A Mechanism for Fatty Acid Inhibition of Glucose Utilization in Liver

ROLE OF XYLULOSE 5-P*

(Received for publication, November 22, 1995, and in revised form, January 25, 1996)

Ye Qi Liu and Kosaku Uyeda
From the Research Service of the Department of Veterans Affairs Medical Center and Department of Biochemistry, The University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75216

The glucose-stimulated rise in Fru-2,6-P$_2$ in liver results from xylulose 5-P activation of a specific protein phosphatase 2A which dephosphorylates Fru-6-P,2-kinase:Fru-2,6-bisphosphatase (Nishimura, M., and Uyeda, K. (1994) J. Biol. Chem. 269, 26100–26106). In order to determine the role of xylulose 5-P in regulating Fru-2,6-P$_2$ in liver, the effect of fatty acids, various hexoses, and hormones was examined in perfused rat liver and in intact rats. When 24-h starved rat livers were perfused with acetate, butyrate, or propionate, Fru-2,6-P$_2$ and xylulose 5-P decreased to the same extent and at similar rates. The activity ratios of the kinase and the phosphatase changed in a reciprocal manner, indicating that the phosphorylated form of the enzyme was increased by the fatty acids perfusion. The fatty acids caused the similar changes in the metabolites and the phosphorylation state of the bifunctional enzyme in livers of fed animals. Fructose, galactose, or mannose perfusion in starved rat liver increased both Fru-2,6-P$_2$ and xylulose 5-P and converted the bifunctional enzyme to the dephospho form. Both the Fru-2,6-P$_2$ and xylulose 5-P levels in rats fed a high fat diet decreased over 50% compared to that in control rats. These results indicated a close correlation between Fru-2,6-P$_2$ and xylulose 5-P levels and the phosphorylation state of fructose 6-P,2-kinase:fructose 2,6-bisphosphatase. Fatty acid inhibition of glucose metabolism can be explained by a decrease in xylulose 5-P, which lowers xylulose 5-P-activated protein phosphatase 2A activity, resulting in more phosphorylated form of the bifunctional enzyme and consequently lower Fru-2,6-P$_2$.

Fructose 2,6-P$_2$ (Fru-2,6-P$_2$) is the most potent activator of phosphofructokinase, and plays an important role in regulation of glycolysis (reviewed in Ref. 1). Changes in glycolytic rates in liver can be attributed to the changes in concentration of Fru-2,6-P$_2$ under nearly all conditions (reviewed in Refs. 1–3), including hormonal stimulation, diet, starvation, and ischemia (4). The concentration of Fru-2,6-P$_2$ is determined by the relative activities of the kinase and the phosphatase moieties of a bifunctional enzyme, Fru-6-P,2-kinase (Fru-6-P + ATP → Fru-2,6-P$_2$ + ADP) and Fru-2,6-bisphosphatase (Fru-2,6-Pase) (Fru-2,6-P$_2$ → Fru-6-P + P). The liver bifunctional enzyme is regulated by phosphorylation and dephosphorylation which alters the relative activities. Phosphorylation of the bifunctional enzyme is catalyzed by cAMP-dependent protein kinase (Scheme I), and the phosphorylation results in inhibition of Fru-6-P,2-kinase and activation of Fru-2,6-Pase.

The administration of a high concentration of glucose to isolated hepatocytes, the perfusion of high glucose to liver, or the feeding of a high carbohydrate diet to starved (5) rats increases Fru-2,6-P$_2$ (6–8). This rise was attributed to the increased concentration of Fru-6-P (9), which is the substrate for Fru-6-P,2-kinase and an inhibitor of Fru-2,6-Pase. More recently, however, Nishimura et al. (10) demonstrated that the glucose-dependent increase in Fru-2,6-P$_2$ is a result of dephosphorylation of the bifunctional enzyme resulting in activation of the kinase and inhibition of the phosphatase, which is the reversal of the effect of cAMP. The dephosphorylation of the enzyme is catalyzed by a specific protein phosphatase, which is activated specifically by xylulose 5-P (Xu-5-P) (Scheme I) (10). The protein phosphatase has been purified to homogeneity and characterized as a heterotrimeric protein phosphatase 2A (PP2A) (11). Although the PP2A dephosphorylates other substrates, such as pyruvate kinase and phosphorylase a, only the reaction with Fru-6-P,2-kinase:Fru-2,6-Pase as a substrate is stimulated by Xu-5-P (11). Based on these observations, we proposed the mechanism shown in Scheme I (11). According to this scheme, the Fru-6-P$_2$ concentration in liver is determined by the phosphorylation state of Fru-6-P,2-kinase:Fru-2,6-Pase, and the phosphorylation state of the enzyme is governed by the relative activities of protein kinase A and Xu-5-P-activated PP2A. The activities of protein kinase A and Xu-5-P-activated PP2A are controlled by cAMP and Xu-5-P, respectively. We proposed that Xu-5-P may serve as a second messenger, sensing the level of glucose in liver and antagonizing the effect of hormone mediated by cAMP, to stimulate glycolysis by raising Fru-2,6-P$_2$ level by dephosphorylation of the bifunctional enzyme (11).

It is well known that glucose metabolism is inhibited by administration of fatty acid in liver and heart, so called "glucose sparing" effect (12–15). Administration of palmitate and hexanate has been shown to inhibit glycolysis in isolated hepatocytes, and the inhibition is attributed to decreased Fru-2,6-P$_2$ concentration (16, 17). Short chain fatty acids including butyrate, propionate, and acetate also have been shown to have a similar effect (18). Hue et al. (16) suggested that the mechanism for this fall in Fru-2,6-P$_2$ could result from inhibition of the bifunctional enzyme by citrate. These results were obtained before the Xu-5-P-activated PP2A was discovered (17). Thus, the objectives of this current work were 2-fold: (a) examine the
validity of the proposed physiological roles of the Xu-5-P and the Xu-5-P-activated PP2A in regulation of Fru-2,6-P2 in liver; and (b) determine whether the fatty acid sparing effect is related to citrate or Xu-5-P. If this hypothesis were true, one predicts that fatty acid feeding or perfusion with fatty acid would lower Xu-5-P concentration in liver, resulting in inhibition of the Xu-5-P-activated PP2A and thus increase the phospho form of Fru-6-P,2-kinase:Fru-2,6-Pase. The net result would be decreased Fru-2,6-P2 level and inhibition of phosphofructokinase and glycolysis to lower glucose utilization. The results presented herein support the Xu-5-P-mediated mechanism for the fatty acid inhibition of glucose metabolism.

**EXPERIMENTAL PROCEDURES**

Materials—CAMP enzyme immunoassay system was purchased from Amersham Corp. Glucose, sucrose, galactose, deoxyglucose, sodium acetate, n-butyl acid, citrate lyase, and gluconate were purchased from Sigma. d-Mannose was purchased from California Co. for Biochemical Research (Los Angeles, CA), and fructose was obtained from Eastman Kodak (Rochester, NY). Insulin was purchased from Eli Lilly & Co (Indianapolis, IN). Propionic acid was purchased from Aldrich Chemical Co. Propionic acid and butyric acid were neutralized with NaOH before use. Casein, a mineral mixture, and a vitamin mixture were purchased from Harlan Co. (Madison, WI). Metamucil, oils, and lard were obtained from a local grocery store. All other chemicals and reagents were analytical reagent grade and purchased from commercial sources.

Rats and Diets—Male Sprague-Dawley rats (200–250 g) were purchased from Sasco Co. (Omaha, NE). Rats were maintained by feeding ad libitum with the standard NIH diet (4% fat, 24% crude protein, and 45% carbohydrate). Male Sprague-Dawley rats (200–250 g) were purchased from Sasco Co. (Omaha, NE). Rats were maintained by feeding ad libitum with the standard NIH diet (4% fat, 24% crude protein, and 45% carbohydrate). Male Sprague-Dawley rats (200–250 g) were purchased from Sasco Co. (Omaha, NE). Rats were maintained by feeding ad libitum with the standard NIH diet (4% fat, 24% crude protein, and 45% carbohydrate). Male Sprague-Dawley rats (200–250 g) were purchased from Sasco Co. (Omaha, NE). Rats were maintained by feeding ad libitum with the standard NIH diet (4% fat, 24% crude protein, and 45% carbohydrate). Male Sprague-Dawley rats (200–250 g) were purchased from Sasco Co. (Omaha, NE). Rats were maintained by feeding ad libitum with the standard NIH diet (4% fat, 24% crude protein, and 45% carbohydrate).

**RESULTS**

Effect of Fatty Acids—Livers from 24-h starved rats or rats fed with the standard diet were perfused with 40 mM glucose for 10 min followed by acetate (5 mM), propionate (10 mM), or butyrate (5 mM) for 10 min. During the initial perfusion with a high concentration of glucose, the liver Fru-2,6-P2 concentration in the starved rat increased from 2.6 to 8.3 nmol/g (Table I) and from 8.7 to 12.6 nmol/g in the fed rat liver (Table II). The continued perfusion with 40 mM glucose for an additional 10 min did not further increase the Fru-2,6-P2 concentration (data not shown). Glucose, Glu 6-P, Fru-6-P, and Xu-5-P also increased similarly in both groups of the rat livers. Following the initial glucose perfusion the Fru-6-P-2-kinase and Fru-2,6-Pase activity ratios (v/Vmax) were 0.93 and 0.67 in both the starved and ad libitum fed rat livers, respectively, indicating that the bifunctional enzyme was mostly in dephospho form in the glucose-perfused livers. The activity ratio of Fru-6-P-2-kinase at two different concentrations of Fru-2,6-P2 for the phosphorylated and dephosphorylated forms are 1 and 0.4, respectively (4, 10). The activity ratios of Fru-6-P-2-kinase at two different concentriers.
The effect of glucose, acetate, butyrate, and propionate on various metabolites and bifunctional enzyme activities in perfused livers of 24-h starved rats

Livers were perfused with 40 mM glucose for 10 min prior to perfusion with the fatty acids for 10 min (starved rat livers contained 2.6 ± 0.2 nmol/g Fru-2,6-P$_2$, see Table VI). The activity ratio of the bifunctional enzyme and the content of the metabolites were determined as described under "Experimental Procedures." Values are mean ± S.D. (n = 4).

| Enzyme/metabolite | Glucose (40 min) | Acetate (5 min) | Butyrate (10 min) | Propionate (5 min) |
|-------------------|------------------|----------------|-------------------|--------------------|
| Fru-2,6-P$_2$ (nmol/g) | 8.3 ± 0.8 | 5.1 ± 0.6 | 4.7 ± 0.2 | 4.0 ± 0.9 |
| Fru-6-P,2-kinase (v/V$_{max}$) | 0.93 ± 0.03 | 0.56 ± 0.01 | 0.54 ± 0.08 | 0.61 ± 0.09 |
| Fru-2,6-Pase (v/V$_{max}$) | 0.67 ± 0.02 | 0.91 ± 0.04 | 0.92 ± 0.04 | 0.90 ± 0.16 |
| Xu-5-P (nmol/g) | 21 ± 1.4 | 8.9 ± 0.1 | 13 ± 0.7 | 8.3 ± 0.3 |
| cAMP (nmol/g) | 0.8 ± 0.1 | 1.1 ± 0.1 | 1.3 ± 0.2 | 1.2 ± 0.1 |
| GIC (mM) | 14 ± 0.2 | 16 ± 0.5 | 22 ± 1.2 | 0.9 ± 0.1 |
| Glc-6-P (nmol/g) | 107 ± 23 | 37 ± 5 | 36 ± 7 | 32 ± 2.1 |
| Fru-6-P (nmol/g) | 42 ± 2 | 12 ± 2 | 12 ± 2 | 12 ± 2 |
| Glyceraldehyde 3-phosphosphate (nmol/g) | 1.3 ± 0.2 | 0.5 ± 0.2 | 0.6 ± 0.1 | 0.4 ± 0.1 |
| Citrate (nmol/g) | 284 ± 16 | 643 ± 34 | 328 ± 14 | 387 ± 26 |
| Fru-2,6-P$_2$/Xu-5-P | 0.40 | 0.57 | 0.38 | 0.48 |
| Fru-2,6-P$_2$/citrate | 0.009 | 0.008 | 0.014 | 0.010 |

The effect of glucose, acetate, butyrate, and propionate on various metabolites and bifunctional enzyme activities in perfused livers of ad libitum fed rats

Livers were perfused with 40 mM glucose for 10 min prior to perfusion with the fatty acids for 10 min. The activity ratio of the bifunctional enzyme and the content of the metabolites were determined as described under "Experimental Procedures." Values are mean ± S.D. (n = 4).

| Enzyme/metabolite | Glucose (40 min) | Acetate (5 min) | Butyrate (10 min) | Propionate (5 min) |
|-------------------|------------------|----------------|-------------------|--------------------|
| Fru-2,6-P$_2$ (nmol/g) | 12.6 ± 1.2 | 6.7 ± 0.8 | 5.7 ± 0.9 | 7.5 ± 0.4 |
| Fru-6-P,2-kinase (v/V$_{max}$) | 0.93 ± 0.05 | 0.67 ± 0.09 | 0.57 ± 0.06 | 0.55 ± 0.08 |
| Fru-2,6-Pase (v/V$_{max}$) | 0.67 ± 0.03 | 0.89 ± 0.05 | 0.93 ± 0.04 | 0.89 ± 0.01 |
| Xu-5-P (nmol/g) | 171 ± 14 | 77 ± 3 | 84 ± 12 | 82 ± 3 |
| cAMP (nmol/g) | 0.7 ± 0.1 | 1.3 ± 0.2 | 1.3 ± 0.1 | 1.5 ± 0.1 |
| GIC (mM) | 25 ± 2 | 6 ± 1 | 7 ± 1 | 10 ± 1 |
| Glc-6-P (nmol/g) | 196 ± 17 | 149 ± 10 | 152 ± 10 | 166 ± 5 |
| Fru-6-P (nmol/g) | 75 ± 6 | 44 ± 5 | 57 ± 5 | 51 ± 5 |
| Glyceraldehyde 3-phosphosphate (nmol/g) | 22 ± 0.2 | 11 ± 0.2 | 11 ± 0.1 | 10 ± 0.1 |
| Citrate (nmol/g) | 276 ± 12 | 947 ± 56 | 864 ± 63 | 368 ± 22 |
| Fru-2,6-P$_2$/Xu-5-P | 0.074 | 0.087 | 0.068 | 0.091 |
| Fru-2,6-P$_2$/citrate | 0.046 | 0.007 | 0.007 | 0.020 |

Titrations of Fru-6-P are v/V$_{max} = 1$ for a fully dephosphorylated form and v/V$_{max} = 0.4$ for fully phosphorylated form (4, 10).

During the subsequent perfusion of the starved rat livers with acetate, propionate, or butyrate, Fru-2,6-P$_2$ decreased 40–50% and Xu-5-P also decreased 40–60%. The activity ratio of Fru-6-P,2-kinase decreased, while the activity ratio of Fru-2,6-Pase increased, indicating that the enzyme became more phosphorylated. Glucose and hexose-Ps were decreased 70–90% by the fatty acid perfusion, but citrate increased 36–126% (Table I). The ratios of Fru-2,6-P$_2$/Xu-5-P concentration was 90% by the fatty acid perfusion, but citrate increased 36–126% (Table I). The ratios of Fru-6-P,2-kinase decreased, while the activity ratio of Fru-2,6-Pase in both fasted and fed livers (Fig. 1, A and B).

Table II shows that these short chain fatty acids decreased glucose utilization, as indicated by the fact that the glucose and hexose-P levels in the perfused livers dropped significantly. Fru-2,6-P$_2$ and Xu-5-P did not decrease as much as glucose or hexose-Ps, but more importantly, both decreased nearly in concert in both fed and starved rat livers. Furthermore, the fatty acids caused conversion of the dephospho form of Fru-6-P,2-kinase:Fru-2,6-Pase to the phospho-form, judging from the changes in the activity ratios.

Effect of Different Hexoses—To test specificity for glucose, starved and fed rat livers were perfused for 10 min with glucose, fructose, galactose, or mannose. The higher concentration of some of the sugars could not be perfused due to a significant drop in the ATP level in liver (29, 30). The results (Table III) showed that all these hexoses were able to increase the Fru-2,6-P$_2$ and Xu-5-P levels 50–90% and 67–104%, respectively, compared to those perfused with 5 mM glucose in the starved rat livers. The concentration ratios of Fru-2,6-P$_2$/Xu-5-P were the same among all these sugars, in spite of the fact that the Fru-2,6-P$_2$ level doubled with some of the sugars (compare fructose in Table III and galactose in Table IV). The increase in Fru-6-P,2-kinase and the decrease in Fru-2,6-Pase activity ratios corresponded closely with the changes in Fru-2,6-P$_2$ as well as Xu-5-P. However, there was less close correspondence between Fru-2,6-P$_2$, Xu-5-P, or the enzyme activity ratios with
the changes in the glucose, hexose-P, and citrate levels. Thus, these results suggested that other hexoses besides glucose were able to increase the levels of both Xu-5-P and Fru-2,6-P₂ and also stimulate dephosphorylation of the bifunctional enzyme.

In the fed rat livers, similar results were obtained with all the hexoses. However, mannose and 40 mM glucose increased Xu-5-P by 4–9-fold, and Fru-2,6-P₂ level increased 23–39% compared to the liver perfused with 5 mM glucose. Thus, there were large variations in Fru-2,6-P₂/Xu-5-P ratios, especially in the fed rat liver perfused with 40 mM glucose because of an extremely high concentration of Xu-5-P.

Effect of Glucagon and Insulin—Glucagon lowered Fru-2,6-P₂ in livers from starved rats perfused with the hormone (Table V). Glucose and hexose-Ps also decreased by approximately 25%. This decrease in Fru-2,6-P₂ could be attributed to increased phosphorylation of the bifunctional enzyme since the activity ratio of Fru-6-P,2-kinase decreased. Insulin had little effect on those metabolites and on the bifunctional enzyme in the fed livers. The similar lack of effect of insulin has been reported with hepatocytes isolated from fed rat livers (31).

Citrate increased slightly with glucagon administration while Fru-2,6-P₂ decreased 63%, indicating no corresponding changes between these metabolites. The metabolite contents in the liver of the starved rats perfused with glucagon or insulin were not different from those in the control livers (data not shown).

Effect of Feeding High Carbohydrate or High Fat Diet—Starved rats (48 h) were fed various diets for 3 h, killed, and the livers removed immediately for analysis. The starved rats had the lowest concentration of Fru-2,6-P₂ (2.6 nmol/g) (Table VI), as expected, while the ad libitum fed rats contained the highest level of the hexose-P₂ (8.7 nmol/g). Feeding the high sucrose diet after starvation also raised the Fru-2,6-P₂ to near the maximum level (8.3 nmol/g liver). A high fat diet (30% fat) containing 26% starch significantly raised the Fru-2,6-P₂ to 4.4 nmol/g and high fat (30% fat), but a no carbohydrate diet increased it only to 3.6 nmol/g.

The activity ratio of Fru-6-P,2-kinase also showed the corresponding changes, i.e. the highest with ad libitum and high carbohydrate diets, intermediate in high fat with no carbohydrate, and the lowest in the starved rat livers. Opposite effects were seen in the activity ratios of Fru-2,6-Pase. Xu-5-P showed values ranging from 6.3 to 17.3 nmol/g with different diets similar to the values seen with Fru-2,6-P₂ except for rats fed a high sucrose diet in which the Xu-5-P level rose to 52 nmol/g. The Xu-5-P contents in the starved, ad libitum fed, and high carbohydrate diets were very similar to those reported previously by Casazza and Veech (19). The ratios of Fru-2,6-P₂/Xu-5-P were nearly the same in all these livers except those from rats fed with a high sucrose diet in which the Xu-5-P concentration was 3× and 13× higher than the 48-h starved and the ad libitum fed rats, respectively. Glucose and hexose-Ps concentrations in these livers did not show large differences (5.1–9.6 μmol/g) while Fru-2,6-P₂ and Xu-5-P concentrations varied significantly more among different diets. There was no correlational changes between citrate and Fru-2,6-P₂ in these fed rats.

**DISCUSSION**

One of the objectives of this investigation is to obtain in vivo evidence in support of the proposed role for Xu-5-P in regulating Fru-2,6-P₂ which ultimately regulates glucose metabolism in liver. In the present study, we demonstrated that perfusion of livers with short chain fatty acids or feeding high fat diet to whole animals produced a decrease in Fru-2,6-P₂ in liver, which was correlated with increased phosphorylation of the bifunctional enzyme, resulting in inhibition of the kinase and activation of the phosphatase. This increased phosphorylation state of Fru-6-P,2-kinase:Fru-2,6-Pase appeared to be closely correlated to a decreased Xu-5-P level in both the perfused

---

**Table III**

The effect of sugars on metabolites and Fru-6-P,2-kinase and Fru-2,6-Pase in 24-h starved rat livers

The livers were perfused with the sugars for 10 min. The activity ratio of the bifunctional enzyme and the content of the metabolites were determined as described under "Experimental Procedures." Values are mean ± S.D. of four livers for each time point.

| Enzyme/metabolite | Glucose (5 mM) | Glucose (40 mM) | Fructose (5 mM) | Galactose (1 mM) | Mannose (1 mM) | Mannose (5 mM) |
|-------------------|---------------|----------------|----------------|----------------|---------------|---------------|
| Fru-2,6-P₂ (nmol/g) | 2.7 ± 0.2     | 8.3 ± 1.0      | 4.1 ± 0.2      | 4.5 ± 0.1      | 5.2 ± 0.8     |
| Fru-6-P,2-kinase (v/Vₘₚₙ) | 0.55 ± 0.07   | 0.94 ± 0.03   | 0.63 ± 0.09   | 0.73 ± 0.04   | 0.61 ± 0.09   |
| Fru-2,6-Pase (v/Vₘₚₙ) | 0.89 ± 0.06   | 0.68 ± 0.12   | 0.78 ± 0.01   | 0.71 ± 0.02   | 0.75 ± 0.01   |
| Xu-5-P (nmol/g) | 4.7 ± 0.4     | 19.3 ± 2.2     | 7.8 ± 1.0     | 8.2 ± 0.5     | 9.6 ± 1.8     |
| Glc (μmol/g) | 0.9 ± 0.3     | 14 ± 0.1       | 1.2 ± 0.1     | 1.1 ± 0.1     | 1.7 ± 0.4     |
| Glc-6-P (nmol/g) | 71 ± 3        | 107 ± 23       | 78 ± 5        | 74 ± 9        | 77 ± 6        |
| Fru-6-P (nmol/g) | 29 ± 3        | 37 ± 14        | 33 ± 5        | 30 ± 5        | 27 ± 1        |
| Glyceraldehyde 3-phosphate (nmol/g) | 0.6 ± 0.1 | 1.3 ± 0.2 | 0.9 ± 0.2 | 0.8 ± 0.3 | 0.8 ± 0.1 |
| Citrate (nmol/g) | 353 ± 28      | 284 ± 16       | 326 ± 22      | 334 ± 19      | 344 ± 25      |
| Fru-2,6-P₂/Xu-5-P | 0.57          | 0.43           | 0.52          | 0.55          | 0.54          |
| Fru-6-P/Fru-2,6-P₂ | 0.008         | 0.029          | 0.013         | 0.013         | 0.015         |

---

**Figure 1.** Time course of Fru-6-P,2-kinase and Fru-2,6-Pase activity ratios in the livers of starved (A) and fed (B) rats perfused with 10 mM butyrate. The activities of Fru-6-P,2-kinase (●) and Fru-2,6-Pase (○) were determined as described under "Experimental Procedures." Values are mean ± S.D. of four livers for each time point.
livers and the livers of rats fed a high fat diet. Such correlation was not seen with hexose-Ps. A similar correlation between Xu-5-P and Fru-2,6-P2 was seen with the livers perfused with other sugars. Glucagon, which raises cAMP and is known to lower Fru-2,6-P2 (reviewed in Refs. 1-3), also decreased Xu-5-P concentration. Moreover, when the increase in the Fru-2,6-P2 and Xu-5-P concentrations were compared in the livers of rats fed with different diets (shown in Fig. 2, closed circles), the Fru-2,6-P2 level reached the maximum value at 20 nmol/g Xu-5-P and the half-maximum at approximately 10 nmol/g (except those fed a high sucrose diet). The in vivo results were similar to the in vitro results obtained using pure Xu-5-P-activated PP2A (11). When the activation of pure PP2A by varying Xu-5-P was determined (Fig. 2, open circles), $K_{a}^{Xu5P}$ was also 10 $\mu$M, and the maximum activation was obtained above 40 $\mu$M Xu-5-P. Thus, there was remarkable agreement in the correlation of Fru-2,6-P2 and Xu-5-P levels between in vivo and in vitro data and adds credence to our claim that Xu-5-P-activated PP2A plays an important role in the regulation of Fru-2,6-P2 level in liver.

Table IV
The effect of sugars on metabolites and Fru-6-P,2-kinase and Fru-2,6-Pase in perfused livers of ad libitum fed rats
The livers were perfused with various sugars for 10 min. The activity ratio of the bifunctional enzyme and the content of the metabolites were determined as described under “Experimental Procedures.” Values are mean ± S.D. (n = 4).

| Enzyme/metabolite | Glucose (5 mM) | Glucose (40 mM) | Fructose (5 mM) | Galactose (1 mM) | Mannose (5 mM) |
|------------------|---------------|----------------|----------------|-----------------|----------------|
| Fru-2,6-P2 (nmol/g) | 8.7 ± 1.2 | 12.1 ± 1.3 | 8.6 ± 0.3 | 9.4 ± 0.2 | 10.7 ± 0.9 |
| Fru-6-P,2-kinase (v/Vmax) | 0.60 ± 0.04 | 0.93 ± 0.04 | 0.94 ± 0.02 | 0.94 ± 0.04 | 0.73 ± 0.06 |
| Fru-2,6-Pase (v/Vmax) | 0.30 ± 0.04 | 0.62 ± 0.08 | 0.92 ± 0.06 | 0.93 ± 0.01 | 1.14 ± 0.9 |
| Xu-5-P (nmol/g) | 18.6 ± 1.2 | 164 ± 8 | 19.0 ± 2 | 17.9 ± 2 | 71.4 ± 5.9 |
| GIC (nmol/g) | 8.9 ± 0.6 | 247 ± 2.0 | 8.6 ± 1.2 | 8.8 ± 1.0 | 10.6 ± 1.3 |
| Glx-6-P (nmol/g) | 16.0 ± 6 | 196 ± 17 | 162 ± 10 | 165 ± 7 | 182 ± 10 |
| Fru-6-P (nmol/g) | 56 ± 6 | 75 ± 6 | 61 ± 5 | 54 ± 5 | 76 ± 9 |
| Glyceraldehyde 3-phosphate (nmol/g) | 1.3 ± 0.2 | 2.2 ± 0.2 | 1.3 ± 0.2 | 1.3 ± 0.2 | 1.8 ± 0.2 |
| Citrate (nmol/g) | 318 ± 17 | 276 ± 14 | 287 ± 19 | 293 ± 15 | 282 ± 21 |
| Fru-2,6-P2/Xu-5-P | 0.47 | 0.074 | 0.45 | 0.52 | 0.15 |

Table V
The effect of glucagon and insulin on metabolites and Fru-6-P,2-kinase and Fru-2,6-Pase in ad libitum fed rat livers
The livers were perfused with glucagon (1 mM) or insulin (1 mM) in the presence of 5 mM glucagon for 10 min. The activity ratio of the bifunctional enzyme and the content of the metabolites were determined as described under “Experimental Procedures.” All the perfusion solutions contain 5 mM glucose. Values are mean ± S.D. (n = 4).

| Enzyme/metabolite | No hormone | Glucagon (1 mM) | Insulin (1 mM) |
|------------------|------------|----------------|----------------|
| Fru-2,6-P2 (nmol/g) | 8.7 ± 1.2 | 3.2 ± 0.3 | 8.6 ± 0.3 |
| Fru-6-P,2-kinase (v/Vmax) | 0.60 ± 0.04 | 0.46 ± 0.04 | 0.61 ± 0.03 |
| Fru-2,6-Pase (v/Vmax) | 0.30 ± 0.04 | 0.92 ± 0.06 | 0.93 ± 0.01 |
| Xu-5-P (nmol/g) | 18.6 ± 1.2 | 9.4 ± 0.5 | 19.1 ± 1.1 |
| CAMP (nmol/g) | 0.6 ± 0.5 | 1.0 ± 0.03 | 0.6 ± 0.07 |
| GIC (nmol/g) | 8.9 ± 0.6 | 6.8 ± 0.3 | 9.5 ± 0.4 |
| Glx-6-P (nmol/g) | 16.0 ± 6 | 135 ± 7 | 164 ± 4 |
| Fru-6-P (nmol/g) | 56 ± 7 | 43 ± 4 | 56 ± 4 |
| Glyceraldehyde 3-phosphate (nmol/g) | 1.3 ± 0.2 | 0.7 ± 0.1 | 1.4 ± 0.2 |
| Citrate (nmol/g) | 318 ± 17 | 335 ± 14 | 292 ± 18 |
| Fru-2,6-P2/Xu-5-P | 0.47 | 0.34 | 0.45 |

Table VI
The effect of high sucrose, high fat with starch, or high fat without starch diet on the bifunctional enzyme and metabolites in 48-h starved rat livers
The livers were perfused with various sugars for 10 min. The activity ratio of the bifunctional enzyme and the content of the metabolites were determined as described under “Experimental Procedures.” Values are mean ± S.D. (n = 4).

| Enzyme/metabolite | ad libitum standard (n = 6) | 48 h starved (n = 6) | High sucrose (n = 4) | High fat starch (n = 6) | High fat (n = 5) |
|------------------|---------------------------|---------------------|---------------------|-----------------------|----------------|
| Fru-2,6-P2 (nmol/g) | 8.7 ± 0.4 | 2.6 ± 0.2 | 8.3 ± 0.2 | 4.4 ± 0.2 | 3.6 ± 0.3 |
| Fru-6-P,2-kinase (v/Vmax) | 0.93 ± 0.03 | 0.60 ± 0.06 | 0.88 ± 0.03 | 0.88 ± 0.04 | 0.73 ± 0.02 |
| Fru-2,6-Pase (v/Vmax) | 0.65 ± 0.04 | 0.94 ± 0.03 | 0.73 ± 0.01 | 0.68 ± 0.08 | 0.74 ± 0.03 |
| Xu-5-P (nmol/g) | 17.3 ± 1.4 | 4.0 ± 1.7 | 51.9 ± 8.0 | 10.6 ± 2.0 | 6.3 ± 0.9 |
| CAMP (nmol/g) | 0.6 ± 0.03 | 0.8 ± 0.1 | 0.6 ± 0.1 | 0.7 ± 0.1 | 0.7 ± 0.1 |
| GIC (nmol/g) | 6.9 ± 0.3 | 5.1 ± 0.4 | 9.6 ± 0.3 | 7.9 ± 0.7 | 6.7 ± 0.5 |
| Glx-6-P (nmol/g) | 140 ± 6 | 68 ± 6 | 167 ± 7 | 143 ± 7 | 125 ± 9 |
| Fru-6-P (nmol/g) | 46 ± 7 | 25 ± 3 | 63 ± 7 | 52 ± 6 | 48 ± 6 |
| Glyceraldehyde 3-phosphate (nmol/g) | 1.3 ± 0.1 | 0.7 ± 0.1 | 2.7 ± 0.2 | 1.5 ± 0.1 | 1.4 ± 0.1 |
| Citrate (nmol/g) | 292 ± 10 | 387 ± 16 | 329 ± 22 | 381 ± 29 | 360 ± 17 |
| Fru-2,6-P2/Xu-5-P | 0.50 | 0.65 | 0.16 | 0.41 | 0.57 |
| Fru-2,6-P2/Citrate (×106) | 2.9 | 0.08 | 2.8 | 1.2 | 1.0 |
fatty acid perfusion of ad libitum fed livers (Table III), however, are difficult to explain because in these livers Xu-5-P decreased from 171 nmol/g (in high glucose liver) to about 80 nmol/g with the fatty acid perfusion and the Fru-2,6-P2 level decreased from 126 to about 6 nmol/g. If the PP2A was saturated at 20 nmol/g Xu-5-P, one would not expect to see any decrease in Fru-2,6-P2 by fatty acid administration. We cannot offer any reasonable explanation for this observation.

All these in vitro and in vivo results are consistent with our proposal that Xu-5-P serves as the key messenger for glucose or carbohydrate for the increased level of Fru-2,6-P2 in liver by stimulating the specific PP2A to dephosphorylate the bifunctional enzyme. Furthermore, these results indicated that activation of the PP2A is extremely sensitive to the lowest concentration ranges of Xu-5-P in liver (levels comparable to those of Fru-2,6-P2), thus making it suitable for the role of a messenger. Our observation that other sugars alter the Xu-5-P level suggests that any substance, including nucleotide, nucleosides, triose-Ps, etc. which generates this pentose-P, may raise Fru-2,6-P2 by the same mechanism. Thus, our original scheme (11) which showed only glucose, has been modified to include the other sugars as shown in Scheme I.

Another objective of this work was to determine whether the well-known phenomenon of fatty acid inhibition of glucose metabolism can be explained by the Xu-5-P-mediated regulation of the enzyme activity. Previously, this inhibition was attributed to allosteric inhibition of Fru-6-P-2-kinase by citrate (16). Indeed, the citrate level does increase upon fatty acid administration, as was well known before (16, 32, 33) and confirmed here (Table V). However, the correlation between Fru-2,6-P2 and citrate in the livers of rats fed with various diets or in the perfused livers was poor compared to that of Fru-2,6-P2 and Xu-5-P. Moreover, the observation that the decreased Fru-2,6-P2 correlates with increased phosphorylation state of the bifunctional enzyme upon fatty acid administration rules out the citrate inhibition of the enzyme, because citrate would not affect the phosphorylation state of the enzyme. Finally, all the citrate levels reported in the literature represent total cell contents of citrate and not that in cytoplasm where the bifunctional enzyme exists. Thus, we suggest that so-called "fatty acid sparing" cannot be explained by the citrate inhibition of Fru-2,6-P2 synthesis but can be explained by the decreased level of Xu-5-P as a result of fatty acid feeding which results in decreased activity of the PP2A. It has been demonstrated that glycosylation, as measured by lactate production, in isolated hepatocytes requires 5 nmol/g Fru-2,6-P2 (34). If this were true in whole animals, glycosylation must be completely inhibited in the livers of those fed with fatty acids containing 4.4 or 3.6 nmol/g Fru-2,6-P2 (Table VI).

The question then is how Xu-5-P concentration is decreased by fatty acid administration. Xu-5-P is generated in the pentose-P pathway in two ways: (a) from 6-P gluconate and NADP catalyzed by 6-P gluconate dehydrogenase and ribulose 5-P epimerase; and (b) from Fru-6-P and glyceroldehyde 3-P by transketolase reaction. The increased fatty acid oxidation may inhibit both pathways by: (a) decreased NADP/NADPH ratio and (b) decreased Fru-6-P and glyceroldehyde 3-P by inhibition of glycolysis by decreased Fru-2,6-P2. Casazza and Veech (19) have shown that the transketolase reaction (as well as all the other enzyme reactions in the nonoxidative pathway) is in equilibrium in ad libitum fed and starved rats, thus any decrease in Fru-6-P and/or glyceroldehyde 3-P results in decreased Xu-5-P. This points to an interesting synergism between phosphofructokinase and the bifunctional enzyme. The Xu-5-P concentration is autoregulated by the synergistic regulation of these enzymes via changes in the Fru-2,6-P2 concentration. The resulting changes in phosphofructokinase activity would affect both glyceroldehyde 3-P and Fru-6-P concentrations (Scheme II) because Fru-6-P is the substrate for phosphofructokinase and glyceroldehyde 3-P is the product of phosphofructokinase and aldolase (Fru-6-P → Fru-1,6-P2 → glyceroldehyde 3P + dihydroxyacetone-P). Thus, although the Fru-6-P level increases immediately after meal feeding, for example, the Xu-5-P level does not rise simultaneously because glyceroldehyde 3-P remains low due to low Fru-2,6-P2 concentration and low phosphofructokinase activity. The Xu-5-P level remains low until Fru-2,6-P2 begins to form, which activates phosphofructokinase to generate glyceroldehyde 3-P. Thus, both Xu-5-P and Fru-2,6-P2 concentrations are coordinately regulated and depend on each other. This explains the previous observation (10) that Fru-6-P increases immediately in liver perfused with high concentration of glucose, but there is a lag period (4 min) in the Xu-5-P formation. Similarly, it may also explain a lag period of 2–4 h observed in Fru-2,6-P2 formation in the livers of starved rats fed with regular lab chow (35, 36). Previously, this delay was suggested to be related to accumulation of a critical concentration of glycogen before an increase in Fru-2,6-P2 (35). These observations also explain why Xu-5-P, among all the pentose-P in the shunt pathway, is selected as an activator for the PP2A. The answer appears to be that Xu-5-P is a product of the transketolase reaction from Fru-6-P and glyceroldehyde 3-P, both of which are intermediates of glycolysis and considered as the branching point from glycolysis and the initial step in the pentose shunt pathway.

In the fatty acid (short chain) perfused livers, the cAMP concentration increased from 0.7 to 0.8 nmol/g to 1.1–1.5 nmol/g in both starved and fed animals (Tables I and III). Since the phosphorylation state of Fru-6-P,2-kinase:Fru-2,6-Pase is determined by the relative activities of protein kinase A and PP2A (Scheme I), the results may suggest that the phosphorylation of the bifunctional enzyme was increased further by the activation of protein kinase A by increased cAMP. For a number of reasons, however, it is difficult to assess the physiological significance of the increased cAMP level in the fatty acid perfused livers. The cAMP concentration in the freeze-clamped livers represents the value in the total liver and not...
that in the cytoplasm. A similar increase in cAMP was not observed in the fatty acid fed animals (Table VI). Nevertheless, the observation that the phosphorylation state of Fru-6-P,2-kinase:Fru-2,6-Pase was increased by fatty acids in both perfused liver and in the whole animal indicates that either protein kinase A activity remained the same or increased, while PP2A activity was inhibited as a result of lower Xu-5-P concentration. Thus, Xu-5-P and the PP2A appear to play a major role in regulation of this complex pair of reciprocating reactions, protein kinase A and PP2A, and Fru-6-P,2-kinase and Fru-2,6-Pase under these dietary conditions.

It is generally thought that the role of the pentose shunt pathway is to provide the source of: (a) the reducing equivalent in the form of NADPH for fatty acid and steroid biosynthesis and (b) ribose 5-P for nucleic acid and nucleotide biosynthesis. We presented herein the third role of the pathway: production of Xu-5-P which plays a vital role in regulation of glycolysis as well as the overall carbohydrate metabolism in liver.

In summary, we have shown that Fru-2,6-P2 in liver, which is primarily determined by the phosphorylation states of Fru-6-P,2-kinase:Fru-2,6-Pase as we stated before (37), was correlated with changes in Xu-5-P in the livers perfused with different hexoses, fatty acids, and glucagon. A similar correlation between Xu-5-P and Fru-2,6-P2 was demonstrated in rats fed with a high carbohydrate or high fat diet and in starved rats. These in vitro and in vivo results provide evidence in support of the proposed mechanism for regulation of the bifunctional enzyme by phosphorylation mediated by cAMP and dephosphorylation mediated by Xu-5-P.

REFERENCES

1. Uyeda, K., Furuya, E., Richards, C. S., and Yokoyama, M. (1982) Mol. Cell. Biochem. 48, 97–120
2. Pilakis, S. J., Chrisman, T., Burgess, B., McGrane, M., Colosia, A., Pilakis, J. L., Claus, T. H., and El-Maghrabi, M. R. (1983) Adv. Enzyme Regul. 21, 147–173
3. van Schaftingen E. (1987) Biochem. J. 245, 394–401
4. van Schaftingen E. (1982) Adv. Enzymol. Relat. Areas Mol. Biol. 59, 315–395
5. van Schaftingen E. (1987) Biochem. J. 245, 394–401
6. van Schaftingen, E., Hue, L., and Hers, H. G. (1980) Biochem. J. 192, 887–985
7. Hue, L., Blackmore, P. F., and Exton, J. H. (1981) J. Biol. Chem. 256, 8900–8903
8. Nishimura, M., El-Maghrabi, R., Pilakis, S. J., and Claus, T. H. (1981) Diabetes 30, 1062–1064
9. Hue, L., and Rider, M. H. (1987) Biochem. J. 245, 313–324
10. Nishimura, M., Fedorov, S., and Uyeda, K. (1994) J. Biol. Chem. 269, 26100–26106
11. Nishimura, M., and Uyeda, K. (1995) J. Biol. Chem. 270, 26341–26346
12. Struck, E., Ashmore, J., and Wieland, O. H. (1966) Biochem. Z. 343, 107–110
13. Ross, B. D., Hens, R., and Krbs, H. A. (1967) Biochem. J. 102, 942–951
14. Williamson, J. R., and Krebs, H. A. (1961) Biochem. J. 80, 540–547
15. Randle, P. J., Newsholme, E. A., and Garland, P. B. (1968) Biochem. J. 93, 652–665
16. Hue, L., Maisin, L., and Rider, M. H. (1988) Biochem. J. 251, 541–545
17. Berry, M. N., Phillips, J. W., Henly, D. C., and Clark, D. G. (1993) FEBS Lett. 319, 26–30
18. Veech, R. L., Glimoter, W. L., King, M. T., Balaban, R. S., Costa, J. L., and Eanes, E. C. (1986) Myocardial and Skeletal Muscle Bioenergetics, pp. 617–646, Plenum Publishing, New York
19. Casazza, J. P., and Veech, R. L. (1986) Biochem. J. 236, 635–641
20. Francone, O., Griffinon, G., and Kalopissi, A. D. (1992) Am. J. Physiol. 236, E615–E623
21. Veech, R. L., Guynn, R., and Veloso, D. (1972) Biochem. J. 127, 387–397
22. Uyeda, K., Furuya, E., and Luby, L. J. (1982) J. Biol. Chem. 256, 8394–8399
23. Casazza, J. P., and Veech, R. L. (1980) J. Biol. Chem. 261, 690–698
24. Dagley, S. (1974) Methods of Enzymatic Analysis, pp. 1562–1565, Academic Press, New York
25. Michal, G., and Beutler, H.-O. (1974) Methods of Enzymatic Analysis, pp. 1314–1319, Academic Press, New York
26. Lowry, O. H. and Passonneau, J. V. (1982) A Flexible System of Enzymatic Analysis, pp. 146–218, Academic Press, New York
27. Tomingima, N., Minami, Y., Sakakibara, R., and Uyeda, K. (1993) J. Biol. Chem. 268, 15951–15957
28. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
29. Woods, H. F., Egeksten, L. V., and Krebs, H. A. (1970) Biochem. J. 119, 501–510
30. Nishi, T., Kido, Y., Furuya, E., Tagama, K., and Mori, T. (1989) Jpn. J. Surg. 19, 352–357
31. Richards, C. S., and Uyeda, K. (1982) Biochem. Biophys. Res. Commun. 109, 394–401
32. Randle, P. J., Garland, P. B., Hales, C. N., Newsholme, E. A., Denton, R. M., and Popon, C. J. (1966) Recent Prog. Horm. Res. 22, 1–44
33. Garland, P. B., and Randle, P. J. (1964) Biochem. J. 94, 678–687
34. Hue, L., Sobrinod, F., and Cohen, P. (1985) Biochem. J. 224, 779–786
35. Kuwajima, M., Golden, S., Katz, J., Unger, R. H., Foster, D. W., and McGarry, J. D. (1986) J. Biol. Chem. 261, 2632–2637
36. Holness, M. J., Cook, E. B., and Sugden, M. C. (1988) Biochem. J. 252, 357–362
37. Sakakibara, R., Kitajima, S., and Uyeda, K. (1984) J. Biol. Chem. 259, 41–46