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Differential regulation of two distinct glucose transporter species expressed in 3T3-L1 adipocytes: Effect of chronic insulin and tolbutamide treatment

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ABSTRACT **The HepG2-type glucose transporter (HepG2-GT) is expressed in 3T3-L1 fibroblasts and adipocytes. In contrast, the acutely insulin-regulatable glucose transporter (IRGT) is expressed only in the adipocytes. In the present study, the expression of the IRGT was shown to increase in parallel with the acquisition of acutely insulin-stimulated glucose uptake during differentiation of these cells, whereas the level of the HepG2-GT decreased during the course of differentiation in parallel with a decline in basal glucose uptake. We examined the effects of chronic insulin and tolbutamide treatment on glucose transporter activity in conjunction with the expression of these two glucose transporter species in 3T3-L1 adipocytes. Treatment of adipocytes with insulin, tolbutamide, or both agents in combination increased 2-deoxyglucose uptake, HepG2-GT protein, and HepG2-GT mRNA levels in parallel. The effect of combined insulin/tolbutamide administration on these three parameters was greater than the effect of either treatment alone. In contrast, these treatments either had no significant effect or decreased levels of IRGT protein and mRNA. We conclude that chronic treatment of 3T3-L1 adipocytes with insulin or tolbutamide increases glucose uptake primarily by means of a selective increase in the expression of the HepG2-GT. We suggest that part of the *in vivo* hypoglycemic effect of insulin and sulfonylureas may involve an increased expression of the HepG2-GT.**

Muscle and fat represent a major depot for insulin-stimulated whole-body glucose disposal (1, 2). Glucose transport across the plasma membrane of these insulin-sensitive tissues appears to occur by means of at least two distinct facilitated diffusion transport proteins. The major species, termed the insulin-regulatable glucose transporter (IRGT), is expressed exclusively in fat and muscle tissues (3, 4). The HepG2-type glucose transporter (HepG2-GT) (5), the predominant species expressed in mammalian brain, erythrocytes, and several other tissues, appears to be expressed at low levels in fat and muscle (3, 6–8). Most of the enhancement of glucose transport observed in response to acute insulin stimulation in fat and muscle is believed to occur by means of the rapid translocation of glucose transporters from an intracellular pool to the plasma membrane (9–12). Both the IRGT and the HepG2-GT undergo insulin-dependent translocation in rat and 3T3-L1 adipocytes (3, 13, 14), and thus both presumably contribute to the acute increase in glucose transport observed in the presence of the hormone. However, an investigation of the long-term regulatory effect of insulin on these two distinct glucose transport systems has not been reported. Some studies on insulin resistance have indirectly suggested a negative effect of chronic insulin administration on glucose disposal in humans (15), although claims to the contrary have also been made (16, 17). Most animal studies, however, have indicated that insulin generally exerts a positive regulatory effect, presumably by raising the pool of glucose transporters and by enhancing their translocation to the plasma membrane (18–23).

To date, the so-called "extrapancreatic" mechanisms of action of the sulfonylureas, which can possibly account for their sustained therapeutic effect in type II diabetes, are still obscure. Although reports have linked the efficacy of these drugs to an increased number of insulin receptors (24, 25), the relative importance of this effect is unclear (26, 27). One potential postreceptor mechanism of action of the sulfonylureas is an effect on cellular glucose transport. Studies using various sulfonylureas have yielded conflicting results. Thus, some have shown an alteration of both basal and insulin-stimulated glucose transport rates in target cells (28–31), whereas others, observing only potentiation of the insulin-stimulated transport rate, have suggested that these drugs act by improving the recruitment of translocatable intracellular carriers in response to insulin (32, 33).

The present study was undertaken to examine the levels at which chronic insulin and sulfonylurea treatments affect glucose uptake in 3T3-L1 adipocytes. These cells provide an excellent model system to examine these effects because they closely resemble adipocytes biochemically and morphologically, they respond to chronic insulin and sulfonylurea exposure with increased glucose transport (30, 34–37), and they express both the IRGT and the HepG2-GT, as do insulin-sensitive tissues *in vivo* (3). The results presented herein demonstrate that a major component of chronic insulin and/or tolbutamide effects on glucose transport is the increased expression of the HepG2-GT.

MATERIALS AND METHODS

Cell Culture and Treatment. 3T3-L1 preadipocytes (American Type Culture Collection) were maintained as fibroblasts in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% (vol/vol) bovine calf serum, 2.0 mM glutamine, and antibiotics (penicillin at 50 units/ml and streptomycin at 50 μg/ml). Differentiation was initiated 24–48 hr after confluence by changing the medium to DMEM containing 10% (vol/vol) fetal calf serum, insulin at 5 μg/ml, 0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone, and antibiotics and glutamine as described above. Forty-eight hours later, and at 24- to 48-hr intervals thereafter, cells were fed with DMEM supplemented with 10% fetal calf serum, glutamine, and antibiotics. This resulted in an adipocyte conversion of >95% as judged by the morphological appear-

Abbreviations: IRGT, acutely insulin-regulatable glucose transporter; HepG2-GT, HepG2-type glucose transporter.

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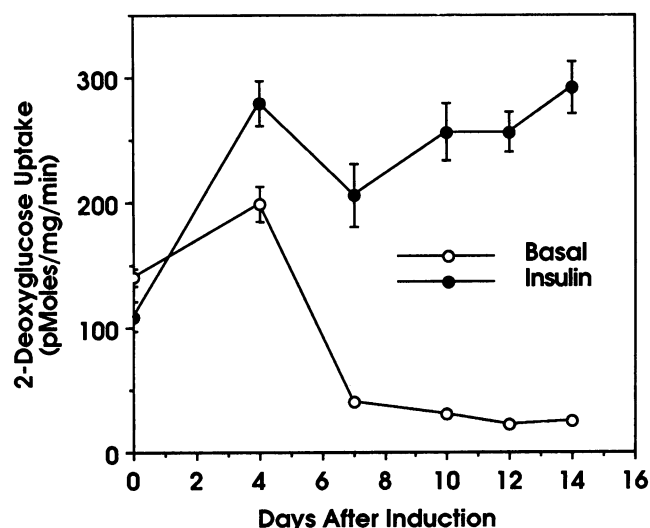

FIG. 1. Time course of the acquisition of acute insulin responsiveness during differentiation of 3T3-L1 cells. Differentiation medium was added to confluent 3T3-L1 fibroblasts on day 0. 2-Deoxyglucose uptake measurements were performed; where indicated, insulin was added to a concentration of 0.1 μM. The results represent the mean ± SEM of three to six determinations of a representative experiment. Noninduced age-matched fibroblasts were examined at the same time points (data not shown).

ance of the cells by phase-contrast microscopy. Experiments were initiated 7 days after the removal of the insulin-containing differentiation medium.

2-Deoxyglucose Uptake. Cells were differentiated in 35-mm dishes and 2-deoxy[^{3}H]glucose uptake (50 μM, 1 μCi/ml; 1 Ci = 37 GBq) was determined at 22°C following minor modifications of a published procedure (38). Uptake measurements were made at 6 min. Non-carrier-mediated uptake was assessed in the presence of 40 μM cytochalasin B. All measurements were done at least in triplicate, and calculations were normalized for protein content. Under all experimental conditions, uptake was linear for at least 15 min. For experiments involving acute insulin stimulation, the basal rate of uptake was determined after preincubating the cells in serum-free DMEM for 1 hr. Serum withdrawal reduces the basal level of transport, presumably by removing insulinomimetic factors that stimulate translocation of internal transporters to the plasma membrane. The acutely stimulated uptake rate was measured after incubating the cells for an additional 20 min in DMEM with 0.1 μM insulin at 37°C. The assay was performed as above with insulin present at all times.

Immunodetection of Glucose Transporter Proteins. 3T3-L1 adipocytes were solubilized by trituration in ice-cold buffer containing 10 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 0.5% sodium deoxycholate, and 1.0 mM of each of the following protease inhibitors: phenylmethanesulfonyl fluoride, N^{α}-(p-tosyl)lysine chloromethyl ketone, and L-1-tosylamido-2-phenylethyl chloromethyl ketone. Insoluble material was removed by centrifugation in a microcentrifuge, and 50–100 μg of the total cellular protein extracts was subjected to SDS/PAGE (10% resolving gel) under nonreducing conditions. Proteins were electrophoretically transferred to nitrocellulose or nylon membranes, and immunodetection was carried out as described (3). Two antibodies were used to detect the HepG2-GT, with identical results. A rabbit antiserum (5) to the purified human erythrocyte glucose transporter was used at a 1:500 dilution. Rabbit antise-

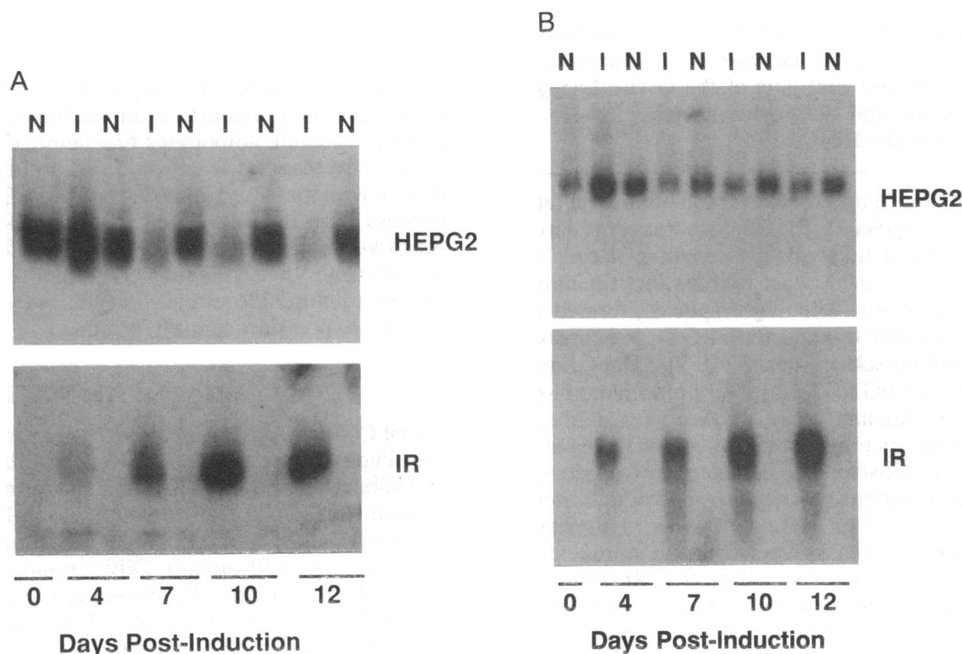

FIG. 2. Levels of HepG2-GT and IRGT protein and mRNA during differentiation of 3T3-L1 cells. Differentiation medium was added to confluent 3T3-L1 fibroblasts on day 0. Control age-matched fibroblasts were treated identically but were not exposed to insulin, dexamethasone, and isobutylmethylxanthine. Greater than 95% of the induced cells had acquired the adipocyte morphology (rounded with intracellular fat droplets) by day 6. Less than 2% of the noninduced cells resembled adipocytes by day 12. Total protein and RNA extracts were prepared from induced (I) and noninduced (N) cells at the indicated time points. (*A*) One hundred micrograms of total cellular protein was subjected to SDS/PAGE and immunoblotting with the HepG2-GT carboxyl-terminal antibody (*Upper*) or the IRGT carboxyl-terminal antibody (*Lower*). Both proteins exhibited sizes of 45–50 kDa relative to soluble protein standards [lactate dehydrogenase, fumarase, and pyruvate kinase (not shown), see Fig. 4*A*]. (*B*) Total RNA (20 μg) from induced (I) and noninduced (N) cells was subjected to Northern blot analysis. The blots were probed with ^{32}P-labeled HepG2-GT (*Upper*) or IRGT (*Lower*) cDNAs. High-stringency wash conditions [65°C, 0.1× SSC (1× SSC = 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0)] were used to prevent cross-hybridization. Both mRNA species exhibited sizes of ≈2.8 kilobases relative to SP6 polymerase-generated RNA size standards (not shown).

rum (R493, ref. 3) raised against a synthetic peptide consisting of the 12 carboxyl-terminal residues of the rat HepG2-GT (rat brain, ref. 8) was purified on a protein A column (Bio-Rad) and used at a concentration of 25 μg of IgG per ml. For detection of the IRGT protein, a rabbit antiserum (R820, ref. 3) raised against the carboxyl terminus of IRGT was used at 25 μg of IgG per ml. The antibodies used in these experiments are specific for the HepG2-GT and the IRGT, respectively, and do not cross-react with the alternate transporter species (3).

RNA Extraction and Northern Blot Analysis. Total RNA was prepared by the guanidium thiocyanate/cesium chloride method (39) and electrophoresed on 1.2% formaldehyde/agarose gels. The samples were then blotted onto nylon, crosslinked by UV irradiation (40), and hybridized to either nick-translated HepG2-GT (5) or IRGT (3) cDNA under high-stringency conditions as previously described (5). The ^{32}P-labeled blots were then subjected to autoradiography. The two cDNA probes are specific for the appropriate mRNA species under these conditions and do not cross-hybridize to a significant extent (see Figs. 2*B* and 4*B*).

Quantitation of Autoradiograms. Quantitation was performed by densitometry on a LKB laser scanner and/or by excision of the bands from the blots and determining the radioactivity in a γ counter or liquid scintillation spectrometer. Each of the quantitation methods was conducted within the linear response range.

RESULTS

Expression of the HepG2-GT and the IRGT During Differentiation of 3T3-L1 Cells. The data in Fig. 1 confirm a previous report (41) that the development of acute insulin responsiveness in 3T3-L1 cells is the result of both a decrease in the basal rate and an increase in the insulin-stimulated rate of glucose uptake. By day 10, the rate of insulin-stimulated glucose uptake increased to ≈8- to 12-fold above the basal level, whereas the response in age-matched fibroblasts varied from ≈1.1- to 2.3-fold above basal over this same time period (data not shown). The highest rate of basal uptake was measured in day 4 adipocytes, 48 hr after the removal of the differentiation medium containing 1.0 μM insulin. This is likely due to the residual increased expression of the HepG2-GT resulting from exposure to the high insulin concentration in the differentiation medium (see below).

Phenotypic differentiation and acquisition of acute insulin responsiveness were associated with the gradual appearance of the IRGT mRNA and protein, along with a decline in the adipocyte content of the HepG2-GT to only 20% that of age-matched fibroblasts by day 10 (Fig. 2). The strongest HepG2-GT mRNA and protein signals were exhibited by day 4 adipocytes. The subsequent decline of the HepG2-GT mRNA in adipocytes. however, did not match the extent of the protein disappearance and dropped to only 50% of the level measured in age-matched fibroblasts (Fig. 2*B*). IRGT was not expressed in untreated age-matched fibroblasts, and the HepG2-GT content of these cells remained reasonably constant throughout the experiment. Note that the relative intensity of the IRGT and HepG2-GT protein bands shown in Fig. 2*A* does not necessarily reflect the relative content of these two proteins within the cells. In fact, quantitation of the relative abundance of these proteins in mature 3T3-L1 adipocytes cultured under the same conditions used in the present experiments indicates that HepG2-GT and IRGT are present at a 1.5:1 molar ratio (D. C. Calderhead and G. E. Lienhard, personal communication). Thus, despite the decrease in the protein with differentiation, the HepG2-GT remains a major form of transporter in 3T3-L1 adipocytes.

Effect of Chronic Insulin and Tolbutamide Treatments on Glucose Transporter Activity, Proteins, and mRNAs. 3T3-L1 adipocytes were treated with either insulin (0.1 μM), tolbutamide (1.5 mM), or a combination of these agents for up to 72 hr. Dose–response studies indicated that these were maximally effective doses of these agents (refs. 30 and 34; data not shown). Following each incubation period, 2-deoxyglucose uptake was measured (Fig. 3), and residual insulin concentrations were assayed by RIA. The data indicate that administration of either insulin or tolbutamide alone resulted in increased glucose transport activity and that these compounds had an additive effect when administered in combination. The effect of each treatment regimen reached a plateau by 48 hr.

A 72-hr incubation period was chosen to look at the maximal effects of these treatments on IRGT and HepG2-GT protein and mRNA levels (Table 1). The treatment regimens were initiated 9 days after induction of differentiation, at which point the IRGT and HepG2-GT proteins and mRNAs had reached their newly acquired steady-state levels in the differentiated adipocytes. Immunoblotting indicated that the functional effects observed were accompanied by an induction of the HepG2-GT protein, whereas no increase in the IRGT protein by either single treatment was demonstrable. The combined treatment actually resulted in a decrease in the IRGT content to 60% that of control (Fig. 4*A*). Northern blot analysis revealed that induction of transporter activity and HepG2-GT protein was paralleled by increases in the steady-state level of the HepG2-GT mRNA (Fig. 4*B*). IRGT mRNA was not affected by insulin treatment alone, whereas tolbu-

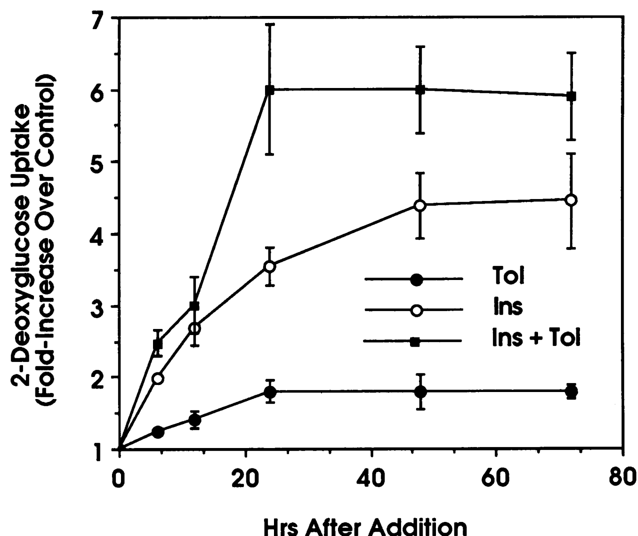

FIG. 3. Time course of the induction of glucose uptake in 3T3-L1 adipocytes following insulin (Ins) and tolbutamide (Tol) treatment. On day 9 following the induction of differentiation, adipocytes were treated with 0.1 μM insulin and/or 1.5 mM tolbutamide. Control adipocytes were maintained in the same medium lacking insulin (<1 pM) and tolbutamide. Basal glucose uptake rates were measured after the indicated incubation periods. Treatment medium was changed every 24 hr. Results are expressed as the means ± SEM of three to five determinations from three independent experiments. In cells treated only with insulin, the insulin concentration in the medium decreased to 35% of the original value during a 24-hr incubation period. In cells treated with both insulin and tolbutamide, the insulin concentration decreased to 21%, 14%, and 8% of the original value 24 hr after the first, second, and third medium changes, respectively. Thus, the average insulin concentration to which the cells were exposed during the treatment period was considerably less than 0.1 μM. The glucose concentration in the medium dropped from 20 mM to 7.8, 6.9, 4.6, and 4.0 mM for control, insulin-, tolbutamide-, and insulin plus tolbutamide-treated cells, respectively. Adipocytes incubated in medium containing from 1 to 10 mM glucose for 24 hr exhibit the same rate of 2-deoxyglucose uptake (unpublished observations).

Table 1. Effect of insulin and tolbutamide on 2-deoxyglucose uptake, glucose transporter protein, and glucose transporter mRNA levels in 3T3-L1 adipocytes

	2-Deoxyglucose uptake, pmol per mg of protein per min (n = 9)	IRGT, fold increase*		HepG2-GT, fold increase*	
		Protein† (n = 3)	mRNA (n = 2)	Protein† (n = 6)	mRNA (n = 7)
Control	128 ± 17‡	—	—	—	—
Insulin	515 ± 41 (4.0)§	1.0 ± 0.2	1.0 ± 0.3	4.1 ± 0.3	3.3 ± 1.2
Tolbutamide	222 ± 23 (1.7)§	0.9 ± 0.1	0.5 ± 0.2	2.0 ± 0.1	2.5 ± 0.2
Insulin + tolbutamide	711 ± 36 (5.6)§	0.6 ± 0.1	0.5 ± 0.3	9.7 ± 0.9	5.2 ± 0.6

3T3-L1 adipocytes were treated with 0.1 μM insulin, 1.5 mM tolbutamide, or both agents for 72 hr. Glucose transport activity was assessed by measurement of 2-deoxyglucose uptake rates. Relative levels of HepG2-GT or IRGT protein and mRNA were determined after excising or scanning bands from Western and Northern blots. Results are presented as means ± SEM or range of independent experiments.

*Relative to untreated controls.

†Insulin, tolbutamide, and insulin + tolbutamide treatments increased total adipocyte protein by 1.5-, 1.0-, and 1.4-fold, respectively.

‡This control uptake value is higher than those shown in Fig. 1 for the control adipocytes because the cells in this case have not been withdrawn from serum prior to the uptake assay to maximize the acute insulin response.

§Values in parentheses represent fold increases relative to untreated controls.

tamide and the combined treatment resulted in a 50% decrease in the level of this message. Actin mRNA levels did not change significantly with any treatment (data not shown). Although increases in the steady-state level of HepG2-GT protein appeared to correlate with observed increases in glucose transport activity under these treatment conditions, a direct quantitative comparison between levels of the two glucose transporter proteins and the 2-deoxyglucose uptake level is not meaningful because of the unknown levels and intrinsic activities of both proteins in the plasma membrane under these conditions. A dose–response study at concentrations ranging from 10 pM (no effect) to 0.1 μM (maximal) gave an ED_{50} of ≈0.7 nM for the chronic effect of insulin on all three levels of HepG2-GT gene expression (data not shown). However, it should be noted that it is difficult to accurately estimate the effective insulin concentration in these experiments because of degradation of the hormone in the medium (see the legend to Fig. 3). Thus, the true ED_{50} is probably considerably less than 0.7 nM.

DISCUSSION

The studies reported here demonstrate that chronic treatment of 3T3-L1 adipocytes with insulin increases glucose transport by raising the levels of HepG2-GT mRNA and protein, while leaving IRGT unaffected. These results are consistent with previous reports that showed that the effects of chronic insulin on basal glucose uptake activity in 3T3-L1 adipocytes were inhibited by cycloheximide and actinomycin D, suggesting a protein synthesis-dependent process (34, 36). Our results also confirm recent observations by Kosaki *et al.* (42), who reported that insulin positively regulates HepG2-GT mRNA in primary cultures of human fibroblasts. These results are also in agreement with previous studies performed in animal and human models, which indicate a positive regulatory effect for chronic insulin administration on glucose transport and insulin responsiveness, often correlating with evidence for an increased pool of glucose transporters (18–23, 43–45).

Similar to insulin, tolbutamide affected glucose transport activity by increasing levels of HepG2-GT rather than IRGT. This finding confirms observations by Zuber *et al.* (30) and provides additional evidence that some of the extrapancreatic effects of sulfonylureas may be mediated by means of a direct modulation of the glucose transport system in tissues responsible for glucose disposal. Direct enhancement of glucose transport by sulfonylureas, independent of insulin stimulation, has been described in rat adipocytes and BC3H1 myocytes (29, 31). However, a direct effect of sulfonylureas on glucose transport has not been consistently reported (28, 32).

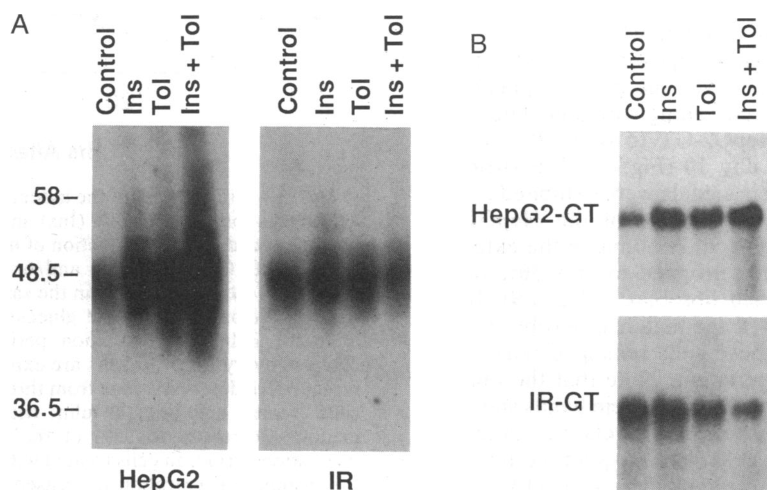

FIG. 4. Changes in HepG2 and IRGT steady-state protein and mRNA levels in response to insulin and tolbutamide treatment. 3T3-L1 adipocytes were treated for 72 hr with insulin (Ins), tolbutamide (Tol), or both agents (Ins + Tol) as described in the legend to Fig. 3. Total protein and RNA extracts were prepared from the treated and control adipocytes. (*A*) One hundred micrograms of total cellular protein was subjected to SDS/PAGE and immunoblotting with HepG2-GT carboxyl-terminal antibody (*Left*) or IRGT carboxyl-terminal antibody (*Right*). Both proteins exhibited sizes of 45–50 kDa relative to soluble protein standards (lactate dehydrogenase, fumarase, and pyruvate kinase; sizes given in kDa). (*B*) Total RNA (25 μg) was subjected to Northern blot analysis. The blots were probed with ^{32}P-labeled HepG2-GT (*Upper*) or IRGT (*Lower*) cDNAs. High-stringency wash conditions (65°C, 0.1× SSC) were used to prevent cross-hybridization. Both mRNA species exhibited sizes of ≈2.8 kilobases relative to SP6 polymerase-generated RNA size standards (not shown).

Jacobs and Jung (33) failed to detect any increase in cytochalasin B binding in a total membrane preparation of isolated rat adipocytes after a 48-hr incubation with glyburide, suggesting no change in the cellular content of total glucose transporters. Given that the IRGT appears to be much more abundant in rat adipocytes than the HepG2-GT (3), these observations are not inconsistent with the data presented in our study.

It is unclear at this point to what extent the regulation of glucose transport in 3T3-L1 adipocytes reflects that of insulin-sensitive tissues *in vivo*, especially muscle. 3T3-L1 is an immortalized cell line that likely expresses higher relative amounts of the HepG2-GT than do adipose or muscle tissue *in vivo*. If we assume, however, that our results with 3T3-L1 cells are applicable to tissues *in vivo* and consider that the HepG2-GT is capable of translocating from an intracellular storage pool to the plasma membrane in response to insulin (13, 14), one might speculate that the alleviation of insulin resistance often reported in type II diabetic patients after insulin therapy (43–45), sulfonylurea treatment (15), or a combination thereof may be due, at least in part, to the preferential effects of these modalities on expression of the HepG2-GT gene in insulin-responsive tissues. Alternatively, because the HepG2-GT is found in many human tissues (7), where its expression may be regulated by insulin and sulfonylureas, it is possible that some of the beneficial therapeutic effects of these agents are the result of improved total body glucose disposal by means of the HepG2-GT. Further study is clearly required to demonstrate this effect *in vivo* and to elucidate its tissue specificity.

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