Supplemental Data

Cleavage of Protein Kinase D Following Acute Hypoinsulinemia Prevents Excessive LPL-mediated Cardiac Triglyceride Accumulation

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RESEARCH DESIGN AND METHODS

Experimental animals

The investigation conforms to the guide for the care and use of laboratory animals published by the US National Institutes of Health and the University of British Columbia. Adult male Wistar rats (260-300 g) were injected i.v. with streptozotocin (STZ), a selective beta cell toxin, which induces hypoinsulinemia, and causes hyperglycemia(1). Two different doses of STZ (55 and 100 mg/kg, dissolved in saline) were used to cause moderate and severe diabetes, and the animals were kept for 4 days. Control animals were injected with saline. Subsequently, hearts were removed for measurement of coronary luminal LPL activity, immunoprecipitation, immunofluorescence, and Western blot. Some D-100 animals were treated twice daily with Z-DEVD-fmk (3.2 mg/kg, i.p.) for 3 days, to inhibit caspase-3 activity. Blood samples were obtained from the tail vein and glucose was determined using a glucometer (Accu-Chek Advantage; Roche). Following centrifugation and its separation, plasma was assayed for insulin (rat ELISA kit), triglyceride (TG; Infinity) and non-esterified fatty acids (NEFA; Wako) using diagnostic kits.

Isolated heart perfusion

Rats were anesthetized with 65 mg/kg sodium pentobarbital i.p. and the heart carefully excised. Following cannulation of the aorta, hearts were perfused retrogradely with Krebs-Henseleit buffer. The rate of coronary flow (7-8 ml/min) was controlled by a peristaltic pump(2). 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 [^{3}H] triolein substrate emulsion(3). The standard assay conditions were 0.6 mM glycerol tri[9,10-^{3}H]oleate (1 mCi/mmol; 1 Ci = 37 GBq), 25 mM PIPES (pH 7.5), 0.05% (w/v) albumin, 50 mM $MgCl_2$, 2% (v/v) heat-inactivated chicken serum (containing the LPL activator, apolipoprotein CII), plus 100 µl of either medium or heart perfusate in a total volume of 400 µl.

Real time-PCR

14-3-3ζ and LPL mRNA expression were analyzed by real-time quantitative PCR using a light cycler and SYBR Green PCR Mix (Roche). Total RNA from hearts (50 mg) was extracted using Trizol (Invitrogen). After spectrophotometric quantification, reverse transcription was carried out using an oligo-(dT) primer and superscript II RT (Invitrogen). The real time PCR mixture with cDNA was amplified using 14-3-3ζ and LPL specific primers. 14-3-3ζ(4): 5'-CACAGCAAGCATACCAAGAA-3'(left) and 5'-AGAATGAGGCAGACAAAGGT-3'(right); LPL(5): 5'-ATCCAGCTGGGCCTAACTTT-3'(left) and 5'-AATGGCTTCTCCAATGTTGC-3'(right). The amplification parameters were set at 95 °C for 1 s and 60 °C for 6 s and 72 °C for 10 s (40 cycles total). The fluorescence signals were analyzed using light cycler relative

quantification (Roche). Relative expression was evaluated by normalizing to 18S-ribosomal RNA.

Isolated cardiac myocytes and transfection

Ventricular calcium-tolerant myocytes were prepared by a previously described procedure(1; 6). To examine the role of PKD on cardiomyocyte LPL, 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% O_2/5% CO_2. Cardiomyocytes were infected with recombinant adenovirus vectors carrying PKD-S744E/S748E (phosphomimetic active form) and PKD-kinase dead (PKD-KD), as described previously(7). Mock infection as a control was performed using EGFP. Infected cells were incubated for a further 36 h before being used for either Western blot, immunofluorescence or measurement of basal and heparin-releasable (heparin 8 U/mL; 1 min) LPL activity. Cardiomyocytes were also transfected with PKD-wild type (Addgene 10808) or PKD-D378A (GenScript; non-cleavable by caspase-3) using lipofectamine 2000 (Invitrogen). These cells were used for Western blot and measurement of basal and heparin-releasable LPL activity.

Silencing of caspase-3 and 14-3-3ζ by siRNA

siRNA (Santa Cruz) transfection in cardiomyocytes was carried out using a kit (Invitrogen)(7). Briefly, in 6-well culture plates, 0.1 x 10^6 cells were plated and subsequently exposed to the siRNA (or scrambled, Scr) solution for 8 h at 37 °C in a CO_2 incubator. Following this, the media was changed to Media 199 and the cells incubated for another 18 h. After transfection of caspase-3 siRNA, cells were treated with or without PAC-1 (10 μM for 30 min), lysed, and subjected to Western blotting. Caspase-3 activity was measured using a fluorometric assay kit that detects cleavage of substrate DEVD-AMC (Biovision). In these cells, LPL activity was determined by adding heparin (8 U/ml for 1 min) to the incubation medium, and the release of surface-bound LPL activity into the medium determined. Bryostatin-1 (Bry, 1 nM for 30 min) was used to activate PKD. In cardiomyocytes transfected with the *14-3-3ζ* siRNA, cells were lysed, the obtained cytosolic fraction incubated with caspase-3 for 30 min, and immunoblotted for detection of PKD.

Western blotting

Western blot was done as described previously(5). Briefly, ventricular tissue (50 mg) or plated myocytes (0.4 x 10^6) were homogenized in ice-cold lysis buffer. Samples were quantified, boiled with loading dye, and 50 μg used in gel electrophoresis. After blotting, membranes were incubated with rabbit PKD, phospho-PKD (Ser 916 or Ser 744/748), cleaved caspase-3 (Asp175), caspase-3, 14-3-3ζ, LPL (5D2 generously provided by Dr. J. Brunzell, University of Washington, Seattle, WA) and VAMP antibodies, and subsequently with secondary goat anti-rabbit or mouse HRP-conjugated antibody. Bands were visualized using an ECL® detection kit, and quantified by densitometry.

Immunoprecipitation

Following STZ, hearts were lysed in lysis buffer (20 mM Tris-HCl, 2 mM EDTA, 5 mM EGTA, 50 mM 2-mercaptoethanol, 25 μg leupeptin, 4 μg aprotinin, pH 7.5) and immunoprecipitated using 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 immunoblotted with anti-14-3-3ζ. Where indicated, GST-PKD (5 μg) or immunoprecipitated

PKD (5 μg) from control heart were incubated with or without caspase-3 (5 units), and full length and cleaved PKD determined using Western blot.

Immunofluorescence

Heart-Hearts were perfused with 10% formalin and kept 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(5). Slides were incubated with chicken anti-bovine LPL antibody (1:400 dilution) for 3 h. After being washed with TBS, slides were incubated with biotinylated rabbit anti-chicken IgG-conjugated Cy3 (1:150 dilution; Chemicon) for 1 h. Slides were visualized using a fluorescent microscope.
Cardiomyocyte-Following transfection, 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 min, and finally rinsed with PBS. Cells were incubated with rabbit polyclonal PKD, mouse monoclonal TGN38, or chicken anti-bovine LPL antibody followed by incubation with secondary antibodies (goat anti-rabbit IgG-FITC, donkey anti-mouse IgG-TR, donkey anti-mouse IgG-FITC, streptavidin-conjugated Cy3 fluorescent probe) to localize PKD (green), TGN38 (Green and Red), and LPL (red) respectively. Slides were visualized using a Zeiss Pascal confocal microscope.

Separation and measurement of cardiac triglyceride

Total cardiac lipids were extracted and solubilized in chloroform: methanol: acetone: hexane (4:6:1:1 v/v/v/v). Separation and quantification of TG was achieved using HPLC (Waters 2690 Alliance HPLC, Milford, MA), as described previously(8). Heart sections were also stained with Oil-Red-O and counter stained with haematoxylin to visualize cardiac lipid accumulation.

Heart fractionation and determination of OXPAT

OXPAT is a lipid-storage droplet protein that is highly enriched in tissues that have high rates of fatty acid β-oxidation(9). To determine whether the cardiac lipid droplet is coated with OXPAT, we fractionated heart tissue by a previously described method(9). Briefly, cardiac tissues were homogenized and centrifuged (2000g for 5 min) with 45% sucrose and lysis buffer. The supernatants obtained were overlaid with 40% and 10% sucrose solutions and subjected to centrifugation at increasing force (2,700-172,000g). The different fractions obtained at a particular force were then subjected to Western blot for OXPAT or used for determination of TG.

Transmission electron microscopy

To assess accumulation of lipid droplets, morphological evaluation of hearts was carried out using transmission electron microscopy(10). Briefly, left ventricular tissue was fixed in 1.5% glutaraldehyde and paraformaldehyde, cut into small blocks (~1 x 0.5 x 0.2 mm), and fixed for 8 h at 4 °C. After washing, tissue was post fixed with 1% osmium tetroxide and further treated with 1% uranyl acetate and dehydrated using increasing concentrations of ethanol (50–100%). Blocks were embedded in epoxy resin and sectioned at ~90 nm. Sections were stained with 1% uranyl acetate and Reynolds lead citrate. Images were obtained with a Hitachi H7600 electron microscope.

Plasma membrane CD36

Heart membrane fractions were isolated by a previously described method using a sucrose density gradient(11). Using Western blot, identification of CD36 protein was done using rabbit polyclonal CD36 as the primary antibody and mouse anti-rabbit horseradish peroxidase as the secondary antibody. Na^+-K^+-ATPase was used as a plasma membrane marker.

Palmitate oxidation in isolated cardiomyocytes

FA oxidation was performed as described previously(12). Following attachment of cardiomyocytes from the different groups to laminin-coated 60-mm center-well organ culture dishes, cells were incubated in glucose-free DMEM containing 0.4 mM palmitic acid, 3% FA-free BSA, and 0.5 μCi [1-^{14}C]palmitic acid. Incubation was carried out for 2 h at 37 °C with 95% O_2-5% CO_2 gassing. 5% KOH was then injected into the center wells, and oxidation stopped by injecting 1 M H_2SO_4 into the incubation buffer. The dishes were sealed, stored at 4 °C overnight, and KOH assessed for $^{14}CO_2$ by scintillation counting. Oxidation was calculated by subtracting $^{14}CO_2$ at zero time from trapped $^{14}CO_2$ after 2 h of incubation. Results are expressed as nmol CO_2/h/mg of protein (BCA Protein Assay Kit). It should be noted that measurement of palmitate oxidation in isolated quiescent cardiomyocytes may not truly reflect what happens in the working heart.

Separation of golgi and measurement of vesicles

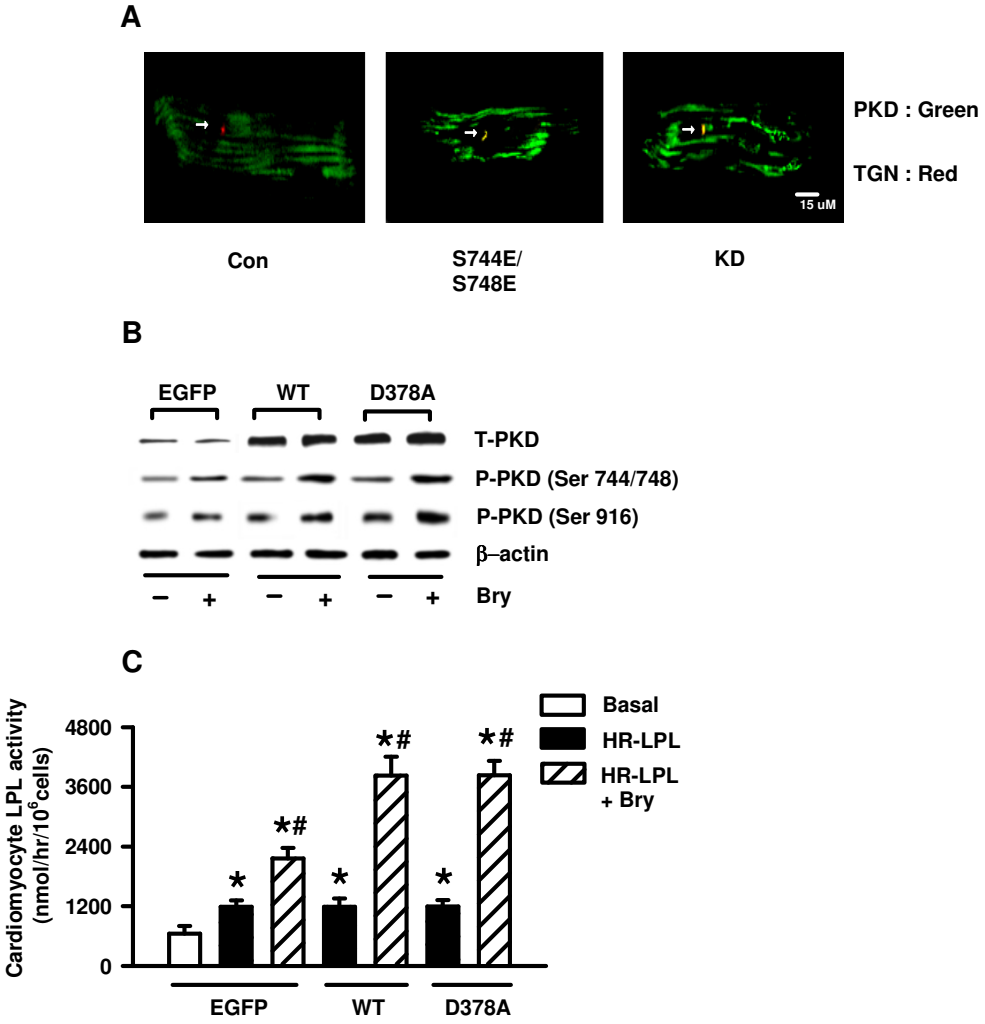
Golgi membranes were isolated from heart tissues using a Golgi isolation kit (Sigma). 5 g of heart tissue was minced and resuspended in 0.25 M sucrose using a Dounce homogenizer. The homogenate was centrifuged at 3,000 g for 15 min. The supernatant was adjusted to 1.25 M by adding 2.3 M sucrose and then overlaid with 1.84 M and 1.1 M sucrose. The density gradient solution was centrifuged at 120,000 g for 3 h. The Golgi enriched fraction was collected at the 1.1/0.25 M sucrose interphase and confirmed using Western blotting with TGN38 antibody (trans-golgi specific marker). Golgi-enriched membranes were diluted with buffer containing 25 mM KCl, 2.5 mM MgSO4, 25 mM Hepes, 0.2 M sucrose, 1 mM dithiothreitol, and 30 μM GTP-γS. GST-PKD (5 μg), caspase-3 (5 units) or cytosol (0.2 mg of protein) from control and D-100 hearts was then added to the membranes, and incubation continued for 15 min at 37 °C(13). After centrifugation (12000 g, 10 min, 4 °C), pellets were re-suspended in the previous buffer plus 0.25 M KCl, and centrifuged at 250,000 g for 1 h to obtain a pellet. Pellets were re-suspended in lysis buffer, and subjected to SDS/PAGE and Western blot for VAMP and LPL.

Materials

[^{3}H]triolein, [1-^{14}C]palmitic acid, and the ECL® detection kit were purchased from Amersham Canada. Heparin sodium injection (Hapalean; 1000 USP U/ml) was obtained from Organon Teknika. PKD, phospho-PKD (Ser 916 or Ser 744/748), cleaved caspase-3 (Asp175), and caspase-3 antibodies were obtained from Cell Signaling (Danvers, MA). 14-3-3ζ, VAMP and β-actin antibodies were obtained from Santa Cruz biotechnology, Inc. (Delaware Avenue, CA). Caspase-3 enzyme, GST (Glutathione S transferases)-PKD protein, and Z-DEVD-fmk were purchased from Biovision. Bryostatin-1 and PAC-1 were from Calbiochem. All other chemicals were obtained from Sigma Chemical.

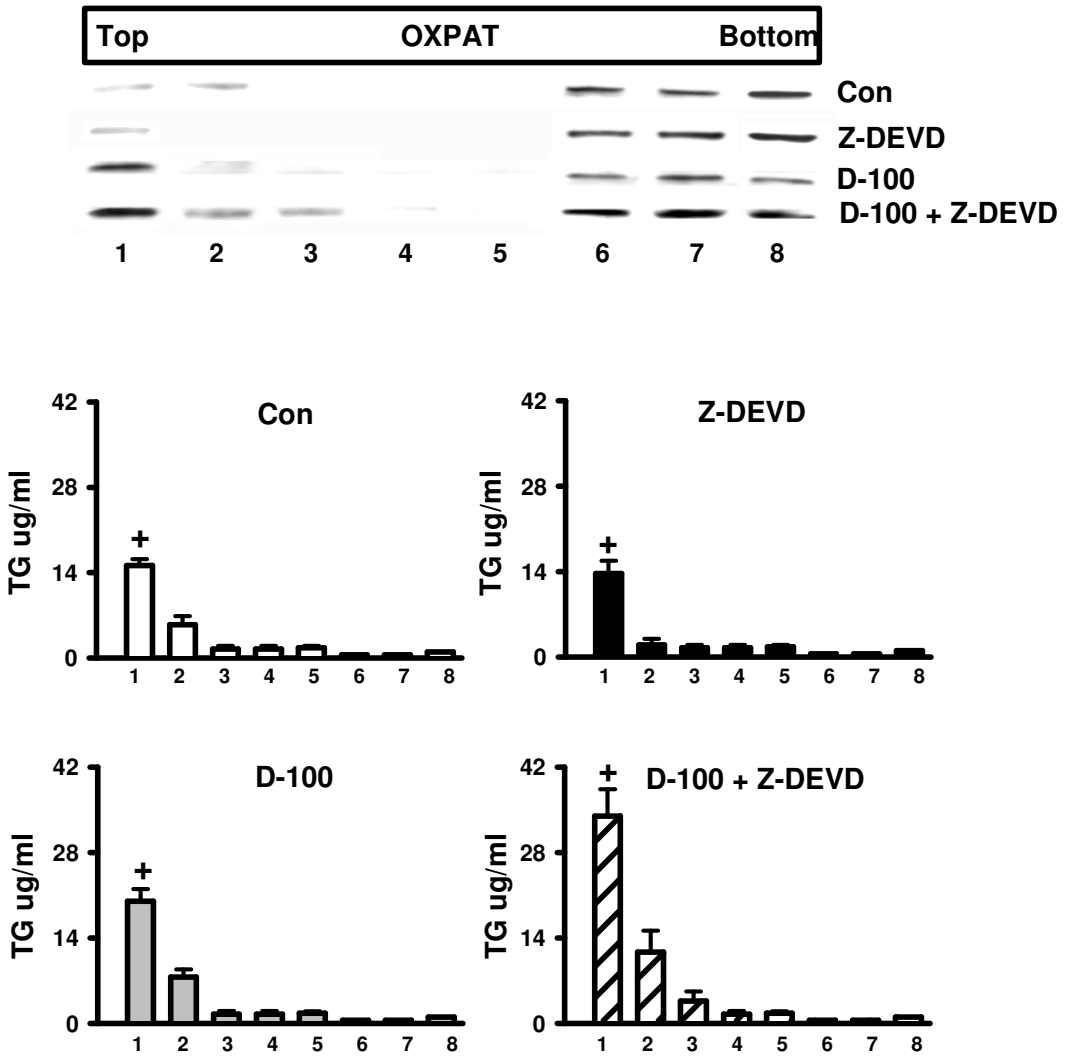
Statistical analysis

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 Figure I. Phosphorylation at Ser 744/748 of PKD is key to increasing cardiomyocyte LPL

Control cardiomyocytes were infected with recombinant adenovirus vector carrying phospho-mimetic (S744E/748E) or kinase-dead (KD) mutants. Infected cells were incubated for a further 36 h before co-localization of PKD and trans-golgi (TGN) was determined using immunofluoresence (arrow). Cardiomyocytes were fixed, incubated with primary antibodies [PKD (Green) and TGN38 (Red)] followed by incubation with secondary antibodies [FITC (Green) and TR (Red)] (A). Cardiomyocytes were also transfected with PKD wild-type (WT) and PKD D378A, and EGFP. Infected cells were incubated for a further 36 h before protein extraction for measurement of total and phosphorylated Ser 744/748 and Ser 916 PKD, in the absence or presence of Bryostatin-1 (B). In these cells, LPL activity 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). Results are the means ± SE of 3-5 cardiomyocyte preparations from different animals. *Significantly different from control; #significantly different from heparin releasable, $P<0.05$.



Online Figure II. Cell fractionation and analysis of TG

Rat heart homogenates from control and D-100 hearts treated or untreated with Z-DEVD were overlaid on sucrose solutions, and the gradients centrifuged (2,700-172,000g). Following fractionation of heart tissue using sucrose density gradients, the different fractions obtained at a particular force were subjected to Western blot for OXPAT (top panel). The different fractions obtained at a particular force were then used for determination of TG (lower panel). Results are the means ± SE of 3-5 rats in each group. $^{+}$Significantly different from all other groups, $P<0.05$.

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