Endothelial Heparanase Regulates Heart Metabolism by Stimulating Lipoprotein Lipase Secretion From Cardiomyocytes

Ying Wang, Dahai Zhang, Amy Pei-Ling Chiu, Andrea Wan, Katharina Neumaier, Israel Vlodavsky, Brian Rodrigues

Objective—After diabetes mellitus, transfer of lipoprotein lipase (LPL) from cardiomyocytes to the coronary lumen increases, and this requires liberation of LPL from the myocyte surface heparan sulfate proteoglycans with subsequent replenishment of this reservoir. At the lumen, LPL breaks down triglyceride to meet the increased demand of the heart for fatty acid. Here, we examined the contribution of coronary endothelial cells (ECs) toward regulation of cardiomyocyte LPL secretion.

Approach and Results—Bovine coronary artery ECs were exposed to high glucose, and the conditioned medium was used to treat cardiomyocytes. EC-conditioned medium liberated LPL from the myocyte surface, in addition to facilitating its replenishment. This effect was attributed to the increased heparanase content in EC-conditioned medium. Of the 2 forms of heparanase secreted from EC in response to high glucose, active heparanase released LPL from the myocyte surface, whereas latent heparanase stimulated reloading of LPL from an intracellular pool via heparan sulfate proteoglycan–mediated RhoA activation.

Conclusions—Endothelial heparanase is a participant in facilitating LPL increase at the coronary lumen. These observations provide an insight into the cross-talk between ECs and cardiomyocytes to regulate cardiac metabolism after diabetes mellitus. (Arterioscler Thromb Vasc Biol. 2013;33:894-902.)

Key Words: heart ■ heparanase ■ high glucose ■ lipoprotein lipase ■ RhoA

After diabetes mellitus, the heart has an increased reliance on fatty acids (FAs) for generation of energy.1 The majority of these FAs come from breakdown of circulating triglyceride-rich lipoproteins, a process catalyzed by lipoprotein lipase (LPL) located at the luminal side of vascular endothelial cells (ECs).2–4 ECs do not express LPL. In the heart, LPL is synthesized in cardiomyocytes and secreted to heparan sulfate proteoglycans (HSPGs).8 We have shown that increased coronary LPL in animal models of type 1 diabetes mellitus, an effect that was evident in the absence of any change in myocyte LPL gene expression.6,7 We concluded that the augmented coronary LPL was a result of increased secretion of the enzyme from myocytes toward the coronary lumen. Regarding secretion, LPL is first transported from an intracellular pool in the myocyte to the cell surface, where it binds to heparan sulfate proteoglycans (HSPGs).8 We have shown that this intracellular transport depends on actin cytoskeleton polymerization, a process that is magnified after diabetes mellitus.9,10 The subsequent process by which myocyte surface LPL is translocated to the coronary lumen, in addition to its replenishment after this onward movement, has not been completely elucidated.

Myocyte surface HSPGs serve as a temporary docking site and an auxiliary reservoir of LPL. HSPGs are proteoglycans bearing HS side chains attached to specific serine residues of a protein core.11 Core proteins can be attached to the cell surface through a glycosylphosphatidyl inositol anchor in case of glypican, or can traverse the membrane as observed with the syndecan family.12,13 The HS side chains are polymers of repeating disaccharides which interact with multiple ligands, including antithrombin, fibroblast growth factor, and LPL.14–16 Thus, for LPL to translocate from myocytes to the vascular lumen, cleavage of myocyte surface HSPGs is required to release the sequestered LPL. It should be noted that in addition to its ligand binding property, any change in the conformation of HSPGs can also induce intracellular signals. For example, clustering of syndecan-4 can stimulate protein kinase C-α (PKCα),17 whereas shear stress results in ERK1/2 activation through HSPG-mediated mechanotransduction.18 Thus, it is possible that with cleavage of myocyte surface HSPGs and...
the resultant release of LPL, intracellular signals could be generated in myocytes to help reload this surface LPL pool.

Heparanase is an endoglucuronidase of special interest because it can cleave HS at low-sulfation sites, liberating sequestered ligands from surface HSPGs. It has also been shown that through surface HSPGs, heparanase can trigger intracellular signal pathways including Src, Akt, and p38 MAPK. Hence, heparanase could help LPL secretion by (1) releasing sequestered LPL from myocyte surface for onward movement to the vascular lumen and (2) provoking signals in myocytes to move LPL from an intracellular pool to replenish the surface reservoir. In the heart, heparanase is synthesized in ECs as a latent 65-kDa form, and processed in lysosomes to become a 50-kDa active enzyme. Interestingly, increased secretion of active heparanase is evident from EC in response to high glucose. Thus, after hyperglycemia, these cells could promote LPL secretion from cardiomyocytes via heparanase. The present study investigated the role of endothelial heparanase in mediating the cross-talk between EC and cardiomyocytes to increase LPL secretion after hyperglycemia.

Materials and Methods

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

Results

RhoA Activation Is Involved in Increasing Cardiomyocyte LPL Secretion

Animals injected with diazoxide (DZ) developed hyperglycemia within 1 hour after injection, and blood glucose remained high at 4 hours (control, 6.5±0.6; DZ, 19.2±2.7 mmol/L, P<0.01). LPL at the coronary lumen determines FA delivery to the underlying myocytes. We perfused hearts with heparin to release LPL from this location, and consistent with our previous observations, LPL activity was increased in hyperglycemic hearts (Figure 1A). Within 30 minutes, 4 U insulin effectively inhibited the development of hyperglycemia in DZ animals (DZ+In, 7.6±1.4; DZ, 21.5±4.2 mmol/L, P<0.01), and normal glycemia remained until the animals were killed. Insulin attenuated the increase of coronary LPL activity in these DZ hyperglycemic animals (Figure 1A). As RhoA has been reported to regulate actin cytoskeleton remodeling, an event that could affect LPL secretion, we tested RhoA activation in diabetic hearts. On activation, RhoA shifts from the cytosolic to the particulate fraction and binds to GTP. In DZ hearts, an increased RhoA in the particulate fraction was observed, which was inhibited by insulin treatment (Figure 1B). Similar to DZ animals, D55 animals also have higher coronary LPL activity, which was accompanied by RhoA activation in these hearts (Figure I in the online-only Data Supplement). To determine
whether RhoA activation can induce LPL secretion from myocytes in vitro, cells were incubated with lysophosphatidic acid (LPA). After 2 hours of LPA, an increased amount of LPL was released into the medium (Figure 1C). RhoA activation by LPA was confirmed in cardiomyocyte as GTP-RhoA increased immediately 1 minute after LPA, and declined with time (Figure 1C, inset). One effect of RhoA activation is actin cytoskeleton polymerization, which we observed as an increase in the formation of F actin in the presence of LPA (Figure 1D). Given that LPL secretion relies on stress fibers to move from an intracellular pool to the myocyte surface, we inhibited actin cytoskeleton polymerization using Cy and found that the impact of LPA on LPL secretion was abolished (Figure 1E). As the effect of LPA on LPL secretion was reproducible in myocytes in which protein synthesis was inhibited (Figure 1E, inset, right panel), our data suggest that the increased LPL secretion observed with LPA is not a consequence of augmented protein synthesis, but likely attributable to increased LPL trafficking. Thus, RhoA activation could contribute toward augmented LPL secretion, possibly via actin cytoskeleton remodeling.

**ECCM Stimulates LPL Secretion From Cardiomyocytes**

After diabetes mellitus, ECs are the first cells exposed to hyperglycemia and could potentially release multiple factors

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**Figure 2.** Endothelial cell–conditioned media (ECCM) increases lipoprotein lipase (LPL) secretion from cardiomyocytes. ECCM was applied to cardiomyocytes, and at the indicated times, LPL activity released into the medium determined. Cardiomyocytes were also incubated with 5.5 mmol/L glucose (normal glucose [NG]) and 25 mmol/L glucose (high glucose [HG]) as controls (CON; A). After 30 minutes incubation with NG or ECCM, LPL remaining on the myocyte surface was released by incubating cells with 8 U/mL heparin for 3 minutes, and LPL activity was determined (A, inset, top). Cardiomyocytes were treated with 5.5 mmol/L glucose DMEM (CON) or ECCM for 30 minutes, and the medium was collected. This medium was then incubated with increasing concentrations of very low density lipoprotein-triglyceride (0–0.8 mmol/L) and the concentration of released free fatty acids determined after 30 minutes (A, inset, bottom). *P<0.05, **P<0.01, compared with NG, n=3 to 5. Isolated cardiomyocytes were treated with 5.5 mmol/L glucose (CON), ECCM, or 10 mU/mL heparin for 30 minutes. After this, surface LPL were depleted by incubating these cells with 8 U/mL heparin for 3 minutes. To validate depletion of this surface LPL, 8 U/mL heparin was given and surface LPL activity determined (B, left). Following a wash with PBS, cells were allowed to recover in 5.5 mmol/L glucose DMEM for 2 hours. LPL activity recruited to the myocyte surface was then determined (B, right). *P<0.05, compared with CON, n=3.
affecting cardiomyocyte metabolism. For this reason, we incubated myocytes with high glucose–treated EC–conditioned medium (ECCM). Interestingly, ECCM released myocyte LPL within 30 minutes, an effect that was more significant after 1 hour. High glucose itself had no impact on LPL (Figure 2A). As this increase in medium LPL was accompanied by a reciprocal decrease in LPL activity remaining at the myocyte surface (Figure 2A, inset, upper panel), we concluded that ECCM is capable of releasing myocyte surface LPL. Importantly, this ECCM-released LPL was catalytically active and able to breakdown exogenous very low density lipoprotein-triglyceride to FA (Figure 2A, inset, bottom panel). In addition to its ability to release LPL, we also tested whether ECCM could stimulate the replenishment of LPL when the enzyme at the myocyte surface is depleted. Myocytes were pretreated with ECCM or heparin (10 mU/mL), followed by a bolus dose of heparin (8 U/mL) to deplete surface LPL, as confirmed in Figure 2B (left panel). After a 2-hour recovery, myocytes pretreated with ECCM were able to recruit significantly more LPL activity to the surface (Figure 2B, right panel), an effect not observed with 10 mU/mL heparin. Considering that RhoA is involved in augmented LPL secretion from myocytes, we tested the effect of ECCM on RhoA activation. An increase in GTP-bound RhoA was observed within 5 minutes in response to ECCM, reaching a peak at 15 minutes, and declined to basal levels after 30 minutes (Figure 3A). We did not see a similar effect with 25 mmol/L glucose or 10 mU/mL heparin (data not shown). The downstream effect of RhoA activation, actin cytoskeleton polymerization, was also augmented with ECCM, an effect that was abolished in the presence of the Rho-associated protein kinase inhibitor Y-27632 (Figure 3B). Hence, our data suggested that ECCM can stimulate both the release, and likely through RhoA-mediated actin cytoskeleton polymerization, replenishment of LPL at the myocyte surface.

Effect of ECCM on LPL Is Related to the Presence of Heparanase

As LPL at the cardiomyocyte surface resides on HSPGs, its release by ECCM could be a consequence of cleavage of these binding sites by heparanase. As anticipated, ECCM contained a higher amount of both latent (65 kDa) and active (50 kDa) heparanase (Figure 4A), with a reciprocal decrease in the intracellular content of this enzyme (Figure IIIC in the online-only Data Supplement). The increase in active heparanase protein mirrored the higher heparanase activity in this medium (Figure II in the online-only Data Supplement). In vivo, both latent and active heparanase also increased in the interstitial space of hearts from DZ animals (Figure 4B), where coronary ECs are exposed to high glucose. When heparanase in the ECCM was immunoprecipitated by an antiheparanase antibody, thereby reducing the amount of both latent and active heparanase (Figure 4C, inset), the LPL releasing effect of ECCM was compromised (Figure 4C). When bovine coronary artery ECs were exposed to high glucose for 2 consecutive periods of 30 minutes, the amount of heparanase released into the medium diminished during the second incubation (Figure IIIA in the online-only Data Supplement). In addition, the ability of this ECCM from the second incubation to release myocyte surface LPL also decreased (Figure IIIB in the online-only Data Supplement).

Latent and Active Heparanase Play Different Roles in LPL Secretion

Because high glucose stimulates the release of both latent and active heparanase from EC, we tested the roles of these 2 forms on LPL secretion using purified heparanase. Active, but not latent, heparanase caused the release of LPL (Figure 4D), and this effect of active heparanase was dose dependent (Figure IV in the online-only Data Supplement). When heparin was added to remove LPL from the myocyte surface, active heparanase was unable to release LPL (Figure 4E), suggesting that LPL released by active heparanase is from the myocyte surface. Unexpectedly, RhoA activation was not observed with active heparanase, which only responded to the latent form of the enzyme (Figure 4F). Our data imply that active heparanase releases LPL from the myocyte surface, whereas latent heparanase may move LPL from an intracellular store to the surface.

Activation of RhoA by Latent Heparanase Depends on HSPGs and PKCα

To examine the mechanism of RhoA activation by latent heparanase, we considered whether the integrity of the myocyte surface HSPGs is required for this signal mechanotransduction.
As expected, removal of HS by heparinase III blocked RhoA activation by latent heparanase (Figure 5A). Interestingly, the effect of latent heparanase on RhoA activation and cytoskeleton polymerization was abolished by the PKCα/β inhibitor Gö6976 (Figure 5B and 5C), whereas phorbol 12-myristate 13-acetate, a conventional PKC activator, had effects similar to that seen with latent heparanase (Figure V in the online-only Data Supplement). Furthermore, the RhoA activation effect observed with latent heparanase was only attenuated in myocytes with reduced PKCα expression, but not in cells in which PKCβ was specifically inhibited (Figure VI in the online-only Data Supplement). In normal myocytes, syndecan-4 is distributed on myocyte surface in a dispersed manner. On addition of latent heparanase and antisyndecan-4 antibody, syndecan-4 appeared clustered on the surface (Figure 5D, arrow), an effect not seen with active heparanase.

LPL Secretion Is Increased When Myocytes Are Cocultured With ECs Exposed to High Glucose

To simulate diabetes mellitus in vitro, myocytes were cocultured with ECs. As a limited number of myocytes can be seeded in the coculture system, the intrinsic LPL activity at the surface of these myocyte is low. Hence, we used purified LPL added exogenously to amplify this surface pool. Two hundred micrograms of purified LPL was sufficient to saturate HSPGs binding sites on the myocyte surface (Figure 6A, inset). Using this amount of LPL, we exposed the coculture system to normal and high glucose. A significantly higher medium LPL activity was detected from the lower chamber in the presence of high glucose (Figure 6A). To examine whether the released LPL from myocytes is ultimately recruited onto the apical side of EC, we tested LPL activity on the EC surface after 2 and 4 hours with high glucose. In the normal glucose coculture, we did not observe any change in EC surface LPL activity at 2 or 4 hours. With high glucose, a robust increase in EC surface LPL activity was evident after 4 hours (Figure 6B). Notably, high-glucose coculture also caused actin polymerization (within 1 hour; Figure 6C, inset), that was preceded by RhoA activation (within 30 minutes) in myocytes (Figure 6C).

**Discussion**

Cardiac muscle has a high demand for provision of energy which is obtained from oxidation of an assortment of different substrates including lactate, ketone bodies, glucose, and FAs. Of these substrates, FA is the favorite and major fuel of the heart. The dominant source of FA is from breakdown of circulating triglyceride-rich lipoproteins (chylomicrons and very low density lipoprotein) by LPL at the coronary lumen. Hence, cardiac LPL is of crucial importance for regulating energy supply in the heart. This enzyme is synthesized in myocytes and secreted to the vascular lumen. After diabetes mellitus, the heart increases its demand for FAs as a result of
impaired glucose utilization. As a consequence, LPL activity at the coronary lumen is expected to increase. Accordingly, in our animal model of hyperglycemia (DZ) and type 1 diabetes mellitus (D55), we observed increased LPL activity at this location, an event that we have previously shown to occur in the absence of any change in (1) LPL-specific activity, (2) LPL gene expression, or (3) the number of coronary lumen binding sites. We concluded that this augmented LPL at the vascular lumen is likely a consequence of increased secretion from myocytes.

LPL secretion requires trafficking from an intracellular pool to myocyte surface HSPGs, followed by translocation to the vascular lumen. Our previous studies have indicated that the trafficking component requires actin cytoskeleton polymerization, which can be achieved by RhoA activation. In fact, when isolated hearts were perfused with LPA, a RhoA activator, increased coronary LPL activity was detected. RhoA is a small GTP-binding protein, with inactive RhoA associated with GDP and sequestered in the cytosol. On activation, GDP is replaced by GTP and GTP-RhoA shifts to the plasma membrane. Subsequently, through its downstream effector Rho-associated protein kinase, activation of RhoA will ultimately induce actin cytoskeleton polymerization. We observed that the increase in LPL activity at the coronary lumen in hyperglycemic DZ and diabetic D55 hearts were both accompanied by RhoA activation. As RhoA activation by LPA also promoted LPL secretion in cardiomyocytes through actin cytoskeleton polymerization, our data suggest that this GTP-binding protein is an important contributor to move LPL to the myocyte surface.

The mechanism by which LPL leaves the myocyte surface to move to the coronary lumen is not completely understood. At the myocyte surface, LPL is sequestered to HSPGs via an ionic interaction. Thus, its release is possible by negatively charged heparin. However, cleavage of HS, or shedding of the extracellular part of HSPGs is a more likely event for LPL to leave the surface in vivo. Interestingly, on exposure of cardiomyocytes to ECCM, LPL is released into the medium with a reciprocal decrease in surface LPL activity, suggesting that ECCM is likely releasing LPL from the myocyte surface. An additional observation noticed with ECCM was its ability to assist in LPL replenishment when the surface pool was depleted. We attributed this effect to its ability to activate RhoA in cardiomyocytes in a pattern similar to that seen with LPA. In both cases, after reaching a peak, the amount of GTP-RhoA declined as it is normally converted to a GDP-bound form after activation of downstream effectors. Intriguingly, heparin had no effect on RhoA or LPL replenishment, implying that simple displacement of LPL is not sufficient to trigger the replenishment process, and that ECCM is likely altering the conformation of HSPGs.

An enzyme of special relevance that is known to cleave HS of HSPGs is heparanase. This enzyme is synthesized in the EC as a latent 65-kDa form, which possesses no catalytic activity. After its secretion, heparanase binds to EC surface HSPGs and is internalized into lysosomes. There it is cleaved into a 50-kDa active form and stored until it is secreted in response to stimulation such as adenosine, ADP, and ATP. In the present study, increased secretion of both latent and active heparanase into the medium was also observed when ECs were exposed to high glucose. As both forms of heparanase were augmented in the interstitial space of hearts from DZ animals, it suggests that hyperglycemia is effective in triggering coronary endothelial heparanase release. We have previously demonstrated that high glucose–induced secretion

![Figure 5](https://example.com/figure5.png)
of active heparanase from EC occurs through activation of P2Y receptors that initiates stress fiber formation (across which heparanase-containing cargos are transported) and disruption of cortical actin cytoskeleton (to allow heparanase to be released into the extracellular space). Whether the same mechanism applies to release of latent heparanase is unknown because we cannot rule out the possibility that high glucose could also block endocytosis of latent heparanase from the EC surface, or is capable of directly detaching the latent enzyme from surface HSPGs. Irrespective of the release mechanism, our study is the first to demonstrate that it is heparanase within ECCM that is responsible for detachment of LPL from the myocyte surface.

Although active heparanase is predicted to release surface LPL by cleaving HS, latent enzyme could also facilitate LPL release. As expected, only active heparanase initiated release of LPL from the myocyte surface. Surprisingly, this release was not accompanied by RhoA activation. In fact, it was latent heparanase that accounted for the RhoA activation seen with ECCM. Growing evidence has demonstrated that heparanase also possesses activity-independent effects, for example, activation of Src during cell adhesion. Hence, our data suggest that active heparanase detoxifies LPL from the myocyte surface HSPGs, whereas latent heparanase activates RhoA to move intracellular LPL to replenish this released reservoir. Studies on HSPG-mediated signaling have focused on syndecan-4, a transmembrane HSPG with its cytoplasmic domain capable of activating and stabilizing PKCα, and syndecan-4–dependent PKCα activation has been indicated in fibroblast growth factor signaling. Moreover, neuronal Thy-1–induced cell spreading requires RhoA-mediated actin cytoskeleton polymerization, a suggested downstream event after syndecan-4–dependent PKCα activation. Our study also indicated that the RhoA activation induced by latent heparanase is mediated by PKCα. In U87 MG human glioma cells, clustering of syndecan-4 by latent heparanase or an antiheparanase antibody was sufficient to induce cell spreading, a process involving PKC activation. In this coculture, activation of RhoA was measured in myocytes after 30 minutes, and compared with cells that did not have EC in the top chamber (−EC; C). F-to-G actin ratio was also tested at 30 and 60 minutes (C, inset). P<0.05, compared with NG, n=3. D, How EC regulates cardiac metabolism after diabetes mellitus is summarized. TG indicates triglyceride.

Figure 6. Heparanase is key for regulating lipoprotein lipase (LPL) secretion from myocytes to endothelial cells (ECs) after diabetes mellitus. A coculture was performed using EC in the insert (top) and cardiomyocytes in the well (bottom). Cardiomyocytes were loaded with increasing amount of purified LPL for 30 minutes, and LPL activity bound to the myocyte surface was determined (A, inset). Myocytes were loaded with 200 µg purified LPL for 30 minutes and washed with PBS to remove unbound LPL. Inserts with EC were placed on top, and the coculture was exposed to normal glucose (NG, 5.5 mmol/L) or high glucose (HG, 25 mmol/L). Medium was collected from the lower chamber after 30 minutes, and LPL activity was determined (A). After 2 and 4 hours, LPL activity at the EC surface was also released and tested by incubating these cells with 8 U/mL heparin for 3 minutes (B). In this coculture, activation of RhoA was measured in myocytes after 30 minutes, and compared with cells that did not have EC in the top chamber (−EC; C). F-to-G actin ratio was also tested at 30 and 60 minutes (C, inset). *P<0.05, compared with NG, n=3. D, How EC regulates cardiac metabolism after diabetes mellitus is summarized. TG indicates triglyceride.
In summary, heparanase is a key mediator of the cross-talk between EC and cardiomyocyte to increase LPL secretion and eventually minimize cardiac lipotoxicity observed with chronic diabetes mellitus (Figure 6D).

Acknowledgments

Y. Wang conceived the idea, generated most of the data, and wrote the manuscript. D.H. Zhang, A.P.L.C., A. Wan, and K. Neumaier helped with obtaining some of the data. B. Rodrigues helped with writing the manuscript. I. Vlodavsky (Cancer and Vascular Biology Research Center, Israel) assisted with valuable suggestions and the preparation of highly purified latent and active heparanase.


This work was supported by an operating grant from the Canadian Diabetes Association.

Disclosures

None.

References

After diabetes mellitus, the heart switches to use more fatty acid for energy supply, and this is achieved through rapid augmentation of lipoprotein lipase at the coronary lumen. Previous studies have shown that the amount of coronary lipoprotein lipase depends on secretion from myocytes, a process that is regulated by multiple signaling pathways in these cells. Our data suggest that after hyperglycemia, endothelial cells, through the release of heparanase, can signal to cardiomyocytes to increase lipoprotein lipase secretion from these cells. Given that endothelial cells are the first to be exposed to hyperglycemia, this cross-talk allows the heart to predominantly use fatty acids for energy supply. Chronically, this excess fatty acid delivery to the heart may explain lipotoxicity and ultimately cardiomyopathy after diabetes mellitus.
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Arterioscler Thromb Vasc Biol. 2013;33:894-902; originally published online March 7, 2013; doi: 10.1161/ATVBAHA.113.301309
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Materials and Methods

Experimental Animals
The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by NIH and the University of British Columbia. Male Wistar rats (250-320 g) were injected with diazoxide (DZ), a K⁺ channel opener which reduces insulin secretion and induces acute hyperglycemia. Animals remain hyperglycemic for up to 4 h following a single injection. DZ was administered i.p. (100 mg/kg), animals were anesthetized using 65 mg/kg i.p. pentobarbital after 4 h, and hearts removed. To induce chronic hyperglycemia, rats were injected i.v. with 55 mg/kg streptozotocin (STZ). Animals (D55) were kept for 4 days.

Reagents and Antibodies
Diazoxide (DZ, Cat# D9035), Oleoyl-L-α-lysophosphatidic acid (LPA, Cat# L7260), Cytochalasin D (Cat# C8273), Cyclohexamide (Cat# C7698), Y-27632 (Cat# Y0503), Gö6976 (Cat# G1171), Phorbol 12-myristate 13-acetate (PMA, Cat# P8139)) were obtained from Sigma. Heparin (HEPALEAN, 1000 U/ml) was from Organon, Canada. PKC β inhibitor was obtained from Calbiochem (Cat# 539654-500UG). It inhibits PKC βI and PKC βII with IC₅₀ of 21 and 5 nM, respectively. Heparinase III (IBEX technologies, Cat# 50-012-001) was purified from recombinant Flavobacterium heparinum. [³H]-triolein was purchased from Amersham Canada. Purified active and latent heparanase were prepared as described. Anti-LPL 5D2 antibody was a kind gift from Dr. J. Brunzell, University of Washington, Seattle. Anti-heparanase antibody mAb 130 was from InSight (Rehovot, Israel, Cat# INS-26-1-0000-21), which recognizes both the active (50 kDa) and latent form (65 kDa) of heparanase. Anti-RhoA antibody was purchased from Santa Cruz (Cat# sc-418). The RhoA activation G-LISA assay kit (Cat# BK124) and F-actin/G-actin assay kit (Cat# BK037) were obtained from Cytoskeleton (Denver, CO). HTRF heparanase activity assay kit was obtained from Cisbio (Cat# 61BHSKAA). To measure free fatty acid released from VLDL-TG breakdown, an NEFA-C assay kit was purchased from Wako (Cat# 999-75406).

Isolated Heart Perfusion
To measure coronary LPL, hearts were perfused retrogradely with heparin (5 U/ml). Coronary effluents were collected (for 10 s) at different time points over 5 min. LPL activity in each fraction was determined, plotted against time, and coronary LPL activity was presented as area under the curve over 5 min. A modified Langendorff retrograde perfusion was used to isolate active and latent heparanase in the interstitial effluent.

Isolation of Cardiac Myocytes
Ventricular calcium-tolerant myocytes were prepared by a previously described procedure.

Endothelial Cell Culture
Bovine coronary artery endothelial cells (bCAECs, Clonetics) and Rat aortic endothelial cells (RAOEC, Cell Applications) were cultured at 37°C in a 5% CO₂ humidified incubator alone or co-cultured with adult rat cardiomyocytes. bCAECs from the 5th to the 8th passage were used. RAOEC at passage 6 were used.

Isolation of Particulate Fraction
To determine RhoA activation in vivo, particulate fraction from ventricles were prepared as described previously.
**Endothelial Cell Conditioned Medium (ECCM)**
bCAECs were incubated with high glucose (25 mM) DMEM for 30 min. This medium was collected as ECCM. Cardiomyocytes were incubated with ECCM for 30 min, and the medium collected to study its lipolytic activity using VLDL-TG as a substrate.8

**Endothelial cell heparanase in response to high glucose**
Following treatment of bCAECs with 5 or 25 mM glucose DMEM for 30 min, cells were collected and intracellular latent and active heparanase measured using Western blot. To test whether consecutive exposure to high glucose can eventually deplete heparanase secretion, bCAECs were treated with 25 mM glucose DMEM for 30 min, medium removed (1st release), and the cells exposed to a second 30 min incubation with 25 mM glucose DMEM (2nd release). Medium from both 1st and 2nd release were concentrated to detect latent and active heparanase. In a separate experiment, bCAECs that were pre-treated with 25 mM glucose DMEM for 30 min (1st release) were placed in the upper chamber of a co-culture system with isolated cardiomyocytes at the bottom. These cardiomyocytes had themselves been pre-incubated with purified LPL to saturate surface binding sites. 25 mM glucose DMEM (2nd release) was then applied to the co-culture system, and medium collected from the bottom chamber after 30 min to test LPL activity released from cardiomyocytes. Results were compared to a co-culture system in which bCAECs was not pre-treated with high glucose.

Rat aortic endothelial cells were incubated with either 5 or 25 mM glucose DMEM, and active heparanase secreted into the medium was determined by Western blot. RAOEC conditioned medium was also applied to myocytes to release surface LPL. 30 min after treatment, LPL activity released into the medium was determined.

**LPL Activity**
To release LPL bound to surface HSPGs, cells were incubated with 8 U/ml heparin for 3 min. This concentration and time is sufficient to completely deplete surface LPL.9,10 LPL activity was determined by measuring in vitro hydrolysis of [3H]triolein substrate.11

**LPL expression in cardiomyocytes**
Myocytes from both control and DZ hearts were isolated, and intracellular LPL protein in these cells determined by Western blot. Gene expression of LPL was measured using Taqman assay (Invitrogen, Rn00561482_m1). Gene expression was normalized to β-actin (Rn00667869_m1).

**Treatments**
1 h after DZ injection, animals were injected with 4 U of a rapid-acting insulin (Humulin R, Eli Lilly Canada Inc) through the tail vein. Blood glucose was monitored every 30 min up to 3 h, animals were killed and hearts removed for determination of coronary LPL activity and RhoA activation. 4 h after DZ injection, these animals were killed and hearts perfused with heparin to determine coronary LPL activity.

To study the effect of RhoA activation on LPL secretion, control myocytes were incubated with lysophosphatidic acid (LPA, 100 nM-1 µM) in the presence or absence of 1 µM cytochalasin D (Cy, an actin polymerization inhibitor). To rule out the effect of protein synthesis on LPL secretion, 50 µM of the protein synthesis inhibitor cyclohexamide (CHX) was applied to myocytes 1 h before and during treatment with LPA, and LPL secretion into the medium determined. RhoA activation was tested in myocytes treated with ECCM, or 1 µg/ml purified latent or active heparanase at the indicated times. To study the role of HSPGs and PKCα in RhoA activation, 10 IU/L
heparinase III and 5 mM Gö6976 was used to digest heparan sulfate of HSPGs and inhibit PKCα/β in cardiomyocytes, respectively. To study the specific effect of PKCα and β on RhoA activation in the presence of latent heparanase, siRNAs specific for PKCα were designed and synthesized by Invitrogen (Carlsbad, CA, USA; 5’-UGAAGAAGCGGCGAUGAAUUGUG-3’). Lipofectamine RNAi/MAX (Invitrogen, Cat# 13778030) were used to transfect siRNAs for PKCα or control unrelated siRNA into cardiomyocytes (at a final concentration of 50 nM). 24 h after transfection, medium was changed to Media 199 and following an additional 24 h, cells were incubated with 1 µg/ml latent heparanase for 15 min and RhoA activation in myocytes measured. In another experiment, myocytes were pre-incubated with 200 nM PKCβ inhibitor, 1 h before and during latent heparanase treatment, and RhoA activation in myocytes measured. To investigate the role of RhoA in ECCM-mediated actin polymerization, 10 µM Y-27632 was used to block ROCK, the downstream effector of RhoA. Isolated myocytes were also treated with 1 µM PMA (a conventional PKC activator) for 0, 5, 15, and 30 min, and RhoA activation determined using a G-LISA assay kit. Actin cytoskeleton polymerization in these cells was also determined 1 h following PMA treatment.

**G-LISA Assay**

G-LISA assay was performed according to manufacturer’s instruction (Cytoskeleton). The active GTP-RhoA was detected by reading the absorbance at 490 nm.

**Actin Polymerization**

Actin polymerization was evaluated by measuring the filamentous to globular actin (F-actin/G-actin ratio) using an assay kit from Cytoskeleton.

**Western Blot**

Western blot was carried out as described previously\(^7\). In some experiments, samples were concentrated by TCA precipitation, or Amicon centrifuge filter (Millipore, Cat# UFC503008) before detection of heparanase or LPL.

**Immunofluorescence**

Isolated cardiomyocytes were treated with 1 µg/ml active or latent heparanase for 30 min, and cells probed with anti-syndecan-4 antibody (H-140, Santa Cruz, Cat# sc-15350) to visualize syndecan-4 (green) stained by Alexa488. To induce syndecan-4 clustering, an antibody against the extracellular domain of syndecan-4 (H-17, Santa Cruz, Cat# sc-33912) was used as a positive control\(^12,13\). 4,6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei, and slides visualized using a Confocal microscope.

**Statistical Analysis**

Values are means ± SE. Wherever appropriate, one-way ANOVA followed by the Bonferroni test was used to determine differences between group mean values. The level of statistical significance was set at \(P<0.05\).
References


Supplemental Material

Figure legends

Supplement Figure I. Increased LPL activity in STZ-induced diabetic hearts is associated with RhoA activation. Male Wistar rats (250-320 g) were injected i.v. with 55 mg/kg STZ (D55), and animals kept for 4 days before hearts were removed. Particulate fractions were isolated from whole heart homogenates of control (CON) and D55 animals, and RhoA recruitment to the particulate fraction determined by Western blot (A). In a separate experiment, hearts were also perfused with heparin (5 U/ml) to release coronary LPL. Coronary effluents were collected (for 10 s) at different time points over 5 min, and LPL activity in each fraction determined. The results are presented as area under the curve for heparin-released LPL activity over 5 min (B). *P<0.05, compared to CON, n=3.

Supplement Figure II. High glucose induced release of active heparanase. bCAECs were treated with 5.5 (NG) or 25 (HG) mM glucose DMEM for 30 min, and medium collected to measure heparanase activity with a kit (A). *P<0.05, compared to NG, n=4.

Supplement Figure III. High glucose affects heparanase secretion from endothelial cells. bCAECs were treated with 25 mM glucose DMEM for 30 min, medium removed (1st HG), and the cells exposed to a second 30 min incubation with 25 mM glucose DMEM (2nd HG). The amounts of latent and active heparanase in the medium of the 1st and 2nd HG treatment were measured by Western blot (A). *P<0.05, compared to 1st HG release, n=3. In a co-culture system, cardiomyocytes placed in the bottom chamber were pre-incubated with 200 µg purified LPL for 30 min to saturate myocyte surface HSPGs. Cells were washed with PBS to remove unbound LPL. bCAECs that were pre-treated with 25 mM glucose DMEM for 30 min were then placed in the upper chamber of the co-culture system. 25 mM glucose DMEM was applied to the co-culture system, and the medium from the bottom chamber collected after 30 min to determine LPL activity released from cardiomyocytes. Results were compared to a co-culture system in which bCAECs was not pre-treated with high glucose. **P<0.01, n=3 (B). bCAECs were treated with either 5 (NG) or 25 (HG) mM glucose DMEM for 30 min, and intracellular heparanase determined by Western blot (C). *P<0.05, compared to NG, n=3 (C). RAOEC were incubated with either 5 (NG) or 25 mM (HG) glucose DMEM for 30 min, and active heparanase released into the medium determined using Western blot (D, inset). RAOEC conditioned medium (NG and HG) was used to treat myocytes for 30 min, and LPL activity released determined. *P<0.05, compared to NG, n=3 (D).

Supplement Figure IV. Active heparanase releases myocyte surface LPL in a dose-dependent manner. Isolated myocytes were incubated with increasing concentrations of active (A-HEPA) or latent (L-HEPA) heparanase for 30 min, and LPL activity released into the medium determined. 5.5 mM glucose DMEM was used as control (CON).

Supplement Figure V. Activation of RhoA by PMA leads to actin cytoskeleton polymerization. Isolated myocytes were treated with 1 µM PMA for the indicated times, and RhoA activation determined using a G-LISA assay kit (A). Actin cytoskeleton polymerization in these cells was determined 1 h following PMA treatment (B). *P<0.05, **P<0.01, compared to CON, n=3.

Supplement Figure VI. PKCα is important for RhoA activation by latent-heparanase. In a 6-well plate, isolated myocytes from control rats (CON) were transfected with siRNA specific for PKCα at 100 or 200 nM or control unrelated siRNA (SCR, 200 nM). 24 h following transfection, medium was changed to Media 199 and following an additional 24 h, cells were collected to Western Blot for PKCα (A). *P<0.05, compared to SCR, n=3. In a separate experiment, myocytes were transfected with 200 nM siRNA for PKCα (+) or 200 nM control unrelated siRNA (-), before being exposed to 1 µg/ml latent heparanase for 15 min. GTP-bound RhoA in these myocytes were determined (B). 200 nM PKCβ inhibitor was given to myocytes 1 h before and
during incubation with latent heparanase for 15 min, and RhoA activation also determined in these myocytes (C). Results were from 3 experiments using different control animals. *$P<0.05$, compared to control, #$P<0.05$, compared to control treated with L-HEPA.

**Supplement Figure VII. DZ does not change LPL expression in the heart.** Following injection of DZ for 4 h, myocytes from both control (CON) and DZ hearts were isolated, and intracellular LPL protein (A) and mRNA (B) levels determined by Western blot and Taqman (B), respectively. $n=3$. 


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