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Prevention of Dexamethasone-induced Insulin Resistance by Metformin

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ABSTRACT. This study investigates the effect of the antidiabetic drug metformin on dexamethasone-induced hyperglycaemia and insulin resistance in mice. Normal mice were treated with dexamethasone (2.5 mg/kg/day p.o.) plus metformin (250 mg/kg/day p.o.) and pair-fed to those receiving dexamethasone alone. Metformin reduced the extent of dexamethasone-induced hyperglycaemia and decreased insulin resistance as indicated by an improved insulin-hypoglycaemia test. Metformin-treated mice also showed increased basal glucose uptake into isolated diaphragm (by 38%), soleus (by 19%) and deep (red) quadriceps (by 31%). Measurements in the quadriceps showed that the increase in glucose uptake occurred without increasing either the mRNA levels or total cellular membrane abundance of the GLUT1 or GLUT4 glucose transporter isoforms. Thus metformin can ameliorate dexamethasone-induced hyperglycaemia and insulin resistance in part by increasing glucose disposal into skeletal muscle. Since this was achieved in quadriceps muscle without increasing mRNA or total membrane abundance of GLUT1 or GLUT4, it is possible that metformin might influence the intrinsic activity of glucose transporters, as well as altering their intracellular translocation. BIOCHEM PHARMACOL **56**;9:1145–1150, 1998.

KEY WORDS. metformin; dexamethasone; insulin resistance; glucose transport; glucose transporters; hyperglycaemia

Excess secretion or administration of glucocorticoids causes hyperglycaemia and hyperinsulinaemia, associated with insulin resistance in hepatic and peripheral tissues [1–3]. In skeletal muscle, glucocorticoids reduce the rate of basal and insulin-stimulated glucose uptake, although the chronic accumulation of glycogen is increased [4–6]. Glucocorticoid-induced insulin resistance is attributed mainly to a postreceptor defect of insulin action [3, 4], but this does not decrease the insulin-sensitive glucose transporter isoform GLUT4 mRNA or GLUT4 abundance in muscle membranes [5–7].

Metformin is an antihyperglycaemic agent used in the treatment of non-insulin dependent (Type 2) diabetes mellitus [8]. It counters insulin resistance, in part by improving insulin-mediated glucose uptake and utilization by skeletal muscle [9–11]. This may involve a direct action of metformin on the pathways of insulin action distal to insulin-receptor binding, and indirect effects due to a general improvement in the metabolic environment [10, 12, 13]. In adipocytes metformin increased insulin-stimulated translocation of GLUT4 and GLUT1 into the plasma membrane [14, 15], and in cultured muscle cells metformin increased the abundance of GLUT1 in the plasma membrane [16] without increasing the total cellular complement of these transporter isoforms in either tissue.

This study in mice investigates whether metformin can protect against the hyperglycaemia and insulin resistance induced by the glucocorticoid dexamethasone. Skeletal muscle is quantitatively the main site of insulin-mediated glucose disposal [17], and the regulation of glucose transporters in this tissue shows considerable differences from that observed in adipose tissue and cultured cells [18, 19]. Hence, this study measures glucose uptake into isolated skeletal muscle, and the mRNA and membrane abundance of GLUT1 and GLUT4.

MATERIALS AND METHODS

Animals

Male homozygous lean (+/+) mice from the Aston colony carrying the *ob* gene were used at 15 weeks of age. Mice were maintained at 22 ± 2° with a 12-hr light:dark cycle, and fed economy rodent breeder diet (Lillico).

Chemicals

Dexamethasone sodium phosphate was from Sigma, 2-deoxy-D-[1-^{3}H] glucose (11.1 Ci/mmol) and L-[1-^{14}C] glucose (55 Ci/mmol) from Amersham International, metformin from Lipha Pharmaceuticals, reagents for insulin radioimmunoassay from Linco and Hisafe II scintillant from Fisons.

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The cDNA probes used were a 436-bp *Bam*HI fragment of rat GLUT1 and a 1.3-kb *Eco*RI/*Sac*I fragment of rat GLUT4 (both kindly provided by G.I. Bell, Howard Hughes Medical Institute, University of Chicago, Chicago, IL). The housekeeping probe used to correct for gel loading was a 209-bp *Pst*I fragment of mouse 18S rRNA (kindly provided by D. Edwards, Faculty of Medicine, University of Calgary, Alberta, Canada). Other chemicals were of analytical and molecular biology grade and obtained from Sigma or BDH.

Treatment Protocol

Mice were randomized to three groups matched for body weight and plasma glucose. The groups were treated with (a) dexamethasone 2.5 mg/kg/day, (b) dexamethasone 2.5 mg/kg/day plus metformin 250 mg/kg/day or (c) control (untreated). Drugs were added to the drinking water, and treatment was continued for 10 days. Because high dosages of dexamethasone reduce food intake, all groups were pair-fed to the dexamethasone group [5], typically about 4 g/mouse/day for the first three days of treatment, and about 5 g/mouse/day thereafter.

In Vivo *Measurements*

Blood samples were taken at 10:00 a.m. from the tail tip of nonfasted mice for determination of plasma glucose [20] and plasma insulin [21]. Insulin hypoglycaemia tests were conducted in 12-hr fasted mice: blood samples were taken at 10, 20 and 40 min after i.p. administration of 0.5 u/kg of human Actrapid insulin (Novo-Nordisk) and the rate of plasma glucose disappearance (%/min) was calculated as $69.3/t_{1/2}$ [22].

2-Deoxyglucose Uptake by Isolated Muscles

Nonfasted mice were killed by cervical dislocation, and hemi-diaphragms, soleus muscle, and strips of deep (red) quadriceps femoris muscle were rapidly excised, washed, weighed and preincubated for 10 min in incubation buffer without isotopes. Tissues were then incubated with gentle agitation for 30 min at 30° in 2 mL of oxygenated (95% O_2, 5% CO_2) Krebs–Henseleit bicarbonate buffer containing 0.1% BSA, 2 mM pyruvate, 1 mM 2-deoxy-D-glucose, 0.1 μCi 2-deoxy-D-[1-^{3}H] glucose and 0.01 μCi of L-[1-^{14}C] glucose. After incubation tissues were blotted, dissolved in 0.5 mL of 1 M NaOH, diluted in scintillant and counted for ^{3}H and ^{14}C radioactivity. Tissue uptake of 2-deoxy-D-glucose was corrected for the extracellular compartment (L-glucose) and expressed as nmol/g/h.

GLUT1 and GLUT4 Protein and mRNA

Red quadriceps muscle was isolated for determination of GLUT1 and GLUT4 abundance in total membranes, and GLUT1 and GLUT4 mRNA. Membranes were prepared from fresh tissue which was weighed, minced and homogenized (Ultra Turrax, high speed for 30 sec) in ice-cold buffer containing 0.25 M sucrose, 50 mM Tris-HCI (pH 7.5), 1.25 mM EGTA, 5 mM sodium azide, 1 mM phenylmethylsulphonyl fluoride and aprotinin (30 KIU/mL). The homogenate was centrifuged at 1,200 *g* for 10 min at 4°, the supernatant saved, pellet resuspended in 5 mL of buffer, rehomogenised (10 sec) at 4°, and recentrifuged as above. The supernatant was pooled with the previous supernatant, centrifuged at 4° for 10 min at 9,000 *g*, and the supernatant centrifuged at 4° for 1 hr at 100,000 *g*. The pellet was suspended in 2 ml buffer and stored at −70°.

Western Blot Analysis

Samples of the membrane extract were loaded (20 μg/lane) onto a 12% polyacrylamide gel containing SDS with a 4% stacking gel [23]. The proteins were transferred electrophoretically to HybondECL nitrocellulose membranes (Amersham). Nonspecific binding was blocked by incubating membranes in PBS with 10% dried milk for 30 min at room temperature. After washing, the blots were incubated for 1 h with a polyclonal antibody to GLUT1 (Chemicon) at a dilution of 1/1000 in PBS containing 3% dried milk. Blots were washed and incubated for 1 hr with a horseradish peroxidase conjugated secondary antibody and for 1 min with chemiluminescent substrate (HRPL, luminescent kit, National Diagnostics) following the manufacturers instructions. Light emission was measured using x-ray film exposed for 5 min. The developed film was scanned using a laser densitometer (Molecular Dynamics), and results corrected to normalise for loading based on protein determination (performed on aliquots of the samples using the Bio-Rad protein assay kit, Bio-Rad). Following washing, membranes were incubated as above using a monoclonal antibody to GLUT4 (Biogenesis Ltd.).

RNA Analysis

Total RNA was extracted from muscle samples (100 μg) using a Ribolyser (Hybaid Ltd.) set at speed 5 for 1 × 20 sec and 1 × 10 sec using the Ribolyser kit and following the manufacturers instructions. Samples were taken up in diethylpyrocarbonate treated sterile distilled water, and the RNA quantitated using a Genequant (Pharmacia Biotech). Aliquots of 20 μg total RNA were loaded onto a 0.8% agarose gel containing 2.2 mmol/liter of formaldehyde and run at 80 V for 2 hr. The RNA was transferred to Hybond N^+ nitrocellulose membrane (Amersham) by capillary Northern blotting. The RNA was cross-linked with UV light.

The membrane was prehybridised in a solution containing 50% formamide, 5 × SSPE (1 × SSPE = 0.15 mol/liter of sodium chloride, 10 mmol/liter of sodium dihydrogen orthophosphate, 1 mmol/liter of ethylenediaminetetra-acetic acid (EDTA), pH 7.4), 5 × Denhardt's solution (1 g per litre each of Ficoll, polyvinylpyrrolidone and BSA fraction

TABLE 1. Body weight, plasma glucose and insulin, and insulin-stimulated glucose disappearance in mice treated for 10 days with dexamethasone (2.5 mg/kg/day) and metformin (250 mg/kg/day)

	Control	Dexamethasone	Dexamethasone and Metformin
Change in body weight (g)	+2.0 ± 0.9	−3.5 ± 1.3†	−3.5 ± 1.0†
Plasma glucose (mmol/L)	8.0 ± 0.4	11.6 ± 0.6†	8.7 ± 0.3‡
Plasma insulin (ng/mL)	0.74 ± 0.11	2.98 ± 0.22†	2.14 ± 0.34†
Insulin-stimulated glucose disappearance (%/min)	1.40 ± 0.20	0.67 ± 0.13*	1.08 ± 0.21

Values are mean ± SEM. N = 8.
*$P < 0.05$ vs. control.
†$P < 0.01$ vs. control.
‡$P < 0.01$ vs. dexamethasone.

V), 5 mmol/liter of sodium phosphate, 1% SDS, 1 mmol/liter of EDTA and 100 μg of denatured, sonicated salmon sperm DNA at 42°. The membrane was sequentially probed with radiolabeled GLUT1, GLUT4 and ribosomal 18S cDNAs prepared by the method of Feinberg and Vogelstein [24] to a specific activity of ~10^8 cpm/μg using deoxycytidine 5′[α^{32}P]triphosphate (Amersham). Following overnight hybridisation at 42° the membranes were washed with 2 × SSC (1 × SSC = 0.3 mol/liter of sodium chloride, 0.03 mol/liter of sodium citrate, pH 7.0), 1% SDS, twice with 0.1 × SSC, 1% SDS at 55° for 0.5 hr and rinsed with 2 × SSC, 1% SDS. The membrane was wrapped in plastic film and placed in an autoradiograph cassette with intensifying screens at −70° for 5 days. Membranes were stripped with 80% formamide, 1% SDS and 1 × SSC at 65° for 1 hr prior to re-probing. The autoradiographs were scanned by laser densitometry (Molecular Dynamics Inc.).

Statistical Analysis

Data are expressed as mean ± SEM or medians plus interquartile ranges for non-normally distributed results. Analysis was performed using Student's unpaired *t*-test, one way analysis of variance, or Kruskal–Wallis test as appropriate. Differences were considered to be significant if $P < 0.05$.

RESULTS

In Vivo *Measurements*

Treatment of mice for 10 days with dexamethasone (2.5 mg/kg/day) induced a catabolic effect indicated by an 11% fall in body weight (Table 1). In contrast, pair-fed control mice showed a 6% gain in body weight, while metformin (250 mg/kg/day) did not alter the dexamethasone-induced fall in body weight. Dexamethasone-treated mice were both hyperglycaemic and hyperinsulinaemic, indicating the presence of insulin resistance. However, metformin reduced the extent of the hyperglycaemia and hyperinsulinaemia, consistent with a reduction of insulin resistance. This was substantiated by an insulin hypoglycaemia test showing that the rate of glucose disappearance was significantly reduced by dexamethasone (by 50%) but not when concurrently treated with metformin (Table 1).

2-Deoxyglucose Uptake by Isolated Muscles

Glucose uptake into diaphragm, soleus and deep quadriceps femoris muscle was assessed using ^{3}H-2-deoxyglucose which is transported into muscle similarly to glucose, but not metabolized beyond phosphorylation. Experiments were conducted without added insulin to reflect the condition of the muscle at the time of isolation, when GLUT1 and GLUT4 were measured. Weights of the muscles studied were not significantly altered by the treatments (data not shown). Dexamethasone reduced glucose uptake into diaphragm by 50% ($P < 0.001$), but did not significantly reduce glucose uptake into soleus and quadriceps (Table 2). However administration of metformin with dexamethasone increased glucose uptake compared with dexamethasone alone in all three muscles (by 19% in soleus, 31% in quadriceps and 38% in diaphragm, $P < 0.05$).

GLUT1 and GLUT4 Abundance and mRNA

The abundance of GLUT1 and GLUT4 in crude total membrane preparations of red quadriceps femoris muscle was not significantly altered by either dexamethasone alone or dexamethasone plus metformin (Fig. 1A). As reported previously [25, 26], both GLUT1 and GLUT4 exhibit a number of *N*-glycosylated bands on Western blots ranging from approximately 43–66 kDa (Fig. 1A). Whilst dexamethasone treatment appeared to be associated with a

TABLE 2. 2-Deoxyglucose uptake by hemi-diaphragm, soleus and quadriceps femoris muscle of mice treated for 10 days with dexamethasone (2.5 mg/kg/day) and metformin (250 mg/kg/day)

	2-Deoxyglucose uptake (nmol/g/h)		
	Control	Dexamethasone	Dexamethasone and Metformin
Diaphragm	291 ± 24	146 ± 12†	202 ± 7*§
Soleus	275 ± 10	255 ± 10	304 ± 11§
Quadriceps	101 ± 5	95 ± 5	125 ± 9‡

Values are mean ± SEM, N = 6.
*$P < 0.01$.
†$P < 0.001$ vs. control.
‡$P < 0.05$.
§$P < 0.01$ vs. dexamethasone.

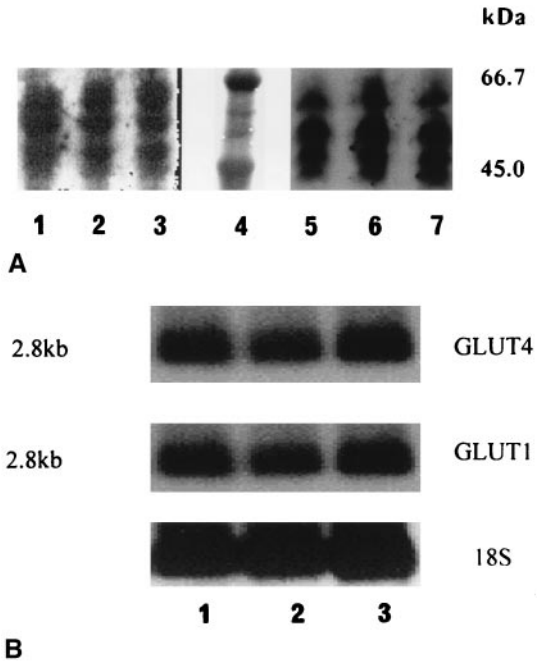

FIG. 1. GLUT1 and GLUT4 membrane abundance (A) and mRNA (B) in deep red quadriceps femoris muscle of mice treated for 10 days with dexamethasone (2.5 mg/kg/day) and metformin (250 mg/kg/day). (A). Representative Western blots probed for GLUT1 (lanes 1–3) and GLUT4 (lanes 5–7). Control lanes 1 and 5; Dex lanes 2 and 6; Dex + Met lanes 3 and 7. Lane 4 is a protein ladder. (B). Representative autoradiograph of Northern blots. Control lane 1; Dex lane 2; Dex and Met lane 3.

reduction in the abundance of both transporter isoforms compared with controls, this failed to reach statistical significance (Table 3). The mRNA from both transporter isoforms gave bands of approximately 2.8 kb as previously reported [27] (Fig. 1B). In contrast to the protein levels there appeared to be an increase in mRNA for both transporter isoforms induced by dexamethasone treatment which was reversed by the combined treatment with dexamethasone and metformin (Table 4), but again this failed to reach statistical significance.

DISCUSSION

This study presents evidence that metformin can reduce the severity of dexamethasone-induced hyperglycaemia and insulin resistance. This was associated with increased glucose uptake by skeletal muscle, and measurements made in deep (red) quadriceps muscle indicated that this was achieved without increasing either the membrane abundance or biosynthesis of GLUT1 and GLUT4.

TABLE 3. Protein expression of glucose transporter isoforms GLUT1 and GLUT4 in isolated membranes of red quadriceps femoris muscle determined by Western blot analysis

	Control (N = 9)	Dexamethasone (N = 6)	Dexamethasone and Metformin (N = 12)
GLUT1	1.35 (1.74)	1.10 (1.54)	1.36 (0.98)
GLUT4	1.64 (3.93)	1.12 (2.41)	0.99 (3.2)

Data are expressed as arbitrary optical density units corrected for protein loading. Values are medians with interquartile ranges in parenthesis.

Kruskal–Wallis H for GLUT1 = 0.36, $P = 0.83$; for GLUT4 = 1.62, $P = 0.44$, both NS.

TABLE 4. Expression of mRNA for glucose transporter isoforms GLUT1 and GLUT4 in snap frozen samples of red quadriceps femoris muscle assessed by Northern blot analysis

	Control (N = 9)	Dexamethasone (N = 6)	Dexamethasone and Metformin (N = 12)
GLUT1	2.39 ± 0.19	2.83 ± 0.18	2.30 ± 0.18
GLUT4	2.67 ± 0.21	3.38 ± 0.33	2.61 ± 0.20

Data are expressed as arbitrary optical density units corrected for loading by the level of expression of 18S rRNA. Values are mean ± SEM.

ANOVA $F_{2,24}$ for GLUT1 = 1.89, $P = 0.17$; for GLUT4 = 2.61, $P = 0.09$, both NS.

Because a high dosage of dexamethasone (e.g. 2.5 mg/kg/day used herein) initially reduces food intake [28, 29], both the control and dexamethasone-metformin groups were pair-fed to the dexamethasone group. This excluded a difference in energy intake, because starvation and glucose deprivation are known to increase the potential for basal glucose transport in muscle together with increased expression of GLUT1 and GLUT4 [18, 30, 31].

Despite the characteristic hyperglycaemia, hyperinsulinaemia and insulin resistance induced by glucocorticoid excess [3, 7], the present study has confirmed that GLUT4 protein and mRNA levels in quadriceps muscle were not reduced compared with controls [5, 6]. A previous study has also shown that dexamethasone does not alter the distribution of GLUT4 between intracellular sites and the plasma membrane in either the basal or the insulin-stimulated state, suggesting that the accompanying decrease in glucose uptake might be due to a decrease in the abundance of another glucose transporter isoform (e.g. GLUT1), or to a defect of inherent transporter activity [7]. Herein we found that neither GLUT4 nor GLUT1 abundance in quadriceps muscle membrane was significantly decreased by dexamethasone. This is reminiscent of recent observations in other insulin-resistant states such as obesity and noninsulin-dependent diabetes mellitus in which decreased glucose uptake into skeletal muscle cannot be explained by a decrease in GLUT1 or GLUT4 [32–34].

Dexamethasone may cause atrophy of type 2B muscle fibers, increasing the proportion of type 1 and 2A fibers in mixed muscles [28], which are richer in GLUT4 than type 2B [35, 36]. Deep red quadriceps has a high proportion of type 2A fibers [37], which may reduce possible interference from a change in fibre composition by dexamethasone. Metformin is not known to affect muscle fibre composition, and there was no effect of metformin on body weight compared with the dexamethasone group. It is well established that metformin treatment can reduce hyperglycaemia in part by reducing hepatic glucose output [8, 11, 13]. Metformin has also been shown to improve muscle glucose

uptake in several states of insulin resistance [8–10]. This may be facilitated by a reduction in fatty acid oxidation, increased glycogenesis and increased glucose oxidation [11]. The lack of weight gain appears to reflect the ability of metformin to exert effects on various tissues to increase glucose turnover, predominantly in the splanchnic bed [22, 38, 39].

Thus, although dexamethasone only significantly reduced basal glucose uptake in diaphragm, co-administration with metformin significantly increased glucose uptake in all three muscles examined (Table 2), yet there was no significant increase in either the total cellular membrane abundance (Table 3, Fig. 1A) or mRNA levels (Table 4, Fig. 1B) of GLUT1 or GLUT4. Accepting that the sensitivity of the Western blots cannot accurately detect small changes in glucose transporter expression, the data suggest that metformin was able to increase glucose uptake without increasing either new synthesis of GLUT1 or GLUT4 or numbers of these transporters in the muscle membranes. Whether this is representative of other skeletal muscles cannot be deduced from this experiment, but the ability of metformin to increase glucose uptake was seen in all three muscles studied. Also, metformin prevented the significant deterioration of insulin-induced hypoglycaemia produced by dexamethasone (Table 1). This indicates a widespread effect of metformin on insulin-sensitive sites of glucose disposal (i.e. a range of skeletal muscles), probably supported by decreased hepatic glucose output [8, 13]. While metformin increases the plasma membrane abundance of GLUT1 and GLUT4 in adipocytes [14, 40] and GLUT1 in cultured muscle cells [16], this does not necessarily correspond to the situation in skeletal muscle, as noted in many other circumstances [18, 19]. Indeed, the differential localization of GLUT1 (sarcolemma) and GLUT4 (sarcolemma and transverse tubules) in muscle, and the identification of insulin-sensitive and insulin-insensitive pools of GLUT4 suggests that the control of glucose transporters is not the same in muscle as adipose tissue [41].

Various agents (including dexamethasone) appear to alter glucose transport independently of glucose transporter synthesis or overall membrane abundance [7, 42, 43]. This raises the possibility of a change in the intrinsic activity of these transporters, for instance differential *N*-glycosylation of the transporters has been shown to alter the transport activity [25]. Because metformin treatment can improve glucose transport into muscle without a measurable increase in GLUT1 or GLUT4 synthesis or overall membrane abundance, it is possible that in addition to affecting the intracellular translocation of glucose transporters, metformin may be capable of altering the intrinsic activity of certain glucose transporters.

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