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GLIPENTIDE AND GLUCOSE METABOLISM IN ISOLATED RAT HEPATOCYTES*

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Abstract—Glipentide, a second generation sulfonylurea, raised the cellular concentration of fructose 2,6-bisphosphate in isolated rat hepatocytes. Parallel to accumulating this regulatory metabolite, glipentide inhibited basal gluconeogenesis and increased the rate of L-lactate production, as well as the metabolic flux through the 6-phosphofructo 1-kinase reaction. Tolbutamide elicited similar metabolic effects to those reported for glipentide, although the latter sulfonylurea was about 10 times more potent. The biochemical mechanism by which sulfonylureas promote the accumulation of fructose 2,6-bisphosphate in hepatocytes seems to be related to a significant increase of the hexose 6-phosphate pool (glucose 6-phosphate plus fructose 6-phosphate), together with the activation of 6-phosphofructo 2-kinase and inactivation of fructose 2,6-bisphosphatase, enzyme activities responsible, respectively, for the synthesis and degradation of fructose 2,6-bisphosphate.

It is well established that extrapancreatic actions are implicated in the hypoglycemic effect of sulfonylureas in chronic treatments [1, 2]. In connection with this, clinical studies have shown that reduction of hepatic glucose production is among the most relevant extrapancreatic actions of these drugs [3–5]. According to these findings, it has been demonstrated that sulfonylureas inhibit gluconeogenesis from different precursors in experiments carried out in isolated perfused livers [6–8] or in isolated liver cells [9]. More recently, our group has evidenced that both chlorpropamide [10] and glipizide [11] stimulate L-lactate production and inhibit gluconeogenesis in isolated rat hepatocytes by raising the intracellular content of fructose 2,6-bisphosphate (F-2,6-P_2). It must be mentioned that this regulatory metabolite plays a relevant role in the control of hepatic glucose metabolism [12].

In this work, we have extended the study of the action of sulfonylureas on hepatic glucose metabolism to glipentide, a second-generation sulfonylurea, comparing its effects to those exerted by tolbutamide. We have also further investigated the mechanism by which these drugs provoke the accumulation of F-2,6-P_2 in liver cells.

Our results show that, in isolated rat hepatocytes, glipentide accelerates both the rate of L-lactate production and the metabolic flux through 6-phosphofructo 1-kinase reaction by increasing the cellular concentration of F-2,6-P_2. Parallel to raising the levels of this regulatory metabolite, glipentide partially blocks the conversion of L-lactate to glucose. The elevation of F-2,6-P_2 in liver cells caused by sulfonylureas seems to be mediated, at least in part, by the increase of the cellular content of hexose 6-phosphates, together with the activation of 6-phosphofructo 2-kinase and the inactivation of fructose 2,6-bisphosphatase, enzyme activities which mediate, respectively, the synthesis and degradation of F-2,6-P_2 [12].

MATERIALS AND METHODS

Reagents. Collagenase and the auxiliary enzymes were purchased from Boehringer Mannheim (Mannheim, F.R.G.). (U-^{14}C)L-lactate and (3-^{3}H)glucose were supplied by the Radiochemical Centre (Amersham, U.K.). Glipentide (*N*-(-4-beta-(*o*-anisamidethyl) - benzenesulphonyl) - *N'* - cyclopentyl carbamide, research code name UR-661) was the generous gift of Laboratorios J. Uriach y Cia. (Barcelona, Spain). Tolbutamide (lot 529115) was kindly supplied by Boehringer Mannheim. Fructose 2,6-bisphosphate used as standard was from Sigma Chemical Co. (St. Louis, MO). The remaining reagents were from Boehringer, Sigma or Merck (Darmstadt, F.R.G.).

Animals. Male Wistar rats from our inbred colony, weighing 200–300 g, were used. The animals were maintained on a standard chow (A.40, Panlab, S.L., Barcelona, Spain) and water *ad libitum*. In some experiments, 24-h fasted rats were used.

Methods. Hepatocytes were isolated by perfusion of the liver with collagenase, and were incubated in Krebs–Henseleit medium in the presence of 10 or 20 mM glucose, as reported elsewhere [13]. Hepatocyte F-2,6-P_2 was measured by the ability of this metabolite to activate potato tuber PP_i:fructose 6-phosphate 1-phosphotransferase [14], according to the method previously described [10]. For the determination of other glycolytic intermediates, aliquots

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of cell suspensions were taken at the indicated times and immediately mixed with 1 vol. of ice-cooled 10% $HClO_4$. After neutralization [10], L-lactate, glucose 6-phosphate and fructose 6-phosphate were measured in the extracts [15, 16].

Glycolytic flux through 6-phosphofructo 1-kinase was estimated by measuring the rate of tritium release from (3-^{3}H)glucose into the incubation medium, according to Hue *et al.* [17]. To carry out this measurement, radioactive glucose was incorporated into the incubation medium (0.5 μCi/ml); 30 min later, 0.5-ml aliquots of cell suspensions were taken in duplicate and processed as indicated by Bontemps *et al.* [18] in order to separate formed ^3H_2O from the radioactive glucose. Gluconeogenesis was estimated by the conversion of radioactive L-lactate from a mixture of (U-^{14})L-lactate/pyruvate (4 mM/0.4 mM; 0.25 μCi/μmol) to glucose, following the method described by Felíu *et al.* [19].

The active non-phosphorylated form of hepatocyte 6-phosphofructo 2-kinase, as well as the total activity of this enzyme, were measured in crude hepatocyte extracts, as indicated by Bartrons *et al.* [20]. The active form of hepatocyte fructose 2,6-bisphosphatase was measured by the disappearance of F-2,6-P_2, following basically the method described by Van Schaftingen *et al.* [21], with some modifications. At selected times, 2-ml aliquots of the cell suspension were taken; after centrifugation (1000 g × 1 min), cell pellets were homogenized with 3 vol. of a medium containing 20 mM glycerol-2-phosphate, 50 mM Hepes, 100 mM KCl, 1 mM DTT and 10 mM EDTA, pH 7. The homogenate was centrifuged at 10,000 g × 15 min at 4°; 50-μl aliquots of the resulting supernatant were used to assay fructose 2,6-bisphosphatase activity at 37°. The incubation medium contained, in a final volume of 250 μl, 5 mM glycerol-2-phosphate, 1 mM ATP, 3 mM $MgCl_2$, 100 mM KCl, 50 mM Hepes, 5 μM fructose 2,6-bisphosphate and 0.5 mM NADP, pH 7.1. At 0, 2, 5 and 10 min of incubation, samples of 50 μl were taken and immediately pipetted into test tubes containing 150 μl of 2 M NaOH. Then, F-2,6-P_2 was assayed as indicated before.

Protein was assayed by the method of Lowry *et al.* [22], using bovine serum albumin as standard; 1 g of packed hepatocytes corresponded to 220 ± 5 mg of protein.

Statistical significance of differences between values was calculated by the paired Student's *t*-test. The differences were considered statistically significant when the *P* value was less than 0.05.

RESULTS

As shown in Fig. 1A, addition of glipentide (0.2 mM) to hepatocytes isolated from fed rats, and incubated in the presence of 10 mM glucose, caused a gradual increase of the cellular F-2,6-P_2 levels from a value of 13.6 ± 1.8 nmol/g of cells at zero time to a value of 32.3 ± 3.9 nmol/g of cells after 40 min of incubation (N = 4 experiments; $P < 0.01$). At this time, the concentration of F-2,6-P_2 in glipentide-treated cells was increased about 1.7-fold with respect to that measured in control hepatocytes. Parallel to increasing F-2,6-P_2 levels, glipentide

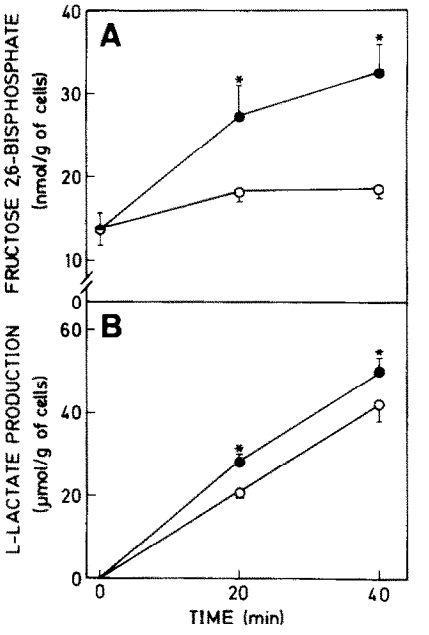

Fig. 1. Time-course studies of the effect of glipentide (0.2 mM) on F-2,6-P_2 concentration (A) and on the rate of L-lactate production (B), in hepatocytes isolated from fed rats. Cells were incubated in Krebs–Henseleit medium in the presence of 10 mM glucose. Values are means ± SEM of 6–10 experiments. Paired Student's *t*-test vs control hepatocytes; *$P < 0.05$; (○) saline; (●) glipentide.

accelerated the rate of L-lactate production (Fig. 1B).

Glipentide (0.1 mM) also increased the cellular concentration of F-2,6-P_2 in hepatocytes isolated from 24-hr fasted rats and incubated with either 10 or 20 mM glucose (Table 1). Again, the accumulation of F-2,6-P_2 was associated with a stimulation of the rate of L-lactate production, as well as with an acceleration of the glycolytic flux through the 6-phosphofructo 1-kinase reaction, estimated on the basis of ^3H_2O formation from (3-^{3}H)glucose. As also shown in Table 1, tolbutamide (1 mM) caused similar metabolic effects as those elicited by a 10-times smaller concentration of glipentide. A good correlation was established between hepatocyte levels of F-2,6-P_2 and the rate of either L-lactate production or tritiated water formation from (3-^{3}H)glucose ($r = 0.71$, N = 15, $P < 0.01$ and $r = 0.82$, N = 15, $P < 0.001$, respectively, for hepatocytes incubated with 20 mM glucose).

Figure 2 shows that the effect of glipentide on the cellular content of F-2,6-P_2 was dose-dependent. A small but statistically significant increase of hepatocyte F-2,6-P_2 was already observed at 0.1 mM glipentide, as compared to that found in hepatocytes incubated in the absence of sulfonylurea (11.9 ± 1.9 vs 9.1 ± 1.6 nmol/g of cells; N = 4 experiments; $P < 0.05$). Figure 2 also shows that the increase of hepatocyte F-2,6-P_2 levels caused by glipentide was accompanied by an also dose-dependent inhibition of the gluconeogenic flux from a mixture of (U-^{14}C)L-lactate and pyruvate (4 and 0.4 mM, respectively). Thus, in the presence of 0.2 mM glipentide, gluco-

Table 1. Effect of glipentide and tolbutamide on F-2,6-P_2 levels, L-lactate production and ^3H release from (3-^3H)glucose, in hepatocytes isolated from 24-hr fasted rats

		Fructose 2,6-bisphosphate (nmol/g of cells)	L-Lactate production (μmol/g of cells × 30 min)	Tritium release (μmol (3-^3H)glucose/ g of cells × 30 min)
Glucose 10 mM	Saline	6.1 ± 0.8	2.2 ± 0.1	5.5 ± 0.2
	Glipentide (0.1 mM)	7.3 ± 1.2*	5.7 ± 0.3**	7.4 ± 0.4**
	Tolbutamide (1 mM)	7.5 ± 1.2**	4.8 ± 0.7**	8.2 ± 0.7**
Glucose 20 mM	Saline	7.4 ± 1.3	11.0 ± 2.3	14.6 ± 2.0
	Glipentide (0.1 mM)	12.3 ± 1.6**	19.1 ± 2.2**	17.6 ± 1.4*
	Tolbutamide (1 mM)	11.0 ± 1.9**	26.3 ± 4.1**	22.1 ± 2.1**

Cells were incubated in Krebs–Henseleit medium in the presence of 10 mM or 20 mM glucose. For the measurement of F-2,6-P_2, aliquots of cell suspensions were taken 15 minutes after sulfonylurea additions. Values are the means ± SEM of 8 experiments. Paired Student's *t*-test versus the corresponding saline incubations: *P < 0.05; **P < 0.01.

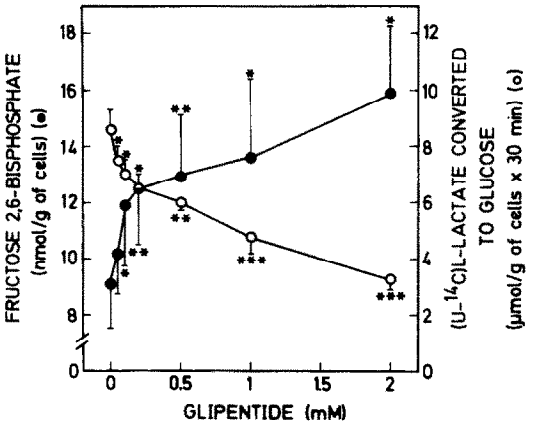

Fig. 2. Effect of different concentrations of glipentide on F-2,6-P_2 levels (●), and on the rate of gluconeogenesis (○), in hepatocytes isolated from fed rats. Cells were incubated in Krebs–Henseleit medium in the presence of 10 mM glucose. Hepatocytes were preincubated under these conditions for 30 min, before the addition of both the sulfonylurea and the gluconeogenic precursors. F-2,6-P_2 was measured in aliquots of cell suspensions taken 15 min after addition of the sulfonylurea. Values are means ± SEM of 4 experiments. Paired Student's *t*-test versus control incubation: *P < 0.05; **P < 0.01; ***P < 0.001.

neogenesis was inhibited by about 25% (6.5 ± 0.5 vs 8.6 ± 0.7 μmol of (U-^{14}C)L-lactate converted to glucose/g of cells × 30 min; N = 4 experiments; P < 0.05). A close inverse correlation can be established between hepatocyte levels of F-2,6-P_2 and the rate of gluconeogenesis ($r = -0.97$; N = 4 experiments; P < 0.001). On the other hand, the fact that glipentide (0.2 mM) did not significantly modify the cellular concentrations of ATP, ADP and AMP indicates that the inhibition of gluconeogenesis is not a consequence of a nonspecific toxic effect of the sulfonylurea on the energetic metabolism of hepatocytes (control hepatocytes: ATP, 2.15 ± 0.1; ADP, 0.32 ± 0.02; AMP, 0.12 ± 0.01; glipentide-treated hepatocytes: ATP, 2.0 ± 0.01; ADP, 0.38 ± 0.02; AMP, 0.12 ± 0.01. Values represent the mean ± SEM of 5–6 experiments and correspond to micromoles per gram of cells).

In order to investigate the mechanism by which sulfonylureas increase the cellular concentration of F-2,6-P_2, we studied the influence of both glipentide and tolbutamide on hexose 6-phosphate levels, as well as on 6-phosphofructo 2-kinase and fructose 2,6-bisphosphatase activities. It must be mentioned that these two activities—responsible for the synthesis and degradation of F-2,6-P_2—take place in the same protein molecule, which is hormonally modulated through a phosphorylation/dephosphorylation mechanism [12, 23, 24].

As shown in Table 2, addition of glipentide

Table 2. Effect of glipentide and tolbutamide on the concentrations of fructose 6-phosphate and hexose 6-phosphates in hepatocytes isolated from 24-hr fasted rats.

	Fructose 6-phosphate (nmol/g of cells)	Hexose 6-phosphates (nmol/g of cells)
Saline	39.7 ± 5.3	138.4 ± 15.1
Glipentide (0.2 mM)	50.7 ± 4.8**	166.7 ± 14.3*
Tolbutamide (1 mM)	55.0 ± 3.6*	174.3 ± 9.7**

Hepatocytes were incubated in Krebs–Henseleit medium with 10 mM glucose, in the absence or in the presence of sulfonylureas, for 20 min. Then, aliquots of cell suspensions were taken for the assay of hexose 6-phosphates. Values are means ± SEM of 12 experiments: *P < 0.05; **P < 0.01.

Table 3. Effect of glipentide and tolbutamide on 6-phosphofructo 2-kinase and fructose 2,6-bisphosphatase activities

	6-Phosphofructo 2-kinase (μU/mg of protein)		Fructose 2,6-bisphosphatase (μU/mg of protein)
	Active form	Total activity	
Saline	12.1 ± 2	22.4 ± 2.6	81 ± 13
Glipentide (0.1 mM)	16.2 ± 2.8*	30.0 ± 5.4 (NS)	49 ± 9**
Tolbutamide (1 mM)	16.1 ± 2.1*	27.7 ± 3 (NS)	59 ± 10*

Liver cells were isolated from 24-hr fasted rats and incubated in the presence of 10 mM glucose. Aliquots of cell suspensions were taken 15 min after saline or sulfonylurea addition. Values are means ± SEM of 5 experiments. Paired Student's *t*-test versus saline incubations: *$P < 0.05$; **$P < 0.01$; NS: not significant.

(0.2 mM) or tolbutamide (1 mM) to hepatocytes isolated from 24-hr fasted rats, and incubated with 10 mM glucose, caused a statistically significant increase of hepatocyte fructose 6-phosphate levels, as well as of the total pool of hexose 6-phosphates (fructose 6-phosphate plus glucose 6-phosphate). Furthermore, glipentide (0.1 mM) and tolbutamide (1 mM) increased the amount of 6-phosphofructo 2-kinase present in hepatocytes in "active" form by about 30%, without significant modification of the total activity of this enzyme (Table 3); in contrast, the presence of sulfonylureas in the incubation medium inactivated fructose 2,6-bisphosphatase.

DISCUSSION

As reported for other sulfonylureas [1, 2], extrapancreatic actions also seem to be implicated in the hypoglycemic effects of glipentide. In fact, a lack of temporal correlation between the maximal hypoglycemia and the highest level of insulinemia attained has been observed after acute administration of glipentide to experimental animals [25, 26]. In relation to these findings, *in vivo* [3–5] and *in vitro* [6–11] studies have shown that inhibition of hepatic glucose production is one of the most tenable extrapancreatic actions of sulfonylureas cooperating in their hypoglycemic action.

The foregoing results clearly show that, in isolated rat hepatocytes, glipentide stimulates glycolysis and partially blocks basal gluconeogenesis from L-lactate, by raising the cellular concentration of F-2,6-P_2. All these effects were similar to those previously described for other sulfonylureas [10, 11, 27], reinforcing the concept that all these drugs share a common mechanism of action on the intermediary metabolism. However, glipentide was about 10 times more active than tolbutamide in increasing F-2,6-P_2 levels in isolated rat hepatocytes and, therefore, in accelerating glycolysis and reducing gluconeogenesis. This is in good agreement with the much more potent hypoglycemic action of glipentide observed in animal studies, as compared to that of tolbutamide [26, 27].

Upon comparing data from Fig. 1 and Table 1, it seems that the effect of glipentide on F-2,6-P_2 levels is more marked in hepatocytes isolated from fed rats than in those obtained from 24-hour fasted animals. This quantitative difference could be explained by a possible glycogenolytic action of this drug on hepatocytes from fed rats, as has been demonstrated for tolbutamide in perfused rat hearts [28]. On the other hand, the reduction of glucokinase activity in livers of fasted rats [29] obviously will decrease the utilisation of glucose, as well as the formation of F-2,6-P_2 by hepatocytes derived from these animals, thus masking the stimulatory effect of glipentide. In agreement with this last argument, Table 1 shows that glipentide exerts a more relevant increase of F-2,6-P_2 levels when glucose utilisation is facilitated by incubating the cells with 20 mM glucose.

We have also further investigated the biochemical mechanism which mediates the rise in hepatocyte F-2,6-P_2 caused by sulfonylureas. Our results indicate that both glipentide and tolbutamide provoked a significant increase of the cellular content of F-2,6-P_2, as well as of the total pool of hexose 6-phosphates in hepatocytes isolated from 24-hr fasted rats. Since the fructose 6-phosphate concentration measured in hepatocytes is in the range of the apparent K_m value (20–50 μM) of liver 6-phosphofructo 2-kinase for this substrate [12, 23, 24], it might be expected that the increment of fructose 6-phosphate caused by sulfonylureas provokes an increased rate of F-2,6-P_2 synthesis. Furthermore, the accumulation of fructose 6-phosphate may also cooperate in elevating hepatocyte F-2,6-P_2 since, as reported elsewhere [23, 24], fructose 6-phosphate is a potent inhibitor of fructose 2,6-bisphosphatase activity. In agreement with our findings, Hue *et al.* [30] have observed a direct correlation between the concentration of hexose 6-phosphate and that of F-2,6-P_2 under various experimental conditions in liver.

It remains to be elucidated how sulfonylureas increase hexose 6-phosphate concentrations in isolated liver cells. Under our experimental conditions—hepatocytes isolated from 24-hr fasted rats—cells were depleted of glycogen, extracellular glucose being the exclusive source of glycolytic intermediates. In order to explain our results, we must postulate that sulfonylureas have to stimulate glucokinase activity or block glucose 6-phosphatase to increase the cellular pool of hexose 6-phosphates. This hypothesis is being investigated in our laboratory. At this point, we must indicate that hepatic glucose 6-phosphatase activity was found to be reduced in tolbutamide-treated normal rats [31]. However, whether this effect was directly caused by

the drug, or mediated by the increase of plasma insulin levels was not clearly established.

A second mechanism that seems to be implicated in the effect of sulfonylureas on hepatocyte F-2,6-P_2 levels is related to reciprocal changes of 6-phosphofructo 2-kinase and fructose 2,6-bisphosphatase activities. Our results, which are in agreement with those previously reported by Kaku *et al.* [32], show that both glipentide and tolbutamide increase the amount of the kinase in active form, and simultaneously provoke the inactivation of the phosphatase. These effects occur without significant modification of total 6-phosphofructo 2-kinase activity and hepatocyte cyclic AMP levels (data not shown).

According to these findings, it can be speculated that sulfonylureas may affect the phosphorylation state of the bifunctional enzyme 6-phosphofructo 2-kinase/fructose 2,6-bisphosphatase by a cyclic AMP-independent mechanism, either by stimulation of protein phosphatases acting on this interconvertible enzyme [33, 34], or by blockade of protein kinases which phosphorylate and inactivate the enzyme [23, 24, 35]. In connection with this, it has been shown that tolbutamide may inhibit the activity of cyclic AMP-dependent protein kinase of adipose tissue [36] and parotid gland [37].

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