Cardiomyocyte VEGF Regulates Endothelial Cell GPIHBP1 to Relocate Lipoprotein Lipase to the Coronary Lumen During Diabetes Mellitus

Amy Pei-Ling Chiu, Andrea Wan, Nathaniel Lal, Dahai Zhang, Fulong Wang, Israel Vlodavsky, Bahira Hussein, Brian Rodrigues

Objective—Lipoprotein lipase (LPL)—mediated triglyceride hydrolysis is the major source of fatty acid for cardiac energy. LPL, synthesized in cardiomyocytes, is translocated across endothelial cells (EC) by its transporter glycosylphosphatidylinositol-anchored high-density lipoprotein–binding protein 1 (GPIHBP1). Previously, we have reported an augmentation in coronary LPL, which was linked to an increased expression of GPIHBP1 following moderate diabetes mellitus. We examined the potential mechanism by which hyperglycemia amplifies GPIHBP1.

Approach and Results—Exposure of rat aortic EC to high glucose induced GPIHBP1 expression and amplified LPL shuttling across these cells. This effect coincided with an elevated secretion of heparanase. Incubation of EC with high glucose or latent heparanase resulted in secretion of vascular endothelial growth factor (VEGF). Primary cardiomyocytes, being a rich source of VEGF, when cocultured with EC, restored EC GPIHBP1 that is lost because of cell passaging. Furthermore, recombinant VEGF induced EC GPIHBP1 mRNA and protein expression within 24 hours, an effect that could be prevented by a VEGF neutralizing antibody. This VEGF-induced increase in GPIHBP1 was through Notch signaling that encompassed Delta-like ligand 4 augmentation and nuclear translocation of the Notch intracellular domain. Finally, cardiomyocytes from severely diabetic animals exhibiting attenuation of VEGF were unable to increase EC GPIHBP1 expression and had lower LPL activity at the vascular lumen in perfused hearts.

Conclusion—EC, as the first responders to hyperglycemia, can release heparanase to liberate myocyte VEGF. This growth factor, by activating EC Notch signaling, is responsible for facilitating GPIHBP1-mediated translocation of LPL across EC and regulating LPL-derived fatty acid delivery to the cardiomyocytes. (Arterioscler Thromb Vasc Biol. 2016;36:145-155. DOI: 10.1161/ATVBAHA.115.306774.)

Key Words: heparanase ▪ hyperglycemia ▪ lipoprotein lipase ▪ receptors, Notch ▪ triglycerides

Cardiac muscle requires an abundant supply of energy, provided by oxidation of numerous substrates, primarily fatty acids (FA), and carbohydrates. In the normal heart, nearly 30% of ATP is generated from glucose and lactate, whereas the remaining 70% is produced from FA oxidation. The heart has a limited capacity to synthesize FA, and thus requires an abundance of this substrate. Impaired glucose uptake and oxidation are impaired. Accordingly, the heart switches to almost exclusive FA use and hence requires an abundance of this substrate. Multiple adaptive mechanisms, either whole body or intrinsic...
to the heart, operate to make this achievable. The former include augmented adipose tissue lipolysis (breakdown of stored triglyceride increases circulating FA) and greater plasma hepatic very low-density lipoprotein–triglyceride availability.12 Innate to the cardiac muscle, the uptake of FA is driven by membrane FA transporters (eg, CD36), which are increased after diabetes mellitus.13 Diabetes mellitus also enhances adipose triglyceride lipase, which mobilizes stored triglyceride within cardiomyocytes.14 Finally, we were the first to report a robust expansion in coronary LPL after acute moderate diabetes mellitus.15 This increase in LPL was immediate, unrelated to gene expression, and included exaggerated processing to dimeric, catalytically active enzyme, an obligatory step for ensuing secretion.16 Transfer to the coronary lumen requires movement of LPL to the cardiomyocyte plasma membrane by AMP-activated protein kinase, protein kinase D, and p38 MAPK. Activation of these kinases after diabetes mellitus facilitates LPL vesicle formation, in addition to promoting cytoskeletal rearrangement for secretion onto surface HSPG.17 For its onward movement across the interstitial space to the apical side of vascular EC, detachment of LPL from the myocyte surface is a prerequisite and is likely mediated by enzymatic cleavage of cardiomyocyte surface HSPG by heparanase. We determined that in response to hyperglycemia, ECs secrete active heparanase, an endoglycosidase exceptional in its ability to instigate LPL release.18 What remained unresolved is how this released LPL is transferred from the basolateral to the apical (luminal) side of EC, where the enzyme is functional. Recently, we reported that EC respond to hyperglycemia by increasing GPIHBP1 by mechanisms that are currently unclear. Data from this study suggest a novel mechanism in which heparanase-induced release of vascular endothelial growth factor (VEGF) and activation of Notch signaling is responsible for enhanced GPIHBP1 expression to regulate FA delivery and utilization by the cardiomyocyte.

Materials and Methods
Materials and Methods are available in the online-only Data Supplement.

Results
High Glucose Alters the Expression of Endothelial GPIHBP1
GPIHBP1 is expressed exclusively in ECs,9 and we have previously reported its increase in hearts from diabetic animals.19 To mimic in vivo hyperglycemia, rat aortic ECs were exposed to high glucose. Interestingly, high glucose increased GPIHBP1 mRNA (Figure 1A, left) and protein (Figure 1A, right panel) expression within 12 hours. Notably, this increase in GPIHBP1 was responsible for an amplification of LPL shuffling across EC (Figure 1A, inset). High glucose is an effective stimulus for endothelial heparanase secretion.10 Exposure of rat aortic ECs to high glucose rapidly increased the secretion of both active and latent heparanase into the incubation medium (Figure 1B). The osmolality control mannitol had no effect on medium heparanase (data not shown). Tumor cells, by expressing higher levels of heparanase, are more invasive as secreted heparanase can breakdown extracellular matrix in addition to promoting gene expression related to an invasive phenotype.20 To test this latter property, EC were exposed to heparanase. There was a substantial increase of GPIHBP1 mRNA and protein within 24 hours of incubation with either active (Figure I in the online-only Data Supplement) or latent (Figure 1C) heparanase, suggesting that heparanase may mediate the effects of high glucose on EC GPIHBP1 expression.

Effect of Heparanase on GPIHBP1 Expression Is Associated With VEGF
Cell surface HSPGs anchor a number of different proteins,21 including growth factors, such as VEGF,22 and heparanase is an endoglycosidase exceptional in its ability to instigate release of these bound ligands.23 Using a protein array and EC exposed to high glucose or latent heparanase, we observed secretion of VEGF into the culture medium (Figure 2A, circle). Given a recent unanticipated function of VEGF in regulating EC FA transport,24 we incubated EC with recombinant VEGF and discovered an induction of GPIHBP1 mRNA (Figure 2B, left) and protein (Figure 2B, right) expression within 24 hours. This increased EC GPIHBP1 in response to VEGF was predominantly membrane bound (Figure 2C). To substantiate this observation, we used a specific antibody to inhibit VEGF. As anticipated, GPIHBP1 expression and LPL translocation induced by VEGF were prevented (Figure 2D), suggesting that this growth factor is an effective stimulus for GPIHBP1 expression.

Cardiomyocyte Surface-Bound VEGF Stimulates EC GPIHBP1
GPIHBP1 is highly expressed in EC in vivo. However, EC in culture lose their ability to express this glycoprotein.25 As EC in the heart are closely appositioned to cardiomyocytes, we reasoned that a paracrine influence from cardiomyocytes may be responsible for EC expression of GPIHBP1. To test this possibility, we used a coculture system to mimic the intact heart and determine whether a simple introduction of cardiomyocytes into the vicinity of EC can restore GPIHBP1 expression. As anticipated, an increased EC GPIHBP1 was found in the coculture group (Figure 3A), suggesting paracrine factors from cardiomyocytes influence GPIHBP1 expression. Like EC, myocyte HSPG-anchored proteins can be liberated by heparanase.26 Using a protein array, we also observed secretion of VEGF from myocytes after treatment with latent heparanase (Figure 3B, left panel circle), an effect that was more robust
than heparanase liberation of VEGF from EC (Figure 2A).
Interestingly, this was also reflected in mRNA expression; cardiomyocytes had a stronger VEGF signal compared with EC (Figure 3B, right). More importantly, cardiomyocytes had a significant amount of VEGF located on the cell surface, a pool that could be easily and rapidly (within 15 minutes) displaced by latent heparanase. This effect of latent heparanase on VEGF was likely through ionic displacement, and surprisingly, much more emphatic than heparin (Figure 3C). Confirmation of the influence of myocyte cell surface VEGF on GPIHB1 induction was achieved using heparitinase to remove cardiomyocyte surface HSPG before coculture with EC. This procedure essentially prevented the induction of GPIHB1 and LPL translocation (Figure 3D). As antibodies against both VEGF and its

Figure 1. High glucose–induced increase in glycosylphosphatidylinositol-anchored high-density lipoprotein–binding protein 1 (GPIHB1) expression is associated with secretion of heparanase. Rat aortic endothelial cells (RAOECs) from passage 5 to 8 were treated with 5.5 (control, CON) or 25 (high glucose, HG) mmol/L glucose for 12 h. GPIHB1 mRNA (A, left) and protein (A, right) were determined using TaqMan and Western blot, respectively. In a separate experiment, RAOECs were seeded on transwell inserts and grown until they formed a tight monolayer before treatment with 5.5 or 25 mmol/L glucose for 12 h. These transwell inserts were then placed in a different 6-well plate with DMEM medium containing 10 µg/mL purified lipoprotein lipase (LPL) on the basolateral side. After incubation for 1 h and washing both the apical and basolateral sides (3×) with PBS, DMEM medium–containing heparin (8 U/mL) was used for 3 min to release the apical surface-bound LPL. LPL activity was determined by measuring the in vitro hydrolysis of [3H]triolein substrate, whereas LPL mass was determined using Western blot (A, inset). To measure high glucose release of heparanase, RAOECs were seeded and grown until 80% to 90% confluence, treated with 5.5 or 25 mmol/L glucose for 15 min, and medium collected. The medium was concentrated using an Amicon column at 4°C and subsequently centrifuged for 10 min at 14000g. Active (A-HPA) and latent (L-HPA) heparanase in this concentrated medium were determined by Western blot (B). RAOECs were also incubated with or without recombinant L-HPA (500 ng/mL) for 12 and 24 h, cell lysates were collected, and GPIHB1 mRNA (C, left) and protein (C, right) were determined. *P<0.05, **P<0.01 compared with CON or 0 h, n=3 to 4.
receptor had identical effects on GPIHBP1 expression and LPL translocation as seen with heparitinase (Figure 3E), our data suggest that cardiomyocyte HSPG-bound VEGF is a crucial paracrine factor that regulates EC GPIHBP1 expression.

GPIHBP1 Induction by VEGF Is Through a Delta-Like Ligand 4–Notch Signaling Pathway
Delta-like ligand 4 (DLL4) is a transmembrane ligand for the Notch receptor,26 a key regulator of metabolism.27 As a recent...
report indicated that Notch participates in VEGF regulation of EC FA transport, we tested whether the VEGF-induced GPIHBP1 expression is through the activation of Notch signaling. In response to the angiogenic growth factor, we observed an increased expression of DLL4 (Figure 4A). To investigate the contribution of the DLL4 pathway to GPIHBP1 expression, EC were grown on plates coated with recombinant DLL4. In addition to the traditional downstream target genes (Hey-1 and Hes-1) of the DLL4-Notch pathway (Figure IIA in the online-only Data Supplement) that were upregulated, we discovered a significant augmentation in both the mRNA and protein of GPIHBP1 (Figure 4B). The increase in GPIHBP1 with DLL4 was predominantly membrane bound (Figure 4C). As a specific antibody against DLL4 reduced the increased...
expression of GPIHBP1 observed following DLL4 stimulus (Figure 4D; Figure IIB in the online-only Data Supplement) or VEGF (Figure III in the online-only Data Supplement), our data suggest that the effect of VEGF on GPIHBP1 induction is through DLL4-Notch signaling.

**VEGF-Induced GPIHBP1 Expression Is Decreased by Inhibition of Notch Signaling**

The Notch receptor is a transmembrane protein composed of an extracellular, a transmembrane, and a Notch intracellular domain (NICD). On ligand binding, the Notch receptor undergoes proteolytic cleavage and releases NICD, which enters the nucleus and modifies gene expression.26,27 To substantiate that Notch signaling is involved in induction of GPIHBP1 after VEGF stimulation, we attempted to inhibit NICD translocation using DAPT. As anticipated, the γ-secretase inhibitor reduced NICD (Figure 5A, left), lowered its entry into the nucleus (Figure 5B), and decreased expression of GPIHBP1 (Figure 5A, right panel). Repeating this experiment in our coculture showed similar results. Thus, pretreatment of EC with DAPT, before coculture with cardiomyocytes, lowered NICD (Figure 5C, left) and prevented the increase in GPIHBP1 (Figure 5C, right). These results suggest that the effect of VEGF on GPIHBP1 is through Notch receptor signaling.

**Reduction in Cardiomyocyte VEGF After Diabetes Mellitus Is Associated With a Decrease in GPIHBP1 Expression and LPL Activity at the Vascular Lumen**

Downregulation of myocardial VEGF is a feature of experimental diabetes mellitus.28,29 We used 100 mg/kg streptozotocin (D100) to generate a rat model of severe diabetes mellitus that exhibited both hyperglycemia and hyperlipidemia (Figure IV in the online-only Data Supplement). Diabetic animals demonstrated a robust decrease in serum VEGF (Figure 6A, inset), whereas in hearts isolated from these animals, a significant reduction in VEGF mRNA was observed (Figure 6A). This decrease in VEGF was also reflected in cardiomyocytes from D100 animals. Thus, the myocyte surface pool of VEGF, as determined by release with either heparin or latent heparanase, was significantly lower in D100 compared with control (Figure 6B). To test the hypothesis that diabetic
Cardiomyocytes exhibiting low expression of VEGF on the cell surface may influence the LPL transporter GPIHBP1, we cocultured these cells with EC. Without cardiomyocytes, EC had limited GPIHBP1 expression in vitro, whereas coculture with control myocytes caused a robust induction of GPIHBP1. Interestingly, we found a reduction of GPIHBP1 expression (Figure 6C) with a decrease of NICD (Figure V in the online-only Data Supplement) on coculturing EC with myocytes from the D100 group, suggesting that VEGF on the myocyte surface is important for GPIHBP1 expression. This in vitro data could explain the decrease in LPL activity and mass at the vascular lumen (Figure 6C, inset).

**Discussion**

In diabetes mellitus, when glucose uptake and oxidation are impaired, the heart relies exclusively on FA to generate energy. Given that the molar concentration of FA in lipoprotein–triglyceride is ≈10-fold greater than albumin-bound circulating FA, LPL-mediated hydrolysis of circulating triglyceride is suggested to be the dominant source of FA for cardiac utilization during diabetes mellitus. Previously, we have reported a robust augmentation in coronary LPL activity after acute moderate diabetes mellitus and linked this observation to an increased expression of EC GPIHBP1,

![Diagram](image-url)
The current study examined the potential mechanism by which hyperglycemia amplifies GPIHBP1. Our data describe a crosstalk where high glucose–induced heparanase secretion from EC releases VEGF from cardiomyocytes. The subsequent increase in EC GPIHBP1 is the adaptation required to enhance FA delivery to, and utilization by, the cardiomyocyte during acute diabetes mellitus. With increased severity or duration of diabetes mellitus, the loss of myocyte...
VEGF is associated with a reduction in GPIHBP1, a prelude to a fall in vascular luminal LPL.

Hydrolysis of triglyceride at the vascular lumen is a function of LPL. However, EC at this location has limited expression of this enzyme.2 In the heart, LPL is synthesized in the cardiomyocytes3 and is delivered across the EC by GPIHBP1.4 After moderate diabetes mellitus and an increase in vascular LPL, we previously reported that this effect could be explained by a hyperglycemia-induced increase in GPIHBP1 expression.10 Although the DAG–PKC (diacylglycerol–protein kinase C) pathway has been implicated in a direct hyperglycemia-induced gene expression of other proteins,31 we tested whether an indirect mechanism could also explain this increase in GPIHBP1 by high glucose. We focused on heparanase as tumor cells transform the phenotype of neighboring cells by feeding them heparanase that is capable of modulating their gene expression.32 In addition, high glucose is a strong stimulus for secretion of heparanase from EC.15 In this study, incubating EC in high glucose triggered a rapid release of both active and inactive (latent) heparanase into the medium. Although the HS hydrolyzing ability of active heparanase is well established and can release surface-bound proteins, latent heparanase also has some remarkable properties, including a greater competence for releasing myocyte surface VEGF when compared with active heparanase.33 Interestingly, EC exposed to recombinant latent heparanase showed a substantial time-dependent increase in GPIHBP1 gene and protein. As this effect was also duplicated by active heparanase, our data suggest that high glucose secretion of heparanase could be the unforeseen mechanism by which the EC can increase its expression of GPIHBP1 after diabetes mellitus.

HSPG functions not only as a structural protein but also as an anchor to several proteins that are bound electrostatically to its HS side chains.2,34 Attachment of these bioactive proteins (chemokines, coagulation factors, enzymes like LPL, and growth factors, such as VEGF)24 is a clever arrangement, providing the cell with a rapidly accessible reservoir, precluding the need for de novo synthesis when the requirement for a protein is increased. Given that latent heparanase has a greater competence for liberating cell surface protein when compared with active heparanase,35 we used a cytokine array and found that several proteins increased in the culture medium after incubation of EC with high glucose or latent heparanase. We focused on VEGF as this protein not only regulates angiogenesis35 (which provides the heart with sufficient oxygen to accommodate FA oxidation) but also plays a significant role in FA metabolism.24 The latter effect is through promotion of FA-binding protein 4 expression,24 a FA transporting protein abundantly expressed in microvascular EC in the heart. Interestingly, EC exposed to recombinant VEGF displayed a substantial time-dependent increase in GPIHBP1 gene and protein, effects that were prevented by a VEGF-specific neutralizing antibody. As cardiomyocytes have a stronger VEGF receptor extracellular domain followed by the release of the intracellular domain (NICD), which enters the nucleus and acts as transcription factor to regulate gene expression,27 we tested whether this pathway can regulate FA delivery by controlling the LPL transporter GPIHBP1. Incubation of EC with VEGF increased DLL4 expression. Intriguingly, recombinant DLL4 augmented GPIHBP1 gene and protein, effects that were prevented by a DLL4-specific neutralizing antibody. The increase in GPIHBP1 by VEGF was associated with an induction of NICD and its nuclear translocation, effects that were inhibited by a γ-secretase inhibitor. As coculturing of EC with cardiomyocytes induced similar effects, our data suggest that VEGF is a robust stimulus for GPIHBP1 and does so through Notch receptor signaling.

Attenuated VEGF production in myocardial tissue is a common feature in both patients and animal models of chronic diabetes mellitus.29,30 We generated a rat model of severe diabetes mellitus, where in addition to hyperglycemia, the animals also demonstrate severe hyperlipidemia. In cardiomyocytes from these animals, we observed a reduction in VEGF gene expression, an outcome that was reflected in a decrease in the myocyte surface-bound VEGF pool. Interestingly, coculture of EC with these myocytes that exhibit muted VEGF caused a significant reduction in the expression GPIHBP1. This effect coincided with a decrease in LPL activity at the coronary lumen of the intact heart from these severely diabetic animals. Unlike the model of severe diabetes mellitus (100 mg/kg streptozotocin), moderate (55 mg/kg streptozotocin) diabetes mellitus induced an increase in LPL activity at the coronary vascular lumen (ref), an effect that was associated with elevated VEGF at the myocyte surface (Figure VI in the online-only Data Supplement). Our results implicate a downregulation of myocyte VEGF as a mechanism to decrease EC GPIHBP1, and by extension, vascular luminal LPL. This could serve as a protective mechanism by which the heart can avoid lipid overload.37

Overall, our data suggest that EC, as the first responders to hyperglycemia, can release heparanase to liberate myocyte VEGF. This growth factor, by activating EC Notch signaling, is responsible for facilitating translocation of LPL across EC and regulating LPL-derived FA delivery to the cardiomyocytes. Although this mechanism serves to guarantee FA supply when glucose utilization is compromised, it unintentionally provides a surfeit of FA to the diabetic heart,
sponsoring a setting where FA uptake exceeds the mitochondrial oxidative capacity. The resulting increase in the conversion of FA to potentially toxic FA metabolites, including ceramides, diacylglycerols, and acylceramides, paired with increased formation of reactive oxygen species secondary to elevated FA oxidation, can provoke cardiac cell death (lipotoxicity). Not surprisingly then, cardiac-specific over-expression of LPL causes severe myopathy characterized by lipid oversupply and deposition, muscle fiber degeneration, excessive dilatation, and impaired left ventricular function in the absence of vascular defects, a situation comparable with diabetes. The present study is supported by an operating grant from the Diabetes Association. We are indebted to Paul Hiebert for his help with the graphics. Not surprisingly then, cardiac-specific over-expression of LPL causes severe myopathy characterized by lipid oversupply and deposition, muscle fiber degeneration, excessive dilatation, and impaired left ventricular function in the absence of vascular defects, a situation comparable with diabetes. The present study is supported by an operating grant from the Diabetes Association. We are indebted to Paul Hiebert for his help with the graphics.

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Disclosures

None.

References


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Being the first responders to hyperglycemia, endothelial cells release heparanase to liberate myocyte vascular endothelial growth factor. Vascular endothelial growth factor, by activating endothelial cell Notch signaling, is responsible for lipoprotein lipase translocation across the endothelial cells and regulating lipoprotein lipase–derived fatty acid (FA) delivery to the cardiomyocytes. Although this serves to guarantee FA supply when glucose utilization is compromised, it unintentionally provides a surfeit of FA to the diabetic heart, sponsoring a setting where FA uptake exceeds the mitochondrial oxidative capacity. The resulting increase in FA conversion to potentially toxic metabolites, including ceramides, diacylglycerols, and acylcarnitines, paired with increased formation of reactive oxygen species secondary to elevated FA oxidation, can provoke cell death (lipotoxicity). Paradoxically, with severe or chronic diabetes mellitus, attenuation of myocyte vascular endothelial growth factor will not only compromise angiogenesis but also orchestrate diminished lipoprotein lipase–derived FA supply to the myocardium to cause cardiac failure (neither glucose nor albumin-bound FA can replace the action of lipoprotein lipase).
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Materials and Methods

Experimental animals
The investigation adheres to the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH) and the University of British Columbia. Adult male Wistar rats (250-320 g) were injected with a single dose of streptozotocin (STZ, i.v.), a β cell-specific toxin, which induces hypoinsulinemia. We use different doses of STZ [55 (D55) or 100 (D100) mg/kg] to cause moderate and severe diabetes respectively, and the animals kept for 4-7 days. With D55, animals are insulin deficient, and luminal LPL activity is augmented. Unlike D55, D100 animals also develop hyperlipidemia, and demonstrate a decline in vascular LPL.\(^1,2\) Hyperglycemia was confirmed in blood samples from the tail vein using a glucometer (AccuSoft) and glucose test strips (Accu-Chek Advantage; Roche). In this study, STZ animals were kept for 4 days before hearts were removed for cardiomyocyte isolation.

Serum measurements
Blood samples were collected from animals before termination and serum isolated using centrifugation. Concentrations of triglyceride (Stanbio) and VEGF (R&D) were determined using ELISA diagnostic kits.

Endothelial cell and cardiomyocyte co-culture
Rat aortic endothelial cells (RAOEC, Cell Applications INC.) from passages 6-10 were cultured in growth medium at 37°C in a 5% CO\(_2\) humidified incubator. For co-culture with cardiomyocytes, ECs were seeded on transwell inserts (Falcon, 24 mm diameter; 1.0 µm pore size). On reaching 80-90% confluence, these inserts were placed in a 6-well plate that had cardiomyocytes attached to the bottom. Cardiomyocytes were plated at a density of 200,000 cells/well.

Isolation of cardiomyocytes
Adult male Wistar rats were anesthetized with 65 mg/kg sodium pentobarbital i.p., the thoracic cavity opened, and hearts carefully excised. After cannulation of the aorta, the heart was secured by tying below the innominate artery and retrogradely perfused with Krebs-Henseleit buffer.\(^3\) Ventricular calcium-tolerant myocytes were prepared by a previously described procedure.\(^4\) Briefly, myocytes were made calcium tolerant by successive exposure to increasing concentrations of calcium. Cardiomyocytes were plated at a density of 200,000 cells/well on laminin-coated 6-well culture plates. Cells were maintained in Medium-199 and incubated at 37°C in a 5% CO\(_2\) humidified incubator.

LPL transport and activity assay
To study basolateral to apical transport of LPL, EC were grown on transwell inserts until the cells formed a tight monolayer. The inserts were then placed in 6-well plates. LPL (10 µg/ml) was then added to the bottom chamber. After 1 hour, LPL that translocated to the apical surface of EC was collected by displacement using heparin (8 U/ml) for 3 minutes, and LPL activity determined. LPL activity was determined by measuring \textit{in vitro} hydrolysis of \[^3\text{H}\]triolein substrate.\(^5\) Radiolabeled FA products were extracted and estimated by liquid scintillation counting.
RNA isolation and real-time PCR
Total RNA was isolated from cardiomyocytes or ECs using Trizol, followed by chloroform and isopropanol extraction, washing by ethanol and dissolving in DEPC-H₂O. RNA was reverse transcribed into cDNA with a mixture of primers, oligo-dT, and SuperScript III. cDNA were amplified by TaqMan probes (GPIHBP1, VEGF, Hes-1, Hey-1, and β-actin) in triplicate, using a 7900HT Fast Real-Time PCR system. Gene expression was calculated with the comparative cycle threshold (ΔΔCT) method.

Western blot
To measure protein expression, 20-40 µg proteins were loaded. Membranes were incubated with primary antibodies (1:1000 for anti-GPIHBP1, anti-heparanase, anti-DLL4 and anti-NICD, 1:2000 for anti-β-actin) at 4°C overnight, and subsequently with secondary antibodies (1:2000) at room temperature for 1 hour. To measure medium heparanase, protein was concentrated with an Amicon centrifuge filter (Millipore), followed by the procedures listed above. Protein expression was expressed using β-actin as the loading control.

Rat cytokine antibody array
Medium was collected from EC incubated with either DMEM containing glucose (5.5 or 25 mM) or purified latent heparanase (500 ng/ml) for 12 hours. Medium was also obtained from myocytes incubated with DMEM containing latent heparanase for 12 hours. This medium was centrifuged at 2000 rpm for 5 minutes. Briefly, array membranes were pre-incubated with blocking buffer at room temperature for 1 hour, and then incubated with the above EC or myocyte culture medium for 2 hours. Membranes were washed, incubated with biotinylated antibody, and subsequently streptavidin. Detection of signals was achieved with ECL and Hyperfilm ECL Film (GE Healthcare).

Immunofluorescence staining
To visualize CD31, GPIHBP1 and NICD localization in EC in response to VEGF, cells were seeded on coverslips and grown until 80-90% confluent. Following treatment with or without VEGF (100 ng/ml), cells were washed with cold PBS, and fixed with methanol at 4°C for 1 hour. Following permeabilization with 0.2% Triton X-100 in PBS for 30 minutes, slides were incubated with blocking buffer containing 10% goat serum and 0.2% Triton X-100 in PBS for 30 minutes at room temperature. Coverslips were then incubated with primary antibodies (1:400 for anti-CD31, anti-GPIHBP1 and anti-NICD) at 4°C overnight. Secondary antibodies were used at a dilution of 1:400 and incubated at room temperature for 1 hour. In some experiments, to detect CD31 and GPIHBP1 in EC in response to DLL4, cells were seeded on coverslips pre-coated with recombinant DLL4 (100 ng/ml) for 12 hours, and the above procedures (wash, fixation, permeabilization, blocking, primary/secondary antibodies) repeated.
VEGF ELISA
Medium was collected from cardiomyocytes incubated with either heparin (5 U/ml) or purified latent heparanase (1 or 2 µg/ml) for 15 minutes. This medium was centrifuged at 2000 rpm for 5 minutes, and VEGF concentration determined using Rat VEGF Quantikine ELISA Kit (R&D). Briefly, the sample was incubated in each well containing assay diluent at room temperature for 2 hours. After incubation, each well was washed, conjugated buffer added for 1 hour and subsequently substrates for 30 minutes. Using stop-solution to terminate the reaction, absorbance readings minus background were converted to picograms using standard curves from the kit.

Reagents and antibodies
Chloroform, N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) and isopropanol were purchased from Sigma-Aldrich. Heparin (HEPALEAN, 1000 U/ml) was from Organon, Canada. [3H]-triolein was purchased from Amersham Canada. Anti-heparanase antibody mAb 130 was from InSight (Rehovot, Israel). Anti-GPIHBP1, anti-VEGF, anti-DLL4 and anti-NICD antibodies were obtained from Abcam. Anti-PECAM (CD31) was from Chemicon (Millipore). Anti-β actin (C4), goat anti-mouse and goat anti-rabbit antibodies were purchased from Santa Cruz Biotechnology. Purified active and latent heparanase were a kind gift from Dr. Israel Vlodavsky, Hadassah University Hospital, Jerusalem, Israel. Recombinant VEGF and DLL4 were purchased from R&D. Alexa 555-labeled anti-mouse IgG, Alexa 488-labeled anti-rabbit IgG, Trizol, SuperScript III RT, GPIHBP1 (Rn01503971_g1), VEGF (Rn01511601_m1), Hes-1 (Rn00577566_m1), Hey-1 (Rn00468865_m1), Actin (Rn00667869_m1) probe, and TaqMan fast advanced master mix were obtained from Invitrogen.

Statistical analysis
Values are means ± SE. Wherever appropriate, Mann-Whitney and one-way ANOVA (followed by the Bonferroni test) were used to determine differences between group mean values. The level of statistical significance was set at $P < 0.05$. 
References
Fig. SI
**Fig. S11**

**A**

Bar chart showing gene expression (fold change) for Hey-1 and Hes-1:
- **CON**
- **DLL4**

**B**

Western blot analysis of GPIHBP1 and β-Actin:
- **-**
- **+ IgG**
- **+ VEGF**
- **+ DLL4 Ab**

Bar chart showing GPIHBP1 protein (fold change):
- **CON**
- **DLL4**

* indicates statistical significance.
Fig. SIII
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<th>CON</th>
<th>D100</th>
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<td>Insulin (ng/ml)</td>
<td>2.3 ± 0.4</td>
<td>0.6 ± 0.1 **</td>
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<td>Glucose (mM)</td>
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<td>28.2 ± 4.7 *</td>
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<td>TG (mM)</td>
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<td>2.7 ± 1.0 **</td>
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<td>NEFA (mM)</td>
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Fig. SIV
Fig. SVI