Endothelial Cell Heparanase Taken Up by Cardiomyocytes Regulates Lipoprotein Lipase Transfer to the Coronary Lumen following Diabetes

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Following diabetes, the heart has a singular reliance on fatty acid (FA) for energy production, which is achieved by increased coronary lipoprotein lipase (LPL) that breaks down circulating triglycerides. Coronary LPL originates from cardiomyocytes, and to translocate to the vascular lumen, the enzyme requires liberation from myocyte surface heparan sulfate proteoglycans (HSPGs), an activity that needs to be sustained following chronic hyperglycemia. We investigated the mechanism by which endothelial cells (EC) and cardiomyocytes operate together to enable continuous translocation of LPL following diabetes.

EC were co-cultured with myocytes, exposed to high glucose, and uptake of endothelial heparanase into myocytes determined. Upon uptake, the impact of nuclear entry of heparanase was also investigated. A streptozotocin model of diabetes was used to expand our *in vitro* observations.

In high glucose, EC-derived latent heparanase was taken up by cardiomyocytes via a caveolae-dependent pathway using HSPGs. This latent heparanase was converted into an active form in myocyte lysosomes, entered the nucleus, and upregulated gene expression of matrix metalloproteinase-9. The net effect was increased shedding of HSPGs from the myocyte surface, releasing LPL for its onwards translocation to the coronary lumen.

EC-derived heparanase regulates the ability of cardiomyocyte to send LPL to the coronary lumen. This adaptation, although beneficial acutely, could be catastrophic chronically as excess FA causes lipotoxicity. Inhibiting heparanase function could offer a new strategy for managing cardiomyopathy observed following diabetes.
In diabetes, as glucose uptake and oxidation are impaired, the heart is compelled to use fatty acid (FA) exclusively for ATP generation (1). Multiple adaptive mechanisms, either whole body or intrinsic to the heart, operate to make this achievable, with hydrolysis of triglyceride-rich lipoproteins being the major source of FA to the diabetic heart (2). This critical reaction is catalyzed by the vascular content of lipoprotein lipase (LPL), and we were the first to report significantly higher coronary LPL activity following diabetes (3). In the heart, LPL is synthesized by cardiomyocytes, transported to heparan sulfate proteoglycan (HSPG) binding sites on myocyte surface, and from this temporary reservoir, the enzyme is transferred across the interstitial space to reach endothelial cells (EC) (4; 5). Prior to this transfer, liberation of HSPG-sequestered LPL is a pre-requisite, and is facilitated by heparanase, an EC endoglycosidase that can cleave heparan sulfate (HS) side chains on HSPGs in the extracellular matrix and on the cell surface to release bound proteins (6).

Heparanase is synthesized as a latent 65 kDa precursor. Following its secretion and reuptake (7), it enters the lysosome, and is cleaved into a 50 kDa active form by cathepsin L (8; 9). In this organelle, active heparanase is stored until secreted. In addition to an extracellular function, by entry into the nucleus to regulate histone acetylation/methylation, heparanase is also capable of modulating gene transcription (10; 11). Thus, 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 (12). For example, matrix metalloproteinase-9 (MMP-9) gene expression induced by nuclear heparanase can further degrade extracellular matrix and basement membranes (13). Additionally, MMP-9 also causes accelerated shedding of HSPGs such as syndecan-1 (14; 15), thereby liberating HSPG-bound ligands including angiogenic and growth factors. At present, it is unclear if
heparanase can influence gene transcription in the heart.

In the heart, heparanase is synthesized predominantly in EC, and following diabetes, we reported a high glucose-induced secretion of active heparanase (16). It should be noted that high glucose also stimulated secretion of latent heparanase, whose function in the heart is unknown. Interestingly, human fibroblasts that do not express heparanase, can take up exogenous latent heparanase and convert it into active heparanase (17). Thus, an immediate response to hyperglycemia is an EC secretion of active heparanase to facilitate translocation of LPL from myocyte surface to the coronary lumen (18). Given that the active heparanase pool in EC is limited, and may not meet the demand for a sustained LPL transfer following diabetes, it is possible that latent heparanase could be taken up and converted to active heparanase in cardiomyocytes. Such an effect could turn on MMP-9 expression, thereby stimulating shedding of HSPGs that bind LPL, thus maintaining vascular LPL increase chronically. Results from the present studies suggest that high glucose induced secretion of EC heparanase can regulate gene expression of MMP-9 in cardiomyocytes, an effect that may sustain transfer of LPL to the coronary lumen following chronic diabetes.
RESEARCH DESIGN 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 i.v. with 55 mg/kg streptozotocin (STZ). These animals (D55) were kept for 4 days before isolation of cardiomyocytes. To induce acute hyperglycemia, diazoxide (100 mg/kg, i.p.) was injected to male Wistar rats (250–320 g), and kept for 4 h.

Materials

Rat aortic endothelial cells (RAOEC) were obtained from Cell Applications. Streptozotocin (STZ), filipin, genistein, and chloroquine were obtained from Sigma. Heparin (HEPALEAN, 1000 U/ml) was from Organon, Canada. Heparinase III (IBEX technologies) was purified from recombinant Flavobacterium heparinum. Purified latent heparanase was prepared as described (19). SiRNA for rat MMP-9 was obtained from Qiagen (SI02004247). Control siRNA consisted of a scrambled sequence purchased from Santa Cruz. Lipofectamine RNAiMAX was from Invitrogen. Purified MMP-9 and p-aminophenylmercuric acetate AMPA were from Raybiotech (Norcross, US). Anti-LPL 5D2 antibody was a kind gift from Dr. J. Brunzell, University of Washington, Seattle. Anti-heparanase antibody mAb 130 which recognizes both the active (50 kDa) and latent (65 kDa) forms of heparanase was from InSight (Rehovot, Israel). An antibody that recognize the cleaved form of HSPGs was purchased from Seikagaku (Japan). Rat MMP-9 antibody was from Millipore. All other antibodies were obtained from Santa Cruz. Rat Syndecan-1 ELISA kit was obtained from MyBioSource (San Diego, US). HAT activity kit was obtained from Biovision (Milpitas, US).
Isolation of cardiomyocytes

Ventricular calcium-tolerant myocytes were prepared by a previously described procedure (20). Cardiomyocytes were isolated from adult male Wistar rats (250-320 g). Following isolation, cardiomyocytes were plated on laminin-coated culture dishes and allowed to settle for 3 h. Before treatment, unattached cells were washed away using Medium 199.

Coronary LPL activity

To measure coronary LPL, hearts were perfused retrogradely with heparin (5 U/ml) (3). Coronary effluents were collected (for 10 s/fraction) at different time points over 5 min, and LPL activity in each fraction determined by measuring the in vitro hydrolysis of a sonicated \[^{3}H\]triolein substrate emulsion (3). Coronary LPL activity is calculated as area under curve of LPL activity in each fraction over time.

Endothelial cell culture

RAOEC were cultured at 37 °C in a 5% CO\(_2\) humidified incubator alone or co-cultured with adult rat cardiomyocytes. RAOEC from the 5\(^{th}\) to the 8\(^{th}\) passage were used.

Treatments

To test whether exogenous heparanase can bind and be taken up by cardiomyocytes, isolated myocytes were treated with 500 ng/ml recombinant latent heparanase for different time intervals at 37 °C. This experiment was also conducted at 15 °C to inhibit heparanase uptake. In the presence of 10 IU/ml of heparin to compete for HSPGs binding sites, or 10 IU/L heparinase III to digest HS side chains (2 h), the contribution of myocyte surface HSPGs in heparanase uptake was determined. For studying the mechanism of internalization, 350 mM sucrose or 1 μg/ml filipin was applied to myocytes for 15 min before latent heparanase, to block clathrin-coated pits and caveolae-dependent internalization, respectively (21; 22). 0-25 μM dynasore or 0-100 μM
genistein for 1 h were also used to inhibit dynamin and tyrosine kinase activity respectively, which could be involved in internalization of exogenous heparanase (23; 24). To inhibit lysosomal enzymes, 200 µΜ chloroquine was used and myocytes incubated for 2 h. 10 µM anacardic acid was used to inhibit HAT activity. Purified MMP-9 proenzyme was activated by 10 µM AMPA at 37 ºC for 1 h (25), and added into the myocyte culture medium at a final concentration of 1, 5, and 10 ng/ml for 30 min to validate the effect of MMP-9 on syndecan-1 shedding and LPL release from the cardiomyocyte surface. To knockdown MMP-9 expression, cardiomyocytes were cultured in a 6-well plate, and transfected with 100 pmol siRNA using lipofectamine.

**Uptake of exogenous heparanase**

Following incubation with 500 ng/ml purified latent heparanase, myocytes were washed three times with cold PBS. To determine binding and uptake of heparanase by myocytes, total cell lysates were collected to detect heparanase by Western blot. For measuring internalization of heparanase, plasma membrane was removed by a procedure described previously (24). Briefly, myocytes were lysed in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2 M sucrose, 2 mM EDTA, 2 mM EGTA and protease inhibitor cocktail (Roche), and centrifuged at 10, 000 g at 4 ºC for 10 min. Supernatant was further centrifuged at 100,000 g at 4 ºC for 1 h to spin down the plasma membrane. The resulting supernatant (-PM) containing cytosolic fraction was used to monitor heparanase internalization (24).

**RT-PCR**

Total RNA was extracted from isolated myocytes or RAOEC using Trizol reagent (Invitrogen), and 1 µg total RNA was used for reverse transcription. PCR primers were as follows (26; 27):

Heparanase (forward, 5'-CAAGAACAGCACCTACTCACGAAGC-3'; reverse,
5’-CCACATAAGCCAGCTGCAAGG-3’; 616 bp product);
MMP-9 (forward, 5’-CCCCACTTTTGGAAACGCU3’; reverse, 5’-ACCCACGACGATACAGATGCTGU3’; 686 bp product);
18S rRNA (forward, 5’-CGGCTACCACATCCAAGGAAU3’; reverse, 5’-GCTGGAATTACCGCGGCT-3’; 187 bp product).

**Isolation of lysosomes and nuclear fractions**

Lysosome enriched fractions were isolated using a kit from Sigma. Nuclear and cytosolic fractions were separated using the nuclear/cytosol fractionation kit from Biovision (Milpitas, US). To validate the purity of proteins, we used cytosolic (GAPDH) and nuclear (Histone H3) protein markers to detect their presence in cytosolic and nuclear fractions.

**Western blot**

Western blotting was carried out as described previously (28). In some experiments, cell culture media was concentrated by TCA precipitation, or Amicon centrifuge filter (Millipore) before detection of heparanase or LPL.

**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 $P<0.05$. 

RESULTS

Heparanase present in cardiomyocytes originates predominantly from an exogenous source

In the heart, heparanase is expressed predominantly by endothelial cells. Undeniably, our RT-PCR results indicated that using total RNA from EC as a template, heparanase mRNA was abundant in RAOEC. However, using the same amount of total RNA, heparanase mRNA was immeasurable in freshly isolated cardiomyocytes, implying low transcription activity (Figure 1A, right inset). Regardless of this indeterminable gene expression, heparanase protein, both the 65 kDa latent and the 50 kDa active forms were detected in isolated myocytes (Figure 1A, left inset). We confirmed that the presence of heparanase in cardiomyocytes was not due to EC contamination as we failed to detect the EC marker CD31 in this preparation (Figure 1A, left inset). Interestingly, unlike RAOEC, the dominant heparanase present in cardiomyocytes was the active form (Figure 1A, left inset). Culturing cardiomyocytes for 36 h reduced the content of both heparanase forms, suggesting that the protein cannot be efficiently synthesized de novo when being turned over (Figure 1B). Introducing latent heparanase into the culture medium rapidly increased the level of latent heparanase in the total cell lysate of myocytes (within 5 min), an effect that continued over time. This increase was inhibited when the temperature was lowered to 15 °C. Unlike latent heparanase, the increase of active heparanase was gradual, and only significant after 4 h (Figure 1C), suggesting an intracellular conversion of latent to active heparanase in cardiomyocytes. Our data imply that the heparanase content in cardiomyocytes originates largely from uptake of exogenous protein.
Internalization of latent heparanase by cardiomyocyte is through a caveolae-dependent pathway that requires HSPGs, dynamin and tyrosine kinase activation

Heparanase in the total cell lysates could consist of two parts: heparanase bound to the myocyte surface or that which had been internalized. Samples devoid of plasma membrane were prepared by ultracentrifugation and validated by the absence of membrane protein Na\(^+\)-K\(^+\) ATPase (Figure 2A, right inset). Following incubation of myocytes with latent heparanase, a robust increase of this exogenous protein was observed in the plasma membrane free fraction, indicating that it had been internalized (Figure 2A). Using heparin to competitively inhibit the binding of latent heparanase to HSPGs, we were able to reduce the amount of heparanase that was internalized (Figure 2A). As similar results were seen with heparinase III, that digests the HS chains of HSPGs (Figure 2B), our data implies that exogenous heparanase may be taken up by myocytes via binding to the HS on the cell surface. Downstream, HSPG-dependent internalization can occur through caveloae or clathrin-coated pits. As the internalization of latent heparanase was blocked by filipin, but insensitive to sucrose (Figure 3A), it is likely that HSPGs-mediated endocytosis of heparanase is a caveolae-dependent rather than a clathrin-mediated event. It should be noted that at the concentration of sucrose used, the endocytosis of epidermal growth factor receptor (EGFR), that is typically internalized through formation of clathrin-coated pits (29), was blocked (Figure 3A, right inset). The involvement of dynamin and tyrosine kinase in this endocytotic process was apparent as dynasore (Figure 3B) and genistein (Figure 3C) reduced the amount of heparanase internalized.

Internalized latent heparanase is activated in lysosomes and enters the nucleus

On incubation of cardiomyocytes with latent heparanase, we were able to detect this enzyme in lysosomal fractions within 30 min. With time, latent heparanase in this lysosomal fraction
progressively increased. Unlike latent heparanase, lysosomal active heparanase only increased at a later time point (after 3 h) (Figure 4A). As this increase in lysosomal active heparanase was prevented by chloroquine, which inhibits lysosomal proteases, our data suggests lysosomal conversion of latent to active heparanase in cardiomyocytes (Figure 4B).

**Nuclear entry of heparanase is accompanied by increased matrix metalloproteinase 9 (MMP-9) expression**

Assuming that conversion of latent to active heparanase in the lysosomes is to fulfill a biological function, we determined the nuclear content of heparanase, and observed the presence of active heparanase in this organelle even in the absence of exogenous heparanase addition. However, following addition of latent heparanase into culture medium, there was a dramatic increase of both latent and active heparanase in the nucleus after 4 h (Figure 5A). In agreement with previous studies (13), addition of active heparanase to nuclear fractions of cardiomyocytes increased HAT activity (Figure 5B). Importantly, nuclear entry of active heparanase was associated with an increase in MMP-9 expression, 18 h after heparanase treatment (Figure 5C). This increase in MMP-9 could be inhibited using the HAT activity inhibitor anacardic acid (Figure 5D). Using purified MMP-9, this sheddase was capable of detaching syndecan-1 from the myocyte surface (Figure 6A, upper inset), together with LPL (Figure 6A, lower inset). In line with its augmented expression, MMP-9 secretion into the medium also increased following latent heparanase incubation (Figure 6A), which was accompanied by an accelerated shedding of syndecan-1 (Figure 6B), and release of HSPG-bound LPL (Figure 6C). As knockdown of MMP-9 effectively damped these effects (Figure 6B and C), our data suggest that the heparanase/MMP-9 axis may play an important role in facilitating LPL translocation from cardiomyocytes to EC.
Increased coronary LPL activity after diabetes is related to endothelial heparanase-induced MMP-9 expression in cardiomyocytes

In the heart, heparanase is predominantly expressed in EC. As we previously reported (18), glucose stimulated secretion of both the latent and active forms of heparanase from EC (Figure 7A). Additionally, co-culturing of cardiomyocytes with EC in the presence of high glucose increased the amount of latent heparanase in cardiomyocytes. As this effect was absent when EC were removed from the top chamber (Figure 7B), our data suggest that cardiomyocytes can take up endothelial-derived heparanase, a process accelerated by high glucose. We reasoned that following prolonged hyperglycemia, such an uptake would result in augmented conversion of latent to active heparanase, together with its nuclear entry. Indeed, cardiomyocytes from D55 animals demonstrated a 1.5 fold increase in the nuclear content of active heparanase (Figure 7C), that was associated with cleavage of nuclear HSPGs (Supplement Figure 1A). Notably, similar to our in vitro observations, MMP-9 expression (Figure 7D and Supplement Figure 1B) and shedding of syndecan-1 (Figure 7E) in D55 myocytes was significantly higher compared to control. As increased coronary LPL activity was observed in D55 hearts (Figure 7F), our data implies that endothelial cells promote sustained translocation of LPL from cardiomyocytes to the coronary lumen via a heparanase/MMP-9 axis.
DISCUSSION

To maintain its energy supply following impaired glucose utilization during diabetes, the heart has a higher demand for FA, and much of it is provided by accelerated LPL-mediated hydrolysis of plasma TG at the coronary lumen. Although functional at the apical side of EC, heart LPL is synthesized in cardiomyocytes, resides at a temporary reservoir on myocyte surface HSPGs, and is fast-tracked to the vascular lumen by high glucose induced release of endothelial heparanase, which cleaves these HSPGs (30). Results from the present study suggest that in addition to this rapid adaptation, cardiomyocytes can take up endothelial heparanase, which activates gene expression to maintain LPL trafficking during chronic hyperglycemia (Figure 8).

Of the multiple cell types in the heart, the EC is likely the major source of heparanase. Surprisingly, in the presence of undetectable heparanase gene expression in cardiomyocytes, we detected protein in these cells that was predominantly active heparanase. Given that the half-life of active heparanase is approximately 30 h (7), and that in vitro culturing of cardiomyocytes for this time decreased its heparanase content, our data suggests that the presence of heparanase in cardiomyocytes is not reliant on de novo synthesis, but likely due to extracellular uptake. We confirmed this process using exogenous latent heparanase, and observed that the uptake was rapid, and likely HSPG-dependent. In CHO cells, latent heparanase triggers clustering of both syndecan-1 and -4 followed by rapid internalization of the heparanase-HSPG complex (31). As we have previously reported clustering of syndecan-4 when cardiomyocytes are exposed to latent heparanase (18), it is possible that such a heparanase-HSPG complex is also responsible for endocytosis of this enzyme in cardiomyocytes. Nevertheless, we cannot rule out the contributive effects of other receptors like low density lipoprotein receptor-related proteins and mannose 6-phosphate receptors in this heparanase
uptake process (32). Of the two novel endocytic pathways, HSPGs are involved more with a
caveolae-dependent rather than clathrin-mediated endocytosis (33; 34). For example,
syndecan-1 has been shown to mediate apoE-VLDL uptake in the human fibroblast cell line
GM00701, a process that was clathrin-independent, but inhibited by nystatin, an inhibitor of the
lipid raft-caveolae endocytosis pathway (35). Our data demonstrate that internalization of
latent heparanase by cardiomyocytes relies on caveolae to form endocytotic vesicles and
dynamin for fission of these vesicles from the plasma membrane. The participation of tyrosine
kinase in this process has also been reported in the syndecan family mediated endocytosis (36;
37). It is believed that clustering leads to redistribution of syndecans to lipid rafts, where their
cytoplasmic domain could be phosphorylated by tyrosine kinase (34; 38). Recruitment of
cortactin to phosphorylated syndecans promotes actin polymerization at the actin cortex, thus
bringing HSPGs-mediated endocytotic vesicles into the cells (39). Collectively, our data imply
that exogenous latent heparanase enters cardiomyocytes through HSPG-dependent caveolae
pathway.

Comparable to cardiomyocytes, cells like fibroblasts which do not express heparanase can
also take up latent heparanase (17). Interestingly, in fibroblasts, this latent heparanase can be
converted into active heparanase, likely through lysosomal processing (8). Our results also
indicate a lysosome enzyme-dependent conversion of latent to active heparanase in
cardiomyocytes. In EC, active heparanase stored in lysosomes can be secreted in response to
high glucose, or enter the nucleus in the presence of fatty acid (40). Data from the current
study demonstrate cardiomyocyte nuclear entry of both latent and active heparanase. Two
potential nuclear localization signals (residues 271–277; PRRKTAK and residues 427–430; KRRK) are present in the human heparanase sequence which could directly mediate its nuclear
entry (41). Additionally, Hsp90 has been proposed as a chaperone that mediates nuclear translocation of heparanase (42). However, the exact mode of heparanase translocation is currently unknown, but of crucial importance given that nuclear protein transport, especially proteins that serve as transcriptional regulators, have been implicated in the pathogenesis of certain diseases (43). In HL-60 cells (42) and bCAECs (40), active heparanase in the nucleus, by regulating gene expression, influences cell differentiation and glucose metabolism, respectively. Likewise, nuclear entry of active heparanase in myocytes was accompanied by an increase in MMP-9 gene expression. It is possible that by cleaving nuclear HSPGs, active heparanase relieves the suppression that HSPGs have on HAT activity, leading to acetylation of histone proteins required to promote gene transcription of MMP-9 (11; 13). This effect has been observed in several cancer cell lines upon transfection of heparanase (13; 44). As MMP-9 is capable of shedding syndecan-1 and -4 to generate soluble ectodomains carrying HSPGs-bound ligands (45), upregulating its expression could conceivably release LPL from the myocyte surface for translocation to the coronary lumen. Our studies illustrate a novel mechanism by which latent heparanase, by modulating myocyte MMP-9 expression, can have long-term impact on LPL trafficking (Figure 8).

Although multiple sources could explain the presence of heparanase in cardiomyocytes, we assumed that it is secondary to uptake of latent heparanase following its secretion from EC. Indeed, high glucose induced secretion of latent heparanase from EC was efficiently taken up by cardiomyocytes in our co-culture system. To extend these observations to a model of diabetes, we used STZ animals that were hyperglycemic for 72 h (D55), and observed accumulation of active heparanase in the myocyte nucleus, together with a robust increase in MMP-9 expression and an accelerated shedding of HSPGs. These animals also exhibited increased coronary LPL
activity with heparin perfusion. Our results suggest that in addition to the high glucose-induced secretion of active heparanase to initiate an immediate transfer of LPL from myocyte surface to the vascular lumen, activation of MMP-9 expression by latent heparanase taken up into the myocytes is pivotal for sustaining trafficking of LPL during chronic hyperglycemia (Figure 8). Interestingly, we have previously shown that when compared to D55, animals with acute hyperglycemia (28.2±2.3 mM compared to control 5.9±1.0 mM) induced by diazoxide (DZ, 4 h) are unable to maintain their high LPL activity at the vascular lumen following enzyme release by heparin (46). As cardiomyocytes from DZ hearts do not increase MMP-9 expression (Supplement Figure 2), the sustained increase of coronary LPL seen with D55 can likely be attributed to upregulation of MMP-9 by latent heparanase.

Overall, results from the present suggest that in response to hyperglycemia, EC-derived heparanase is a key mediator that controls the ability of the cardiomyocyte to send LPL to the coronary lumen. Although this adaptation might be beneficial in the short-term to compensate for energy deficit following diabetes, over a protracted duration, it is potentially catastrophic as superfluous FA causes lipotoxicity in the heart, ultimately leading to diabetic cardiomyopathy (Figure 8). Inhibiting heparanase secretion or function could offer a new strategy for managing the cardiomyopathy following diabetes.
LIMITATIONS

One limitation of this study is the lack of mouse models supporting the role of MMP-9 in regulating coronary LPL. MMP-9 knockout mice are available, and could be potentially used in this study. However, coronary LPL activity remains unchanged in both Type 1 and Type 2 diabetic mouse hearts. This could be a consequence of genetic adaptation or the excessive heart rate in control animals (~600 bpm), which, unlike in humans (parasympathetic tone), is under sympathetic control. This permits prior translocation of LPL from the cardiomyocyte to the coronary lumen to saturate all of the LPL binding sites. Hence, we have largely depended on rats when studying cardiac metabolism and LPL.
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Dr. Brian Rodrigues is the guarantor of this work and, as such, had fully access to all the data in the study and takes responsibility for the integrity of the data and accuracy of the data analysis. YW conceived the idea, generated most of the data and wrote the manuscript. AC, KN, FLW, DHZ, AW, BH and NL helped with obtaining some of the data. BR helped with writing the manuscript. IV (Cancer and Vascular Biology Research Center, Israel) assisted with valuable suggestions and the preparation of highly purified latent and active heparanase. GL (Institute of Cardiovascular Sciences and Key Laboratory of Molecular Cardiovascular Sciences, China) provided valuable suggestions and technical support on this work. The present study is supported by an operating grant from the Canadian Diabetes Association (to BR) and in part by a grant to Dr. Vlodavsky from the Israel Ministry of Health and Dr. Liu from the National Natural Science Foundation. Ying Wang and Dahai Zhang are the recipients of Doctoral Student Research Awards from the Canadian Diabetes Association.

DISCLOSURE

None
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FIGURE LEGEND

Figure 1. **Heparanase present in cardiomyocytes relies on exogenous uptake.** Total cell lysates of RAOEC (EC) and cardiomyocytes (Myo) were extracted to Western blot for latent (L-HEPA) and active heparanase (A-HEPA). To validate the purity of cardiomyocytes, equal amounts of protein from Myo and EC were used to determine the presence of CD31 (A, left panel). 1 µg total RNA from cardiomyocytes and EC was used for reverse transcription. Amplified DNA products from PCR were run on an agarose gel in line with a DNA ladder (M), and a negative control (N, using water to replace PCR product) to detect heparanase gene expression (A, right panel). Cardiomyocytes from control rats were isolated, plated and allowed to equilibrate. Total cell lysates were then collected at 0 or 36 h, and probed for L-HEPA and A-HEPA using Western blot (B). 500 ng/ml purified latent heparanase was added to isolated myocytes and heparanase content in these cells determined at the indicated times by Western blot. To inhibit internalization, myocytes were also incubated with latent heparanase at 15 °C for 15 min (C). Results are the mean ± SE of 3 repeated experiments using different animals. *Significantly different from 0 time point, $P < 0.05$. **Significantly different from 0 time point, $P < 0.01$. #Significantly different from results at 15 min at 37 °C for L-HEPA.

Figure 2. **Internalization of heparanase by cardiomyocytes is mediated by HSPGs.** Isolated cardiomyocytes were treated with 500 ng/ml purified latent heparanase in the absence or presence of 10 IU/ml heparin for 15 min. Cell lysates devoid of plasma membrane (-PM) were used to monitor internalization of latent heparanase (A). To determine the purity of these cell lysates, 10 µg protein from –PM or total cell lysates (Total) was used to Western blot for the plasma membrane marker Na$^+$-K$^+$-ATPase (A, inset, right panel). Isolated myocytes were
untreated (UT) or pre-treated with 10 IU/L heparinase III for 2 h, and subsequently incubated with 500 ng/ml purified latent heparanase for 15 min. Internalization of latent heparanase was determined using –PM fraction (B). Results are the mean ± SE of 3 repeated experiments using different animals. **Significantly different from 0 time point, \( P < 0.01 \). ***Significantly different from 15 min without heparin. *Significantly different from untreated myocytes, \( P < 0.05 \).

**Figure 3.** Internalization of latent heparanase is caveolae-dependent and requires dynamin and tyrosine kinase activity. Isolated cardiomyocytes were incubated with 350 mM sucrose (to inhibit clathrin-coated pit formation) or 1 µg/ml filipin (to inhibit caveolae-dependent internalization) for 15 min before 500 ng/ml purified latent heparanase was added to the medium. Internalization of latent heparanase was measured after 15 min using –PM fractions (A). The inhibitory effect of sucrose on clathrin-coated pit dependent internalization was validated by testing the internalization of epidermal growth factor receptor (EGFR) in myocytes treated with EGF in the presence or absence of sucrose (A, right inset). In a different experiment, isolated myocytes were pre-treated with dynasore (to inhibit dynamin activity, B) or genistein (to inhibit tyrosine kinase activity, C) for 1 h, and internalization of latent heparanase after 15 min determined using Western blot. Results are the mean ± SE of 3 repeated experiments using different animals. *Significantly different from untreated myocytes, \( P < 0.05 \). **Significantly different from untreated myocytes, \( P < 0.01 \).

**Figure 4.** Following uptake exogenous latent heparanase is activated in lysosomes. Isolated myocytes were incubated with 500 ng/ml purified latent heparanase for the indicated times. Fractions rich in lysosomes were isolated to detect heparanase by Western blot. Lysosomal-associated membrane protein 1 (Lamp1) was used as a marker to indicate equal
loading of lysosomal protein (A). Myocytes were pre-incubated with 200 µM chloroquine before latent heparanase was added to the medium. Heparanase in the lysosomal fraction was measured after 3 h (B). Results are the mean ± SE of 3 repeated experiments using different animals. *Significantly different from 0 time point or untreated myocytes, $P < 0.05$.

**Significantly different from 0 time point, $P < 0.01$.

**Figure 5. Nuclear entry of heparanase increases MMP-9 expression.** Isolated myocytes were incubated with 500 ng/ml purified latent heparanase for the indicated times. Nuclear fraction was separated from cytosolic protein to detect heparanase by Western blot (A). To validate the purity of the nuclear fraction, equal amount of protein from both cytosolic and nuclear fractions were Western blotted for GAPDH and histone H3 (A, bottom inset). Nuclear fraction from control myocyte was incubated with (A-HEPA) or without (CON) 200 ng/ml active heparanase for 8 h, and HAT activity measured (B). Total RNA was extracted from cardiomyocytes treated with 500 ng/ml latent heparanase to measure gene expression of MMP-9 using RT-PCR (C). In another experiment, cardiomyocytes were treated 500 ng/ml latent heparanase in the presence or absence of 10 µM anacardic acid for 24 h, and MMP-9 gene expression determined. Cardiomyocytes without any treatment was used as control (D). *Significantly different from 0 time point or control, $P < 0.05$. **Significantly different from 0 time point or control, $P < 0.01$. #Significantly different from L-HEPA in the absence of anacardic acid, $P < 0.05$.

**Figure 6. MMP-9 releases LPL from myocyte surface by shedding HSPGs.** Purified MMP-9 proenzyme was activated by incubation with 10 µM AMPA at 37 °C for 1 h, and added to myocyte culture medium at the indicated concentrations. Both syndecan-1 and LPL released into the medium was determined after 30 min (A, upper and lower inset, respectively). MMP-9
secretion into the medium was determined 24 h after cardiomyocytes were incubated with latent heparanase (A). 100 pmol siRNA for MMP-9 (siRNA) was transfected into cardiomyocytes in a six well plate, and the efficacy of transfection was validated 24 h after transfection by Western blot (B, inset). Transfection of a scrambled sequence was used as a negative control (SCR). These transfected cardiomyocytes were incubated in the presence or absence (CON) of 500 ng/ml latent heparanase for 24 h, and shedding of Syndecan-1 together with release of LPL into the medium determined (B and C, respectively). Results are the mean ± SE of 3 repeated experiments using different animals. *Significantly different from control, \( P < 0.05 \). **Significantly different control, \( P < 0.01 \). #Significantly different from SCR, \( P < 0.05 \).

**Figure 7. Following diabetes, endothelial cells influence coronary LPL activity through a heparanase/MMP-9 axis.** RAOEC were incubated with 5.5 mM (NG), or 25 mM (HG) glucose DMEM for 30 min. The amount of latent and active heparanase released into the medium was determined by Western blot (A). Results are the mean ± SE of 3 repeated experiments. *Significantly different from NG-treated RAOEC, \( P < 0.05 \). A co-culture was carried out using cardiomyocytes in the well (bottom) with (+EC) RAOEC on the insert (top), and the co-culture was exposed to NG or HG for 2 h. The amount of L-HEPA in cardiomyocytes was measured by Western blot. Cardiomyocytes were also treated with NG or HG DMEM without RAOEC (-EC) as a control (B). Results are the mean ± SE of 3 repeated experiments. *Significantly different from NG-treated co-culture system, \( P < 0.05 \). Animals were made diabetic by injecting 55 mg/kg STZ and kept for 4 days (D55). The amount of active heparanase in the nuclear fractions of myocytes isolated from control and D55 heart was measured by Western blot (C). MMP-9 gene expression was also determined in these myocytes (D). Shedding of syndecan-1 into the culture medium over 24 h was detected by
ELISA (E). LPL activity at the coronary lumen of both control and D55 hearts were measured by perfusing the heart with 5 U/ml heparin. 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 (AUC) for heparin-released LPL activity over 5 min (F). Results are the mean ± SE of 3 repeated experiments using different animals. *Significantly different from control, $P < 0.05$. **Significantly different from control, $P < 0.01$.

**Figure 8.** Endothelial cells affect cardiomyocyte metabolism through heparanase-induced release of LPL. In the presence of hyperglycemia, there is release of both active and latent heparanase from endothelial cells. Active heparanase can cause an immediate release of myocyte HSPG-bound LPL. However, latent inactive heparanase can be taken up by cardiomyocytes via HSPGs and converted into active heparanase in the lysosomes. Upon entry into the nucleus, active heparanase, by modulating HAT activity, promotes gene expression of MMP-9, which cleaves the ectodomain of myocyte surface HSPGs such as syndecan-1. By this mechanism, HSPGs-bound LPL is liberated from myocyte surface to translocate to the coronary lumen. The ultimate effect would be increased TG hydrolysis, over-abundant delivery of FA to the cardiomyocytes, and eventually lipotoxicity.
Figure 1. Heparanase present in cardiomyocytes relies on exogenous uptake. Total cell lysates of RAOEC (EC) and cardiomyocytes (Myo) were extracted to Western blot for latent (L-HEPA) and active heparanase (A-HEPA). To validate the purity of cardiomyocytes, equal amounts of protein from Myo and EC were used to determine the presence of CD31 (A, left panel). 1 µg total RNA from cardiomyocytes and EC was used for reverse transcription. Amplified DNA products from PCR were run on an agarose gel in line with a DNA ladder (M), and a negative control (N, using water to replace PCR product) to detect heparanase gene expression (A, right panel). Cardiomyocytes from control rats were isolated, plated and allowed to equilibrate. Total cell lysates were then collected at 0 or 36 h, and probed for L-HEPA and A-HEPA using Western blot (B). 500 ng/ml purified latent heparanase was added to isolated myocytes and heparanase content in these cells determined at the indicated times by Western blot. To inhibit internalization, myocytes were also incubated with latent heparanase at 15 ºC for 15 min (C). Results are the mean ± SE of 3 repeated experiments using different animals. *Significantly different from 0 time point, P < 0.05. **Significantly different from 0 time point, P < 0.01. #Significantly different from results at 15 min at 37 ºC for L-HEPA.
Figure 2. Internalization of heparanase by cardiomyocytes is mediated by HSPGs. Isolated cardiomyocytes were treated with 500 ng/ml purified latent heparanase in the absence or presence of 10 IU/ml heparin for 15 min. Cell lysates devoid of plasma membrane (-PM) were used to monitor internalization of latent heparanase (A). To determine the purity of these cell lysates, 10 µg protein from -PM or total cell lysates (Total) was used to Western blot for the plasma membrane marker Na+K+-ATPase (A, inset, right panel). Isolated myocytes were untreated (UT) or pre-treated with 10 IU/L heparinase III for 2 h, and subsequently incubated with 500 ng/ml purified latent heparanase for 15 min. Internalization of latent heparanase was determined using -PM fraction (B). Results are the mean ± SE of 3 repeated experiments using different animals. **Significantly different from 0 time point, P < 0.01. ##Significantly different from 15 min without heparin. *Significantly different from untreated myocytes, P < 0.05.
Figure 3. Internalization of latent heparanase is caveolae-dependent and requires dynamin and tyrosine kinase activity. Isolated cardiomyocytes were incubated with 350 mM sucrose (to inhibit clathrin-coated pit formation) or 1 µg/ml filipin (to inhibit caveolae-dependent internalization) for 15 min before 500 ng/ml purified latent heparanase was added to the medium. Internalization of latent heparanase was measured after 15 min using –PM fractions (A). The inhibitory effect of sucrose on clathrin-coated pit dependent internalization was validated by testing the internalization of epidermal growth factor receptor (EGFR) in myocytes treated with EGF in the presence or absence of sucrose (A, right inset). In a different experiment, isolated myocytes were pre-treated with dynasore (to inhibit dynamin activity, B) or genistein (to inhibit tyrosine kinase activity, C) for 1 h, and internalization of latent heparanase after 15 min determined using Western blot. Results are the mean ± SE of 3 repeated experiments using different animals. *Significantly different from untreated myocytes, P < 0.05. **Significantly different from untreated myocytes, P < 0.01.
Figure 4. Following uptake exogenous latent heparanase is activated in lysosomes. Isolated myocytes were incubated with 500 ng/ml purified latent heparanase for the indicated times. Fractions rich in lysosomes were isolated to detect heparanase by Western blot. Lysosomal-associated membrane protein 1 (Lamp1) was used as a marker to indicate equal loading of lysosomal protein (A). Myocytes were pre-incubated with 200 µM chloroquine before latent heparanase was added to the medium. Heparanase in the lysosomal fraction was measured after 3 h (B). Results are the mean ± SE of 3 repeated experiments using different animals. *Significantly different from 0 time point or untreated myocytes, P < 0.05. **Significantly different from 0 time point, P < 0.01.
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254x190mm (96 x 96 DPI)
Supplemental Figure 1. Cleaved form of HSPGs and the expression of MMP-9 increased in cardiomyocytes from diabetic heart. Cardiomyocytes were isolated from control and D55 animals. Nuclear fractions were extracted from these myocytes, and the cleaved form of HSPGs was determined by Western blot (A). MMP-9 expression was also determined in the whole cell lysates by Western blot (B). Results are the mean ± SE of 3 repeated experiments using different animals. *Significantly different from control, $P < 0.05$. 
Supplement Figure 2. MMP-9 expression in diazoxide-induced hyperglycemic animals.

Animals were injected with 100 mg/kg diazoxide i.p. and kept for 4 h (DZ). RNA was extracted from isolated myocytes to determine MMP-9 expression. Results are the mean ± SE of 3 repeated experiments using different animals.