Metabolic control of hepatic gluconeogenesis during exercise

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1. Prolonged exercise increased the concentrations of the hexose phosphates and phosphoenolpyruvate and depressed those of fructose 1,6-bisphosphate, triose phosphates and pyruvate in the liver of the rat. Since exercise increases gluconeogenic flux, these changes in metabolite concentrations suggest that metabolic control is exerted, at least, at the fructose 6-phosphate/fructose 1,6-bisphosphate and phosphoenolpyruvate/pyruvate substrate cycles. 2. Exercise increased the maximal activities of glucose 6-phosphatase, fructose 1,6-bisphosphatase, pyruvate kinase and pyruvate carboxylase in the liver, but there were no changes in those of glucokinase, 6-phosphofructokinase and phosphoenolpyruvate carboxykinase. 3. Exercise changed the concentrations of several allosteric effectors of the glycolytic or gluconeogenic enzymes in liver; the concentrations of acetyl-CoA, ADP and AMP were increased, whereas those of ATP, fructose 1,6-bisphosphate and fructose 2,6-bisphosphate were decreased. 4. The effect of exercise on the phosphorylation–dephosphorylation state of pyruvate kinase was investigated by measuring the activities under conditions of saturating and subsaturating concentrations of substrate. The submaximal activity of pyruvate kinase (0.5 mM-phosphoenolpyruvate), expressed as percentage of $V_{\max}$, decreased in the exercised animals to less than half that found in the controls. These changes suggest that hepatic pyruvate kinase is less active during exercise, possibly owing to phosphorylation of the enzyme, and this may play a role in increasing the rate of gluconeogenesis.

There is considerable evidence that the rate of gluconeogenesis is increased during prolonged exercise. Ahlborg *et al.* (1974) and Ahlborg & Felig (1982) measured the splanchnic uptake of glucose precursors during exercise in human subjects and found that gluconeogenesis was stimulated by as much as 10-fold. During the third hour of exercise at an intensity of 60% of maximum aerobic capacity, the uptake of gluconeogenic substrates could account for approx. 60% of the total splanchnic output of glucose. Shaw *et al.* (1976), using radioisotopic methods, confirmed that gluconeogenesis is increased by exercise. They determined the rate of conversion of [^{14}C]glycerol into glucose in exercising dogs and concluded that exercise increased gluconeogenesis approx. 9-fold. Several investigators have also demonstrated that exercise increases gluconeogenesis in rat kidney-cortex slices incubated *in vitro* (Krebs & Yoshida, 1963; Sanchez-Medina *et al.*, 1972).

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Since the rates of adipose-tissue lipolysis and net protein degradation are increased during exercise (Refsum & Strømme, 1974; Décombaz *et al.*, 1979; Dohm *et al.*, 1980, 1982), it is likely that glycerol and amino acids are available in increased quantities. With the increased supply of substrates and the increased hepatic uptake of glycerol and amino acids, gluconeogenesis is a significant source of glucose during exercise.

The changes in blood metabolite concentrations and the altered rate of gluconeogenesis that occur during prolonged exercise are very similar to those that occur during starvation. A considerable amount of work has been performed on the control of hepatic gluconeogenesis in starvation. It is known that changes in allosteric effectors (e.g. acetyl-CoA activation of pyruvate carboxylase), changes in maximum activities of key non-equilibrium enzymes, changes in the state of phosphorylation of pyruvate kinase and changes in the concentration of fructose 2,6-bisphosphate probably play a role in increasing the rate of gluconeogenesis in starvation (Pilkis *et al.*, 1982). How far these mechanisms

operate in prolonged exercise has been investigated in the present work by measuring the maximal activities of key enzymes, concentrations of key metabolites and the state of phosphorylation of regulatory enzymes.

Materials and methods

Chemicals and enzymes

All enzymes were purchased from Boehringer Corp. (London), London W5 2TZ, U.K. [$U\text{-}^{14}\text{C}$]-Glucose and [$U\text{-}^{14}\text{C}$]glucose 6-phosphate were obtained from The Radiochemical Centre, Amersham, Bucks. HP7 9LL, U.K. Other chemicals were obtained from BDH Chemicals, Poole, Dorset BH12 4NN, U.K., and from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.

Source and treatment of animals

Male Sprague-Dawley rats weighing approx. 200 g were obtained from OLAC, Blackthorn, Bicester, Oxon, U.K., and given water and commercial lab chow *ad libitum*. On the day of the experiment, rats were assigned randomly to control (rested) and exercised groups. Rats in the exercised group ran on a rodent treadmill, the speed of which was increased slowly for approx. 10 min, after which they ran at 28 m/min for the following 80 min, with a rest for 2 min after every 20 min of running. The rats tolerated this regimen well and were not exhausted at the end of the exercise period. Immediately after the rats had completed the exercise they were killed by cervical dislocation. The liver was quickly removed and either placed on ice for enzyme-activity measurement or freeze-clamped between aluminium tongs cooled in liquid N_2 for assay of metabolic intermediates. Control rats were killed at approximately the same time as the exercised group.

Extraction of liver and assay of metabolites

After freeze-clamping, the frozen liver samples were stored under liquid N_2 until required for extraction. The frozen liver was powdered in a mortar and pestle under liquid N_2 and was then homogenized in 0.7 M- HClO_4 in a Polytron homogenizer [the ratio of tissue to HClO_4 was 1:2 (w/v)]. The precipitate was removed by centrifugation at 1000 g for 10 min, and the supernatant was neutralized with 3.0 M- K_2CO_3 containing 0.5 M-triethanolamine. Samples were kept on ice for 30 min and then the precipitate of KClO_4 was removed by centrifugation at 1000 g for 10 min. The supernatant was used for metabolite assays, which were completed on the same day as preparation of the extract. However, a different extraction procedure was used for the measurement of fructose 2,6-bisphosphate (see Hue *et al.*, 1982).

Acetyl-CoA was assayed by the method de-

scribed by Decker (1974), ADP and AMP were assayed by that of Jaworek *et al.* (1974), glucose 6-phosphate and fructose 6-phosphate as described by Lang & Michal (1974), ATP was assayed by the method of Lamprecht & Trautschold (1974), citrate as described by Dagley (1974), phosphoenolpyruvate and pyruvate were measured by the method of Czok & Lamprecht (1974), fructose 1,6-bisphosphate, dihydroxyacetone phosphate and glyceraldehyde 3-phosphate by the method of Michal & Beutler (1974), glycerol 3-phosphate was assayed by that of Michal & Lang (1974), and lactate as described by Gutman & Wahlefeld (1974).

A semi-quantitative method was used for the measurement of the concentration of fructose 2,6-bisphosphate by monitoring the degree of activation of purified phosphofructokinase by extracts of livers from rested and exercised rats (Kuwajima & Uyeda, 1982). Commercially available rabbit muscle phosphofructokinase was used for the assay, and the assay mixture contained 50 mM-triethanolamine/HCl (pH 7.4), 100 mM-KCl, 5 mM- MgCl_2 , 3.0 mM-ATP, 0.15 mM-NADH, 0.5 unit of aldolase/ml, 0.5 unit of glycerol 3-phosphate dehydrogenase/ml and 12.5 units of triose phosphate isomerase/ml. Activity was measured at fructose 6-phosphate concentrations of 0.05 mM and 5.0 mM (V_{max}).

Extraction of liver and assay of enzyme activities

Liver was extracted [the ratio of tissue to extraction medium was 1:10 (w/v)] in a Polytron homogenizer at setting 4 for 15 s and the homogenate was centrifuged for 2 min (Eppendorf model 3200 centrifuge). The supernatant was used for all enzyme assays except pyruvate carboxylase.

For assay of phosphoenolpyruvate carboxykinase, the extraction medium contained 30 mM-mercaptoethanol. For assay of pyruvate carboxylase, the extraction medium contained 50 mM-triethanolamine, 0.3 M-sucrose and 1 mM-EDTA, pH 7.2. The homogenates were centrifuged (8000 g, for 15 min) and the pellets were resuspended in an equal volume of homogenization buffer plus 0.1% (w/v) Triton X-100. The resuspended pellets were used for assay of the enzyme. For assay of glucokinase, fructose bisphosphatase and glucose 6-phosphatase, the extraction buffer contained 50 mM-triethanolamine, 0.3 M-sucrose and 1 mM-EDTA, pH 7.2.

Enzymes were assayed either spectrophotometrically at 25°C (with a Gilford recording spectrophotometer, model 240) or radiochemically at 30°C (with a Beckman scintillation counter, model 7500). Fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase were assayed spectrophotometrically by the method described by Crabtree *et al.* (1972). Pyruvate carboxylase was

assayed spectrophotometrically as described by Martin & Denton (1970). Glucokinase and glucose 6-phosphatase were both assayed radiochemically (Stanley, 1980; Surholt & Newsholme, 1981).

The activities of pyruvate kinase and 6-phosphofructokinase were measured at both saturating and subsaturating concentrations of phosphoenolpyruvate and fructose 6-phosphate respectively as described by Feliu *et al.* (1976) and Castaño *et al.* (1979). The maximal activity of phosphofructokinase was measured at 5.0mM-fructose 6-phosphate and that for pyruvate kinase was determined at 5.0mM-phosphoenolpyruvate. Tissue extracts were prepared by homogenization of freeze-clamped liver in a buffer containing 50mM-triethanolamine/HCl (pH 7.4), 100mM-KF and 1mM-EDTA. The homogenates were centrifuged and the supernatants were used in the assays. Freeze-clamped livers were stored in liquid N₂ for no more than 6h, and the activities of phosphofructokinase and pyruvate kinase were determined immediately after the tissue extract was prepared.

Results and discussion

Exercise decreased the concentrations of pyruvate and of fructose 1,6-bisphosphate and increased those of phosphoenolpyruvate and hexose monophosphates (Table 1). Since exercise increases the rate of hepatic gluconeogenesis (see the introduction), these changes indicate that the net rates of conversion of pyruvate into phosphoenolpyruvate and fructose bisphosphate into fructose 6-phosphate have been stimulated by external regulation (i.e. by factors other than their pathway substrates; see Newsholme & Crabtree, 1979). The factors that may be responsible are discussed below.

Maximal enzyme activities

An increase in the maximum activities of key gluconeogenic enzymes and/or a decrease in those of the glycolytic enzymes (owing to changes in enzyme concentration via effects on protein synthesis and/or degradation) could account for the increase in gluconeogenic flux. Enzyme activities (Table 2) have been expressed as both units/g of liver or units/mg of protein for ease of comparison with similar data in the literature. However, because of loss of liver weight and protein during acute exercise (Kasperek *et al.*, 1982), interpretation of changes in enzyme activity are difficult. On the basis of units/mg of protein, the maximal activities of glucose 6-phosphate, fructose 1,6-bisphosphatase, pyruvate kinase and pyruvate carboxylase were all slightly increased by exercise, but when expressed as units/total liver protein there are no statistically significant changes. It seems that changes in maximal enzyme activities play little or no role in the increased rate of gluconeogenesis during exercise.

Phosphorylation of regulatory enzymes

It is well established that hepatic pyruvate kinase and 6-phosphofructokinase exist in phosphorylated and dephosphorylated forms (Feliu *et al.*, 1976; Castaño *et al.*, 1979) and that a change in the phosphorylation state plays a role in control of gluconeogenesis during starvation. The effect of exercise on the phosphorylation state of these enzymes was investigated by measuring the activities under conditions of saturating and subsaturating concentrations of substrate. For pyruvate kinase, exercise caused a decrease in the activity measured at low substrate concentration (0.5mM-phosphoenolpyruvate; control activity was

Table 1. Concentrations of metabolites in livers of control and exercised rats

Concentrations are presented as means ± S.E.M., with at least six animals in each group. A statistically significant difference (Student's *t* test) between control and exercised animals is indicated by **P* < 0.05.

Metabolite	Metabolite concn. (μmol/g)		Exercised as % of control
	Control	Exercised	
Glucose 6-phosphate	0.34 ± 0.03	0.55 ± 0.05*	162
Fructose 6-phosphate	0.073 ± 0.005	0.110 ± 0.009*	151
Fructose 1,6-bisphosphate	0.021 ± 0.002	0.012 ± 0.002*	57
Dihydroxyacetone phosphate	0.030 ± 0.002	0.015 ± 0.002*	50
Glycerol 3-phosphate	0.34 ± 0.03	0.24 ± 0.02*	69
Glyceraldehyde 3-phosphate	0.0064 ± 0.0014	0.0015 ± 0.0008*	23
Phosphoenolpyruvate	0.075 ± 0.010	0.127 ± 0.013*	169
Pyruvate	0.031 ± 0.004	0.016 ± 0.002*	52
Lactate	1.54 ± 0.21	0.89 ± 0.08*	58
Acetyl-CoA	0.025 ± 0.002	0.032 ± 0.003*	128
Citrate	0.23 ± 0.03	0.24 ± 0.03	104
ATP	2.27 ± 0.17	1.54 ± 0.10*	68
ADP	0.93 ± 0.13	1.17 ± 0.18*	126
AMP	0.32 ± 0.07	0.64 ± 0.10*	200

Fig. 2. Relative activities of 6-phosphofructokinase as a function of fructose 6-phosphate concentrations in livers of control and exercised rats

6-Phosphofructokinase was assayed as described in the Materials and methods section at 1.0 mM-ATP concentration and, where indicated, 2.0 mM-AMP was included in the assay. A statistically significant difference (Student's *t* test) between control and exercised animals is indicated by **P* < 0.05. ○, Extracts from control rats; ●, extracts from exercised rats; △, extracts from control rats assayed in the presence of AMP; ▲, extracts from exercised rats assayed in the presence of AMP.

tration of allosteric regulators. Pyruvate kinase and 6-phosphofructokinase are activated *in vitro* by fructose 1,6-bisphosphate, so that the decreased concentration of this metabolite in liver of exercised animals (Table 1) may decrease the activities of these enzymes, although the concentration *in vivo* may be too low to be effective (Hers & Van Schaftingen, 1982). Acetyl-CoA is an activator of pyruvate carboxylase, and the increase in acetyl-CoA with exercise should increase the enzyme activity. These changes in concentrations of allosteric regulators are consistent with increased gluconeogenic flux, although no studies on the changes in the concentrations of these metabolites in the compartment of the cell in which the specific enzyme is located have been made.

The adenine and guanine nucleotides are known to be allosteric effectors of several key enzymes of glycolysis and gluconeogenesis, so that if the concentration ratio ATP/ADP decreased this would be expected to increase the activities of the key glycolytic enzymes and decrease those of gluconeogenesis. Exercise decreased the ATP/ADP con-

centration ratio and also raised the concentration of AMP. These changes in the adenine nucleotide concentrations are not consistent with an increased flux in gluconeogenesis, and suggest that other regulators must play a major role in inhibition of the glycolytic enzymes and stimulation of those of gluconeogenesis. The conclusion that changes in adenine nucleotide concentrations do not play a major role in regulating hepatic gluconeogenesis is supported by the results of Start & Newsholme (1968), who demonstrated that ATP was decreased and AMP increased in livers of starved rats where gluconeogenic flux is known to be increased.

One question raised by the changes in the concentrations of the adenine nucleotides concerns the cause(s) of these changes. One of the simplest explanations would be anoxia caused by the decrease in blood flow to the liver that is known to occur during exercise (Ahlborg & Felig, 1982). However, since there was no large change in the [NAD⁺]/[NADH] ratio ([lactate]/[pyruvate] was 41 ± 6 in rested and 58 ± 7 in exercised rats; not statistically different), it does not seem likely that anoxia would play a large role in altering the energy charge of the liver during exercise.

It has been shown that fructose 2,6-bisphosphate is a potent stimulator of phosphofructokinase (Van Schaftingen *et al.*, 1980) and an inhibitor of fructose 1,6-bisphosphatase (Pilkis *et al.*, 1981). The maximal activities are not affected by fructose 2,6-bisphosphate, but the concentration of substrate required to give half-maximum activity is changed.

In the present study a semi-quantitative assay for fructose 2,6-bisphosphate has been performed. This involves addition of extracts from livers of rested and exercised rats to a purified preparation of muscle 6-phosphofructokinase and observing the degree of stimulation of activity at low concentration of fructose 6-phosphate (that is, when ATP is inhibitory). Extracts from livers of control rats resulted in considerable stimulation of 6-phosphofructokinase activity (measured at low fructose 6-phosphate concentration). This effect was removed by acid treatment of the liver extract, demonstrating that the stimulation was in fact due to fructose 2,6-bisphosphate (Van Schaftingen *et al.*, 1980). Extracts from liver of exercised rats resulted in less stimulation of 6-phosphofructokinase activity than that caused by control extract (Table 3). This finding suggests that the hepatic concentration of fructose 2,6-bisphosphate is decreased during endurance exercise and that this could play a major role in the stimulation of the activity of fructose bisphosphatase and inhibition of that of 6-phosphofructokinase.

The results of this study suggest that the increase in gluconeogenic flux that occurs during exercise is the result of increases in the activities of

Table 3. *Effect of liver extracts from control and exercised rats on the activity of purified muscle 6-phosphofructokinase*

Samples (0.1 ml) of liver extracts (1:10, w/v) were added to purified muscle phosphofructokinase and the activity was assayed at 0.05 mM- (v) and 5.0 mM- ($V_{\max}$) fructose 6-phosphate as described in the Materials and methods section. Values are presented as means $\pm$ S.E.M., for four animals in each group. The difference between controls and exercised was statistically significant (Student's *t* test) ($P < 0.05$).

	Relative activity ($v/V_{\max}$)
No extract	6.5 $\pm$ 0.3
Extract from control rats	29.5 $\pm$ 1.5
Extract from exercised rats	18.0 $\pm$ 1.5

pyruvate carboxylase, fructose 1,6-bisphosphatase and glucose 6-phosphatase and diminished activities of phosphofructokinase and pyruvate kinase. Of the regulatory controls studied the most important changes seem to be decreases in the concentrations of fructose 2,6-bisphosphate and fructose 1,6-bisphosphate and the phosphorylation of pyruvate kinase to decrease its activity at physiological substrate concentration.

It is interesting to speculate on the possible 'signals' that produce these biochemical changes. Since the phosphorylation of pyruvate kinase and phosphofructokinase is under the control of cyclic AMP-dependent protein kinase, it seems likely that the exercise-induced increase in gluconeogenesis may be mediated by elevated concentrations of cyclic AMP. Running in rats is known to increase cyclic AMP in the liver, especially after 60 min of running (Winder *et al.*, 1979). An increase in liver cyclic AMP would also be consistent with the decreased concentration of fructose 2,6-bisphosphate, since it has been demonstrated that changes in the activity of 6-phosphofructo-2-kinase are also mediated through enzyme phosphorylation by cyclic AMP-dependent protein kinase (El-Maghrabi *et al.*, 1982). The hormones most likely to cause an increase in liver cyclic AMP during exercise are noradrenaline, adrenaline and glucagon, since the concentrations of these hormones are elevated by prolonged exercise (Galbo, 1981) and their physiological effects are mediated through cyclic AMP.

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