Materials and Methods

Experimental Animals
The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by NIH and the University of British Columbia. Male Wistar rats (250-320 g) were injected with diazoxide (DZ), a K⁺ channel opener which reduces insulin secretion and induces acute hyperglycemia. Animals remain hyperglycemic for up to 4 h following a single injection¹.². DZ was administered i.p. (100 mg/kg), animals were anesthetized using 65 mg/kg i.p. pentobarbital after 4 h, and hearts removed. To induce chronic hyperglycemia, rats were injected i.v. with 55 mg/kg streptozotocin (STZ). Animals (D55) were kept for 4 days.

Reagents and antibodies
Diazoxide (DZ, Cat# D9035), Oleoyl- L-α-lysophosphatidic acid (LPA, Cat# L7260), Cytochalasin D (Cat# C8273), Cyclohexamide (Cat# C7698), Y-27632 (Cat# Y0503), Gö6976 (Cat# G1171), Phorbol 12-myristate 13-acetate (PMA, Cat# P8139) were obtained from Sigma. Heparin (HEPALEAN, 1000 U/ml) was from Organon, Canada. PKC β inhibitor was obtained from Calbiochem (Cat# 539654-500UG). It inhibits PKC βI and PKC βII with IC⁵₀ of 21 and 5 nM, respectively. Heparinase III (IBEX technologies, Cat# 50-012-001) was purified from recombinant Flavobacteriumheparinum. [³H]-triolein was purchased from Amersham Canada. Purified active and latent heparanase were prepared as described³. Anti-LPL 5D2 antibody was a kind gift from Dr. J. Brunzell, University of Washington, Seattle. Anti-heparanase antibody mAb 130 was from InSight (Rehovot, Israel, Cat# INS-26-1-0000-21), which recognizes both the active (50 kDa) and latent form (65 kDa) of heparanase. Anti-RhoA antibody was purchased from Santa Cruz (Cat# sc-418). The RhoA activation G-LISA assay kit (Cat# BK124) and F-actin/G-actin assay kit (Cat# BK037) were obtained from Cytoskeleton (Denver, CO). HTRF heparanase activity assay kit was obtained from Cisbio (Cat# 61BHSKAA). To measure free fatty acid released from VLDL-TG breakdown, an NEFA-C assay kit was purchased from Wako (Cat# 999-75406).

Isolated Heart Perfusion
To measure coronary LPL, hearts were perfused retrogradely with heparin (5 U/ml)⁴. Coronary effluents were collected (for 10 s) at different time points over 5 min. LPL activity in each fraction was determined, plotted against time, and coronary LPL activity was presented as area under the curve over 5 min. A modified Langendorff retrograde perfusion was used to isolate active and latent heparanase in the interstitial effluent⁵.

Isolation of Cardiac Myocytes
Ventricular calcium-tolerant myocytes were prepared by a previously described procedure⁶.

Endothelial Cell Culture
Bovine coronary artery endothelial cells (bCAECs, Clonetics) and Rat aortic endothelial cells (RAOEC, Cell Applications) were cultured at 37°C in a 5% CO₂ humidified incubator alone or co-cultured with adult rat cardiomyocytes⁵. bCAECs from the 5th to the 8th passage were used. RAOEC at passage 6 were used.

Isolation of Particulate Fraction
To determine RhoA activation in vivo, particulate fraction from ventricles were prepared as described previously⁷.
Endothelial Cell Conditioned Medium (ECCM)

bCAECs were incubated with high glucose (25 mM) DMEM for 30 min. This medium was collected as ECCM. Cardiomyocytes were incubated with ECCM for 30 min, and the medium collected to study its lipolytic activity using VLDL-TG as a substrate.

Endothelial cell heparanase in response to high glucose

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

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

LPL Activity

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

LPL expression in cardiomyocytes

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

Treatments

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

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

**G-LISA Assay**

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

**Actin Polymerization**

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

**Western Blot**

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

**Immunofluorescence**

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

**Statistical Analysis**

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


