

In Vitro Effects of a Sulfonylurea on Insulin Action in Adipocytes

POTENTIATION OF INSULIN-STIMULATED HEXOSE TRANSPORT

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ABSTRACT The mechanism(s) by which the oral sulfonylurea, tolazamide, exerts its extrapancreatic hypoglycemic effects was studied using rat epididymal adipose tissue maintained 20–44 h in the presence or absence of the drug. Insulin binding, hexose transport and glucose metabolism were compared in adipocytes isolated from the cultured tissue. In contrast to earlier reports that suggested that sulfonylureas alter the binding of insulin, neither receptor number nor affinity were changed by tolazamide treatment. The uptake of the glucose analogs 2-deoxyglucose and 3-O-methylglucose in the absence of insulin (i.e., basal) was also unchanged. However, exposure to tolazamide resulted in a potentiation of the stimulatory effects of insulin by ~30% at each hormone concentration assayed (0.4–40 ng/ml). This potentiation was dependent on the tolazamide concentration (0.003–0.30 mg/ml), with a maximal effect observed at therapeutic levels. A tolazamide analog lacking hypoglycemic activity *in vivo* was found not to enhance either basal or insulin-stimulated uptake *in vitro*. Conversion of 0.1–5.0 mM glucose to CO₂ and total lipids in the presence of insulin was also potentiated by tolazamide treatment. The inability of the drug to directly stimulate basal glucose uptake was paralleled by its lack of effect on glucose metabolism. At 50 mM glucose, where transport is no longer rate-limiting, tolazamide did not potentiate metabolism in the absence or the presence of insulin.

These studies demonstrate that tolazamide *in vitro* alters postreceptor insulin action without influencing the receptor, and suggests insulin-stimulated hexose transport as the cellular process responsible for the hypoglycemic effect of sulfonylureas in adipose tissue.

INTRODUCTION

Over the past three decades, a number of substituted sulfonylureas have been widely used as hypoglycemic

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agents in the control of maturity-onset diabetes, a condition of relative insulin deficiency. Administration of these drugs acutely stimulates insulin secretion without significantly enhancing insulin biosynthesis (1). However, chronic studies demonstrate that plasma insulin levels usually return to or even fall below pretreatment levels but the hypoglycemic effect persists (2–4). These latter observations are compatible with the suggestion that these drugs potentiate the effects of endogenous circulating insulin on target tissues. The extrapancreatic effects of sulfonylureas on several aspects of insulin action have been assessed in cells or tissues obtained from humans or animals following treatment (3–6). For example, tolbutamide treatment enhances the stimulatory effect of insulin on hexose transport in muscle (5). In addition, several reports indicate that these drugs increase insulin receptor number and/or affinity (3, 4, 6). However, the influence of binding changes on insulin sensitivity was not examined in these studies because either plasma membrane fractions (6) or cells not responsive to insulin (3, 4) were used. Although these studies demonstrate enhanced insulin binding or action following treatment with sulfonylureas, they also emphasize the difficulty in defining direct effects of these drugs in light of the numerous substrate and hormonal changes that accompany improvement of the diabetic state. In the present report, we used an organ maintenance system for adipose tissue to better characterize the cellular basis of the direct effect of the sulfonylurea tolazamide.

METHODS

Adipose tissue culture and cell isolation. Epididymal fat pads were removed from male Sprague-Dawley rats (150–225 g), minced under sterile conditions, and placed in Parker's medium 199 containing 1% (wt/vol) bovine albumin and 0.3 mg HEPES/ml, pH 7.4. Approximately 20 pieces, each weighing ~10 mg, were placed in a culture dish and incubated at 37°C for 2, 20, or 44 h under an atmosphere of 95% O₂/5%

CO₂. During this period the fat tissue was incubated in the absence or presence of 0.003–0.30 mg/ml of the Na salt of tolazamide [1-(hexahydro-1H-azepin-1-yl)-3-(*p*-tolysulfonyl)-urea]. This concentration range was chosen because it encompasses therapeutic plasma levels (0.03 mg/ml) observed in man (7). In addition to tolazamide, an analog lacking hypoglycemic activity *in vivo* [1-butyl-3-(*p*-carboxyphenylsulfonyl)urea] was also tested (personal communication, Dr. Paul O'Connell, The Upjohn Co., Kalamazoo, Mich.). Following the incubation, the minced tissue was washed with Krebs-Ringer phosphate buffer, pH 7.4, which contained 3% (wt/vol) bovine albumin, and isolated adipocytes were prepared by collagenase digestion (8). The cell suspensions were maintained in this buffer at pH 7.4 after washing to remove the collagenase. The fat tissue that had been cultured in the presence of tolazamide or its analog was also exposed to the appropriate drug during isolation of adipocytes and subsequent experiments. Aliquots from the same cell suspensions were used to assay binding, transport, and metabolism. Neither tolazamide nor its analog affected cell morphology, cytosolic space (9), or the number of cells isolated. There was no degradation of tolazamide during culture as determined by gas-liquid chromatography (10).

Insulin binding. Monoiodinated ¹²⁵I-labeled insulin (1 Ci/μmol) was prepared by the chloramine-T method and purified on talc (11). Binding of the labeled hormone (0.6 ng/ml) was determined by measuring its displacement with native insulin (0–5,000 ng/ml). In these assays, adipocytes (0.3-ml aliquots, corresponding to ~10 mg lipid or 1.25 × 10⁸ cells) were incubated with the labeled insulin for 60 min at room temperature in the presence of varying concentrations of native insulin. The incubation was terminated by centrifugation for 15 s at 10,000 *g* through 100 μl of silicone oil as has been previously described (12). Degradation of ¹²⁵I-labeled insulin was assayed by measuring its precipitability in 5% (wt/vol) trichloroacetic acid. Specific binding was determined by taking the difference between total binding of labeled hormone and that amount remaining bound in the presence of a large excess (5,000 ng/ml) of native hormone.

Hexose transport. The activity of the glucose transport system was assayed by measuring the rates of uptake of the glucose analogs 2-deoxyglucose and 3-*O*-methylglucose. 2-Deoxy-D-[1-³H]glucose (final concentration 0.1 mM, sp act 12 μCi/μmol) was added to 0.3-ml aliquots of the adipocyte preparation following a 1-h preincubation of the cells in the presence of varying concentrations of insulin (0–40 ng/ml) at 37°C in an atmosphere of 95% O₂/5% CO₂. The labeled sugar was exposed to the cells for 60 s at 37°C and was rapidly separated at the end of the incubation by centrifugation through oil. Alternatively, following preincubation with insulin (0 or 40 ng/ml) the cells were exposed to 3-*O*-[¹⁴C]methyl-D-glucose (0.1 mM, sp act 3.6 μCi/μmol) for 10 s prior to termination of transport by addition of cytocholasin b (final concentration 50 μM), which specifically blocks the glucose transport system (13). Medium and cells were again separated by centrifugation through oil. Additional incubations were carried out in which cytocholasin b had been added before either of the labeled glucose analogs to correct for sugar trapped but not transported by the cell pellets. The fat cell pellets were placed in plastic vials and dissolved in 10% Triton-X 100 before the addition of scintillation fluid and determination of radioactivity.

Glucose metabolism. Incorporation of glucose into ¹⁴CO₂ and [¹⁴C]triglycerides was monitored through the use of D-[U-¹⁴C]glucose (0.1–50.0 mM, sp act 0.03–3.00 μCi/μmol, depending on the total glucose concentration). Adipocytes were incubated in plastic vials stoppered with rubber caps in which center wells were inserted. These aliquots from the

cell suspensions (0.3 ml diluted with Krebs-Ringer solution to a final volume of 2.0 ml) were incubated with the labeled glucose for 2 h at 37°C under an atmosphere of 95% O₂/5% CO₂ in the absence or presence of 40 ng insulin/ml. The incorporation of glucose into its metabolites was terminated by addition of 0.2 ml of 6 N H₂SO₄ injected through the rubber stopper into the bottom of the vial, and ¹⁴CO₂ trapped by injection of 0.2 ml of hyamine hydroxide into the center well. The remaining medium was then extracted by the Dole's procedure (14) to determine total lipids. All incubations were performed in triplicate and corrected for appropriate blank values using cell-free samples of buffer.

Materials. Crystalline porcine insulin was a gift of the Eli Lilly & Co. (Indianapolis, Ind.). The Upjohn Co. supplied the Na salt of tolazamide and its inactive analog. Worthington Biochemical Corp. (Freehold, N. J.) supplied crude collagenase (Type I), and bovine albumin fraction V was obtained from Reheis Co., Inc. (Kankakee, Ill.). 2-Deoxy-D-[1-³H]-glucose, 3-*O*-[¹⁴C]methyl-D-glucose, and D-[U-¹⁴C]glucose were supplied by Amersham Corp. (Arlington Heights, Ill.). Carrier-free Na¹²⁵I was supplied by New England Nuclear (Boston, Mass.).

RESULTS

As illustrated in Fig. 1, exposure to 0.30 mg tolazamide/ml for 20 h did not influence the binding of [¹²⁵I]-iodoinsulin or the ability of unlabeled insulin to compete for binding with the iodinated hormone. Scatchard analysis of these data indicated no difference in insulin receptor number or affinity, and insulin degradation (11) during the assay (2–10%) was unaffected by the drugs. In other experiments, insulin binding was un-

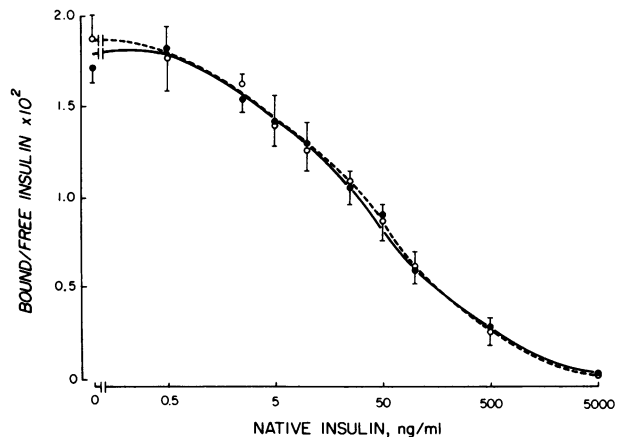


FIGURE 1 Specific insulin binding to adipocytes prepared from tissue cultured for 20 h in the absence (●) and presence (○) of tolazamide (0.30 mg/ml). Specific insulin binding at 24°C for 60 min was measured by using the oil centrifugation procedure previously reported (e.g. 12) Total, specific, and nonspecific binding were the same in both groups of cells. The concentrations of native insulin necessary to displace 50% of the labeled hormone were not significantly different by paired *t*-testing (25.3 ± 8.7 ng/ml vs. 28.5 ± 12.3 ng/ml for the control and tolazamide-treated cells, respectively). The values represent the mean ± SEM of four experiments.

altered by exposure to 0.003 or 0.03 mg tolazamide/ml, or by an additional 24 h of incubation. The inactive analog was also without effect on binding, or any other aspect of insulin action. In addition, the down-regulation of insulin binding by insulin (60 ng/ml), as shown previously in this system (15), was not affected by concomitant incubation with 0.30 mg/ml of the sulfonylurea (Fig. 2).

Transport of 2-deoxyglucose, a glucose analog not metabolized beyond phosphorylation, was not significantly different in the absence of insulin (basal) for the control and tolazamide-treated (0.30 mg/ml) cells (Fig. 3). In contrast, a significant enhancement of insulin-stimulated hexose transport was observed at each concentration of insulin used (0.4–40 ng/ml). The average increase in stimulated transport corresponded to $30.8 \pm 2.6\%$ (mean \pm SEM, $P < 0.005$). Although the insulin responsivity was greater in sulfonylurea-treated cells, the concentrations of insulin causing 50%

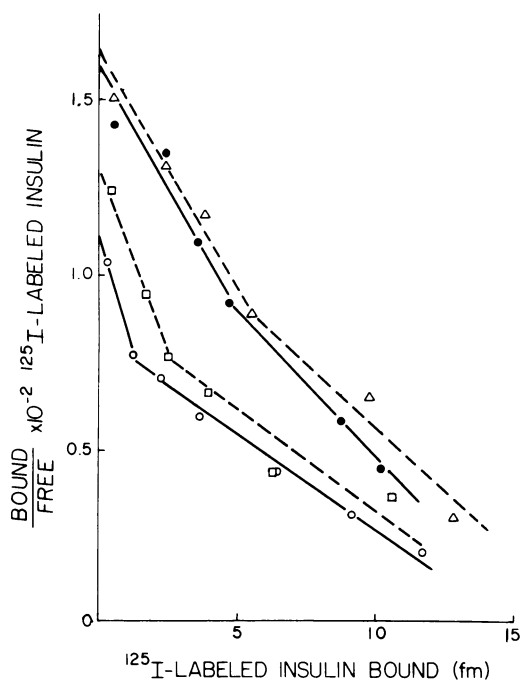


FIGURE 2 Effect of tolazamide (0.3 mg/ml) on down-regulation by insulin (60 ng/ml). These curves were generated by Scatchard analysis of the specific binding of ^{125}I -labeled insulin to cells prepared from insulin-treated tissue in the absence (\circ) or presence (\square) of tolazamide and non-insulin-treated tissue in the absence (\bullet) or presence (Δ) of tolazamide. The total and specific amounts of hormone bound in the presence of 0.6–10.6 ng insulin/ml were significantly different for each of the insulin-treated vs. noninsulin-treated conditions at $P < 0.05$ by paired t analysis, but were not significantly different for the corresponding tolazamide-treated vs. nontolazamide treated conditions. The results represent the mean of three separate experiments.

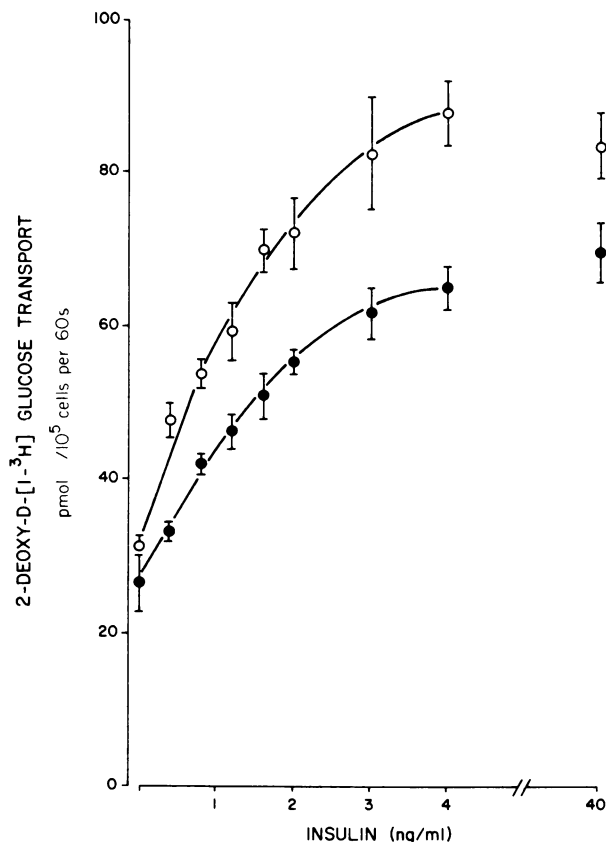


FIGURE 3 Insulin enhancement of hexose uptake by tolazamide-treated (\circ) (0.30 mg/ml) and untreated (\bullet) cells. The rate of 2-deoxy-D-[1- ^3H]glucose uptake (0.1 mM, sp act 12 $\mu\text{Ci}/\mu\text{mol}$) was measured at 37°C for 60 s. Each point represents the mean \pm SEM of eight separate experiments. Neither the basal values for the two conditions nor the insulin concentrations that caused one-half maximal stimulation (1.2 \pm 0.1 ng/ml vs. 1.1 \pm 0.1 ng/ml for the control and tolazamide-treated cells, respectively) were significantly different. At each concentration of insulin assayed, hexose transport was greater in tolazamide-treated cells ($P < 0.05$ by the paired t test).

maximal uptake were the same. Potentiation appeared to be dependent on the tolazamide concentration during culture: at 0.003 mg/ml, maximally insulin-stimulated 2-deoxyglucose uptake was 12% greater than the control ($P < 0.05$), and at 0.03 mg/ml the same effect as found for 0.30 mg/ml was observed. Culture for an additional 24 h did not change the response observed at 20 h. Uptake of 3-O-methylglucose, a glucose analog that is transported through the glucose transport system, but not metabolized by fat cells, and is a pure index of uptake phenomena (13) was also assayed in adipocytes prepared from tissue cultured in the presence or absence of tolazamide. As for the 2-deoxyglucose assay, basal 3-O-methylglucose uptake values

were not significantly altered by tolazamide treatment, however the ability of a maximally effective insulin concentration to stimulate transport was again significantly potentiated ($P < 0.05$). After 10 s of exposure to the glucose analog, values for 3-O-methylglucose uptake were as follows; in the absence of insulin, 8.4 ± 2.8 pm/10³ cells for the untreated cells vs. 11.8 ± 6.7 pm for the tolazamide-treated cells, and 23.0 ± 6.8 pm vs. 45.1 ± 13.9 pm for the corresponding conditions in the presence of 40 ng insulin/ml.

The influence of tolazamide on hexose transport was reflected by glucose metabolism. At glucose concentrations of 0.1–5.0 mM, where transport was rate-limiting (16), insulin-stimulated conversion of glucose to CO₂ and total lipids was greater in adipocytes exposed to tolazamide (Fig. 4). Insulin was present at a concentration of 1,000 μU/ml, so that metabolic responsiveness could be assessed under conditions of maximal stimulation. At each glucose concentration, basal levels of metabolism were not significantly altered. The proportions of metabolites formed were the same in the tolazamide-treated and untreated cells. In contrast, at 50 mM glucose, where transport is no longer rate-limiting (16), no effect of prior exposure to tolazamide was apparent. Thus, it would appear that the changes in metabolism observed were due predominantly to changes in transport, rather than alter-

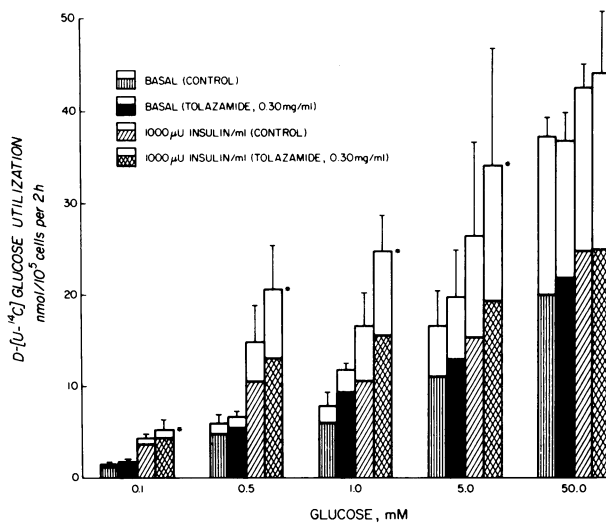


FIGURE 4 Glucose utilization in adipocytes prepared from tissue cultured in the presence or absence of tolazamide (0.30 mg/ml) for 20 h. Isolated cells were incubated for 2 h at 37°C in the absence or presence of 1,000 μU insulin/ml (40 ng/ml) and 0.1–50.0 mM U-[¹⁴C]-D-glucose (0.30–3.0 μCi/μmol); and formation of CO₂ (clear portion of bar) and total lipids (filled portion of bar) was determined. The values represent the mean ± SEM of three experiments. Asterisks denote statistically significant differences in total utilization between the control and tolazamide treated cells by the paired *t* test at $P < 0.05$.

ations in metabolic pathways or capacities. In separate experiments, tolazamide treatment of noncultured adipocytes for 2 h or tissue cultured with tolazamide for 2 h before cell isolation indicated that the drug did not influence insulin binding, basal and insulin-stimulated glucose transport, or metabolism (data not shown).

DISCUSSION

Potentiation of insulin action on target tissues by a sulfonylurea has been described in both liver and muscle. Colwell (17) found that tolbutamide enhanced insulin-stimulated glucose removal by the liver, and Feldman and Lebovitz (5) reported that the same drug administered in vivo potentiated the effect of a submaximal insulin dosage on the skeletal muscle transport system of mice. These studies along with others showing that enhancement of plasma insulin levels is not necessary for the hypoglycemic action of sulfonylureas (2–4), demonstrate that these drugs can exert their insulinlike effects at nonpancreatic sites. It is evident from our studies that tolazamide acts directly to modulate insulin action in adipocytes, and that this effect involves events distal to the receptor. These findings are not in accord with in vivo studies demonstrating that sulfonylureas increase insulin binding (3, 4, 6). This lack of agreement may be due to our use of a different sulfonylurea, but could also be attributable to changes in the ambient insulin concentration arising in vivo, which could secondarily influence the insulin receptor. For example, Beck-Nielsen et al. (3) showed that administration of glibenclamide for 10 d caused a greater reduction in the ambient insulin concentration of obese diabetics than did treatment with an equivalent diet alone. In addition, Duckworth et al. (2) reported that glyburide elicited hypoglycemic effects in some adult diabetics after 1 yr of treatment despite concomitant decreases in glucose-induced insulin secretion. In this regard, in vitro (15) and in vivo (18) studies have shown that insulin can regulate its own receptor, and more specifically that increased insulin binding is observed in states of reduced plasma insulin, e.g., fasting (19). Therefore, it seems reasonable that the enhanced insulin binding seen with long-term administration of sulfonylureas may be a function of the concurrent decrease in plasma insulin levels (2, 3) rather than a primary effect of the drugs. In addition, the relative influence of drug-induced binding changes observed in vivo on potentiation of insulin action remains unestablished.

In contrast to our findings in adipose tissue, Prince and Olefsky (20) recently reported that glyburide increases insulin receptor number and partially inhibits down-regulation of receptors by insulin in cultured human fibroblasts. They suggest that sulfonyl-

ureas increase insulin binding by inhibiting internalization of the hormone-bound receptor. This hypothesis is based on observations showing that tolbutamide inhibits clustering and presumably internalization of α_2 -macroglobulin (21), a protein which occupies the same endocytotic vesicles as receptor-bound insulin and epidermal growth factor (22). This inhibition may reflect the drug's ability at very high concentrations to partially decrease the activity of transglutaminase, an enzyme thought to play a role in the internalization process (21). However, later work from the same laboratory indicates that some inhibitors of transglutaminase were without effect on epidermal growth factor endocytosis, suggesting that epidermal growth factor and α_2 -macroglobulin do not share the same pathway of internalization, despite occupying the same vesicles (23). Although insulin internalization has recently been linked to transglutaminase activity in human fibroblasts, it was found not to be important in IM-9 lymphocytes (24). Several reports also suggest that the internalization process may be of little significance in mediating insulin action in adipose tissue. For example, Jarett and Smith (25) did not find coated pits on adipocyte plasma membranes when assayed by electron microscopy, and have also reported that clustering of receptors in fat cells is not related to biologic response (26). Taken together with our data, these studies suggest that tissue-specific differences may exist for potentiation of insulin action by sulfonylureas.

We have identified insulin-stimulated hexose transport, in the absence of binding changes, as the cellular process responsible for the hypoglycemic effect of sulfonylureas in adipose tissue, however, the mechanism(s) remains unknown. Our inability to observe changes acutely suggests that protein synthesis is required for this process. The finding that tolazamide significantly affects insulin-stimulated but not basal uptake is consistent with the theory that these two systems are distinct. This concept is supported by studies showing different temperature dependencies of the two process (27) and our observations that growth hormone inhibits only basal hexose transport (28), and that dexamethasone reduces basal transport prior to inhibiting insulin-stimulated uptake (29). In this regard, evidence has been presented suggesting that insulin stimulation of hexose transport involves translocation of carrier systems from intracellular pools to the plasma membrane (30). The possibility that sulfonylureas facilitate this phenomenon warrants further investigation.

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