Protein Kinase D Is a Key Regulator of Cardiomyocyte Lipoprotein Lipase Secretion After Diabetes

Min Suk Kim, Fang Wang, Prasanth Puthanveetil, Girish Kewalramani, Elham Hosseini-Beheshti, Natalie Ng, Yanni Wang, Ujendra Kumar, Sheila Innis, Christopher G. Proud, Ashraf Abrahani, Brian Rodrigues

Abstract—The diabetic heart switches to exclusively using fatty acid (FA) for energy supply and does so by multiple mechanisms including hydrolysis of lipoproteins by lipoprotein lipase (LPL) positioned at the vascular lumen. We determined the mechanism that leads to an increase in LPL after diabetes. Diazoxide (DZ), an agent that decreases insulin secretion and causes hyperglycemia, induced a substantial increase in LPL activity at the vascular lumen. This increase in LPL paralleled a robust phosphorylation of Hsp25, decreasing its association with PKCδ, allowing this protein kinase to phosphorylate and activate protein kinase D (PKD), an important kinase that regulates fission of vesicles from the golgi membrane. Rottlerin, a PKCδ inhibitor, prevented PKD phosphorylation and the subsequent increase in LPL. Incubating control myocytes with high glucose and palmitic acid (Glu+PA) also increased the phosphorylation of Hsp25, PKCδ, and PKD in a pattern similar to that seen with diabetes, in addition to augmenting LPL activity. In myocytes in which PKD was silenced or a mutant form of PKCδ was expressed, high Glu+PA were incapable of increasing LPL. Moreover, silencing of cardiomyocyte Hsp25 allowed phorbol 12-myristate 13-acetate to elicit a significant phosphorylation of PKCδ, an appreciable association between PKCδ and PKD, and a vigorous activation of PKD. As these cells also demonstrated an additional increase in LPL, our data imply that after diabetes, PKD control of LPL requires dissociation of Hsp25 from PKCδ, association between PKCδ and PKD, and vesicle fission. Results from this study could help in restricting cardiomyocyte LPL translocation, leading to strategies that overcome contractile dysfunction after diabetes. (Circ Res. 2008;103:0-0.)

Key Words: heat shock protein ■ protein kinase C ■ hyperglycemia ■ hyperlipidemia ■ vesicles

Cardiac muscle has a high demand for energy and uses multiple substrates, including fatty acid (FA), carbohydrate, amino acids, and ketones.1 Among these substrates, carbohydrate and FA are the major sources from which the heart derives most of its energy. In a normal heart, whereas glucose and lactate account for approximately 30% of energy provided to the cardiac muscle, 70% of ATP generation is through FA oxidation.2 FA delivery and utilization by the heart involves: (1) release from adipose tissue and transport to the heart after complexing with albumin,3 (2) provision through breakdown of endogenous cardiac triglyceride (TG) stores,4 (3) internalization of whole lipoproteins,5 and (4) hydrolysis of circulating TG-rich lipoproteins to FA by lipoprotein lipase (LPL) positioned at the endothelial surface of the coronary lumen.6 The molar concentration of FA bound to albumin is ≈10-fold less than that of FA in lipoprotein-TG,7 and LPL-mediated hydrolysis of circulating TG-rich lipoproteins to FA is suggested to be the principal source of FA for cardiac utilization.8 Coronary endothelial cells do not synthesize LPL.9 In the heart, this enzyme is produced in cardiomyocytes and subsequently secreted onto heparan sulfate proteoglycan (HSPG) binding sites on the myocyte cell surface.10 From here, LPL is transported onto comparable binding sites on the luminal surface of endothelial cells.11

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 exclusively using FA for energy supply.12 It does this by increasing its LPL activity at the coronary lumen.8 We have examined LPL biology in the diabetic heart and have determined that the augmented activity13 is: (1) not the result of increased gene expression,13 (2) unrelated to an increase in the number of capillary endothelial HSPG binding sites,13 (3) acutely (hours) regulated by short-term changes in insulin,14 and (4) functionally relevant and capable of hydrolyzing lipoprotein-TG.15 More recently, we have examined the contributions of the endothelial cell and the cardiomyocyte in enabling this increased enzyme at the vascular lumen.
At the endothelial cell, we reported that TG16 and lipoprotein breakdown products like lysophosphatidylcholine, likely through their release of heparanase, enabled myocyte HSPG cleavage and transfer of LPL toward the coronary lumen. Within the myocyte, recruitment of LPL to the cell surface was controlled by stress kinases like AMPK and p38 MAPK, which allowed for actin cytoskeleton polymerization and provision of a network that facilitated LPL movement. In the present study, we determined the mechanism that controls cardiac LPL vesicular trafficking after diabetes. Our data suggest that protein kinase D (PKD) activation is essential for LPL vesicle formation and its movement to the cardiomyocyte plasma membrane, for eventual translocation to the coronary vascular lumen.

### Materials and Methods

An expanded version of the materials and methods has been described in the online Data Supplement available at http://circres.ahajournals.org. In this supplement, we have explained our animal model of diabetes, perfusion of isolated hearts, isolation of cardiomyocytes, assay for LPL activity, adenoviral transfection.
of cardiac cells, Western Blotting, immunoprecipitation, immunofluorescence, gene silencing, and measurement of cardiac diacylglycerol.

**Results**

**Characterization of the Model of Acute Diabetes Induced With Diazoxide**

Subsequent to injection of DZ, blood glucose levels increased and were significantly higher after 1 and 4 hours (supplemental Table I). Changes in plasma parameters with DZ also included significant and rapid increases in FA (supplemental Table I). Retrograde perfusion of hearts with heparin resulted in release of LPL into the coronary perfusate. Compared to control rat hearts, there was a substantial increase in LPL activity at the vascular lumen after 1 and 4 hours of DZ (supplemental Table I). We have previously shown that this change in LPL activity was independent of shifts in mRNA, and was dependent on its lowering of insulin rather than its direct effects on the heart or blood pressure. Incubation of cardiomyocytes with DZ (1 mg/mL; calculated concentration in vivo) had no influence on heparin releasable (HR) LPL activity (supplemental Figure I).

**Mechanism of Activation of PKD in Hearts From Animals With Hyperglycemia**

Recently, we have determined that diabetes increases the phosphorylation of Hsp25. In the present study, we duplicated this result (Figure 1A) and additionally show that Hsp25 phosphorylation enables PKC\(\delta\) to dissociate from Hsp25 (Figure 1B). As Hsp25 binds directly to ser-643 of PKC\(\delta\), its separation permits PKC\(\delta\) phosphorylation (Figure 1C), with an associated increase in its activity. PKC\(\delta\) has been suggested to regulate PKD activity in intestinal epithelial cells. Interestingly, in addition to PKC\(\delta\) phosphoryla-

**Figure 2.** Inhibition of PKD phosphorylation reduces the diabetes induced augmentation of coronary LPL activity. Animals were pretreated with rottlerin for 45 minutes before administration of diazoxide (DZ). Animals were killed 4 hours subsequent to DZ injection and hearts isolated for determination of PKD and LPL. Total and phosphorylated PKD were assessed using Western blotting (A). B, Representative photograph showing the effect of rottlerin on LPL immunofluorescence as visualized by fluorescent microscopy. Majority of LPL in the DZ heart was present at the coronary lumen (arrows). To estimate this LPL, hearts were isolated and perfused in the nonrecirculating retrograde mode with heparin. Coronary effluents were collected (for 10 s) at different time points over 10 minutes (only the peak value is shown). Results are the means±SE of 3 to 5 rats in each group. *Significantly different from control; #Significantly different from DZ alone, \(P<0.05\).

**Figure 3.** Mimicking diabetes in vitro using high glucose and fatty acid increases cardiomyocyte phosphorylation of Hsp25, PKC\(\delta\), and PKD. Cardiomyocytes were plated on laminin-coated culture plates. Cells were maintained using Media-199 for 16 hours. Subsequently, glucose (Glu, 20 mmol/L) and palmitic acid (PA, 1.5 mmol/L) were added to the culture medium. At the indicated times, protein was extracted to determine both total and phosphorylated Hsp25 (A), PKC\(\delta\) (B), and PKD (C) using Western Blotting. Data are means±SE n=3 myocyte preparations from different animals. *Significantly different from Con; #Significantly different from Glu+PA-1 hour, \(P<0.05\).
tion, DZ also augmented PKD phosphorylation at 1 and 4 hours after injection (Figure 1E). This increase in PKD phosphorylation paralleled a robust attachment of phospho-PKC/ to PKD (Figure 1D). Injection of STZ precipitated overt hyperglycemia (supplemental Table I). Compared to control hearts, LPL activity increased at the vascular lumen after 1 and 7 days of STZ (Figure 1F). As observed with DZ, this increase in LPL activity with STZ diabetes closely paralleled activation of PKD (Figure 1G).

Role of PKD in Diabetes Induced Augmentation of LPL Activity
PKD assists in protein transport from the golgi to plasma membrane.27 We hypothesized that after DZ, activation of PKD facilitates LPL vesicular movement to the cardiomyocyte cell surface and eventually to the vascular lumen, and that its inhibition should reduce LPL activity at this location. In the absence of specific inhibitors of PKD, we used rottlerin, an inhibitor of PKCδ. Treatment of rottlerin for 1 hour decreased PKD phosphorylation that is produced after DZ (Figure 2A). More importantly, the remarkable increase in LPL immunofluorescence (Figure 2B) or activity (Figure 2C) at the vascular lumen after 4 hours of DZ was also reduced by preincubation with rottlerin.

Simulation of Diabetes Promotes LPL Trafficking to the Cardiomyocyte Plasma Membrane
We duplicated the hyperglycemia and hyperlipidemia observed after DZ by incubating control myocytes with high glucose and palmitic acid (Glu/PA). 20 mmol/L glucose with 1.5 mmol/L PA increased the phosphorylation of Hsp25 (Figure 3A), PKC/ (Figure 3B), and PKD (Figure 3C) in a pattern similar to that seen with diabetes induced by DZ. Once activated, PKD regulates formation of transgolgi vesicles and facilitates their movement to the plasma membrane with help of vesicle associated membrane protein (VAMP).28 High Glu/PA brought about both PKD and VAMP translocation, as measured by Western blotting and confocal microscopy (Figure 4A and 4B). Interestingly, this milieu also augmented cardiomyocyte cell surface HR-LPL activity (Figure 4C) and protein (Figure 5B, middle panel). Independently, high glucose or PA (supplemental Figure IV), and mannitol with PA (Figure 4C) had no effect on cardiomyocyte LPL trafficking. Additionally, high glucose in the

Figure 4. High glucose and fatty acid induced translocation of PKD is associated with increasing cardiomyocyte heparin-releasable LPL activity. After high glucose and palmitic acid added to the culture medium for 1 and 2 hours, respectively, cardiomyocyte homogenates were prepared. Homogenates were subjected to cytosolic and membrane separation. Identification of total PKD protein was carried out using polyclonal rabbit PKD as the primary and goat antirabbit horseradish peroxidase as the secondary antibody (A). Representative photograph showing the effect of high glucose and PA on PKD immunofluorescence as visualized by a Zeiss Pascal confocal microscope (B). Cardiomyocytes were fixed, incubated with primary antibodies (PKD and VAMP) followed by incubation with secondary antibodies (FITC [green] and TR [red]). LPL activity in control and high glucose/mannitol and PA treated cardiomyocytes was determined by adding heparin (8 U/mL for 1 minute) to the incubation medium, and the release of surface-bound LPL activity into the medium determined (C). Data are means±SE, n=3 myocyte preparations from different animals. *Significantly different from Con; #Significantly different from Glu+PA-1 hour, P<0.05.
presence of physiologically relevant concentrations of PA (0.5 mmol/L) did not change HR-LPL activity, which only increased with 1 and 1.5 mmol/L PA (supplemental Figure II). Finally, unlike PA, oleic acid (1.5 mmol/L) in the presence of high glucose had no influence in increasing HR-LPL activity (supplemental Figure III).

Silencing of PKD Prevents Cardiomyocyte LPL Recruitment Observed With High Glu+PA
To confirm the relationship between PKD and LPL, we used siRNA to silence PKD expression in isolated cardiomyocytes. We first validated successful PKD inhibition using Western blotting (Figure 5A, inset). In myocytes in which PKD was silenced, high Glu+PA had no influence on total PKD, which remained low in the PKD knockdown cells (Figure 5A). Interestingly, in myocytes in which PKD was silenced, high Glu+PA was incapable of increasing LPL immunofluorescence (Figure 5B) and activity (Figure 5C) at the cardiomyocyte cell surface.

Hsp25 Impedes the Action PMA to Phosphorylate PKCδ
As high Glu+PA induced a 1.4-fold increase in DAG (Con-2241±146, Glu+FA-3136±360 ng/10^6 cells; P<0.05), which is known to activate PKCδ, we incubated control myocytes with PMA, a DAG mimetic. Under our conditions, PMA was unable to phosphorylate PKCδ (Figure 6B). We hypothesized that as Hsp25 masks the catalytic site of PKCδ, silencing of Hsp25 would permit PMA to promote phosphorylation of PKCδ. We validated successful Hsp25 inhibition using Western blotting (Figure 6A, inset). In myocytes in which Hsp25 was silenced, PMA had no influence on total Hsp25, which remained low in the Hsp25 knockdown cells (Figure 6A). Surprisingly, in these cells, PKCδ phosphorylation increased (Figure 6B) with an associated amplification in its interaction with PKD (Figure 6C). As predicted, in myocytes in which Hsp25 was silenced, PMA was now able to induce significant phosphorylation of
PKCδ (Figure 6B). More importantly, in the presence of PMA, Hsp25 knockdown cells demonstrated a strong interaction between PKCδ and PKD (Figure 6C).

**Directly Activating PKD Enlarges the Cardiomyocyte Cell Surface LPL Pool**

Given the relationship between PKD and LPL movement, we determined PKD phosphorylation in Hsp25 silenced myocytes. Simply knocking down Hsp25 augmented PKD phosphorylation (Figure 7A), accelerated its translocation to the plasma membrane (Figure 7B), and enlarged LPL immunofluorescence (Figure 7B) and activity at the cardiomyocyte cell surface (Figure 7C). In these cells, PMA brought about an even greater phosphorylation and translocation of PKD (Figure 7A and 7B) with associated enlargement in LPL immunofluorescence (Figure 7B) and activity (Figure 7C).

**PKD Phosphorylation Requires Prior Activation of PKCδ**

PKCδ expression increased after infection with adenoviral vectors encoding WT and DN PKCδ (Figure 8A). We have previously reported that overexpression of the DN PKCδ does not increase its activity.22 In PKCδ DN myocytes, high glucose and PA failed to increase phospho PKD (Figure 8A) and completely abrogated the increase in HR-LPL activity (Figure 8B).

**Discussion**

During diabetes, when cardiac glucose uptake, glycolysis, and pyruvate oxidation are impaired, the heart rapidly adapts to using FA exclusively for ATP generation.30 This change can occur not only as a consequence of increased FA supply (attributable to an increased release of FA from adipose tissue and hydrolysis of TG-rich lipoproteins by LPL), but also through an intrinsic adaptation/maladaptation to elevated FA levels.27 We demonstrate that, with phosphorylation of PKCδ, its association with PKC and PKD is reduced, enabling PKCδ phosphorylation and activation. As PKCs (δ, ε, θ, η) can directly associate with the pleckstrin homology domain of PKD, allowing for its phosphorylation and activation,26 we determined the interaction between PKCδ and PKD. Interest-
1,2-DAG,42 whose downstream signaling includes activation of the PKC super family of enzymes.41,43 We measured levels of this lipid intermediate and report a 1.4-fold increase in myocytes treated with high Glu+PA. Interestingly, similar to DZ, the increase in LPL in myocytes treated with high Glu+PA closely mirrored the increase in phosphorylation of PKCδ and PKD. The zinc-finger domain of PKD is known to interact with transgolgi membranes, allowing for fission of vesicles.27,44 In addition, activation of PKD is also suggested to promote recruitment of VAMP,28 which helps with vesicle exocytosis and movement along the actin filament network to reach the cell surface. As silencing of PKD prevented the ability of high Glu+PA to increase cell surface LPL, our data suggest that by increasing DAG and promoting activation PKCδ, PKD is the eventual trigger that enables the cardiomyocyte LPL secretory pathway to turn on.

An alternate strategy to activate PKC is to use PMA, a DAG mimetic that is known to phosphorylate PKC.41,45 Interestingly, in cardiomyocytes, we were unable to observe activation of PKCδ by PMA, an observation that was previously reported.46 In this setting, PMA was also unable to affect PKD. Given the observation that Hsp25 can prevent activation of PKCδ through a direct interaction,29 we knocked down Hsp25 using siRNA. In myocytes in which Hsp25 was silenced, PKCδ and PKD phosphorylation increased, and so did LPL activity. Interestingly, when these myocytes were now exposed to PMA, there was a further phosphorylation of PKCδ, an appreciable association between PKCδ and PKD, and a vigorous activation of PKD. As these cells also demonstrated an additional increase in cell surface LPL activity, and as a mutant form of PKCδ prevented PKD activation and increase in LPL activity observed with high glucose and palmitic acid, our data imply that after diabetes, PKD control of cardiomyocyte LPL activity requires dissociation of Hsp25 from PKCδ, activation of PKCδ by DAG, association between PKCδ and PKD, and vesicular transport of LPL.

In summary, after diabetes, when cardiac glucose utilization is impaired, the heart undergoes metabolic transformation wherein it switches energy production to exclusive β-oxidation of FA. One way by which this process is made possible is through amplification of coronary LPL, thereby allowing uninterrupted FA supply to the diabetic heart. Recruitment of LPL to the cardiomyocyte cell surface and eventually the vascular lumen could represent an immediate compensatory response by the heart to guarantee FA supply. The mechanism underlying this process embraces myocyte increase in actin cytoskeleton polymerization (through an AMPK/p38 MAPK pathway)18 and PKD control of LPL vesicle formation and movement (Figure 8C). Increasing FA uptake through overexpression of cardiac human LPL46 or fatty acid transport protein,47 or augmenting FA oxidation through overexpression of cardiac PPAR-α48 or long-chain acyl CoA synthase,49 results in a cardiac phenotype resembling diabetic cardiomyopathy. Thus, results from the present study could help in restricting or slowing cardiac LPL translocation and could lead to strategies that overcome contractile dysfunction after diabetes.
Limitations of the Study
One limitation of this and other studies4,50–54 is that when examining the lipotoxic effects of palmitic acid on the heart, most studies have used in vitro incubations with 1 to 1.5 mmol/L PA to duplicate the plasma concentration of total FFA observed with diabetes. Given that albumin-bound PA only makes up a fraction of the total plasma FFA, the concentration of PA used may be higher than the actual circulating amount bound to albumin. However, it should be noted that 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 palmitic acid containing lipoproteins (47.5% of total fatty acids released),55 also play some role in the provision of PA. This is particularly important as: (1) LPL increases in the DZ diabetic heart, (2) the molar concentrations of FA in lipoproteins are approximately 10-fold higher than that of FA bound to albumin,7 and (3) circulating plasma TG concentrations increase after DZ. As intracellular TG and membrane phospholipids are also potential sources of PA, a true measure of the effects of PA and glucose on cardiac LPL would only be possible if all of these sources of PA are determined after diabetes induced by DZ.

Acknowledgments
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References
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Supplement

Detailed Materials and Methods

Experimental animals

The investigation conforms to the guide for the care and use of laboratory animals published by the US NIH and the University of British Columbia. Adult male Wistar rats (260-300 g) were injected with diazoxide (DZ). This agent, either through selective K+ ATP channel opening or inhibitory effects on aerobic energy generation, decreases insulin secretion and causes hyperglycemia. DZ (100 mg/kg) was administered i.p., and animals were euthanized at 1 and 4 h after injection. Subsequently, hearts were removed for measurement of coronary luminal LPL activity, immunoprecipitation, immunofluorescence, and Western Blot. To inhibit PKD, some rats were injected with rottlerin (10 μM), 40 mins prior to administration of DZ. To validate our results using DZ, rats were made diabetic with streptozotocin (STZ, 55 mg/kg i.v.). Animals were kept for 1 (acute) or 7 (chronic) days after STZ, at which time they were killed and hearts removed for measurement of LPL activity and PKD.

Isolated heart perfusion

Rats were anesthetized with 65 mg/kg sodium pentobarbital i.p. and the heart carefully excised. Following cannulation of aorta, hearts were perfused retrogradely with Krebs-Henseleit buffer. The rate of coronary flow was controlled by a peristaltic pump. To measure coronary endothelium-bound LPL, the perfusion solution was changed to buffer containing heparin (5 U/ml). The coronary effluent was collected in timed fractions (10 sec) over 5 min, and assayed for LPL activity by measuring the hydrolysis of a [3H]triolein substrate emulsion.

Isolated cardiac myocytes

Ventricular calcium-tolerant myocytes were prepared as described previously. To examine the influence of high glucose and FA or phorbol 12-myristate 13-acetate (PMA; a protein kinase C activator) on cardiomyocytes, cells were plated on laminin-coated 6-well culture plates (to a density of 200,000 cells/well). Cells were maintained using Media-199, and incubated at 37°C under an atmosphere of 95% O2/5% CO2 for 16 hours. Subsequently and where indicated, 20 mM glucose and 1.5 mM albumin bound palmitic (PA) or oleic (OA) acid (molar ratio 1:2; 1-2 h) or 1 μM PMA (15 min) were added to the culture medium. BSA-FA solutions were prepared by first dissolving the FA in ethanol and then adding appropriate amounts to media to obtain the required molar ratio of BSA to FA. In some experiments, 20 mM mannitol was used as osmolarity control. Following the indicated times, cells were used either for Western Blot, immunoprecipitation, immunofluorescence or measurement of basal and heparin-releasable (heparin 8 U/mL; 1 min) LPL activity. To determine translocation of PKD, myocytes were homogenized in ice-cold buffer A (20 mM Tris-HCl, 2 mM EDTA, 5 mM EGTA, 50 mM 2-mercaptoethanol, 25 μg leupeptin, and 4 μg aprotinin, pH 7.5) and centrifuged for 1 h at 35000 rpm; the supernatant was used as the cytosolic fraction. The pellet was resuspended in buffer B.
(1% NP-40, 0.1% SDS, 0.5% deoxycholic acid and 5 mM EGTA, pH 7.5), sonicated for 30 s, and centrifuged at 35000 rpm for 1 h; the supernatant was used as the membrane fraction.

**Adenoviral gene transfer**

Cardiomyocytes were infected with recombinant adenovirus vectors carrying wild-type (WT) PKCδ and dominant-negative (DN) PKCδ (K376A), as described previously. Mock infection as a control was performed using LacZ. Infected cells were incubated for a further 36 h before treatment in the absence or presence of high glucose and PA (2 h).

**Western blotting**

Western blot was carried out as described previously. Briefly, ventricular tissue (50 mg) or plated myocytes (0.4 x 10⁶) were homogenized in ice-cold lysis buffer. Samples were diluted, boiled with sample loading dye, and 50 μg used in SDS-polyacrylamide gel electrophoresis. After blotting, membranes were blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween-20. Membranes were incubated with rabbit Hsp25, phospho-Hsp25 (S86), PKCδ, phospho-PKCδ (ser-643), PKD, and phospho-PKD (ser-744/748) antibodies, and subsequently with secondary goat anti-rabbit HRP-conjugated antibody. Reaction products were visualized using an ECL® detection kit, and quantified by densitometry.

**Immunoprecipitation**

Following DZ or treatment of plated cardiomyocytes with PMA (1 µM), lysates were immunoprecipitated using PKCδ or PKD antibodies overnight at 4°C. The immunocomplex was pulled down with protein A/G-sepharose for 1 h, and then heated for 5 min at 95°C. The immunocomplex was separated into two equal portions, each of which was immunoblotted with anti-Hsp25 and PKCδ or phospho-PKCδ and PKD.

**Immunofluorescence**

Heart-Hearts were placed in 10% formalin for 24 h. After formalin fixation and paraffin embedding, 3-µm sections were cut on silane-coated glass slides. Immunostaining was carried out as described before. Slides were incubated with chicken anti-bovine LPL antibody (1:400 dilution) overnight. After being washed with TBS, slides were incubated with biotinylated rabbit anti-chicken IgG (1:150 dilution; Chemicon) and streptavidin-conjugated Cy3 fluorescent probe (1:1,000 dilution) for 1 h. Slides were visualized using a fluorescent microscope.

Cardiomyocyte-Following incubation with high glucose and PA or PMA at the indicated times, myocytes were fixed for 10 min with 4% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS for 3 min, treated with PBS containing 1% BSA for 20 minutes, and finally rinsed with PBS. Cells were incubated with rabbit polyclonal PKD and goat polyclonal vesicle associated membrane protein (VAMP) or chicken anti-bovine LPL antibody followed by incubation with secondary antibodies [goat anti-rabbit IgG-FITC, donkey anti-goat IgG-TR, streptavidin-conjugated Cy3 fluorescent probe] to localize PKD (green), VAMP (red), and LPL (red) respectively. Slides were visualized using a Zeiss Pascal confocal microscope.

**Silencing of PKD and Hsp25 by siRNA**

siRNA transfection in cardiomyocytes was carried out using a kit from Santa Cruz. Briefly, in 6-well culture plates, 0.1 x 10⁶ cells were plated and subsequently exposed to the siRNA (or scrambled, Scr) solution for 8 h at 37°C in a CO₂ incubator. Following this, the media was changed to Media 199 and the cells incubated for another 18 h. Subsequently, high glucose and
PA or PMA were added to the culture medium, and LPL activity (released by heparin) and immunofluorescence, and PKD, Hsp25 and PKCδ (using Western Blot and immunoprecipitation) were determined.

**Separation and measurement of cardiac diacylglycerol**

Total cardiac lipids were extracted and solubilized in chloroform:methanol:acetone:hexane (4:6:1:1 v/v/v/v). Separation of 1,2-DAG was achieved using HPLC (Waters 2690 Alliance HPLC, Milford, MA) as described previously\(^{14}\).

**Plasma measurements**

Following DZ, blood samples from the tail vein were collected over a period of 4 h, and blood glucose determined using a glucometer (AccuSoft) and glucose test strips (Accu-Chek Advantage). At varying intervals, blood was also acquired in heparinized glass capillary tubes. Blood samples were immediately centrifuged and plasma was collected and assayed for non-esterified fatty acid using a diagnostic kit (Wako).

**Materials**

\(^3\text{H}\)triolein and the ECL® detection kit were purchased from Amersham Canada. Heparin sodium injection (Hapalean; 1000 USP U/ml) was obtained from Organon Teknika. PKCδ, phospho-PKCδ (ser-643) and PKD antibodies were obtained from Santa Cruz biothechnology, Inc. (Delaware Avenue, CA). Hsp25 and phospho-Hsp25 antibodies were obtained from GeneTex®, Inc. (San Antonio, TX). Phospho-PKD (ser-744/748) and GAPDH were obtained from Cell Signaling (Danvers, MA). Rottlerin was purchased from Calbiochem. All other chemicals were obtained from Sigma Chemical.

**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 \(P < 0.05\).
**Online Table I. General characteristics of the experimental animals**

<table>
<thead>
<tr>
<th></th>
<th>Glucose (mM)</th>
<th>FA (mM)</th>
<th>LPL activity (nmol/ml/h)</th>
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</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.9 ± 0.8</td>
<td>0.43 ± 0.07</td>
<td>398 ± 59</td>
</tr>
<tr>
<td>DZ 1hr</td>
<td>14.7 ± 1.2*</td>
<td>1.48 ± 0.08*</td>
<td>712 ± 43*</td>
</tr>
<tr>
<td>DZ 4hr</td>
<td>17.5 ± 0.4*</td>
<td>1.5 ± 0.1*</td>
<td>1568 ± 89*</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4 animals in each group. Animals were treated with diazoxide (DZ-1 and 4 hr), blood samples collected, and plasma glucose and fatty acids (FA) determined. At the indicated times with DZ, hearts were also isolated and coronary luminal LPL released with heparin. Some animals were also made diabetic with streptozotocin (STZ) and kept for 1 (plasma glucose 18.7 ± 1.1* ) and 7 (plasma glucose 19.6 ± 2* ) days. Although plasma FA was not measured following STZ, LPL activity in these animals is documented in Fig. 1. *Significantly different from untreated control, P < 0.05.
Online Description of Results

Online Figure I. Direct effects of diazoxide on cardiomyocyte LPL

Previously, the issue of whether the increase in LPL activity following diazoxide (DZ) could be a result of a direct effect of this agent on the heart or a consequence of a drop in insulin was addressed\(^8\). Confirmation that the effects of DZ are likely due to diabetes was supported by the observation that exogenous insulin was able to reverse the DZ induced augmentation of luminal LPL\(^8\). Nevertheless, we have also tested the direct effects of DZ on isolated cardiomyocyte LPL. Cardiomyocytes were plated on laminin-coated 6-well culture plates (to a density of 200,000 cells/well). Cells were maintained using Media-199, and incubated at 37°C under an atmosphere of 95% O\(_2\)/5% CO\(_2\) for 16 hours. Subsequently, DZ (1 mg/ml; calculated concentration \textit{in vivo}) was added to the culture medium for 4 h and myocyte basal LPL activity released into the medium was measured. To release surface-bound LPL activity, heparin (8 U/mL; 1 min) was added to the culture plates, aliquots of cell medium removed, separated by centrifugation, and assayed for LPL activity. Our data suggest that incubation of cardiomyocytes with DZ had no influence on basal or heparin releasable LPL activity (Online Fig. I).

Online Figure II. Dose dependent effects of palmitic acid on cardiomyocyte LPL in the presence of normal and high glucose

Cardiomyocytes were incubated with normal (NG, 5 mM) or high (HG, 20 mM) glucose and increasing concentrations of albumin bound palmitic acid (PA, 0-1.5 mM). With HG and physiologically relevant concentrations of PA (0.5 mM), no change in heparin releasable LPL activity was observed. However, HG with 1 and 1.5 mM PA increased heparin releasable LPL activity (Online Fig. II). PA in the presence of NG did not influence LPL activity (Online Fig. II).

Online Figure III. Comparing the effects of oleic acid and palmitic acid on cardiomyocyte LPL

We tested the effects of high glucose and albumin bound oleic acid (OA; 1.5 mM) on cardiomyocyte LPL. Interestingly, unlike PA, oleic acid in the presence of high glucose had no influence in increasing heparin releasable LPL activity (Online Fig. III). Other studies have documented that when high glucose is combined with either palmitic or oleic acid, oleic acid is unable to increase DAG to levels comparable to that seen with palmitic acid\(^15\).

Online Figure IV. Independent effects of high glucose and palmitic acid on cardiomyocyte LPL trafficking

Independently, in isolated cardiomyocytes, high glucose or PA had no influence on PKCd and PKD phosphorylation or heparin releasable LPL activity (Online Fig. IV).
Online Figure I. Diazoxide has no direct effect on isolated cardiomyocyte LPL. Cardiomyocytes were plated on laminin-coated culture plates. Cells were maintained using Media-199, and incubated at 37°C under an atmosphere of 95% O₂/5% CO₂ for 16 hours. Subsequently, DZ (1 mg/ml; calculated concentration in vivo) was added to the culture medium for 4 h and myocyte basal LPL activity released into the medium was measured. To release surface-bound LPL activity, heparin (8 U/mL; 1 min) was added to the culture plates, aliquots of cell medium removed, separated by centrifugation, and assayed for LPL activity. Data are means ± SE; n=3 myocyte preparations from different animals.

Online Figure II. Cardiomyocyte heparin releasable LPL activity only increases in the presence of high glucose and palmitic acid. Laminin coated cardiomyocytes were treated with normal (NG, 5 mM) or high (HG, 20 mM) glucose and increasing concentrations of albumin bound palmitic acid (PA, 0-1.5 mM). After 2h, basal and heparin releasable LPL in the medium was determined using radio labeled triolein. Data are means ± SE; n=3 myocyte preparations from different animals.

Online Figure III. Unlike palmitic acid, high glucose and oleic acid does not increase cardiomyocyte heparin releasable LPL. Cardiomyocytes were treated with high glucose (20 mM) and either palmitic (1.5 mM) or oleic (1.5 mM) acid. After 2 h, basal and heparin releasable LPL in the medium was measured. Data are means ± SE; n=3 myocyte preparations from different animals. PA, palmitic acid; OA, oleic acid.

Online Figure IV. High glucose and palmitic acid alone have no effect on cardiomyocyte LPL trafficking. Cardiomyocytes were treated with high glucose (20 mM) or palmitic acid (1.5 mM) alone. At the indicated times, protein was extracted to determine both total and phosphorylated PKCδ (A, C) and PKD (B, D) using Western Blotting. To release surface-bound LPL activity, heparin was added to the culture plates, aliquots of cell medium removed, separated by centrifugation, and assayed for LPL activity (E). Data are means ± SE; n=3 myocyte preparations from different animals. PA, palmitic acid.
Cardiomyocyte LPL activity (nmol/hr/10^6 cells)

Online Figure III

Phosphorylation of PKC-δ

A

Phosphorylation of PKD

B

C

D

E

Online Figure IV
Online References


