Direct In Vitro Effect of a Sulfonylurea to Increase Human Fibroblast Insulin Receptors

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A B S T R A C T We have studied the effects of the oral sulfonylurea agent glyburide to modulate insulin receptors on nontransformed human fibroblasts in tissue culture. When glyburide was added to monolayers of human fibroblasts, a dose-dependent increase in the number of cell surface receptors was observed with a maximum effect (19% increase) seen at 1 µg/ml glyburide. Insulin can induce a loss of insulin receptors in these cells, and when fibroblasts are exposed to 100 ng/ml insulin for 6 h, ~60% of the initial complement of cell surface receptors are lost. When the process of insulin-induced receptor loss (or down regulation) was studied in the presence of glyburide, the drug exerted a marked inhibitory effect on this regulatory process. Thus, glyburide inhibited insulin-induced receptor loss in a dose-dependent fashion, and the maximally effective drug concentration (1 µg/ml) inhibited 34% of the receptor loss. These studies demonstrate a direct in vitro effect of this oral hypoglycemic agent to increase the number of cell surface insulin receptors or prevent their loss, presumably by slowing the rate of receptor internalization. These findings may explain the well known extrapancreatic effect of sulfonylurea agents to improve insulin-mediated tissue glucose metabolism.

INTRODUCTION

Insulin resistance is a widely described characteristic feature of adult onset, nonketotic, or type II diabetes mellitus (1), and this decrease in insulin effectiveness plays an important role in maintaining the hyperglycemic state. Insulin binding to receptors has been studied in this condition and several groups have reported that insulin receptors are decreased in type II diabetic patients (2–5). In subjects with chemical diabetes (3) there is a significant relationship between decreased insulin receptors and in vivo insulin resistance; however, this relationship is less clear in patients with fasting hyperglycemia (3, 5). Nevertheless, it is apparent that decreased insulin receptors, at least in part, contribute to the in vivo insulin resistance seen in this condition.

Sulfonylurea drugs have a well known effect to ameliorate hyperglycemia. Although these compounds can acutely stimulate insulin release if administered orally or intravenously, when given orally for a period of several months an improvement in glucose tolerance is often observed in the absence of any demonstrable increase in plasma insulin levels (6–8). This phenomenon is commonly referred to as the "extrapancreatic effect of the sulfonylureas" (6–8). Despite a fair-sized literature, the mechanism of this extrapancreatic action is unknown. We have previously demonstrated that when adult, nonketotic diabetic patients are treated for several months with a sulfonylurea, insulin binding to receptors on circulating monocytes increases (9). This effect has subsequently been confirmed using a wide variety of sulfonylurea agents in both animals (10) and man (11). All of the above studies were carried out in vivo, and to date no direct in vitro effect of these compounds has been demonstrated. Consequently, the mechanism whereby these drugs lead to an increase in cellular insulin receptors is unknown and it is not even clear whether this is a direct drug effect or related to some secondary metabolic event.

Human fibroblasts represent an excellent model to study the in vitro effects of the drug because they represent a nontransformed human cell line, which can be chronically manipulated in tissue culture. In the current studies, we have investigated the effects of glyburide, a second generation sulfonylurea agent, on insulin receptors of cultured human fibroblasts. The results demonstrate that this drug directly increases fibroblast insulin receptors and inhibits the ability of insulin to induce receptor loss.

METHODS

Materials. Minimal essential medium (MEM 140-1500) and trypsin were purchased from Gibco Laboratories, Div.

Grand Island Biological Co., Grand Island, N. Y. Fetal calf serum and bovine serum albumin were purchased from Reheis Chemical Co., Kankakee, Ill. Hepes and Tricine (N-Tris [hydroxymethyl]methylglycine), were purchased from Sigma Chemical Co. (St. Louis, Mo.). Na-^{125}I (carrier free) was purchased from New England Nuclear (Boston, Mass.). Single component crystalline porcine insulin was the kind gift of Dr. Ronald Chance, Eli Lilly and Co. (Indianapolis, Ind.). Micronase (glyburide) was obtained from the Upjohn Co., Kalamazoo, Mich.

Cell culture. Normal human fibroblasts obtained from forearm punch biopsy or from foreskin resection were cultured at 37°C, 100% humidity, and under 5% Co_2. The growth medium used was minimal essential medium pH 7.4 supplemented with 10 mM Hepes, 26 mM $NaCHO_3$, 1 mg/liter biotin, and 10% fetal calf serum. Cells were routinely subcultured (1–3 split) every 6 d in 25-ml flasks and reached confluence in 4 d. For experiments, confluent cells were subcultured (1–2 split) into 60 × 15-mm plastic dishes and were used on the 6th d after subculture. After complete trypsinization, documented by visualization under light microscopy, cell counts were determined in all experiments in control and treated dishes by use of a Coulter counter (model ZB, Coulter Electronics, Inc., Hialeah, Fla.). Cell counts between different subcultures ranged from 0.8 to 1.4 × 10^6 cells/dish. However experiments were always performed within the same subculture, which yielded cell counts with a mean coefficient of variation of 3%. Cell viability always was >95% as determined by trypan blue exclusion. Neither insulin nor glyburide resulted in a difference in cell number or viability in the experimental dishes.

Experimental procedure. Binding experiments were performed with cultured human fibroblast monolayers in 60 × 15-mm plastic dishes. After aspiration of growth media, monolayers were washed twice with 3 ml of 22°C binding buffer (minimal essential medium, pH 7.4, with 27 mM Hepes, 25 mM Tricine, 1% bovine serum albumin). Then 0.2–0.3 ng/ml ^{125}I-insulin, insulin standards, other reagents, and buffer were added to a total volume of 2 ml and the dishes were incubated in a shaking water bath (50 oscillations per minute) for 3 h at 16°C. The reaction was terminated by repetitive washing with ice-cold modified Hanks' buffer. Next, the monolayers were extracted and solubilized in 3 ml of 1 N NaOH and this solution containing all cell-associated radioactivity was counted in an automatic gamma counter. All data are normalized to a cell concentration of 10^6 cells/dish. With this approach nonspecific binding averaged 20±2% of the total counts bound, and specific binding in control cells averaged 6.6±0.5 fmol/10^6 cells at an insulin concentration of 0.3 ng/ml.

^{125}I-insulin was prepared to a specific activity of 80–150 μCi/μg by a modification (12) of the method of Freychet et al. (13) as previously described (2).

RESULTS

Fig. 1 demonstrates the effect of glyburide on ^{125}I-insulin binding to human fibroblasts in monolayer. Cells were incubated in media alone, or media containing 100, 500, or 1,000 ng/ml glyburide for 6 or 48 h at 37°C. After washing, ^{125}I-insulin binding was measured at 16°C for 3 h. As can be seen, a dose-dependent increase in insulin binding occurred at both time periods. The maximal effect was observed at the highest concentration, 1 μg/ml glyburide, which yielded a 15% (P < 0.025) and 19% (P < 0.05) increase in binding at 6 and 48 h, respectively. Scatchard
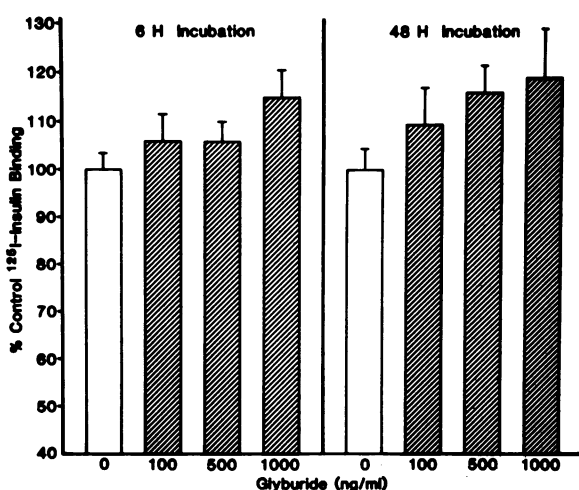

FIGURE 1 Effect of glyburide on subsequent ^{125}I-insulin binding to human fibroblasts in monolayer. Cells were incubated with various concentrations of glyburide (hatched bars) or with media alone (open bars) for 6 or 48 h at 37°C. Following washing, cells were incubated with ^{125}I-insulin (0.2 ng/ml) for 3 h at 16°C, and insulin binding was measured. The amount of ^{125}I-insulin bound in the presence of 100 ng/ml native porcine insulin was considered nonspecific binding and all data are corrected for this factor, and normalized to 10^6 cells. The absolute percent ^{125}I-insulin specifically bound in control cells was 0.197±0.017 and 0.145±0.008, respectively, following the 6- and 48-h incubation period. Data represented the mean (±SE) of five separate experiments.

analysis of studies conducted over a range of insulin concentrations demonstrated that the drug-induced increase in insulin binding seen in Fig. 1 was entirely the result of an increase in receptor number (data not shown).

Fig. 2 demonstrates the effect of glyburide on insulin-mediated receptor loss. Cells were incubated at 37°C for 6 or 48 h with insulin-free media, media containing 100 ng/ml insulin, or 100 ng/ml insulin plus various concentrations of glyburide. After washing to remove all extracellular unbound insulin, and dissociation of receptor-bound insulin in insulin-free media (37°C, 1 h), ^{125}I-insulin binding was measured at 16° for 3 h. The indicated concentrations of glyburide were present during the incubation and dissociation phases, but not during the binding phase of the experiment. Preincubation with media alone had no effect on insulin binding, whereas preincubation with 100 ng/ml insulin led to a 50 and 60% decrease at 6 and 48 h, respectively, and Scatchard analysis showed that this effect was due to an insulin-induced decrease in receptor number. When cells were incubated with 100 ng/ml insulin plus various concentrations of glyburide, a dose-dependent inhibition of receptor loss is seen. Thus, incubation with 100 ng/ml insulin plus 1 μg/ml glyburide for 6 h resulted in only 66% of the effect seen with insulin alone, representing a 34% (P < 0.01)

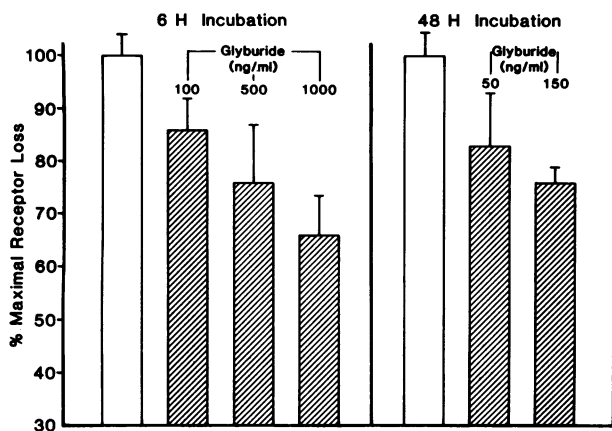**6 H Incubation**   **48 H Incubation**

FIGURE 2 Effect of glyburide on insulin-mediated receptor loss in human fibroblasts in monolayer. Cells were incubated with media alone or media containing 100 ng/ml insulin. Insulin led to a decrease in the absolute percent specifically bound from 0.197±0.017 to 0.098±0.008 (50% decrease) and from 0.145±0.008 to 0.058±0.003 (60% decrease) at 6 and 48 h, respectively. For purposes of data presentation this amount of receptor loss is taken as 100% (open bars) and the degree of receptor loss in the presence of insulin plus glyburide (hatched bars) is expressed as a percentage of the effect seen with insulin alone. Data represent the mean±SE for five separate experiments.

inhibition of insulin-mediated receptor loss. As can be seen in Fig. 2, a longer incubation with glyburide led to a greater inhibition of receptor loss, when similar drug concentrations are compared. For example, a 48-h incubation with 150 μg/ml glyburide resulted in a 24% ($P < 0.025$) inhibition of receptor loss, whereas 6 h incubation with 100 ng/ml glyburide led to only a 14% inhibition. Thus, the inhibitory effects of this sulfonylurea compound on insulin-induced receptor loss are both dose and time dependent.

DISCUSSION

In this report we have demonstrated a direct effect of a sulfonylurea, glyburide, to increase insulin receptors in nontransformed cultured human fibroblasts. In addition, this drug markedly inhibited the ability of insulin to induce a loss of cell surface insulin receptors. All of these effects were observed at concentrations within the therapeutic plasma concentration range (14).

The potential clinical significance of these findings is reasonably straightforward and may clarify the cellular mechanism of the extrapancreatic action of these drugs, as well as help elucidate the cause of the insulin resistance in patients with type II diabetes mellitus. By increasing insulin binding and by attenuating the insulin-mediated loss of cell surface receptors, sulfonylureas should lead to an increased sensitivity to endogenous and exogenous insulin. In this regard, we have recently demonstrated that after

sulfonylurea treatment, overall in vivo insulin resistance decreases in type II diabetic patients (15). Further studies designed to correlate the effect of these agents on insulin receptors with the effect on in vivo insulin action in individual subjects should lead to a more quantitative understanding of the role of decreased insulin receptors in the insulin resistance of this condition.

It seems likely that the current findings may explain the extrapancreatic effect of the sulfonylureas, but the biochemical basis underlying the cellular mechanism whereby these drugs increase insulin receptors is unknown. However, the current results, coupled with recent observations by Haigler et al. (16) and Davies et al. (17) suggest a possible mechanism. Thus, it has been proposed that the concentration of cell surface insulin receptors reflects a dynamic state of equilibrium between receptor degradation or internalization and receptor synthesis and reinsertion, and recent work from our own laboratory using cultured human fibroblasts is consistent with this formulation (18). After initial cell surface binding, insulin receptor complexes can be internalized by absorptive endocytosis, and several groups (17, 19, 20) have suggested that this pathway may mediate the process of insulin-induced receptor loss. Davies et al. (17) have found that transglutaminase, an intracellular enzyme found in many cell types, plays an essential role in receptor mediated endocytosis of α2-macroglobulin and polypeptide hormones. This enzyme covalently links occupied receptors in specialized areas of the plasma membrane termed coated pits, and this facilitates internalization. In addition, these studies have shown that a variety of competitive inhibitors of transglutaminase also inhibit hormone-induced endocytosis of receptors. Interestingly, the sulfonylurea tolbutamide, inhibits fibroblast transglutaminase (17) and this effect correlates with the drug's ability to inhibit clustering, an early step in hormone-induced endocytosis of receptors. Thus, in the steady state a drug-induced decrease in transglutaminase activity would lead to a decrease in the rate of receptor internalization, while the rate of receptor insertion remains the same, leading to a modest increase in surface insulin receptors (Fig. 1). When cells are exposed to high concentrations of insulin, the rate of receptor internalization is augmented and, by inhibiting transglutaminase activity, glyburide could slow this process, attenuating the ultimate decrease in the concentration of surface receptors. Because the rate of receptor internalization is much greater in the presence of media insulin, the effects of the drug on insulin-mediated receptor loss (Fig. 2) would be more prominent than on basal levels of insulin receptors.

It should be noted that human fibroblasts are not a quantitatively important target tissue for insulin action

and do not play a significant role in overall glucose homeostasis. However, these cells do possess high affinity, specific insulin receptors that exhibit kinetic and functional properties analogous to those of receptors studied on classic target tissues (18, 20). Therefore, it seems likely that the current findings in fibroblasts are reflective of the events occurring in other insulin target tissues.

In summary, the above studies demonstrate for the first time a direct effect of a sulfonylurea to enhance insulin binding and inhibit insulin-mediated receptor loss in vitro. These findings may explain the extra-pancreatic effects of sulfonylurea drugs. A better understanding of the mechanisms of action of these drugs could help: (a) elucidate the mechanisms of the insulin resistance in type II diabetes mellitus, (b) improve our therapeutic understanding of the use of these drugs in the treatment of diabetes, and (c) lead to the development of newer more efficacious drugs with a more specific mechanism of action.

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