained with antibodies against Pecam-1, and the number of Pecam-1-positive vessels was quantified (C). D through F, Number of Pecam-1-positive vessels per cell nucleus (D), average area of Pecam-1 positive vessels (E), and total area covered by Pecam-1 positive vessels (F) in αMyHC-VEGF-B167 and WT littermates. The values are presented as averages ± SD ***P<0.001.

![Table](http://circres.ahajournals.org/doi/abs/10.1161/CIRCRESAHA.108.186786)

**Table.** Echocardiographic Analysis of Three- and Twelve-Month-Old Transgenic Mice and Wild-Type Littermates

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Three Months</th>
<th>Twelve Months</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>TG</td>
<td>WT</td>
</tr>
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<td>IVS (mm)</td>
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<td>0.9±0.1</td>
</tr>
<tr>
<td>IVSs (mm)</td>
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<td>1.9±0.1</td>
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<td>LVs (mm)</td>
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<tr>
<td>LVPWd (mm)</td>
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<td>0.9±0.1</td>
</tr>
<tr>
<td>LV PWs (mm)</td>
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<td>1.6±0.2</td>
</tr>
<tr>
<td>MWTd (mm)</td>
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<td>0.9±0.1</td>
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<tr>
<td>MWTs (mm)</td>
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<td>1.7±0.1</td>
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<tr>
<td>LV FS (%)</td>
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<td>LV EF (%)</td>
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<tr>
<td>LV mass (mg)</td>
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<tr>
<td>HR (beats/min)</td>
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<td>416±90</td>
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</table>

Results are expressed as means±SD. *P<0.05 vs WT (n=8–10 in each group). d indicates diastole; s, systole; IVS, interventricular septum; LV, left ventricle; LVPW, left ventricle posterior wall; MWT, mean wall thickness; FS, fractional shortening; EF, ejection fraction; HR, heart rate.
but, instead, hypertrophy of the cardiac muscle without significant changes in pumping function. Because the transgenic blood pressure and heart rate were decreased, the hypertrophy was not a consequence of increased workload but, rather, caused by an intrinsic metabolic effect induced by cardiomyocyte expression of VEGF-B. Although the transgenic mice had larger myocardial capillaries, their tolerance to cardiac ischemia and reperfusion, as well as to Ang II–induced myocardial stress was not compromised. However, evidence was obtained of altered cardiac lipid metabolism, ceramide accumulation, gradual mitochondrial damage, and increased death of the transgenic mice. The unexpected metabolic and trophic effects of VEGF-B in the myocardium...
and the absence of major angiogenic effects are striking for a member of the VEGF family.

Several studies, including our present results, indicate that VEGF-B is not a major angiogenesis-inducing factor. Instead, VEGF-B may have the ability to potentiate neovascularization in pathological conditions. In this regard, VEGF-B differs greatly from placenta growth factor, which also binds to VEGFR-1 and neuropilin-1 and induces angiogenesis in various tissues, including the heart. The skin, VEGF-B overexpression only slightly increased dermal capillary density. In the VEGF-B overexpressing hearts, vessel density was decreased because of vessel displacement by hypertrophic cardiomyocytes, whereas vessel diameter was greater so that the total capillary area per cardiomyocyte was increased. VEGF-B staining decorated cardiomyocytes and the pericellular basement membranes, indicating that the transgene was abundantly expressed, and consistent with the finding that VEGF-B

had been shown to promote minimal angiogenesis. In this work, VEGF-B167 binds to pericellular heparan sulfate. The lack of a significant angiogenic effect is consistent with earlier reports where VEGF-B was expressed in endothelial cells and shown to promote minimal angiogenesis.

We detected a progressively increasing heart to body weight ratio in the transgenic mice, which was associated with increased thickness of the cardiac muscle wall. The reason for the increased cardiac mass appeared to be hypertrophy of the cardiomyocytes as determined by increased cell size, and the transgenic hearts lacked areas of fibrosis or edema. In contrast to forms of human hypertrophic cardiomyopathy, parallel sarcomere orientation was preserved in the transgenic hearts. Increased β-adrenergic stimulation and systemic hypertension with associated work overload are known to induce a hypertrophic response in the left ventricle, and angiogenesis is known to be essential for overload-induced adaptive cardiac hypertrophy, whereas inhibition of angiogenesis leads to heart failure. The VEGF-B–induced hypertrophy, however, was not related to these conditions as the heart rate and blood pressure were lower in the transgenic mice, and hypertrophy comprised all parts of the myocardium. Otherwise, the anatomy of the heart and the great vessels was normal. The myocardial hypertrophy in the VEGF-B–overexpressing hearts contrasts with the loss-of-function phenotype of the VEGF-B knockout mice, which have smaller hearts, and is consistent with the recent report that adenoviral VEGF-B treatment promotes cardiomyocyte hypertrophy after myocardial infarction.

We observed that intracellular vacuoles accumulate in the cardiomyocytes of the transgenic mice. With electron microscopy these were found to be structures left behind from damaged mitochondria, suggesting that mitochondrial energy metabolism is disturbed in the VEGF-B–overexpressing hearts. As cardiomyocytes mainly use lipids as an energy source, it was logical to analyze their lipidomic profiles. Indeed, lipidomic analysis revealed significant changes in the lipid composition of the hearts and suggested a likely reason for the cardiac hypertrophy. Previous studies have indicated that excess transport of fatty acids can induce cardiac hypertrophy, which converts to lipotoxicity when the mitochondria cannot use all of the available lipids. In particular, ceramide accumulation is known to induce mitochondrial damage and to play a critical role in the pathogenesis of lipotoxic cardiomyopathy. The increased lipolysis of triacylglycerols is the likely reason for their observed depletion in transgenic hearts, and would also explain the elevated ceramide levels. Higher availability of fatty acids resulting from lipolysis would lead to increased demand for mitochondrial β-oxidation, whereas the excess pool of saturated long-chain fatty acids would provide substrate for de novo ceramide synthesis.

Cardiac hypertrophy has also been described following overexpression of long-chain acyl-coenzyme A synthetase in the heart. In this case, toxic lipid species accumulated in the hearts. Altered lipid metabolism seems to be the likely cause for the cardiac hypertrophy in the VEGF-B transgenic hearts, and, via increased ceramide accumulation, this would gradually lead to cardiomyocyte damage.

Ceramides are also involved in triggering macroautophagy. The intracellular vacuoles in the transgenic hearts suggest ongoing autophagy, which is a normal process in the heart for removal of damaged cytosolic components. This process is linked both to cardioprotection and lipolysis. A major portion of myocardial intracellular triacylglycerol hydrolysis is lysosomal, and lysosomes are involved in autophagic formation. Moreover, increasing the autophagic capacity of cardiomyocytes decreased apoptosis induction on ischemia/reperfusion. These 2 processes, autophagy and lipolysis, both related to lysosomal activities, may link the present observations of protection against ischemic damage, a decrease in myocardial triacylglycerols, and an increase in ceramide. There appears to be a fine balance between the proapoptotic and protective actions of ceramide, because ceramide has been shown to attenuate hypoxic cell death. Elevated levels of ceramide could thus at least partly account for the improved cell survival after ischemia.

Hearts isolated from αMyHC-VEGF-B167 mice showed some protection against cellular damage after ischemia/reperfusion, as evidenced by less LDH release. Nevertheless, there was no significant difference between the αMyHC-VEGF-B167 mice and controls in the mechanical work output 25 minutes after the beginning of reperfusion, although, during the first 10 minutes, there seemed to be a lag in attaining the basal work output. The causal connection between the subdued commencement of contractions on reperfusion and the decrease in LDH release is unclear. However, in isolated hearts, lower pressure during commencement of reperfusion has been found to protect against injury. We monitored the redox state of mitochondria during variable workload and substrate availability, but no systematic effects of the VEGF-B167 overexpression were found (E.L. and I.E.H., unpublished observations, 2004). The response of the redox state of myocardial cytochrome aa3 to workload transitions was normal, and the redox level at extrapolated zero workload was similar in the αMyHC-VEGF-B167 and control hearts, indicating no difference in mitochondrial energy coupling or basal metabolic rate. Interestingly however, VEGF-B expression is correlated with high metabolic activity in tissues, suggesting that its expression is associated with energy metabolism. A recent study has also indicated that the metabolic sensor peroxisome proliferator-activated receptor-γ coactivator 1α (PGC-1α) controls VEGF
expression and angiogenesis in ischemic tissues. These results shed new light on an important interplay between nutrient demand, metabolism, and vascular supply.

The changes observed in the VEGF-B167 overexpressing cardiomyocytes may be triggered by receptor activation in endothelial cells, followed by an as yet undetermined signaling or metabolite transfer to the cardiomyocytes. The finding that VEGF-B alters cardiomyocyte mitochondria and lipid metabolites in the heart in the absence of overt vessel growth is a new finding for an angiogenic factor. Although, in our case, the excess of VEGF-B ultimately leads to tissue damage, the potential of VEGF-B to regulate cardiomyocyte energy metabolism during shorter treatment periods may turn out to be useful in attempts to salvage myocardial insufficiency where the myocardial energy sources are defective.

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Disclosures
None.

References


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Supplemental Figure Legends

Online Figure I. Expression of the transgenes and minimal angiogenic effects of VEGF-B on skin vasculature. (A) The expression of the K14-VEGF-B transgene was analyzed by Northern blotting of RNA isolated from the skin of the K14-VEGF-B and littermate control mice with a probe recognizing VEGF-B mRNA. (B) The proper expression of the K14-VEGF-B transgene was confirmed by immunohistochemistry of paraffin sections of the skin with antibodies against mouse VEGF-B. The skin vasculature was analyzed by whole-mount staining of ear skin with antibodies against the panendothelial marker Pecam-1. (C) Number of Pecam-1 stained vessels and their branching points per microscopic high-power field (hpf). **, p < 0.05. (D) The expression of the αMyHC-VEGF-B167 transgene was analyzed by RT-PCR (left) and Northern blotting (right) of RNA isolated from the hearts of αMyHC-VEGF-B167 and littermate control mice.

Online Figure II. Telemetric analysis of blood pressure. Day- and night-time systolic (A) and diastolic (B) blood pressure of αMyHC-VEGF-B167 and wild type mice as a function of time. Note the decreasing trend in the blood pressure after the probe insertion procedure (on day 0).

Online Figure III. Analysis of mitochondrial changes in the hearts of αMyHC-VEGF-B167 mice by electron microscopy. Transmission electron micrographs displaying mitochondrial morphology in the hearts of six-month-old αMyHC-VEGF-B167 (A) and wildtype (B) mice. (C) Magnification of boxed area in A, featuring accumulating vacuoles and swollen mitochondria with altered cristae (arrows). (D) Electron micrograph from a two-month-old αMyHC-VEGF-B167 mouse heart showing progressive mitochondrial degeneration. Scale bar = 2 µm.
Online Figure IV. Levels of cardiac ceramides with different acyl chain lengths. The ratio of ceramides to sphingomyelin in the hearts of female αMyHC-VEGF-B<sub>167</sub> and wild type mice. **, p < 0.05.

**Supplemental Results**

**VEGF-B is minimally angiogenic in transgenic mouse skin.** Previous studies have shown that when overexpressed under the K14 promoter in transgenic mouse skin, VEGF and PIGF induce an angiogenic phenotype and VEGF-C and VEGF-D a lymphangiogenic phenotype<sup>1-4</sup>. We generated transgenic mice overexpressing VEGF-B under the K14 promoter and confirmed transgene expression by Northern blotting and immunohistochemistry (Supplemental Figure 1, A-B). The mice developed normally, appeared healthy, were fertile, and had a normal life span. The skin of the ears, snout and paws had a normal pinkish blush, and hair growth over the body was normal, without any signs of spontaneous skin lesions or abnormalities, for up to 1.5 years of age. VEGF-B overexpression only slightly increased vessel density (42 ± 5 vessels/mm<sup>2</sup> in K14-VEGF-B mice versus 35 ± 4 vessels/mm<sup>2</sup> in WT mice, n = 10, p < 0.05) and vessel branching (392 ± 74 branchpoints/mm<sup>2</sup> in K14-VEGF-B mice versus 331 ± 54 branchpoints/mm<sup>2</sup> in WT mice, n = 7, p < 0.05) in the skin (Supplemental Figure 1C). These vessels had a normal morphology, size, patterning and mural cell coating as analyzed by immunostaining for smooth muscle alpha-actin (SMA; data not shown). Thus, VEGF-B only minimally promoted angiogenesis when overexpressed in the skin.
Supplemental Materials and Methods

Generation of the transgenic mice. To generate the K14-VEGF-B transgenic mice, DNA from the human VEGF-B gene corresponding to nucleotides 745-5059 of Genbank accession number AF468110 was cloned into the K14 expression vector (kindly provided by Dr. Elaine Fuchs5), and one non-initiating upstream ATG was mutated into a GTG. The expression cassette was excised from the vector backbone and injected into fertilized mouse oocytes of FVB background. The mice were PCR-genotyped using tail DNA with the primers 5’-TCTCCAGCCTGATGCCCT-3’ and 5’-GGACTTGGTGCTGCCAGTG-3’.

To generate a heart specific transgene, the recessed 3'-ends of the EcoRI fragment from a human VEGF-B167/pCRII vector6 were filled in with the Klenow fragment of DNA polymerase I and ligated to the Sal I-opened and Klenow filled-in αMyHC promoter expression vector7 (a kind gift from Dr. Jeffrey Robbins). The expression cassette was excised from the vector backbone with BamHI and injected into fertilized mouse oocytes of FVB background. The mice were genotyped by PCR of tail DNA with the primer pair 5’-TCTCCAGCCTGATGCCCT-3’ and 5’-GCCATGTGTACCTTCGCAG-3’.

The expression of the transgene was confirmed by subjecting total RNA to Northern blotting and hybridization with a 32P-labeled VEGF-B cDNA probe (nucleotides 1-382, Genbank Accession No U48800), by RT-PCR using the primer pair 5’-TCTCCAGCCTGATGCCCT-3’ and 5’-CTAAGCCCCGCCCCTGGC-3’, and by immunohistochemistry and Western blotting. The phenotypes were analyzed from two αMyHC-VEGF-B founder lines expressing the transgene. All experiments
involving mice were approved by the Provincial State Office of Southern Finland and carried out in accordance with institutional guidelines.


**Immunohistochemistry.** For quantification of blood vessels in the heart, 7 µm sections of 4% paraformaldehyde (PFA) fixed, paraffin-embedded mouse tissues were deparaffinized, rehydrated, and pretreated with trypsin (0.25 mg/ml trypsin in 9 mM CaCl₂, 50 mM Tris pH 7.8) for 30 minutes at 37°C, and the endogenous peroxidase was inactivated with 3% H₂O₂ in methanol. After blocking, the slides were incubated with purified rat anti-mouse Pecam-1 (MEC13.3, BD Pharmingen) at a concentration of 0.625 µg/ml overnight at +4°C, washed, and incubated with biotinylated rabbit anti-rat IgG at a concentration of 1.7 µg/ml (BA-4001, Vector Laboratories) for 30 min at room temperature. The slides were stained using the TSA-kit (NEN Life Sciences/PerkinElmer Life and Analytical Sciences) according to the manufacturer’s instructions. The number and size of the Pecam-1 stained vessels were quantified from four photomicrographs per mouse photographed with an Olympus AX70 microscope and DP50 Camera (Olympus) using the ImageJ program (National Institutes of Health). The number of Pecam-1 positive endothelial cell nuclei per blood vessel cross-section was quantified using Pecam-1/hematoxylin staining (four photomicrographs per mouse). Results are expressed as average ± SD.

8 µm cryosections of frozen tissues were fixed in acetone, blocked, and incubated with rabbit anti-mouse collagen IV (Cosmo Bio), rabbit anti-laminin-1 antiserum (a kind gift from Päivi Liesi), anti-rat VEGFR-1 (5B12, ImClone Systems Incorporated), goat anti-rat neuropilin-1 (AF566, R&D Systems) or goat anti-human VEGF-B (AF751, R&D Systems) primary antibodies for 2 hours. Intracellular VEGF-B staining was observed when using microwave pretreatment of slides in DakoCytomation Low
pH Target Retrieval Solution. AlexaFluor488-conjugated anti-rabbit and AlexaFluor594-conjugated anti-goat (Molecular Probes) antibodies were used for detection. Zeiss Axioplan2 epifluorescence microscope (Zeiss) was used for imaging. Cardiomyocyte sizes in transgenic and control hearts were quantified from four correspondingly located laminin-1 stained high-power field photomicrographs per heart using the Axiovision program (Zeiss).

Mouse ears were dissected for whole-mount immunostaining, fixed in 4% PFA, and incubated with antibodies against Pecam-1, followed by detection with peroxidase-labeled IgGs (Dako). For quantification of branch points and vessel densities, 24 optical fields (400x) per mouse, sampled randomly in the center and periphery of the ear, were photographed with a digital camera. For quantification of vessel densities, prints of the images were divided by horizontal lines into five equal sectors and all crossing points of the vessels with the horizontal lines were counted. Vessel branching was quantified from the same prints by counting the branch points. Values are presented as average ± SD.

Echocardiography. Transthoracic echocardiography was performed using an Acuson Ultrasound System (Sequoia™ 512) and a 15-MHz linear transducer (15L8) (Acuson) or a VEVO 779 Ultrasound System (VisualSonics). Mice were anesthetized with fentanyl citrate 8 µg/10 g, fluanisone 250 µg/10 g and midazolam 125 µg/10 g. Normal body temperature was maintained during the examination with a warming pad and lamp.

Using two-dimensional imaging, a short axis view of the left ventricle at the level of the papillary muscles and two-dimensionally guided M-mode recordings through the anterior and posterior walls of
the left ventricle were obtained. Left ventricular end-systolic (LVDs) and end-diastolic (LVDd) dimensions as well as thickness of the interventricular septum (IVS) and left ventricular posterior wall (LVPW) in diastole (d) and systole (s) were measured from the M-mode tracings. Left ventricular shortening fraction (LVFS) was calculated from the M-mode left ventricular dimensions using the following equation: LVFS (%) = [(LVDd-LVDs) / LVDd] x 100. Ejection fraction (EF) was also calculated from the M-mode dimensions using the equation: EF (%) = [(LVDd)3 – (LVDs)3 / LVDd3] x 100. Functional left ventricular mass was calculated using the equation: LVmass = 1.055 x [(IVSd + LVd + LVPWd)3 – LVDd3]. All the measurements were made from three subsequent cycles and calculated as the mean of these three measurements. Results are expressed as mean ± SD.

Function of the mitral, tricuspid, aortic and pulmonary valves was evaluated by using color flow mapping and pulsed Doppler.

*Telemetric analysis.* During the telemetric measurements, the mice were housed one per cage in a thermostatically controlled environment at 23±2°C and relative humidity of 50-70%. Mice were allowed free access to chow and drinking water, available ad libitum. The room was artificially illuminated from 7am to 7pm. The heart rate and mean arterial pressure were recorded from the left carotid artery using TA11PA-C20 telemetric implants as previously described, except that xylazine instead of promazine was used for anaesthesia. Mice were allowed a recovery period after surgery and data collection was started on the fifth day when the normal daily circadian rhythm of the mice had returned. The data were sampled continuously day and night for two weeks, every 5 min for 10 s. The presented values, average ± SD, are from a 24-hour period during the fifth day after the start of the measurements. No significant changes were noted in the telemetric variables, 24 h mean daily values of the blood pressure, or heart rate after the fifth day.
Measurements of mitochondrial redox state and tolerance of short-term ischemia in the isolated, perfused hearts. The mice were sacrificed by decapitation after cervical dislocation. The aorta was immediately cannulated and perfusion commenced in situ with ice-cold perfusion fluid. The heart was dissected out and perfusion continued with Krebs-Henseleit buffer consisting of 118.5 mmol/L NaCl, 4.7 mmol/L KCl, 2.5 mmol/L CaCl₂, 0.25 mmol/L Ca-EDTA, 1.2 mmol/L MgSO₄, 1.2 mM KH₂PO₄, 25 mmol/L NaHCO₃ and 10 mmol/L glucose (pH 7.4, 37°C) with a pressure of 100 cm H₂O (9.81 kPa) and the medium gassed with O₂/CO₂ (19:1).

The heart was enclosed in a thermostatic, light-tight chamber equipped with fiber optics for simultaneous epicardial readout of reflectance spectrum changes at 605 and 630 nm and fluorescence at 520 nm with excitation at 460 nm by means of a three-channel spectrophotometer-fluorometer⁹. The photometric data were collected via a data acquisition card (PCI-6024E, National Instruments) and stored on a PC at two-second intervals. Left ventricular pressure was monitored through the ventricular wall by inserting a saline-filled Teflon cannula connected to a Statham P231D pressure transducer linked to a Statham SP1400 pressure monitor. The pressure wave signal was led to a Lab-PC+ data acquisition card (National Instruments) and heart function parameters (heart rate, peak systolic pressure, diastolic pressure and peak pressure development) were calculated on-line with custom designed software and stored on a PC at 4 s intervals. Coronary flow was measured during the experiments by using a drop counter with an analog output. Oxygen consumption was calculated from the arteriovenous concentration difference multiplied by coronary flow. Venous effluent from the heart was collected in 1 min aliquots and lactate dehydrogenase washout measured as described¹⁰. The values are presented as mean ± SE.
Angiotensin II-induced pressure overload. AngII was administered to mice via subcutaneous osmotic Alzet minipumps (Scanbur AB) for 1 week (0.1 mg/kg/h). Mice were sacrificed one week later. Echocardiographic analysis of cardiac dimensions was carried out before the treatment and before sacrifice using a Vevo 779 high-resolution in vivo imaging system (VisualSonics).

Electron microscopy. Tissue samples from the left ventricle were fixed with 2% glutaraldehyde, postosmicated and embedded in epon. Semithin sections were stained with toluidine blue, and on the basis of initial analysis under light microscope, regions of interest were selected for thin 100 nm sectioning and analysis using a JEOL 1400 EX Transmission Electron Microscope equipped with Morada CCD Camera (Olympus SIS).

Lipidomic analysis of heart tissue. Hearts were perfused with PBS upon excision, dissected, and snap-frozen. Corresponding pieces (5-9 mg) were mixed with an internal standard mixture containing 1-heptadecanoyl-sn-glycero-3-phosphocholine, N-(heptadecanoyl)-sphing-4-enine, 1,2-diheptadecanoyl-sn-glycero-3-phosphocholine, 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine and 1,2,3-triheptadecanoyl-sn-glycerol at a concentration level of 0.5-1 µg/sample and 200 µl chloroform:methanol (2:1). The tissues were homogenized with grinding balls in a Mixer MILL at 25 Hz for 2 min and 50 µl of 0.9% NaCl was added. The samples were vortexed for 2 min and after 30 min standing, centrifuged at 10 000 rpm for 3 min. The labeled lipid standard mixture was added into the separated lipid extracts (1 µg/sample) before ultra performance liquid chromatography-mass spectrometric analysis.

Lipid extracts were analyzed on a Waters Q-Tof Premier mass spectrometer (Waters, Inc., Milford, MA) combined with an Acquity Ultra Performance LC™ (UPLC). The column (at 50°C) was an
Acquity UPLC™ BEH C18 10 x 50 mm with 1.7 µm particles. The solvent system included A. ultrapure water (1% 1M NH₄Ac, 0.1% HCOOH) and B. LC/MS grade acetonitrile/isopropanol (5:2, 1% 1M NH₄Ac, 0.1% HCOOH). The gradient started from 65% A / 35% B, reached 100% B in 6 min and remained there for the next 7 min. There was a 5 min re-equilibration step before the next run. The flow rate was 0.200 ml/min and the injected amount 1.0 µl (Acquity Sample Organizer, Waters, Inc., Milford, MA). Reserpine was used as the lock spray reference compound. The lipid profiling was carried out using ESI+ mode and the data was collected at mass range of m/z 300-1200 with scan duration of 0.2 sec. The data was processed by using MZmine software v. 0.60 \(^{11}\) and the lipid identification was based on an internal spectral library\(^{12}\). Results are presented as mean ± SEM.

Statistical analysis of data. Two-group comparison was made with the Student’s two-tailed t-test by using the method of summary measures\(^{13}\) when appropriate.
References


