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Inhibition of Fructose-6-Phosphate,2-Kinase by *N*-Bromoacetylethanolamine Phosphate *In Vitro* and *In Vivo*[1]

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Fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase (Fru-6-P,2-kinase/Fru-2,6-BPase), a bifunctional enzyme, catalyzes the synthesis and degradation of a potent activator, fructose-2,6-bisphosphate (Fru-2,6-P_2), of phosphofructokinase, and has been postulated to be an important enzyme in the regulation of glycolysis in mammalian tissues. The purpose of this study was to determine whether or not *N*-bromoacetylethanolamine phosphate (BrAcNHEtOP), a specific active site-directed inactivator of Fru-6-P,2-kinase, is useful for studies on the role of Fru-6-P,2-kinase in the regulation of glycolysis *in vivo*. BrAcNHEtOP inactivated purified recombinant rat testis-type Fru-6-P,2-kinase as well as Fru-6-P,2-kinase in a rat liver extract, with half maximum inactivation concentrations of 2 and 15 mM, respectively, on 30 min incubation at 30°C. The increases in Fru-6-P,2-kinase activity and the Fru-2,6-P_2 concentration in livers, prepared from fasted rats, induced by high glucose (50 mM) perfusion were suppressed in parallel after pre-perfusion with 1 to 10 mM BrAcNHEtOP, dose-dependently. Five hours after intraperitoneal injection of BrAcNHEtOP (50 to 150 mg/kg) into mice, the Fru-6-P,2-kinase activity and Fru-2,6-P_2 concentration in livers had decreased in parallel, dose-dependently. These effects continued for 24 h and were accompanied by decreases in the fructose-1,6-bisphosphate, triose phosphates, and lactate contents, although the contents of glucose-6-phosphate and fructose-6-phosphate did not change. These results suggested that BrAcNHEtOP inactivates Fru-6-P, 2-kinase, resulting in a decrease in the Fru-2,6-P_2 level, which causes inactivation of phosphofructokinase and consequently inhibition of glycolysis in liver. Furthermore, the suppressed levels of Fru-6-P,2-kinase activity and metabolites in mice livers were sustained by daily injection of BrAcNHEtOP for 4 days, and body weight gain was also suppressed during the administration of BrAcNHEtOP. These results suggested that BrAcNHEtOP will be a useful reagent for studying the role of Fru-6-P,2-kinase *in vivo*.

Key words: bifunctional enzyme, fructose-2,6-bisphosphatase, fructose-6-phosphate,2-kinase, glycolysis, inhibitor.

Fructose-2,6-bisphosphate (Fru-2,6-P_2) is a potent activator of phosphofructokinase, one of the key regulatory enzymes in glycolysis (*1-3*), and is also an inhibitor of fructose-1,6-bisphosphatase (*4, 5*). The synthesis and degradation of Fru-2,6-P_2 are catalyzed by a bifunctional enzyme, fructose-6-phosphate,2-kinase/fructose-2,6-bisphosphatase (Fru-6-P,2-kinase/Fru-2,6-BPase) (*6-8*). There are several Fru-6-P,2-kinase/Fru-2,6-BPase isozymes, that differ in tissue distribution (*8-14*). The importance of a bifunctional enzyme for regulation of phosphofructokinase (PFK) activity as well as the glycolytic rate has been postulated in different tissues in various animals. There is a relationship between the glucose-dependent increases in glycolysis and Fru-2,6-P_2 in various tissues studied so far. For example, the rate of glycolysis in liver seems to be controlled by the Fru-2,6-P_2 level *via* phosphorylation/dephosphorylation of Fru-6-P,2-kinase/Fru-2,6-BPase (reviewed in Refs. *15-17*). The stimulation of gluconeogenesis in fasted rat liver or glucagon-treated liver involves inhibition of glycolysis, which results from a decrease in Fru-2,6-P_2. This effect of Fru-2,6-P_2 results from the phosphorylation of Fru-6-P,2-kinase/Fru-2,6-BPase by cAMP-dependent protein kinase. On the other hand, a high concentration of glucose increases the glycolytic rate by increasing Fru-2,6-P_2, and the mechanism underlying glucose-induced Fru-2,6-P_2 synthesis was recently determined to be due to dephosphorylation of the bifunctional enzyme by a protein phosphatase type 2A (*18, 19*). However, these results were obtained predominantly

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Abbreviations: BrAcNHEtOP, *N*-bromoacetylethanolamine phosphate; EDTA, ethylenediaminetetraacetic acid; Fru, fructose; Fru-6-P, fructose-6-phosphate; Fru-6-P,2-kinase, fructose-6-phosphate,2-kinase; Fru-1,6-P_2, fructose-1,6-bisphosphate; Fru-2,6-P_2, fructose-2,6-bisphosphate; Fru-2,6-BPase, fructose-2,6-bisphosphatase; Glc-6-P, glucose-6-phosphate; PFK, phosphofructokinase.

through *in vitro* experiments involving purified Fru-6-P, 2-kinase/Fru-2,6-BPase, cultured cells and *in situ* perfusion systems, and not through *in vivo* studies. Generally, it has been considered that lipogenesis may be enhanced by acceleration of glycolysis, and an *in vivo* but not an *in vitro* study will been able evaluation of the contribution of Fru-2,6-P_2 to lipogenesis *via* glycolysis. For an *in vivo* study on the role of Fru-2,6-P_2, the administration of specific inhibitors, *e.g.*, suicide inhibitors, for Fru-6-P, 2-kinase must be useful. *N*-Bromoacetylethanolamine phosphate (BrAcNHEtOP), originally developed as a synthetic substrate analog for Fru-1,6-bisphosphate aldolase (*20*), is a specific active site-directed inactivator of Fru-6-P,2-kinase *in vitro* (*21*). This compound is a competitive inhibitor of Fru-6-P,2-kinase with respect to Fru-6-P without affecting Fru-2,6-BPase. The mechanism underlying the inhibition may comprise alkylation of an active site histidine or lysine (*22*).

In the present study, we have assessed the inhibitory effect of BrAcNHEtOP on Fru-6-P,2-kinase *in vitro* using purified recombinant rat testis-type Fru-6-P,2-kinase, *in situ* using a rat liver perfusion system, and *in vivo* by *ip* injection of BrAcNHEtOP into mice. The results obtained in this study indicated that BrAcNHEtOP significantly inhibits Fru-6-P,2-kinase not only *in vitro* but also *in vivo*, resulting in a decrease in glycolytic flux.

MATERIALS AND METHODS

Materials—BrAcNHEtOP was synthesized according to the method described by Hartman *et al.* (*20*). Rabbit muscle phosphofructokinase (PFK) was prepared as described previously (*23*). Recombinant rat testis Fru-6-P,2-kinase/Fru-2,6-BPase was prepared as described (*24*). Male mice (ddY strain) weighing 18–20 g and male rats (Wistar strain) weighing 200 g were obtained from Ohtsubo Animal Co. (Nagasaki). Phosphoglucose isomerase, glucose-6-phosphate dehydrogenase, hexokinase, rabbit muscle aldolase, triosephosphate isomerase, fructose-1,6-bisphosphate (Fru-1,6-P_2), fructose-6-phosphate (Fru-6-P), NADP, and NADH were purchased from Boeringer-Mannheim (Tokyo). Glucose and a triacylglycerol assay kit were obtained from Wako Pure Chem. (Osaka). All other reagents were of reagent grade and obtained from commercial sources.

Rat Liver Perfusion—Twenty-four hour-fasted rats were anesthetized by injection of sodium pentobarbital (60 mg/kg), and then their livers were perfused for 10 min with Krebs-Hensereit bicarbonate buffer equilibrated with 95% O_2, 5% CO_2 containing 5.6 mM glucose and the indicated concentrations of BrAcNHEtOP (pre-perfusion), after which the perfusion solution was changed to the buffer containing 50 mM glucose, followed by perfusion for an additional 10 min. After perfusion, a piece of the left lobe of the liver was quickly cut out and freeze-clamped in liquid nitrogen. The frozen liver samples were ground in liquid nitrogen and stored at −70°C until use.

Administration of BrAcNHEtOP—Various doses of BrAcNHEtOP dissolved in saline were injected, once a day or daily, into the mice intraperitoneally. At the indicated times, blood and livers were removed from mice anesthetized with sodium pentobarbital. Blood was collected from a femoral artery and the livers were quickly freeze-clamped in liquid nitrogen. The frozen liver samples were ground in liquid nitrogen. The serum and ground liver samples were stored at −70°C until use.

Fru-2,6-P_2 Measurement—Fru-2,6-P_2 was extracted from the frozen tissue powder with 0.1 M glycine/NaOH buffer, pH 10.0 (1 : 3 w/v), and then assayed by the method of Uyeda *et al.* (*1*).

Preparation of Liver Homogenates for the Enzyme Assay—Frozen liver samples were homogenized in ice-cold 50 mM Tris-phosphate buffer, pH 8.0 (1 : 2 w/v), containing 0.5 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM phenylmethylsulfonylfluoride, 1 mM benzamidine, 0.1% aprotinin, and 1% polyethylene glycol 300 using a Ultra Turrax homogenizer (IKA-WERK). The homogenates were centrifuged at 20,000 × *g* for 20 min. Aliquots of the supernatant solutions were used for each enzyme assay.

Measurement of Fru-6-P,2-Kinase Activity—Fru-6-P,2-kinase activity was assayed according to the method of Tominaga *et al.* (*24*). The reaction mixture comprised, in a final volume of 90 μl: 100 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 5 mM ATP, 1 mM Fru-6-P, and 10 mM $MgCl_2$. The reaction was started by the addition of 10 μl of enzyme solution. The mixture was incubated at 30°C, and after various intervals aliquots (10 μl) were transferred to 90 μl of 50 mM Tris-HCl, pH 8.0, and then heated at 90°C for 1 min to stop the reaction. Suitable aliquots of the heated reaction mixture were assayed for Fru-2,6-P_2 as described above. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of Fru-2,6-P_2 per min under these conditions.

Measurement of Fru-2,6-BPase Activity—The reaction mixture comprised 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 50 μM NADP, 17 μM Fru-2,6-P_2, 0.4 unit of desalted Glc-6-P dehydrogenase, and 1 unit of phosphoglucose isomerase. The reaction was initiated by the addition of Fru-2,6-BPase, and was followed at room temperature fluorometrically at excitation and emission wavelengths of 354 and 452 nm, respectively.

Measurement of Aldolase Activity—Aldolase activity was measured according to the method of Rajkumar *et al.* (*25*) with slight modification. The incubation mixture comprised, in a final volume of 1.0 ml: 50 mM Tris-HCl, pH 7.5, 0.1 mM NADH, and 50 μg of a glycerophosphate dehydrogenase-triosephosphate isomerase mixture. The reaction was started by the addition of 2 mM Fru-1,6-P_2 and the rate of the decrease in absorbance at 340 nm was recorded spectrophotometrically. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the degradation of 1 μmol of Fru-1,6-P_2 per min under these conditions.

Measurement of PFK Activity—PFK activity was assayed under the optimum assay conditions according to Uyeda *et al.* (*23*). The incubation mixture comprised 50 mM Tris-HCl, pH 8.0, 0.1 mM EDTA, 6 mM $MgCl_2$, 4 mM $(NH_4)_2SO_4$, 0.16 mM NADH, 1 mM Fru-6-P, and 100 μg of a aldolase-glycerophosphate dehydrogenase-triosephosphate isomerase mixture. The reaction was started by the addition of the PFK solution and the rate of the decrease in absorbance at 340 nm was recorded spectrophotometrically. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μmol of Fru-1,6-P_2 per min under these conditions.

Metabolite Measurement—Intermediates of glycolysis in the livers were extracted with 0.6 M perchloric acid (1 : 5 w/v). The extracts were neutralized with 1 M KOH and then centrifuged at 5,000 × *g* for 10 min. The supernatants were subjected to each assay. All intermediates were assayed by enzymatic analysis. Glucose-6-phosphate (Glc-6-P) and Fru-6-P were assayed using glucose-6-phosphate dehydrogenase and phosphoglucose isomerase according to Michal (*26*). Fru-1,6-P_2 and triose phosphates were measured using aldolase, triosephosphate isomerase and glyceraldehyde dehydrogenase according to Michal (*27*). Lactate was measured by the method of Noll (*28*).

Assay Methods for Serum Lactate, Triacylglycerol, and Glucose—Serum lactate was measured by the method of Noll (*28*). Serum triacylglycerol and glucose were measured using the glycerol-3-phosphate oxidase and glucose oxidase methods, respectively.

Data Analysis—The results are expressed as means ± SEM. Following analysis of variance for repeated measurements of the overall data to assess the statistical significance, the differences between the individual mean values in different groups were analyzed by means of Student's *t*-test by Dunnett's test. The difference was considered to be significant at $p < 0.05$.

Other Methods—Protein concentrations were determined by the Bradford method (*29*) with bovine serum albumin as a standard.

RESULTS

Effect of BrAcNHEtOP on Purified Fru-6-P,2-Kinase and Fru-6-P,2-Kinase in Liver Extracts—It has been reported that BrAcNHEtOP is an irreversible competitive inhibitor of Fru-6-P,2-kinase with respect to Fru-6-P, the half maximum inactivation concentrations for purified Fru-6-P,2-kinases being 1 to 2 mM (*21, 22*). First, we determined the effectiveness of the synthesized BrAcNHEtOP using purified recombinant rat testis Fru-6-P,2-kinase/Fru-2,6-BPase as a model enzyme, and confirmed that the compound inhibited Fru-6-P,2-kinase activity, with a half maximum inactivation concentration of approximately 2 mM, on 30 min incubation at 30°C. On the other hand, Fru-2,6-BPase, aldolase, and PFK were not affected by BrAcNHEtOP treatment (Fig. 1). The inhibitory effect of BrAcNHEtOP on Fru-6-P,2-kinase and other enzymes activity in crude liver extracts was also checked. As shown in Fig. 2, the crude mouse liver extracts and various concentrations of BrAcNHEtOP were incubated, and the remaining enzyme activities were determined. BrAcNHEtOP showed dose-dependent inhibition of Fru-6-P,2-kinase, the half maximum inactivation concentration of BrAcNHEtOP being about 15 mM. However, neither aldolase nor PFK activity was affected by this reagent at the same concentration. The need of a higher concentration of BrAcNHEtOP for the inactivation of Fru-6-P,2-kinase in crude liver extracts may be due to degradation of the compound by contaminating lysosomal enzymes.

Effect of BrAcNHEtOP on Fru-6-P,2-Kinase Activity and the Metabolite Content in Perfused Liver—It has been observed that, when livers prepared from fasted rats are perfused with a high concentration of glucose (40 mM), Fru-6-P,2-kinase is activated and the Fru-2,6-P_2 content increases in liver compared to in the case of perfusion with a low concentration of glucose (5.6 mM) (*18*). We used this perfusion system to determine the more direct effect of BrAcNHEtOP on liver, *i.e.* whether or not BrAcNHEtOP inhibits both the activation of Fru-6-P,2-kinase and the increase in the Fru-2,6-P_2 content in liver induced by high glucose perfusion was investigated. As shown in Fig. 3a, the increases in the Fru-2,6-P_2 content and Fru-6-P,2-kinase activity on high glucose perfusion (50 mM) were suppressed by pre-perfusion with 1 to 10 mM BrAcNHEtOP, dose-dependently. The basal Fru-2,6-P_2 content and Fru-6-P,2-kinase activity level decreased beyond 15 mM BrAcNHEtOP. Although the increasing levels of Glc-6-P and Fru-6-P on high glucose perfusion were not affected by pre-

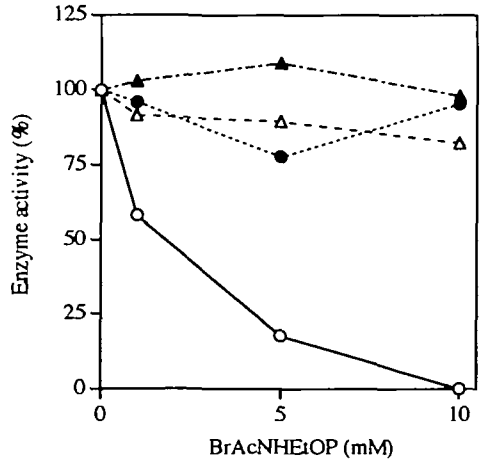

Fig. 1. **Effect of BrAcNHEtOP on the activities of Fru-6-P,2-kinase/Fru-2,6-BPase, PFK, and aldolase.** Recombinant rat testis Fru-6-P,2-kinase/Fru-2,6-BPase (2.9 mU/ml, ○, and 1.3 mU/ml, ●), rabbit muscle PFK (2.4 U/ml, △), and rabbit muscle aldolase (4.5 U/ml, ▲) were incubated with increasing concentrations of BrAcNHEtOP at 30°C for 30 min, and then the residual activities were determined.

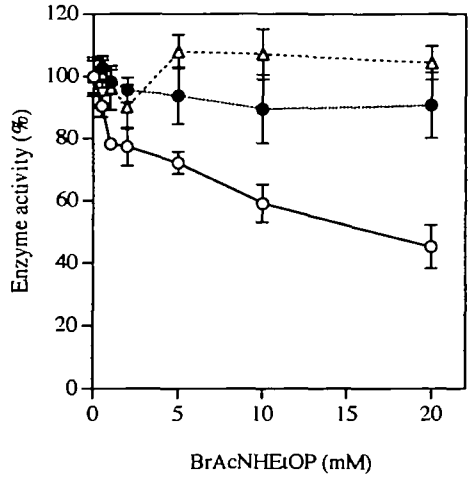

Fig. 2. **Effect of BrAcNHEtOP on the activities of Fru-6-P,2-kinase, PFK, and aldolase in mouse liver extracts.** Mouse liver extracts were incubated with increasing concentrations of BrAcNHEtOP at 30°C for 30 min, and then the residual activities of Fru-6-P,2-kinase (○), PFK (●), and aldolase (△) were determined. Each point is the mean ± SEM for three experiments.

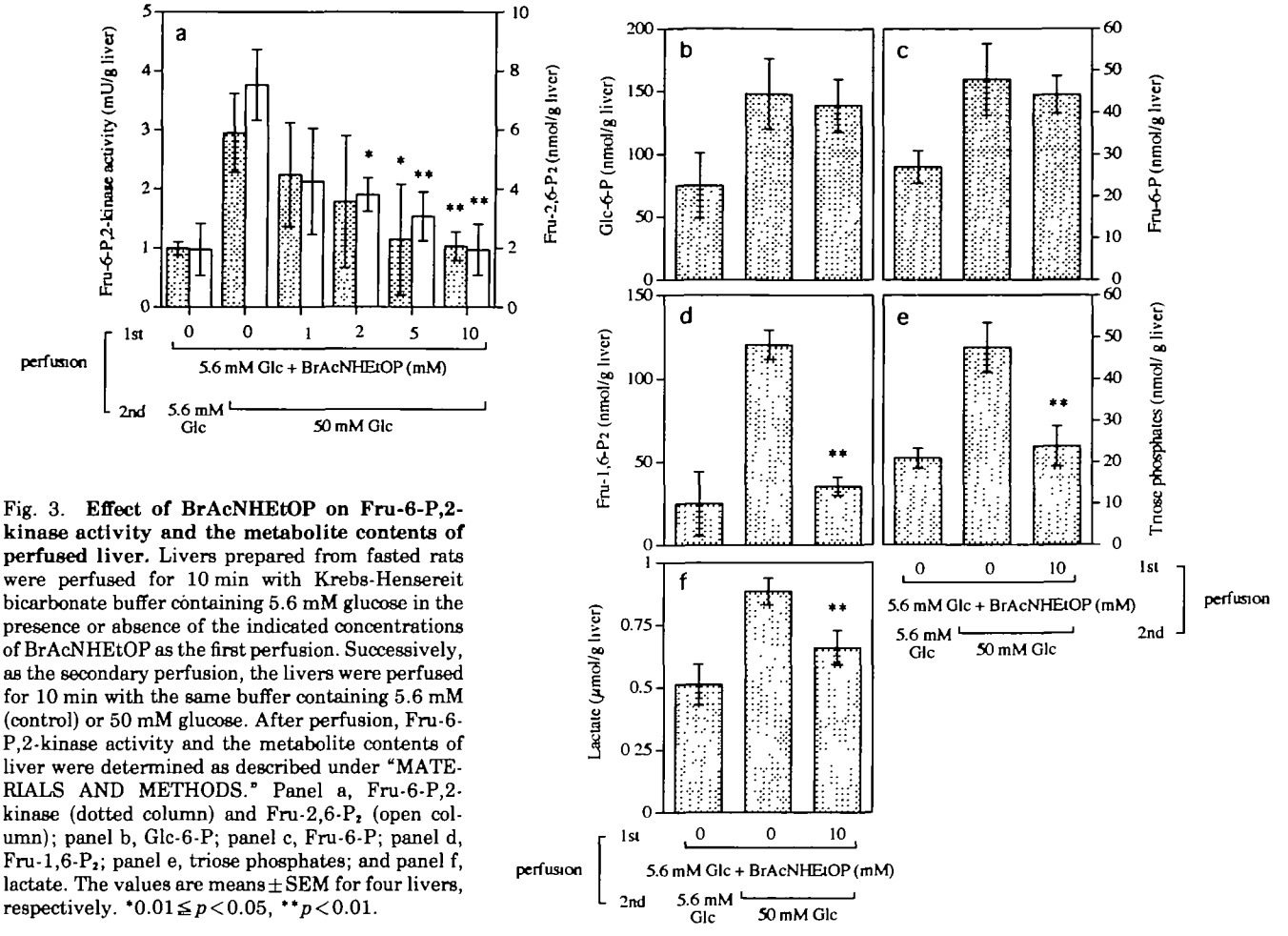

Fig. 3. **Effect of BrAcNHEtOP on Fru-6-P,2-kinase activity and the metabolite contents of perfused liver.** Livers prepared from fasted rats were perfused for 10 min with Krebs-Hensereit bicarbonate buffer containing 5.6 mM glucose in the presence or absence of the indicated concentrations of BrAcNHEtOP as the first perfusion. Successively, as the secondary perfusion, the livers were perfused for 10 min with the same buffer containing 5.6 mM (control) or 50 mM glucose. After perfusion, Fru-6-P,2-kinase activity and the metabolite contents of liver were determined as described under "MATERIALS AND METHODS." Panel a, Fru-6-P,2-kinase (dotted column) and Fru-2,6-P_2 (open column); panel b, Glc-6-P; panel c, Fru-6-P; panel d, Fru-1,6-P_2; panel e, triose phosphates; and panel f, lactate. The values are means ± SEM for four livers, respectively. $^*0.01 \leq p < 0.05$, $^{**}p < 0.01$.

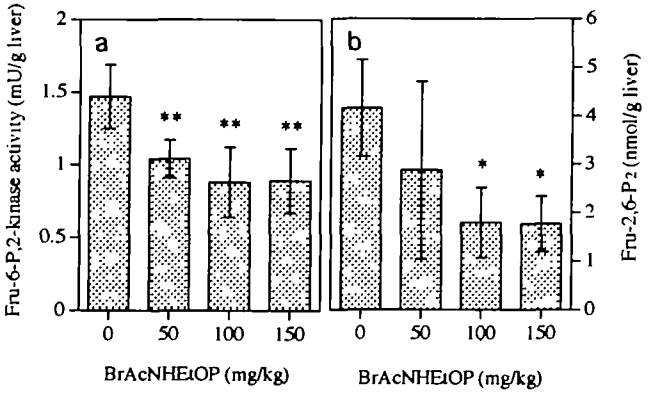

Fig. 4. **Effect of a single administration of increasing doses of BrAcNHEtOP on Fru-6-P,2-kinase activity and the Fru-2,6-P_2 content of liver.** BrAcNHEtOP (50, 100, and 150 mg/kg) was *ip* injected into mice, and after 5 h their livers were removed to assay Fru-6-P,2-kinase activity (panel a) and the Fru-2,6-P_2 content (panel b). The values are means ± SEM for four livers, respectively. $^*0.01 \leq p < 0.05$, $^{**}p < 0.01$.

perfusion with 1 to 10 mM BrAcNHEtOP, the increases in the concentrations of Fru-1,6-P_2, triose phosphates and lactate on high glucose perfusion were suppressed by pre-perfusion with BrAcNHEtOP (Fig. 3, b-f).

Effect of a Single Administration of BrAcNHEtOP—Various concentrations of BrAcNHEtOP were administered to male mice intraperitoneally. Five hours after the administration, the mice were sacrificed and their livers were removed immediately. The Fru-6-P,2-kinase activities and Fru-2,6-P_2 contents of the livers were determined (Fig. 4). Fru-6-P,2-kinase activity was decreased by BrAcNHEtOP administration in a dose-dependent manner. The Fru-2,6-P_2 level also decreased, corresponding to the decrease in Fru-6-P,2-kinase activity. Figure 5a shows the time-dependent changes in the Fru-6-P,2-kinase activity and Fru-2,6-P_2 levels in liver after the administration of BrAcNHEtOP (150 mg/kg). Fru-6-P,2-kinase activity in the livers of BrAcNHEtOP-administered mice had decreased to about two-thirds of the control level at 2 h after treatment, and the effect continued for 24 h. The recovery of Fru-6-P,2-kinase activity was observed from 30 h later, and the activity reached the control level at 48 h after the treatment (data not shown). The Fru-2,6-P_2 content of the livers of BrAcNHEtOP-administered mice also decreased, and the change in the Fru-2,6-P_2 level was accompanied by a change in the Fru-6-P,2-kinase level. Figure 5, b-f, shows the effects of BrAcNHEtOP treatment on the levels of glycolytic intermediates in liver. The Glc-6-P and Fru-6-P levels were not changed by BrAcNHEtOP treatment (b and c). However, the Fru-1,6-P_2, triose phosphates, and serum lactate contents of liver decreased compared with the control levels (d-f).

TABLE I. **Effect of repetitive administration of BrAcNHEtOP.** Various doses of BrAcNHEtOP were daily injected *ip* for 4 days. On the fourth day, mice were sacrificed, and Fru-6-P,2-kinase activity and the Fru-2,6-P_2 content of liver, and the lactate and triacylglycerate contents of serum were measured. During the course of the experiments, body weight gain of mice was also monitored.

	Fru-6-P,2-kinase activity (mU/g liver)	Fru-2,6-P_2 (nmol/g liver)	Body weight gain (g/day)	Lactate (mM)	Triacylglycerol (mM)
Control	0.821±0.114	3.03±0.12	2.17±0.38	5.49±0.81	1.10±0.06
50 mg/kg	0.363±0.048**	2.70±0.34	1.72±0.24	5.26±0.66	0.73±0.12
100 mg/kg	0.277±0.012**	2.54±0.30	1.03±0.38**	4.02±0.77	0.60±0.02*
150 mg/kg	0.131±0.028**	1.39±0.15**	0.51±0.22**	2.24±0.35**	0.53±0.29*

*$0.01 \leq p < 0.05$, **$p < 0.01$.

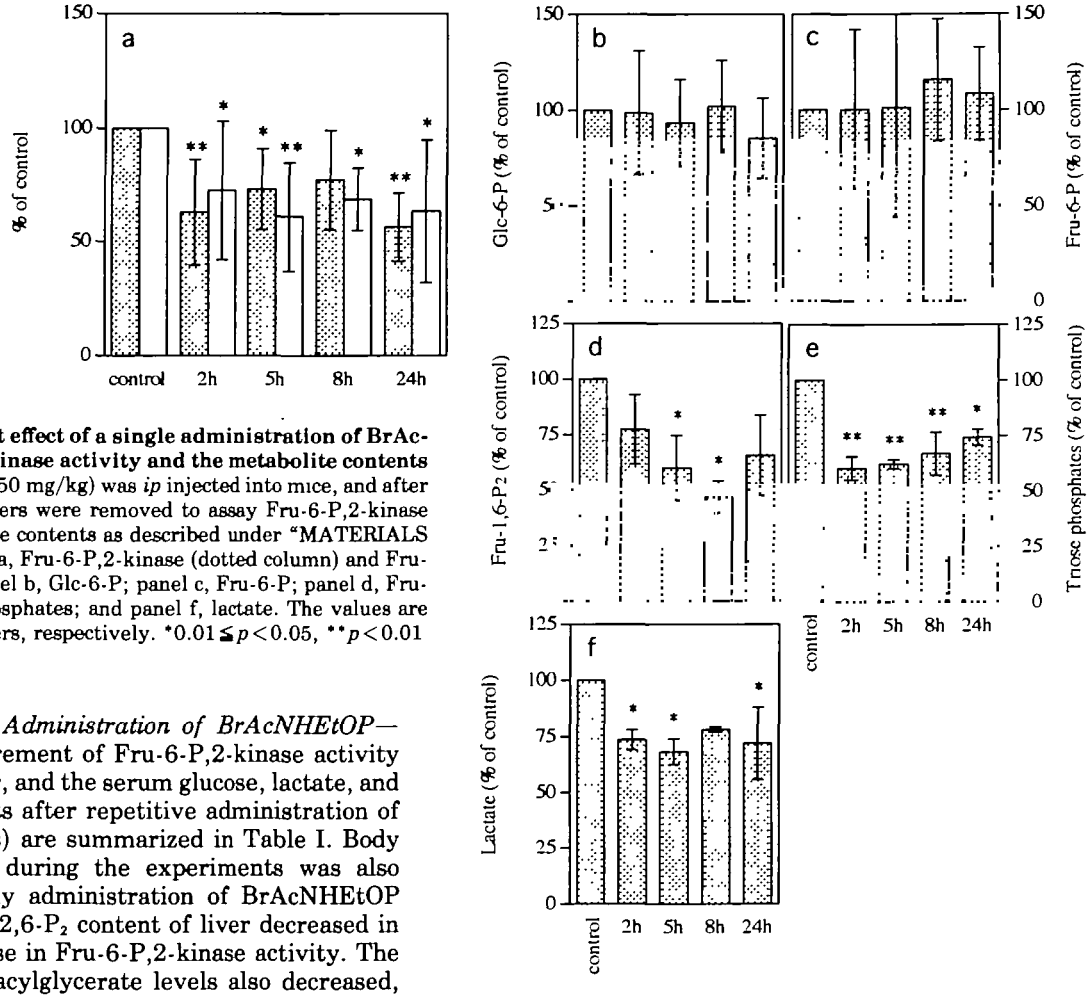

Fig. 5. **Time-dependent effect of a single administration of BrAcNHEtOP on Fru-6-P,2-kinase activity and the metabolite contents of liver.** BrAcNHEtOP (150 mg/kg) was *ip* injected into mice, and after 2, 5, 8, and 24 h their livers were removed to assay Fru-6-P,2-kinase activity and the metabolite contents as described under "MATERIALS AND METHODS." Panel a, Fru-6-P,2-kinase (dotted column) and Fru-2,6-P_2 (open column); panel b, Glc-6-P; panel c, Fru-6-P; panel d, Fru-1,6-P_2; panel e, triose phosphates; and panel f, lactate. The values are means±SEM for four livers, respectively. *$0.01 \leq p < 0.05$, **$p < 0.01$

Effect of Repetitive Administration of BrAcNHEtOP— The results of measurement of Fru-6-P,2-kinase activity and Fru-2,6-P_2 in liver, and the serum glucose, lactate, and triacylglycerol contents after repetitive administration of BrAcNHEtOP (4 days) are summarized in Table I. Body weight gain of mice during the experiments was also monitored. After daily administration of BrAcNHEtOP (150 mg/kg), the Fru-2,6-P_2 content of liver decreased in parallel with a decrease in Fru-6-P,2-kinase activity. The serum lactate and triacylglycerate levels also decreased, but no notable change in the serum glucose level was observed. Furthermore, body weight gain of mice was inhibited during BrAcNHEtOP administration.

DISCUSSION

Many studies concerning the relationship between glucose metabolism and regulation of PFK activity have been reported. Various allosteric effectors for PFK, such as ATP, ADP, AMP, citrate, P_i, and Fru-2,6-P_2, are known. Among these effectors, especially in liver, Fru-2,6-P_2 is believed to be the most important one and not only enhances PFK activity but also inhibits fructose-1,6-bisphosphatase (*1-5*). Up to now, this regulation of glycolysis has best been explained by the level of this stimulator, Fru-2,6-P_2 (reviewed in Refs. *30-32*). Thus, the specific inhibitor of Fru-6-P,2-kinase, which catalyzes this reaction of Fru-2,6-P_2 synthesis, must be a potent tool for determining the physiological role of Fru-2,6-P_2 in glucose and consequent metabolism. *N*-Bromoacetylethanolamine phosphate (BrAcNHEtOP) was originally synthesized as an inhibitor of Fru-1,6-bisphosphate aldolase (*20*). It has been revealed that this compound is a competitive and irreversible inhibitor of Fru-6P,2-kinase with respect to Fru-6-P without affecting to Fru-2,6-BPase (*21*). The purpose of this study was to determine whether or not BrAcNHEtOP is useful for studying the roles of Fru-2,6-P_2 and Fru-6P,2-kinase in the regulation of glycolysis *in vivo*. In this study, we focused on the effect of BrAcNHEtOP on liver metabolism *in vitro* and *in vivo* using liver homogenates, a liver perfusion system and whole animals.

In vitro experiments (Figs. 1 and 2) showed that BrAcNHEtOP inhibited Fru-6-P,2-kinase but not aldolase or PFK. To determine the direct effect of BrAcNHEtOP on liver cells, fasted rat livers were perfused in the presence of BrAcNHEtOP. In the fasted state and on glucagon stimulation, liver Fru-6-P,2-kinase is phosphorylated by cAMP dependent protein kinase and its activity is thus kept at a low level, which causes a low level of Fru-2,6-P_2 in liver (*8, 33, 34*). When such livers prepared from fasted rats are perfused with a high concentration of glucose, Fru-6-P,2-kinase is activated and the Fru-2,6-P_2 level increases (*18*). This activation of Fru-6-P,2-kinase has been estimated to be due to dephosphorylation of Fru-6-P,2-kinase by a protein phosphatase type 2A (*19*). Under these perfusion conditions, pre-perfusion with BrAcNHEtOP before high glucose perfusion apparently diminished the glucose effect (Fig. 3, a and b), indicating that incorporated BrAcNHEtOP or its metabolites affected Fru-6-P,2-kinase and thus inhibited its activity. Also the metabolite concentrations in liver, such as those of Glc-6-P, Fru-6-P, Fru-1,6-P_2, triose phosphates, and lactate, are usually increased by high glucose perfusion, which was confirmed in this study. The increased levels of Glc-6-P and Fru-6-P did not change on pre-perfusion with BrAcNHEtOP, but the metabolite levels in the post-PFK reaction in glycolysis, such as those of Fru-1,6-P_2, triose phosphates and lactate, were significantly decreased. These results indicated that BrAcNHEtOP or its metabolites are incorporated into liver cells and bound to inhibit Fru-6-P,2-kinase, which causes a decrease in the Fru-2,6-P_2 level, inactivates PFK and depresses glycolytic flux, successively.

When BrAcNHEtOP was *ip* administered to mice, the liver Fru-6-P,2-kinase activity was inhibited and the level of Fru-2,6-P_2 decreased in a dose-dependent manner (Fig. 4). The effects of single BrAcNHEtOP administration to mice on the Fru-6-P,2-kinase activity and the level of Fru-2,6-P_2 in liver continued for almost 24 h (Fig. 5). Similar to in the case of perfusion experiments, the levels of Glc-6-P and Fru-6-P did not change with the treatment, however, the levels of Fru-1,6-P_2, triose phosphates, and lactate decreased, indicating that glycolysis was suppressed due to a decrease in PFK activity in liver. These results indicated that BrAcNHEtOP can be used as an effective inhibitor of glycolysis *via* Fru-6-P,2-kinase inhibition.

To determine the chronic effect of BrAcNHEtOP, BrAcNHEtOP was injected into mice repetitively at 24 h intervals. After 4 days, the Fru-6-P,2-kinase activity and Fru-2,6-P_2 content in liver, and the serum lactate and triacylglycerol contents were sustained significantly at low levels by repetitive injection of BrAcNHEtOP (150 mg/kg). Furthermore, during the administration of BrAcNHEtOP, body weight gain was suppressed. These results suggested that repetitive administration of BrAcNHEtOP to mice affected not only inhibition of glycolysis but also lipid metabolism, causing suppression of body weight gain. Enhanced lipogenesis (*35, 36*) and an elevated Fru-2,6-P_2 level (*37*) in livers of genetically obese mice have been observed. Liver glycolysis provides C3 units for the synthesis of lipids and thus is an important component of the control of lipogenesis. It has been considered that the increased concentration of Fru-2,6-P_2 in the livers of obese mice contributes to the increased lipogenesis by providing an increased supply of lactate and pyruvate (*35*). Our data revealing suppression of body weight gain by Fru-6-P,2-kinase inhibitor administration supported the above consideration.

It is not known whether or not BrAcNHEtOP itself is incorporated into cells. However, the results obtained in this study indicated that BrAcNHEtOP or its derivatives are indeed incorporated into cells and inhibit Fru-6-P,2-kinase activity. There is a possibility that the phosphate residue of BrAcNHEtOP may be hydrolyzed by some esterase and may be derivatized in cells. Indeed, synthesized *O*-methylated BrAcNHEtOP was effective in inhibiting Fru-6-P,2-kinase *in vitro* (data not shown). Investigation of the metabolism of BrAcNHEtOP after *in vivo* administration is in progress.

In conclusion, BrAcNHEtOP is a potent inhibitor of Fru-6-P,2-kinase not only *in vitro* but also *in vivo*. In BrAcNHEtOP treated mice, the glycolysis rate is significantly decreased. Work is in progress to elucidate more details of the effects of the inhibitor on normal and abnormal animals, and of metabolism of the inhibitor.

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