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Fat body fructose-2,6-bisphosphate content and phosphorylase activity correlate with changes in hemolymph glucose concentration during fasting and re-feeding in larval *Manduca sexta*

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Abstract

Fasting of second-day fifth instar larval *Manduca sexta* leads to a rapid decrease in hemolymph glucose concentration from 3.39 ± 0.29 to 0.33 ± 0.06 mM in 1 h, along with a decrease in the fructose-2,6-bisphosphate content in the fat body (from 5.92 ± 0.31 to 2.80 ± 0.47 nmol fructose-2,6-bisphosphate/g fat body in 3 h) and activation of fat body glycogen phosphorylase (from 16% to 55–65% phosphorylase a). During re-feeding an increase in the glucose level in the hemolymph was observed (from 0.36 ± 0.05 to 3.91 ± 0.36 mM in 3 h), along with an increase in the fructose-2,6-bisphosphate level in the fat body (from 2.88 ± 0.47 to 6.66 ± 0.42 nmol fructose-2,6-bisphosphate/g fat body in 3 h) and inactivation of fat body glycogen phosphorylase (from 56% to 16% phosphorylase a). These data are consistent with the hypothesis that a decrease in hemolymph glucose both activates fat body glycogen phosphorylase and causes a decrease in fat body fructose-2,6-bisphosphate content. Both of these changes would favor conversion of stored glucose to trehalose in the fat body. When second-day larvae were decapitated, the changes in hemolymph glucose and fat body fructose-2,6-bisphosphate were very similar to those observed in fasting whole insects. These data are consistent with a direct role for glucose in controlling carbohydrate metabolism in *Manduca sexta*. © 2001 Elsevier Science Ltd. All rights reserved.

Keywords: *Manduca sexta*; Fat body; Hemolymph; Glucose; Fructose-2,6-bisphosphate; Octopamine; Adipokinetic hormone

1. Introduction

Trehalose is the major sugar in insect hemolymph, reaching concentrations between 1 and 2% (for a review see Becker et al., 1996). Trehalose is synthesized in the fat body (Becker et al., 1996), which is the main organ of energy metabolism in insects, combining the functions

of vertebrate liver and adipose tissue (for a review see Law and Wells, 1989). In vertebrates, glucose is the main sugar in the blood and the liver plays a primary role in glucose homeostasis (for a review see Hers, 1990). There are significant parallels between the synthesis and release of glucose from the liver and the synthesis of trehalose by the fat body (Becker et al., 1996). Glucose production by the liver is regulated by both hormonal and nonhormonal mechanisms. Glucagon is released from the pancreas in response to a low blood glucose concentration and stimulates glycogen breakdown and glucose release by the liver (Hers, 1990). In insects the adipokinetic/hypertrehalosemic hormones, which are released from the corpora cardiaca, stimulate the degradation of glycogen and the synthesis of trehalose in the fat body (Gäde, 1996; Becker et al., 1996). Octopamine, the major biogenic amine found in invertebrates (Axelrod and Saavedra, 1977) acts both as a neu-

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rotransmitter, modulating the release of peptide hormones from the corpus cardiacum (Passier et al., 1995), and as a hormone with direct energy store mobilizing activity on the fat body (Fields and Woodring, 1991).

In both vertebrates and insects the peptide hormones lead to activation of phosphorylase via a phosphorylation cascade, which ultimately leads to production of glucose-6-phosphate (Hers, 1990; Becker et al., 1996). In insects the glucose-6-phosphate can either enter glycolysis or trehalose synthesis, while in vertebrates it either enters glycolysis or is used to produce free glucose. Thus, in both insects and vertebrates it is necessary to restrict the flux of glucose-6-phosphate into glycolysis in order to divert it into trehalose or glucose production, respectively. In both vertebrates (Van Schaftingen, 1993) and insects (Becker and Wegener, 1998) inhibition of glycolysis is achieved by lowering the concentration of fructose-2,6-bisphosphate (F-2,6-P₂), the major allosteric regulator of phosphofructokinase-1, the key regulated enzyme of glycolysis (Van Schaftingen, 1993). In vertebrates the concentration of F-2,6-P₂ is lowered because glucagon stimulates the phosphorylation of the bifunctional enzyme phosphofructokinase-2/fructose-2,6-bisphosphatase, which activates the phosphatase activity, which converts F-2,6-P₂ into fructose-6-phosphate (Hers, 1990). It is not known whether the same mechanism occurs in insects.

In vertebrates the nonhormonal regulation of glucose homeostasis is controlled by the concentration of glucose in the blood (Hers, 1990). At high concentrations, glucose stimulates glycogen synthesis by promoting dephosphorylation of phosphorylase a and glycogen synthase, which inhibits glycogen breakdown and promotes glycogen synthesis. At the same time glucose promotes glycolysis by activating synthesis of F-2,6-P₂ because glucose is converted into xylulose-5-phosphate, which is an allosteric activator of the phosphatase that converts phosphofructokinase-2/fructose-2,6-bisphosphatase into the kinase form (Nishimura et al., 1994; Nishimura and Uyeda, 1995), which leads to synthesis of F-2,6-P₂. As the blood glucose level decreases, these activating effects of glucose are lost and glycogen breakdown occurs (Hers, 1990).

Although trehalose is the main carbohydrate in insect hemolymph, there is also a significant concentration of glucose (Friedman, 1985), but to our knowledge, the potential role of hemolymph glucose in regulating trehalose synthesis has not been previously studied. Because trehalose must be converted into glucose before it can enter the cell (Becker et al., 1996), it seemed possible that the hemolymph glucose concentration might be an indicator of the status of carbohydrate metabolism in insects. Here we show that changes in hemolymph glucose concentrations correlate with the content of fat body F-2,6-P₂ and the activity of fat body glycogen phos-

phorylase during fasting or re-feeding in *Manduca sexta* larvae.

2. Material and methods

2.1. Insects

M. sexta larvae from a colony maintained in this laboratory were reared according to Bell and Joachim (1976) on a high wheat germ diet (Reinecke et al., 1980) using a 16 h light/dark cycle (lights on at 7 a.m.) at 25°C. Animals were synchronized at the end of the fourth larval instar by the appearance of head capsule apolysis (Truman et al., 1973). Second-day fifth instar larvae were usually used. In some experiments larvae were ligated with dental floss between the second and third pair of prolegs. Ligations were done at this position so as to have a nearly equal distribution of fat body between the two ligated halves. Immediately after ligation, insects were decapitated, and these abdomens were used to analyze the effect of removing the influence of the brain, corpora cardiaca, corpora allata and prothoracic glands (Siegert, 1992). During fasting, fecal pellets were removed to prevent coprophagy. Re-feeding was carried out after larvae had been fasted for 3 h.

2.2. Fat body extracts

Larvae were pinned in a dish and cut open along the dorsal midline. The fat body was washed with 0.15 M NaCl and then removed. The fat body cells were disrupted by homogenization in an alkaline solution (50 mM NaOH) for determination of the F-2,6-P₂ content or in a buffer containing 50 mM MOPS-TRIS, pH 7.2, 5 mM NaF, 5 mM EDTA, 1 mM EGTA, and 1 mM dithiothreitol for determination of glycogen phosphorylase activity.

2.3. Determination of the fat body fructose-2,6-bisphosphate content

Fat bodies from three separate insects for each point were dissected and individually weighed and homogenized in 50 mM NaOH (0.1 g tissue to 1 ml), employing a Teflon glass homogenizer (15 strokes). The homogenates were heated for 5 min at 80°C and then cooled. The homogenates were centrifuged (20 min, 12 000g) and the supernatants were neutralized with an ice-cold solution containing 20 mM Hepes and 1 mM acetic acid to pH 7.5–8.0. These viscous solutions were centrifuged again to remove precipitated proteins. The supernatants were assayed for F-2,6-P₂ content on the basis of its ability to activate the pyrophosphate-dependent 6-phosphate phosphotransferase from potato tubers (EC 2.7.1.90) using a coupled enzymatic system containing 0.5 U aldo-

lase, 5 U triosephosphate isomerase, 2 U glycerol-3-phosphate dehydrogenase and 10 U pyrophosphate-dependent 6-phosphate phosphotransferase from potato tubers (Van Schaftingen, 1984). Standard conditions were 50 mM Tris-HCl, pH 8.0, 2 mM $MgCl_2$, 1 mM fructose-6-phosphate, 0.15 mM NADH and 0.5 mM sodium pyrophosphate.

2.4. Determination of the fat body glycogen phosphorylase activity

Fat bodies from three different insects for each point were dissected, individually homogenized as described above and centrifuged (20 min, 12 000g). The supernatant was used for determination of glycogen phosphorylase activity in the direction of glycogen breakdown by coupling the production of glucose-1-phosphate to the reduction of $NADP^+$ using phosphoglucomutase and glucose-6-phosphate dehydrogenase. The assay medium was the same as used by Arrese et al. (1995). Standard conditions for measuring total phosphorylase activity were: 40 mM potassium phosphate buffer (pH 7.0), 5 mM imidazole, 2 mM NaEDTA, 1.4 mM dithiothreitol, 5 mM magnesium acetate, 2 mM AMP, 4 μ M glucose-1,6-bisphosphate, 0.6 mM $NADP^+$, 2 mg/ml glycogen (free from AMP), 4 U of phosphoglucomutase, and 0.8 U of glucose-6-phosphate dehydrogenase. Phosphorylase activity was measured under the same conditions except that 5'AMP was omitted. Values for the active phosphorylase are expressed as the percentage of total phosphorylase activity. Endogenous $NADP^+$ -dehydrogenase activity (e.g. glucose-6-phosphate dehydrogenase) present in the homogenate supernatant results in $NADP^+$ reduction that must be corrected for by a blank made without adding enzymes, glycogen and AMP. If this correction is not made, the activation of phosphorylase in the supernatant is overestimated (Arrese et al., 1995).

2.5. Determination of hemolymph glucose concentration

Hemolymph (100 μ l) from three different insects for each point were individually mixed with 500 μ l 0.3 N perchloric acid and the precipitate was removed by centrifugation (10 min, 12 000g). The supernatants were used for the determination of hemolymph glucose concentrations (Siegert, 1987).

2.6. Chemicals

The substrates for the enzymatic assays and the enzymes (phosphoglucomutase, glucose-6-phosphate dehydrogenase, hexokinase, pyrophosphate-dependent 6-phosphate phosphotransferase, aldolase, triosephosphate isomerase, glycerol-3-phosphate dehydrogenase) and

coenzymes were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

2.7. Statistics

Data are means \pm SE of three independent determinations using three different insects for each point. Differences were evaluated for statistical significance using Student's *t* test.

3. Results and discussion

3.1. Effect of fasting on the concentration of hemolymph glucose

In second-day fifth instar larvae the hemolymph glucose concentration decreases very rapidly after the onset of fasting, and after only 1 h the glucose concentration decreased from 3.39 ± 0.29 to 0.33 ± 0.06 mM (Fig. 1(a)). This decrease was not dependent on factors from the brain, because in ligated/decapitated larvae the time course was similar (Fig. 1(b)). *M. sexta* larvae feed nearly constantly, pausing only to molt (Reinecke et al., 1980). However, throughout development larvae do stop eating for short periods of time. First- and second-day fifth instar larvae were observed to stop feeding for 15–45 min, which is enough time to cause a rapid decrease in the glucose level in the hemolymph (Fig. 1(a)). Therefore, in these experiments, only larvae that had been actively feeding for at least 5 min were selected for fasting studies.

3.2. Effect of fasting on the F-2,6- P_2 content of the fat body

Fig. 2 shows that during fasting the level of F-2,6- P_2 in the fat body of second-day fifth instar larvae also decreased. This decrease in F-2,6- P_2 content was also not under control by the brain, because in ligated/decapitated larvae the observed time course was the same (Fig. 2(b)).

3.3. Effect of re-feeding on the concentration of hemolymph glucose and the content of fat body F-2,6- P_2

During re-feeding, the glucose concentration in the hemolymph increased from 0.36 ± 0.05 to 3.91 ± 0.36 mM in 3 h (Fig. 3). At the same time the F-2,6- P_2 content of fat body also increased (Fig. 4). Taken together, these data (Figs. 1–4) suggest that the level of F-2,6- P_2 in the fat body is under the influence of the hemolymph glucose concentration. As described in the introduction, there is evidence that glucose can regulate the level of F-2,6- P_2 by influencing the activity of phosphofructokin-

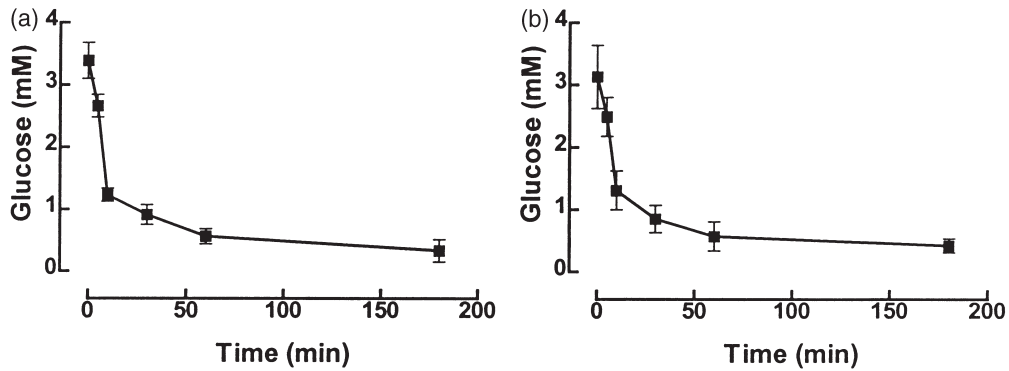


Fig. 1. Changes in hemolymph glucose concentration during fasting of *M. sexta* larvae. Second day-fifth instar larvae (a) were fasted and after different times hemolymph was collected and the glucose concentration was determined. In (b), insects were ligated and decapitated and then after different times hemolymph was collected and the glucose concentration was determined. Values are means \pm SEM of three independent determinations.

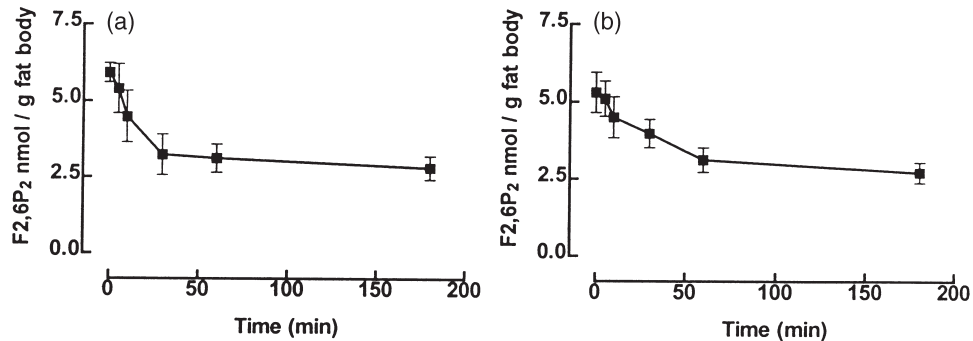


Fig. 2. The F-2,6-P₂ content in the larval fat body during fasting. Second-day fifth instar larvae (a) were starved and after different times the fat bodies were dissected and the F-2,6-P₂ content was determined. In (b), insects were ligated and decapitated, then after different times the fat bodies were dissected and the F-2,6-P₂ content was determined. Values are means \pm SEM of three independent determinations.

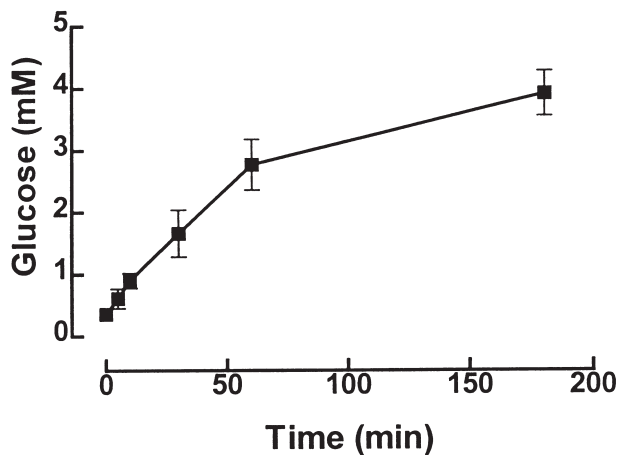


Fig. 3. The effect of re-feeding on the glucose concentration in larval hemolymph. After fasting for 3 h, second-day fifth instar larvae were re-fed. After different times, hemolymph was collected and the glucose concentration was determined. Values are means \pm SEM of three independent determinations.

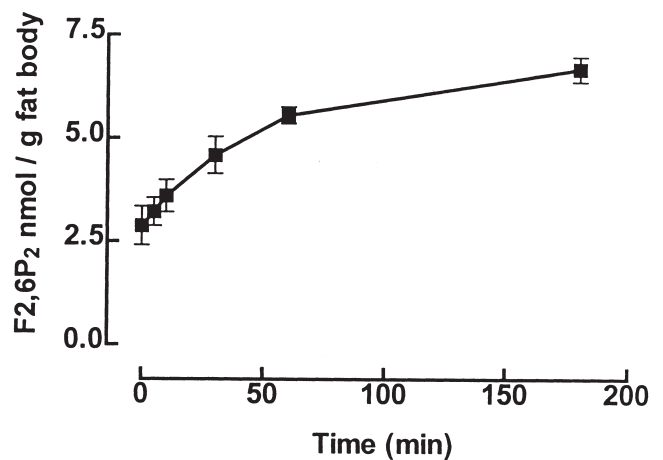


Fig. 4. The effect of re-feeding on the F-2,6-P₂ content in the larval fat body. After fasting for 3 h, second-day fifth instar larvae were re-fed. After different times, fat bodies were dissected and F-2,6-P₂ content was determined. Values are means \pm SEM of three independent determinations.

ase-2/fructose-2,6-bisphosphatase via the metabolic product xylulose-5-phosphate. The possible participation of xylulose-5-phosphate in the increase of fat body F-2,6-P₂ content during re-feeding remains to be determined.

3.4. Activation and inactivation of glycogen phosphorylase after fasting and re-feeding

During fasting, glycogen phosphorylase was activated (Fig. 5). On the other hand, during re-feeding, we observed inactivation of glycogen phosphorylase (Fig. 5). These data agree with previous observations in which it was shown that feeding fasted larvae for 2–5 min was long enough to inactivate fat body glycogen phosphorylase (Siegert and Mordue, 1992).

3.5. Correlation between the hemolymph glucose concentration and fat body F-2,6-P₂ content and fat body phosphorylase activity

In Fig. 6 we have plotted the data for fat body F-2,6-P₂ content from Figs. 2 and 4 against the data for the hemolymph glucose concentration from Figs. 1 and 3. This linear plot suggests a direct correlation between the two. Such a relationship would be consistent with glucose directly affecting the level of F-2,6-P₂ by altering the activity of phosphofructokinase-2/fructose-2,6-bisphosphatase—a high glucose concentration would favor the phosphofructokinase-2 activity, while the fructose-2,6-bisphosphatase activity would be favored at low glucose concentration. The increase in the glucose concentration in the hemolymph could also favor an increase in the glycolytic intermediates. Hexose-phosphates, such as glucose 6-phosphate and fructose 6-phosphate, increased in a dose-dependent manner when rat liver was incubated with glucose (Nishimura et al., 1994). In this

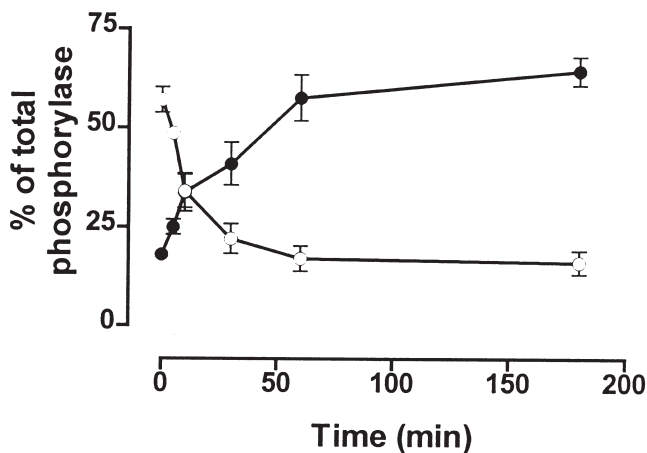


Fig. 5. Effect of fasting or re-feeding on fat body glycogen phosphorylase activity. At different times after the onset of fasting (●) or re-feeding (○), the activation of glycogen phosphorylase was determined as the percentage of the total phosphorylase activity. Values are means±SEM of three independent determinations.

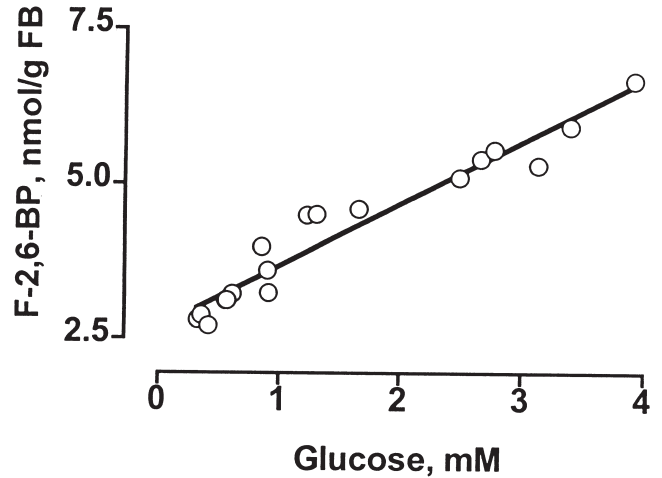


Fig. 6. Correlation of the fat body F-2,6-P₂ content with the hemolymph glucose concentration. For this plot the data of Figs. 1–4 were used. The linear least squares regression lines had $r^2=0.9385$, $P<0.0001$.

regard, an interdependence of glycolytic and pentose cycle intermediates in fed rats has been shown in vertebrates (Casazza and Veech, 1986). Because the activity of the pentose phosphate pathway depends directly on the concentration of glucose, it seems reasonable to suggest that this regulation could be exerted through xylulose-5-phosphate.

Fig. 7 shows the correlation of the activity of fat body phosphorylase with the concentration of hemolymph glucose. These data are consistent with a role for glucose in inactivating phosphorylase, as is the case for the vertebrate enzyme.

In summary, the fact that changes in hemolymph glucose and fat body F-2,6-P₂ during fasting are not affected by decapitation suggests, but does not prove, that there may be a direct role for glucose in controlling carbo-

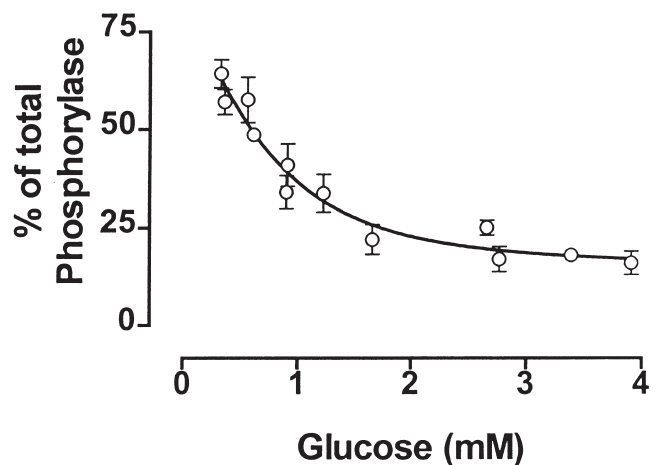


Fig. 7. Correlation of the percentage of the total fat body phosphorylase as activated phosphorylase with the hemolymph glucose concentration. For this plot the data of Figs. 1a, 3 and 5 were used.

hydrate metabolism in *M. sexta*. More work will be required to determine whether the situation in insects is indeed similar to that in vertebrates.

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