Dexamethasone-Induced Impairment in Skeletal Muscle Glucose Transport Is Not Reversed by Inhibition of Free Fatty Acid Oxidation

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Our previous studies suggested a possible role for the glucose–free fatty acid (FFA) cycle, ie, preferential utilization of FFA by muscle at the expense of glucose, in dexamethasone (DEX)-induced insulin resistance. To determine whether this resistance could be reversed by inhibiting FFA utilization, we used etomoxir, a potent inhibitor of mitochondrial FFA oxidation. Male Sprague-Dawley rats were injected subcutaneously with 1 mg/kg DEX or the vehicle every other day for 10 days, and half of each group was administered 10 mg/kg etomoxir by gavage once per day and 1 hour before the experiment. As expected, etomoxir treatment increased serum FFA levels and inhibited FFA oxidation by diaphragm in vitro. Administration of etomoxir decreased serum glucose and insulin concentrations under basal conditions in both control and DEX-treated animals, implying enhanced insulin sensitivity. DEX treatment significantly increased endogenous glucose production and decreased whole-body glucose disposal, as well as 2-deoxyglucose (2-DG) uptake by skeletal muscle during euglycemic-hyperinsulinemic clamps. Administration of etomoxir led to small but significant increases in glucose disposal rates of both control (14%) and DEX (23%) groups, but had no effect on residual endogenous glucose production. Thus, DEX-induced insulin resistance was marginally ameliorated but not completely reversed by etomoxir. Depressed 2-DG uptake by individual muscle tissues observed in the present study in conjunction with the absence of free intracellular glucose in muscle tissue following glucose-insulin infusion strongly suggests that the primary defect in glucose metabolism is at the level of transport. Neither overall abundance of the insulin-sensitive glucose transporter (GLUT-4) in skeletal muscle nor its distribution between intracellular stores and plasma membrane were modified by DEX treatment, either under basal conditions or in response to acute insulin stimulus. These results suggest a defect(s) in the inherent activity of plasma membrane-bound GLUT-4 as the likely mechanism for DEX-induced insulin resistance.

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IT IS WELL-DOCUMENTED that glucocorticoid excess decreases glucose utilization and stimulates insulin and glucagon secretion, lipolysis, proteolysis, and hepatic glucose production (reviewed in McMahon et al[1]). Although glucocorticoids can modulate insulin action at both binding and postbinding steps, most of the evidence in the literature suggests a postbinding defect(s) as the major determinant of glucocorticoid-induced insulin resistance.[2] In a previous study,[3] we demonstrated an impairment in insulin-mediated glucose uptake in vivo in rats treated long-term with dexamethasone (DEX). The antilipolytic action of insulin was blunted both in vivo and in vitro. Oxidation of free fatty acids (FFA) by diaphragm removed from DEX-treated animals was enhanced in vitro. Decreased glucose disappearance rates in DEX-treated rats, as measured by glucose-insulin tolerance tests, were restored to normal values by a prior decrease of FFA levels with nicotinic acid, an antilipolytic agent. Collectively, these data suggested a possible role for the glucose-FFA cycle,[4,5] ie, preferential utilization of FFA by muscle in vivo at the expense of glucose, in DEX-induced insulin resistance.[3] In the present investigation, evidence for this hypothesis was sought by examining the effect of inhibiting FFA oxidation on insulin-mediated glucose metabolism in DEX-treated rats. For this purpose, we chose etomoxir, a newly described and potent inhibitor of carnitine palmitoyltransferase I, a key enzyme for the transport of long-chain acyl coenzyme A into the mitochondria and their metabolism.[6,7] Furthermore, the possibility that reduced glucose uptake in DEX-treated rats is a reflection of diminished levels or impaired translocation of the insulin-sensitive glucose transporter (GLUT-4) in skeletal muscle was evaluated.

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MATERIALS AND METHODS

Animals and Treatment

Animal studies were performed in accordance with the *National Institutes of Health Guide for the Care and Use of Laboratory Animals.* Male Sprague-Dawley rats weighing 200 to 220 g obtained from Bantin & Kingman (Fremont, CA) were housed at 21° ± 2°C with 12-hour alternating light-dark cycles and fed standard laboratory chow. Rats were injected subcutaneously with either 1 mg/kg DEX-phosphate (LyphoMed, Rosemont, IL) or saline every other day for five doses. Half of each group were administered by gavage 10 mg/kg etomoxir (ethyl-2-[6-(4-chlorophenoxy)-hexyl]oxirane-2-carboxylate), obtained from Dr H.P.O. Wolf (Byk Gulden Pharmaceutical, Konstanz, Germany) and suspended in 0.5% methylcellulose, once every day and 1 hour before the experiment, and the other half of each group received vehicle. Thus, there were four groups of animals: control, etomoxir-treated, DEX, and DEX plus etomoxir–treated. The animals were killed 2 days after the last injection of DEX. Food intake of animals receiving both DEX and etomoxir was determined in preliminary studies, and the other three groups were pair-fed with this group.

Whole-Body Glucose Utilization Study

A constant tracer infusion procedure was used for measuring glucose turnover as described previously.[8] After an overnight fast, the animals were anesthetized with Inactin (100 mg/kg intraperitoneally; Byk Gulden Pharmaceutical, Konstanz, Germany), and through a ventral midline neck incision, the left carotid artery and right jugular vein were catheterized with PE50 tubing for blood sampling and infusion, respectively. Infusion lines from different

pumps converged into a small manifold assembly connected to the jugular catheter (4-in long PE50 tubing, 0.01 in ID) through a 30-gauge needle. Since the void volume of the assembly unit and catheter was less than 20 μL, the response of blood glucose to changes in glucose infusion rate was virtually instantaneous. Animals were kept warm by an isothermal pad and given a tracheotomy with a small plastic T-tube connected to low-flow oxygen. After obtaining a baseline blood sample, a primed (0.2 mL)-continuous (0.02 mL/min) infusion of (3-^{3}H)glucose (10 μCi/mL) was started to measure glucose turnover rate. A primed-continuous infusion of porcine insulin (5 mU · kg^{-1} · min^{-1}) was begun simultaneously to acutely increase and maintain plasma insulin concentration at submaximal level while infusing somatostatin (1.5 μg · kg^{-1} · min^{-1}) to inhibit endogenous insulin secretion. Plasma glucose concentration was kept constant at 140 mg/dL by determining plasma glucose every 7 to 8 minutes and empirically adjusting the infusion rate of a 20% glucose solution.

Plasma samples for glucose specific activity and insulin concentrations were obtained at 10-minute intervals between 60 and 90 minutes after the start of the infusion, and the mean of these values defined steady-state plasma levels. FFA levels were measured in the 90-minute samples. The total amount of blood withdrawn including the final sample was 1.7 mL, which is less than 10% of the blood volume[9] of animals studied. The rate of glucose turnover was calculated from the non–steady-state derivation of Steele's equation,[10] which takes into account fluctuations in plasma glucose specific activity. The rate of appearance is equal to the rate of glucose utilization under clamp conditions, and the difference between the utilization rate and the exogenous glucose infusion rate represents residual endogenous glucose production.

Glucose turnover is known to be reduced by 20% to 30% under halothane[11] and pentobarbital[12] anesthesia. A similar decrease probably occurs in Inactin-treated rats, although to our knowledge there is no published report on the effect of Inactin on glucose metabolism. It has been shown that a 30- to 40-minute equilibration period after induction of anesthesia is necessary to establish a new steady state.[12] This criterion was routinely fulfilled in our experiments, so the observed effects of DEX and/or etomoxir refer to the new steady-state conditions under Inactin anesthesia. Furthermore, even though anesthesia may reduce glucose turnover by 20% to 30%, differences among the four groups were due to the various treatments, since all animals were anesthetized.

Measurement of In Vivo Tissue Glucose Utilization Index

Euglycemic-hyperinsulinemic clamps were performed as described earlier, except that 3-^{3}H-glucose infusion was omitted. Sixty minutes after the start of the clamp, a tracer dose of the non-metabolizable glucose analog, 2-deoxy-(1-^{3}H)-D-glucose ([^{3}H-2DG] New England Nuclear, Boston, MA), was injected as a bolus via the carotid artery (30 μCi per rat). Blood samples were collected at 2, 5, 10, 20, 30, and 45 minutes after injection for determination of plasma glucose and plasma tracer concentrations.[13,14] Plasma insulin level was measured in samples obtained before administration of ^{3}H-2DG and at the end of the study. Anesthesia was then deepened, and individual muscle tissues were rapidly dissected and frozen in liquid N_2 before determination of ^{3}H-2DG-6-phosphate content.[13,14]

Glucose utilization in individual tissues was calculated by a method extensively discussed and validated previously.[13-15] The amount of ^{3}H-2DG-6-phosphate per milligram of tissue (dpm/mg tissue) was divided by the integral of the ratio of the concentrations of ^{3}H-2DG/glucose (dpm/mg glucose) measured in arterial plasma during the last 45 minutes. Since the values were not corrected by the discrimination factor (lumped constant) for 2DG in glucose metabolic pathways,[13,14] the results represent an index rather than absolute values of glucose utilization. Since glucose and 2DG are transported by the same carrier in skeletal muscle,[16,17] hexokinase efficiently phosphorylates all glucose transported across the cell membrane under most conditions,[18] and 2-DG uptake is linear for 60 minutes,[13] it is valid to equate ^{3}H-2DG uptake with glucose transport.

FFA Oxidation by Diaphragms In Vitro

To measure rates of oxidation of FFA by muscle tissue, quarter-diaphragms removed from ad libitum–fed rats were weighed and incubated in duplicate for each rat in 2 mL Krebs-Ringer bicarbonate buffer, pH 7.4, at 37°C for 2 hours under 95% O_2–5% CO_2. The incubation medium contained 1 mg/mL glucose, 0.1 μCi (5.26 nmol/mL) ^{14}C-palmitate (which had been complexed to 5% fatty acid–poor albumin), and 1 mg/mL fatty acid–poor serum albumin. The reaction was terminated by addition of H_2SO_4, and $^{14}CO_2$ released from ^{14}C-palmitate oxidation was trapped in an NCS tissue solubilizer (Amersham, Arlington Heights, IL) and counted. The amount of FFA oxidized was calculated from specific activity of ^{14}C-palmitate in the medium.

To determine FFA levels in diaphragms, tissue pieces weighing 10 to 20 mg were homogenized in 6 mL chloroform:heptane: methanol (25:25:1 vol/vol) and FFA concentrations in the extract were analyzed.[19]

Preparation of Skeletal Muscle Membrane Fractions

Ad libitum–fed control and DEX-treated rats were anesthetized with Inactin, and a catheter was placed in the carotid artery. After obtaining a baseline blood sample, insulin (1 U/kg) was administered via the artery, and 15 minutes later, a blood sample was collected. Anesthesia was deepened, and muscles (gastrocnemius, soleus, and biceps femoris) from one leg were removed rapidly and placed in ice-cold homogenizing buffer containing 0.25 mol/L sucrose, 20 mmol/L HEPES, pH 7.6, 0.2 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride, and 5 mmol/L NaN_3. Different membrane fractions were prepared simultaneously from this fresh tissue using published methods.[20,21] The level and subcellular distribution of GLUT-4 were also determined in the basal state in additional subsets of control and DEX-treated rats.

Muscle was trimmed free of fat and connective tissue, weighed, minced, disrupted by polytron homogenizer (Brinkman PT-10, Westbury, NY) with two 10-second bursts at setting 8, and further homogenized with a Potter-Elvehjem tissue grinder. The homogenate was filtered through a layer of 40-mesh wire. An aliquot of the homogenate was subjected to sequential centrifugation at 1,000 × *g* for 10 minutes and 9,000 × *g* for 10 minutes. The resulting supernatant was centrifuged at 227,000 × *g* for 1 hour to pellet the total membrane (plasma membrane and microsome) fraction. The remaining homogenate was centrifuged at 48,000 × *g* for 20 minutes and the resulting supernatant at 227,000 × *g* for 1 hour to pellet the intracellular membrane (microsome) fraction. Plasma membranes were prepared by resuspending the pellet of the 48,000 × *g* centrifugation in homogenizing buffer. Concentrated KCl and Na pyrophosphate were added to the suspension to a final concentration of 300 and 25 mmol/L, respectively, to solubilize contractile proteins, and after vigorous mixing the solution was centrifuged at 227,000 × *g* for 1 hour. The pellet was suspended in 34% sucrose, layered in a discontinuous gradient (45%, 38%, 34%, 32%, 30%, 27%, and 12% sucrose), and centrifuged for 16 hours at 68,000 × *g*. Fractions were collected from the gradient at the 12%/27% and 27%/30% sucrose interfaces, combined, diluted with water, and centrifuged at 227,000 × *g* for 1 hour to yield the plasma membrane pellet. The various membrane fractions were resuspended in homogenizing buffer and stored at −80°C.

GLUT-4 Protein Analysis by Western Blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of membrane proteins was performed on 12% acrylamide gel.[22] The samples contained 0.12 mol/L β-mercaptoethanol and were not heated before electrophoresis. Aliquots (10 μg protein) of the rat heart membrane pool were included in each gel to normalize results from different gels. Prestained molecular size markers were included in parallel lanes for determination of apparent molecular weight. Electrophoresis and electrophoretic transfer of proteins to nitrocellulose membranes were performed with materials and apparatus from Bio-Rad Laboratories (Richmond, CA). The blots were washed with agitation for 5 minutes in TBS-Triton (150 mmol/L NaCl, 10 mmol/L Tris hydrochloride, pH 7.4, and 0.1% Triton X-100) and then blocked with TBS-Triton containing 5% nonfat dry milk for 1 hour. The blots were then incubated overnight in TBS-Triton-milk containing antibody against GLUT-4 (East Acres Biologicals, Southbridge, MA) at a dilution of 1:500. The blots were washed for 5 minutes six times in TBS-Triton and incubated for 1 hour in TBS-Triton-milk containing 0.2 μCi/mL (^{125}I)protein A (New England Nuclear, Boston, MA). After six more washes (5 min/wash) in TBS-Triton, (^{125}I)protein A bound to immunoglobulin was detected by autoradiography and quantified either by counting in a gamma counter in the basal state or by an image analyzer (Ambis, San Diego, CA) after insulin stimulation. Bound radioactivity was linear with the amount of protein loaded per lane.

Analytical Methods

Glucose concentrations in serum were determined by a Beckman glucose analyzer (Irvine, CA). Serum insulin level was measured by a double-antibody technique using a kit supplied by ICN (Irvine, CA). Serum (3-^{3}H)-glucose radioactivity was determined in supernatants after precipitating protein with perchloric acid and removing ^3H_2O by evaporation. FFA levels in serum and muscle extract were determined by the colorimetric method described by Falholt et al.[19] Protein content of membrane fractions was determined by the method of Bradford.[23] K^+-stimulated *p*-nitrophenol phosphatase (K*p*NPPase), which cofractionates with but is less labile than Na^+,K^+-ATPase, is considered an appropriate plasma membrane marker specific for sarcolemma. This activity was assayed with and without 20 mmol/L KCl[20] to determine purity of the various membrane fractions.

Materials

The following items were obtained from the indicated sources: porcine insulin from Sigma (St Louis, MO), somatostatin from Bachem (Torrance, CA), (3-^{3}H)-glucose and (^{14}C)-palmitate from ICN (Irvine, CA), and Inactin and etomoxir from Byk-Gulden (Konstanz, Germany). All other chemicals were of reagent grade and obtained from standard suppliers.

Statistics

Statistical analysis were performed either by multiple ANOVA or by Student's *t* test, as applicable, and $P \leq .05$ was considered significant.

RESULTS

Basal Parameters

DEX treatment resulted in weight loss and elevated levels of plasma glucose and insulin (Table 1). On the other hand, etomoxir treatment significantly decreased circulating concentrations of both glucose and insulin. However, DEX-treated animals receiving etomoxir had higher plasma glucose and insulin levels versus control animals receiving etomoxir, suggesting the persistence of insulin resistance due to DEX treatment.

Table 1. Physiological Characteristics of Control and DEX-Treated Rats With and Without Etomoxir

Characteristic	Control		DEX-Treated	
	−Etomoxir	+Etomoxir	−Etomoxir	+Etomoxir
Weight gain (g/d)*	1.8 ± 0.4	2.0 ± 0.3	−0.5 ± 0.2	−1.5 ± 0.4
Food intake (g/d)	26.3 ± 0.5	25.8 ± 0.4	26.6 ± 0.5	25.4 ± 0.5
Plasma glucose (mg/dL)*†	138 ± 4.1	110 ± 6.5	164 ± 2.9	132 ± 6.3
Plasma insulin (μU/mL)*†	39.4 ± 3.0	21.4 ± 2.9	68.4 ± 4.0	35.9 ± 6.0

NOTE. Values are the mean ± SE of 17 to 22 animals per group.
*Significant effect of DEX ($P < .001$ by multiple ANOVA).
†Significant effect of etomoxir ($P < .001$ by multiple ANOVA).

To ensure that the protocol of oral administration of etomoxir used in the present study was effective in inhibiting FFA oxidation, the rate of oxidation of ^{14}C-palmitate in vitro by diaphragms isolated from the four groups of rats was determined. Etomoxir treatment decreased FFA oxidation in muscles from both control and DEX groups (Table 2). DEX treatment by itself increased FFA oxidation, confirming our previous observations.[3] To ascertain that these results were not due to different intracellular levels of FFA causing different tissue-specific activities, which in turn can lead to spurious increase or decrease in ^{14}C-palmitate oxidation, we measured FFA levels in quarter-diaphragms. In animals not receiving etomoxir, tissue FFA level was higher in the DEX group, resulting in lower specific activity of the FFA pool; however, specific activity would have to be higher to explain the enhanced rate of FFA oxidation by muscles removed from the DEX group. Etomoxir treatment had no effect on tissue FFA level in control rats, but decreased the level in the DEX group; thus, specific activity of the FFA pool was either unaltered or increased, whereas only lower specific activity would be compatible with decreased FFA oxidation in etomoxir-treated rats. Differences in tissue FFA levels therefore cannot account for the effect of DEX or etomoxir on FFA oxidation by diaphragm. In agreement with the inhibition of FFA oxidation in vitro, etomoxir administration significantly enhanced basal serum FFA levels in both control and DEX-treated groups (Fig 1).

Table 2. Oxidation of FFA by Diaphragm In Vitro

Parameter	Control		DEX-Treated	
	−Etomoxir	+Etomoxir	−Etomoxir	+Etomoxir
^{14}C-palmitate oxidized (nmol/g tissue/2 h)*	5.4 ± 0.4	4.4 ± 0.2	6.6 ± 0.3†	3.7 ± 0.2
Tissue FFA level (mEq/g)	3.1 ± 0.2	3.1 ± 0.1	4.1 ± 0.4†	3.0 ± 0.2‡

NOTE. Values are the mean ± SE of 7 animals per group. All animals were fed ad libitum until tissue removal.
*Significant effect of etomoxir ($P < .001$) by multiple ANOVA.
†$P < .05$ *v* control rats not receiving etomoxir (Student's *t* test).
‡$P < .05$ *v* DEX group not receiving etomoxir (Student's *t* test).

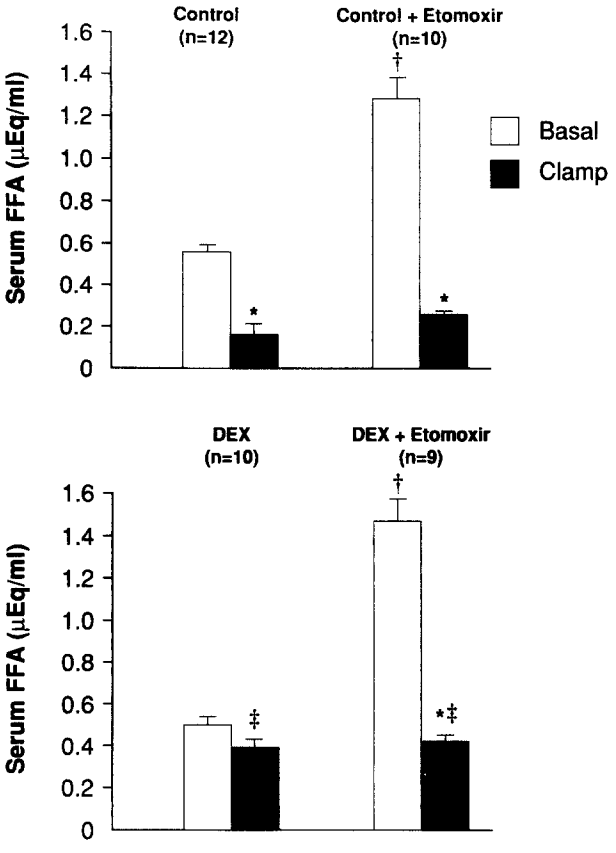

Fig 1. Serum FFA levels under basal and euglycemic-hyperinsulinemic clamp conditions. Values are the mean ± SE of the number of animals shown in parentheses. *$P < .001$ *v* corresponding basal value by paired *t* test. †Significant effect of etomoxir treatment on basal FFA concentration ($P < .001$) by multiple ANOVA. ‡Significant effect of DEX on clamp FFA levels *v* respective controls ($P < .001$) by Student's *t* test.

Insulin-Stimulated Metabolism

Effects of etomoxir administration on insulin-mediated whole-body glucose metabolism in control and DEX-treated rats are illustrated in Fig 2. Steady-state plasma concentrations of glucose were comparable in four groups of animals studied. However, steady-state plasma insulin levels were higher in the DEX group with or without etomoxir treatment. DEX treatment decreased the glucose disposal rate and the amount of exogenous glucose needed to maintain euglycemia and increased the glucose production rate despite elevated plasma insulin levels. Administration of etomoxir increased both the glucose disposal rate (22.6 ± 1.1 to 25.8 ± 1.0 mg · kg^{-1} · min^{-1} in controls and 14.5 ± 0.7 to 17.8 ± 0.7 in DEX group) and the amount of glucose infusate necessary to maintain euglycemia, but had no effect on residual glucose production. Although etomoxir treatment increased glucose disposal of the DEX group, the rate was still considerably lower than in control rats with or without etomoxir treatment. Thus, DEX-induced insulin resistance was only partially reversed by etomoxir.

The antilipolytic effect of insulin during the clamp study is depicted in Fig 1. Clamp FFA values were markedly reduced from baseline in controls, controls receiving etomoxir, and the DEX group receiving etomoxir, but not in the DEX group. Blunting of the antilipolytic action of insulin in the DEX group confirms our previous observations using the pancreatic suppression test.[3] Clamp FFA levels in the DEX group with or without etomoxir treatment were increased as compared with respective controls.

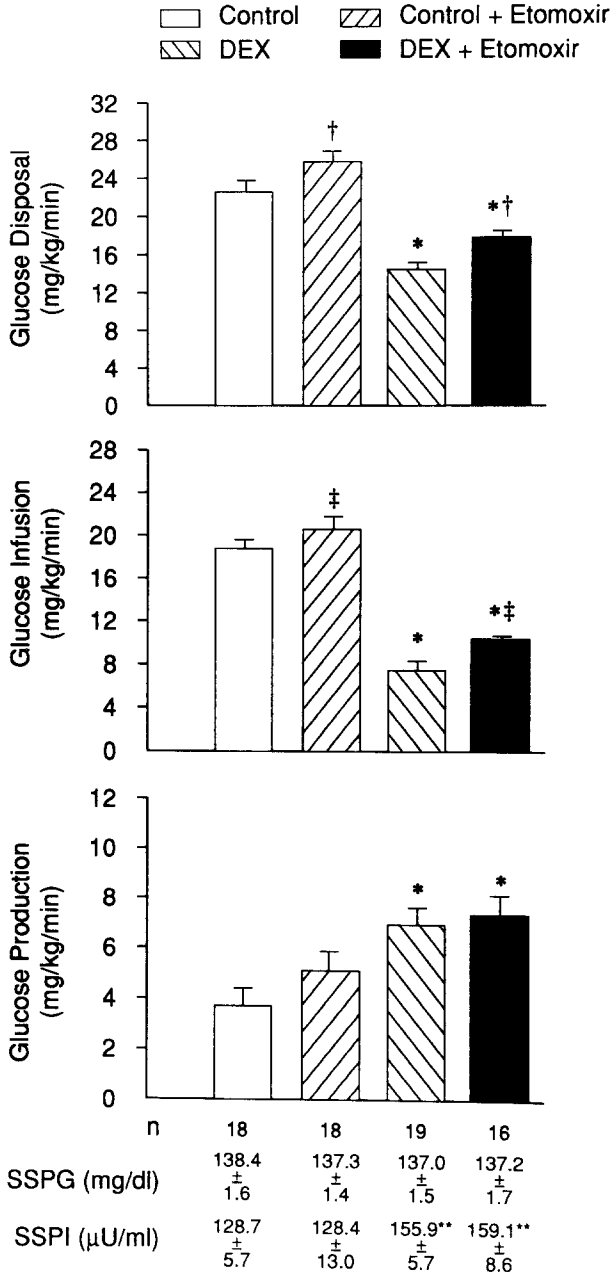

Fig 2. Effect of DEX and/or etomoxir treatment on glucose kinetics during insulin clamp. SSPG and SSPI, steady-state plasma glucose and insulin concentrations, respectively. Values represent the mean ± SE of the indicated number of animals. Statistical analysis was performed by multiple ANOVA. †Highly significant effect of etomoxir on glucose disposal ($P < .001$). ‡Significant effect of etomoxir on glucose infusion rate ($P < .03$). *Highly significant effect of DEX ($P < .001$). **Significant effect of DEX ($P < .05$).

Glucose Utilization by Skeletal Muscle In Vivo

Since skeletal muscle, representing 35% to 40% of total body weight in the rat,[24] is responsible for the majority of insulin-mediated glucose metabolism,[25,26] the reduction in whole-body glucose disposal due to DEX treatment noted earlier most likely reflects decreased glucose metabolism in skeletal muscle. To confirm this, we used a technique that combines a euglycemic-hyperinsulinemic clamp with the uptake of ^{3}H-2DG, a nonmetabolizable glucose analog, into individual muscles under steady-state conditions. The results summarized in Table 3 clearly show that DEX treatment significantly reduced insulin-mediated glucose transport in four individual muscles studied, ie, biceps, gastrocnemius, soleus, and plantaris, despite elevated steady-state plasma insulin levels (183.5 ± 17.6 *v* 132 ± 10.3 μU/mL, $P < .05$); steady-state plasma glucose levels were similar in the two groups (163.9 ± 9.8 *v* 146.7 ± 1.7 mg/dL).

GLUT-4 Protein Analysis in Skeletal Muscle Membranes

We next sought to determine whether decreased GLUT-4 content and/or defective translocation of the transporter were responsible for DEX-induced impairment in glucose transport. To that end, control and DEX-treated rats were treated short-term with insulin, and skeletal muscle membrane fractions were prepared. (In our hands, GLUT-4 content of muscle plasma membranes is doubled following insulin stimulation in control rats[22].) Basal glucose concentrations were similar in the two groups, but basal insulin levels were higher in the DEX group (Table 4). Glucose levels following insulin injection were higher in the DEX group, although insulin concentrations did not differ between the two groups. Thus, insulin resistance was evident in DEX-treated rats whose muscles were processed for GLUT-4 analysis 15 minutes after receiving 1 U/kg insulin via the artery.

K*p*NPPase activity, a plasma membrane marker, was greatly enriched (40- to 50-fold) in the plasma membrane fraction in comparison to the crude homogenate (Table 5). In addition, K*p*NPPase specific activity was approximately 11- to 13-fold greater in plasma membranes than in intracellular membranes. These data are in general agreement with published values in the literature. [20,21,27] No significant differences in marker enzyme specific activity or enrichment were observed in plasma membranes or intracellular membranes from the two groups of animals. There was also no difference in protein content of either the crude homogenate (149 ± 9.3 *v* 165 ± 10.1 mg/g tissue, n = 8) or the plasma membrane fraction (48.8 ± 8.2 *v* 56.4 ± 8.6 μg/g tissue, n = 8) between control and DEX groups.

Proteins of the different membrane fractions were separated by gel electrophoresis and analyzed by immunoblotting with antiserum to GLUT-4. In each fraction, this antiserum recognized a single major band migrating with an apparent molecular weight in the 45- to 50-kd range (blots not shown) and corresponding to GLUT-4 as reported by others.[21,28,29] Relative abundance of GLUT-4 in the various fractions is summarized in Table 6. DEX treatment did not affect the overall concentration of GLUT-4 (in total membrane fraction) or the distribution of GLUT-4 between intracellular and plasma membranes either under basal conditions or after an acute insulin stimulus.

Table 3. Effect of DEX Treatment on Insulin-Mediated Glucose Utilization by Skeletal Muscle During Euglycemic-Hyperinsulinemic Clamp (ng · mg tissue^{-1} · min^{-1})

Muscle	Control (n = 8)	DEX-Treated (n = 7)	*P*
Biceps	9.6 ± 1.8	4.6 ± 0.7	<.025
Gastrocnemius	8.7 ± 1.7	3.2 ± 0.5	<.01
Soleus	8.8 ± 1.9	3.6 ± 0.4	<.02
Plantaris	10.3 ± 2.0	3.8 ± 0.4	<.01

NOTE. Values represent the mean ± SE of 7 to 8 animals per group.

Table 4. Response of Glucose to Acute Insulin Injection in Control and DEX-Treated Rats

Time	Control (n = 5)	DEX-Treated (n = 7)	*P*
Glucose (mg/dL)			
0 min	155.7 ± 11.4	155.3 ± 5.7	NS
15 min	56.8 ± 2.4	79.1 ± 4.0	<.001
Insulin (μU/mL)			
0 min	30.4 ± 3.0	53.3 ± 4.6	<.005
15 min	1,337 ± 175	1,824 ± 219	NS

NOTE. Glucose and insulin concentrations were determined in blood samples obtained before and 15 minutes after administration of 1 U/kg insulin into the carotid artery of anesthetized animals. Values represent the mean ± SE of the indicated number of animals.

DISCUSSION

DEX-Induced Insulin Resistance

Results of the euglycemic-hyperinsulinemic clamp study (Fig 2) clearly indicate in vivo insulin resistance due to DEX at both hepatic and peripheral loci. Glucose utilization indices, as measured by ^{3}H-2DG uptake under steady-state conditions, were significantly lower in DEX-treated animals (Table 3), confirming that glucose uptake in skeletal muscle is a major target of DEX action. The significantly higher steady-state plasma insulin levels in the clamp study could be due to either a reduced metabolic clearance rate of insulin in DEX-treated animals or an incomplete suppression of endogenous insulin secretion. The antilipolytic effect of insulin during the clamp was blunted in the DEX group (Fig 1). These data confirm and extend our

Table 5. Specific Activity and Enrichment of K*p*NPPase in Muscle Membrane Fractions

K*p*NPPase Activity (nmol · mg^{-1} · 20 min^{-1})	Control	DEX-Treated
Homogenate specific activity	49.2 ± 9.0	57.4 ± 12.0
Intracellular membranes		
Specific activity	196.8 ± 26.0	156.8 ± 18.3
Enrichment (-fold)	4.4 ± 0.7	2.9 ± 0.5
Plasma membranes		
Specific activity	2,147 ± 132	2,035 ± 222
Enrichment (-fold)	50.1 ± 8.6	39.5 ± 5.8

NOTE. Values are the mean ± SE of 8 animals per group. There were no significant differences between control and DEX groups.

Table 6. GLUT-4 Content in Muscle Membrane Fractions (cpm/10 μg protein)

Group	Total Membrane	Internal Membrane	Plasma Membrane
Basal*			
Control (n = 4)	307 ± 26	383 ± 55	1,295 ± 229
DEX-treated (n = 4)	296 ± 35	532 ± 57	1,656 ± 244
Insulin-stimulated†			
Control (n = 4)	886 ± 36	1,154 ± 152	8,558 ± 609
DEX-treated (n = 4)	980 ± 65	1,458 ± 134	9,880 ± 1,578

NOTE. There were no significant differences between control and DEX groups.

*Quantified by counting GLUT-4 band in gamma counter.

†Quantified by an image analyzer.

previous observations using pancreatic suppression tests to measure insulin sensitivity.[3]

Glucose-FFA Cycle and DEX-Induced Insulin Resistance

The concept of substrate competition, whereby enhanced availability and oxidation of FFA leads to an impairment in glucose oxidation, termed the glucose-FFA cycle, was proposed over 30 years ago by Randle et al.[4,5] According to this proposal, an increased rate of FFA oxidation suppresses glycolysis and glucose oxidation by increasing the mitochondrial ratio of acetyl coenzyme A to coenzyme A, which in turn inhibits several key enzymes in the glycolytic and citric acid cycles. A number of human studies have confirmed that elevated levels of lipid oxidation are associated with impaired oxidative glucose disposal.[30-33] In a more recent study, glucose uptake measured by positron emission tomography was found to be reduced 25% to 31% in femoral and arm muscles by elevated plasma FFA levels.[34] However, in other reports,[35] FFA was not found to inhibit glucose oxidation at constant rates of glucose uptake. Moreover, Jenkins et al[15] observed stimulation of glucose uptake and muscle glycogen synthesis by high FFA concentrations in the presence of insulin in vivo in rats. Thus, the interaction between glucose and FFA is complex and appears to be dependent on the experimental conditions.

The blunting of insulin-mediated FFA suppression in vivo (Fig 1) and the attenuated antilipolytic effect of insulin on epinephrine-induced lipolysis by adipose tissue in vitro,[3] coupled with enhanced FFA oxidation by muscles removed from DEX-treated rats (Table 2), suggested a role for the glucose-FFA cycle in steroid-induced insulin resistance. If such a mechanism were operative, inhibition of FFA oxidation would be expected to relieve inhibition of glucose oxidation and thereby reverse DEX-induced insulin resistance. This hypothesis was tested by examining the effect of inhibiting FFA oxidation by etomoxir on basal and insulin-mediated glucose metabolism. Etomoxir treatment decreased basal glucose and insulin levels in both control and DEX-treated groups, implying enhanced insulin sensitivity (Table 1). In other studies, etomoxir treatment has been shown to reduce blood glucose levels in fasted pigs,[36] streptozocin-induced diabetic rats,[37] and non–insulin-dependent diabetic patients.[38] However, insulin resistance was evident in the DEX group despite etomoxir treatment, since their insulin and glucose concentrations were higher as compared with the control group receiving etomoxir.

Etomoxir treatment resulted in a small but significant stimulation of insulin-mediated glucose disposal in both control (14% increase) and DEX-treated (23% increase) rats (Fig 2); however, insulin resistance due to DEX was not completely reversed by etomoxir administration. An association between elevated plasma FFA oxidation and excessive hepatic glucose production rates is well recognized.[39] Activation of pyruvate carboxylase by acetyl coenzyme A resulting from FFA oxidation is the mechanism of stimulation of gluconeogenesis by FFA oxidation.[40] Conversely, inhibition of FFA oxidation, and hence of acetyl coenzyme A levels, would be expected to decrease gluconeogenesis. In previous studies, etomoxir treatment of rats[41] and pigs[36] with normal glucose tolerance improved glucose clearance without any effect on hepatic glucose production. In contrast, inhibition of FFA oxidation by etomoxir decreased glucose production in streptozocin-induced diabetic rats[42] and in type II diabetic patients.[38] In the current study, etomoxir had no effect on glucose production during euglycemic-hyperinsulinemic clamp in either control or DEX-treated rats. Thus, the beneficial effect of etomoxir on glucose disposal observed here must be due to its action at the periphery.

Since the completion of our study, two reports dealing with the glucose–fatty acid cycle have appeared and are relevant to the present discussion. In one study,[43] cortisone pellets (150 mg/250 to 270 g body weight) were implanted that increased basal serum FFA levels threefold in 2 days (basal serum FFA level was not altered by DEX treatment over a 10-day period in the present study, Fig 1), and etomoxir (50 mg/kg) was administered intragastrically twice per day for 2 days. Under these conditions, etomoxir increased whole-body glucose disposal of steroid-treated rats by 33%, but not control rats. In the present study, etomoxir treatment increased whole-body glucose disposal of both control and DEX groups. In another study,[44] acute blockade of FFA oxidation by methyl palmoxirate had no significant effect on whole-body glucose disposal under either basal or hyperinsulinemic clamp conditions. Whereas ^{3}H-2DG uptake was elevated in heart and diaphragm, it was suppressed in soleus and red quadriceps and unaffected in several other muscles. Thus, the interaction between glucose and FFA metabolism is complex and may not have a simple unifying explanation.

GLUT-4 Content and Translocation

Since DEX-induced insulin resistance could not be completely reversed by inhibition of FFA oxidation, we considered the possibility that impaired glucose transport into muscle tissue might be the primary defect in this insulin-resistant model. This line of reasoning derives support from the following considerations. First, the glucose transport system appears to be the rate-limiting determinant of glucose action by muscle in basal, euglycemic, and hyperglycemic states.[26,45-47] Second, we have previously[3] shown that following glucose-insulin infusion, levels

of substrates and products of the rate-determining reactions of glycolysis were reduced to the same extent (22% to 26%) in muscle from DEX-treated rats compared with controls without any measurable intracellular glucose, suggesting that impaired glucose transport into muscle is the primary lesion in steroid-induced insulin resistance. If hexokinase activity relative to transport were limiting, intracellular glucose would have accumulated and been detected. Third, DEX treatment inhibited insulin-induced uptake of 3-*O*-methyl glucose (an analog that is not phosphorylated) to the same extent as that of 2DG (an analog that is phosphorylated but not metabolized further) by soleus muscle in vitro.[29] Finally, under conditions of hyperinsulinemia and euglycemia, muscle 2DG uptake reflects glucose transport,[48] and hence impaired ^{3}H-2DG uptake by muscle in DEX-treated rats (Table 3) directly implicates a defect in glucose transport.

Several lines of evidence suggest that the insulin-regulatable glucose transporter isoform, GLUT-4, is mainly responsible for insulin-mediated glucose uptake in tissues such as adipose, skeletal muscle, and heart.[49] Translocation of GLUT-4 from intracellular stores to the plasma membrane appears to be the major mechanism of action of insulin[21,50,51]; however, activation of preexisting cell surface–bound GLUT-4 by insulin, as shown in adipocytes,[52,53] may also occur in muscle tissue.[54] Thus, there are several potential mechanisms[55] by which insulin-mediated glucose uptake may be impaired in DEX-treated rats: reduction in overall GLUT-4 abundance, decreased translocation of GLUT-4 from intracellular membrane to plasma membrane, partial fusion or altered configuration of transporters in the plasma membrane, and defects in the activation of surface-bound GLUT-4 necessary for full stimulation of glucose transport. In this study, we determined two factors, namely transporter abundance and transporter distribution, 15 minutes after intravenous administration of 1 U/kg insulin. Insulin resistance was evident under these conditions of maximal insulin stimulation (Table 4). With a similar protocol, Barnard et al[56] demonstrated reduction in glucose transport and cytochalasin B binding in sarcolemma vesicles isolated from streptozocin-induced diabetic rats. Neither the overall abundance of GLUT-4 protein as measured in the total membrane fraction nor the distribution of the transporter between intracellular and plasma membranes after insulin stimulus were affected by DEX treatment (Table 6). Likewise, DEX treatment had no effect on GLUT-4 content or distribution under basal conditions. A similar lack of glucocorticoid effect on GLUT-4 content in skeletal muscle under basal conditions was observed in another study.[29] Whereas insulin-stimulated glucose uptake is primarily mediated by GLUT-4, basal glucose uptake is mainly under the influence of GLUT-1, an isoform not subject to regulation by insulin.[55]

Although overall GLUT-4 abundance (in crude homogenates) was quantified in numerous studies investigating glucose homeostasis under various conditions,[55] compartmentalization of the transporter between intracellular stores and the plasma membrane in skeletal muscles has been addressed in only a few investigations on insulin resistance,[56-58] none of which examined glucocorticoid action. Since translocation of the transporter represents an important regulatory step in glucose transport, we determined the relative distribution of GLUT-4 between membrane fractions in skeletal muscle tissue. Although glucose uptake was impaired in DEX-treated rats, GLUT-4 concentration in the plasma membrane was not decreased. This suggests incomplete fusion of the transporter into the plasma membrane, altered configuration, and decreased activity of the transporter as possible causes of steroid-induced insulin resistance. Similarly, insulin resistance in spontaneously hypertensive rats was not found to be due to either reduced GLUT-4 levels or defective translocation in skeletal muscle.[57] However, defects in the action of insulin to stimulate muscle transporter translocation have been observed in streptozocin-diabetic rats[56] and obese Zucker rats.[58] Recent studies have shown that additional components of the muscle cell surface (eg, transverse tubules) are enriched in GLUT-4 and that short-term insulin treatment induces not only an increase in plasma membrane GLUT-4 content (90%) but an even more pronounced increase (180%) in GLUT-4 content of rat skeletal muscle transverse tubules.[59,60] Whether DEX treatment results in reduced concentration of transverse tubule GLUT-4 remains to be determined.

In conclusion, inhibition of FFA oxidation by etomoxir did not completely reverse steroid-induced insulin resistance. Impairment of ^{3}H-2DG uptake by muscle in DEX-treated rats under euglycemic-hyperinsulinemic clamp conditions, coupled with our previous demonstration[3] of a lack of free intracellular glucose in muscle tissue subsequent to glucose-insulin infusion, suggested that the primary defect in insulin-mediated glucose uptake is at the level of glucose transport. Neither the overall content of GLUT-4 nor insulin-stimulated translocation of the transporter to the plasma membrane of skeletal muscle were modified by DEX treatment. These results suggest a defect(s) in the inherent activity of plasma membrane bound GLUT-4 as the likely explanation for DEX-induced insulin resistance.

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