Modulation by a Sulfonylurea of Insulin-dependent Glycogenesis, but Not of Insulin Binding, in Cultured Rat Hepatocytes

Evidence for a Postreceptor Mechanism of Action

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SUMMARY

To detect potential direct effects of the sulfonylurea glyburide on hepatic carbohydrate metabolism, we tested whether the drug was capable of modulating insulin binding and glycogenesis in primary cultured hepatocytes. After 24-h culture under serum- and hormone-free conditions, cells were incubated with or without 10^{-8} M insulin and/or glyburide (0.1–5.0 μg/ml) for another 24 h. Then, specific ^{125}I-insulin binding and basal and insulin-stimulated glycogen synthesis were determined. Acute addition of glyburide to previously untreated cells did not modulate any of these parameters. Incubation for 24 h with 2 μg/ml of glyburide did not affect the DNA and protein content of the dishes. Cellular glycogen content and basal glycogenesis also remained unchanged by glyburide in hepatocytes incubated in the absence of insulin, but glycogen content was increased and basal glycogen synthesis decreased in insulin-pretreated cells. In contrast, glyburide increased insulin-stimulated glycogenesis in a dose-dependent fashion in both insulin-pretreated and control cells by enhancing responsiveness, but not sensitivity, toward insulin. Pretreating hepatocytes with 10^{-8} M insulin caused a 40% reduction in specific insulin binding. Glyburide did not modulate insulin binding or degradation in control cells nor was insulin-induced regulation of insulin receptors affected. These results demonstrate a direct dose-dependent effect of a sulfonylurea on an insulin action toward hepatic carbohydrate metabolism, and suggest that this effect is mediated by a postreceptor mechanism. DIABETES 33:285–290, March 1984.

Sulfonylureas are widely used in the treatment of non-insulin-dependent diabetes mellitus (NIDDM). When acutely administered, they stimulate the release of insulin from the endocrine pancreas.[1-3] Chronic application of these agents, however, always ameliorates glucose intolerance of NIDDM while the effects on insulin secretion are controversial. Both an increase[4,5] and decrease[6-9] of insulin secretion were observed, the latter favoring the concept of an "extrapancreatic action" of the sulfonylureas.[10-12] Several groups demonstrated an increase in the concentration of insulin receptors on circulating mononuclear cells of sulfonylurea-treated NIDDM patients[13,14] or on rat or mice liver plasma membranes after application of such agents in vivo.[15-17] In vitro studies using chemically defined systems, which alone would allow detection of direct effects of sulfonylureas on insulin binding and action, are sparse and the results controversial. While Prince and Olefsky[18] demonstrated an increase in insulin binding to cultured human fibroblasts and an inhibition in insulin-induced downregulation of the receptor by glyburide, Vigneri et al.[19] were unable to detect such actions of glyburide and various related sulfonylureas in this and other cell types. Maloff and Lockwood[20] recently described potentiation by tolazamide of insulin-stimulated hexose transport in cultured adipocytes, while insulin binding was not affected. In perfused livers from fed rats, Blumenthal[21] found that chlorpropamide acutely enhanced the inhibition by insulin of glucagon-stimulated glucose release. However, he did not check for eventual effects of this agent on insulin binding, nor was it possible to discriminate between changes in insulin sensitivity and insulin responsiveness.

Therefore, we investigated the effect of glyburide, a second-generation sulfonylurea, on insulin binding and action on hepatic carbohydrate metabolism in a chemically defined system of primary cultured hepatocytes. Our results demonstrate that glyburide potentiates insulin-stimulated glycogenesis in normal and insulin downregulated liver cells, while insulin binding and insulin-induced receptor regulation were not affected. This establishes the existence of a direct

Preliminary results of parts of this work were presented at the Annual Meeting of the European Society for Clinical Investigation, Luxembourg, April 22–24, 1982, and were published in abstract form [Eur. J. Clin. Invest. 1982; 12 (Suppl.):12].
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Received for publication 8 June 1983.

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action of glyburide on hepatic carbohydrate metabolism via a postreceptor mechanism.

MATERIALS AND METHODS

Materials. Plastic culture dishes were purchased from Nunc, Wiesbaden, FRG. Collagenase, enzymes, and substrates for biochemical determinations were from Boehringer, Mannheim, FRG. Monoiodinated porcine ^{125}I-(Tyr A14)-insulin and unlabeled purified pork insulin were generous gifts of Bruce Frank through the courtesy of Dr. Enzmann, Eli Lilly and Company, Bad Homburg, FRG. D-^{14}C(U)-glucose (348 mCi/mmol) was obtained from New England Nuclear, Dreieich, FRG. Glyburide (HB 419) was obtained from Hoechst, Frankfurt, FRG. Stock media for hepatocyte culture were purchased from Seromed, Munich, FRG; glutamine and antibiotics from Gibco, Grand Island, New York; and fetal calf serum (FCS) from Paesel, Frankfurt, FRG. Bovine serum albumin (BSA) from Behringwerke, Marburg, FRG was dialyzed extensively against Earl's balanced salt solution before use. All other chemicals used were of analytic grade.

Animals. Male Wistar rats (Chbb strain, Thomae, Biberach, FRG) were fed a commercial rat chow (Fundel, Ulm, FRG) ad libitum. They were adapted to a controlled lighting cycle, with the dark period lasting from 7 p.m. to 7 a.m., for at least 1 wk. After a 24-h fast, rats weighing 150–180 g were anesthetized with pentobarbital sodium for hepatocyte isolation between 9 a.m. and 10 a.m.

Hepatocyte isolation and culture. Hepatocytes were isolated by recirculating liver perfusion in situ using a recently described modification procedure[22–24] of Berry and Friend.[25] Only cell suspensions of >90% viability, as judged by trypan blue exclusion, were suspended in Dulbecco's MEM supplemented with 5% FCS, 10 mM glucose, 2 mM lactate, 2 mM glutamine, essential and non-essential amino acids, 40 mM $NaHCO_3$ (pH 7.4), 50 μU/ml penicillin, and 50 μg/ml streptomycin. Suspensions were then plated onto plastic culture dishes at a density of 30,000 cells/cm^2. Viable cells attached to the culture dish within 1 h of incubation at 37°C in a humidified atmosphere of 92% air/8% CO_2. Inoculation medium was replaced by serum- and hormone-free medium supplemented with 1 mg/ml BSA at 4 h after seeding. At 24 h the medium was removed and new medium with or without insulin (10^{-8} M) and/or glyburide at the indicated concentrations was added. Insulin was again added at 30, 36, and 42 h. At 48 h, cultures were washed three times with serum- and hormone-free medium, with 20-min incubations at 37°C after each washing step. After the third wash, cells were incubated for another 30 min in control medium before insulin binding and glycogen synthesis were studied. This procedure allowed for complete dissociation of any prebound insulin, since no difference in ^{125}I-insulin binding was detected between control cells and hepatocytes pretreated with 10^{-7} M unlabeled insulin for 30 min (data not shown).

Insulin binding and degradation. Insulin binding and degradation by the monolayers were assessed in complete control medium with 10 mM glucose and 1 mg/ml BSA at 37°C under 92% humidified air/8% CO_2, in order to create conditions identical to those of the glycogen synthesis assay. In experiments studying the association kinetics, hepatocytes were incubated for 5–120 min with tracer amounts of ^{125}I-insulin (10^{-10} M). Parallel cultures were run with an excess amount of unlabeled insulin (10^{-6} M) to correct for unspecific binding (10–15% of total hormone bound). After the respective incubation periods, the incubation medium was aspirated and an aliquot analyzed for TCA (10%)-soluble and TCA-precipitable radioactivity to correct for labeled degradation products released into the medium. The monolayers were washed five times with ice-cold, phosphate-buffered saline, then solubilized by 1 ml of 0.1% SDS, and finally precipitated with 10% TCA to determine cell-associated TCA-soluble and -insoluble radioactivity.[26] Alternatively, cells were extracted by 1 ml of 4 M urea, 1 M acetic acid, and 0.1% Triton X-100 and analyzed by gel filtration over Sephadex G-50 fine (1.8 × 30 cm), equilibrated and eluted with the same buffer.[27,28] Both methods gave similar results for the analysis of cell-associated intact and degraded label after 30-min incubations, since the proportion of radioactivity eluting in the void volume with a high-molecular-weight fraction that is also precipitated by TCA amounts to only 2.2 ± 0.31% (mean ± SEM, N = 5). Binding data were corrected for cell-associated degraded radioactivity and for unspecific binding in the presence of 10^{-6} M unlabeled insulin. Both binding and degradation data were normalized to the DNA estimated from triplicate parallel dishes in each experimental group.

Glycogen synthesis. Monolayers were incubated at 37°C with serum- and hormone-free medium supplemented with 10 mM glucose (0.1 μCi/μmol) and 1 mg/ml BSA, with or without various concentrations of insulin. Both control conditions and 10^{-7} M insulin gave linear incorporation of labeled glucose into cellular glycogen over a period of at least 4 h. Incubation periods of 2 h were used in routine experiments. Hepatocytes were then washed four times with ice-cold, phosphate-buffered saline with 40 mM unlabeled glucose. Then, cells were homogenized by sonication in 0.33 N KOH. Fifty microliters of carrier glycogen (40 mg/ml) were added to a 200-μl aliquot of the cell homogenate and brought to 35% KOH. Glycogen was extracted by incubation at 95°C for 30 min, followed by precipitation with 95% ethanol in the cold overnight. The precipitate containing the glycogen was sedimented at 13,000 × *g* for 6 min and washed twice with ethanol. The final precipitate was solubilized in water and the radioactivity was determined in duplicate from 200-μl aliquots in 10 ml of LUMAGEL (Baker, Deventer, Holland). Control plates were run at 4°C to correct for unspecifically associated label. Data are presented as nmol glucose incorporated into glycogen/μg of DNA/h.

Chemical determinations. Glycogen was extracted from the monolayers as described for the glycogen synthesis assay, except that glucose was omitted from the washing buffer and no carrier glycogen was added. The final ethanol precipitate was solubilized in 50 mM sodium azide (pH 4.5) and digested with amyloglucosidase. After centrifugation, glucose was determined with the glucokinase/glucose-6-phosphate dehydrogenase method (Boehringer, Mannheim, FRG). Results are expressed as nmol glucose/μg DNA.

DNA was determined from aliquots of the cell homogenate by incubation with 0.33 N KOH at 37°C for 1 h followed by precipitation in the cold (0°C) with TCA at a final concentration of 5%. After centrifugation, the pellet containing the DNA was resuspended in 1 ml 5% TCA and the DNA extracted for 15 min at 95°C. TCA-insoluble material was removed by

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centrifugation and the DNA in the supernatant was determined by the diphenylamine reaction.[29]

Protein was determined according to Lowry et al.[30]

Statistics. At the least, triplicate plates were analyzed for each experimental condition from each rat. Results are given as means ± SEM of 4 or 5 rats. Statistical evaluations were done utilizing Student's *t* test for paired samples when one experimental value was compared with one control, and by means of Dunnett's modification of the *t* test for multiple processes when more than one mean was compared with the same control.[31]

RESULTS

DNA, protein, and glycogen content. Preincubating cells with glyburide at 0.2 μg/ml and 2.0 μg/ml between 24 and 48 h of culture did not affect the DNA, protein, and glycogen contents of the dishes at 48 h. Similarly, the improved cell maintenance by insulin resulting in increased DNA and protein contents (13.2 ± 0.9 versus 11.5 ± 0.7 μg DNA/dish and 1.61 ± 0.10 versus 1.07 ± 0.18 mg protein/dish in insulin pretreated versus control cells, means ± SEM of 4 separate experiments, $P < 0.05$) was not modulated by the drug. However, it slightly but significantly augmented the increase in cellular glycogen content caused by 10^{-8} M insulin (control: 37.61 ± 1.59 nmol glucose/μg DNA; insulin: 42.63 ± 1.92 nmol/μg DNA; insulin + 2 μg/ml glyburide: 48.11 ± 2.06 nmol/μg DNA, N = 4; $P < 0.05$ for insulin versus control and insulin + glyburide versus insulin).

Insulin binding and degradation. Glyburide did not affect the time course of ^{125}I-insulin binding or degradation in this system, regardless of whether 0.2 or 2.0 μg/ml were added directly to the binding assay (data not shown). Similarly, preincubation of the cells for 24 h with 2 μg/ml glyburide did not alter insulin association and degradation kinetics of the hepatocyte monolayers (data not shown).

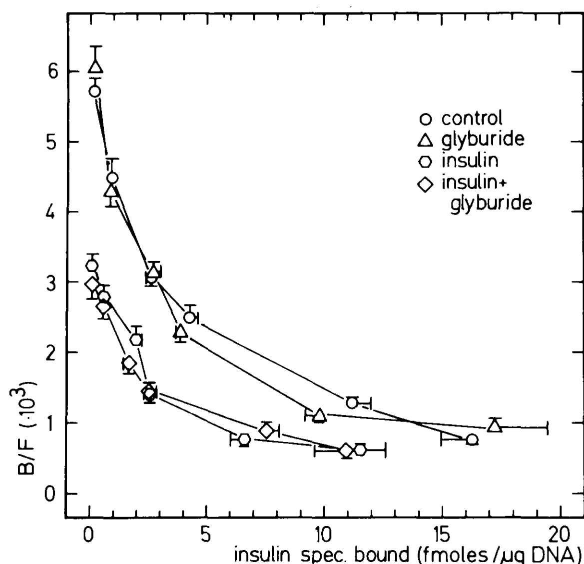

FIGURE 1. Effect of insulin and glyburide on insulin binding in cultured hepatocytes. Cells were preincubated with or without insulin (10^{-8} M) and/or glyburide (2 μg/ml) between 24 and 48 h of culture. Thereafter, specific insulin binding was assessed as described in METHODS. The graph gives a Scatchard analysis of the binding data normalized to DNA (means ± SEM of 5 different experiments).

To answer the question as to whether glyburide could modulate the number or affinity of insulin receptors of cultured hepatocytes, cells were preincubated with and without glyburide (2 μg/ml) and/or insulin (10^{-8} M) between 24 and 48 h of culture. Scatchard analysis[32] of the binding data obtained from 30-min incubations with 2×10^{-11} M labeled insulin and with or without various concentrations of unlabeled hormone yielded the typical curvilinear Scatchard plot indicative of negative cooperativity of one class of binding sites or of two classes of receptors with different ligand affinity (Figure 1). Preincubating the cells with insulin resulted in significant downregulation of the receptors with an obvious decrease in receptor concentration and no change in affinity. At all concentrations between 2×10^{-11} and 10^{-8} M insulin, binding was significantly ($P < 0.05$ or less) decreased by about 40%. Insulin binding and insulin-induced downregulation of insulin receptors were not affected by pretreating hepatocytes with 2 μg/ml glyburide (Figure 1).

Glycogen synthesis. To check whether glyburide might modulate insulin actions on hepatic carbohydrate metabolism, incorporation of ^{14}C-glucose into glycogen was studied in the absence and presence of the sulfonylurea. Addition of 2 μg/ml glyburide at 48 h to previously untreated hepatocyte cultures did not alter basal or insulin-stimulated rates of incorporation of ^{14}C-glucose into glycogen (data not shown).

Similarly, basal glycogen synthesis remained essentially unchanged by preincubating cells between 24 and 48 h with 2 μg/ml of glyburide (Figure 2A). However, insulin-stimulated glycogen synthesis was significantly increased in glyburide-pretreated cells compared with untreated control cultures at all insulin concentrations tested (Figure 2A and C, $P < 0.05$ at 10^{-11} and 10^{-10} M, $P < 0.01$ at 10^{-9} to 10^{-7} M). At 10^{-7} M insulin, glyburide enhanced the response to the hormone by 154%. Insulin sensitivity remained unchanged with an ED_{50} of $1.2 \pm 0.3 \times 10^{-9}$ M in glyburide-treated cells and $1.4 \pm 0.2 \times 10^{-9}$ M in control hepatocytes.

Preincubating hepatocytes with 10^{-8} M insulin for 24 h significantly reduced the basal rate of ^{14}C-glucose incorporation into glycogen from 0.365 ± 0.021 nmol · μg DNA^{-1} · h^{-1} in control cells to 0.282 ± 0.014 nmol · μg DNA^{-1} · h^{-1} ($P < 0.01$, Figure 2A and B). In addition, these cells were both less insulin-sensitive and -responsive (Figure 2B and D). Insulin pretreatment increased the insulin concentration effecting half-maximal stimulation of glycogen synthesis from $1.4 \pm 0.2 \times 10^{-9}$ M in control cells to $9.0 \pm 0.5 \times 10^{-9}$ M ($P < 0.01$). Glycogen synthesis was stimulated maximally to 187 ± 38% of basal or by 0.245 ± 0.093 nmol · μg DNA^{-1} · h^{-1} in insulin-pretreated cells (Figure 2B and D), but to 225 ± 11% or by 0.456 ± 0.050 nmol · μg DNA^{-1} · h^{-1} in control cultures (Figure 2A and C, $P < 0.05$).

Preincubation with 10^{-8} M insulin plus 2 μg/ml glyburide caused a further decrease of basal glycogen synthesis (0.215 ± 0.015 versus 0.282 ± 0.014 nmol · μg DNA^{-1} · h^{-1} with insulin preincubation, $P < 0.01$). At merely 10^{-8} and 10^{-7} M insulin, insulin-stimulated glycogen synthesis was increased over cultures preincubated with insulin alone (Figure 2B). However, when the absolute data were expressed in terms of Δ glucose incorporation (insulin minus basal) or as percent of basal rates (Figure 2D) it became apparent

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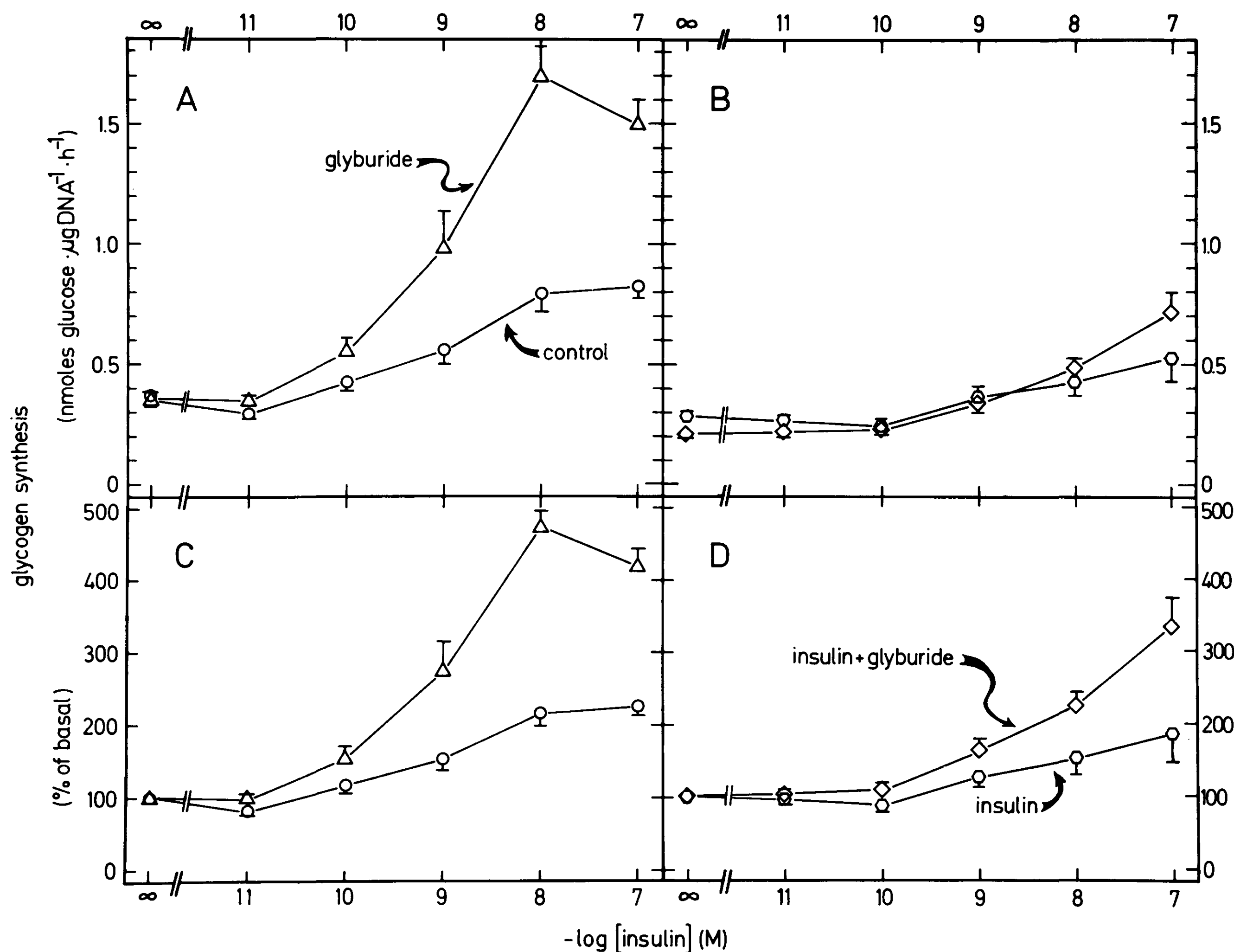

FIGURE 2. Long-term effect of insulin and glyburide on glycogen synthesis in cultured hepatocytes. Cells were cultured in the presence or absence of 10^{-8} M insulin and/or 2 µg/ml glyburide between 24 and 48 h after seeding. Then, glycogen synthesis from ^{14}C-glucose (10 mM, 0.1 µCi/µmol) was determined for 2 h. Data are given as nmol glucose incorporated into glycogen/µg DNA/h in control (A) or insulin-pretreated cells (B), and as the percentage of basal glycogenesis in the absence of insulin in control (C) or insulin-pretreated hepatocytes (D). Results represent the means ± SEM of 5 different experiments.

that glyburide preincubation increased insulin responsiveness in downregulated cells at all insulin concentrations $\geq 10^{-9}$ M ($P < 0.05$ at 10^{-9} M, $P < 0.01$ at 10^{-8} and 10^{-7} M). Insulin sensitivity ($ED_{50} = 7.1 \pm 0.6 \times 10^{-9}$ M) was not significantly different from hepatocytes pretreated with insulin alone. Similar results were obtained when glycogen synthesis was measured at 5 mM glucose (data not shown).

Cells were preincubated with various concentrations of glyburide (0.1–5.0 µg/ml) to investigate whether this potentiation of insulin action was dose-related. Insulin-stimulated glycogen synthesis was significantly increased over control cells at all glyburide concentrations ≥0.5 µg/ml ($P < 0.05$ at 0.5 µg/ml, $P < 0.01$ at ≥1 µg/ml). Maximal effects were observed at 2 µg/ml and half-maximal responses were elicited by 0.66 ± 0.03 µg/ml of the drug (Figure 3).

DISCUSSION

The mechanisms by which the sulfonylureas exert their extrahepatic effects are still largely unknown. The present study investigated the effect of glyburide, a potent second-generation sulfonylurea, on insulin binding and insulin action toward glycogenesis in the liver as the prime regulator of glucose homeostasis. Our data provide the first evidence for a direct effect of a sulfonylurea on the action of insulin on hepatic carbohydrate metabolism mediated by a postreceptor mechanism.

After completion of this work, Salhanick et al.[33] reported a direct effect of the sulfonylurea tolazamide on insulin-stimulated lipogenesis in a similar system of cultured hepatocytes. While these authors studied the effect of tolazamide on the long-term action of insulin (16 h) on lipogenesis within the first 20 h of culture, the present study investigated the effect of glyburide on an acute action (2 h) of insulin on glucose metabolism after preincubation with the drug between 24 and 48 h of culture. Nevertheless, the results of both studies are largely compatible in demonstrating insulin-dependent effects of two different sulfonylureas on two different metabolic pathways regulated by insulin that appear to be mediated by postreceptor mechanisms.

Our data are also in accordance with the experiments of Maloff and Lockwood[20] who demonstrated an insulin-dependent effect of tolazamide on hexose transport in isolated

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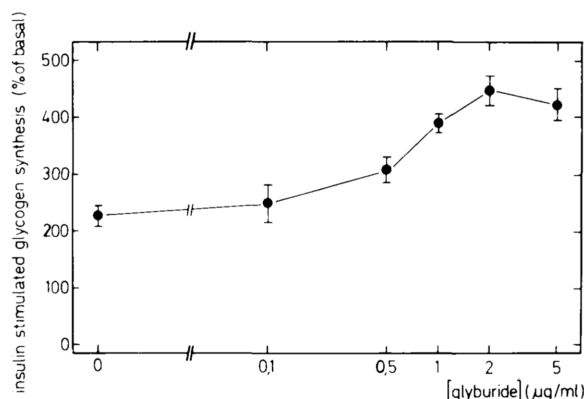

FIGURE 3. Dose-response relation of the action of glyburide on insulin-stimulated glycogen synthesis. Hepatocytes were pretreated with or without various concentrations of glyburide between 24 and 48 h of serum- and hormone-free culture. Then, glycogen synthesis from ^{14}C-glucose (10 mM, 0.1 µCi/ml) was determined in the presence and absence of 10^{-7} M insulin for 2 h. Data represent the means ± SEM of 5 different experiments.

adipocytes while insulin binding remained unchanged. Insulin binding of isolated adipocytes was not modulated by tolbutamide.[34]

The present results demonstrating the absence of direct effects of glyburide on insulin binding and insulin-induced downregulation of insulin receptors is in contrast to the findings of Prince and Olefsky,[18] who showed a 19% increase in insulin binding and a 34% inhibition of insulin-induced receptor loss in cultured human fibroblasts. However, Vigneri et al.[19] were unable to reproduce these results, and Salhanick et al.[33] estimated the small (12%) increase in high-affinity binding observed only in hepatocytes cultured in the absence of insulin as of questionable physiologic significance. Earlier studies investigated the effects of sulfonylureas on insulin binding by in vivo application of the drug for various lengths of time and thereafter analyzing insulin binding in mononuclear cells[13,14] or liver membranes.[15-17] Since sulfonylureas are not only capable of increasing insulin secretion but might also affect the pancreatic secretion[35-37] and hepatic extraction[38] of glucagon, alterations of insulin binding in such in vivo protocols can hardly be ascribed to direct effects of the sulfonylurea.

The present investigation also documented insulin-dependent effects of glyburide on hepatocytes cultured under conditions that downregulated insulin binding by 40%. Parallel to the decrease in insulin binding, sensitivity of glycogen synthesis toward insulin was significantly reduced. In addition, the basal rate of incorporation of ^{14}C-glucose into glycogen and insulin responsiveness was decreased in these monolayers. Whether this is causally related to the slight but significant (13.3%) increase in cellular glycogen remains to be clarified. While increasing the responsiveness toward insulin in downregulated cells, glyburide further decreased basal glycogenesis. This was accompanied by a further 12.8% increase in glycogen content compared with hepatocytes treated with insulin alone. A similar parallel decrease in insulin binding and insulin sensitivity concerning glycogen synthesis after preincubation with insulin has been described by Capeau et al.[39] in cultured Zajdela rat hepatoma cells. Although these cells also decreased their basal glycogen synthesis after insulin preincubation, insulin responsiveness (expressed as percent of basal glycogenesis) remained unaltered. Amatruda et al.[40] demonstrated a decrease in sensitivity to insulin of cultured hepatocytes after exposure to insulin for 16 h, while the responsiveness of aminoisobutyric acid uptake to insulin was unaltered. In the same system, responsiveness of lipogenesis to insulin was significantly enhanced in downregulated cells while insulin sensitivity remained unchanged. In accordance with these data, the present study demonstrated that the responsiveness of a target cell toward insulin is a function of postbinding events rather than of insulin binding, and that basal glycogen synthesis may be regulated independently of the insulin responsiveness of this metabolic pathway.

Peripheral glyburide levels in the range of 0.5 nmol/ml serum (≈0.25 µg/ml) result from a single oral dose of 7.5 mg glyburide.[41] After chronic application of 5–20 mg glyburide daily for at least 1 yr, steady-state concentrations of up to 1500 nM (≈0.75 µg/ml) were estimated in the peripheral serum of NIDDM patients with normal renal function.[42] Since the drug administered orally enters the circulation via the portal venous system, and because it is metabolized by the liver,[43] sinusoidal concentrations might be even higher. Therefore, the concentrations producing significant effects in the present in vitro study may well be within the range presented to the liver in vivo.

In conclusion, the present investigation demonstrates a potentiation of insulin-dependent glycogenesis by the direct action of the sulfonylurea glyburide and suggests that this action is mediated by a postreceptor mechanism. It offers one possible explanation for the "extrapancreatic effect" of the sulfonylureas postulated from clinical observations.

ACKNOWLEDGMENTS

We are greatly indebted to Sabine Steudter and Sylvia Höhn for their excellent technical assistance. The typing of the manuscript by Rita Kley is gratefully acknowledged. This study was supported by the Deutsche Forschungsgemeinschaft (SFB 112, A 10).

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