Endothelial heparanase secretion after acute hypoinsulinemia is regulated by glucose and fatty acid

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Wang F, Kim MS, Puthanveetil P, Kewalramani G, Deppe S, Ghosh S, Abrahani A, Rodrigues B. Endothelial heparanase secretion after acute hypoinsulinemia is regulated by glucose and fatty acid. Am J Physiol Heart Circ Physiol 296: H1108–H1116, 2009. First published February 13, 2009; doi:10.1152/ajpheart.01312.2008.—Following diabetes, the heart increases its lipoprotein lipase (LPL) at the coronary lumen by transferring LPL from the cardiomyocyte to the endothelial lumen. We examined how hyperglycemia controls secretion of heparanase, the enzyme that cleaves myocyte heparan sulphate proteoglycan to initiate this movement. Diazoxide (DZ) was used to decrease serum insulin and generate hyperglycemia. A modified Langendorff technique was used to separate coronary from interstitial effluent, which were assayed for heparanase and LPL. Within 30 min of DZ, interstitial heparanase increased, an effect that closely mirrored an augmentation in interstitial LPL. Endothelial cells were incubated with palmitic acid (PA) or glucose, and heparanase secretion was determined. PA increased intracellular heparanase, with no effect on secretion of this enzyme. Unlike PA, glucose dose-dependently lowered endothelial intracellular heparanase, which was strongly associated with increased heparanase activity in the incubation medium. Preincubation with cytochalasin D or nocodazole prevented the high glucose-induced depletion of intracellular heparanase. Our data suggest that following hyperglycemia, translocation of LPL from the cardiomyocyte cell surface to the apical side of endothelial cells is dependent on the ability of the fatty acid to increase endothelial intracellular heparanase followed by rapid secretion of this enzyme by glucose, which requires an intact microtubule and actin cytoskeleton.

diabetes; diazoxide; high glucose; endothelial cell; cytoskeleton

Since uninterrupted contraction is a unique feature of the heart, cardiac muscle has a high demand for provision of energy (2). Under normal physiological conditions, hearts can utilize multiple substrates, including fatty acid (FA), carbohydrate, amino acid, and ketone bodies. Among these substrates, carbohydrate (~30%) and FA (~70%) are the major sources from which the heart derives most of its energy (29). FA delivery and utilization by the heart involves 1) release from adipose tissue and transport to the heart after complexing with albumin (19), 2) provision through the breakdown of endogenous cardiac triglyceride (TG) stores (23), 3) internalization of whole lipoproteins (14), and 4) hydrolysis of circulating TG-rich lipoproteins (very low density lipoproteins and chylomicrons) to FA by lipoprotein lipase (LPL) positioned at the endothelial surface of the coronary lumen (4). Despite this critical function, coronary endothelial cells do not synthesize LPL (6). In the heart, this enzyme is produced in cardiomyocytes and subsequently secreted onto heparan sulphate proteoglycan (HSPG) binding sites on the myocyte cell surface (11). From here, LPL is transported onto comparable binding sites on the luminal surface of endothelial cells (3). At the lumen, LPL actively metabolizes the TG core of lipoproteins to FA; these released FA are then transported into the heart for numerous metabolic and structural functions.

LPL synthesis and activity is regulated in a tissue-specific manner by various physiological and pathophysiological conditions such as fasting and diabetes. During fasting, with ensuing hypoinsulinemia, LPL activity decreases in adipose tissue but rapidly increases in the heart (28); as a result, FA from circulating TG are diverted away from storage to meet the metabolic demands of the heart. Hence, LPL fulfills a gatekeeping function by regulating the supply of FA to meet the metabolic demands of different tissues. Using retrograde perfusion of the heart with heparin to displace coronary LPL, we also found significantly elevated luminal LPL activity following diabetes (27). We determined that the increased enzyme in the diabetic heart is 1) not the result of increased gene expression (25), 2) likely unrelated to an increase in the number of capillary endothelial HSPG binding sites (25), 3) functionally relevant and capable of hydrolyzing lipoprotein-TG (30), and 4) acutely (hours) regulated by short-term changes in insulin levels (31). More recently, we have been studying the contribution of the cardiomyocyte in enabling this increased enzyme to the vascular lumen using an acute model of hypoinsulinemia induced with diazoxide (DZ) (16, 17). This agent produces stable hyperglycemia within 1 h and is a useful model to examine rapid changes in cardiac LPL observed during diabetes (25). In myocytes from DZ-treated hearts, LPL vesicle fission was controlled by protein kinase D (17), whereas recruitment of LPL to the cell surface was controlled by stress kinases like AMPK and p38 MAPK that allowed for actin cytoskeleton polymerization (16).

In the heart, the mechanisms that regulate LPL translocation from the cardiomyocyte surface HSPG to the endothelial lumen are still unclear. Heparanase, an endoglucuronidase, specifically cleaves HSPG and facilitates the release of several bioactive proteins including growth factors, cytokines, and LPL (24, 38). Heparanase is synthesized as a latent 65-kDa enzyme that undergoes secretion followed by HSPG-mediated reuptake. After undergoing proteolytic cleavage (in lysosomes...
lungs still attached. After the aorta was cannulated and tied below the left anterior vena cava was ligated below the azygous vein (31). Rats were anesthetized, the thoracic cavity was opened, and Langendorff retrograde perfusion technique was used (8, 9, 15, 25, 26). DZ (100 mg/kg ip) was administered, animals were euthanized after 1 to 4 h, and animal hearts were removed for measurement of the hydrolysis of [3H]triolein. For modified Langendorff perfusion, rats were

Materials and Methods

Animal model. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health and the University of British Columbia and was approved by the Animal Care and Use Committee (Protocol No. A08-0627). Adult male Wistar rats (260–300 g) were obtained from the University of British Columbia Animal Care Unit. Previously, we have reported that an acute reduction in insulin (hours) is associated with an increase in LPL at the coronary lumen (31). To examine LPL under conditions where insulin levels are rapidly manipulated, we used DZ, a selective KACp channel opener. In the pancreas, opening of the KACp channel hyperpolarizes the β-cell membrane and decreases intracellular calcium and insulin secretion is rapidly inhibited (within 1 h). After an intraperitoneal injection of DZ, stable hyperglycemia develops within 1 h and persists for 4 h and blood glucose remains 2 to 3 times higher than normal. Changes in plasma parameters with DZ also included significant and rapid increases in FA and TG, alterations that are reversed by treatment with insulin (25, 26).

Overall, we have repeatedly demonstrated that DZ is a useful model for the inadequately controlled patient with type 1 diabetes (16, 17, 25, 26). DZ (100 mg/kg ip) was administered, animals were euthanized after 1 to 4 h, and animal hearts were removed for measurement of LPL activity and Western blot analysis.

Plasma measurements. Rats were injected with DZ at 10 AM (fed state). Following DZ, blood samples from the tail vein were collected over a period of 4 h, and blood glucose was determined using a glucometer (AccuSoft). At varying intervals, blood was also acquired in heparinized glass capillary tubes. Blood samples were immediately centrifuged, and plasma was collected and assayed for FA using a diagnostic kit (NEFA; Wako).

Isolated heart perfusions. For Langendorff perfusion, rats were anesthetized with 65 mg/kg ip pentobarbital sodium, the thoracic cavity was opened, and the heart was carefully excised. After the aorta was cannulated and tied below the innominate artery, hearts were perfused retrogradely by the noncirculating Langendorff technique with Krebs-Ringer-HEPES buffer as described previously (27). To measure endothelium-bound LPL, perfusion solution was changed to buffer containing 1% FA free bovine serum albumin (BSA) and heparin (5 U/ml). The coronary effluent was collected in timed fractions over 10 min and assayed for LPL activity by measuring the hydrolysis of [1H]triolein. For modified Langendorff perfusion, to separate the coronary from the interstitial effluent, a modified Langendorff retrograde perfusion technique was used (8, 9, 15, 31). Rats were anesthetized, the thoracic cavity was opened, and the left anterior vena cava was ligated below the azygos vein followed by ligation of the right anterior vena cava. The hearts were then carefully excised with the aorta, inferior vena cava, and lungs still attached. After the aorta was cannulated and tied below the innominate artery, hearts were perfused retrogradely by the noncirculating Langendorff technique. The right and left branches of the pulmonary artery were cut before they entered the lungs, and the two branches were then trimmed off at their junction. Subsequently, the inferior vena cava and branches of the right and left pulmonary veins were ligated, the lungs were removed, and the pulmonary artery was cannulated and tied. At this time, most of the perfusate (~98% to 99%) starts flowing through the pulmonary cannula (coronary perfusate), whereas a small amount of fluid (~1% to 2%) drips down to the apex of the heart (interstial transudate). The coronary and interstitial effluents were collected separately in timed fractions and frozen until assayed for heparanase and LPL using Western blot.

Immunohistochemistry. Immediately on excision, control and DZ rat hearts were stored in 10% Formalin for 48 h and processed for immunolocalization of heparanase as described previously (39). Briefly, sections were incubated with the heparanase antibody MAb130 (1:30 dilution) overnight at room temperature in a humid chamber and further incubated for 1 h with the secondary horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (1:200 dilution). Color was developed by an AEC kit, followed by counter staining with Mayer’s hematoxylin. Slides were mounted and visualized using a Leica fluorescent microscope (Wetzlar, Postfach, Germany). Absence of staining was observed when the primary antibody was omitted and replaced by 1% goat serum.

Endothelial cell culture. Both coronary artery endothelial cells (bCAECs; Clonetics) were cultured in endothelial growth medium (EGM) supplemented with EGM-MV BulletKit (Lonza) at 37°C in a 5% CO₂ humidified incubator. Cells from the 5th to the 8th passage were used for the experiment. Cells (0.5 × 10⁶) were placed in six-well plates and grown to confluence. Subsequently and where indicated, albumin-bound palmitic acid (PA; 0.25–1 mM) or oleic acid (1 mM; molar ratio, 1:6) or high glucose (HG; 15–25 mM) was added to the culture medium for 30 min. BSA-FA solutions were prepared by first dissolving the FA in ethanol (500 μl) and then adding appropriate amounts to media to obtain the required molar ratio of BSA to FA. Mannitol (20 mM; osmolarity control) or 5 ng/ml TNF-α (positive control) was also added to the culture medium for 30 min. In some experiments, to simulate hyperglycemia induced by DZ, bCAECs were also incubated for 30 min with 1 mM PA before exposure to 25 mM glucose for another 30 min. Following separation of medium from the cells, heparanase was determined in both cell lysates (using Western blot) and medium (using an activity assay kit).

Cells were also used for immunofluorescent detection of heparanase. Finally, intracellular heparanase was measured after preincubations with cytochalasin D (an actin polymerization inhibitor) or nocodazole (a microtubule disrupting agent) for 10 min followed by PA (1 mM, for 30 min) or HG (25 mM, for 30 min).

Western blot analysis. Western blot was carried out as described previously (17). Briefly, ventricles or plated bCAECs were homogenized in ice-cold lysis buffer. After centrifugation at 5,000 g for 20 min, the protein content of the supernatant was quantified using a Bradford protein assay. Samples were diluted and boiled with sample loading dye, and 50 μg was used in SDS-PAGE. After blotting, membranes were blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween 20. Membranes were incubated with either anti-heparanase MAb130 (that recognizes both 50 and 65 kDa heparanase forms; only the active 50-kDa heparanase data are presented) or anti-LPL antibodies or secondary antibodies. Reaction products were visualized using an ECL detection kit and quantified by densitometry.

Immunofluorescence. Following incubation with PA (1 mM), glucose (25 mM), or TNF-α (5 ng/ml) for 30 min, cells were fixed for 10 min with 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized with 0.1% Triton X-100 in PBS for 3 min, treated with PBS containing 1% goat serum for 1 h, and finally rinsed with PBS. Cells were incubated with anti-heparanase MAb130 antibody followed by incubation with Texas Red-conjugated donkey anti-mouse antibody to localize heparanase. 4,6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei. Slides were visualized using a fluorescent microscope.

Nuclear localization of heparanase. After incubation of bCAECs with PA, cells were scraped and washed twice with 0.5 ml PBS.
**RESULTS**

Coronary lumen LPL increases after acute DZ injection. We have previously reported that 100 mg/kg DZ caused a rapid decline in serum insulin within 1 h (26). In this study, blood glucose levels increased within 30 min and reached a maximum after 2 h of DZ administration (Fig. 1A). Therefore, all subsequent experiments were carried out using DZ administered for up to 2 h. Changes in plasma parameters with DZ also included a significant and rapid increase in FA (Fig. 1B). Notably, the near maximum level of FA was reached earlier than the peak for glucose. In a preliminary experiment, to determine the kinetics of LPL upregulation at the vascular lumen, some DZ-treated hearts were isolated at 30–120 min, and LPL activity was measured. The increase in LPL activity at the vascular lumen (as measured by retrograde perfusion of hearts with heparin) became apparent as early as 30 min subsequent to the injection of DZ, peaked at 60 min, and was maintained for an additional 120 min (Fig. 1C and inset).

Rapid release of heparanase is associated with changes in interstitial LPL. We used the modified Langendorff perfused heart to separate the coronary perfusate from interstitial fluid and measured coronary and interstitial heparanase (the 50-kDa active form) and LPL. Within 30 min of DZ, interstitial heparanase increased approximately twofold (Fig. 2A). Unlike the changes observed in interstitial heparanase, we were unable to detect heparanase in the coronary outflow of either control or DZ-treated hearts (data not shown). Extending the duration of DZ for 2 h lowered interstitial heparanase to below control values (Fig. 2A). Since the measurement of whole heart heparanase using Western blot and immunohistochemical evaluation indicated a time-dependent loss of heparanase protein (Fig. 2B) over the duration of DZ for 2 h [more specifically from within the endothelial cells (Fig. 2C)], our data suggest that acute hyperglycemia has a robust influence on secretion (into the interstitial space) and subsequent attenuation of stored heparanase. Previously, endothelial heparanase has been shown to
FAs facilitate endothelial intracellular heparanase accumulation. To examine the mechanism by which DZ increases heparanase secretion, we incubated endothelial cells with increasing concentrations of PA, up to the peak circulating FA concentrations seen with DZ. Unexpectedly, concentrations that varied from 0.75 to 1 mM increased intracellular heparanase (Fig. 3A). This effect was not exclusive to PA since 1 mM oleic acid also had a similar outcome (Fig. 3A, bottom inset). Immunofluorescent detection of intracellular heparanase revealed a novel observation. In control conditions, intracellular heparanase was located predominately around the nucleus (Fig. 3B, top row). However, high concentrations of PA (that resembled the peak circulating concentrations seen with DZ) increased the nuclear staining of heparanase (Fig. 3B, bottom row). Quantification using Western blot validated the increased nuclear content of heparanase following PA (Fig. 3B, inset). Conversely, measurement of heparanase activity in the incubation medium demonstrated that PA had no effect in secretion of this enzyme (Fig. 3A, top inset).

Dose-dependent secretion of endothelial heparanase by glucose. Unlike FAs, glucose dose-dependently lowered endothelial intracellular heparanase, with significant changes observed after 20 mM glucose (Fig. 4A). This decrease in intracellular heparanase was strongly associated to increased heparanase activity in the incubation medium as the concentration of glucose was raised (Fig. 4B). Confirmation of the effects of HG (25 mM) on heparanase secretion was done using immunofluorescence. Compared with control, HG caused the intracellular heparanase located predominately around the nucleus (Fig. 4C, top row) to disperse toward the plasma membrane (Fig. 4C, middle row). The osmolarity control mannitol had no effect on intracellular (Fig. 4A, inset) or medium (Fig. 4B, inset) heparanase, whereas the positive control, TNF-α, had effects similar to HG; it lowered intracellular heparanase (Fig. 4A, inset), increased medium heparanase activity (Fig. 4B, inset), and dispersed heparanase located around the nucleus toward the plasma membrane (Fig. 4C, bottom row).

Simulating DZ-induced increase in substrates in vitro augments endothelial heparanase secretion. Given the opposite effects of FA and HG on endothelial heparanase, we incubated bCAECs with FA or HG independently and compared the results with endothelial cells first exposed to PA and then to...
HG (PA + HG). The latter group was expected to simulate the temporal changes seen with DZ; earlier peak in FA was followed by a delayed maximum glucose concentration. Interestingly, in the presence of HG, PA failed to increase endothelial intracellular heparanase (Fig. 5A). More importantly, this pattern of incubation produced an even greater increase in medium heparanase activity when compared with HG alone (Fig. 5B).

Endothelial cytoskeleton mediates both FA and HG-induced changes in heparanase. The actin cytoskeleton has been implicated in managing heparanase processing (22). To determine whether PA or HG elicits F-actin polymerization, we quantitated F-actin and G-actin cellular fractions using Western blot. An increase in F-actin-to-G actin ratio indicates actin polymerization. Both PA and HG (and TNF-α) increased the F-/G-actin ratio, an effect that was absent when using mannitol (Fig. 6A and inset). Preincubation of bCAECs for 10 min with cytochalasin D prevented the PA-induced accumulation and the HG-induced depletion of intracellular heparanase (Fig. 6B). Using nocodazole to disrupt microtubules, the effects of PA and HG on intracellular heparanase were dissimilar. Although pretreatment with nocodazole prevented the HG-induced depletion of heparanase, it had no influence in affecting the PA-induced accumulation of heparanase (Fig. 6C).

DISCUSSION

The earliest change that occurs in the diabetic heart is altered energy metabolism, where in the presence of lower glucose utilization, the heart switches to predominantly using FAs for energy supply (1). One means by which this is achieved is through rapid augmentation of LPL (a key enzyme, which

Fig. 3. Dose-dependent effect of palmitic acid (PA) on endothelial heparanase. Bovine coronary artery endothelial cells (bCAECs; 0.5 × 10⁶ cells) were grown to 80–90% confluence and incubated either in the absence or presence of 0.25–1.0 mM albumin-bound PA (1:6) for 30 min. Following separation of medium from the cells, heparanase was determined in cell lysates (A) and incubation medium (top inset). A, bottom inset: influence of oleic acid (OA; 1 mM for 30 min) on heparanase in cell lysates. Results are means ± SE for 6 rats/group and are expressed as ratio to control. Control and PA-treated bCAECs were fixed, permeabilized, and double stained with anti-heparanase MAb 130 (red) and 4,6-Diamidino-2-phenylindole (DAPI; blue; B). The merged image of heparanase and nucleus is described in the Merge column. Scale bar = 25 μm. Data are from a representative experiment done twice. B, inset: nuclear heparanase after bCAECs were treated with 1 mM PA for 30 min. *Significantly different from control, P < 0.05.
hydrolyzes lipoproteins to release FA) at the coronary lumen (25–27, 30). Given that vascular endothelial LPL is acquired from the cardiomyocyte, we previously examined the mechanisms by which DZ-induced hypoinsulinemia increases cardiomyocyte cell surface LPL, a reservoir that can rapidly augment coronary luminal LPL when the need for FA is intensified. In the myocyte, we reported that recruitment of LPL to the cell surface was controlled by protein kinase D (which regulated LPL vesicle fission) (17), whereas stress kinases like AMPK and p38 MAPK allowed for actin cytoskeleton polymerization (16), providing the cardiomyocyte with an infrastructure to facilitate LPL movement. In the present study, our data demonstrate that following DZ-induced hypoinsulinemia, heparanase is released into the interstitial compartment; the subsequent heparanase-induced cleavage of cardiomyocyte surface

Fig. 4. Dose-dependent effect of glucose on endothelial heparanase. bCAECs (0.5 × 10⁶ cells) were grown to 80–90% confluence and incubated either in the absence or presence of glucose (15–25 mM) for 30 min. Following separation of medium from the cells, heparanase was determined in both cell lysates (A) and medium (B). The insets depict the influence of mannitol (Mnt; 20 mM) and TNF-α (5 ng/ml) on heparanase in cell lysates (A) and medium (B). Results are means ± SE of 3 separate experiments and are expressed as ratio to control. *Significantly different from control, P < 0.05. HG, high glucose (25 mM).

Fig. 5. Dual effect of PA and glucose on endothelial heparanase. bCAECs were incubated either in the absence or presence of PA (1 mM) or glucose (HG; 25 mM) for 30 min. To simulate the substrate change induced by DZ, bCAECs were also incubated for 30 min with PA before exposure to 25 mM glucose for another 30 min (PA + HG). Following separation of medium from the cells, heparanase was determined in both cell lysates (A) and medium (B). Results are means ± SE of 3 separate experiments and are expressed as ratio to control. *Significantly different from control; #Significantly different from HG, P < 0.05.
LPL could result in the eventual transfer of LPL to the vascular endothelial lumen. However, it must be acknowledged that at present, we have no direct evidence that this released interstitial heparanase actually caused the observed increase in interstitial LPL.

In coculture experiments using bovine endothelial cells and adipocytes, endothelial cells secreted compounds with heparanase-like activity that released adipocyte LPL, promoting its transfer to the luminal endothelial surface (24). Interestingly, this heparanase secretion following lysophosphatidylcholine was polarized, with preferential secretion toward the basolateral rather than the apical side of endothelial cells (24). If this mechanism was also to occur in the heart, we hypothesized that hyperglycemia would increase the amount of heparanase in the interstitial space. Using a modified Langendorff heart perfusion that separates coronary from interstitial fluid, we established for the first time that following DZ, heparanase indeed increased rapidly (within 30 min of DZ) in the interstitial compartment, an effect that was lost by 2 h. This secretion of active heparanase into the interstitial space paralleled the observations that with increased duration of hyperglycemia (2 h), the residual content of heparanase in the whole heart, endothelial cells, and interstitial fluid decreased. In HEK-293 cells, the time required for the conversion of latent to active heparanase requires at least 4 h (13). Hence, following secretion, 2 h of hyperglycemia may be insufficient for either latent heparanase to be synthesized or active heparanase to be regenerated and could explain this reduction in activity over time. Interestingly, the peak increase in interstitial heparanase closely mirrored the amplification in interstitial LPL, suggest-
ing that following DZ, it is this rapid secretion of endothelial heparanase that initiates the release of subendothelial myocyte cell surface LPL. Once released, this bolus amount of interstitial LPL has to traverse the endothelial cell to reach HSPG binding sites at the coronary lumen. Since this process is unlikely to occur rapidly, it could explain the discrepancy between interstitial heparanase and LPL after 2 h of DZ. Our results imply that following hyperglycemia, the secretion of endothelial heparanase into the interstitial compartment may contribute to LPL translocation from its site of synthesis (cardiomyocyte) to its site of action (endothelial lumen). It should be noted that other methods have also been proposed to explain the transfer of LPL from the cardiomyocyte to the endothelial cell and include vectorial movement of enzyme along a continuous network of HSPG that extends from myocyte to endothelial cells (33). Future experimentation will have to determine the relative importance of interstitial heparanase as an LPL translocation mechanism.

Since immunohistochemical localization of heparanase in the heart revealed a predominant expression in endothelial cells and depletion with the progression of hyperglycemia, we attempted to examine the mechanism of endothelial heparanase secretion. bCAECs were incubated with concentrations of PA or glucose that duplicated the plasma concentrations of these substrates observed after DZ. Unexpectedly, with increasing concentrations of PA, intracellular heparanase increased within 30 min, with no release into the medium observed even at the highest concentration of PA used (1 mM). This effect was not exclusive to PA since the unsaturated oleic acid also had a similar outcome. Visualization of the enzyme after treatment with 1 mM PA illustrated a predominant perinuclear and nuclear localization, an observation confirmed by nuclear isolation followed by Western blot. This acute effect of PA is likely through an increased reuptake of the latent 65-kDa enzyme followed by lysosomal activation (13) and nuclear translocation; heparanase has been reported to have two potential nuclear localization sequences (32). At present, the motive behind this FA-induced nuclear compartmentalization of heparanase is unclear. In hepatocytes, ~12% of the total heparan sulfate pool is located within the nucleus (12). It is possible that within the endothelial nucleus, heparanase can facilitate heparan sulfate degradation, a feedback safety mechanism to limit the expression of endothelial HSPG and eventually LPL-derived FA. Alternatively, the nucleus can serve as a storage reservoir for heparanase. Overall, our data suggest that high FA, by increasing perinuclear and nuclear heparanase, may limit the hydrolysis of circulating TG by LPL to avoid excess FA delivery to cardiomyocytes.

In human microvascular endothelial cells, the inflammatory cytokine TNF-α has been shown to facilitate heparanase secretion (7). In the absence of an effect of FA on endothelial heparanase secretion, we tested the influence of glucose in mediating heparanase release. Both TNF-α and glucose lowered endothelial intracellular heparanase (there was a robust dispersion of perinuclear heparanase toward the plasma membrane), with a related increase in heparanase activity in the incubation medium. At present, it is unclear whether this glucose-induced secretion of heparanase is through the apical or basolateral (or both) side of endothelial cells. To more closely mimic the temporal changes in substrates observed with DZ, bCAECs were first incubated with PA before the addition of HG. The addition of HG to cells pretreated with PA prevented intracellular accumulation of heparanase. More importantly, under these conditions, the amount of heparanase released into medium was higher than that observed with glucose alone, suggesting that FA can increase the amount of intracellular heparanase available for subsequent release by glucose. Taken together with the observation that DZ increases heparanase release into the cardiac interstitial space, our data suggest that HG is the predominant reason for endothelial heparanase secretion. At present, the mechanism by which HG initiates heparanase release is unclear. At least in HEK-293 cells, nucleotides mediate heparanase secretion by stimulation of P2Y receptors and activation of PKC signaling (34), mechanisms that are currently being tested in our system. Irrespective of the mechanism, HG-dependent secretion of heparanase will facilitate cardiomyocyte HSPG cleavage and LPL release, translocation of LPL to the vascular lumen, and increased provision of TG-derived FA when cardiac utilization of glucose is compromised.

Cellular heparanase is present predominantly in lysosomes (39), whose exocytosis is highly regulated and dependent on both microtubules and the actin cytoskeleton (5). In cardiomyocytes, the G-actin-to-F-actin ratio is approximately 10:90, whereas nonactivated platelets may have an 80:20 G-actin-to-F-actin ratio. We have previously reported that following acute hyperglycemia, the increase in the cardiomyocyte F-actin-to-G-actin ratio is a function of a reduction in G-actin; we could not detect any increase in F-actin since this was the overwhelming form of actin in the cardiomyocyte (16). bCAECs treated with HG demonstrated an increase in the F-actin-to-G actin ratio, indicating actin polymerization. Interestingly, HG had little influence on G-actin with F-actin being the key element in cytoskeleton rearrangement. Other studies also report that postconfluency has little influence on G-actin in bovine aortic endothelial cells, with F-actin being the key constituent involved in cytoskeleton alteration (10). Since the actin polymerization inhibitor, cytochalasin D, prevented the HG-induced depletion of intracellular heparanase, an effect similar to that observed using nocodazole to disrupt microtubules, our data suggest that both microtubules and actin cytoskeleton are important means by which HG enables heparanase secretion. It should be noted that the actin cytoskeleton also plays a key role in promoting insulin-induced GLUT4 translocation (35) and in managing myocyte LPL secretion (16, 17). PA also increased actin polymerization, which has previously been shown to facilitate the uptake of latent heparanase that is processed into an active form and stored in the lysosomal compartment (22). Since pretreatment with cytochalasin D prevented the intracellular accumulation of heparanase, whereas nocodazole had no influence on this PA-induced build-up of heparanase, our data suggest that only the actin cytoskeleton is responsible for this effect of PA on intracellular heparanase.

In summary, following DZ-induced hypoinsulinemia, multiple mechanisms are turned on in the heart to increase vascular lumen LPL. These include an increased transfer of LPL to the cardiomyocyte cell surface and subsequent translocation to the apical side of endothelial cells. The latter process is dependent on the ability of FA to increase endothelial intracellular heparanase followed by the rapid secretion of this endothelial enzyme by glucose. Given that augmented levels of heparanase activity have been reported in plasma and urine of patients with
diabetic nephropathy (20) and overexpression of cardiac human LPL results in a cardiac phenotype resembling diabetic cardiomyopathy (18), results from the present study could help in restricting or slowing cardiac LPL translocation and could lead to strategies that overcome cardiac dysfunction following diabetes.

GRANTS

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REFERENCES