Rapid Publications

Tolbutamide Stimulates Fructose-2, 6-Bisphosphate Formation in Perfused Rat Liver

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SUMMARY

Effect of tolbutamide on liver fructose-2,6-bisphosphate (F-2,6-P_2) was examined in isolated perfused rat liver in situ with a flow-through method. Tolbutamide (1 mM) gradually increased liver F-2,6-P_2 level from 7.4 ± 1.6 to 21.2 ± 1.6 pmol/mg wet wt for 20 min perfusion. The increase of liver F-2,6-P_2 induced by tolbutamide was dose dependent and was significantly observed at 10 min perfusion. The maximum plateau level of F-2,6-P_2 induced by 16.7 mM glucose was further increased with 1 mM tolbutamide. Glucagon (10^{-11} M) decreased the elevated level induced by 16.7 mM glucose, but this effect was completely inhibited with 2 mM tolbutamide. Cyclic AMP level of the liver throughout the perfusion with tolbutamide did not change. Carboxytolbutamide or gliclazide perfusion did not change significantly the liver F-2,6-P_2 level; however, the results suggest that tolbutamide may increase the liver F-2,6-P_2 level by affecting the phosphorylation state of fructose-6-phosphate, 2-kinase/fructose-2,6-bisphosphatase through cyclic AMP–dependent protein kinase, resulting in the stimulation of glycolysis and the inhibition of gluconeogenesis in the liver. Thus, the extrapancreatic action and the mechanism of action of different sulfonylureas may differ. DIABETES *33*:495–498, May 1984.

It has been clearly demonstrated that acute or short-term administration of sulfonylurea stimulates insulin secretion both in animal models[1] and in man,[2,3] and the poor correlation between this insulin secretory effect and the effect of long-term sulfonylurea treatment of diabetes[4] has raised the argument concerning the presence of an extrapancreatic action of the drug. Many extrapancreatic actions of sulfonylurea were proposed, including amelioration of the deranged carbohydrate metabolism in the liver.[5] Tolbutamide has been reported to inhibit hepatic glucose output in dogs[6] and chickens,[7] to increase the myocardial glycolytic flux,[8] and to potentiate glucose uptake of the perfused rat hind limb by insulin.[9] Chlorpropamide decreases hepatic glucose production in man[5] and decreases hepatic glucose release[10] or glycogenolysis[11] in the rat. However, the details of the extrapancreatic action of sulfonylurea, including its mechanism, still remain to be clarified.

Recently, fructose-2,6-bisphosphate (F-2,6-P_2) was found to be the most potent activator of liver phosphofructokinase.[12,13] It was reported that the level of this activator was regulated by the synthesizing (fructose-6-phosphate, 2-kinase) and degrading (fructose-2,6-bisphosphatase) enzymes. Their activities are determined by the phosphorylation state of the enzyme depending on cyclic AMP and/or Ca^{2+}-calmodulin–dependent protein kinase.[14–17] On the other hand, it has been reported that tolbutamide inhibits cyclic AMP–dependent protein kinase in adipose tissue[18] and the parotid gland.[19] In addition, Kramer et al.[8] reported that tolbutamide stimulated anaerobic metabolism by an activation of both phosphofructokinase and phosphorylase on the basis of the determination of key metabolic intermediates in rat perfused heart.

These observations motivated us to investigate the effect of tolbutamide on liver F-2,6-P_2 level. The present study demonstrates that tolbutamide stimulates F-2,6-P_2 accumulation and releases glucagon suppression of F-2,6-P_2 level using isolated perfused rat liver.

MATERIALS AND METHODS

Phosphofructokinase, aldolase, triose-phosphate isomerase, glycerin-3-phosphate dehydrogenase, and fructose-6-phosphate were purchased from Boehringer Mannheim (West Germany). NADH, dithiothreitol, fructose-2,6,-bisphosphate, and tolbutamide were from Sigma Co. (St. Louis, Missouri). Carboxytolbutamide was a gift from Hoechst (Japan) and gliclazide was from Dainippon Pharmaceutical Co., Japan. All reagents were of analytic grade.

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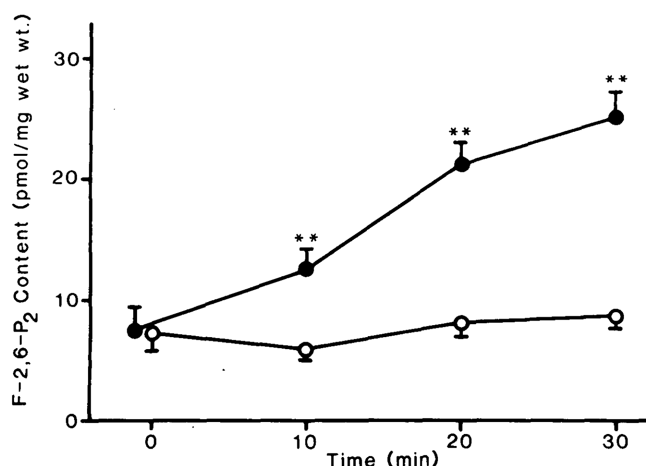

FIGURE 1. Time course of 1 mM tolbutamide stimulation of F-2,6-P_2 in the liver. Liver was perfused with KRB buffer containing 5 mM glucose (○——○) or 5 mM glucose and 1 mM tolbutamide (●——●). Each value represents the mean ± SE of 5 separate experiments. **$P < 0.01$.

Male Wistar rats weighing 200–250 g after overnight fast were anesthetized with sodium pentobarbital. Isolated liver perfusion in situ was performed with a flow-through method by a modification of the method of Sugano et al.[20] All vessels coming into the liver were ligated except the portal vein, which was cannulated with a polyethylene catheter, and the abdominal vena cava was incised. The thoracic vena cava was incised at the beginning of perfusion to allow perfusate to flow freely. The basal medium used for perfusion consisted of Krebs-Ringer bicarbonate buffer (pH 7.4) containing 5 mM glucose. The medium was warmed to 37°C in a water bath, continuously gassed with 95% O_2 and 5% CO_2, and introduced to the portal vein with a peristaltic pump (Tokyo Rikakiki Co., Japan). All experiments were carried out after 10 min pre-perfusion with the basal medium, and the flow rate was adjusted to 30 ml/min. A small piece of liver (about 100 mg) was biopsied and immediately frozen in acetone–dry ice at the start and the indicated time of perfusion, and stocked at −80°C until assay of F-2,6-P_2. Several different parts of liver obtained at the same time during perfusion showed similar F-2,6-P_2 content.

The frozen sample of liver was placed in 1180 μl of 0.2 M ice-cold Tris-HCl buffer (pH 7.2), containing 0.5 mM EGTA and 5 mM $MgCl_2$, and 120 μl of 0.5 N NaOH was added. The sample was homogenized at first with a glass homogenizer and then with a sonifier. The homogenate was heated at 80°C for 20 min and centrifuged at 50,000 × *g* for 30 min; the supernatant was used for F-2,6-P_2 determination. F-2,6-P_2 was assayed based on its ability to release ATP inhibition of muscle phosphofructokinase as described previously by Furuya and Uyeda.[12] The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.5), 0.2 mM EDTA, 10 mM ATP, 0.16 mM NADH, 0.4 U aldolase, 2.4 U triose-phosphate isomerase, and 0.4 U glycerin-3-phosphate dehydrogenase. The sample (20 μl) was added to 0.98 ml of the reaction mixture in an assay cuvette, and reaction velocity was spectrophotometrically recorded at 37°C. The amount of F-2,6-P_2 was determined by comparison with standards containing a known concentration of F-2,6-P_2. In each assay, triplicate determination was done. The activation of phosphofructokinase was linear over the range of 0–50 nM of F-2,6-P_2, and tolbutamide (1 mM) did not interfere with this assay system.

RESULTS

The mean basal level of F-2,6-P_2 of the liver was 7.3 ± 1.4 pmol/mg wet wt. Figure 1 shows the effect of 1 mM tolbutamide on F-2,6-P_2 level in the perfused liver. The F-2,6-P_2 level of the liver perfused with 5 mM glucose did not change significantly for the 30-min perfusion period, but that of livers perfused with 5 mM glucose and 1 mM tolbutamide gradually increased throughout the 30-min perfusion. The significant increase of F-2,6-P_2 was observed at 10 min perfusion, and this effect of tolbutamide was dose dependent as shown in Figure 2.

The gradual increase of F-2,6-P_2 induced by 16.7 mM glucose reached a plateau level at 15 min, and thereafter the level did not change significantly throughout the latter half (15 min) of the perfusion period as shown in Table 1. After obtaining a small piece of liver for the determination of F-2,6-P_2 content at 20 min perfusion with 16.7 mM glucose, the liver was further perfused for 10 min with 16.7 mM glucose in the presence or absence of tolbutamide and/or glucagon. At the end of the perfusion, liver F-2,6-P_2 was determined again. As shown in Table 1, tolbutamide increased the F-2,6-P_2 level of liver, which seemed to have reached the maximum level with 16.7 mM glucose. Glucagon (10^{-11} M) decreased the plateau level of liver F-2,6-P_2, which had been increased by 16.7 mM glucose, but 2 mM tolbutamide completely inhibited this glucagon effect and, moreover, increased F-2,6-P_2 content above the plateau level.

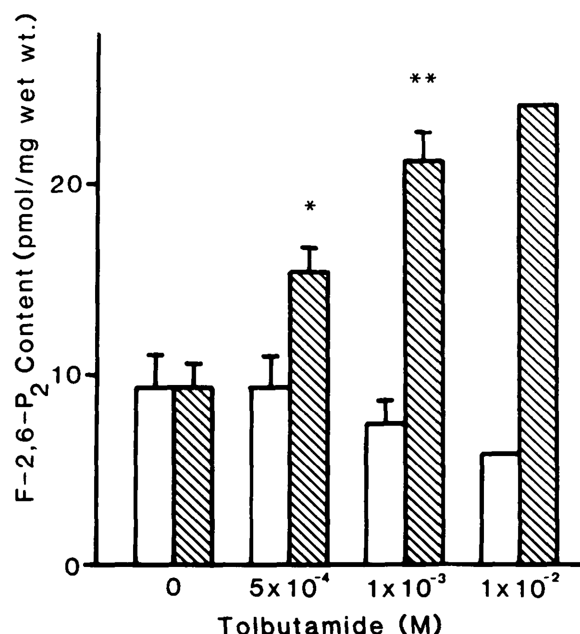

FIGURE 2. Effect of various concentrations of tolbutamide on liver F-2,6-P_2 formation. F-2,6-P_2 concentration was determined at 0 (open bars) and 20 min (hatched bars) of perfusion in the same liver. Each value represents the mean ± SE of five separate experiments except one with 10 mM tolbutamide (N = 2). *$P < 0.02$; **$P < 0.01$.

TABLE 1
Effect of tolbutamide and/or glucagon perfusion on F-2,6-P_2 content increased by 16.7 mM glucose

	Liver F-2,6-P_2 (pmol/mg wet wt)					
	Perfusion period (min)					
	0	5	10	15	20	30
16.7 mM Glucose	5.0 ± 0.91	6.3 ± 0.3	12.7 ± 2.0	20.1 ± 1.0	22.5 ± 0.8	20.7 ± 1.2
Additions						
Tolbutamide (1 mM)					23.8 ± 2.4	31.8 ± 2.9*
Glucagon (10^{-11} M)					23.4 17.0	12.8 10.9
Glucagon (10^{-11} M) + tolbutamide (2 mM)					21.6 ± 1.4	33.9 ± 2.8*

The liver was initially perfused with 16.7 mM glucose for 20 min and further perfused for 10 min with tolbutamide and/or glucagon in the presence of 16.7 mM glucose. F-2,6-P_2 was determined at 20 and 30 min of the perfusion. Each value represents the mean ± SE of five separate experiments except glucagon perfusion (N = 2).
*$P < 0.01$.

The perfusion of gliclazide at the concentration of 1 mM did not change the level of liver F-2,6-P_2, and the perfusion of 1 mM of carboxytolbutamide marginally increased it as shown in Table 2. Cyclic AMP level of the liver throughout the perfusion with tolbutamide did not change (data not shown).

DISCUSSION

The present study clearly demonstrated that tolbutamide increased the F-2,6-P_2 concentration in the liver with dose dependency, and released glucagon suppression of the F-2,6-P_2 level. Tolbutamide gradually increased the F-2,6-P_2 concentration in a manner similar to the effect of a high concentration of glucose. However, tolbutamide perfused with 16.7 mM glucose increased the F-2,6-P_2 level more than glucose alone, suggesting that its effect was unlike that of glucose.

Glucagon has been reported to decrease the F-2,6-P_2 level rapidly by inhibition of fructose-6-phosphate, 2-kinase and stimulation of fructose-2,6-bisphosphatase through phosphorylation depending on cyclic AMP–dependent protein kinase.[16,21] Pilkis et al. reported that the synthesizing and degrading activity of F-2,6-P_2 belongs to a single protein fructose-6-phosphate, 2-kinase/fructose-2,6-bisphosphatase, and this bifunctional enzyme was a substrate for cyclic AMP–dependent protein kinase but not for phosphorylase kinase, and further, that insulin opposed the action of glucagon and epinephrine on liver F-2,6-P_2 by affecting the phosphorylation state of this bifunctional enzyme.[22] Although there are contradictory results,[7,10] the extrapancreatic action of sulfonylurea is reported as being based on its ability to potentiate insulin action.[23-25] Our observations suggest, however, that the effect of tolbutamide is not through the potentiation of insulin action, but through its own stimulatory effect, for the liver was isolated from the general circulation and the perfusate did not contain insulin.

A high concentration of tolbutamide (10 mM) has been reported to inhibit cyclic AMP–dependent protein kinase in adipose tissue and the parotid gland, resulting in the inhibition of lipolysis and amylase secretion, respectively. We confirmed that 10 mM tolbutamide decreased the protein phosphorylation augmented by exogenous cyclic AMP in the liver cytosol using SDS-polyacrylamide gel electrophoresis and autoradiography (data not shown), and that tolbutamide did not change cyclic AMP concentration of the liver throughout perfusion.

Therapeutically effective levels of tolbutamide had been reported to be 8–18 mg/dl.[26,27] The concentration of tolbutamide used in our experiments (1 mM = 27 mg/dl) is reasonable considering the higher concentration of tolbutamide in the portal vein when orally administered.

Carboxytolbutamide, which is a metabolite of tolbutamide without hypoglycemic action, showed marginal stimulation of F-2,6-P_2 formation, although only two experiments were done, and gliclazide did not affect F-2,6-P_2 level in the liver. Therefore, the extrapancreatic action and the mechanism of action of sulfonylurea may differ in each sulfonylurea.

Based on our studies, we suggest that tolbutamide may increase the F-2,6-P_2 level by affecting the phosphorylation state of fructose-6-phosphate, 2-kinase/fructose-2,6-bis-

TABLE 2
Effect of tolbutamide, carboxytolbutamide, and gliclazide on F-2,6-P_2 content of the liver for 20 min perfusion

	Liver F-2,6-P_2 (pmol/mg)	
	Perfusion period (min)	
Sulfonylurea	0	20
None	7.3 ± 1.4	8.2 ± 1.1
Tolbutamide (1 mM)	7.4 ± 1.6	21.2 ± 1.6*
Carboxytolbutamide (1 mM)	6.4 9.1	12.7 13.5
Gliclazide (1 mM)	8.7 4.3	3.4 5.7

Each value represents the mean ± SE of five separate experiments except carboxytolbutamide and gliclazide (N = 2).
*$P < 0.01$.

phosphatase through cyclic AMP–dependent protein kinase, resulting in the stimulation of glycolysis and inhibition of gluconeogenesis in the liver.

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REFERENCES

[1] Grodsky, G. M., Epstein, G. H., Fanska, R., and Karam, J. H.: Pancreatic action of the sulfonylureas. Fed. Proc. 1977; 36:2714–19.

[2] Saltzer, H. S.: Quantitative effects of glucose, sulfonylureas, salicylate and indole-3-acetic acid on the secretion insulin activity into pancreatic venous blood. J. Clin. Invest. 1962; 41:289–300.

[3] Yalow, R. S., Black, H., Villazon, M., and Berson, S. A.: Comparison of plasma insulin levels following administration of tolbutamide and glucose. Diabetes 1960; 9:356–62.

[4] Vallence-Owen, J., Joplin, G. F., and Fraser, R.: Tolbutamide control of diabetes mellitus: clinical responsiveness and insulin reserve. Lancet 1959; 2:584–86.

[5] Best, J. D., Judzewitsch, R. G., Pfeifer, M. A., Beard, J. C., Halfer, J. B., and Porte, D., Jr.: The effect of chronic sulfonylurea therapy on hepatic glucose production in non-insulin-dependent diabetes. Diabetes 1982; 31:333–38.

[6] Ishida, T., Chou, M. C. Y., Lewis, R. M., Hartley, J., Entman, M., and Field, J. B.: The effect of tolbutamide on hepatic extraction of insulin and glucagon and hepatic glucose output in anesthetized dogs. Endocrinology 1981; 109:443–50.

[7] Dandy, R. W., Martin, I. K., and Gibson, W. R.: Effects of pancreatectomy, tolbutamide and insulin on glucose fluxes in chickens. J. Endocrinol. 1982; 94:429–41.

[8] Kramer, J. H., Lampson, W. G., and Schatter, S. W.: Effect of tolbutamide on myocardial energy metabolism. Am. J. Physiol. 1983; 245:313–19.

[9] Daniels, E. L., and Lewis, S. B.: Acute tolbutamide administration alone or combined with insulin enhances glucose uptake in the perfused rat hind limb. Endocrinology 1982; 110:1840–42.

[10] Blumenthal, S. A., and Whitmer, K. R.: Hepatic effect of chlorpropamide: inhibition of glucagon-stimulated gluconeogenesis in perfused livers of fasted rats. Diabetes 1979; 28:646–50.

[11] Pagasta, G., Kaldor, A., and Vizi, E. S.: The inhibitory effect of sulfonylurea derivatives on liver glycogenolysis increased by catecholamines. J. Pharm. Pharmacol. 1970; 22:447–51.

[12] Furuya, E., and Uyeda, K.: An activation factor of liver phosphofructokinase. Proc. Natl. Acad. Sci. USA 1980; 77:5861–64.

[13] Pilkis, S. J., El-Maghrabi, M. R., Pilkis, J., Claus, T. H., and Cumming, D. A.: Fructose 2,6-bisphosphate—a new activator of phosphofructokinase. J. Biol. Chem. 1981; 256:3171–74.

[14] El-Maghrabi, M. R., Fox, E., Pilkis, J., and Pilkis, S. J.: Cyclic AMP-dependent phosphorylation of the liver 6-phospho-fructokinase/fructose 2,6-bisphosphatase. Biochem. Biophys. Res. Commun. 1982; 106:794–802.

[15] Furuya, E., Yokoyama, M., and Uyeda, K.: Regulation of fructose-6-phosphate 2-kinase by phosphorylation and dephosphorylation: possible mechanism for coordinated control of glycolysis and glycogenolysis. Proc. Natl. Acad. Sci. USA 1982; 79:325–29.

[16] Richards, C. S., and Uyeda, K.: Hormonal relation of fructose-6-P, 2-kinase and fructose-2,6-P_2 by two mechanisms. J. Biol. Chem. 1982; 257:8854–61.

[17] Pilkis, S. J., Walderhaug, M., Murray, K., Beth, A., Venkataramu, S. D., Pilkis, J., and El-Maghrabi, M. R.: 6-Phospho-fructo-2-kinase/fructose-2,6-bisphosphatase from rat liver. J. Biol. Chem. 1983; 258:6135–41.

[18] Wray, H. L., and Harris, A. W.: Adenosine 3′,5′-monophosphate-dependent protein kinase in adipose tissue. Biochem. Biophys. Res. Commun. 1973; 53:291–94.

[19] Kanamori, T., Hayakawa, T., and Nagatsu, T.: Adenosine 3′,5′-monophosphate-dependent protein kinase and amylase secretion from rat parotid gland. Biochem. Biophys. Res. Commun. 1974; 57:394–98.

[20] Sugano, T., Suda, K., Shimada, M., and Oshino, N.: Biochemical and ultrastructural evaluation of isolated rat liver systems perfused with a hemoglobin-free medium. J. Biochem. 1978; 83:995–1007.

[21] Uyeda, K., Furuya, E., Richard, C., and Yokoyama, M.: Fructose-2,6-P_2, chemistry and biological function. Mol. Cell. Biochem. 1982; 48:97–120.

[22] Pilkis, S. J., Christman, T. D., El-Maghrabi, M. R., Colosia, A., Fox, E., Pilkis, J., and Claus, T. H.: The action of insulin on hepatic fructose 2,6-bisphosphate metabolism. J. Biol. Chem. 1983; 258:1495–1503.

[23] Lebovitz, H. E., Feinglos, M. N., Bucholtz, H. K., and Lebovitz, F. L.: Potentiation of insulin action: a probable mechanism for the anti-diabetic action of sulfonylurea drugs. J. Clin. Endocrinol. Metab. 1977; 45:601–604.

[24] Putnam, W. S., Anderson, D. K., Jones, R. S., and Lebovitz, H. E.: Selective potentiation of insulin-mediated glucose disposal in normal dogs by the sulfonylurea glipizide. J. Clin. Invest. 1981; 67:1016–23.

[25] Salhanick, A. I., Konowitz, P., and Amatruda, J. M.: Potentiation of insulin action by a sulfonylurea in primary culture of hepatocytes from normal and diabetic rats. Diabetes 1983; 32:206–12.

[26] Baird, J. D., and Dunchan, L. J. P.: An analysis of the hypoglycemic response to tolbutamide. Scott. Med. J. 1957; 2:341–50.

[27] Stowers, J. M., Mahler, R. F., and Hunter, R. B.: Pharmacology and mode of action of the sulfonylureas in man. Lancet 1958; 1:278–83.