Cardiomyocyte lipids impair β-adrenergic receptor function via PKC activation

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1Divisions of Preventive Medicine and Cardiology, Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York; 2Center for Translational Medicine and George Zallie and Family Laboratory for Cardiovascular Gene Therapy, Department of Medicine, Thomas Jefferson University, Philadelphia, Pennsylvania; 3Department of Pharmacology, Columbia University College of Physicians and Surgeons, New York, New York; and 4Department of Pharmaceutical Sciences, College of Pharmacy, Nova Southeastern University, Fort Lauderdale, Florida

Submitted 30 September 2010; accepted in final form 3 December 2010

Drosatos K, Bharadwaj KG, Lymeropoulos A, Ikeda S, Khan R, Hu Y, Agarwal R, Yu S, Jiang H, Steinberg SF, Blaner WS, Koch WJ, Goldberg IJ. Cardiomyocyte lipids impair β-adrenergic receptor function via PKC activation. Am J Physiol Endocrinol Metab 300: E489–E499, 2011. First published December 7, 2010; doi:10.1152/ajpendo.00569.2010.—Normal hearts have increased contractility in response to catecholamines. Because several lipids activate PKCs, we hypothesized that excess cellular lipids would inhibit cardiomyocyte responsiveness to adrenergic stimuli. Cardiomyocytes treated with saturated free fatty acids, ceramide, and diacylglycerol had reduced cellular cAMP response to isoproterenol. This was associated with increased PKC activation and reduction of β-adrenergic receptor (β-AR) density. Pharmacological and genetic PKC inhibition prevented both palmitate-induced β-AR insensitivity and the accompanying reduction in cell surface β-ARs. Mice with excess lipid uptake due to either cardiac-specific overexpression of anchored lipoprotein lipase, PPARγ, or acyl-CoA synthetase-1 or high-fat diet showed reduced inotropic responsiveness to dobutamine. This was associated with activation of protein kinase C (PKC)-α or PKCβ. Thus, several lipids that are increased in the setting of lipotoxicity can produce abnormalities in β-AR responsiveness. This can be attributed to PKC activation and reduced β-AR levels.

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Cell treatments with lipids. AC16 cells were grown in DMEM-F-12 medium containing FBS (10%) and antibiotics (1%) and were incubated at 37°C in a humidified atmosphere containing 5% CO2–95% air until 85–90% confluent. GFP-B2AR cells were grown in DMEM containing FBS (10%) and antibiotics (1%). RPCM were grown as described elsewhere (41). RPCM were incubated at 37°C in a humidified atmosphere containing 2% CO2–98% air. After washing, each cell type was incubated in 1% FBS-containing medium, in which 1% BSA was added, along with LCFA, ceramide, or DAG, and cells were incubated for 14 h.

**Cellular neutral lipid staining.** Intracellular neutral lipids were stained with Oil Red O, as described previously (55).

**DAG and ceramide measurement.** LCFA-treated AC-16 cells were harvested, and pellets were weighed and assayed for DAG and ceramide levels, using the diacylglycerol kinase method as described (45).

**RNA purification and gene expression analysis.** Total RNA was purified from cells using the TRIzol reagent according to the instructions of the manufacturer (Invitrogen). cDNA was synthesized using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen). cDNA was analyzed with quantitative real-time PCR that was performed with SYBR Green PCR Core Reagents (Agilent Technologies, Santa Clara, CA). Incorporation of the SYBR green dye into the PCR products was monitored in real time with an Mx3000 sequence detection system (Stratagene, La Jolla, CA). Samples were normalized against β-actin. The sequences of the primers are provided in Supplemental Table S1 (Supplemental Material for this article is available online at the AJP-Endocrinology and Metabolism web site).

**Cyclic AMP assay.** Control and LCFA-treated cells were washed with Krebs-Ringer buffer and stimulated with 100 nM isoproterenol in PBS. The concentration of cyclic AMP (cAMP) was determined with the CatchPoint Cyclic-AMP Fluorescent Assay Kit according to the instructions of the manufacturer (Molecular Devices, Sunnyvale, CA). Control treatments were performed with either PBS or 100 nM forskolin in PBS. Obtained values were normalized with total protein concentration as determined with the Modified Lowry Protein Assay Kit (Thermo Scientific, Fremont, CA).

**Protein isolation.** Isolated heart tissues or cells were homogenized in PBS containing protease inhibitors and phosphatase inhibitors (Roche, Indianapolis, IN). Membrane and cytosolic fractions were separated by ultracentrifugation. Twenty micrograms from each fraction was applied to SDS-PAGE and transferred onto nitrocellulose membranes. Antibodies were obtained from the following suppliers: PKCo, Millipore (Billerica, MA); PKCβ, -δ, -ε, and -ζ, Santa Cruz Biotechnology (Santa Cruz, CA).

**Immunoprecipitation.** Immunoprecipitation of specific PKC isoforms was performed with protein A/G Plus UltraLink resin (Thermo Scientific) according to the instructions of the manufacturer.

**PKC activity assay.** PKC activity was assessed with the nonradioactive PKC Kinase Activity Assay Kit according to the instructions of the manufacturer (Molecular Devices, Sunnyvale, CA). Control treatments were performed with either PBS or 100 nM forskolin in PBS. Obtained values were normalized with total protein concentration. The sequences of the primers are provided in Supplemental Table S2. siRNA oligos were transfected in the cells with the siPORT NeoFX Transfection Agent according to the instructions of the manufacturer (Applied Biosystems). Cells were assayed 24–48 h posttransfection.

**Animals.** All procedures involving animals were approved by the Institutional Animal Care and Use Committee at Columbia University. Mice were maintained under appropriate barrier conditions in a 12:12-h light-dark cycle and received food and water ad libitum. The animals that were used for this study were C57BL/6 mice and mice expressing specifically in cardiomyocytes a GPl-anchored lipoprotein lipase [α-mysian heart chain (MHC)-LpL<sub>GPI</sub>], peroxisome proliferator-activated receptor-γ (α-MHC-PPARγ), or acyl-CoA synthetase (α-MHC-ACS). All mice were on the C57BL/6 background, except for the α-MHC-ACS mouse model that was on the FVB background. All studies were performed on the different genotypes with littermates as controls. Male C57BL/6 mice were fed either a high-fat diet containing 60% kcal as fat (Diet no. 12492; Research Diets, New Brunswick, NJ) or regular chow diet for 23 wk, at the end of which they were catherized to assess heart function as described below. Hearts from these mice were harvested, flash-frozen, and stored at −80°C until further use. These hearts were then analyzed for PKCε and PKCδ protein expression in the membrane and cytosolic fractions, as described above. All analyses involving animals were performed with at least four mice per experimental group.

**Echocardiography.** Two-dimensional echocardiography was performed on 10- to 12-wk-old male mice (n = 6–7/group) anesthetized with isoflurane (Sonos 5500 system; Philips Medical Systems, Andover, MA) (59). Echocardiographic images were recorded in a digital format. Images were then analyzed offline by a single observer blinded to the murine genotype (64).

**In vivo cardiac function measurements.** Mice from the different groups were anesthetized using pentobarbital sodium (50–100 mg/kg ip). The right carotid artery was cannulated with a 1.4-F Millar MIKRO-TIP catheter/pressure transducer (SPR-671) (Millar Instruments, Houston, TX), and the catheter was advanced into the left ventricle. Another catheter (an 0.012-in. silicone tubing attached to a 30-gauge needle) was inserted into the femoral vein for administration of drugs. After completion of all the surgical procedures, the animal was allowed to stabilize for ~15 min, followed by recording of basal hemodynamic parameters. Myocardial responses to increasing doses of dobutamine (1, 3, 10, and 30 μg/kg administered at 1- to 2-min intervals were determined. Data calculation was achieved using the PowerLab software (AD Instruments, Colorado Springs, CO).

**Saturation ligand binding.** Plasma membranes from excised mouse hearts or from AC-16 cells were prepared, and saturation ligand binding was performed as described previously (36), using <sup>125</sup>I-CYP (iodocyanopindolol; PerkinElmer, Waltham, MA) for β-AR density measurement. Data were analyzed by nonlinear regression analysis using GraphPad Prism (GraphPad Software, La Jolla, CA).

**Plasma catecholamine measurements.** Plasma epinephrine (Epi) and norepinephrine (NE) levels were determined by ELISA and performed on mouse plasma samples with the BI-CAT EIA kit (ALPCO Diagnostics, Salem, NH), as described previously (36, 63). For these analyses, mice were anesthetized by inhalation of isoflurane.

**Lipid extraction and liquid chromatography-mass spectrometry analyses.** Lipid extraction was performed as described previously (38), with minor modifications.

All experiments were carried out on a Waters Xevo TQ MS Acquity UPLC system (Waters, Milford, MA). The system was controlled by Mass Lynx Software 4.1. The sample was maintained at 6°C in the autosampler, and 7.5 μl was loaded onto a Waters Acquity UPLC BEH Phenyl column (3 mm inner diameter × 100 mm with 1.7 μm particles), preceded by a 2.1 × 5 mm guard column containing the same packing material. The column was maintained at 40°C throughout the analysis. The UPLC flow rate was continuously 300 μl/min in a binary gradient mode with the following mobile phase: initial flow
conditions were 85.5% solvent A [H2O, containing 0.05% triethylamine (TEA)] and 14.5% solvent B (acetonitrile, containing 0.05% TEA); solvent B was increased linearly to 42.5% over a 7-min period, and this was followed by a reduction of solvent B starting at 7.5 min and continuing through 8 min. Fatty acyl-CoAs of interest eluted between 3.5 and 6.5 min. Positive ESI-MS/MS was performed using the following parameters: capillary voltage, 3.8 kV; source temperature, 150°C; desolvation temperature, 500°C; desolvation gas flow, 1,000 l/h; and collision gas flow, 0.15 ml/min. The optimized cone voltage was 58 V, and collision energy for neutral loss and multiple reactions monitoring mode was 34 eV. For multiple reactions monitoring analysis, the following transitions were employed: myristoyl-CoA 978.4 → 471.4 m/z, palmitoleoyl-CoA 1,004.3 → 497.3 m/z, palmitoyl-CoA 1,006.4 → 499.5 m/z, heptadecanoyl-CoA 1,020.4 → 513.5 m/z, linolenoyl-CoA 1,028.4 → 521.4 m/z, linoleoyl-CoA 1,030.4 → 523.4 m/z, oleoyl-CoA 1,032.4 → 525.5 m/z, stearoyl-CoA 1,034.4 → 527.5 m/z, arachidonoyl-CoA 1,054.4 → 547.4 m/z, eicosanoyl-CoA 1,062.4 → 555.4 m/z, and docosahexaenoyl-CoA 1,078.4 → 571.4 m/z.

Statistical analysis. All data on cardiac function assessed by in vivo catheter were analyzed by repeated-measures two-way ANOVA. Comparisons between two groups were performed using unpaired two-tailed Student’s t-tests. All values are presented as means ± SE. Differences between groups were considered statistically significant at P < 0.05.

RESULTS

PA reduced β-AR responsiveness in cardiomyocytes. To determine whether lipids could directly affect β-AR responsiveness in cardiomyocytes, we treated AC-16 cells with 0.4 mM palmitic acid (PA) or oleic acid (OA) in serum-free medium for 14 h. Oil Red O staining of AC-16 cells treated with 0.4 mM palmitic acid (PA) or oleic acid (OA) for 14 h. (A) Oil Red O staining of AC-16 cells treated with 0.4 mM palmitic acid (PA) or oleic acid (OA) for 14 h. (B) and (C) triglyceride (TG) and free fatty acid (FFA) levels in OA- and PA-treated AC-16 cells. (D) Brain natriuretic peptide (BNP) mRNA levels determined by quantitative real-time (qRT)-PCR analysis; n = 7. * P < 0.01 compared with cells that were not treated with LCFA. (E) cAMP levels in LCFA-treated cells stimulated with 100 nM isoproterenol for 15 min; n = 6. ** P < 0.005 compared with cells that were not treated with LCFA. (F) cAMP levels in LCFA-treated cells stimulated with 100 nM isoproterenol for 15 min; n = 6. ** P < 0.005 compared with cells that were not treated with LCFA. (G) β-AR distribution in OA- and PA-treated cells as monitored by confocal microscopy. (H) Western blots of PKCα, PKCβ, PKCδ, PKCe, and PKCζ protein levels in membrane (M) and cytosolic (C) fractions obtained from PA-treated AC-16 cells. (I) and (J) total (I) and isoform-specific (J) PKC activity in AC-16 cells treated with 0.4 mM PA for 14 h; n = 3, P < 0.05 compared with control cells. (K) Western blots of PKCα and PKCβ protein in membrane (M) and cytosolic (C) fractions obtained from PA- or OA-treated AC-16 cells.
mM PA or OA along with 1% BSA (molecular ratio LCFA/BSA = 2.64). Both LCFA increased intracellular TG content (Fig. 1A); PA increased TG eightfold, and OA increased TG 10.4-fold (Fig. 1B). Intracellular nonesterified fatty acids increased 5.9-fold with both PA and OA (Fig. 1C). As expected, PA addition increased palmitoyl-CoA, and OA increased oleoyl-CoA levels in the cells, whereas combination of PA and OA increased both palmitoyl- and oleoyl-CoA levels (Supplemental Fig. S1).

Although both LCFA increased neutral lipid accumulation within the cells, only PA increased the expression of brain natriuretic peptide (BNP), a marker of heart failure, by 94% (Fig. 1D). Similarly, only PA treatment reduced isoproterenol-stimulated cell lysate cAMP production by 67% (Fig. 1E). This observation indicates that the PA-induced defect in β-AR function did not correlate with TG accumulation. Stimulation of the AC-16 cells with forskolin, an adenyl cyclase activator, for 15 min profoundly increased cAMP levels in PA-treated and untreated cells (Supplemental Fig. S2), attributing reduced β-AR responsiveness to a defect of the receptor rather than downstream signaling molecules.

PA treatment alters β-AR cell surface density and leads to abnormal receptor distribution. Treatment of AC-16 cells with PA and OA reduced basal β-AR density by 59 and 68%, respectively (Fig. 1F). Ligand-dependent internalization of β-AR following isoproterenol stimulation markedly decreased β-AR density in control AC-16 cells (84%). Isoproterenol treatment also decreased β-AR density in OA-treated cells (60%). In contrast, isoproterenol did not decrease β-AR density in PA-treated AC-16 cells. Thus, receptor trafficking was defective with PA but not OA.

Because β-ARs cannot be visualized in cardiomyocytes, we studied HEK-293 cells with GFP-tagged β2-ARs. Consistent with the radioligand binding assay, both PA and OA treatments reduced cell surface β-ARs. However, only PA treatment compromised intracellular sequestration of the GFP-tagged β-AR (Fig. 1G).

**Fig. 2. Role of ceramide in PA-mediated lipotoxicity in AC-16 cells.** A: ceramide levels determined by diacylglycerol kinase assay in lipid extracts from control (CTRL) and 0.4 mM PA- and 0.4 mM OA-treated cells for 14 h. Control cells were treated with methanol. B: BNP mRNA levels determined by qRT-PCR analysis in cells treated with 10 μM of C6 ceramide for 14 h; n = 4. *P < 0.05. C: intracellular cAMP levels in AC-16 cells treated with 10 μM of C6 ceramide for 14 h and stimulated with 100 nM isoproterenol for 15 min; n = 6. **P < 0.01; 1-fold corresponds to 15.5 nM cAMP. D: intracellular cAMP levels in isoproterenol-stimulated AC-16 cells treated with 0.4 mM PA + 0.2 μM myriocin for 14 h and stimulated with 100 nM isoproterenol for 15 min; n = 6. *P < 0.05 compared with control cells that were not treated with LCFA and myriocin; 1-fold corresponds to 23.0 nM cAMP. E: Western blots of PKCα and PKCδ in membrane and cytosolic fractions of AC-16 cells treated with 10 μM C6 ceramide.
PKCα and PKCδ are activated in PA-treated AC-16 cells. Western blots of membrane fractions from PA-treated AC-16 cells showed that PKCα and PKCδ, but not PKCβ, PKCε, or PKCζ, were increased (Fig. 1H and Supplemental Fig. S3). Total PKC activity increased 70% in PA-treated cells (Fig. 1I). As expected, increased membrane-bound PKCα and PKCδ protein levels were associated with greater PKCα and PKCδ activity (Fig. 1J). On the other hand, membrane-bound PKCα and PKCδ levels were not affected in OA-treated AC-16 cells (Fig. 1K).

Ceramide inhibits β-AR responsiveness to isoproterenol and triggers PKCα and PKCδ translocation to the membrane. Treatment of AC-16 cells with PA increased ceramide levels 3.4-fold, whereas OA treatment caused a smaller increase (2-fold) (Fig. 2A). These levels of ceramide (Fig. 2A) do not exceed those found in lipotoxic hearts (34, 44). Treatment of cells with 1,2-dioctanoyl-sn-glycerol increased BNP mRNA 76% (Fig. 3B), whereas similar treatment with 1,2-dipalmitoyl-sn-glycerol, which does not enter the cells (15, 46), did not affect BNP levels (Fig. 3C). 1,2-Dioctanoyl-sn-glycerol treatment resulted in complete lack of responsiveness of the β-AR to isoproterenol (Fig. 3D) and increased membrane-bound PKCα and PKCδ (Fig. 3E and Supplemental Fig. S5).

PKC pathway mediates the effects of lipotoxicity on β-AR function. PKC activation was inhibited when AC16 cells were incubated with a combination of PA and Ro-31-8220, a general PKC-inhibitor (Fig. 4A). The PKC inhibitor treatment of PA-treated cells increased isoproterenol-stimulated intracellular cAMP 2.9-fold (Fig. 4B), reduced membrane-associated PKCs, and normalized cell surface levels and distribution of β-ARs (Figs. 1G and 4C). siRNA inhibition of PKCα or PKCδ
expression increased cAMP levels by 59 and 92%, respectively (Fig. 4, D–F).

**PA inhibits cAMP production in primary cardiomyocytes.** Treatment of adult rat primary cardiomyocytes with either 0.4 mM PA or 0.4 mM OA increased neutral lipid accumulation, as shown by Oil Red O staining (Fig. 5 A). However, PA but not OA reduced β-AR responsiveness to isoproterenol, as shown by cAMP production (Fig. 5 B). This was not altered by supplementation of PA with glutathione or salubrinal, inhibitors of oxidative stress (40) and ER stress-mediated apoptosis (5), respectively (Fig. 5 B). However, combined treatment of the cells with 0.4 mM PA and 0.2 mM of OA restored β-AR responsiveness to isoproterenol (Fig. 5 B).

α-MHC-LpL<sub>GPI</sub> hearts show reduced response to β-AR agonist. Hearts from α-MHC-LpL<sub>GPI</sub> mice were studied at baseline and after stimulation with increasing doses of the β-AR agonist dobutamine. These mice accumulate lipids in their hearts, whereas plasma lipids are normal (67). Cardiac fatty acyl-CoAs measured by LC-MS/MS in these mice compared with littermate controls showed similar fatty acyl-CoA distribution (Supplemental Fig. S6). Cardiac performance in a wild-type (WT) and α-MHC-LpL<sub>GPI</sub> mouse is shown as LVdP/dt[the 1st-order derivative of the left ventricular pressure (LVP) waveform] in Fig. 6. Surprisingly, 4-mo-old α-MHC-LpL<sub>GPI</sub> mice with no baseline heart dysfunction by either echocardiography (Supplemental Fig. S6) or direct catheterization (baseline data in Fig. 6, A and B) failed to respond to increasing doses of dobutamine (Fig. 6, A and B). Moreover, lusitropic response, the ability of the myocardium to relax in response to dobutamine, was impaired in those mice (Fig. 6 C) compared with the WT mouse. Nine-month-old α-MHC-LpL<sub>GPI</sub> mice with heart dysfunction, as expected, failed to respond to the dobutamine (Supplemental Fig. S7).

α-MHC-LpL<sub>GPI</sub> mouse hearts have reduced β-AR density. Saturation ligand-binding assay in membrane fractions isolated from hearts of 4-mo-old α-MHC-LpL<sub>GPI</sub> mice showed that
β-ARs were reduced by 30% compared with littermate control mice (Fig. 6D). Plasma Epi and NE levels were not increased in α-MHC-LpLGPI mice (Fig. 6E).

α-MHC-LpLGPI hearts have increased membrane-bound PKCα and PKCβ levels. We found increased membrane-associated PKCα and PKCβ in the α-MHC-LpLGPI hearts compared with WT (Fig. 6F and Supplemental Fig. S8). Cytosolic PKCα was not significantly altered in α-MHC-LpLGPI hearts, whereas cytosolic PKCβ was increased (Fig. 6F and Supplemental Fig. S8). Thus, cardiac lipotoxicity is associated with limited responsiveness to catecholamines that is accompanied by reduced β-AR density and increased membrane-bound PKCα and PKCβ, indicating PKC activation.

Other lipotoxic models also exhibit defective responses to β-AR agonist. Cardiac-specific overexpression of nuclear receptor PPARγ (55) or ACS (11) results in significant cardiac lipid accumulation, left ventricular dysfunction, and premature death. Since we observed defective β-adrenergic response in the α-MHC-LpLGPI hearts, we next determined whether this defect occurs in other cardiac lipotoxic models. Impairment in the contractile response to increasing doses of dobutamine was found in both the α-MHC-PPARγ (Fig. 7A) and α-MHC-ACS (Fig. 7B) models. The difference was not as profound in the α-MHC-ACS mice (FVB) compared with the α-MHC-LpLGPI and α-MHC-PPARγ (C57BL6) mice, which may be attributable to differences between the C57BL6 and FVB background strains (32). Moreover, high-fat-fed mice also exhibited a blunted response to increasing doses of dobutamine similar to the genetic models of cardiac lipotoxicity compared with the Chow-fed controls (Fig. 7C).

Western blots of proteins isolated from cardiac tissue showed activation of PKC in α-MHC-PPARγ, α-MHC-ACS, and high-fat-fed mouse hearts. However, they differed in that membrane PKCβ was increased in the α-MHC-PPARγ (Fig. 7D and Supplemental Fig. S9), whereas membrane-associated PKCα was increased in α-MHC-ACS hearts (Fig. 7E and Supplemental Fig. S9). Protein analysis for hearts obtained from high-fat-fed mice showed significant activation of PKCα (Fig. 7F and Supplemental Fig. S9).

**DISCUSSION**

Intracellular lipid accumulation leads to a number of detrimental effects, such as alterations in insulin signaling (10, 50), endoplasmic reticulum stress (3, 60), apoptosis (11, 25, 57), and mitochondrial dysfunction (51). Hearts are the most energy-requiring organ of the body and normally rely on lipids for their major source of energy (58). However, when lipids accumulate in the heart they can lead to heart dysfunction and premature death (27, 66). The causes of cardiac dysfunction in lipotxicities are unclear. LCFA, particularly PA, are considered to be a more potent cause of lipotoxicity than unsaturated LCFA, such as OA. This has been attributed to the accumulation of either DAG (10, 68) or ceramide (10, 42) and/or other lipids (56). We hypothesized that lipid-mediated abnormalities in cellular pathways would alter the normal physiological responses to stress. Indeed, our data show that intracellular lipid accumulation inhibits normal β-AR signaling. This appears to be via stimulation of PKCs, leading to reduced numbers and defective internalization of β-ARs. Moreover, these abnormal cellular processes were found in several models of lipotoxic heart disease.

We used cardiomyocytes to prove that β-AR function is compromised by lipids and that this occurs via activation of...
PKCs. PA treatment of AC-16 cells, derived from human ventricular cardiomyocytes (14), and adult rat primary cardiomyocytes reduced isoproterenol-induced cAMP production, the downstream read out of β-AR activation. OA addition prevented PA-induced β-AR insensitivity. This is thought to be due to greater incorporation of PA into TG rather than its conversion to other more toxic lipids (33, 37). Ligand-binding assays in AC-16 cells and confocal microscopy of GFP-tagged β2-AR HEK-293 cells (39) showed that PA-treated cells had reduced cell surface β-AR density and defective ligand-dependent internalization.

β-ARs are members of the G protein-coupled receptor family. Reduced β-AR activity can occur due to a reduction in β-ARs or reduced signaling because the receptors are inactivated by phosphorylation. GRK2 phosphorylates the β-AR to ignite the homologous desensitization of the receptor. Phosphorylated β-AR dissociates from the G protein complex and undergoes internalization (18, 19, 21, 69). This is a key step required either for β-AR dephosphorylation and resensitization (31) or for its proteasomal degradation (53). Failing hearts demonstrate reduced cardiac β-AR-mediated responsiveness to catecholamines and abnormal myocardial β-AR signaling (8), which coincides with increased production of catecholamines (36). Because α-MHC-LpL<sub>GPI</sub> mice used for this study did not have increased plasma catecholamines, we questioned whether the β-AR desensitization involved a second process. β-ARs can be desensitized by a ligand-independent cascade that is called heterologous desensitization and is triggered by direct phosphorylation of the receptor by several protein kinases, including PKCs (54). Membrane-associated PKCs were increased in both in vitro and in vivo lipotoxic environments, with reduced β-AR numbers on the membrane and impaired β-AR responsiveness. Furthermore, inhibition of PKC signaling by pharmacological or genetic means restored the presence of the receptor on the membrane and its function. The exact molecule(s) that causes cellular lipotoxicity has not been determined. Saturated FA-induced toxicity may be mediated by intracellular accumulation of either DAG (10, 68) or ceramide (10, 42). Treatment of the AC-16 cells with DAG reproduced the inhibitory effect of PA on β-AR responsiveness to isoproterenol. Treatment of the AC-16 cells with ceramide also reproduced the PA-mediated inhibition of β-AR responsiveness to isoproterenol. Unexpectedly, incubation of PA-treated AC-16 cells with myriocin, a de novo ceramide biosynthesis inhibitor, totally restored the function of the β-AR, as shown by cAMP levels following isoproterenol stimulation. Previous data from our group have shown that myriocin does not reduce by cAMP levels following isoproterenol stimulation. Previous data from our group have shown that myriocin does not reduce

Fig. 6. Impaired β-AR responsiveness to dobutamine in lipotoxic hearts of mice expressing specifically in cardiomyocytes a GPI-anchored lipoprotein lipase [α-myosin heavy chain (MHC)-LpL<sub>GPI</sub>], A: LVdP/dt (1st-order derivative of the left ventricular pressure waveform) recording in response to increasing doses of dobutamine in 4-mo-old wild type (WT) and α-MHC-LpL<sub>GPI</sub> mice. Dobutamine was injected via the femoral vein every 1–2 min, and the LVP waveform was recorded via a pressure transducer/catheter in the left ventricle; n = 4/genotype. B: LVdP/dmax as an index of cardiac contractility to increasing doses of dobutamine in 4-mo-old WT and α-MHC-LpL<sub>GPI</sub> mice. Data computation was achieved using the PowerLab Software; n = 4/genotype. C: LVdP/dmin as an index of myocardial relaxation to increasing doses of dobutamine in 4-mo-old WT control and α-MHC-LpL<sub>GPI</sub> mice; n = 4/genotype. D: membrane β-AR density in 4-mo-old WT and α-MHC-LpL<sub>GPI</sub> hearts measured by saturation-ligand binding assay; n = 4–5/genotype. E: mouse plasma norepinephrine (NE) and epinephrine (Epi) levels measured by ELISA after a 4-h fast; n = 6–7. F: Western blots of PKC<sub>α</sub> and PKC<sub>δ</sub> in the membrane and cytosolic fractions of hearts of 4-mo-old WT and α-MHC-LpL<sub>GPI</sub> mice; n = 3/genotype.

PKCs. PA treatment of AC-16 cells, derived from human ventricular cardiomyocytes (14), and adult rat primary cardiomyocytes reduced isoproterenol-induced cAMP production, the downstream read out of β-AR activation. OA addition prevented PA-induced β-AR insensitivity. This is thought to be due to greater incorporation of PA into TG rather than its conversion to other more toxic lipids (33, 37). Ligand-binding assays in AC-16 cells and confocal microscopy of GFP-tagged β2-AR HEK-293 cells (39) showed that PA-treated cells had reduced cell surface β-AR density and defective ligand-dependent internalization.

β-ARs are members of the G protein-coupled receptor family. Reduced β-AR activity can occur due to a reduction in β-ARs or reduced signaling because the receptors are inactivated by phosphorylation. GRK2 phosphorylates the β-AR to ignite the homologous desensitization of the receptor. Phosphorylated β-AR dissociates from the G protein complex and undergoes internalization (18, 19, 21, 69). This is a key step required either for β-AR dephosphorylation and resensitization (31) or for its proteasomal degradation (53). Failing hearts demonstrate reduced cardiac β-AR-mediated responsiveness to catecholamines and abnormal myocardial β-AR signaling (8), which coincides with increased production of catecholamines (36). Because α-MHC-LpL<sub>GPI</sub> mice used for this study did not have increased plasma catecholamines, we questioned whether
the membrane, as has been shown by others (22). Nonetheless, our data suggest that either DAG or ceramide has the potential to disrupt normal β2-AR function.

PKCs have been implicated as pathogenic intermediates in lipotoxicity and T2DM. PKCβ is activated in hyperglycemia and might be responsible for several diabetes complications (2). PKCε has been associated with proapoptotic signaling when upregulated in cardiomyocytes (13). The most analogous defect to that in the heart is skeletal muscle insulin signaling by PKCδ, a process initiated by excess myocellular DAG (28). PKCα, PKCβ, PKCε, PKCζ, and PKCγ are expressed in cardiomyocytes (30). Among these isoforms, PKCα and PKCδ were activated in PA-treated AC-16 cells. Using a nonspecific PKC inhibitor, we abrogated the PA-mediated reduction in β2-ARs and the defective isoproterenol-induced cAMP production. Moreover, siRNA inhibition of either PKCα or PKCδ ameliorated the defect. Thus, both PKCα and PKCδ are mediators of lipid-induced impairment of β2-AR responsiveness to catecholamines.

To assess whether cardiac lipid-mediated inhibition of β2-AR function occurs in vivo, responsiveness to dobutamine was studied in three genetically engineered cardiolipotoxic mice, α-MHC-LpLGP, α-MHC-PPARγ, and α-MHC-ACS. These mouse models have been shown to have elevated cardiac ceramide and DAG levels (34, 44, 55). These genetic animal models of normal, or almost normal in the case of the α-MHC-ACS mice, basal heart function as well as high-fat-fed C57BL/6 mice reproduced defective contractility following increasing dobutamine doses. The activated PKC isoform differed in these models. Thus, the type of lipid or its intracellular localization might lead to defects via different members of the PKC family.

T2DM and obesity are associated with increased lipid accumulation in the heart and reduced cardiac function, which eventually results in heart failure (61, 66). In addition to defective heart mechanics, abnormalities in regulation of heart rhythms are a major risk factor for adverse outcomes (62). Failing hearts demonstrate reduced cardiac β2-AR-mediated responsiveness to catecholamines and abnormal myocardial β2-AR signaling (9, 65). This defect is mitigated by the use of β-blockers, which prevent catecholamine-mediated β2-AR desensitization in heart failure patients. We found another cause of cardiac adrenergic dysfunction that might be present in patients with T2DM and metabolic syndrome. Lipotoxic hearts from several models have increased PKCα and PKCδ activation and defective β2-AR responsiveness that predates the development of heart failure.

Defective β2-adrenergic signaling alone causes cardiomyopathy (47). β2-AR function is crucial in cardiac contractility and response to normal physiological stress, such as during exer-
cise (48). Moreover, human studies suggest that patients with T2DM who are likely to have lipotoxic injury have abnormalities of cardiac rhythm (62). Our data show that cardiac lipid accumulation compromises β-AR responsiveness to catecholamines. This effect may be mediated by both ceramide and DAG. Activation of PKCs and PKCβ is associated with defective β-AR function, and inhibition of PKCs improved the responsiveness of the receptor. These data implicate PKCs as suitable targets for the treatment of cardiac dysfunction in obese and diabetic individuals. Moreover, our studies suggest a common pathological process, lipid induction of PKCs, as a cause of cellular dysfunction in lipotoxicity.

ACKNOWLEDGMENTS

We thank Dr. John P. Morrow and Chad Trent for providing us with adult rat primary cardiomyocytes, Dr. Mercy M. Davidson for the AC-16 cells, and Dr. Graeme Milligan for giving us the GFP-β-AR cells.

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