Vol. 101, No. 1, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 16, 1981 Pages 104-111

FRUCTOSE 1,6-BISPHOSPHATASE IN RAT LIVER CYTOSOL: ACTIVATION AFTER GLUCAGON TREATMENT IN VIVO AND INHIBITION BY FRUCTOSE 2,6-BISPHOSPHATE IN VITRO

Stéphanie Mörikofer-Zwez, Franziska B. Stoecklin and Paul Walter
Department of Biochemistry, University of Basel
Vesalianum, Vesalgasse 1, CH-4051 Basel, Switzerland

Received June 8,1981

SUMMARY: 1. After intraperitoneal injection of glucagon, the activity of fructose 1,6-bisphosphatase is increased in subsequently isolated liver cytosol from fed rats. The glucagon effect is time-dependent and is maximal 15-20 minutes after injection. At 20 minutes the activation is 44 ± 6% as compared to the controls (mean ± S.E.M., n = 16). 2. Fructose 1,6-bisphosphatase is shown to be inhibited by fructose 2,6-bisphosphate prepared according to Van Schaftingen and Hers ((1980) Biochem. Biophys. Res. Commun. 96, 1524-1531). The K_i of the enzyme for fructose 2,6-bisphosphate is about 0.1 µM in control animals and is approximately doubled after injection of glucagon.

INTRODUCTION

The fructose 6-phosphate/fructose 1,6-bisphosphate substrate cycle is one of the regulatory points controlling glycolytic vs. gluconeogenic fluxes. Glucagon, which activates gluconeogenesis from a number of substrates (for review see 1), has been shown to inhibit phosphofructokinase and thereby glycolytic flux (2-6). However, the complementary activation of fructose 1,6-bisphosphatase, which would directly enhance gluconeogenic flux, is still a matter of debate (1,3,6). Taunton et al. (7) reported a 30-40% activation of fructose 1,6-bisphosphatase in rat liver 5 minutes after in vivo application of glucagon through the portal vein. However, Söling et al. (3) could not reproduce these results. On the other hand, Chatterjee and Datta (8) observed a 100% increase of the enzyme activity in mouse liver 15 minutes after injection of glucagon into the tail vein. Furthermore, in rat hepatocytes, Clark et al. (2) observed a 73% increase of flux through fructose 1,6-bisphosphatase in response to added glucagon. In contrast to these results, Rognstad and Katz (5) found only small effects of glucagon on fructose 1,6-bisphosphatase in rat hepatocytes after addition of a 10 times higher hormone concentration than used by Clark et al. (2).

In a previous paper we have investigated the regulation of fructose 1,6-bisphosphatase in isolated rat liver cytosol under near-physiological condi-

0006-291X/81/130104-08$01.00/0

tions (9). The same technique was used in the present paper, and the results show that the activity of fructose 1,6-bisphosphatase was markedly increased after application of glucagon in vivo.

Recently phosphofructokinase has been shown to be activated by a low molecular weight compound (10-12). In isolated hepatocytes, this substance increases in response to glucose and decreases following glucagon administration (10,13). This compound has been identified as fructose 2,6-bisphosphate (14-16). Since the regulation of fructose 1,6-bisphosphatase would be expected to be complementary to variations of phosphofructokinase activity, the effect of fructose 2,6-bisphosphate on fructose 1,6-bisphosphatase activity was tested with liver cytosol from glucagon-treated and control rats.

MATERIALS AND METHODS

Fed male albino Wistar rats from the Swiss Vitamine Institute in Basel weighing 200-280 g were used. 16 hours prior to hormone treatment, the animals were transferred in pairs to small cages (volume: 6 liters) and were kept overnight in a quiet room with free access to food and water. In these cages, the rats were anaesthetized between 8 and 9 a.m. with methoxyflurane (Metofane^{R}, Pittman-Moore Inc., Washington Crossing, USA) and were then injected intraperitoneally with either a freshly prepared solution of glucagon (Sigma, St. Louis, USA)in 0.9% NaCl containing 1% bovine serum albumine, pH 10.5 (500 µg/kg body weight) or a corresponding amount of hormone vehicle. Unless indicated otherwise, the animals were killed by decapitation 15 minutes after injection while still being under narcosis. Liver cytosols were prepared as described previously (9) with the following modifications: 1 g of liver was homogenized with 20 ml of homogenization buffer additionally containing 100 µM FMN (Sigma, Grade I) (unless indicated otherwise). The resulting cytosol was not diluted further.

Initial rates of glucose 6-phosphate formation were measured as described previously (9) with either glyceraldehyde 3-phosphate, fructose 1,6-bisphosphate or fructose 6-phosphate as substrate in presence of 0.4 mM $MgSO_4$. With fructose 6-phosphate or fructose 1,6-bisphosphate as substrate, initial rates were read immediately after substrate addition, whereas with glyceraldehyde 3-phosphate initial activity was read after a lag phase (9) of 90 seconds. No chelators were added to the assay medium but the glassware was washed with EDTA to avoid interference by Zn^{2+} and Ca^{2+} (9,17). Fructose 2,6-bisphosphate was synthetized, purified and assayed as described by Van Schaftingen and Hers (14). For the calculation of results, 1 ml of cytosol was considered equivalent to 48 mg liver wet weight. The final concentration of cytosol in the assay corresponded to 3.2 mg liver wet weight/ml.

RESULTS

Effect of glucagon treatment in vivo on fructose 1,6-bisphosphatase activity

The rate of glucose 6-phosphate formation was measured with liver cytosol from control rats and from rats pretreated with glucagon. With fructose 1,6-bisphosphate as substrate, the rate of glucose 6-phosphate formation was increased by 34% 15 minutes after hormone injection whereas the rate with fruc-

Table I. Effect of glucagon on glucose 6-phosphate formation from fructose 1,6-bisphosphate, fructose 6-phosphate or glyceraldehyde 3-phosphate

Substrate	Rate of glucose 6-phosphate formation		Change of activity
	Control	Glucagon-treated	
	$\mu moles \cdot min^{-1} \cdot g\ liver^{-1}$		%
Fructose 1,6-bisphosphate	1.71 ± 0.14 (4)	2.38 ± 0.14* (6)	+ 34 ± 4 (6)
Fructose 6-phosphate	5.39 ± 0.57 (4)	5.09 ± 0.21 (6)	+ 2 ± 5 (6)
Glyceraldehyde 3-phosphate	1.64 ± 0.15 (5)	2.26 ± 0.19* (6)	+ 34 ± 7 (6)

Initial rates of glucose 6-phosphate formation were determined as described under Materials and Methods. Initial substrate concentrations were 0.2-0.3 mM glyceraldehyde 3-phosphate, 18 µM fructose 1,6-bisphosphate or 16 µM fructose 6-phosphate. Results are expressed as mean ± S.E.M. for the number of animals given in brackets; p vs. controls (unpaired data): * <0.02.

tose 6-phosphate remained unchanged (Table I). Since phosphofructokinase is not operative in the present system (9) these observations clearly show that fructose 1,6-bisphosphatase is activated by glucagon. The extent of this activation is comparable to the one observed by Taunton et al. (7) but is somewhat lower than the value reported by Clark et al. (2) for isolated hepatocytes. Initial rates of glucose 6-phosphate formation as well as the hormone effect were equal with either fructose 1,6-bisphosphate or glyceraldehyde 3-phosphate as substrate (Table I). This observation shows that in the present system fructose 1,6-bisphosphatase is the rate-limiting enzyme. Since fructose 1,6-bisphosphatase activity is difficult to measure accurately with physiological concentrations of fructose 1,6-bisphosphate as substrate (<20 µM (18)), in the subsequent experiments 0.2-0.3 mM glyceraldehyde 3-phosphate was used as substrate yielding a constant fructose 1,6-bisphosphate concentration of about 10 µM (9).

As shown in Fig. 1, the activation of fructose 1,6-bisphosphatase by glucagon was time-dependent. Maximal activation of 44 % was reached 20 minutes after hormone injection and then decreased again. Whether this decrease was due to an antagonistic effect of insulin released in response to glucagon (19) has not been studied. Taunton et al. (7) reported maximal activation of fructose 1,6-bisphosphatase to occur already 5 minutes after hormone injection. This much

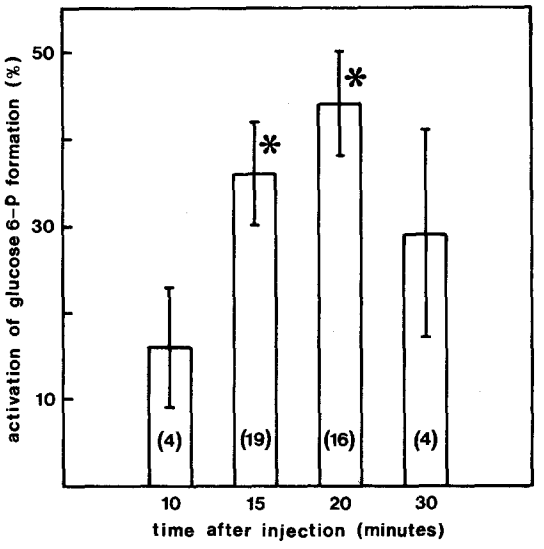

Fig. 1. Activation of fructose 1,6-bisphosphatase: time-dependence of the glucagon effect in vivo. Fructose 1,6-bisphosphatase activity was measured with 0.2-0.3 mM glyceraldehyde 3-phosphate as substrate in liver cytosol from rats injected intraperitoneally with either glucagon or hormone vehicle and decapitated after the indicated time intervall (see Materials and Methods). Activation (%) was calculated for each glucagon-treated rat as compared to the control(s) within the same experiment. Results are expressed as mean ± S.E.M. for the number of glucagon-treated rats given in brackets. p vs. control (paired data): * <0.001.

shorter activation time may be due to the different route of application (portal vein vs. intraperitoneal injection).

In vitro activation of fructose 1,6-bisphosphatase in isolated cytosol

As mentioned in Materials and Methods, 100 µM FMN were routinely added during the preparation of the liver cytosols. When FMN was omitted, the activity of fructose 1,6-bisphosphatase in isolated cytosol rapidly increased after centrifugation. This in vitro activation was variable and results of three separate preparations are shown in Fig. 2. In experiments with liver cytosols from glucagon-treated rats, no in vitro activation could be observed (Fig. 2).

FMN has been shown by Kuo et al. (20) to be an inhibitor of cyclic AMP-dependent protein kinase. The fact that it suppresses the time-dependent in vitro activation of fructose 1,6-bisphosphatase in liver cytosol from control rats (Fig. 2) suggests the involvement of protein phosphorylation in this activation. This conclusion is supported by the observation that in cytosol from glucagon-treated animals FMN had no effect (Fig. 2).

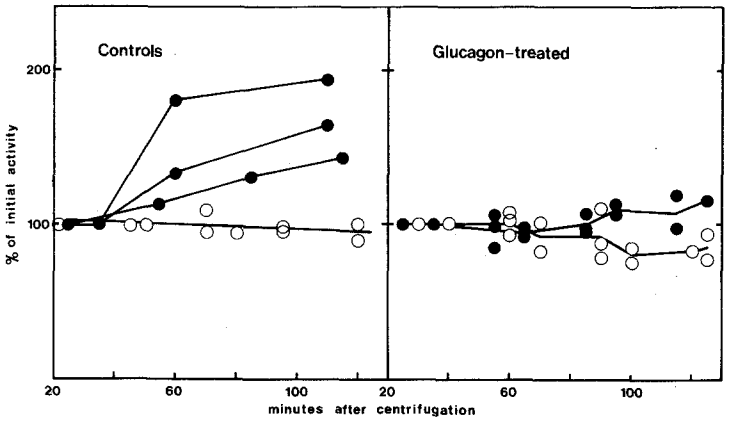

Fig. 2. Activation of fructose 1,6-bisphosphatase in vitro. Effect of FMN. Fructose 1,6-bisphosphatase activity was determined with 0.2-0.3 mM glyceraldehyde 3-phosphate as substrate at different times after centrifugation in liver cytosols from rats injected intraperitoneally with either glucagon or vehicle only. Cytosols were prepared in presence (○) or absence (●) of 100 µM FMN (see Materials and Methods). Results from 3 controls and 5 glucagon-treated animals are shown. The first measured activity after isolation of each cytosol was taken as 100 %.

It should be noted that the in vivo activation of fructose 1,6-bisphosphatase (Table I, Fig. 1) can only be observed if FMN is added to the cytosol preparations. In the absence of FMN, erroneously high values of enzyme activity are measured in cytosol from control rats and the glucagon effect in vivo is not apparent. This may be one of the reasons why differing results on the activation of fructose 1,6-bisphosphatase by glucagon in vivo have been obtained (3,7,8).

Effect of fructose 2,6-bisphosphate on fructose 1,6-bisphosphatase activity

As shown in Fig. 3, fructose 1,6-bisphosphatase activity from both glucagon-treated and control rats was significantly inhibited by fructose 2,6-bisphosphate (Fig. 3a). This effect was more marked with cytosol from controls than from glucagon-treated animals. A rearrangement of these data (Fig. 3b) shows that the inhibitor constant K_i for fructose 2,6-bisphosphate is approximately doubled in response to glucagon in vivo. This agrees well with the observation of Furuya and Uyeda (11) that phosphorylation of phosphofructokinase doubles the activation constant K_a for a "low molecular weight activation factor", which probably corresponds to fructose 2,6-bisphosphate. The concentration of the fructose 2,6-bisphosphate solution used in the present experiments has been estimated on the basis of its activating effect on phosphofructokinase

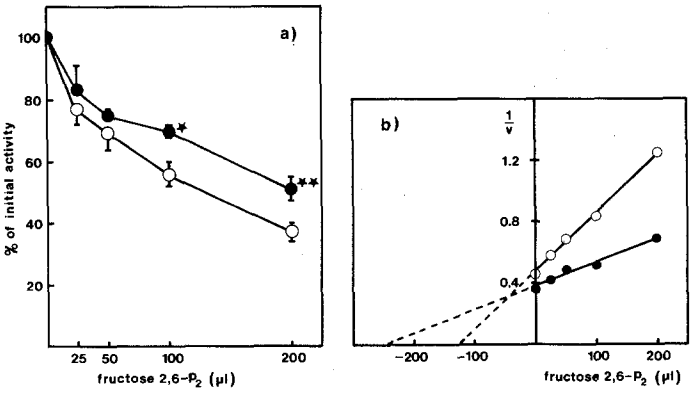

Fig. 3. Inhibition of fructose 1,6-bisphosphatase by fructose 2,6-bisphosphate in cytosol from glucagon-treated rats and from controls. Fructose 1,6-bisphosphatase activity was measured with 0.2-0.3 mM glyceraldehyde 3-phosphate as substrate in liver cytosol from rats injected intraperitoneally with either glucagon (●) or hormone vehicle (○) (see Materials and Methods). Fructose 2,6-bisphosphate (fructose 2,6-P_2, approx. 2.3 µM) was added to the assay medium (final volume 3 ml) in the indicated amounts. In Fig. 3a results are expressed as mean ± S.E.M. from 3 (glucagon) or 4 (controls) independent experiments. p (glucagon-treated vs. control) ★ <0.02 ★★ <0.05. The results were recalculated for Fig. 3b using the measured activity expressed as µmoles·min^{-1}·g $liver^{-1}$ (Dixon plot).

(14) to be about 2.3 µM. The apparent K_i for fructose 1,6-bisphosphatase from control animals as calculated from Fig. 3b, would thus be 0.09 µM. This value agrees well with the K_a of 0.1 µM reported for the activation of phosphofructokinase (14) and is within the concentration range for fructose 2,6-bisphosphate observed in rat liver (15).

DISCUSSION

The results show that on one hand fructose 1,6-bisphosphatase activity is enhanced after injection of glucagon to fed rats and on the other hand that the enzyme activity in vitro can be inhibited by added fructose 2,6-bisphosphate. It could be postulated that the observed activation of the enzyme after glucagon treatment is the result of differing fructose 2,6-bisphosphate concentrations in the isolated cytosols. However, this mechanism is not very probable since the cytosols are diluted more than 300-fold for the measurement of the enzyme activity. It is more likely that the change in enzyme activity is a result of a phosphorylation by a cyclic AMP-dependent protein kinase as has been suggested

by Riou et al. (21). It is furthermore possible that fructose 2,6-bisphosphate is tightly bound to the enzyme and that the amount of this bound inhibitor varies depending on the phosphorylation state of the enzyme, thereby regulating its activity. This interpretation is supported by the observation that the affinity for the inhibitor decreases upon glucagon treatment.

Together with recently published data on phosphofructokinase (10-12), our results suggest a reciprocal regulation of the fructose 6-phosphate/fructose 1,6-bisphosphate substrate cycle by glucagon involving enzyme modification as well as changes in the concentration of fructose 2,6-bisphosphate.

ACKNOWLEDGEMENT

This work was supported by grants of the Swiss National Science Foundation. The excellent technical assistance of Miss C. Kocher is gratefully acknowledged.

REFERENCES

1. Pilkis, S.J., Park, C.R., and Claus, T.H. (1978) Vitamines and Hormones 36, 383-460.
2. Clark, M.G., Kneer, N.M., Bosch, A.L., and Lardy, H.A. (1974) J. Biol. Chem. 249, 5695-5703.
3. Söling, H.S., Brand, I., Imesch, E., Unger, C., Lück, H.J., and Kuhn, A. (1978) Hormones and Cell Regulation (Dumont, J., and Nunez, J. eds.), Vol. 2, pp 209-225, Elsevier, Amsterdam
4. Pilkis, S., Schlumpf, J., Pilkis, J., and Claus, T.H. (1979) Biochem. Biophys. Res. Commun. 88, 960-967.
5. Rognstad, R., and Katz, J. (1980) Arch. Biochem. Biophys. 203, 642-646
6. Van Schaftingen, E., Hue, L., and Hers, H.G. (1980) Biochem. J. 192, 263-271.
7. Taunton, O.D., Stifel, F.B., Greene, H.L., and Herman, R.H. (1974) J. Biol. Chem. 249, 7228-7239.
8. Chatterjee, T., and Datta, A.G. (1978) Biochem. Biophys. Res. Commun. 84, 950-956.
9. Mörikofer-Zwez, S., and Walter, P. (1979) Biochem. Biophys. Res. Commun. 91, 1182-1189.
10. Van Schaftingen, E., Hue, L., and Hers, H.G. (1980) Biochem. J. 192, 887-895.
11. Furuya, E., and Uyeda, K. (1980) J. Biol. Chem. 255, 11656-11659.
12. Claus, T.H., Schlumpf, J.R., Raafat El-Maghrabi, M., Pilkis, J., and Pilkis, S.J. (1980) Proc. Natl. Acad. Sci USA 77, 6501-6505.
13. Richards,C.S., and Uyeda, K. (1980) Biochem. Biophys. Res. Commun. 97, 1535-1540.
14. Van Schaftingen, E., and Hers, H.G. (1980) Biochem. Biophys. Res. Commun. 96, 1524-1531.
15. Van Schaftingen, E., Hue, L., and Hers, H.G. (1980) Biochem. J. 192, 897-901.
16. Pilkis, S.J., Raafat El-Maghrabi, M., Pilkis, J., Claus, T.H., and Cumming, D.A. (1981) J. Biol. Chem. 256, 3171-3174.

17. Tejwani, G.A., Pedrosa, F.O., Pontremoli, S., and Horecker, B.L. (1976) Proc. Natl. Acad. Sci USA 73, 2692-2695.
18. Veech, R.L., Raijman, L., Dalziel, K., and Krebs, H.A. (1969) Biochem. J. 115, 837-842.
19. Goldfine, I.D., Roth, J., and Birnbaumer, L. (1972). J. Biol. Chem. 247, 1211-1218.
20. Kuo, J.F., Krueger, B.K., Sanes, J.R., and Greengard, P. (1970) Biochim. Biophys. Acta 212, 79-91.
21. Riou, J.-P., Claus, T.H., Flockhart, D.A., Corbin, J.D., and Pilkis, S.J. (1977) Proc. Natl. Acad. Sci USA 74, 4615-4619.