Cardiac glycogen accumulation following dexamethasone is regulated by AMPK

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**Running Head:** AMPK control of cardiac glycogen

**Key Words:** Glucocorticoids, Insulin resistance, GLUT4, Glycogen synthase

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ABSTRACT

Glycogen is an immediate source of glucose for cardiac tissue to maintain its metabolic homeostasis. However, its excess brings about cardiac structural and physiological impairments. Previously, we have demonstrated that in hearts from dexamethasone (DEX) treated animals, glycogen accumulation was enhanced. We examined the influence of AMPK on glucose entry and glycogen synthase as a means of regulating the accumulation of this stored polysaccharide. Following DEX, cardiac tissue had limited contribution towards the development of whole body insulin resistance. Measurement of GLUT4 at the plasma membrane revealed an excess presence of this transporter protein at this location. Interestingly, this was accompanied by an increase in GLUT4 in the intracellular membrane fraction, an effect that was well correlated to an increased GLUT4 mRNA. Both total and phosphorylated AMPK increased following DEX. Immunoprecipitation of AS160 followed by Western blotting demonstrated no change in Akt phosphorylation at Ser473 and Thr308 in DEX treated hearts. However, there was a significant increase in AMPK phosphorylation at Thr172, which correlated well with AS160 phosphorylation. In DEX hearts, there was a considerable reduction in the phosphorylation of glycogen synthase, whereas GSK-3-β phosphorylation was augmented. Our data suggest that AMPK mediated glucose entry, combined with activation of glycogen synthase and reduction in glucose oxidation (Qi, D., et al. Diabetes 53:1790, 2004), act together to promote glycogen storage. Should these effects persist chronically in the heart, they may explain the increased morbidity and mortality observed with long term excesses in endogenous or exogenous glucocorticoids.
On metabolic demand, glycogen, a mobilized storage form of glucose, is readily broken down to yield glucose moieties. The major rate limiting enzymes involved with the metabolism of glycogen include glycogen synthase and phosphorylase. For its synthesis, the core protein glycogenin acts as a primer for the attachment of UDP-glucose moieties, promoting the growth of this polymer (31). Glycogen synthase catalyzes the attachment of UDP-glucose to the non-reducing end of already formed glycogen (36). Glycogen breakdown is mediated by glycogen phosphorylase. The activity of these enzymes is regulated by both phosphorylation and allosteric stimulation (24).

Glycogen is an immediate source of glucose for cardiac tissue to maintain its metabolic homeostasis (3). However, its excess has been suggested to bring about structural and physiological impairments including an ionic imbalance, a change in pH and stimulation of pathways leading to hypertrophic signaling. Thus, glycogen accumulation that is associated with mutation of AMPK has been reported to cause cardiac hypertrophy, conduction system failure and ventricular arrhythmias (1). In glycogen storage diseases like Pompe disease, characterized by deficiency of debranching enzyme function, an excessive accumulation of cardiac glycogen leads to left ventricular hypertrophy and subsequent failure (55).

Glucocorticoids have widespread use as anti-inflammatory and immunosuppressive agents (47). However, both excess endogenous and exogenous glucocorticoids are known to contribute towards cardiovascular complications (39, 48). These cardiac abnormalities could be secondary to glucocorticoid induced insulin resistance and Type 2 diabetes, and alterations in cardiac metabolism. With the latter, we have previously demonstrated that in hearts from dexamethasone (DEX) treated animals, amplification of lipoprotein lipase (LPL) provided the heart with excessive fatty acids (FA) that are known to induce cardiomyopathy (43, 53, 57).
Glycogen accumulation was also enhanced in these hearts. We rationalized that this increase in glycogen was a consequence of compromised glucose oxidation observed in DEX treated hearts (43). In the present study, we examined whether additional factors related to glycogen synthesis like glucose entry and glycogen synthase also play a role in the accumulation of this stored polysaccharide. Our data suggest that in the presence of intact insulin signaling, AMPK mediated glucose entry, combined with activation of glycogen synthase and the previously reported reduction in glucose oxidation, act together to promote glycogen storage. Should these effects persist chronically, they may explain the increased morbidity and mortality observed with long term excesses in endogenous or exogenous glucocorticoids.
MATERIALS 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 obtained from the UBC Animal Care Unit. The synthetic glucocorticoid hormone dexamethasone (DEX; 1 mg/kg) or an equivalent volume of ethanol was administered by i.p. injection to non-fastened rats, and the animals were euthanized at 4 h. In the human body, the basal daily secretion of cortisol is approximately 6-8 mg/m$^2$ (in a 70 kg adult male, this translates to approximately 0.2 mg/kg). In response to stress, cortisol release is increased up to 10-fold of the basal value (2 mg/kg) (13). For exogenous administration, the dosing with corticosteroids depends on the disease condition, and varies from 75-300 mg/day (approximately 1-4 mg/kg). Previous studies using the euglycemic-hyperinsulinemic clamp have determined that this dose of DEX induces whole-body insulin resistance within 4 h (20, 43).

Euglycemic-hyperinsulinemic clamp. Whole-animal insulin resistance was assessed using a euglycemic-hyperinsulinemic clamp, as described previously (43). This procedure involves the simultaneous intravenous infusion of insulin (HumulinR; 3 mU/min/kg) (to inhibit endogenous hepatic production) and d-glucose (50%) for 3 h; the quantity of exogenous glucose required to maintain euglycemia is a reflection of the net sensitivity of target tissues (mainly skeletal muscle) to insulin. At regular intervals, a small amount of blood taken from the tail vein was analyzed for glucose (using a glucometer; AccuSoftTM AdvantageTM). Glucose infusion rate (GIR) was adjusted accordingly to maintain euglycemia.

Tissue specific response to insulin. To assess tissue specific insulin resistance, skeletal muscle (gastrocnemius and soleus from hind leg) and heart from control and 4 h DEX treated
animals were evaluated for total and phospho IRS-1 and Akt, prior to and after 15 min of injecting a rapid acting insulin into the tail vein (8 U, HumulinR) using Western Blot (18, 23). At this early time point, there was no significant reduction in blood glucose with insulin.

*Mycocardial glycogen content.* Frozen cardiac tissue was powdered, weighed, incubated at 85 °C (10 min) with 1N NaOH, and followed by neutralization with 1N HCl. The neutralized sample was subjected to further acid hydrolysis with 6N HCl (85 °C, 2 h) (32). After neutralization with 5N NaOH, samples were subjected to glucose analysis using a glucokinase assay kit. Presence of cardiac glycogen was confirmed by visualization after periodic acid-Schiff (PAS) staining (21). Where indicated, animals were administered Mifepristone (RU-486, 20 mg/kg), one hour prior to DEX. After 4h of DEX, hearts were isolated, frozen and powdered tissue used to measure cardiac glycogen. We have previously reported that phosphorylation of AMPK was inhibited by acute intralipid infusion (19). To determine the effect of AMPK inhibition on cardiac glycogen accumulation, animals were anaesthetized with sodium pentobarbital, and the left jugular vein cannulated. Intralipid (IL 5%; 1.2 ml/kg/h) or vehicle (saline) was then infused over a period of 6h, following which hearts were removed for determination of glycogen. In some animals, DEX was administered 2h after the IL infusion was initiated.

*Subcellular compartmentalization of GLUT4.* Membrane fractions were isolated by a previously described method using sucrose density gradient (14). With Western blot, identification of GLUT4 protein was done by using rabbit polyclonal GLUT4 as the primary and mouse anti-rabbit horseradish peroxidase as the secondary antibody. Na⁺-K⁺ ATPase was used as a plasma membrane marker.
Immunoprecipitation and Western blotting. Following DEX treatment, heart homogenates (500 µg of protein) were immunoprecipitated using a rabbit monoclonal total AS 160 antibody (3 h, 4°C). The immunocomplex was pulled down with protein A/G-sepharose for 3 h, separated and boiled for 5 min at 95 °C in Laemmli buffer, and subjected to SDS-PAGE. Western blotting using antibodies against AS160, PAS (Phospho (Ser/Thr) Akt substrate antibody, Phospho-Akt (Ser-473, Thr-308), total and Phospho-AMPKα (Thr-172) and Phospho-ACC (Ser-79) was then performed as described previously (2). Measuring the phospho form of AMPK, ACC, glycogen synthase and glycogen synthase kinase-3-β is a surrogate for estimation of their activities. This was done using Western blot. Reaction products were visualized using an ECL® detection kit, and quantified by densitometry.

Measurement of mRNA. mRNA levels were measured using quantitative real time PCR. cDNA was synthesized from 1 µg RNA and purified using a sample purification kit (QIAGEN). RNA levels were determined from standard curves generated for each primer. The sample run was carried out for 40 cycles. The oligonucleotide primers were as follows: GLUT4 mRNA, forward 5’-GGGCAAGGAACACAACAGT-3’, reverse 5’-TGGAGGGGAACAAGAAAGTG-3’; AMPK α1 mRNA, forward 5’-GCAGAG AGA TCC AGA ACC TG-3’, reverse 5”-CTC CTT TTC GTC CAA CCT TCC-3; AMPK α-2 mRNA, forward 5’-GCTGTGGGATCGCCAAATTAT-3’, reverse 5’-GCATCAGCAGAGTTG CAATA-3’. Sample amplifications were done with the help of a fluorescent SYBER green dye (Roche Applied Science) in a Roche Applied Science Light Cycler system. β-actin was used as an internal reference.

G-6-P content. G-6-P was determined in perchloric acid extracts of frozen ventricular tissue using a standard spectrophotometric technique (6, 26).
**Glucose uptake in cardiomyocytes.** Ventricular calcium-tolerant myocytes were prepared by a previously described procedure (41). Cells were plated on laminin-coated culture plates (60 mm; to a density of 300,000 cells/well). Glucose uptake was evaluated using radiolabeled 2-deoxyglucose (DOG) (9). Briefly, myocytes were incubated in a glucose-free DMEM containing 0.2% FA-free BSA and pyruvate (1.0 mM) as the energy source. DEX (100 nM) was then added to the incubation media for 20 min. A radioactive mixture (containing 5 µCi of 2-deoxy-[³H]glucose) was added to the plates, and incubation continued for another 10 min. Following removal of buffer, cardiomyocytes were washed with cold PBS (2x), lysed using NaOH, and lysates used to determine the radioactivity. 2-DOG uptake is expressed as nanomoles per milligram per min.

**Materials.** ECL® detection kit was purchased from Amersham Canada. Akt, phospho-Akt (Ser-273/Thr -308), AMPK, Phospho-AMPKα, glycogen synthase (GS), Phospho-GS, GSK-3-β, Phospho-GSK-3-β, AS160, PAS, Na⁺-K⁺ ATPase and GAPDH antibodies were obtained from Cell Signaling (Danvers, MA). GLUT4 antibody was purchased from Abcam Inc (Cambridge, MA). All other chemicals were obtained from Sigma Chemical.

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

Cardiac tissue has limited influence on whole-body insulin resistance induced by DEX. We have previously reported that injection of DEX for 4 h was not associated with either hyperinsulinemia or hyperglycemia (43). Nevertheless, using the euglycemic-hyperinsulinemic clamp, a direct measure of insulin sensitivity, DEX lowered the glucose infusion rate necessary to maintain euglycemia (Fig. 1A). We assessed the effects of DEX on the responses of skeletal muscle and cardiac tissue to insulin. In skeletal muscle, both basal and insulin stimulated phosphorylation of IRS-1 (Fig. 1C) and Akt (Fig. 1B) were reduced after 4 h of DEX. These effects were not observed in cardiac tissue, which demonstrated a normal response to insulin when IRS-1 (Fig. 1E) and Akt (Fig. 1D) phosphorylation were measured. Following 4h of DEX, total IRS-1 and Akt did not change in skeletal and cardiac muscle when compared to control (Fig. 1 B-D). Thus, following DEX, cardiac tissue has limited contribution towards the development of whole body insulin resistance.

Buildup of cardiac glycogen following DEX is coupled to GLUT4 translocation. Even though DEX did not impede cardiac insulin signaling, we have previously reported a decline in cardiac glucose oxidation following DEX (43). In the presence of lower glucose oxidation, we hypothesized that glucose entering into the heart would be converted into glycogen. Indeed, DEX induced an approximately 2-fold increase in the cardiac glycogen content as measured enzymatically (Fig. 2A) or by histochemical staining (Fig. 2B). This effect of DEX on glycogen was partially related to receptor activation as although RU-486 reduced the DEX induced increase in cardiac glycogen, the levels observed were still higher than control (CON 37.8±0.50, DEX 69.7±1.5, DEX+RU 44.6±2.1 μg/g dry weight; *P*<0.05). In addition to a reduction in glucose oxidation, it was unclear whether changes in glucose transport could also contribute
towards the accumulation of glycogen. Interestingly, DEX treated hearts exhibited a higher G-6-P content compared to control (CON 2.67±0.16, DEX 3.31±0.20 μmol/g dry weight; \(P<0.05\)). More importantly, after 4 h of DEX, measurement of GLUT4 at the plasma membrane revealed an excess presence of this transporter protein at this location (Fig. 3A). Usually, this observation is accompanied by a decrease in GLUT4 in the intracellular pool. However, in the present study, the DEX induced increase in plasma membrane GLUT4 was also accompanied by an increase in GLUT4 in the intracellular membrane fraction (Fig. 3B). This latter effect was well correlated to an increased GLUT4 mRNA (Fig. 3B, inset). DEX also increased glucose uptake directly in isolated cardiomyocytes (CON 4.2±0.85, DEX 9.86±1.74 nmol/mg/min; \(P<0.05\)).

**DEX augments both total and phosphorylated cardiac AMPK.** In addition to Akt signaling, cardiac GLUT4 translocation is also controlled by AMPK (45). In the presence of a normal Akt signal, we measured AMPK and report an increase in both total (Fig. 4C) and phosphorylated AMPK phosphorylation (Fig. 4A) following 4 h of DEX. This change in AMPK total protein was accompanied by a modest but insignificant increase in AMPK\(\alpha\)-2 (Fig. 4E), but a significant increase in AMPK\(\alpha\)-1 (Fig. 4D) gene expression. Once activated, AMPK phosphorylates and inactivates ACC, facilitating FA oxidation. We measured ACC phosphorylation as a measure of AMPK activity. ACC phosphorylation was significantly increased in DEX hearts compared to CON (Fig. 4B). To substantiate the role of AMPK in DEX induced accumulation of cardiac glycogen, IL was used to inhibit AMPK phosphorylation. IL lowered cardiac glycogen accumulation that is observed after DEX (CON 37.4±1.8, DEX 66.2±2.0, DEX+IL 45.9±0.7, μg/g dry weight; \(P<0.05\)).

*Phosphorylation of Akt substrate of 160 kDa (AS160) is mainly regulated by AMPK.* AS160 regulates GLUT4 translocation to the plasma membrane by retaining this transporter in
intracellular membranes, a function that is lost upon its phosphorylation (22). The major upstream regulators of AS160 phosphorylation include Akt and AMPK. Immunoprecipitation of AS160 followed by Western blotting demonstrated no change in Akt phosphorylation at Ser473 and Thr308 in DEX treated hearts (Fig. 5, A and B). However, there was a significant increase in AMPK phosphorylation at Thr172 (Fig. 5C), which correlated well with AS160 phosphorylation, as reflected by an increase in PAS (measure of phosphorylation at Ser and Thr sites of AS160) (Fig. 5D).

*Glycogen synthase undergoes robust dephosphorylation with acute administration of DEX.* In addition to the contributions by GLUT4 delivered glucose and reduction of glucose oxidation towards glycogen synthesis following DEX, enzymatic control of this stored polysaccharide is also an important factor that controls its accumulation. Glycogen synthase, the rate-limiting enzyme for glycogen synthesis, is activated upon dephosphorylation (26). In DEX treated hearts, there was a considerable reduction in the phosphorylation of glycogen synthase (Fig. 6, lower panel). The major upstream kinase that regulates glycogen synthase phosphorylation is GSK-3-β, whose activity in turn is reduced by its phosphorylation. As GSK-3-β phosphorylation was augmented (Fig. 6, upper panel), our data suggest that cardiac glycogen accumulation following DEX is also dependent on the phosphorylation states of these two rate-limiting enzymes.
DISCUSSION

Chronically, increased levels of endogenous glucocorticoids are known to cause Cushing’s syndrome, a condition that is characterized by obesity, insulin resistance, increased lipid mobilization, and hypertension (28, 58). Exogenous delivery of glucocorticoids as anti-inflammatory and immunosuppressive agents is also associated with myocardial failure when administered chronically (35). We attempted to examine the acute effects of DEX specifically related to cardiac metabolism, given the injurious effects that excess triglyceride (30) and glycogen (1) have on the heart. We reported an enlargement in the coronary LPL pool, with a subsequent increase in FA delivery and augmented cardiac TG accumulation (20, 42, 43). With carbohydrate metabolism, DEX promoted the expression of PDK4, which is known to inhibit PDH and pyruvate flux, and hence glucose oxidation (43). Under these circumstances, we proposed that glucose disposal occurred by its conversion to glycogen. In the present study, our data suggest that AMPK mediated glucose entry, combined with activation of glycogen synthase and the previously reported reduction in glucose oxidation (43), act together to promote glycogen storage.

With insulin resistance, metabolism in multiple organ systems including the heart is altered, which is believed to be an important factor in increased morbidity and mortality (5, 7). Measurement of insulin sensitivity using the euglycemic-hyperinsulinemic clamp revealed the presence of whole body insulin resistance with acute DEX treatment, suggesting that any change in cardiac metabolism that is exhibited by DEX may be a consequence of the prevalent reduction of insulin sensitivity. Nevertheless, we also determined the responses of the skeletal muscle and the cardiac tissue to insulin and unlike skeletal muscle, cardiac tissue responded normally to insulin. Under these conditions, it is possible that the effects of DEX on cardiac metabolism are
also related to direct effects of this glucocorticoid on the heart. Glucocorticoids work through multiple mechanisms to bring about their desired effects. These include a specific cytosolic receptor mediated event, and specific and non-specific membrane bound receptor mediated effects through which glucocorticoids bring about genomic and non-genomic outcomes (8, 10, 11). Given our recently reported observation that DEX activated AMPK (a metabolic switch that plays an important role in maintaining cellular energy homeostasis) phosphorylation in isolated cardiomyocytes within 60 min (20), and the use of RU486 in our current study to reduce cardiac glycogen accumulation, our data suggest that the effects of DEX on cardiac metabolism in vivo may be linked to its direct impact on the heart. At present the mechanism by which DEX increases phosphorylation of AMPK is unknown and could include changes in the AMP/ATP ratio (17) or activation of a Ca\(^{2+}\)/calmodulin-dependent protein kinase kinase (49).

AMPK is known to inhibit anabolic and promote catabolic processes leading to conservation of cellular ATP levels. It does so through multiple mechanisms including increased delivery and metabolism of both glucose and fatty acids (16, 29, 45). In this context, and related to glycogen, AMPK is known to inhibit the formation of this storage form of glucose in skeletal muscle (40, 50, 56). In the heart, both low flow ischemia (37) and exercise (34) increase AMPK activity, that is correlated to a reduction in glycogen content. Unexpectedly, our results suggest that accumulation of glycogen with DEX was associated with increased phosphorylation of AMPK, a phenomenon that is observed in transgenic models of AMPK activation. In these models, mutation of regulatory gamma sub units (\(\gamma1R70Q\), \(\gamma2N488I\), \(\gamma2R531G\)) increased AMPK activation and facilitated glycogen accumulation (1, 12, 15, 27). It is possible that the intrinsic property of DEX to block glucose oxidation counteracts the ability of AMPK to prevent the storage of this carbohydrate. An additional explanation for this occurrence could be related to
the effect of AMPK to decrease malonyl CoA, thereby removing its inhibition on CPT-1 and promoting fatty acid oxidation (42). The resultant blockade of glucose oxidation, as suggested by the Randle hypothesis, could explain the increase in glycogen storage (44). Whatever the mechanism, our data suggests that with carbohydrate metabolism, AMPK phosphorylation in the presence of DEX is associated with an anabolic function.

Under basal conditions, only a small percentage of GLUT4 resides at the plasma membrane, with the remaining fraction being redistributed in endosomal recycling and GLUT4 storage compartments (25, 46). Translocation of this transporter protein from the intracellular pool to the plasma membrane is regulated largely by the PI3 kinase-Akt pathway, in addition to AMPK. These kinases, by phosphorylating and inactivating AS160 (which has multiple phosphorylation motifs at the serine and threonine residues), removes the constraint that AS160 has on GLUT4, allowing the trafficking of this transporter to the membrane surface (22, 51, 52). Indeed, the increase in plasma membrane GLUT4 with DEX was well correlated to an increase in PAS, a measure of phosphorylation at Ser and Thr sites of AS160. To determine the contribution of Akt and AMPK towards this increased AS160 phosphorylation, we used immunoprecipitation to pull down the trimeric complex containing phospho-Akt, phospho-AMPK and AS-160, and immunoblotted for the respective phosphoproteins. The present study suggests that following DEX, in the presence of normal insulin signaling, AMPK mediated phosphorylation of AS160 is the predominant factor that controls cardiac GLUT4 movement.

Increased substrate availability plays an important role in glycogen synthesis. Thus, the increase in glucose uptake and G-6-P following DEX could be an important contributing feature in glycogen accumulation. Other factors include alterations in glucose oxidation (43) and changes in enzymes that control glycogen synthesis or breakdown. In skeletal muscle, glycogen
regulation by GSK-3-β and GS is well established. AMPK is known to phosphorylate GS making it prone to further phosphorylation by casein kinase-1 and GSK-3-β leading to its inactivation (40, 50). The relationship between AMPK, GSK-3-β and glycogen has yet to be resolved in the heart. For example, Mora et al. showed that cardiac glycogen levels are regulated independently of insulin's ability to phosphorylate GSK-3-β and stimulate GS (33). In the present study, phosphorylation of GS after DEX administration decreased, an effect closely associated with an increase in GSK-3-β phosphorylation. This effect of DEX on GS in the presence of increased AMPK phosphorylation was uncommon. As AMPK was recently shown to activate GS through a GSK-3-β dependent pathway in HepG2 cells (54), our data suggest that through multiple mechanisms, AMPK activation with DEX is associated with glycogen storage.

In summary, acute DEX administration was associated with significant accumulation of myocardial glycogen. One way by which this process is made possible is through an AMPK mediated augmentation of glucose uptake, coupled to its regulation of glycogen synthase, a key enzyme involved in glycogen synthesis. Given the contribution of glycogen storage in eliciting cardiac hypertrophy, ventricular arrhythmias and conduction system disorders, results from the present study could help in limiting the deleterious effects of long term excesses in endogenous or exogenous glucocorticoids on the heart.

**Limitation of the study.** When examining the effects of AMPK inhibition on the heart, studies have used dominant negative AMPK overexpression, or SiRNA to decrease AMPK activity. One limitation of this study is the use of intralipid to inhibit AMPK. This is because IL, in addition to its effects on AMPK, is also known to effect insulin signaling, and hence glucose metabolism, by decreasing IRS (tyrosine) and Akt (serine) phosphorylation in liver and skeletal
muscle (4, 38). Thus, the decrease in cardiac glycogen following DEX+intralipid could be a result of both AMPK inhibition and a reduced insulin signaling.
ACKNOWLEDGEMENTS

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FIGURE LEGENDS

FIG. 1. Dexamethasone effects on whole body and tissue specific insulin resistance. Following injection of vehicle or DEX for 4 h, whole-body insulin resistance was assessed using a euglycemic-hyperinsulinemic clamp (A). Insulin (HumulinR; 3 mU/min/kg) and d-glucose (50%) were continuously delivered (by a cannula inserted into the left jugular vein) for 3 h. At regular intervals, blood samples taken from the tail vein were analyzed for glucose using a glucometer. Glucose infusion rate (GIR) was adjusted accordingly to maintain euglycemia. To determine tissue specific insulin resistance, skeletal muscle (gastrocnemius and soleus from hind leg) (B and C) and heart (D and E) from control and 4 h DEX treated animals were evaluated for phospho IRS-1 (Tyr-989) and Akt (Ser-473) and total IRS-1 and Akt, prior to and after 10 min of injecting rapid acting insulin into the tail vein (8 U, HumulinR) using Western Blot. Results are the means ± SE of 3-5 rats in each group. *Significantly different from control; †Significantly different from all other groups; ‡Significantly different from control given insulin; §Significantly different from respective basal (without insulin), P < 0.05.

FIG. 2. Cardiac glycogen accumulation following dexamethasone. Cardiac glycogen was determined: a) as glucose residues using a glucokinase method after acid hydrolysis, and b) by histochemical analysis of cross sections of ventricular tissue using PAS-staining (B). Results are the means ± SE of 5 rats in each group. *Significantly different from control, P < 0.05.

FIG. 3. Subcellular localization of GLUT4 protein. Following DEX, heart homogenates were prepared and subjected to plasma (A) and intracellular (B) membrane separation using sucrose gradient. Identification of GLUT4 protein was carried out using rabbit polyclonal GLUT4 as the primary and mouse anti-rabbit horseradish peroxidase as the secondary antibody. Na⁺-K⁺ ATPase was used as a plasma membrane marker. Quantitative real-time PCR enabled
determination of GLUT4 mRNA in hearts from control and DEX treated animals. Results are the means ± SE of 3-5 rats in each group. *Significantly different from control, \( P < 0.05 \). CON-control; DEX-dexamethasone; IM-intracellular membrane; PM-plasma membrane.

FIG. 4. Changes in AMPK protein (total and phosphorylated) and gene expressions in hearts isolated from DEX treated animals. Following DEX, total (C) and phosphorylated (A) AMPK-\( \alpha \) and phosphorylated ACC (C) were measured using rabbit AMPK-\( \alpha \), phospho-AMPK (Thr 172) and Phospho-ACC (Ser-79) antibodies respectively. AMPK-\( \alpha 1 \) and AMPK-\( \alpha 2 \) gene expressions were measured using quantitative real-time PCR (D and E). Results are the means ± SE of 3-5 rats in each group. *Significantly different from untreated control, \( P < 0.05 \).

FIG. 5. AMPK regulation of Akt substrate of 160 kDa (AS160). Animals were treated with DEX, and at 4 h, hearts from control and DEX treated animals were isolated. To examine the association between AS160, Akt and AMPK, AS160 was first immunoprecipitated using a total AS160 antibody. The immunocomplex was then immunoblotted with anti phospho-Akt (Ser473, A; Thr308, B), anti phospho-AMPK (Thr172, C), anti PAS (Ser/Thr, D), and anti-AS160 (inset). Results are the means ± SE of 3-5 rats in each group. *Significantly different from untreated control, \( P < 0.05 \). IP-immunoprecipitation.

FIG. 6. Enzyme regulation of cardiac glycogen synthesis subsequent to administration of DEX. Animals were killed 4 h subsequent to DEX injection and hearts isolated for determination of phospho (Ser 9) (top panel) and total GSK-3-\( \beta \) and phospho (Ser 641) (bottom panel) and total glycogen synthase (GS) using Western Blot. Results are the means ± SE of 3-5 rats in each group. *Significantly different from untreated control, \( P < 0.05 \).

FIG. 7. Schematic mechanism of how DEX regulates cardiac glycogen through AMPK. In the presence of intact insulin signaling (normal Akt function), DEX, through its effects in enabling
AMPK phosphorylation, regulates GLUT4 translocation by its action on AS160. The subsequent influx of glucose augments the cardiac content of G-6-P. This effect, combined with phosphorylation and inactivation of GSK-3-β, together with dephosphorylation and activation of glycogen synthase, acts in unison to increase cardiac glycogen content.
REFERENCES


Fig. 2

**A**

Cardiac glycogen (μg/g dry wt of tissue)

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**B**

Micrographs of cardiac tissue for CON and DEX groups at 5x and 10x magnification.
**Fig. 5**

**A**

Akt Phosphorylation-Ser473 (AU)

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Akt Phosphorylation-Thr308 (AU)

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**C**

AMPK Phosphorylation-Thr172 (AU)

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* indicates statistical significance.