Altered cardiac fatty acid composition and utilization following dexamethasone-induced insulin resistance

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GLUCOCORTICOIDS ARE WIDELY USED as anti-inflammatory and immunosuppressive agents. However, glucocorticoid therapy is often associated with serious adverse effects, including dyslipidemia, impaired insulin sensitivity, and cardiovascular disease (38). Increasing evidence from clinical and experimental studies (20, 29) has established that metabolic abnormalities play a crucial role in the development of heart disease. Under physiological conditions, the heart acquires most of its energy from metabolism of glucose and fatty acid (FA), with the latter being the major substrate consumed by cardiac tissue (29). During metabolic stresses, such as diabetes and insulin resistance, characterized by inadequate glucose utilization, cardiac FA consumption supersedes glucose oxidation. In the heart, elevated FA use has been implicated in a number of metabolic, morphological, and mechanical changes and, more recently, in "lipotoxicity" (30). During lipotoxicity, when the capacity to oxidize FA is saturated, FA accumulates and can, either by itself or via production of second messengers, such as ceramides, provoke cell death (30).

Previously, we (27) reported that a single dose of the synthetic glucocorticoid hormone dexamethasone (Dex) induced whole body insulin resistance within 4 h. Hearts from those animals showed high pyruvate dehydrogenase kinase-4 (PDK-4), an enzyme that inactivates pyruvate dehydrogenase complex and subsequently attenuates glucose oxidation (27). DEX-treated hearts also demonstrated enlargement of coronary lipoprotein lipase (LPL), the enzyme that mediates hydrolysis of circulating lipoproteins to FA and is suggested to be the principal source of FA for cardiac utilization (27). The present study was designed to evaluate the fate of FA delivered to the heart following DEX treatment.

MATERIALS AND METHODS

Experimental animals. The investigation conformed to the Guide For the Care and Use of Laboratory Animals published by the National Institutes of Health and the University of British Columbia (animal care certificate no. A00-0291). Male Wistar rats (250–300g) were obtained from the University of British Columbia Animal Care Unit and fed a standard laboratory diet (PMI Feeds, Richmond, VA) and water ad libitum. The synthetic glucocorticoid hormone DEX (1 mg/kg) or an equivalent volume of ethanol was administered intraperitoneally, and the animals were killed after 4 and 8 h, respectively.

Euglycemic hyperinsulinemic clamp. Whole animal insulin resistance was assessed using a euglycemic hyperinsulinemic clamp. Briefly, after treatment with DEX, anesthetized rats were administered insulin (Humulin R, 30 mU·min⁻¹·kg⁻¹) and d-glucose (50%) continuously for 1 h; glucose infusion was initiated 4 min after commencement of insulin infusion. Throughout the procedure, circulating blood glucose was monitored by analysis of blood obtained from the tail vein (using a glucometer; AccuSoft Advantage). M value represents the average glucose infusion rate measured over the last 30 min of the euglycemic hyperinsulinemic clamp.

Postheparin plasma lipolytic activity. Plasma LPL activity in the fed state and in response to a heparin injection was determined in...
control (CON) and Dex rats as described previously (35). Heparin (25 U/ml) was injected into the jugular vein of lightly anesthetized (20 mg/kg pentobarbital sodium ip) rats, and blood samples were collected after 10 min. Plasma was separated and stored at −70°C until it was assayed for LPL activity. Plasma lipase activity was determined by first measuring total lipase (hepatic + LPL) activity in 5 μl of plasma sample. Hepatic lipase activity was measured by incubating plasma with 1 M NaCl (at room temperature for 10 min before being exposed to substrate) and conducting the assay in the absence of apolipoprotein CII (10) to suppress LPL activity. Plasma LPL activity was calculated as the difference between total and hepatic lipase activity.

**Trition WR-1339.** WR-1339, a nonionic detergent, physically alters lipoproteins, making them inaccessible for LPL-mediated hydrolysis (5). When injected intravenously, newly synthesized TGs accumulate in the plasma. Rats were injected (iv) with WR-1339 (25% wt/vol solution in normal saline to give a dose of 600 mg/kg body wt). WR-1339 was injected 30 min before Dex administration, and blood samples were collected at 2 and 4 h after the injection. Plasma was separated, and the TG concentration was measured.

**Plasma TG.** Blood samples from the tail vein were collected at varying intervals in heparinized glass capillary tubes. Blood samples were immediately centrifuged, and plasma was collected and assayed. A diagnostic kit was used to measure triglyceride (TG, Thermo Electron).

**Separation and characterization of cardiac lipids.** Total cardiac lipids were extracted and solubilized in chloroform-methanol-ace-tone-hexane (4:6:1:1 vol/vol/vol/vol). Separation of TG and FA was achieved using HPLC (Waters 2690 Alliance HPLC, Milford, MA) equipped with an autosampler and column heater. FAs were quantified as their respective methyl esters with the use of heptadecanoic acid (17:0) as the internal standard, with a Varian 3400 GLC equipped with a flame ionization detector, a Varian Star data system, and an SP-2330 capillary column (30 m x 0.25 mm; Supelco, Bellefonte, PA). Values of cardiac FA and TG were expressed as micrograms per milligram of protein.

**Measurement of Δ-6 desaturase activity.** Δ-6 desaturase plays a key role in the synthesis of arachidonic acid (AA) from linoleic acid (LA). The total activity of this enzyme is reflected by the sum of all desaturation products from LA (16). Briefly, following 4 and 8 h of Dex, hearts were removed and microsomes prepared as described previously (16); 0.5 mg of microsomal protein were reacted with 200 μmol 18:2(n-6) with 0.1 μCi of [14C]18:2(n-6) at 37°C for 20 min. Following reaction termination and FA extraction, the resulting methyl esters were dissolved in petroleum ether (bp 30–60°C) with a mixture of unlabeled methyl ester carriers (100 μg each) and then separated according to their unsaturation by argentation TLC on silica gel. The plates were developed in toluene acetone, and the bands were visualized under UV light. The quantification of desaturation products was performed by liquid scintillation spectrometry with quench correction and conversion to dpm. Recovery of radioactivity from the TLC plates was >95%.

**Cardiac glucose and FA oxidation.** To measure glucose oxidation, isolated hearts were perfused for 30 min with Krebs-Henseleit buffer in the working mode at a preload of 11.5 mmHg and an afterload of 80 mmHg, as previously described (27). Rates of glucose oxidation were quantitatively measured by collection of 14CO2 liberated from [U-14C]glucose at the pyruvate dehydrogenase reaction and in the citric acid cycle. To measure cardiac palmitate oxidation, hearts from halothane (2–3%)-anesthetized rats were perfused in the working mode with modified Krebs-Henseleit buffer (including 1.0 mM [9,10-3H]palmitate prebound to 3% BSA, 5.5 mM glucose, 2.0 mM calcium, and 100 U/l insulin) at a preload of 11.5 mmHg, as described previously (11). An afterload of 80 mmHg was maintained, and samples of perfusate and heartomydrioxine were taken every 10 min for measurement of FA oxidation.

**Cardiac PDK-4 and AMPK gene expression.** Heart PDK-4 and AMPK gene expression were measured using RT-PCR. Briefly, total RNA from cardiac tissues was extracted using TRIzol (Invitrogen), and reverse transcription was carried out using an oligo(dT) primer and SuperScript II RT (Invitrogen). cDNA was amplified using PDK-4-(reported previously) (27) and AMPK [5′-GCTGTTG- GATCGCAAAATTAT-3′ (left) and 5′-GCATCGAGCTGAGT- GCCATA-3′ (right)]-specific primers. The β-actin gene was amplified as an internal control using 5′-TGTTGGTATGGGTTCA-GAAGG-3′ (left) and 5′-ATCTGTCAAGGATCGCTT GG-3′ (right). The amplification parameters were set at 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min for a total of 30 cycles. The PCR products were electrophoresed on a 1.7% agarose gel containing ethidium bromide. Expression levels were represented as the ratio of signal intensity for PDK-4 and AMPK mRNA relative to β-actin mRNA.

**Western blotting for AMPK and ACC.** Phosphorylation of AMPK increases its activity ~50- to 100-fold (32, 33) and subsequently phosphorlates and inactivates ACC. To determine total and phosphorylated AMPKα and ACC, whole cell homogenates were isolated as described previously (2). Briefly, hearts were ground under liquid nitrogen, and 50 mg were homogenized. After centrifugation at 5,000 g for 20 min, the protein content of the supernatant was quantified using a Bradford protein assay. Samples were diluted and loaded with sample loading dye, and 50 μg were used in SDS-PAGE. After being transferred, membranes were blocked in 5% skim milk in Tris-buffered saline containing 0.1% Tween-20. Membranes were incubated with either rabbit AMPKα, phospho-AMPK (Thr172), phospho-ACC (Ser79; Cell Signaling), or actin antibodies (Santa Cruz Biotechnology). Subsequently, a secondary goat anti-rabbit horseradish peroxidase-conjugated antibody was used, and the membranes were visualized by using enhanced chemiluminescence detection. Measuring the phospho form of AMPK is a surrogate for estimation of its activity.

**Materials.** [3H]triolein was purchased from Amersham Canada. Heparin sodium injection (Hapalean, 1,000 USP U/ml) was obtained from Oraongan Teknika. All other chemicals were obtained from Sigma Chemical.

**Statistical analysis.** Values are means ± SE. Wherever appropriate, one-way ANOVA followed by Tukey’s or Bonferroni tests or the unpaired and paired Student’s t-tests were used to determine differences between group mean values. The level of statistical significance was set at P < 0.05.

**RESULTS**

**Acute Dex induces insulin resistance.** Our previous study reported that Dex for 4 h induced whole body and cardiaco-specific insulin resistance (27). In the present study, we confirmed and extended this finding. Thus, following 1 mg/kg Dex injection and using the euglycemic hyperinsulemic clamp, our data revealed that the glucose infusion rate necessary to maintain euglycemia was lower after both 4 and 8 h of Dex treatment (Fig. 1A). This whole body insulin resistance was also associated with a heart-specific increase in PDK-4 gene expression (Fig. 1B) and a reduction of cardiac glucose oxidation (Fig. 1C).

**High plasma TG clearance following Dex is due to amplified lipolytic activity.** LPL, the rate-limiting enzyme in TG clearance, controls catabolism of TG-rich lipoproteins (3, 4). To test whether Dex influences whole body lipolytic activity, basal and postheparin plasma was obtained from CON and Dex-treated animals. Both basal and postheparin plasma LPL activity increased at 4 and 8 h after Dex (Fig. 2A). Given this increase in lipolytic activity, we evaluated plasma TG at varying times (0–8 h). Interestingly, plasma TG declined progressively and remained low at 8 h following Dex (Fig. 2B).
To exclude the possibility that the Dex-induced reduction in plasma TG is a consequence of decreased lipoprotein secretion from the liver, both CON and Dex animals were treated with WR-1339. A 20-fold increase in circulating TG was observed following injection of this detergent, with no statistical difference between the CON and Dex-treated groups (Fig. 2).

**FA composition in the heart following Dex.** As incubation of circulating lipoproteins with LPL predominantly releases palmitic acid (36), we measured the cardiac FA composition following Dex. Palmitic (Fig. 3A) and oleic (Fig. 3B) acid levels were higher after 4 h of Dex and decreased to CON levels within 8 h. More interestingly, polyunsaturated FAs demonstrated a drop in LA and γ-linolenic acid (LNA), with an increase in AA after acute Dex injection (Fig. 3, table).

**Fig. 1.** Acute dexamethasone (Dex) induces insulin resistance and alters cardiac glucose oxidation. A: after 1 mg/kg Dex injection for 4 and 8 h, whole animal insulin resistance was assessed using a euglycemic hyperinsulinemic clamp. M value represents the average glucose infusion rate measured over the last 30 min of the euglycemic hyperinsulinemic clamp. B: to identify changes in glucose metabolism specifically in the heart, we evaluated pyruvate dehydrogenase kinase-4 (PDK-4). PDK-4 gene expression was measured using RT-PCR. Expression levels were represented as the ratio of signal intensity for PDK mRNA relative to β-actin mRNA. C: glucose oxidation was determined as described in MATERIALS AND METHODS. Results are means ± SE of 3–4 animals in each group. *P < 0.05 vs. control (CON).

**Fig. 2.** High plasma triglyceride (TG) clearance after Dex is due to amplified lipolytic activity. A: plasma lipoprotein lipase (LPL) activity in the basal state, and in response to a heparin injection, was determined in CON and Dex rats. Heparin was injected into the jugular vein of lightly anesthetized rats, and blood were samples collected after 10 min. Plasma lipase activity was determined by first measuring total lipase (hepatic + LPL) activity. Incubating plasma with 1 M NaCl and conducting the assay in the absence of apolipoprotein CII to suppress LPL activity measured hepatic lipase activity. Plasma LPL activity was calculated as the difference between total and hepatic lipase activity. B: a diagnostic kit was used to measure plasma TG over time. C: in a different experiment, 30 min before Dex, WR-1339 was administered, rats were killed after 4 and 8 h, and plasma TG was measured. Results are means ± SE of 5 animals in each group. *P < 0.05 vs. CON. PHPLA, postheparin plasma lipolytic activity.
Measurement of cardiac Δ-6 desaturase showed a decrease in activity following Dex (Fig. 4).

Dex alters cardiac TG and increases FA oxidation. Tissue FA clearance occurs through both storage as TG and oxidation. At 4 h, DEX augmented cardiac TG accumulation (Fig. 5). However, this increase in tissue TG could not be maintained, such that at 8 h following Dex, TG declined to CON levels (Fig. 5). At an afterload of 80 mmHg, palmitate oxidation after 4 and 8 h of DEX was higher than CON (Fig. 6). The increase in palmitate oxidation following Dex was unrelated to any change in cardiac Peroxisome proliferator-activated receptor (PPAR)α gene expression (data not shown).

Acute DEX influences cardiac AMPK and phosphorylates ACC. After 4 h of Dex, an approximately twofold increase of AMPK phosphorylation was observed, which was maintained until 8 h (Fig. 7C). Interestingly, this change in AMPK phosphorylation paralleled a rise in total AMPK protein (Fig. 7B) and gene expression (Fig. 7A). Once activated, AMPK phosphorophylates and inactivates ACC, the predominant isof orm in the heart (33). As ACC catalyzes the conversion of acetyl-CoA to malonyl-CoA, AMPK, by phosphorylating ACC, is able to decrease malonyl-CoA and minimize its inhibition of FA oxidation (33). After 4 and 8 h of DEX, phosphorylation of ACC increased (Fig. 8).

DISCUSSION

Both endogenous (25, 31) and exogenous glucocorticoids (40, 41) impair insulin sensitivity, contributing to generation of the metabolic syndrome, including insulin resistance, obesity, and hypertension. In the present study, both 4- and 8-h Dex treatment decreased glucose infusion rate during the euglycemic hyperinsulinemic clamp. This whole body insulin resistance was associated with augmented expression of cardiac PDK-4 and a reduction in the rate of cardiac glucose oxidation.

Plasma TG levels are maintained through uptake from the gut, secretion from the liver, and clearance by vascular endothelium-bound LPL. By use of WR-1339, the decrease in plasma TG following Dex was likely not related to decreased lipoprotein secretion from the liver. At the lumen, LPL actively metabolizes the TG core of lipoproteins to FA, which are then transported into the underlying tissue for numerous metabolic and structural functions. Through such a role, LPL activity directly affects the level of circulating lipoprotein TG. For example, in transgenic rabbits that have global overexpression of LPL, attenuation of hypertriglyceridemia is observed (18). Given the association between glucocorticoids and LPL gene expression in the heart and adipose tissues (9, 23, 27), we measured postheparin plasma lipolytic activity. Both basal and heparin-releasable LPL increased after 4 and 8 h of Dex. More importantly, this increased LPL was related to a progressive clearance of plasma TG, which remained low, even at 8 h of Dex. It is unclear whether this effect of Dex on TG is beneficial. Systemic overexpression of LPL has been reported to ameliorate insulin resistance (17, 21). However, it should be noted that despite this reduction in TG, Dex was still associated with whole body insulin resistance.

AMPK, a heterotrimeric enzyme, plays a key role in regulation of cardiac metabolism (42). AMP binding to the AMPK γ-subunit and Thr172 phosphorylation by AMPK kinase increases AMPK activity (42). Under physiological and pathological conditions like exercise, fasting, and ischemia that change the AMP/ATP ratio, AMPK is phosphorylated and...
activated in the absence of any change in total AMPK protein (12, 14). Various drugs, including 5-aminoimidazole-4-carboxamide-1-β-4-ribofuranoside and metformin, also activate AMPK without altering total protein (1). In the present study, our data for the first time demonstrate that acute Dex treatment, through transcriptional regulation, augments total AMPK protein and thus phosphorylation. Glucocorticoids have previously been reported (38) to influence the transcription of 1% of the entire genome in humans.

Recently, AMPK has been implicated in FA delivery to cardiomyocytes through its regulation of CD36 (22). Given the importance of LPL in providing hearts with FA (3) and the observation that incubation of circulating lipoproteins with LPL predominantly releases palmitic acid (47.5% of total FAs released) (36), we predicted that, following Dex, total cardiac palmitic acid should increase. Indeed, palmitic (and oleic) acid in the heart increased almost twofold following 4 h of Dex. Whether this increase is also associated with hydrolysis of esterified pools (endogenous TG and phospholipids) has yet to be determined. At 8 h, levels of palmitic and oleic acid returned to normal and are possibly reflective of the low circulating TG or increases in FA oxidation. In the heart, AMPK activation is known to promote FA oxidation (8). AMPK phosphorylates ACC and subsequently lowers malonyl-CoA. Decreased malonyl-CoA increases carnitine palmitoyltransferase I, a rate-limiting enzyme in mitochondria, and FA oxidation is amplified (8). In the present study, acute Dex promoted ACC phosphorylation and increased palmitate oxidation, perhaps through its effects on AMPK. On the other hand, even though cardiac PPARα expression was unchanged after Dex, the possibility that Dex-induced metabolic changes were due to PPARα activation by elevated cellular FAs cannot be excluded.

During diabetes and insulin resistance, to compensate for the diminished contribution of glucose as an energy source, cardiac energy production occurs largely from FAs, which are supplied in excess to the heart (27, 29). However, the heart and other nonadipose tissues have inadequate ability to handle excess lipids. Given that FA oxidation is likely operating at maximum in Dex-treated hearts (in the normal heart, 70% of Fig. 4. Cardiac Δ-6 desaturase activity. After 4 and 8 h of Dex, hearts were removed, and microsomes were prepared and reacted with 200 μmol 18:2(n-6) with 0.1 μCi [14C] 18:2(n-6) at 37°C for 20 min. Top: after reaction termination and FA extraction, the resulting methyl esters were dissolved and separated by argentation TLC on silica gel. Plates were developed in toluene acetone, and bands were visualized under UV light. Bottom: quantification of desaturation products was performed by liquid scintillation spectrometry with quench correction and conversion to dpm. Results are means ± SE of 4 animals in CON and 8-h DEX groups and 2 animals in 4-h Dex group. *P < 0.05 vs. CON.

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Fig. 5. TG in the heart after Dex. Separation of TG was achieved using HPLC. These values were expressed as micrograms per milligram of protein. Results are means ± SE of 4 animals in each group. *P < 0.05 vs. CON.

Fig. 6. Palmitate oxidation increases in Dex-treated hearts. Hearts were perfused in the working mode with Krebs-Henseleit buffer at a preload of 11.5 mmHg. An afterload of 80 mmHg was maintained, and samples of perfusate and hyamine hydroxide were taken every 10 min for measurement of FA oxidation. Values are means ± SE of 4 rats in each group. *P < 0.05 vs. CON.
energy production is already being obtained through oxidation of FA (29), the excess cardiac FA is likely channeled towards TG synthesis. Measurement of cardiac TG showed high levels at 4 h after Dex but was normalized at 8 h. Because ACC phosphorylation and palmitate oxidation remained high at 8 h, our data suggest that the drop in TG likely occurred due to persistent intracellular oxidation of FA and/or the presence of diminished circulating lipoprotein TG. At present, it is unclear whether this drop in cardiac TG would also occur under conditions of hyperlipidemia, which would be expected to maintain elevated intracellular TG. Given the clinical prevalence of glucocorticoid use, it is possible that, should this occur, elevated FA delivery and subsequent TG synthesis may result in a number of metabolic, morphological, and mechanical changes and, eventually, in “lipotoxicity” (30).

Unlike saturated FAs necessary for ATP generation, polyunsaturated FAs (PUFAs) are also required to manufacture and repair cell membranes (15, 24) and regulate functions like heart rate, blood pressure, and clotting (6, 19). In an effort to determine whether Dex influences cardiac PUFAs composition, we measured the cardiac FA species and established a drop in LA and LNA with an increase in AA. Given the function of glucocorticoids to inhibit phospholipase A2 (37), the increase in cardiac AA was unexpected. It is possible that, because Dex decreased LA and LNA acid over time, these FAs are being either oxidized or converted to AA. Other studies have reported that in rat testis, Dex can stimulate Δ-6 desaturase (34), the rate-limiting enzyme for converting LA to AA. In the present study, because Dex inhibited cardiac Δ-6 desaturase, it is likely that the decrease in LA and LNA is due to increased FA oxidation. At present, the mechanism for the increase in cardiac AA is unknown. Irrespective of the mechanism, excess amounts of AA are known to alter insulin signaling and sensitivity (15), and induce cell death (26, 28), directly through the mitochondrial permeability transition (39) or indirectly through conversion of AA to toxic byproducts like hydroxyeicosatetraenoic (7) and epoxycosatrienoic acids (6, 19). Unlike ω-6 FAs, Dex had limited effects on ω-3 FAs like docosahexaenoic acid and eicosapentaenoic acid, reported to protect the heart from cardiovascular disease (13).

In summary, acute Dex-induced insulin resistance increases plasma lipolytic activity and rapidly clears circulating TG. The FAs entering the heart are either stored as TG or oxidized. FA
oxidation occurs through activation of AMPK and subsequent phosphorylation of ACC. In addition to saturated FA, Dex also influences the cardiac composition of PUFAs, with the most significant change being the increase in AA acid. Given the detrimental effects of high FA oxidation, TG storage, and AA acid accumulation, our data suggest that the acute effects of Dex on cardiac metabolism may be associated with the increased cardiovascular risk following chronic therapy.

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