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Sulphonylureas in Vitro Do Not Alter Insulin Binding or Insulin Effect on Amino Acid Transport in Rat Hepatocytes

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Summary. The effects of four sulphonylureas (gliclazide, glibenclamide, chlorpropamide and glipizide) on insulin binding and insulin action were studied in vitro using primary cultured rat hepatocytes. Cells were cultured for 20 h in the absence or presence of the sulphonylurea. The binding of insulin to rat hepatocyte monolayers was not altered in cells previously exposed to gliclazide at 0.7, 7.0 or 70 μg/ml; and to glibenclamide, chlorpropamide, or glipizide at 0.1, 1.0 and 10 μg/ml. Insulin-induced down regulation was not affected by a simultaneous exposure of hepatocyte monolayers to any of the four agents. The stimulatory effect of insulin on α-aminoisobutyric acid uptake by the cells was not modified following exposure to the drugs. These studies indicate that the sulphonylureas tested do not have a direct effect on insulin receptors in hepatocytes; and that, in vitro, they do not alter the post-receptor events involved in the insulin-induced stimulation of amino acid transport in these cells.

Key words: Insulin receptors, aminoacid transport, primary cultures of hepatocytes, sulphonylureas.

There is some evidence that part of the therapeutic effects of sulphonylureas on glucose metabolism is due to an extrapancreatic action of the drugs, but the mechanism involved in this action is unclear at present. It has been suggested that sulphonylureas, administered in vivo [1–4] or employed in vitro [5], may enhance insulin action through increasing the hormone binding to its receptors; in these studies, the possible consequence of this increase in binding on the hormone biological function was not specifically investigated. It has, however, been reported recently that insulin effect is enhanced while insulin binding remains unaltered following exposure of adipose tissue to tolazamide in vitro [6]. Indeed, a causal implication of insulin receptors in any extrapancreatic effect of sulphonylureas remains to be demonstrated.

The present study was designed to seek a direct effect of sulphonylureas on the liver in vitro. The effects of gliclazide, glibenclamide, glipizide and chlorpropamide on insulin binding and insulin effect on amino acid transport in primary cultured rat hepatocytes were investigated.

Materials and Methods

Chemicals

α-Amino(1-^{14}C)isobutyric acid (specific activity, 60 Ci/mol), and (^{3}H)inulin (specific activity, 500 Ci/mol) were purchased from the Radiochemical Centre, Amersham, Bucks, UK; Na^{125}I was obtained from the Commissariat à l'Energie Atomique, Saclay, France. Porcine monocomponent insulin was a gift from the Novo Research Institute, Copenhagen, Denmark and from Novo France (Paris). Waymouth's MB 752/1 medium, penicillin, streptomycin and fetal calf serum were from Grand Island Biological Company, Grand Island, New York; defatted bovine serum albumin (BSA, Fraction V) was from Sigma Chemicals, St. Louis, Missouri. Gliclazide was provided by Dr. C. Nathan, Laboratoires Servier, Paris. Chlorpropamide and glipizide were obtained from Laboratoires Pfizer, Orsay, France. Glibenclamide was from Laboratoires Hoechst, Paris. All other reagents were of the best grade commercially available.

Primary Cultured Hepatocytes and Incubation Conditions

All experiments were performed with primary cultures of adult rat hepatocytes, prepared as previously described [7], except that cells were plated at a concentration of 1×10^6/ml. After 4 h at 37 °C in the presence of 10% fetal calf serum (attachment period), hepatocyte monolayers were incubated for 20 h in serum-free Waymouth's medium in the presence or absence of insulin at 3 μmol/l and/or the sulphonylurea at varying concentrations. The medium was changed twice during the 20-h incubation period, with insulin and/or the sulphonylurea being reintroduced with fresh medium at each change. In some experiments, the level of gliclazide in the incubation medium at each medium change was determined by high performance liquid chromatography and was found to represent 50%–80% of the initial amount of the drug. After the final removal of the medium at the end of the 20-h incubation period, hepatocyte monolayers were extensively washed in insulin-free or drug-free medium at 37 °C before the determination of insulin binding and transport studies. The washing procedure, which consisted of three successive incubations (each with a duration of 25 min) in insulin-free medium at 37 °C, was designed to dissociate receptor-bound insulin in hepatocyte monolayers previously exposed to the hormone for 20 h. This procedure was observed to

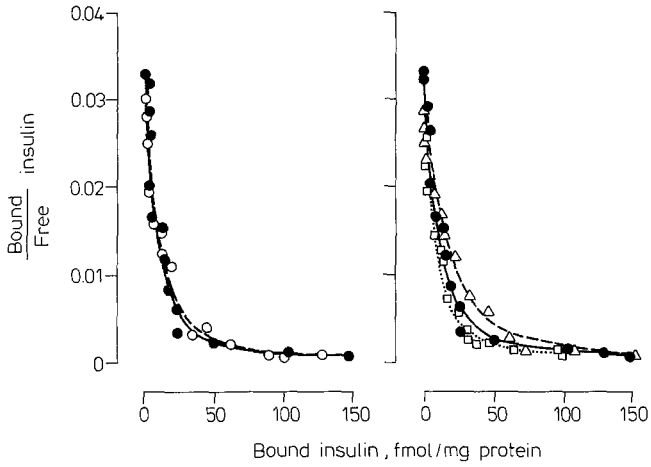

Fig. 1. Scatchard analysis of insulin binding to hepatocyte monolayers following exposure for 20 h to gliclazide. Insulin binding was measured at 37 °C under steady state conditions as described in Materials and Methods. The data correspond to a representative experiment; each point is the mean of triplicate determinations. Key: ●——● control, O-----O gliclazide (0.7 μg/ml) □·····□ gliclazide (7.0 μg/ml) △-----△ gliclazide (70 μg/ml)

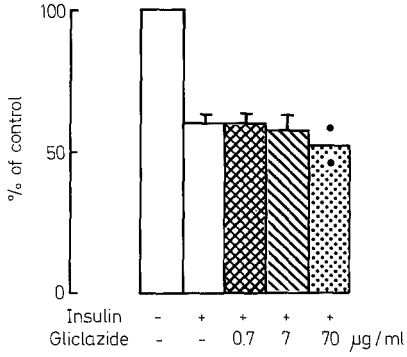

Fig. 2. Effect of gliclazide on insulin-induced down regulation in hepatocyte monolayers. Hepatocytes were incubated for 20 h without or with insulin at 3 μmol/l in the absence or presence of gliclazide at 0.7, 7.0 and 70 μg/ml. Control cells were incubated under the same conditions, except that insulin was added 10 min before the washing procedure, as described in Materials and Methods. Insulin binding was measured at 37 °C (1 h-incubation) with ^{125}I-insulin (0.3 ng/ml) and data were corrected for non-specific binding. Results are expressed as a percentage of respective controls, and represent the mean ± SEM of three to four separate experiments except for gliclazide at 70 μg/ml where each dot represents separate experiments with each point run in quadruplicate. SEM of replicates ranged from 2 to 5% of each value

cause the removal of about 90% of cell-associated insulin. Determination of insulin concentration in the medium by radioimmunoassays and rebinding studies indicated that 30%–70% of the hormone was intact at the end of each incubation period.

Insulin Binding Studies

Insulin was iodinated to a specific activity of 200–250 μCi/μg using a modification [referred to as the second modification in 8] of the chloramine T method; ^{125}I-insulin was purified by gel filtration (Sephadex G50 Fine). Under our conditions of iodination the binding properties of radiolabelled insulin were indistinguishable from those of A 14 monoiodinated insulin [9]. Binding assays were performed as previously described [7, 10]. Briefly, after the washing procedure hepatocyte monolayers were resuspended in Krebs-Ringer bicarbonate (KRB) buffer containing 1% bovine serum albumin (BSA), bacitracin (0.8 mg/ml) and gentamicin (50 μg/ml), and incubated for 1 h at 37 °C with 0.3 ng/ml of ^{125}I-insulin in the absence or presence of increasing concentrations of unlabelled hormone; previous studies have shown that under these conditions binding reaches a steady state [7]. Unless indicated otherwise, binding assays were performed in the absence of the sulphonylurea. In order to account for the residual receptor occupancy that occurs despite the extensive washing procedure in hepatocyte monolayers previously exposed to insulin at 3 μmol/l, control experiments were performed in which cells were 'acutely' exposed to the hormone (3 μmol/l) for 10 min before the washing steps. All binding data have been corrected for non-specific binding as previously described [7], and expressed per mg of cell protein. Non-specific binding represented 10%–20% of total insulin binding.

Amino Acid Transport Studies

The effect of insulin on amino acid transport in hepatocyte monolayers was investigated by use of α-aminoisobutyric acid (AIB), a non-metabolisable analogue of alanine. After the washing procedure that followed the 20-h incubation period, monolayers were incubated in the same buffer as that described above for binding studies, in the absence or presence of various insulin concentrations. After 3 h of incubation at 37 °C, transport assays were performed as previously described [7] by adding (^{14}C)AIB and (^{3}H)inulin for 30 min. All transport data have been corrected for extracellular trapping (assessed by (^{3}H)inulin), and expressed per mg of cell protein. In some experiments, the effect of insulin on AIB uptake was studied in the presence of the sulphonylurea.

Statistical Analysis

Statistical analysis was performed using the Student's t-test for unpaired comparisons.

Results

Insulin Binding

The binding of insulin to hepatocyte monolayers was measured under steady state conditions at 37 °C using ^{125}I-insulin and varying concentrations of unlabelled insulin. Scatchard analysis (Fig. 1) of these data revealed no detectable change in receptor number or affinity in hepatocytes previously exposed to gliclazide (0.7; 7.0; and 70 μg/ml) for 20 h, compared with controls. Results were the same when the sulphonylurea remained present throughout the completion of the binding assay, or when the drug was present only during the assay itself.

In order to investigate a possible effect of gliclazide on the insulin-induced loss of insulin binding ('down regulation'), hepatocyte monolayers were first exposed to insulin (3 μmol/l, i.e. 400 mU/l) without or with gliclazide (0.7; 7.0; and 70 μg/ml) for 20 h at 37 °C. As shown in Figure 2, a previous exposure of the cells to insulin reduced subsequent insulin binding by about 45% compared with controls. At all three concentrations tested, gliclazide failed to affect the insulin-induced loss of insulin binding (Fig. 2).

The effect of other sulphonylureas on insulin binding was also tested by incubating primary cultured hep-

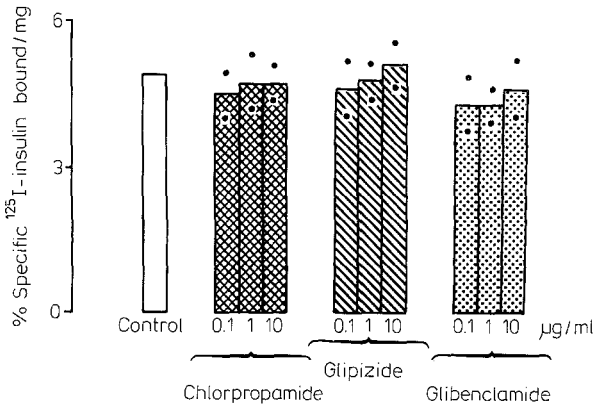

Fig. 3. Effects of chlorpropamide, glipizide and glibenclamide on insulin binding to hepatocyte monolayers. Cells were exposed for 20 h to the sulphonylurea at 0.1, 1.0 and 10 µg/ml, and the specific binding of ^{125}I-insulin (0.3 ng/ml) was measured (1 h at 37 °C). Data are the mean of two representative experiments where each point was determined in quadruplicate. Within each experiment SEM of replicates ranged from 2 to 5% of each value

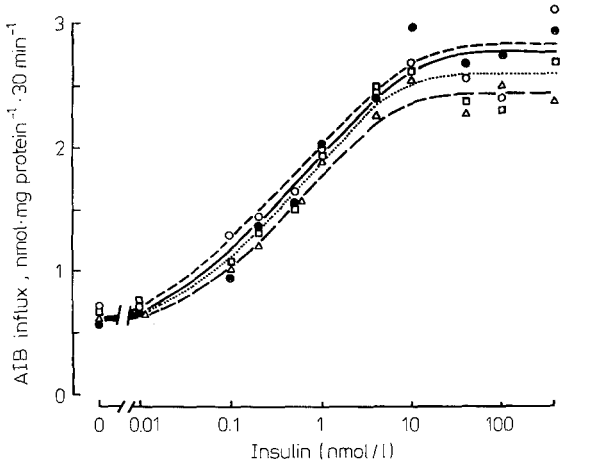

Fig. 4. Dose-response curves of insulin stimulation of α-amino isobutyric acid (AIB) uptake in hepatocyte monolayers following exposure to gliclazide. After exposure to gliclazide at 0.7, 7.0 and 70 µg/ml for 20 h, cells were incubated for 3 h in the presence of various concentrations of insulin. The uptake of AIB (0.1 mmol/l) was then measured as described under Materials and Methods. Results have been expressed as nmol of AIB taken up per mg of cell protein over 30 min. Each value is the mean of two separate experiments where each point was run in triplicate. Key: ●——● control, ○-----○ gliclazide (0.7 µg/ml), □·····□ gliclazide (7.0 µg/ml), △-----△ gliclazide (70 µg/ml)

Table 1. Effect of glipizide, glibenclamide and chlorpropamide on insulin-induced 'down regulation'

Sulphonylurea (at 10 µg/ml)	% ^{125}I-insulin specifically bound mg protein	
	Control cells	Insulin-treated cells
None	6.3 ± 0.1	3.0 ± 0.2
Glipizide	6.1 ± 0.1	3.0 ± 0.1
Glibenclamide	6.6 ± 0.2	3.1 ± 0.2
Chlorpropamide	6.5 ± 0.2	3.0 ± 0.1

Each value is the mean ± SEM of four determinations

Table 2. Effect of glipizide, glibenclamide and chlorpropamide on insulin stimulation of AIB transport

		α-aminoisobutyric acid uptake (nmol · mg protein^{-1} · 30 min^{-1})		
		Basal	Insulin (100 nmol/l)	Increment
Experiment 1				
Control		0.50 ± 0.04	1.56 ± 0.07	1.06
Glipizide	0.1	0.61 ± 0.02	1.61 ± 0.16	1.00
(µg/ml)	1.0	0.68 ± 0.05	1.64 ± 0.05	0.96
	10	0.69 ± 0.06	1.74 ± 0.06	1.05
Experiment 2				
Control		0.30 ± 0.05	0.94 ± 0.05	0.64
Glibenclamide	0.1	0.22 ± 0.01	0.94 ± 0.02	0.72
(µg/ml)	1.0	0.40 ± 0.02	1.10 ± 0.09	0.70
	10	0.21 ± 0.01	0.90 ± 0.08	0.69
Experiment 3				
Control		0.44 ± 0.04	1.25 ± 0.06	0.81
Ethanol	0.02%	0.34 ± 0.03	1.28 ± 0.08	0.94
	0.25%	0.34 ± 0.03	1.65 ± 0.09	1.31
Chlorpropamide	0.1	0.42 ± 0.02	1.41 ± 0.07	0.99
(µg/ml)	1.0	0.43 ± 0.04	1.58 ± 0.04	1.15
	10	0.41 ± 0.04	1.50 ± 0.09	1.09

Cells were incubated for 20 h in the absence or presence of the sulphonylurea, and insulin was added for 3 additional hours. The uptake of α-aminoisobutyric acid was measured at 0.1 mmol/l as described under Materials and Methods. Results are expressed as mean ± SEM of three determinations. In experiment 3, the small effect observed at 1 and 10 µg/ml was similar to that observed with the diluent alone, i.e., 0.02 and 0.25% ethanol, respectively

atocytes with chlorpropamide, glipizide, or glibenclamide (glyburide), for 20 h before the binding assay. Again, insulin binding was not affected by this previous exposure of the cells to any of these sulphonylureas (Fig. 3). As observed with gliclazide, the insulin-induced loss of insulin binding was not altered by glipizide, chlorpropamide, or glibenclamide at 10 µg/ml (Table 1).

Amino Acid Transport

The effect of insulin on amino acid transport was investigated in hepatocyte monolayers previously exposed for 20 h to gliclazide at 0.7; 7.0; and 70 µg/ml. Maximal effects of insulin (hormone at 100 nmol/l) were not statistically different ($p > 0.05$, $n = 4$) in control cells and in gliclazide treated hepatocytes. As depicted in Figure 4, dose responses of the hormone's effect on AIB influx in cells pre-treated with gliclazide were similar to that observed in controls. In all conditions tested, the half-maximal response was elicited by insulin at about 0.5 nmol/l. It was also observed that the presence of gliclazide with insulin during the 3 h incubation preceding the assay of AIB transport did not alter the stimulation effect of the hormone on AIB influx.

In similar manner, pre-treating hepatocyte monolayers with glipizide, glibenclamide, or chlorpropamide (0.1; 1.0; and 10 µg/ml) for 20 h did not affect the sub-

sequent stimulation of AIB influx by a maximally effective concentration (100 nmol/l) of insulin (Table 2). Similar observations were made when a submaximally stimulating concentration (0.4 nmol/l) of insulin was employed.

Discussion

The present study has revealed that the number and the affinity of insulin receptors in primary cultured rat hepatocytes are not affected following a 20 h exposure of these cells to the sulphonylureas gliclazide, glibenclamide, chlorpropamide, and glipizide. We have also shown that these sulphonylureas do not interfere with the ability of insulin to regulate its own receptor. Our findings are in agreement with recent reports by Maloff and Lockwood [6] in rat adipose tissue, and by Vigneri [12] in three human cell lines and H35 rat hepatoma. These and our results are in contrast with the study by Prince and Olefsky [5] who reported a slight increase (about 20%) in insulin binding and a partial inhibition of insulin-induced down regulation in cultured human fibroblasts following exposure to glyburide (i.e. glibenclamide). We have no explanation for this discrepancy, except that differences may exist among tissues regarding the action of sulphonylureas. Thus, despite the fact that insulin receptors in liver membranes increase following reatment of the animal with a sulphonylurea in vivo [2, 3], we have been unable to demonstrate a direct effect of four different sulphonylureas on insulin binding in hepatocytes, suggesting that the increase in hepatic insulin receptors observed after administration in vivo is due to a secondary rather than a primary effect of the drugs.

The present study was also designed to search for a direct, albeit post-receptor, effect of sulphonylureas on insulin action in liver. Our results indicate that the stimulatory effect of insulin on amino acid influx in hepatocytes is not enhanced by the previous exposure of the cells to a broad range of concentrations of gliclazide, glibenclamide, glipizide, or chlorpropamide. These results are at variance with studies in vivo which have shown that administration of tolbutamide [13], glipizide [14, 15], or glibenclamide [4], enhances insulin action but, as discussed above regarding binding data, such effects may be secondary rather than primary. Our results also differ from those reported by Blumenthal [16] using the isolated perfused liver, and from the observation by Maloff and Lockwood [6] who reported that tolazamide in vitro enhances the insulin stimulation of hexose transport in adipose tissue. Since, we investigated the effects of four sulphonylureas on amino acid transport in hepatocytes, whereas Maloff and Lockwood examined the effect of another sulphonylurea on glucose transport in adipocytes, it is difficult to compare the two studies. Moreover, sulphonylureas may specifically alter the action of insulin on carbohydrate metabolism by a post-receptor effect, without affecting other biological responses.

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