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# Expression of an insulin-responsive glucose transporter (GLUT4) minigene in transgenic mice: Effect of exercise and role in glucose homeostasis

**(insulin resistance/cyclic AMP/arachidonic acid/fasting/diabetes mellitus)**

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**ABSTRACT** **The effects of a GLUT4 mini-transgene (containing 7 kb of 5′ flanking and 1 kb of 3′ flanking sequence and all exons and introns of the GLUT4 gene as well as a small foreign DNA tag) and of exercise training on expression of GLUT4 and glycemic control in mice were investigated. Transgenic mice harboring the minigene expressed ≤2-fold the normal level of GLUT4 mRNA and protein in skeletal (gastrocnemius) muscle and adipose tissue. This modest tissue-specific increase in GLUT4 expression led to an unexpectedly rapid blood glucose clearance rate following oral glucose administration. In nontransgenic animals exercise caused a 1.5-fold increase in expression of GLUT4 mRNA and protein as well as a significant improvement of glycemic control. In transgenic animals harboring the minigene exercise increased expression of GLUT4 mRNA and protein derived from the minigene and endogenous gene and led to a further improvement of glycemic control. These findings indicate that the cis-regulatory element(s) controlling exercise-induced expression of the GLUT4 gene is located within the nucleotide sequence encompassed by the GLUT4 minigene. The fact that glycemic control is markedly improved by a relatively low level of expression of GLUT4 caused by the transfected minigene and is further enhanced by exercise in transgenic animals demonstrates that GLUT4 plays a pivotal role in glucose homeostasis *in vivo*. Of the effectors—i.e., cAMP, insulin, and arachidonic acid—known to down-regulate expression of GLUT4 by 3T3-L1 adipocytes in culture, only the decline in circulating arachidonate level *in vivo* correlated with up-regulation of GLUT4 caused by exercise.**

Insulin stimulates glucose uptake by activating translocation of the insulin-responsive glucose transporter (GLUT4) from an intracellular vesicular compartment(s) to the plasma membrane (1, 2), a process that occurs only in skeletal and heart muscle cells and in adipocytes (3). In theory, a defect in any component of the signal transmission pathway between the insulin receptor and GLUT4 could lead to insulin resistance of the type associated with non-insulin-dependent diabetes (type 2). Recent studies indicate that a decrease in the expression of GLUT4 is associated with insulin resistance in adipose tissue. Resistance of glucose uptake to insulin by adipocytes is closely correlated with a decreased GLUT4 level in several animal models of insulin resistance—e.g., the aged/obese SD rat (4), rats fed a high-fat diet (5), and the genetically obese Zucker rat (6). Likewise, adipocytes from non-insulin-dependent diabetes mellitus patients express markedly decreased levels of GLUT4 message and protein (7), although the levels in muscle are unaffected (8). In contrast, exercise training leads to increased levels of GLUT4 message and protein in muscle and adipose tissue and to the reversal of insulin resistance (9, 10). Nuclear run-on assays show that the exercise-induced increase of GLUT4 mRNA in muscle is due to a transient rise in the rate of transcription of the GLUT4 gene (11).

To gain a better understanding of the mechanism(s) by which expression of the GLUT4 gene is regulated, we recently produced transgenic mice that express a murine GLUT4 minigene in an appropriate tissue-specific manner (12). The present paper shows that a relatively low level of expression of the GLUT4 minigene causes an unexpectedly large increase in the rate of glucose disposal after oral glucose administration in exercise-trained and nonexercised animals. The fact that exercise training markedly activates expression of the minigene indicates that the minigene contains the cis-regulatory element(s) that mediates exercise-induced expression.

## EXPERIMENTAL PROCEDURES

**Transgenic Mice and Transcript Detection.** Heterozygous transgenic mice harboring the 14-kb GLUT4 minigene were produced as described (12). The 281-bp tag inserted into the 3′ untranslated region of the gene permitted transcript detection without affecting translation of the minigene mRNA. The RNase protection analysis employed an antisense RNA probe that generates protected fragments of 433 bp and 399 bp corresponding to the minigene transcript and of 182 bp corresponding to the endogenous GLUT4 transcript (12). RNA transcripts were quantitated with an Image analyzer (BAS 2000, Fuji).

**Southern Blotting and Gene Copy Number.** The DNA probe corresponding to the 281-bp chloramphenicol acetyltransferase (CAT) tag insert of the minigene (12) used for Southern blot analysis was labeled with [$\alpha$-$^{32}$P]dCTP by the random priming method (13). To estimate the minigene copy number, chromosomal DNA from transgenic mice prepared as described by Wigler *et al.* (14) was digested overnight with *Bam*HI and then electrophoresed in 0.8% agarose gels. Following transfer to Hybond membranes (Amersham) and hybridization with the CAT tag probe, the copy number of the integrated minigene was quantitated with an Image analyzer using a *Bam*HI-restricted plasmid containing the GLUT4 minigene as reference standard.

**Exercise Training.** Mice (8–15 weeks of age) were exercise trained by forced swimming in plastic barrels (24-cm diameter) filled with water to a depth of 25 cm and maintained at 34°C. Eight to 10 mice were exercised together in each barrel. To adapt mice to the exercise protocol, all animals swam for 10 min three times daily for 3 days. After adaptation, the swimming program was shifted to 30-min periods four times daily

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Abbreviations: BAT, brown adipose tissue; WAT, white adipose tissue; CAT, chloramphenicol acetyltransferase.

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separated by 5-min rest periods. This program was continued for 3 weeks. Eighteen to 22 hr after the terminal exercise session, tissues were isolated by rapid dissection and snap-frozen in liquid nitrogen, and RNA was isolated for RNase protection assays or immunoblotting. Mice were allowed free access to a laboratory mouse chow (with 11%, 60%, and 29% of total calories from fat, carbohydrate, and protein, respectively) and water. For analysis of tissue cAMP and plasma insulin and arachidonic acid for exercise-trained animals, 40 female C57BL/6 mice were subjected to the exercise protocol. At 1, 3, 6, and 24 hr after the last exercise session (during which the mice had access to food), the mice were sacrificed. Blood was drawn from the inferior vena cava, and plasma was analyzed for insulin and arachidonic acid. For determination of cAMP tissues were homogenized and analyzed immediately.

**Immunoblotting.** Crude membrane fractions from skeletal (gastrocnemius) muscle, brown adipose tissue (BAT), and white adipose tissue (WAT) were prepared as described (15). It should be noted that the membrane fractions isolated from skeletal muscle also contained large amounts of insoluble contractile proteins. Proteins separated by SDS/PAGE were electrophoretically transferred to Immobilon (Millipore) and immunoblotted with antibodies directed against the C-terminal amino acid sequence of GLUT4 and then $^{125}$I-labeled protein A as described (15).

**Oral Glucose Tolerance Test.** After an overnight fast, D-glucose (1 mg/g of body weight) was administered by stomach tube. Blood samples were obtained before and 30, 60, and 120 min after glucose administration. Blood glucose levels were measured, using a TIDEX glucose analyzer (Miles).

**Other Analyses and Methods.** Tissue cAMP levels were determined by an ELISA procedure using the supplier's (Amersham) protocol. Immunoreactive insulin was measured by radioimmunoassay using human insulin as standard. Arachidonic acid was analyzed by gas/liquid chromatography. Protein was assayed using the Micro BCA protein assay reagent kit (Pierce). Statistical comparisons were made with Student's $t$ test as appropriate. Statistical significance is defined as $P < 0.05$. Values are mean ± SE.

## RESULTS AND DISCUSSION

The present investigation was undertaken to determine the effect on glucose homeostasis of expressing a GLUT4 minigene (12) in transgenic mice. The 14-kb minigene (containing 7 kb of 5′ flanking and 1 kb of 3′ flanking sequence and all exons and introns of the mouse GLUT4 gene plus a 281-bp CAT tag in the 3′ untranslated region for transcript identification; see ref. 12) was used to establish several heterozygous transgenic mouse lines, two of which were used for the experiments described in this paper. The numbers of copies of the minigene per cellular genome were 28 and 30 for the two cell lines (i.e., lines 1 and 2, respectively). Mice expressing the transgene exhibited no differences in growth rate, adult body weight, or adipose (epididymal or parametrial) tissue mass compared to nontransgenic control animals.

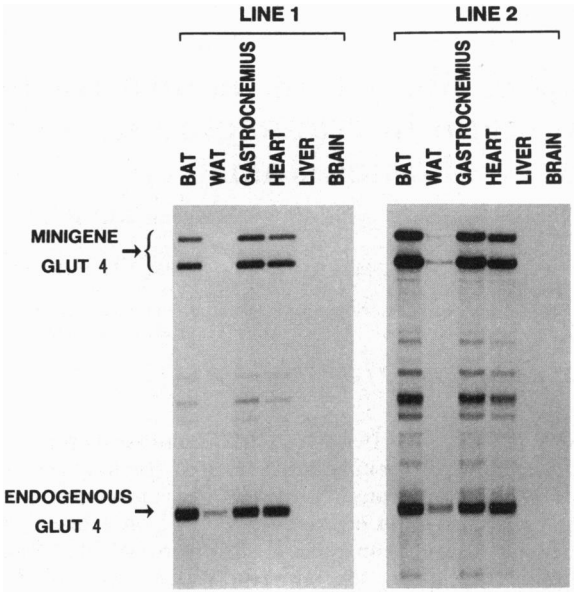


FIG. 1. Expression of minigene and endogenous GLUT4 mRNAs in transgenic mice harboring a GLUT4 minigene. RNA isolated from the indicated tissues of male heterozygous transgenic mice (lines 1 and 2; copy numbers 28 and 30 per genome, respectively) was subjected to RNase protection analysis as described (12). The 433-bp and 399-bp protected fragments correspond to the GLUT4 minigene transcript and the 182-bp protected fragment corresponds to the endogenous GLUT4 transcript. Several bands observed between 399 bp and 182 bp appear to be degraded fragments of the minigene-protected fragment.

The transgene exhibited an appropriate tissue-specific pattern of expression (Fig. 1). Like endogenous GLUT4 mRNA,

Table 1. Effect of exercise training on expression of minigene and endogenous GLUT4 mRNA from transgenic mice

| | | | %* | | | |
|---|---|---|---|---|---|---|
| | | | Nonexercised | | Exercised | |
| Tissue | Line | Sex | Minigene GLUT4 mRNA | Endogenous GLUT4 mRNA | Minigene GLUT4 mRNA | Endogenous GLUT4 mRNA |
| Gastrocnemius | 1 | ♀ | 117 ± 18 | 100 ± 11 | 276 ± 16† | 144 ± 11† |
| | 2 | ♀ | 173 ± 10 | 100 ± 7 | 497 ± 50† | 165 ± 12† |
| | 2 | ♂ | 178 ± 20 | 100 ± 15 | 502 ± 41† | 151 ± 8† |
| | NT | ♀ | — | 100 ± 1 | — | 153 ± 1† |
| BAT | 1 | ♀ | 63 ± 16 | 100 ± 8 | 248 ± 48† | 220 ± 22† |
| | 2 | ♀ | 147 ± 15 | 100 ± 13 | 666 ± 70† | 270 ± 43† |
| | NT | ♀ | — | 100 ± 4 | — | 162 ± 5† |
| WAT | 1 | ♀ | 37 ± 10 | 100 ± 8 | 89 ± 14† | 172 ± 15† |
| | 2 | ♀ | 50 ± 13 | 100 ± 19 | 144 ± 27† | 152 ± 30 |
| | 2 | ♂ | 17 ± 8 | 100 ± 4 | 44 ± 6† | 140 ± 12† |
| | NT | ♀ | — | 100 ± 2 | — | 143 ± 7† |

Data are not corrected for differences in size of the protected probe hybridized to minigene versus endogenous GLUT4 mRNA. Each data point entry represents four or five mice. Values are mean ± SE. NT, nontransgenic.

*Relative to endogenous GLUT4 mRNA of nonexercised controls.

†Statistically significant at $P < 0.05$ compared to nonexercised controls.

GLUT4 minigene mRNA was expressed in BAT and WAT, skeletal (gastrocnemius) muscle, and heart but not in liver and brain. RNase protection assays of RNA from these tissues gave rise to the protected fragments of 433 bp and 399 bp derived from the minigene message and to a 182-bp protected fragment from the endogenous GLUT4 message (12). The levels of expression of the endogenous and minigene messages were low in WAT compared to BAT, skeletal muscle, and heart (Fig. 1). For reasons not presently understood, expression of GLUT4 minigene mRNA in WAT from female mice was consistently 2.5- to 3.0-fold higher, relative to the endogenous message, than in WAT from male mice (Table 1 and results not shown).

Expression of GLUT4 minigene mRNA gave rise to increased levels of expression of GLUT4 protein—i.e., 2-fold, 2.3-fold, and 1.3-fold increases—over the levels in skeletal muscle, BAT, and WAT, respectively, of nontransgenic controls (Fig. 2). It should be noted that the level of GLUT4 protein in muscle, relative to the levels in BAT and WAT in the same animal (Fig. 2), is underestimated since equivalent amounts of crude membrane protein from each tissue were compared and muscle (but not BAT and WAT) membrane fractions contained substantial amounts of nonmembrane contractile proteins.

As exercise increases expression of GLUT4 by activating transcription of the gene (11), it was of interest to ascertain whether the cis-acting regulatory element(s) that mediates exercise-induced expression of the GLUT4 gene is located within the 14-kb GLUT4 minigene. Thus, mice harboring the minigene and nontransgenic control mice were subjected to an intensive swimming exercise program over a 3-week period and then were subjected to analysis. That the exercise-inducible cis-element(s) is present in the minigene is indicated by the fact that exercise training caused an increase in the level of minigene mRNA in gastrocnemius muscle, BAT, and WAT of female and male animals (Fig. 3 and Table 1). Moreover, the

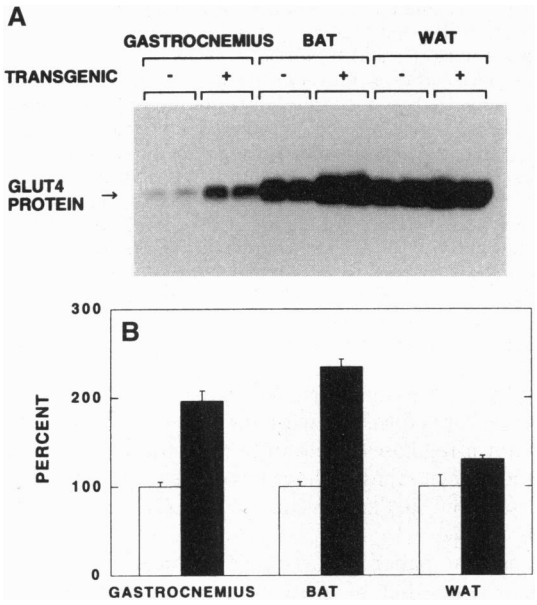


FIG. 2. Expression of GLUT4 protein in tissues from mice harboring a GLUT4 minigene. Tissues from female heterozygous transgenic mice (line 2; +) and their control littermates (−) were extracted and subjected to SDS/PAGE (40 μg of protein per lane). GLUT4 was detected by immunoblotting and quantitated. (*A*) Representative autoradiogram. (*B*) Relative amount of GLUT4 from each tissue in transgenic mice (shaded bars) expressed as a percentage of that in the same tissue from nontransgenic mice (open bars). Results represent the mean ± SE for four mice. The increased levels of GLUT4 in tissues from transgenic mice versus those from nontransgenic mice were statistically significant at $P < 0.05$.

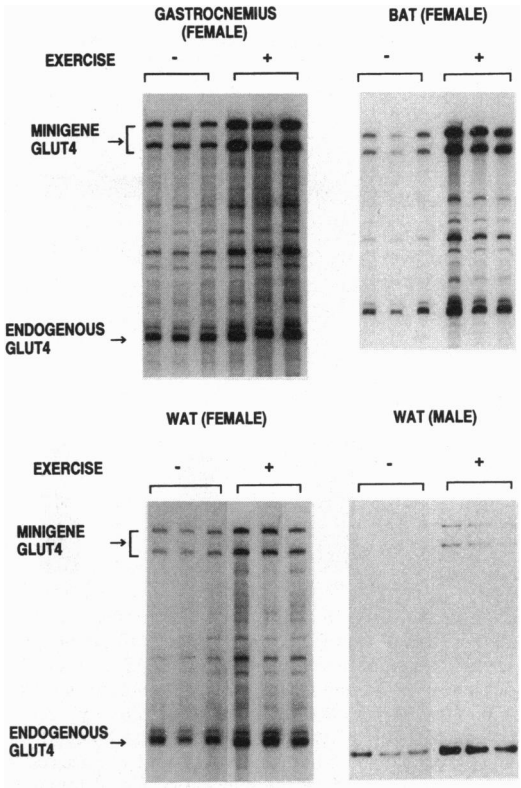


FIG. 3. Effect of exercise training on the expression of minigene and endogenous GLUT4 mRNA by muscle and adipose tissue from transgenic mice. Line 2 transgenic mice were divided into two groups, one subjected to the exercise protocol (+) and the other not (−). RNAs isolated from gastrocnemius skeletal muscle, BAT, and epididymal or parametrial WAT were subjected to RNase protection assays. The 433-bp and 399-bp protected fragments correspond to the GLUT4 minigene message and the 182-bp protected fragment corresponds to endogenous GLUT4 message. Autoradiograms of gastrocnemius, BAT, and WAT are shown for three female mice from each group and of WAT for three male mice from each group.

magnitude of the increase of message level induced by exercise was considerably greater for the GLUT4 minigene message (2.4- to 2.8-fold in muscle) than for the endogenous GLUT4 message (1.4- to 1.6-fold in muscle) (Table 1). The fact that exercise induced substantial increases of the GLUT4 minigene message in two independent transgenic mouse lines (Fig. 3) shows that the exercise effect was not merely the result of a favorable site of integration of the minigene into the mouse genome. It can be concluded, therefore, that the cis-regulatory element(s) responsible for exercise-induced expression of the endogenous GLUT4 gene is located within the 14-kb minigene. In nontransgenic animals the exercise-induced increase in endogenous GLUT4 mRNA (Table 1) led to corresponding increases in the level of GLUT4 protein—i.e., 1.6-fold in gastrocnemius muscle, 1.3-fold in BAT, and 1.5-fold in WAT (Fig. 4). Moreover, exercise training of mice harboring the GLUT4 minigene, which led to increased expression of minigene mRNA (Fig. 3 and Table 1), further increased the expression of GLUT4 protein (Fig. 4 *A* and *B*).

To assess the functional consequences of increased tissue (skeletal and heart muscle and adipose) levels of GLUT4 caused by expression of the GLUT4 minigene and by exercise, glucose tolerance tests were performed. As shown in Fig. 5 *A* and *C*, expression of the minigene, which led to an ≈2-fold increase in the GLUT4 level in skeletal muscle of control (not exercised) mice, caused a marked improvement of glycemic control following oral glucose administration. A glucose tolerance test with a homozygous mouse derived from a mating of line 2 heterozygotes showed further improvement of glycemic control (results not

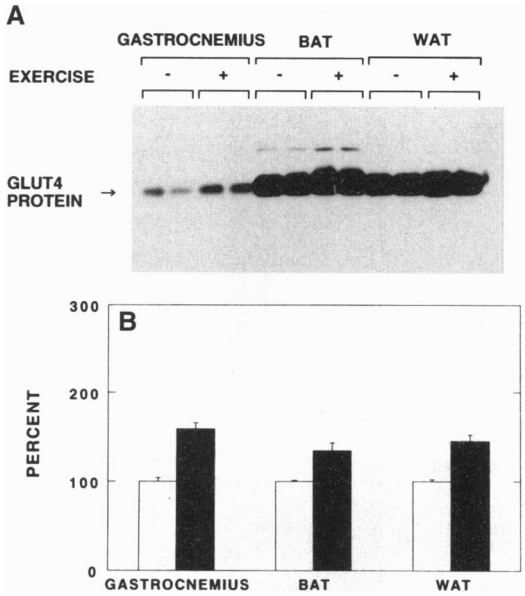


FIG. 4. Effect of exercise training on the expression of GLUT4. Tissue extracts from exercise-trained (+) and sedentary control nontransgenic (−) mice were subjected to SDS/PAGE (40 μg of protein per lane) and immunoblotted, and the amount of GLUT4 was quantitated. (*A*) Representative autoradiogram. (*B*) Amount of GLUT4 of each tissue in exercise-trained mice (shaded bars) expressed as a percentage of that for sedentary control mice (open bars). Results are the mean ± SE of the mean for each of four mice. The amount of GLUT4 protein in each tissue from exercise-trained mice was statistically significantly higher ($P < 0.05$) than that from sedentary control mice.

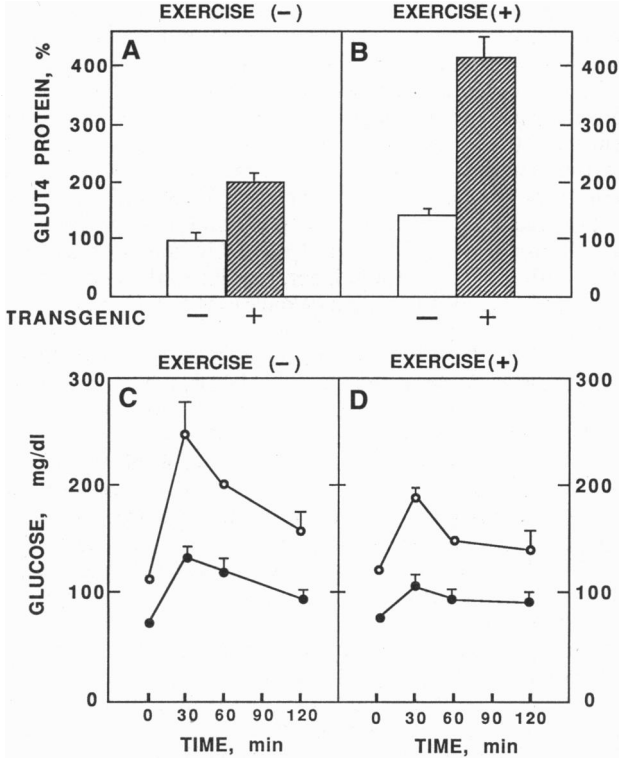


FIG. 5. Effect of expression of the GLUT4 minigene and of exercise training on GLUT4 protein level in skeletal muscle and on glycemic control. (*A*) Skeletal (gastrocnemius) muscle from female line 2 transgenic mice harboring the GLUT4 minigene (hatched bars) and nontransgenic mice (open bars) were either subjected to the exercise protocol or not (five mice in each of the four groups). Muscle extracts were subjected to SDS/PAGE and immunoblotted, and the amount of GLUT4 protein was expressed in percent relative to that of nontransgenic sedentary control mice. (*A* and *B*) Amount of GLUT4 protein expressed as percent relative to that of nontransgenic sedentary control mice. (*C* and *D*) Results of glucose tolerance tests on transgenic mice harboring the minigene (●) or nontransgenic mice (○) that were subjected or not subjected to the exercise protocol. Nonexercised line 2 transgenic and nontransgenic littermates were fasted overnight, after which D-glucose (1 mg/g of body weight) was administered orally by stomach tube (*C*). Overnight fasting of exercised mice began 4–6 hr following the last exercise session, after which oral glucose was administered (*D*). Plasma glucose levels were determined at the times indicated. The results of one (out of three) representative experiments are shown. Results are the mean ± SE of the mean obtained for the four groups, each of which included three mice. All glucose levels for the transgenic mice were significantly ($P < 0.05$) lower than those for nontransgenic mice.

shown). Improved glycemic control was also caused by expression of GLUT4 directed by the minigene in exercise-induced animals (Fig. 5 *B* and *D*). The initial fasting blood glucose level and the peak level achieved after glucose administration were substantially reduced in animals harboring the minigene. As expected, exercise training of control (nontransgenic) mice caused an increase (≈1.5-fold) of tissue GLUT4 protein (Fig. 5 *A* and *B*), comparable to that of GLUT4 mRNA (Table 1), and led to a significant improvement of glycemic control (Fig. 5 *C* and *D*). Likewise, with mice harboring the GLUT4 minigene, exercise caused increased expression of GLUT4 protein (Fig. 5 *A* and *B*) and a smaller, but consistent, improvement of glycemic control (Fig. 5 *C* and *D*).

cAMP (16), insulin (17), and arachidonic acid (18) are known to down-regulate expression of GLUT4 mRNA and protein in fully differentiated 3T3-L1 adipocytes. Similarly, reduced levels of GLUT4 mRNA and protein correlate with changes in the levels of cAMP in adipose tissue (19) and plasma insulin (7), which accompany insulin-dependent diabetes mellitus (type 1) and insulin-resistance associated with obesity-linked non-insulin-dependent diabetes mellitus (type 2), respectively. It was of interest, therefore, to determine whether exercise-induced changes in the levels of these effectors correlate in a similar manner with the observed changes in expression of GLUT4 mRNA. Thus, RNA from tissues of exercise-induced and control mice, obtained 1, 3, 6, and 24 hr after the last exercise training session, was subjected to RNase protection analysis. As illustrated in Table 2, plasma insulin levels were increased substantially (i.e., ≈40% compared with nonexercised controls) by exercise training at all time points, while blood arachidonic acid levels were consistently decreased, but to a lesser extent (8–24%), by exercise. No significant changes, however, occurred in the cAMP levels of gastrocnemius, BAT, and WAT. Fasting sedentary mice for 48 hr, however, which served as a positive control, substantially increased cAMP levels and decreased GLUT4 mRNA levels in BAT and WAT but not in gastrocnemius muscle (Table 3). The latter results are consistent with our previous finding (16) that cAMP down-regulates transcription of the GLUT4 gene in 3T3-L1 adipocytes and findings by others (20) that fasting has little effect on the expression of the GLUT4 message in skeletal muscle.

The present paper reports the important, though unexpected, finding that a relatively small increase—i.e., ≤2-fold—in the level of GLUT4 protein in the appropriate tissues (skeletal muscle, heart, BAT, and WAT) of transgenic mice harboring a GLUT4 minigene causes a dramatic improvement of glycemic control following oral glucose administration (Fig. 5). While an improvement in glycemic control due to expression of a GLUT4 transgene had been reported previously (21), in that study the levels of GLUT4 protein, also expressed in the appropriate tissues, were >10-fold greater than by nontransgenic controls. Although the transgenic mice used in the present study harbored multiple copies—i.e., as many as 30

Table 2. Effect of exercise on plasma insulin, plasma arachidonate, and tissue cAMP levels

| Effector | Control (not exercised) | Time after last exercise, hr | | | |
|---|---|---|---|---|---|
| | | 1 | 3 | 6 | 24 |
| Insulin, μunits/ml | 5.2 ± 0.2 | 7.2 ± 0.5* | 7.5 ± 0.5* | 6.8 ± 0.8* | 7.0 ± 0.7* |
| Arachidonate, μg/ml | 111 ± 8 | 92 ± 3* | 102 ± 11 | 98 ± 2 | 84 ± 4* |
| cAMP, pmol/mg of protein | | | | | |
| Gastrocnemius | 264 ± 35 | 285 ± 38 | 229 ± 31 | 255 ± 28 | 260 ± 33 |
| WAT | 294 ± 29 | 235 ± 18 | 233 ± 23 | 284 ± 36 | 212 ± 17 |
| BAT | 689 ± 87 | 698 ± 33 | 582 ± 66 | 723 ± 32 | 916 ± 168 |

*Statistically significant at $P < 0.05$ compared to nonexercised or fed controls. Each data point entry represents the average of 5–15 mice.

copies per genome—of the transgene (i.e., a GLUT4 minigene that contained 7 kb of 5′ and ≈1 kb of 3′ flanking sequence, all exons and introns of the murine GLUT4 gene, and a 281-bp CAT tag inserted into the 3′ untranslated region), they expressed relatively low levels of GLUT4 protein (Fig. 5*A*). Our previous study (12) with the GLUT4 minigene transfected into 3T3-L1 adipocytes and transgenic mice suggested that the minigene lacked a cis-regulatory element required for maximal levels of expression. Based on the copy number of the minigene relative to the endogenous gene and their relative levels of expression (mRNAs, Table 1), corrected for differences in the sizes of the $^{32}$P-labeled protected fragments in RNase protection assays, it is calculated that the level of expression per copy of the minigene is ≤1/20th that of the endogenous gene. Nevertheless, the minigene exhibits normal regulation in other respects—i.e., it exhibits tissue-specific expression (Fig. 1 and Table 1), differentiation-induced expression in 3T3-L1 preadipocytes (12), cAMP-induced down-regulation (12), and exercise-induced expression (Fig. 5 and Table 1). Thus, the GLUT4 minigene possesses the appropriate cis-regulatory elements for these physiological responses.

From these and other studies (21) it can be concluded that GLUT4 plays an important role in glycemic control. Relatively small perturbations of tissue levels of GLUT4 lead to substantial changes in fasting blood glucose level and the rate of clearance of plasma glucose following glucose administration. Thus, the GLUT4 gene constitutes a rationale target for intervention in disease states, such as non-insulin-dependent diabetes mellitus (type 2), in which glycemic control is abnormal. Although prevailing evidence (22) indicates that skeletal muscle plays the dominant role in glucose disposal, exercise is the only means presently available for altering expression of the GLUT4 gene in this tissue.

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Table 3. Effect of fasting on tissue cAMP levels and GLUT4 mRNA

| Tissue | cAMP, pmol/mg of protein | | GLUT4 mRNA, % (relative to fed control) | |
|---|---|---|---|---|
| | Fed | Fasted | Fed | Fasted |
| Gastrocnemius | 274 ± 13 | 315 ± 17 | 100 ± 9 | 126 ± 11 |
| WAT | 247 ± 18 | 414 ± 28* | 100 ± 18 | 38 ± 8* |
| BAT | 471 ± 20 | 663 ± 23* | 100 ± 15 | 54 ± 5* |

*See footnote * in Table 2.

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