Chronic growth hormone treatment in normal rats reduces post-prandial skeletal muscle plasma membrane GLUT1 content, but not glucose transport or GLUT4 expression and localization

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Whether skeletal muscle glucose transport system is impaired in the basal, post-prandial state during chronic growth hormone treatment is unknown. The current study was designed to determine whether 4 weeks of human growth hormone (hGH) treatment (3.5 mg/kg per day) would impair glucose transport and/or the number of glucose transporters in plasma membrane vesicles isolated from hindlimb skeletal muscle of Sprague–Dawley rats under basal, post-prandial conditions. hGH treatment was shown to have no effect on glucose influx ($V_{\max}$ or K_m) determined under equilibrium exchange conditions in isolated plasma membrane vesicles. Plasma membrane glucose transporter number (R_o) measured by cytochalasin B binding was also unchanged by hGH treatment. Consequently, glucose transporter turnover number ($V_{\max}/R_o$), a measure of average glucose transporter intrinsic activity, was similar in hGH-treated and

control rats. hGH did not change GLUT4 protein content in whole muscle or in the plasma membrane, and muscle content of GLUT4 mRNA also was unchanged. In contrast, GLUT1 protein content in the plasma membrane fraction was significantly reduced by hGH treatment. This was associated with a modest, although not significant, decrease in muscle content of GLUT1 mRNA. In conclusion, high-dose hGH treatment for 4 weeks did not alter post-prandial skeletal muscle glucose transport activity. Neither the muscle level nor the intracellular localization of GLUT4 was changed by the hormone treatment. On the contrary, the basal post-prandial level of GLUT1 in the plasma membrane was reduced by hGH. The mRNA data suggest that this reduction might result from a decrease in the synthesis of GLUT1.

INTRODUCTION

There is considerable interest in the use of human growth hormone (hGH) in the treatment of diseases other than growth hormone deficiency [1]. Therefore, it is important to understand stimulatory and inhibitory effects of long-term hGH administration on target tissues. Insulin-stimulated skeletal muscle glucose uptake is one of the functions impaired by chronic growth hormone treatment, both in humans [2] and in rats [3]. Since glucose transport is the rate-limiting step for glucose uptake in this tissue under physiological circumstances [4], abnormalities in the glucose transport system might play a relevant role in the reduction of glucose utilization. In rats, it has been demonstrated that glucose transport in isolated muscle is impaired during chronic GH stimulation, both in the absence of insulin [5] and after insulin stimulation [5,6]. However, insulin-stimulated glucose transport under these conditions appears to result from a decrease in non-insulin-dependent glucose transport, rather than a decreased response to insulin [5].

These *in vitro* determinations of glucose transport were made after incubation of isolated muscles at the same defined insulin concentration for GH-treated and control rats. However, chronic GH treatment induces a compensatory increase in the plasma insulin concentration [5–7]. Therefore, it is possible that skeletal muscle glucose transport after GH treatment would be normal if measured under *in vivo* conditions. In this regard, *in vivo* data in humans have demonstrated normal basal whole-body glucose disposal after GH treatment [2], suggesting that basal glucose

transport may not be altered by GH. Thus, whether skeletal muscle glucose transport is impaired in the basal, post-prandial state during GH treatment is unknown. The first aim of this study was to investigate this question in rats chronically treated with hGH. For this purpose, we measured the glucose transport $V_{\max}$ under conditions of equilibrium exchange in plasma membrane vesicles isolated under basal post-prandial conditions from hindlimb muscle of control and 4-week-hGH-treated rats.

Glucose transport across the plasma membrane in skeletal muscle tissue is regulated by two glucose transporters: GLUT4 and GLUT1. Although skeletal muscle glucose transport has been reported to be reduced after GH treatment [5,6], no changes were found [5,7] in the total content of GLUT4 or GLUT1 proteins in this tissue. Since the whole muscle content of GLUT4 or GLUT1 may not correlate with the number of functional transporters localized to the plasma membrane, a second goal of this study was to determine whether GH treatment results in a specific decrease in the number of plasma membrane glucose transporters. The total number of glucose transporters in a plasma membrane preparation from skeletal muscle of hGH-treated rats was measured by cytochalasin B (CB) binding. In addition, since a reduction of glucose transport could result from decrease in the average intrinsic activity of the transporters [8], the carrier turnover number [8,9], an index of average transporter activity in the plasma membrane ($V_{\max}/R_o$), was calculated to clarify whether a reduction of transporter intrinsic activity contributes or is responsible for the impaired glucose transport. Since CB binding measures the total number of glucose trans-

Abbreviations used: CB, cytochalasin B; hGH, human growth hormone; R_o , plasma membrane glucose transporter number; KpNPPase, K⁺-stimulated *p*-nitrophenol phosphatase.

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porters without discriminating between different isoforms, we also measured the content of GLUT4 and GLUT1 transporters in the same plasma membrane preparation by Western blotting. Finally, to gain insight into the potential role of changes in the synthesis of glucose transporters following hGH treatment the muscle content of GLUT4 and GLUT1 mRNAs was determined.

MATERIALS AND METHODS

Animals and experimental protocol

Fourteen female Sprague–Dawley rats (body weight 239 ± 4 g), obtained from Charles River Breeding Laboratories (Wilmington, MA, U.S.A.), were housed at 23 °C with a 12 h light/dark cycle (06:00h–18:00h) and allowed free access to food and water. The animals were matched for body weight and divided into two groups: hGH group ($n = 7$) received 3.5 mg/kg per day of hGH via subcutaneous injections twice per day for 4 weeks; and the control group ($n = 7$) was left untreated during the same period. After 4 weeks, the animals were studied between 10:00h and 17:30h. Since they were consistently fed the night before the experiment, we consider them as under post-prandial physiological conditions. They were killed by heart excision under anaesthesia (ketamine hydrochloride 50 mg/kg plus xylazine 10 mg/kg intraperitoneally) and blood was collected for measurements of plasma glucose, insulin and hGH. The quadriceps muscle was quickly exposed, isolated and immediately frozen in liquid N₂ and later used for mRNA determinations. Soleus, gastrocnemius and extensor digitorum longus muscles were rapidly exposed and dissected from both legs, taking care to remove fat, nerves and connective tissue. The latter muscles were minced and homogenized in 250 mM sucrose, 20 mM Hepes, pH 7.4, at 4 °C, with Polytron and then Potter–Elvehjem homogenizers. The resulting homogenate was rapidly frozen in liquid N₂ and stored at –80 °C until used for the plasma membrane preparation. Since the hGH-treated animals demonstrated an increase in muscle weight, we used whole individual muscles (soleus, gastrocnemius and extensor digitorum longus) pooled together for the plasma membrane preparation, rather than equal weights of muscle tissue.

Plasma membrane preparation

After collecting the homogenates from all the animals, plasma membranes were prepared as described previously [10]. Briefly, the homogenates were diluted, the final volume measured, and KCl and sodium pyrophosphate were added to final concentrations of 300 mM and 25 mM respectively. After centrifugation for 1 h at 227 000 *g*, the resuspended pellet was incubated with DNase for 1 h at 30 °C and recentrifuged. The resulting pellet was resuspended in 34 % (w/v) sucrose, 20 mM Hepes for a 16 h centrifugation in a sucrose step gradient. The plasma membranes were collected from the 12/27 and 27/30 % sucrose interfaces. The membranes in these fractions were pelleted by centrifugation, resuspended in the sucrose/Hepes buffer to a final protein concentration of 2–4 mg/ml, and stored in liquid N₂ for later use.

Protein and marker enzyme assays

Homogenate and plasma membrane protein content was determined for each preparation using the Coomassie Brilliant Blue method (Bio-Rad Laboratories, Richmond, CA, U.S.A.) as described by Bradford [11] with BSA as standard. K⁺-stimulated *p*-nitrophenol phosphatase (KpNPPase) specific activity was assayed in the absence or presence of 20 mM K⁺ as a plasma membrane marker [12].

Plasma assays

Plasma glucose concentrations were determined with a YSI Glucose Analyser (Yellow Springs Instruments Company, Inc., Yellow Springs, OH, U.S.A.). Insulin concentrations were measured by radioimmunoassay [13]. hGH was measured by ELISA [14].

Glucose transport

The uptake of D-[¹⁴C (U)]glucose and L-[1-³H (N)]glucose in plasma membrane vesicles was determined under equilibrium exchange conditions at 25 °C using a rapid filtration technique [8,9]. Membranes were pre-equilibrated with a Hepes-buffered Krebs–Ringer solution containing 1, 5, 10, 20, 40, or 60 mM L- and D-glucose. Uptake was measured at four time points for each preparation (0–5 s). Transport was initiated by combining an aliquot of the membranes with Hepes-buffered Krebs solution and 1–60 mM D- and L-glucose with 6 μ Ci of L-[N-³H]glucose and 1.6 μ Ci of D-[U-¹⁴C]glucose. Transport was stopped by the addition of Hepes-buffered Krebs solution containing 0.2 mM phloretin at 4 °C. The membranes were rapidly loaded on to a filter (Millipore HA 0.45 μ m), washed, and the filter and adhering membranes analysed by liquid scintillation counting using quench correction for the dual label. Facilitated transport was calculated by subtracting the initial rate of L-glucose influx from that of D-glucose. $V_{\max}$ and K_m were determined using non-linear least-squares fit of the data.

CB binding

Equilibrium D-glucose-inhibitable [³H]CB binding was measured and the concentration of glucose transporters determined by a modification of the method of Wardzala et al. [15] as described previously [10]. Scatchard plots were generated from binding studies in which membranes were incubated with CB in the presence or absence of 500 mM D-glucose. Cytochalasin E (2 μ M) was present to decrease non-specific binding. Bound CB was separated from free CB by centrifugation. Tracer amounts of [¹⁴C]urea were used to correct for [³H]CB trapped in the pellet. The total number of glucose transporters (R_o) and the dissociation constant (K_d) were determined from a linear plot derived by subtraction along the radial axes of binding curves generated in the presence of D-glucose from those in the absence of D-glucose.

Carrier turnover number

The carrier turnover number [8,9], an index of glucose transported per transporter, was calculated by dividing the glucose transport $V_{\max}$ obtained at 25 °C by the total number of glucose transporters, both measured in the same plasma membrane vesicles ($V_{\max}/R_o$). Carrier turnover number represents the average activity of transporters in the plasma membrane fraction. Therefore, changes in transporter isoforms in the plasma membrane, if they had different intrinsic activity, would result in altered average carrier turnover number even if no change of individual transporter intrinsic activity occurred. In addition, since no reliable markers for microsomal membranes containing glucose transporters are available, the degree of contamination of these membranes in the plasma membrane fraction cannot be evaluated. This theoretical contamination might potentially lead to an underestimation of the carrier turnover number.

RNA extraction and Northern blot analysis

Total muscle RNA was extracted by the acid guanidinium thiocyanate–phenol–chloroform method [16]. An aliquot (20 μ g)

of total RNA per sample was denatured and electrophoresed in a 1% formaldehyde agarose gel to assess RNA integrity by ethidium bromide staining of 28 S and 18 S ribosomal subunit bands. The RNA was transferred to nylon membranes (Gene Screen Plus, Dupont, NEN Products, Boston, MA, U.S.A.) by capillary blotting overnight in $10 \times \text{SSC}$ ($1 \times \text{SSC}$: 0.15 M NaCl/0.015 M sodium citrate) or by direct application of RNA on to membranes using a dot-blot vacuum manifold apparatus (Scheicher and Schuell, Keene, NH, U.S.A.). RNA in nylon membranes was immobilized by UV-cross-linking and hybridized using ^{32}P -labelled GLUT4 cDNA (Dr. G. I. Bell, Howard Hughes Medical Institute, Chicago, IL, U.S.A.), or GLUT1 cDNA (Dr. G. I. Bell), or 18 S ribosomal cDNA (Dr. P. Bachierre, Centre de Recherche de Biochimie et Genetique Cellulaires, Toulouse Cedex, France). The 18 S ribosomal cDNA probe was used to correct for total RNA loading differences. Blots were hybridized overnight at 42 °C and washed according to methods described by the manufacturer of the nylon membranes. Bands or dots corresponding to specific cDNAs were quantified with a Phosphorimager (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Western blotting

Homogenate (200 μg of protein) and plasma membrane (50 μg for GLUT4, 80 μg for GLUT1) fractions were subjected to SDS/PAGE, and resolved proteins were transferred to nitrocellulose filters [17,18]. Incubations were carried out in Tris-buffered saline (20 mM Tris, 250 mM NaCl, pH 7.5) at 22 °C unless otherwise indicated. The nitrocellulose transfer membranes were blocked in Tris-buffered saline containing 0.5% Tween 20, 3% BSA and 0.01% sodium azide. For GLUT1 determination, Carnation non-fat dried milk (2% final concentration) was added to this blocking buffer. To identify GLUT4 or GLUT1 proteins, the transfer membranes were incubated overnight with either a polyclonal antibody GT4 at a concentration of 10 $\mu\text{g}/\text{ml}$ (produced in our laboratory from a synthetic peptide corresponding to a 15-amino-acid C-terminal sequence in rat muscle GLUT4 and purified on a peptide affinity column) or a polyclonal antibody produced from a synthetic peptide corresponding to a 12-amino-acid C-terminal sequence in rat brain GLUT1 (East-Acres Biologicals, Southbridge, MA, U.S.A.) at the dilution 1:200, followed by incubation with ^{125}I -labelled Protein A (ICN, Irvine, CA, U.S.A.). The nitrocellulose filters were analysed and bands corresponding to specific glucose transporters were quantified by Phosphorimager (Molecular Dynamics). For each gel, serial dilutions of a microsomal membrane stock were run as an internal standard to allow a comparison of samples run in separate gels and to determine linearity.

Statistical analysis

Data were analysed by unpaired *t* test. All data are reported as means $\pm$ S.E.M.

RESULTS AND DISCUSSION

The characteristics of the rats studied are shown in Table 1. Compared with control animals, body weight was significantly higher in the hGH group at the end of the fourth week of treatment. Mean plasma glucose and insulin concentrations were elevated also, although this increase did not reach statistical significance. As expected, the level of plasma hGH was markedly elevated in the animals treated with injections of this hormone. Although the range of hGH level was wide (300–20400 ng/ml),

Table 1 General characteristics of the rats studied

	Body weight (g)	Plasma glucose (mg/dl)	Plasma insulin ($\mu\text{units}/\text{ml}$)	hGH plasma level (ng/ml)
Control group	273 $\pm$ 8	188 $\pm$ 5	84 $\pm$ 22	—
hGH group	328 $\pm$ 10*	221 $\pm$ 29	140 $\pm$ 61	6027 $\pm$ 3250

* $P < 0.001$ versus control group.

Table 2 GLUT4 protein, GLUT4 and GLUT1 mRNA levels in rat skeletal muscle

	Homogenate GLUT4 protein (arbitrary units)	GLUT4 mRNA (arbitrary units)	GLUT1 mRNA (arbitrary units)
Control group	100 $\pm$ 11	100 $\pm$ 8	100 $\pm$ 9
hGH group	100 $\pm$ 6	94 $\pm$ 21	78 $\pm$ 7

all the animals had high plasma levels of hGH and increased growth rates compared with the controls.

The muscle content of GLUT4 protein measured in muscle homogenates and the content of GLUT4 and GLUT1 mRNAs in quadriceps muscles of animals from the two groups are shown in Table 2. hGH treatment did not change the whole muscle content of GLUT4 protein. Likewise, muscle GLUT4 mRNA content was similar in the control and in the hGH-treated group. These results, which confirm the work of others [5,7], indicate that hGH treatment did not change whole muscle content of GLUT4 protein or mRNA levels. In the same muscle, a 22% decrease in GLUT1 mRNA content was detected (Table 2). Although this reduction did not reach statistical significance ($P < 0.08$ with one-tail *t* test), it suggests that hGH may decrease muscle GLUT1 mRNA. A previous paper has shown no change in muscle GLUT1 mRNA content in rats bearing a GH-secreting tumour [7], possibly because the GH plasma level was much lower than in the current study.

The characteristics of the muscle used for plasma membrane preparation, and the recovery and enrichment of KpNPPase activity, an enzyme marker for sarcolemma, in the final plasma membrane fraction relative to the starting homogenate are shown in Table 3. Following hGH treatment, muscle wet weight was significantly increased compared with controls. Nevertheless, the muscle homogenate protein content was similar, indicating that the increase in muscle weight was not due to an increase in protein. The recovery and enrichment of KpNPPase activity were similar in the two groups, indicating that quality and purity of the plasma membrane fractions did not differ.

Data on glucose transport V_{max} in the experimental groups are shown in Table 4. Glucose transport V_{max} , under conditions of equilibrium exchange, provides a measure of the activity of the transporters located in the plasma membrane. The V_{max} was slightly, but not significantly, lower in the hGH-treated group. Similarly, the K_m of glucose transport was not different between the two groups (38.8 ± 7.9 and 32.6 ± 7.8 mM in controls and hGH-treated animals respectively). These results are in contrast with previous reports [5,6], which demonstrated a significant decrease in glucose transport in isolated epitrochlearis or soleus muscles at moderate insulin levels following chronic hGH treatment. However, we measured glucose transport in muscle plasma membranes obtained from animals with their individual

Table 3 Muscle weight and protein content and enzymic characterization of skeletal muscle homogenates (HM) and plasma membrane (PM) fractions of the rats studied

	Muscle weight (g)	HM total protein (mg)	PM total protein (mg)	PM KpNPPase activity (nmol/mg per 30 min)	KpNPPase recovery (%)	KpNPPase enrichment (fold)
Controls	5.14 ± 0.13	843 ± 36	2.76 ± 0.2	4242 ± 175	7.9 ± 0.5	24.6 ± 2.1
hGH group	6.11 ± 0.17*	886 ± 22	2.91 ± 0.18	4396 ± 350	8.3 ± 0.3	26.1 ± 2.3

* $P < 0.05$ versus control group.**Table 4** Glucose transport $V_{\max}$, total glucose transporter number, and carrier turnover number in skeletal muscle plasma membrane vesicles from control or hGH-treated rats

	Glucose transport $V_{\max}$ (nmol/mg per s)	Total transporter number (pmol/mg)	Carrier turnover number (s^{-1})
Controls	8.62 ± 2.0	12.64 ± 0.80	699 ± 172
hGH group	6.61 ± 1.07	13.54 ± 1.12	499 ± 89

Table 5 Skeletal muscle plasma membrane (PM) GLUT4 or GLUT1 from control or hGH-treated rats

	PM GLUT4 (arbitrary units)	PM GLUT1 (arbitrary units)
Control group	100 ± 16	100 ± 7.0
hGH group	133 ± 29	76 ± 8.68*

* $P = 0.05$ versus control group.

in vivo levels of insulin, glucose, free fatty acids or other potentially important factors. We have previously demonstrated [8] that an insulin stimulation may induce a 130 % increase in the glucose transport $V_{\max}$ (due to an increase in both R_o and carrier turnover number). In the previous studies, a single *in vitro* incubation medium (insulin, 3-*O*-methylglucose, absence of any D-glucose, etc.) was used for both control and hGH-treated rats [5,6]. Although this represents the usual method of choice for measuring differences in isolated muscles, our approach allowed us to obtain information on the function of glucose transporters that should more accurately reflect their state *in vivo*.

Our results support the conclusion that basal, post-prandial plasma membrane glucose transport activity is unchanged by chronic treatment with hGH. These results are consistent with the previous observation in humans that basal *in vivo* glucose disposal is unchanged after hGH treatment [2]. Since *in vivo* glucose disposal is largely determined by the amount of glucose utilized by peripheral tissues, isolated muscle glucose uptake data should be analysed in terms of the total amount of glucose taken up by the entire mass of muscle. The transport of glucose in the incubated muscle in previous studies was reported to be impaired when expressed per unit of intracellular volume [5] or gram of muscle [6]. However, since muscle weight is increased by chronic GH stimulation, the glucose transported by the entire mass of muscle could have been normal. Unfortunately, such a calculation was not performed, and the muscle weights were not provided.

In the same plasma membrane vesicles used for the transport measurements, we determined the total number of glucose transporters by CB binding. As illustrated in Table 4, the same number of glucose transporters were present in the plasma membrane of the skeletal muscle of the two groups. The K_d values for CB binding were 136 ± 10 and 148 ± 16 nM in the control and hGH-treated animals respectively (P not significant). The carrier turnover number, an index of average intrinsic activity of the glucose transporters in the plasma membrane vesicles, also was not changed by hGH treatment (Table 4), although the mean was modestly decreased. From these results, we conclude that the number and average activity of the

transporters in the plasma membrane in the basal post-prandial state were not changed by chronic hGH treatment.

To determine whether the unchanged total number of glucose transporters in the plasma membrane reflected oppositely directed changes in the content of GLUT4 and GLUT1, we measured the plasma membrane contents of these individual transporter isoforms by Western blotting (Table 5). There was a modest but statistically insignificant increase ($P < 0.16$, one tail) in plasma membrane GLUT4 content in the hGH group, and the plasma membrane level of GLUT1 was significantly reduced ($P = 0.05$) in the hGH group. Since muscle weight was significantly larger in the hGH-treated group (Table 3), we also calculated the total amount of GLUT1 present on the plasma membrane of the whole muscles by using the KpNPPase recovery data. This total amount of GLUT1, expressed per gram of muscle, was significantly lower in the hGH-treated rats versus the controls (64 ± 6 % of the controls, $P = 0.02$ versus controls). This finding is consistent with data obtained from adipocytes [19] indicating that GH treatment reduces GLUT1 content in these cells. Since GLUT1 is postulated to be responsible for basal glucose transport in skeletal muscle cells, we hypothesize that the previously observed reduction of glucose transport in the absence of insulin [5,6] could be due to a reduction of GLUT1 content in the plasma membrane. GLUT1 is less abundant than GLUT4, and this may explain why we did not detect a significant change in the total number of glucose transporters in the plasma membrane.

In conclusion, hGH treatment for 4 weeks did not induce a change in basal post-prandial skeletal muscle glucose transport activity. The muscle level, the intracellular localization, and the synthesis of GLUT4 were unchanged by the GH treatment. In contrast, the basal post-prandial level of GLUT1 in the plasma membrane was reduced by GH. Our results suggest that this reduction might be due to decreased mRNA levels and thus decreased synthesis of GLUT1.

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