Insulin-like and Insulin-enhancing Effects of the Sulfonylurea Glyburide on Rat Adipose Glycogen Synthase

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SUMMARY

The effects of long-term exposure of cultured rat adipose tissue to glyburide were examined on glycogen synthase activity. Glyburide alone caused an increase in the activity ratio (low glucose-6-P/high glucose-6-P) of glycogen synthase, and enhanced insulin's activation of the enzyme. The glyburide effects were time dependent, requiring fat pieces to be exposed to the drug for at least 10–20 h. The glucose concentration in the culture medium was also important: optimal concentrations of glucose were 10–20 mM. Glyburide acted to shift the insulin dose-response curve to the left by a factor of 2.5, but did not enhance the effects of maximal concentrations of the hormone. The Ka of the glyburide effects was about 2.0 μM. If glucose was omitted during the 20-min incubation with or without insulin, the increase in the activity ratio of glycogen synthase by glyburide was unaffected, but the enhancement of insulin action was reduced. Because these data indicate that glyburide's actions are glucose dependent, we propose that the sulfonylurea is probably acting to increase glucose transport, thus allosterically increasing the activity of a synthase phosphatase by glucose-6-P. The net result of this would be increased dephosphorylation and activation of glycogen synthase. DIABETES 1985; 34:281–86.

Sulfonylureas are important drugs for the management of hyperglycemia in type II diabetes.[1] Although the stimulation of insulin release from the pancreatic B-cells is felt to be a primary mechanism by which these drugs function,[2] a growing body of literature exists that documents a variety of extrapancreatic effects, both in vivo[3,4] and in vitro.[5,6] Among the chronic extrapancreatic effects reported are enhancement of insulin's stimulation of: (1) glucose transport in rat[3] and mouse[7] diaphragm and adipose tissue,[8] (2) lipogenesis in hepatocytes[9] and adipose tissue,[4] and (3) glycogen synthesis from glucose in cultured hepatocytes.[5] On the other hand, data on the extrapancreatic effects after acute treatment with these drugs have tended to be contradictory. For example, acute treatment of mouse[7] or rat[10] diaphragms with sulfonylureas did not alter insulin's effect on hexose transport, while in perfused rat hindlimb,[11] not only was insulin-stimulated uptake enhanced by these drugs, but so also was basal uptake of hexose. Additionally, contradictory results have been reported concerning sulfonylureas' ability to increase the number of insulin receptors after chronic exposure to the drugs.[8,12]

One of insulin's best-defined intracellular targets is the enzyme glycogen synthase, a key regulatory point in the synthesis of glycogen. It has been well established that this enzyme is hormonally controlled via covalent phosphorylation-dephosphorylation.[13,14] The observation that insulin could increase the proportion of active dephosphorylated glycogen synthase in adipocytes in the presence or absence of glucose[15,16] allowed the dissection of insulin's action into two separate but mutually reinforcing mechanisms: (1) a glucose-independent mechanism, which can be activated directly by concanavalin A, anti-insulin receptor antibody, and trypsin, and presumably involves the generation of putative insulin mediators; and (2) a glucose-dependent mechanism, which can be activated by H_2O_2, increased pH, and oxidized glutathione, and which depends on the enhanced formation of the regulator glucose-6-P (see ref. 17 for review).

Because of the key role played by glycogen synthase, and the intricate mechanisms controlling it, it was of interest to determine whether sulfonylurea treatment might affect this enzyme's activity, either directly or indirectly. In the present report, the glucose-dependent, insulin-like, and insulin-enhancing effects of the second generation sulfonylurea glyburide on glycogen synthase from rat adipose tissue are presented.

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MATERIALS AND METHODS

Materials. Collagenase (type CLS) was from Worthington Biochemical Corp. (Freehold, New Jersey). UDP-[^{14}C]-glucose was prepared as previously described.[18] Bovine serum albumin (BSA) was obtained from Sigma (St. Louis, Missouri), and prescreened according to Lawrence et al.[16] All culture material was obtained through the Tissue Culture Core lab of the University of Virginia Diabetes Research and Training Center. Glyburide was kindly provided by Dr. John Wheeler of Upjohn Company (Kalamazoo, Michigan), and all other materials were of the highest quality available.

Animals. Adult, male, Sprague-Dawley rats (170–180 g), obtained from Hilltop Laboratories, were fed standard rat chow ad libitum.

Preparation of glyburide. A 2-mM stock solution of glyburide was prepared fresh daily in 0.1 M glycine (pH 11). After dilution to the desired concentration in Dulbecco's modified Eagle's medium plus 1% BSA (DMEM), 3% BSA/Kreb's-Ringer phosphate (KRP: 10 mM Na_2PO_4, 128 mM NaCl, 5.2 mM KCl, 1.4 mM $CaCl_2$, 1.4 mM $MgSO_4$, pH 7.4), or 1% BSA/KRP, the pH was readjusted to 7.4.

Culture of fat pieces. After cervical dislocation, the rat epididymal fat pads were removed in a sterile manner and placed in sterile 3% BSA/KRP (30°C, pH 7.4). Under a laminar flow hood, the fat pads were washed with fresh 3% BSA/KRP, then transferred in batches of 5–8 to 25-ml Petri dishes containing 40 ml DMEM plus 1% BSA with or without glyburide present. The DMEM contained 25 mM glucose unless otherwise indicated. Each pad was snipped into pieces of about 8–9 cm^3. The Petri dishes were incubated at 37°C under 90% air/10% CO_2 for 20 h, unless otherwise indicated. After culture, the fat pieces were washed three times with 3% BSA/KRP, then adipocytes were prepared by the method of Rodbell,[19] with or without glyburide present. Adipocytes were filtered through nylon mesh, washed three times in 3% BSA/KRP, then suspended in an equal volume of 1% BSA/KRP (pH 7.4) plus 5 mM glucose with or without glyburide. Adipocytes prepared by this method averaged 80% viability and 60% yield compared with adipocytes prepared from fresh adipose tissue, as evidenced by the ability to exclude trypan blue.

Incubation of adipocytes with insulin and glyburide. One milliliter of adipose suspension was added to 1 ml of 1% BSA/KRP plus 5 mM glucose with or without insulin and/or glyburide (see tables for concentrations). The incubation with insulin was carried out in a shaking 37°C water bath for 20 min, and was terminated by adding 2 ml of 1% BSA/KRP to the cell suspension, pouring the suspension into glass homogenizing tubes (Thomas, size AA), briefly centrifuging (200 × *g*, 10 s) to collect the cells on the surface of the liquid, removing the infranatant by suction, and freezing the cell pellet by immersing the homogenizing tube in liquid nitrogen. The frozen pellets were stored in liquid nitrogen until they were processed.

Homogenization of adipocytes and assay of glycogen synthase. The frozen adipocyte pellets were homogenized in 0.5 ml of 10 mM tricine buffer containing 10 mM EDTA, 100 mM KF, and 0.1% mercaptoethanol (pH 7.5 at 4°C). The homogenates were centrifuged for 2 min at 8500 × *g* (7°C) in a Beckman Microfuge B. The infranatants were removed from beneath the lipid layer with a syringe, and served as the source of glycogen synthase. Glycogen synthase was assayed by measuring the incorporation of [^{14}C]-glucose from UDP-[^{14}C]-glucose into glycogen using the more sensitive low glucose-6-P/high glucose-6-P method described by Guinovart et al.[20] The reaction mixture (60 μl) contained 0.2 mM UDP-[^{14}C]-glucose (about 250,000 cpm/μmol), 0.1 mM (low) or 10 mM (high) glucose-6-P, plus 6.7 mg glycogen, 33 mM Tris, 58 mM KF, and 13 mM EDTA (pH 7.8). After 20 min of incubation at 30°C, the reaction was terminated by the filter paper method of Thomas et al.[21] or by the rapid filtration method of Oron and Larner.[22] Both methods gave equivalent results.

During the initial phases of these investigations, it was noted that an incomplete precipitation of glycogen occurred when these assays were terminated by either procedure (N. Altan, V. M. Altan, and C. F. W. Schwartz, unpublished observations). Inclusion of the ethanol-soluble salt LiBr was found to remedy this problem, and thus all glycogen synthase assays reported here used 10 mM LiBr in the termination step. After extensive washing of the filters carrying the precipitated glycogen, the amount of [^{14}C]-glucose incorporated into the glycogen was determined using a liquid scintillation counter. The results are expressed in terms of the glycogen synthase activity ratio, which is an indicator of the amount of active glycogen synthase (measured with low glucose-6-P mixture) divided by the total glycogen synthase activity (measured with high glucose-6-P mixture).

Data presentation. Although within each experiment the effects of insulin and glyburide were very consistent (see Table 1), from day to day the absolute values for glycogen synthase activity and its activation by these agents varied widely. This problem has been reported previously for adipocytes.[23–25] To minimize the variations, we have elected to present the data as the difference from the control value (Figure 1, A and B) or in terms of a ratio of glyburide versus control treatments (Tables 2 and 3). It will be readily apparent that the standard deviations can be quite large (see, for example, Table 3), but we feel that with the number of experiments involved and the statistical analyses, the conclusions drawn are supportable.

Statistics. The significance of the results were evaluated using Student's *t*-test.

RESULTS

Many of the reports of extrapancreatic effects of sulfonylureas have indicated the necessity for prolonged exposure of the animal or tissue to the drug (see, for example, refs. 3 and 8); thus, our initial studies with glyburide were carried out on cultured fat pieces derived from the epididymal fat pads. Table 1 illustrates a typical experiment. It can be seen that, after 20 h of culturing fat pieces with the drug, glyburide (40 μM) had not only enhanced the increase in the activity ratio of glycogen synthase observed in the presence of submaximal insulin (124%), but had also stimulated the basal activity ratio in the absence of insulin (70%). The effect of insulin alone was 56%. The effect of glyburide was not due to a direct effect on glycogen synthase, since no change in the activity ratio was observed in homogenates of adipocytes prepared with or without glyburide present, or in the activity ratio of purified rabbit skeletal muscle glycogen synthase exposed to the drug (data not shown). Additionally, filtration

TABLE 1
Activation of glycogen synthase by chronic glyburide treatment, and potentiation of insulin action*

Glyburide (40 μM)	Insulin (6.7×10^{-10} M)	Glycogen synthase activity ratio
–	–	0.082 (±0.003)
–	+	0.130 (±0.003)
+	–	0.139 (±0.004)
+	+	0.184 (±0.004)

Number of determinations = 2.
*Fat pieces were cultured in DMEM containing 25 mM glucose in the absence or presence of glyburide (40 μM) for 20 h, then adipocytes prepared and incubated with or without insulin and/or glyburide for 20 min in KRP plus 5 mM glucose (METHODS). The glycogen synthase activity ratios were then determined.

of adipocyte extracts over Sephadex G-25 did not alter the observed results (data not shown), suggesting that this was a covalent change, and not an apparent change due to altered concentrations of metabolites.

The enhancement of both the basal and the insulin-stimulated glycogen synthase activity ratios by glyburide was time dependent (Figure 1A), requiring 10–20 h of drug exposure in the culture system for significant stimulation to occur. Because enhanced glucose transport has been implicated in the mechanism of the extrapancreatic effects of sulfonylureas,[8] the effects of varying glucose concentrations in the culture medium were tested. Although it was not possible to culture the fat pieces in a glucose-lacking medium, decreasing the concentration of glucose to 2.5 mM reduced glyburide's efficacy by twofold compared with 25 mM glucose (Figure 1B) ($P < 0.02$). There was no significant difference between glyburide treatments of fat pieces in 2.5 mM or 10 mM glucose. Omission of glucose during the final 20-min incubation without insulin did not alter glyburide's activation of glycogen synthase, but markedly reduced the drug's effect to further enhance the activation of this enzyme when insulin was present (Table 2). The enhancement effect could be recovered if glucose was restored during the incubation (data not shown).

As shown in Figure 2, glyburide treatment of fat pieces in DMEM containing 25 mM glucose for 20 h caused a significant shift to the left in the dose-response curve for insulin stimulation of glycogen synthase activity. This effect could be seen whether the fat pieces were cultured in the presence of 5, 10, or 25 mM glucose, but the response was optimal at the higher glucose concentration (data not shown). In the presence of maximal glyburide (40 μM), the stimulation of glycogen synthase was observable at insulin concentrations as low as 6.7×10^{-11} M. There was no potentiation by glyburide at saturating insulin concentrations (6.7×10^{-9} M). The half-maximal concentration of insulin was 6.7×10^{-10} M in the absence of glyburide, and 3.0×10^{-10} M in the presence of glyburide.

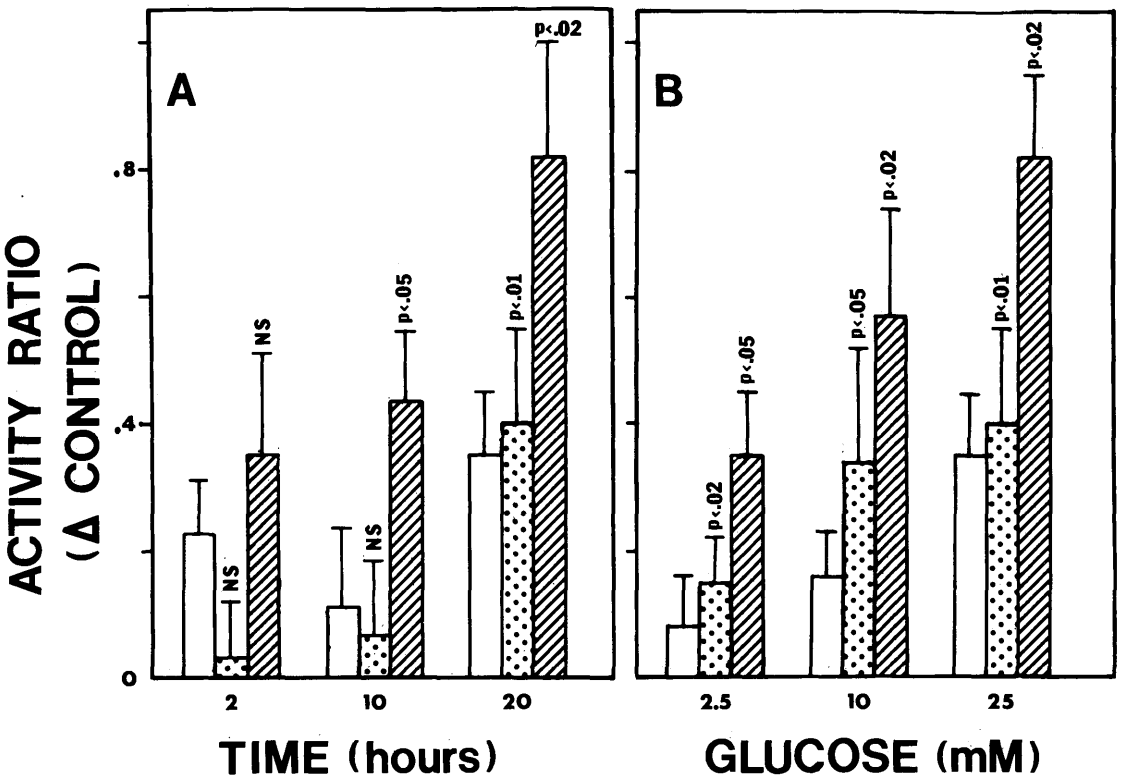

FIGURE 1. Time and glucose dependencies for glyburide-induced activation of glycogen synthase. Fat pieces were cultured in the absence or presence of 40 μM glyburide as described in the text for increasing time (A), or increasing glucose for 20 h (B). Adipocytes were prepared by collagenase digestion as described in the text, and were incubated with or without 6.67×10^{-10} M insulin for 20 min in the presence of 5 mM glucose (with or without 40 μM glyburide). Values are expressed as the difference from control (without exposure to glyburide or insulin). Open bars, insulin treatment; dotted bars, glyburide treatment; and lined bars, glyburide plus insulin treatment. Data represent the mean ± SD of duplicate values from three separate experiments. The indicated statistics are comparisons of glyburide treatments compared with control values or glyburide and insulin treatments compared with insulin values. NS, not significant.

TABLE 2
Activation of glycogen synthase by chronic glyburide treatment*

Glyburide (μM)	Ratio of activity ratios	
	− Insulin	+ Insulin (1.67×10^{-10} M)
0.0	100	100
1.0	115 ± 13 (NS)	95 ± 15 (NS)
5.0	138 ± 23 ($P < 0.05$)	119 ± 27 (NS)
10.0	136 ± 20 ($P < 0.05$)	121 ± 42 (NS)
20.0	161 ± 26 ($P < 0.02$)	145 ± 32 (NS)
40.0	171 ± 22 ($P < 0.01$)	147 ± 35 (NS)

NS = not significant.
*Fat pieces were cultured in the absence or presence of 40 μM glyburide (METHODS). The subsequently prepared adipocytes were incubated with or without insulin and/or glyburide in the *absence* of glucose for 20 min, then processed for glycogen synthase activity ratio determination. The results are the means of duplicate determinations from three separate experiments.

The reciprocal experiment examining the glyburide concentration dependency with and without suboptimal insulin (6.7×10^{-10} M) showed detectable glyburide effects at 0.75 μM, with half-maximal activation occurring at about 2.0 μM (Table 3). In contrast to the effect of glyburide on the insulin dose-response curve (Figure 2), insulin had no significant effect on the glyburide Ka (Table 3).

DISCUSSION

In this article, evidence has been presented that chronic glyburide treatment was not only able to induce an activation of adipocyte glycogen synthase, but was also able to enhance insulin's activation of this enzyme. These chronic effects were time dependent (Figure 1A) and were enhanced with increased glucose concentrations (between 2.5 and 20 mM) during the culture period (Figure 1B). The action of glyburide was concentration dependent (Table 3), and functioned to increase the efficacy of insulin treatment by shifting the insulin dose-response curve to the left (Figure 2).

That chronic sulfonylurea treatment can *enhance* insulin's stimulation of glucose uptake or utilization has been well established in fat,[10] skeletal muscle,[7] and hepatocytes.[9] However, in cultured adipose tissue, no direct effects of sulfonylureas alone on glucose uptake or metabolism were detected by Musbah and Furman,[10] who used glyburide, or by Maloff and Lockwood,[8] who used tolazamide. Both groups did observe a sulfonylurea-enhanced insulin stimulation of glucose utilization by adipocytes. The maximum stimulation of glucose utilization by insulin reported by Musbah and Furman was twofold under conditions in which 500 μU/ml of insulin was a suboptimal dose. In contrast, results from this laboratory and others[16,26] have shown adipocytes to respond maximally to insulin at concentrations of 100–250 μU/ml, with 5–30-fold stimulations. The data presented by Musbah and Furman[10] may therefore have been derived from insulin-insensitive adipocytes; thus, it may have been difficult to observe the smaller direct effects of sulfonylureas. Likewise, the results reported by Maloff and Lockwood[8] for the magnitude of insulin-stimulated glucose utilization (2.5–fold at 1.0 mU/ml insulin) were small compared with the results of others.[16,26] Indeed, a report by Malchoff et al.[27] from this same laboratory showed that adipocytes derived from cultured adipose tissue had a fivefold stimulation of 2-deoxyglucose transport by insulin. It is possible, of course, that the lack of a direct effect reported by Maloff and Lockwood could be due to subclass differences between the sulfonylurea they used (tolazamide) and the one used in the present communication (glyburide). Nevertheless, it is readily apparent from these reports that the culturing of adipose tissue may give rise to cells that are less responsive to insulin. Indeed, even in our system, the basal and insulin-stimulated activities of glycogen synthase in the presence of glucose are somewhat depressed compared with fresh cells, although the magnitude of the insulin stimulation was the same (data not shown). It is possible that the assay system we used (glycogen synthase activation) is less sensitive than glucose uptake or utilization to alterations of the adipose

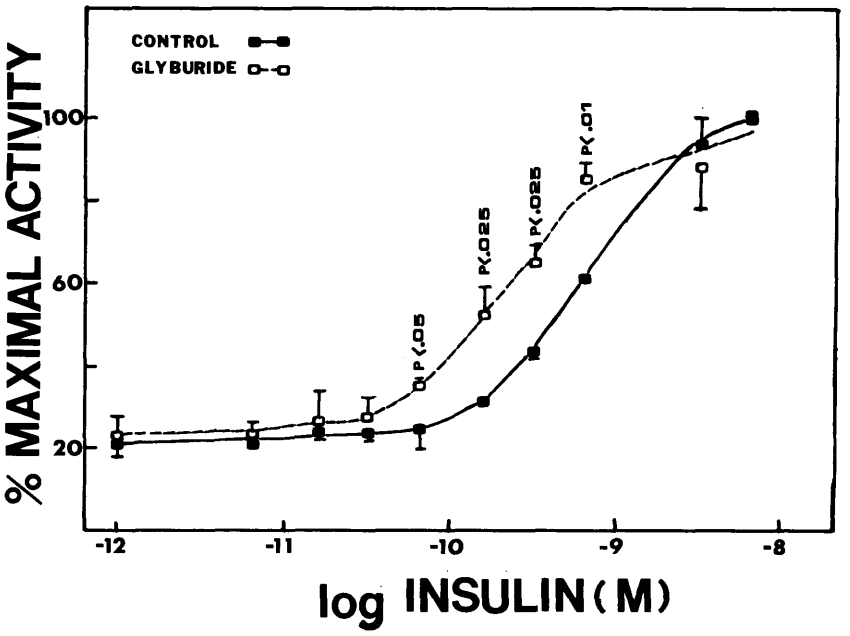

FIGURE 2. Effect of glyburide on the activation of glycogen synthase by varying insulin concentrations. Fat pieces were cultured as described in Figure 1 for 20 h in the presence of 25 mM glucose with or without 40 μM glyburide. After preparation of adipocytes, varying concentrations of insulin were added to the cells in the presence or absence of 40 μM glyburide. All incubations had 5 mM glucose. The basal activity ratio of glycogen synthase (absence of insulin) was 0.04 for these experiments. Data represent the mean ± SD of duplicate values from four separate experiments.

TABLE 3
Activation of glycogen synthase by chronic glyburide treatment: dose response*

A		B	
Glyburide (μM)	Ratio of activity ratios	Glyburide (μM)	Ratio of activity ratios
0.0	100	0.0	100
0.5	113 ± 21 (3) NS	0.5	120 ± 18 (4) NS
0.75	120 ± 6 (3) $P < 0.05$	0.75	143 ± 14 (3) $P < 0.05$
1.0	129 ± 25 (6) $P < 0.01$	1.0	138 ± 30 (5) $P < 0.01$
2.0	130 ± 19 (3) $P < 0.01$	2.0	162 ± 26 (4) $P < 0.05$
5.0	149 ± 8 (5) $P < 0.001$	5.0	194 ± 42 (6) $P < 0.001$
10.0	154 ± 34 (3) $P < 0.05$	10.0	195 ± 56 (4) $P < 0.05$
20.0	165 ± 33 (5) $P < 0.01$	20.0	216 ± 79 (6) $P < 0.01$
40.0	171 ± 27 (4) $P < 0.01$	40.0	208 ± 61 (5) $P < 0.01$

*Fat pieces were cultured for 20 h in DMEM plus 25 mM glucose in the presence of increasing concentrations of glyburide. The derived adipocytes were then incubated in KRP plus 5 mM glucose and increasing glyburide in the absence (A) or the presence (B) of 1.67×10^{-10} M insulin. The values in parentheses are the number of experiments done for each glyburide concentration, each experiment being done in duplicate. NS = not significant.

tissue that may occur in culture. The reports of Musbah and Furman[10] and Maloff and Lockwood[8] both contrast with that of Lopez-Quijada et al.,[28] who presented evidence that the uptake of glucose by adipose tissue was directly stimulated by tolbutamide, as was the synthesis of glycogen from glucose. The magnitude of this latter effect was equivalent to the almost twofold stimulation seen by a suboptimal concentration of insulin (20 μU/ml), which agrees closely with our results.

Because the increases in the glycogen synthase activity ratio reported here were stable to gel filtration (data not shown), the mechanism through which glyburide activated this enzyme could not have involved a simple allosteric activation of the enzyme by glucose-6-phosphate, but could very well have been due to activation via covalent dephosphorylation. Insulin has been proposed to activate glycogen synthase through glucose-dependent and -independent mechanisms.[15,17] The glucose-dependent mechanism was shown by Lawrence and Larner[29] and Oron and Larner[30] to involve a dose-dependent allosteric activation of a synthase phosphatase by increased intracellular levels of glucose-6-P, with the phosphatase then activating glycogen synthase. The results shown in Figure 1B, which indicate a glucose concentration-dependent activation of glycogen synthase by insulin or glyburide plus insulin, are consistent with this hypothesis.

It is conceivable that, in the presence of glucose but without insulin, glyburide acts to stimulate a synthase phosphatase or inhibit a synthase kinase. The net effect of either of these actions would be a decreased phosphorylation state of glycogen synthase, resulting in an increased activity ratio. Wray and Harris[31] reported that tolbutamide was able to inhibit cAMP-dependent protein kinase activity in fat pads, and Osegawa et al.[6] reported that acute treatment with tolbutamide, acetohexamide, or glyburide could inhibit isoproterenol-induced lipolysis while activating low-Km phosphodiesterase in adipocytes. Blumenthal[32] showed that chlorpropamide was able to inhibit glucagon-stimulated glucose production and decrease the concentration of cAMP in perfused rat liver. It is conceivable that glyburide might be acting to counteract β-receptor actions by decreasing cAMP concentrations, perhaps via an enhancement of low-Km phosphodiesterase activity. This would in turn lead to inhibition of cAMP-dependent protein kinase, causing a shift in the equilibrium between phosphorylation/dephosphorylation reactions, and resulting in an increased rate of glycogen synthase dephosphorylation. However, this mechanism cannot totally explain the actions of glyburide in this system.

The ability of glyburide to enhance insulin's activation of glycogen synthase could be due to an increase in the number or the affinity of insulin receptors. Prince and Olefsky[12] have reported that glyburide caused a small increase in the number of insulin receptors in human fibroblasts, and inhibited insulin-induced downregulation of its receptor. However, Fleig et al.[33] were unable to detect any alteration in insulin receptor number or affinity in hepatocytes treated with glyburide, nor could glyburide affect insulin-induced downregulation of its receptor. Maloff and Lockwood[8] were also unable to detect an alteration in insulin receptor number or affinity in adipose tissue cultured with tolazamide, even though this sulfonylurea could potentiate insulin's stimulation of 3-O-methyl glucose uptake. The results presented in Figure 2 of our article, however, could be explained by a glyburide-induced increase in the affinity of insulin receptors for insulin, which would give rise to the observed leftward shift in the dose-response curve. Because insulin binding data were not obtained in our studies, we can do no more than speculate on this point. Obviously, more investigation is required to resolve these differences.

Glyburide's ability in the presence of glucose to activate glycogen synthase and enhance insulin's activation of this enzyme suggests that the drug may be acting through the same glucose-dependent mechanism as insulin. A plausible hypothesis to explain the results could involve the indirect activation by glyburide of the same glucose-6-P–sensitive synthase phosphatase that is activated by insulin via enhanced glucose transport. In the presence of glucose, treatment of fat pieces with glyburide during culture would activate the synthase phosphatase to an extent dependent on the amount of glucose-6-P generated by the enhanced glucose transport, and thus would lead to an increased activation of glycogen synthase. Because the transport of glucose is already enhanced in the presence of glyburide, a subsequent treatment of the adipocytes with a suboptimal

concentration of insulin would result in an apparently greater activation of glycogen synthase when compared with non-glyburide-treated fat pieces. The net effect would be a leftward shift in the insulin dose-response curve (Figure 2). If glucose were to be omitted during the incubation with insulin, two results might be predicted. First, because the fat pieces have already been chronically exposed to glyburide, a direct activation of glycogen synthase will have occurred. When glucose is removed from the incubation medium (as in the 20-min incubation with or without insulin), the glycogen synthase activity ratio will already be elevated compared with non-glyburide-treated tissue (Table 2). Second, in the absence of glucose during the incubation with suboptimal insulin, the glucose-dependent mechanism of insulin action cannot occur,[17] and thus no potentiating effect by glyburide should be detected. The data in Table 2 and Figure 2 are consistent with this second hypothesis. Further studies are required to delineate the actual mechanism(s) involved.

The results presented in this article demonstrate that the sulfonylurea glyburide is capable of exerting direct insulin-like and insulin-potentiating effects on nonpancreatic (adipose) tissue in vitro as measured by the glycogen synthase activity ratio. Because the changes in glycogen synthase activity reported here are stable (and thus probably represent changes in the net phosphorylation state), this enzyme is a very useful marker for glyburide action. Coupled with the use of the very sensitive, low glucose-6-P/high glucose-6-P assay, which allows easy detection of minor changes in the enzyme's activity, we have been able to detect insulin-like effects of glyburide in this system. It would thus be of interest to examine the long-term in vivo effects of this drug on the activity of adipose tissue glycogen synthase in human subjects. Because the effects of glyburide reported in this present work are dependent on the presence of glucose in the extracellular medium, it seems possible that a direct effect of this drug on the glucose transporter may be involved, either increasing the total number of transporters, or causing a redistribution of transporters from intracellular pools.[34,35]

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