Fatty Acid-Induced Nuclear Translocation of Heparanase Uncouples Glucose Metabolism in Endothelial Cells

Fang Wang, Ying Wang, Dahai Zhang, Prasanth Puthanveetil, James D. Johnson, Brian Rodrigues

Objective—Heparanase is an endoglycosidase that specifically cleaves carbohydrate chains of heparan sulfate. We have recently reported that high fatty acid increased the nuclear content of endothelial heparanase. Here, we examined the mechanism and the consequences behind this nuclear translocation of heparanase.

Methods and Results—Bovine coronary artery endothelial cells were grown to confluence and incubated with palmitic acid. Palmitic acid induced rapid nuclear accumulation of heparanase that was dependent on Bax activation and lysosome permeabilization. Heat shock protein 90 was an important mediator of palmitic acid-induced shuttling of heparanase to the nucleus. Nuclear heparanase promoted cleavage of heparan sulfate, a potent inhibitor of histone acetyltransferase activity and gene transcription. A TaqMan gene expression assay revealed an increase in genes related to glucose metabolism and inflammation. In addition, glycolysis was uncoupled from glucose oxidation, resulting in accumulation of lactate.

Conclusion—The results presented in this study demonstrate that fatty acid can provoke lysosomal release of heparanase, its nuclear translocation, activation of genes controlling glucose metabolism, and accumulation of lactate. Given that lactate and inflammation have been implicated in the progression of atherosclerosis, our data may serve to reduce the associated cardiovascular complications seen during diabetes. (Arterioscler Thromb Vasc Biol. 2012;32:00-00.)

Key Words: endothelium ■ energy metabolism ■ lipids ■ metabolism
cardiomyocytes. Unlike high glucose, augmented concentrations of fatty acid increased the nuclear content of heparanase. In the current study, we examined the mechanism behind this nuclear translocation of heparanase in response to fatty acid and the consequences of this relocation. Our data suggest that fatty acids, by inducing lysosome permeabilization, initiates endothelial heparanase nuclear translocation and induction of gene transcription that affects glucose metabolism and inflammation.

Materials and Methods

Endothelial Cell Culture and Treatments
To examine the effect of fatty acid on endothelial heparanase, bovine coronary artery endothelial cells (bCAECs; Clonetics) were incubated with 0 to 1 mmol/L albumin-bound palmitic acid (PA) (molar ratio 1:6) for 30 minutes (A, inset), or 1 mmol/L PA over a period of 30 minutes (A), and heparanase (Hep) in the nuclear fractions determined. The influence of bromohexadecanoic acid (BPA) (1 mmol/L for 30 minutes) on nuclear heparanase was compared to PA in B. Results are mean±SEM for 3 experiments expressed as ratio changes. *Significantly different from 0 minutes/control (CON). #Significantly different from all groups, P<0.05. Cells were stained with LysoTracker (green), antiheparanase mAb 130 (red) and DAPI (blue). The merged image is described in the fourth panel (C). Bar=20 µm. Data are from a representative experiment done 3 times.

Small Interfering RNA Transfection
Small interfering RNA (siRNA) was designed and synthesized by Applied Biosystems. Transfection was carried out using Lipofectamine™ 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

Histone Acetyltransferase Activity Assay
The histone acetyltransferase (HAT) activity of nuclear extracts was measured with a colorimetric HAT assay kit. This assay is based on the acetylation of substrate by active HAT released CoA, which serves to produce NADH that is detected by a tetrazolium dye.

Lactate Assay
Lactate was determined in the culture medium using a lactate assay kit.

Reverse Transcription-Quantitative Polymerase Chain Reaction
Total RNA was extracted using TRIzol (Invitrogen) and reverse transcription-quantitative polymerase chain reaction was performed using the TaqMan gene expression assay on a StepOnePlus system (Applied Biosystems) according to manufacturer’s instructions.

Statistical Analysis
Values are means±SEM. Wherever appropriate, one-way ANOVA followed by the Tukey test was used to determine differences between groups mean values. The level of statistical significance was set at P<0.05.
Results

Fatty Acid Stimulates Rapid Nuclear Accumulation of Heparanase

In endothelial cells, heparanase is located predominantly in the cytoplasm, with a small amount (≈4%) present in the nucleus (Supplement Figure I, available online at http://atvb.ahajournals.org). To simulate hyperlipidemia, bCAECs were incubated with increasing concentrations of PA. PA concentration-dependently increased endothelial nuclear heparanase (Figure 1A, inset). Using the 1 mmol/L concentration, cells were next treated for up to 30 minutes. PA time-dependently increased nuclear heparanase, with an approximately 2-fold augmentation of enzyme observed after 30 minutes (Figure 1A). With prolonged exposure to PA, the amount of nuclear heparanase dropped such that after 120 minutes, levels similar to 0 minutes were observed (data not shown). This nuclear increase was not reflected by a significant reduction in the cytoplasmic content of heparanase (Supplement Figure I). In addition, this effect was not related to metabolism of PA, as 1 mmol/L 2-bromohexadecanoic acid (a nonmetabolizable palmitate analog) also had a similar effect (Figure 1B). Confirmation of the effect of PA on nuclear accumulation of heparanase was done using immunofluorescence. In endothelial cells, heparanase colocalized with lysosomes that had a predominant perinuclear distribution (Figure 1C). PA caused lysosomal heparanase escape and relocation into the nucleus (Figure 1C, bottom panel). Overall, these experiments demonstrate that fatty acids possess an intrinsic ability to facilitate nuclear translocation of heparanase.

Nuclear Translocation of Heparanase is Dependent on Bax-Induced Lysosome Permeabilization

A prerequisite for nuclear entry of heparanase is likely lysosomal disruption with heparanase released into the cytoplasm. Bax activation has been suggested to induce lysosome permeabilization. Incubation with PA or bromohexadecanoic acid significantly increased Bax activation, without changes in total Bax (Figure 2A). Immunofluorescence images confirmed this observation, in addition to demonstrating colocalization of activated Bax with lysosomes (Figure 2B). Interestingly, the isolated lysosomal fraction from PA-treated cells exhibited lower heparanase and a higher content of activated Bax (Figure 2C). Cathepsin B (Ctsb), a lysosomal resident protease used as an indicator for lysosome integrity, was also decreased following PA in isolated lysosomes (Figure 2C). The PA-induced decrease in lysosomal Ctsb coincided with appearance of this lysosomal protease in the cytoplasm (Figure 2D). To further examine the effect of Bax
activation on lysosome permeabilization, we used a Bax channel blocker CN. CN attenuated lysosomal loss of Ctsb (Figure 2D, bottom panel). More importantly, CN prevented the nuclear translocation of heparanase in response to PA (Figure 2E). It should be noted that PA had no effect on caspase 3 activation (Supplemental Figure II, available online at http://atvb.ahajournals.org), suggesting that the increase in lysosomal permeability is not sufficient to trigger apoptosis up to 24 hours. Collectively, these data suggest that PA-induced nuclear translocation of heparanase is dependent on Bax activation and lysosome permeabilization.

Hsp90 Accompanies the Nuclear Translocation of Heparanase
In HL-60 leukemic cells, Hsp90 has been suggested to function as a chaperone for nuclear shuttling of heparanase.15 Immunoprecipitation of cytosolic Hsp90 revealed a robust association between this chaperone and heparanase 15 minutes following PA, an association that tended to decrease with time (Figure 3A). Interestingly, the nuclear presence of Hsp90-bound heparanase following PA also increased after 15 minutes, with further amplification at 30 minutes (Figure 3B). As GA, a specific Hsp90 inhibitor, effectively blocked the nuclear entry of heparanase (Figure 3C), our data indicate that Hsp90 is an important mediator of PA-induced shuttling of heparanase to the nucleus.

Nuclear Heparanase is Associated With Cleavage of Heparan Sulfate (HS)
Heparanase is an endoglycosidase that can degrade HS into small oligosaccharides.5 Using immunostaining with anti-HS mAb 10E4, which targets intact HS chains, and anti-HS mAb 3G10, which detects cleaved HS chains, we examined the effect of PA on nuclear HS.12,16 With 10E4 staining, intact HS presented a cellular distribution that included both the cytoplasm and nucleus. Treatment with PA eliminated the presence of intact HS mainly in the nucleus (Figure 4A), an effect that was accompanied by the appearance of cleaved nuclear HS (Figure 4B). Quantification of this effect using Western blot demonstrated that the increase in cleaved nuclear HS was apparent at 30 minutes after PA, increased further with time, and remained high up to 4 hours (Figure 4C). Interestingly, similar to PA, bromohexadecanoic acid-induced nuclear translocation of heparanase was also associated with HS cleavage (data not shown). Blocking the nuclear entry of heparanase with GA reduced the amount of cleaved nuclear HS observed following PA (Figure 4D). These data suggest that nuclear HS can undergo cleavage following translocation of heparanase into the nucleus.

Heparanase Regulates HAT activity and Genes Related to Glycolysis
Heparan sulfate is a potent inhibitor of HAT activity.17 To determine if HAT activity changes following nuclear translocation of heparanase and HS cleavage, HAT activity in addition to acetylation of histone were evaluated. Using Western blot, we observed increased HAT activity and augmented histone acetylation after 60 minutes of PA (Figure 5A). These effects were attenuated by the Hsp90 inhibitor GA (Figure 5B). An expression assay for genes related to glycolysis revealed an increase in PDK2 and LDHA in endothelial cell treated with PA, effects that were prevented by GA (Figure 5C) or heparanase siRNA (Supplemental Table I, available online at http://atvb.ahajournals.org). It should be noted that in addition to genes controlling glucose metabolism, nuclear entry of heparanase was also associated with an increase in genes related to inflammation, like VCAM1, SERPINE1 and VEGFA, effects that were prevented by GA (Supplemental Figure III, available online at http://atvb.ahajournals.org) or heparanase siRNA (Supplemental Table II, available online at http://atvb.ahajournals.org).

PDK2 Expression and Extracellular Lactate Accumulation Are Regulated by Nuclear Heparanase
PDK can reduce the oxidative breakdown of glucose through its inactivation of PDH. In response to 1 mmol/L PA, both PDK2 gene and protein increased as early as 6 hours, and remained high until 12 hours (Figure 6A). This effect oc-
curred in the absence of activation peroxisome proliferator-activated receptor-α (Supplemental Figure IV, available online at http://atvb.ahajournals.org). This increase in PDK2 was associated with augmented PDH phosphorylation (Figure 6A, inset). Using siRNA, we were successful in reducing total endothelial heparanase (Figure 6B, left panel). In these cells, PA-induced nuclear entry of heparanase and HS cleavage was reduced (Figure 6B, right panel). More importantly, depletion of heparanase completely prevented the PA-induced increases in PDK2 gene and protein, as well as PDH phosphorylation (Figure 6C). Phosphorylation and inactivation of PDH uncouples glycolysis from glucose oxidation, resulting in accumulation of lactate. Measurement of lactate revealed a robust increase in the incubation medium following PA (Figure 6D, inset), an effect that was effectively prevented by GA or heparanase siRNA (Figure 6D). Our data clearly demonstrate an important role of nuclear heparanase on glucose metabolism through its effect on PDK2. Given the immediate effect of fatty acids in translocating heparanase, we determined the cellular location of heparanase. Immunofluorescent images showed that heparanase was colocalized within lysosomes, in close proximity to the nucleus. This perinuclear position of heparanase precludes protracted transit time and could explain the quick nuclear entry of heparanase in response to fatty acids.

**Discussion**

Heparanase, an enzyme that has a predominant cytoplasmic localization, is present mainly in lysosomes. However, in response to high fatty acid, a nuclear localization of this endoglycosidase has also been reported by us in endothelial cells. Given the function of heparanase to cleave HS, and the impact that HS has on gene transcription, the present study focused on the mechanisms by which fatty acid induces nuclear translocation of heparanase. Our data demonstrates that PA triggers lysosome permeabilization, nuclear shuttling of released heparanase by Hsp90, and induction of genes that affect glucose metabolism and inflammation.

In endothelial cells exposed to PA, a rapid nuclear accumulation of heparanase was observed. This nuclear presence of heparanase is not uncommon, and several groups have detected this enzyme in the nucleus of MDA-435 human breast carcinoma cells and U87 glioma cells that were stable transfected with heparanase. As the nonmetabolizable palmitate analog bromohexadecanoic acid had similar effects in mediating nuclear translocation of heparanase, it is unlikely that this nuclear shuttling requires prior oxidation of fatty acids. Given the immediate effect of fatty acids in translocating heparanase, we determined the cellular location of heparanase. Immunofluorescent images showed that heparanase was colocalized within lysosomes, in close proximity to the nucleus. This perinuclear position of heparanase precludes protracted transit time and could explain the quick nuclear entry of heparanase in response to fatty acids.

Given the predominant lysosome localization of heparanase, its prior release from lysosomes would be anticipated...
before its nuclear entry. In HepG2 cells, high fatty acid-induced lysosomal permeabilization is preceded by activation of the proapoptotic protein Bax. A Bax-dependent lysosome disruption is also involved in the α-lactalbumin and oleic acid-induced early leakage of lysosomes in tumor cells. Results from this study also implicate Bax in the PA-induced nuclear entry of heparanase in endothelial cells. Thus, fatty acids induced Bax activation, which colocalized with lysosomes and was associated with release of lysosome contents including heparanase and Ctsb. At present, the mechanism by which fatty acid induces lysosomal permeabilization through Bax is unclear. In mitochondria from human cancer cells, activated Bax undergoes a conformational change, binds to the mitochondrial outer membrane to form complex oligomer pores, and induces membrane potential loss and permeabilization. Interestingly, overexpression of Bcl-XL, which antagonizes Bax pore formation activity, prevented PA-induced lysosomal permeabilization in HepG2 cells. As the Bax channel blocker CN also inhibited lysosomal leakiness in this study, our data provide strong evidence that PA-induced lysosomal permeabilization and release of heparanase is related to Bax induced pore formation.

Although different pathways exist for nuclear import of proteins, common shared features include a nuclear localization sequence, carrier molecules, and translocation through nuclear pore complexes. Human heparanase contains 2 potential nuclear localization sequences (residues 271–277 and 427–430). Regarding carriers, at least with the glucocorticoid receptor, a ubiquitous chaperone protein Hsp90, binds with the glucocorticoid receptor and together with the immunophilin assembly system, forms a transportosome to help glucocorticoid receptor shift from cytoplasm to nucleus. Recently, a role for Hsp90 as a chaperone for nuclear translocation of heparanase has also been reported in HL-60 cells. Consistent with this report, our immunoprecipitation experiments revealed a robust cytoplasmic association between Hsp90 and heparanase, and their time-dependent shuttling into the nucleus. As the Hsp90 inhibitor GA effectively abolished the nuclear presence of heparanase in response to PA, our study suggests that Hsp90 is the likely carrier in nuclear shuttling of heparanase in endothelial cells.

HS are linear polysaccharide chains that require vesicle transport to be transferred onto the cell surface. However, the presence of HS in the nucleus has been reported in various cell types. For example, in primary hepatocytes, 18% of the total intracellular HS pool is located in the nucleus. Our immunofluorescent images also demonstrated staining for intact HS in the nucleus of endothelial cells. With PA, staining for intact nuclear HS was lost, and this coincided with appearance of cleaved HS in the nucleus. It should be noted that although intact HS displayed whole cell distribution, cleaved HS following PA was only evident in the nucleus. One explanation for this observation is the possibility that released heparanase from lysosomes has limited access to intact HS that are transported in vesicles. As GA, which prevented the nuclear entry of heparanase, effectively attenuated the cleavage of nuclear HS, our data suggest that it is the PA-induced nuclear entry of heparanase that facilitates HS cleavage. The unique location of cleaved HS stimulated us to examine nuclear functions of HS. Recently, HS has been reported to be a potent repressor of gene transcription through its inhibitory effect on HAT, the enzyme that acetylates the lysine side chain of histone, allowing access of transcription factors to bind DNA and initiate transcription. Indeed, augmented HAT activity and histone acetylation were observed when nuclear HS was cleaved, effects that were prevented by GA. A broad range of genes are regulated by HAT, including genes related to cell metabolism and inflammation. As our special interest was glucose metabolism that served when nuclear HS was cleaved, effects that were attenuated by GA or heparanase siRNA, our data suggest that PA can influence gene transcription through nuclear entry of heparanase.

Phosphorylation of the PDH complex at its E1 site by PDK has been recognized to inactivate PDH and lower glucose oxidation. In response to starvation and diabetes, PDK expression is adjusted in a tissue-dependent manner; PDK expression increases in heart and skeletal muscle, but not in brain. Of the different isoforms of PDK, PDK2 is a ubiquitously and abundantly present isoenzyme. As PDK2 responds rapidly to control the PDH complex, we examined...
its endothelial expression following PA treatment. PA for 6 hours was sufficient to induce PDK2 mRNA and protein, with a corresponding increase in the phosphorylation of PDH. In skeletal muscle and heart, fatty acid is a well-known stimulator of PDK through activation of peroxisome proliferator-activated receptor α.\textsuperscript{33} As peroxisome proliferator-activated receptor α was unaffected by PA up to 24 hours, and as silencing of heparanase prevented these effects of PA on PDK2 and PDH, our study provides evidence for another potential regulator of PDK in endothelial cells. Inactivation of PDH uncouples glucose oxidation from glycolysis, resulting in increased pyruvate conversion to lactate as observed in this study. As this lactate accumulation was prevented by GA or heparanase siRNA, our present data also suggest a key role for nuclear heparanase in regulation of endothelial glucose metabolism.

Patients with diabetes are predisposed to developing atherosclerosis, and inflammation is considered an important regulatory process in early development of the atheromatous lesion. The critical role of VCAM1, plasminogen activator inhibitor type 1, and VEGFA in the development of atherosclerosis has been reported in different studies.\textsuperscript{34–36} VCAM is expressed on activated endothelial cells, and promotes monocyte and T lymphocyte adhesion and accumulation on the vessel wall. Plasminogen activator inhibitor type 1 is a major inhibitor preventing the process of fibrinolysis and VEGFA actively participates in angiogenesis through multiple mechanisms, including microvessel proliferation and monocyte infiltration. In our study, these inflammatory genes were upregulated following FA-induced nuclear translocation of heparanase. However, whether this effect contributes to the increased susceptibility of patients with diabetes to atherosclerosis requires further investigation.

In conclusion, the results presented in this study demonstrate that fatty acid can provoke lysosomal release of heparanase, its nuclear translocation, and activation of genes controlling glucose metabolism and inflammation. The ensuing uncoupling between glucose oxidation and glycolysis leads to accumulation of lactate. Given that lactate and its lowering of pH has been implicated in the progression of atherosclerosis through its promotion of lipoprotein binding to proteoglycans,\textsuperscript{37} angiogenesis, and plaque rupture,\textsuperscript{9,38} and the critical role of VCAM, plasminogen activator inhibitor type 1, and VEGF in the development of atherosclerosis,\textsuperscript{34,35} these data detailing the novel mechanism by which FA shuttles endothelial heparanase into the nucleus may serve to reduce the associated cardiovascular complications seen during diabetes.

**Limitations**

When examining the effects of PA on the heart, most studies have used up to 1.2 mmol/L PA to duplicate the plasma concentration of total free FA observed with diabetes.\textsuperscript{39,40} In all of our Type 1 models of diabetes (streptozotocin or diazoxide),\textsuperscript{41,42} the plasma concentration of FA reaches 1 mmol/L. We acknowledge that not all of the FA measured in these diabetic models is PA, and that albumin-bound PA may only make up a fraction of the total plasma-free FA. Thus, the 1 mmol/L concentration of PA used in this study may be higher than the actual circulating amount bound to albumin. However, FA derived from the albumin-bound
fraction does not account for all of the FA provided to the heart. Thus, other physiologically relevant sources like hydrolysis of lipoproteins by cardiac LPL, which has a selective affinity toward PA containing lipoproteins (47.5% of total FA released), play some role in the provision of PA. This is particularly important as 1) LPL increases in diabetic hearts, 2) the molar concentrations of FA in lipoproteins are approximately 10-fold higher than that of FA bound to albumin, and 3) circulating plasma triglyceride concentrations increase to 1.4 to 2 mmol/L after diazoxide and streptozotocin. As intracellular triglyceride and membrane phospholipids are also potential sources of PA, a true measure of the effects of PA on heparanase would only be possible if all of these sources of PA are determined. Finally, high concentrations of PA can induce cell toxicity. Indeed, at 1 mmol/L PA, a significant decrease (≈30%) in the number of viable endothelial cells and increase (≈35%) in the release of LDH was observed compared to cells in the absence of PA (Supplemental Figure IX, available online at http://atvb.ahajournals.org). Thus, it is possible that some of the effects of heparanase could be secondary to cell toxicity. Nevertheless, using 0.5 mmol/L PA (which did not significantly affect cytotoxicity) increased expression of genes related to glycolysis and inflammation (Supplemental Table III, available online at http://atvb.ahajournals.org) was also observed. Overall, our data suggests that the effect of heparanase, at least with lower concentrations of PA, is independent of cytotoxicity, whereas with 1 mmol/L PA, cytotoxicity may contribute toward its effect.

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Disclosures

None.

References


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Supplement Material

Materials

Anti-heparanase mAb 130 antibody was obtained from InSight Ltd (Rehovot, Israel). The specificity of this antibody is described in the Supplement. Hsp90 and Caspase 3 antibody was from Cell Signaling. Prohibitin antibody was from Abcam Cambridge, MA. Anti-HS 10E4 and 3G10 antibody was from Seikagaku Corporation (Tokyo, Japan). Phospho-PDHE1α antibody was from Novus Biologicals. All other antibodies were from Santa Cruz. Lysosome isolation kit was from Sigma. HAT and lactate kits were obtained from BioVision. LysoTracker DND-26, Alexa 633-conjugated goat anti-mouse antibodies were from Invitrogen. ECL® detection kit was purchased from Amersham Canada. Anti-Bax mAb 6A7 and Bax channel blocker CN196810 (CN) were purchased from Calbiochem. All other chemicals were obtained from Sigma Chemical.

Endothelial Cell Culture and Treatments

Bovine coronary artery endothelial cells (bCAEC, Clonetics) were cultured in endothelial growth medium supplemented with EGM-MV BulletKit (Lonza) at 37°C in a 5% CO2 humidified incubator. Cells from 5-8th passage were used for all of the experiments described. BSA-PA solutions were prepared by first dissolving PA in ethanol (500 µl) and then adding appropriate amounts to culture medium to obtain the required molar ratio of BSA to FA. A non-metabolizable albumin bound palmitate analog, 2-bromohexadecanoic acid (BPA, 1 mM) was also added to the culture medium.

Isolation of Nuclear, Lysosome and Mitochondrial Fractions

Nuclear, lysosome and mitochondrial fractions were prepared as previously described. Briefly, cells were washed twice with ice-cold PBS, and subsequently lysed in ice-cold buffer A [10 mM
HEPES (pH 7.9), 10 mM KCl, 1.5 mM MgCl₂, 0.5 mM DTT, 0.5 mM PMSF and 0.05 % NP40] for 10 min. After centrifugation (1,500 g, 10 min, 4°C), the supernatant (cytosolic fraction) was separated, and the pellet was sonicated with buffer B [20 mM HEPES (pH 7.9), 0.42 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF and 10% glycerol] for 5 sec. Following centrifugation (15,000 g, 5 min, 4°C), the supernatant (nuclear fraction) was quantified using a Bradford protein assay, and used for Western blot to determine the nuclear content of heparanase. Using an antibody against histone 3 as a nuclear marker, and β-actin as a cytoplasmic marker, we confirmed the purity of nuclear fractions. In addition, antibodies against LAMP-1 and prohibitin excluded the contamination of nuclear fractions by lysosomes and mitochondria (Supplemental Figure 1).

Lysosomal fractions were isolated by differential centrifugation followed by density gradient centrifugation. Briefly, cells were trypsinized and collected by centrifugation (600 g, 5 min, 4°C). After washing twice with ice cold phosphate buffered saline (PBS), cells were sonicated in extraction buffer for 10 sec. Following centrifugation (1,000 g, 10 min, 4°C), the supernatant was collected and further centrifuged at 20,000 g for 20 min. The pellet was resuspended in extraction buffer and loaded on the sucrose gradient. Subsequent to centrifugation (150,000 g, 4 h, 4°C), 500 μl fractions were collected, starting from the top of the gradient and further purified using 250 mM CaCl₂. The purification of lysosomes was confirmed using lysosome-associated membrane protein 1 (LAMP1) antibody. To exclude mitochondrial contamination, cytochrome c oxidase was measured.

For mitochondrial isolation, bCAECs were scraped and suspended in a homogenization buffer [10 mM Tris-HCl (pH 7.5), 10 mM KCl, 0.15 mM MgCl₂, 0.5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), and a 1% mixture of
protease and phosphatase inhibitors], centrifuged at 4000 g for 15 min, and subjected to further lysis and sucrose gradient [10 mM Tris-HCl (pH 7.5), 0.15 mM MgCl$_2$, 250 mM sucrose, 0.5 mM EDTA, 1 mM PMSF, and 1 mM DTT] separation at high speed (20,000 g) for 15 min. The isolated pellet was resuspended using a lysis buffer [10 mM Tris-HCl (pH 7.5), 5 mM MgCl$_2$, 0.5 mM EDTA, 10% glycerol, 0.2 mM PMSF, 1% Triton X-100, and a 1% mixture of protease and phosphatase inhibitors] as described previously. Prohibitin antibody, a mitochondrial marker was used to confirm the purity of mitochondrial fractions.

**Western Blot and Immunoprecipitation**

Western blot and immunoprecipitation were carried out as described previously. Briefly, samples were quantified, boiled with sample loading dye, and 50 µg used in SDS-polyacrylamide gel electrophoresis. Membranes were blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween-20. Membranes were incubated with anti-heparanase mAb130 (recognizes both 50 and 65 kDa heparanase forms; only the active 50 kDa heparanase data is presented), anti-Bax, anti-Bax mAb 6A7 (conformation dependent; only recognizes activated Bax), anti-cathepsin B (Ctsb), anti-lysosomal-associated membrane protein 1 (LAMP-1), anti-histone 3, anti-Hsp90, anti-HS 3G10 epitope (recognizes digested heparan sulfate), anti-Ac-histone 3, anti-pyruvate dehydrogenase kinase 1, 2 and 4 (PDK1, 2 and 4), anti-PPARα, anti-phospho pyruvate dehydrogenase (PDH) and anti-prohibitin antibodies, and subsequently treated with secondary antibodies. Reaction products were visualized using an ECL® detection kit, and quantified by densitometry. Cytosol and nuclear fractions of endothelial cells were immunoprecipitated using anti-Hsp90 antibody for 3 hr at 4°C. The immunocomplex was pulled down with protein A/G-sepharose overnight, and then heated for 5 min with sample loading dye
at 95°C. The immunocomplex was immunoblotted for heparanase and reaction products were visualized using an ECL® detection kit, and quantified by densitometry.

**Immunofluorescence**

Cells were fixed for 10 min with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in phosphate buffered saline (PBS) for 3 min, treated with PBS containing 1% goat serum for 1 h, and finally rinsed with PBS. Cells were incubated with the indicated antibodies (anti-heparanase mAb130, anti-Bax mAb 6A7, mouse monoclonal Ctsb, anti-heparan sulphate 10E4 and 3G10 epitopes). Following washes with PBS (3x), cells were incubated with secondary Alexa 633 (red) conjugated goat anti-mouse antibodies. 4’,6-Diamidino-2-phenylindole (DAPI) was used to stain nuclei and LysoTracker was used to identify lysosomes. Slides were visualized using a Zeiss Pascal Confocal microscope.

**Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)**

After spectrophotometric quantification, reverse transcription was carried out using oligo (dT) primer and superscript II RT (Invitrogen). RT-qPCR was performed using the TaqMan gene expression assay. PDK2 gene was also validated on a Roche Applied Science LightCycler with a commercial SYBR Green kit (Qiagen). The primer of PDK2: 5’-CTGGACCGCTTCTACCTCAG-3’ (left) and 5’-GATGCGTTGATCTCCTGGAT-3’ (right). The amplification program included initial activation at 95°C for 5 min, and 40 cycles of steps (95°C for 5 sec, 75°C for 10 sec, and 62°C for 10 sec). The values were normalized with β-actin as the internal standard.

**Small Interfering RNA (siRNA) Transfection**

100 nM siRNAs were mixed with Lipofectamine™ in Opti-MEM medium for 15 min. bCAECs were cultured with antibiotic-free EGM medium, which contained heparanase siRNA (or
scrambled, scr) solution for 48 h at 37°C in a CO₂ incubator. Following this, cells were treated with 1 mM PA, and nuclear heparanase, cleaved HS, and PDK2 protein or mRNA were determined.

**Lactate Assay**

500 μl medium was collected from 6-well culture plate at the indicated times and centrifuged at 12,000 rpm for 10 min to remove cell debris. Subsequently, 50 μl (10 times diluted) samples were taken to detect lactate concentration at 570 nm in a colorimetric microplate reader.

**Specificity of the heparanase antibody (mAb 130)**

mAb 130 is a protein G affinity purified monoclonal antibody raised against human heparanase. We tested mAb 130 against bovine coronary artery endothelial heparanase. It has been reported that the amino acid sequence of bovine heparanase has a ~82% identity with human heparanase (GenBank accession AF281160). Using HepG2 cells which do not express heparanase (Cancer Letters 193: 83-89, 2003), we transfected GFP-tagged bovine heparanase (GFP-Hep), and immunostained cells with heparanase mAb 130 (Hep mAb 130). Supplemental Figure 7 demonstrates that this antibody cross reacted in proximity to bovine heparanase. In a separate experiment, heparanase mAb 130 antibody was used to detect heparanase in untreated (CON) bovine coronary artery endothelial cells (bCAECs) or cells transfected with heparanase siRNA (Hep siRNA). Heparanase mAb 130 antibody staining was dramatically decreased when heparanase was knocked down (Supplemental Figure 8). Altogether, our data suggested that the heparanase mAb 130 antibody used in our study has the ability to specifically detect heparanase in bCAECs.
Cytotoxicity Assays

The cytotoxic effect of PA was determined by measuring cell proliferation using Cell Titer 96 Aqueous One Solution Proliferation Assay Kit, and release of LDH using CytoTox-One Homogeneous Membrane Integrity Assay Kit according to the manufacturer’s (Promega) instructions.
Supplement Figure I. Cellular distribution of endothelial heparanase. bCAECs were incubated in the absence (CON) or presence of 1 mM palmitic acid (PA) for a period of 30 min, and heparanase (Hep) in the nuclear and cytosolic fractions determined. Histone was used as a nuclear marker. The purity of the nuclear fraction was verified by determining its contamination with cytoplasmic proteins like β-actin, LAMP-1 or prohibitin. The immunoblot is a representative figure of three separate experiments.
Supplement Figure II. PA for up to 24 hr is unable to induce caspase 3 cleavage. bCAECs were incubated with 1 mM PA over a period of 24 hr. At the indicated times, full-length (FL) and cleaved (CL) caspase (Casp) 3 was determined using Western blot. PAC-1 (PAC, 50 μM for 2 hr), a procaspase 3 activator, was used as a positive control. The immunoblot is a representative figure of three separate experiments.
Supplement Figure III. Genes related to inflammation are influenced by PA induced nuclear entry of heparanase. bCAECs were incubated with 1 mM PA up to 24 hr in the absence or presence of 2 μM geldanamycin (GA). RNA was isolated to analyze genes predominantly involved with inflammation using a TaqMan gene expression assay. VCAM1, vascular cell adhesion molecule 1; SERPINE1, plasminogen activator inhibitor type 1; VEGFA, vascular endothelial growth factor A. Data represents fold change relative to control (CON) and is illustrated as mean±SEM for three separate experiments. *Significantly different from CON, #Significantly different from PA; $P<0.05$. 
Supplement Figure IV. PA has no influence on the expression of endothelial PPARα. bCAECs were incubated with 1 mM PA over a period of 24 hr. At the indicated times, PPARα protein was measured using Western blot. Fenofibrate (FF, 50 μM for 24 hr), a PPARα agonist, was used as a positive control. The immunoblot is a representative figure of three separate experiments.
Supplement Figure V. The effect of PA on endothelial PDK 1 and 4 expression. bCAECs were incubated with 1 mM PA over a period of 12 hr. At the indicated times, PDK1 and 4 proteins were measured using Western blot. The immunoblot is a representative figure of three separate experiments.
Supplement Figure VI. Scheme of probable mechanisms of how high FA stimulates nuclear translocation of endothelial heparanase. In response to high FA, endothelial Bax is activated which can provoke lysosome permeabilization and release of heparanase. Using Hsp90 as a chaperone, this endoglycosidase undergoes nuclear translocation and cleaves nuclear HS, which increases histone acetylation and activation of genes controlling glucose metabolism and inflammation.
Supplement Figure VII. Targeting of heparanase mAb 130 antibody with transfected GFP-heparanase in HepG2 cells. HepG2 cells were transfected with 1 µg GFP-tagged ORF clone of Bos Taurus heparanase DNA for 48 hr (GFP-Hep, green). Cells were then subjected to immunofluorescence staining with heparanase mAb 130 antibody (Hep mAb 130, red) followed by Alexa 633 conjugated goat anti-mouse antibody. DAPI was used to stain nucleus and slides were visualized using a Zeiss Pascal Confocal microscope. Bar = 20 µm.
Supplement Figure VIII. Heparanase immunostaining using heparanase mAb 130 antibody in bCAECs transfected with heparanase siRNA. bCAECs transfected with heparanase siRNA (Hep siRNA) for 48 hr were immunostained with heparanase mAb 130 antibody (red) followed by Alexa 633 conjugated goat anti-mouse antibody. DAPI was used to stain nucleus and slides were visualized using a Zeiss Pascal Confocal microscope. Bar = 20 μm.
Supplement Figure IX. Cytotoxic effect of PA on endothelial cells. bCAECs were plated at 2x10^4 cells/well in 96-well plate and treated with 0-1 mM PA for 24 hr. Cytotoxicity was measured using (A) a proliferation assay or (B) LDH assay, respectively. The values represent the mean±SEM of three replicate experiments. *Significantly different from 0 mM PA; P<0.05.
Supplemental Tables

Supplement Table I. Gene Expression Changes in Glycolysis

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<td>Pyruvate dehydrogenase kinase 2</td>
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PA = 1 mM

Supplement Table II. Gene Expression Changes in Inflammation

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PA = 1 mM

Supplement Table III. Effect of two concentrations of PA on Gene Expression

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