Biochem. J. (1985) 231, 217–220 (Printed in Great Britain)

The rate of substrate cycling between fructose 6-phosphate and fructose 1,6-bisphosphate in skeletal muscle from cold-exposed, hyperthyroid or acutely exercised rats

R. A. John CHALLIS,* Jonathan R. S. ARCH† and Eric A. NEWSHOLME*
*Department of Biochemistry, University of Oxford, South Parks Road, Oxford OX1 3QU, U.K., and †Beecham Pharmaceutical Research Division, Biosciences Research Centre, Great Burgh, Yew Tree Bottom Road, Epsom, Surrey KT18 5XG, U.K.

The effects of cold-exposure, the hyperthyroid state and a single exercise bout *in vivo* on the maximal enzyme activities of 6-phosphofructokinase and fructose-1,6-bisphosphatase in vastus lateralis muscle and the rates of fructose 6-phosphate/fructose 1,6-bisphosphate cycling measured in epitrochlearis muscle *in vitro* were investigated. In all cases significant changes in substrate cycling rates were observed, whether in the absence of added hormones *in vitro* (acute exercise), or when stimulated by insulin plus adrenaline (cold-exposure), or with respect to the catecholamine-sensitivity of the cycling rate (the hyperthyroid state).

INTRODUCTION

The precise rate of substrate cycling between fructose 6-phosphate and fructose 1,6-bisphosphate in isolated epitrochlearis muscle of the rat was measured for the first time by using both a dual- and a single-isotope technique by Challiss *et al.* (1984*a*,*c*). These studies demonstrated that the rate of cycling could be increased dramatically by the presence in the incubation medium of adrenaline and insulin.

It has been predicted that the rate of substrate cycling will be increased *in vivo* by several conditions, namely in the hyperthyroid state, during feeding, during cold-exposure and after exercise (Newsholme & Crabtree, 1976; Newsholme, 1978, 1980). With respect to this, it has been shown that the rate of the triacylglycerol/fatty acid cycle in adipose tissue *in vivo* is increased by feeding and by cold-exposure (Brookes *et al.*, 1983). Evidence has also been obtained that the rate of fructose 6-phosphate/fructose 1,6-bisphosphate cycling is increased *in vivo* in the hyperthyroid state (Huang & Lardy, 1981), but in that study it was not established in which tissue(s) the increased cycling occurred. In addition, it has been shown that the rate of this latter cycle is higher in incubated epitrochlearis muscle from fed rats compared with muscles from starved rats (Challiss *et al.*, 1985). Consequently, it was considered important to investigate the effects of cold-exposure, the hyperthyroid state and exercise both on the basal rate of cycling between fructose 6-phosphate and fructose 1,6-bisphosphate and on the adrenaline-stimulated rate of cycling. In order to determine if these conditions could influence the maximal enzymic capacity for cycling, effects on the maximal activities of 6-phosphofructokinase, fructose-1,6-bisphosphatase and also hexokinase were investigated. The results are presented and discussed in this paper.

MATERIALS AND METHODS

Materials

All enzymes, biochemicals and radiochemicals were obtained from the sources given by Challiss *et al.* (1984*a*,*c*). 3,3',5-Tri-iodo-L-thyronine was obtained from Sigma (London) Chemical Co., Poole, Dorset, U.K.

Animal manipulations

Male Wistar rats were obtained from Bantin and Kingman, Hull, U.K., and were maintained in the Department of Biochemistry animal house for at least 5 days before use.

For cold-exposure, animals (170 g; housed in groups of eight with free access to food and water) were transferred to an environment with an ambient temperature of 4 °C for 60 h or 156 h until immediately before they were killed. Hyperthyroidism was induced by intraperitoneal injection of rats (160 g) with tri-iodothyronine (65 μg/100 g body wt.) on 5 consecutive days; on day 6 (24 h after the final injection) animals were killed. Rats (160 g) to be exercised were familiarized with the treadmill on 4 days before the day of the experiment. Animals were run for 90 min at a speed of 28 m/min, with 2 min rest periods every 15 min; at the end of the last 15 min run, animals were immediately killed. This period of exercise was about 50% of that required to cause exhaustion (Dohm *et al.*, 1983).

Assay of maximal enzyme activities

Animals were killed by cervical dislocation, and superficial white and deep red fibres were dissected from the vastus lateralis (Baldwin *et al.*, 1972). Muscle samples were homogenized in a Polytron (setting 5, 2 × 20 s) at 0 °C in 10 vol. of 50 mM-triethanolamine/HCl buffer, pH 7.4, containing 5 mM-$MgCl_2$, 1 mM-EDTA and 20 mM-2-mercaptoethanol. Hexokinase was assayed as described by Zammit & Newsholme (1976), 6-phosphofructokinase as described by Opie & Newsholme (1967) and fructose-1,6-bisphosphatase by the method of Crabtree *et al.* (1972).

Measurement of cycling rates

Fructose 6-phosphate/fructose 1,6-bisphosphate cycling rates were measured in epitrochlearis muscles rapidly removed from rats immediately after they were killed; incubations and subsequent processing of muscles and

separation and analysis of intermediates were carried out as described previously in detail (Challiss *et al.*, 1984*a*,*c*). In all cases the dual-label [5-^{3}H,6-^{14}C]glucose was used (Challiss *et al.*, 1984*a*). For the acute-exercise experiments, the single-isotope randomization method was also used (Challiss *et al.*, 1984*c*).

In addition, rates of lactate, [^{14}C]lactate and [^{14}C]glycogen formation were measured (Challiss *et al.*, 1983), and the rate of glycolysis (*J*) and the flux from hexose monophosphate to glycogen (*b*) calculated. The formulae used to calculate the rates of cycling are given by Challiss *et al.* (1984*b*,*c*).

RESULTS AND DISCUSSION

The effects of acute cold-exposure (for 60 h and 156 h), the hyperthyroid state and a single bout of exercise on maximal enzyme activities in muscle of the rat are shown in Table 1. There are marked differences in response between the superficial ('white') and deeper ('red') muscle of the vastus lateralis, indicating a different response to these conditions according to the type of fibre: the superficial muscle contains a greater proportion of Type IIb fibres and the deeper muscle contains predominantly Type IIa (about 70%) and Type I (about 30%) fibres (Baldwin *et al.*, 1972). For the present investigation, an important observation was an increase in maximal activities of both 6-phosphofructokinase and fructose-1,6-bisphosphatase after 60 h, but not after 156 h, of cold-exposure; this change occurred only in the superficial muscle. This suggests that Type IIb fibres may respond to cold-exposure, at the level of the fructose 6-phosphate/fructose 1,6-bisphosphate cycle, during the early stages of cold-acclimation before brown-adipose-tissue non-shivering thermogenesis has fully adapted. For this reason, the effect of 60 h cold-exposure on the rate of substrate cycling at the level of fructose 6-phosphate was investigated in isolated epitrochlearis muscles incubated *in vitro* by using the dual isotope method described previously (Challiss *et al.*, 1984*a*). The rate of cycling was increased in muscles isolated from 60 h-cold-exposed animals in all three conditions studied (Table 2), that is in the absence of hormonal additions (basal cycling rate), in the presence of insulin and in the presence of insulin plus adrenaline; in this last case the measured cycling rate was 3.5 μmol/h per g of tissue.

It is well established that the thyroid hormones increase the sensitivity of some tissues to β-adrenoceptor stimulation by catecholamines (Malbon *et al.*, 1978; Williams & Lefkowitz, 1983). As catecholamines are known to increase energy expenditure (Steinberg, 1963; Jung *et al.*, 1980), this may be one means by which thyroid hormones can increase metabolic rate. Consequently, the effect of the hyperthyroid state was investigated in the isolated epitrochlearis muscle at sub-saturating and saturating concentrations of adrenaline. The only effect of the hyperthyroid state was to increase significantly the rate of cycling at a sub-saturating (10 nM) concentration of adrenaline compared with the euthyroid control (cycling rates of 1.81 ± 0.19 and 0.90 ± 0.18 μmol/h per g of tissue respectively; Table 2). This finding is therefore consistent with the view that one effect of the hyperthyroid state is to increase the sensitivity of muscle to adrenaline.

For the study of the effect of a single 90 min exercise bout, muscles were dissected out immediately after

Table 1. Effect of cold-exposure, hyperthyroid state and exercise on the maximal activities of hexokinase, 6-phosphofructokinase and fructose-bisphosphatase in the superficial and deep vastus lateralis muscle of the rat

Vastus lateralis was dissected according to Baldwin *et al.* (1972) to give superficial layer (predominantly Type IIb fibres) and deep portion (predominantly Type IIa and Type I fibres). Activities are presented as means ± S.E.M., with numbers of separate animals used given in parentheses. The statistical significance (Student's *t* test) of the difference in activity between control and experimental is given by $^*P < 0.05$.

Vastus lateralis...	Enzyme activities (μmol/min per g fresh wt.)					
	Superficial			Deep		
	Hexokinase	6-Phospho-fructokinase	Fructose-bisphosphatase	Hexokinase	6-Phospho-fructokinase	Fructose-bisphosphase
Control	0.37 ± 0.02 (5)	38.9 ± 2.2 (6)	0.16 ± 0.03 (5)	0.94 ± 0.05 (5)	29.8 ± 2.6 (5)	0.11 ± 0.01 (5)
Cold-exposure (60 h)	0.40 ± 0.03 (6)	45.7 ± 2.0 (6)*	0.27 ± 0.03 (5)*	1.06 ± 0.08 (6)	27.9 ± 2.1 (6)	0.09 ± 0.04 (5)
Cold-exposure (156 h)	0.41 ± 0.03 (4)	37.8 ± 6.6 (4)	0.19 ± 0.04 (5)	1.29 ± 0.11 (5)*	26.6 ± 2.0 (6)	0.08 ± 0.01 (6)
Control	0.42 ± 0.04 (7)	36.8 ± 3.1 (7)	0.18 ± 0.02 (7)	1.04 ± 0.07 (7)	30.3 ± 2.6 (5)	0.10 ± 0.01 (6)
Hyperthyroid	0.63 ± 0.08 (7)*	38.3 ± 4.2 (6)	0.22 ± 0.02 (6)	1.19 ± 0.06 (6)	33.7 ± 3.3 (5)	0.09 ± 0.01 (6)
Control	0.35 ± 0.04 (7)	37.0 ± 3.0 (8)	0.08 ± 0.01 (6)	0.91 ± 0.06 (6)	33.0 ± 1.8 (7)	0.04 ± 0.02 (8)
Acute exercise	0.42 ± 0.07 (7)	36.9 ± 2.5 (7)	0.07 ± 0.01 (6)	0.99 ± 0.03 (6)	20.8 ± 1.9 (7)*	0.04 ± 0.01 (6)

Table 2. Effect of the hyperthyroid state and cold-exposure (60 h) on the ratios of radioactivities in hexose monophosphate and glucose (S_1) and fructose 1,6-bisphosphate and glucose (S_2), the rates of glycolysis and the calculated cycling rate between fructose 6-phosphate and fructose 1,6-bisphosphate in isolated epitrochlearis muscle of the rat

Procedures for the treatment of animals, incubation of muscles *in vitro*, separation of glycolytic intermediates and measurement of radioactivities and rates of glycolysis are described in the Materials and methods section. Glucose (5 mM) was present in all incubations. Insulin, when present, was at a concentration of 1 munit/ml. Cycling rate is calculated as total glycolytic rate $\times (1-S_1)/(S_1-S_2)$. †S_1 and S_2 ratios determined by resolution of sugar phosphates by Dowex-2 (formate form) ion-exchange chromatography. Detritiation of [5-^{3}H,U-^{14}C]glucose 6-phosphate standard was $0.9\pm0.1\%$. Results are presented as means ± S.E.M., with numbers of separate incubations given in parentheses. The statistical significance (Student's *t* test) of the difference between control and experimental groups under identical incubation conditions is indicated by $*P < 0.05$, $**P < 0.01$.

Condition of animal	Hormone	S_1	S_2	$\frac{1-S_1}{S_1-S_2}$	Rate (μmol/h per g of muscle)	
					Glycolysis	Cycling
Control	Insulin	0.947±0.009 (6)†	0.610±0.023 (6)	0.126±0.012 (6)	5.59±0.35 (6)	0.70±0.11 (6)
	Insulin + 10 nM-adrenaline	0.951±0.009 (5)	0.699±0.012 (5)	0.189±0.022 (5)	5.36±0.36 (5)	0.90±0.18 (5)
	Insulin + 1 μM-adrenaline	0.936±0.012 (5)	0.695±0.019 (5)	0.266±0.027 (5)	9.32±0.63 (5)	2.48±0.41 (5)
Hyperthyroid state	Insulin	0.971±0.006 (9)	0.683±0.013 (9)	0.107±0.009 (9)	7.19±0.46 (10)	0.82±0.21 (9)
	Insulin + 10 nM-adrenaline	0.919±0.005 (7)†	0.664±0.021 (8)	0.275±0.023 (7)*	6.59±0.31 (9)	1.81±0.19 (7)**
	Insulin + 1 μM-adrenaline	0.929±0.006 (8)†	0.709±0.025 (8)	0.291±0.033 (8)	8.34±0.59 (9)	2.70±0.46 (8)
Control	—	0.988±0.004 (10)	0.700±0.003 (9)	0.042±0.003 (9)	3.69±0.20 (10)	0.16±0.03 (9)
	Insulin	0.978±0.004 (6)	0.683±0.009 (6)	0.075±0.010 (6)	6.53±0.30 (6)	0.49±0.06 (6)
	Insulin + 1 μM-adrenaline	0.934±0.011 (6)	0.699±0.017 (6)	0.271±0.018 (6)	9.32±0.63 (6)	2.48±0.41 (6)
Cold-exposure (60 h)	—	0.973±0.005 (9)	0.664±0.010 (8)	0.088±0.009 (8)**	3.53±0.18 (8)	0.31±0.05 (8)*
	Insulin	0.964±0.007 (6)	0.659±0.014 (6)	0.118±0.015 (6)*	7.08±0.40 (6)	0.83±0.10 (6)*
	Insulin + 1 μM-adrenaline	0.929±0.012 (6)	0.701±0.011 (6)	0.311±0.014 (6)	11.25±0.38 (6)	3.50±0.39 (6)*

Table 3. Effect of a single exercise bout on the rates of cycling between fructose 6-phosphate and fructose 1,6-bisphosphate determined by using a dual-isotope method or a single-isotope randomization technique in isolated epitrochlearis muscle

Animal treatments, incubations, separations and measurement of radioactivities and calculation of cycling rates were carried out as described in the Materials and methods section. For the dual-isotope method, cycling rates were calculated as glycolytic flux $\times (1-S_1)/(S_1-S_2)$. For the single-isotope method the cycling rate was calculated from the formula glycolytic flux $\times (1+f)p/(1-pf)$, where p is (^{14}C in C-1 of glycogen)/(^{14}C in C-6 of glycogen) and f is assumed from previous observations (Challiss *et al.*, 1984*c*) to be 2.6. Results are presented as means ± S.E.M. for eight observations. The statistical significance (Student's *t* test) of the difference between control and experimental group is indicated by $*P < 0.05$, $**P < 0.02$.

Condition of animal	S_1	S_2	p	$\frac{1-S_1}{S_1-S_2}$	Rate (μmol/h per g of tissue)		
					Glycolysis	Dual-isotope cycling	Single-isotope cycling
Control	0.977±0.011	0.734±0.024	0.015±0.003	0.092±0.014	3.52±0.17	0.32±0.05	0.20±0.03
Acute exercise	0.960±0.006	0.689±0.025	0.035±0.005	0.148±0.019*	3.80±0.23	0.56±0.09*	0.53±0.11**

completion of exercise and incubated according to the normal protocol (i.e. 30 min preincubation followed by a 60 min incubation period). Both the dual-isotope method (Challiss *et al.*, 1984*a*) and the single-isotope randomization technique (Challiss *et al.*, 1984*c*) have been used (Table 3). The cycling rate was increased by 80% as measured by the dual-isotope technique, whereas the single-isotope method revealed a more marked difference of 160% between muscles taken from acutely exercised and sedentary control animals. This difference between the two methods may reflect the fact that the dual-isotope technique measures the cycling rate at the moment of freeze-clamping the incubated muscle (i.e. 90 min after excision), whereas the single-isotope method depends on the accumulation of ^{14}C label in muscle glycogen (i.e. 30–90 min after excision), so that in the latter method the cycling rate is a 'time-average' value for the overall 60 min incubation period. It is suggested from this argument that the cycling rate is higher in muscles from the previously exercised animals during the early part of the incubation period. There is considerable evidence that oxygen consumption is increased after exercise in both man (Hill *et al.*, 1924; Hermansen *et al.*, 1984) and the rat (Gleeson *et al.*, 1982), and it has been suggested that one factor responsible for this extra post-exercise oxygen consumption is an increased rate of substrate cycling (Newsholme, 1978, 1980). The current results may support this hypothesis.

In summary, it has been shown that altering physiological status *in vivo* has a pronounced effect on the rate of cycling between fructose 6-phosphate and fructose 1,6-bisphosphate measured in skeletal muscle *in vitro*, either under basal conditions or after incubation with insulin and/or adrenaline. As has been emphasized previously (Challiss *et al.*, 1984*a*), such findings are qualitatively consistent with the initial postulates of Newsholme & Crabtree (1976), in so far as cycling rates can be markedly altered, either *in vitro* or after manipulation *in vivo*. However, quantitatively the observed changes in cycling rates measured *in vitro* would make only a small contribution to enhancement of sensitivity of the rate of fructose 6-phosphate phosphorylation to changes in concentrations of effector molecules, primarily because the cycling rate/flux ratio is small (it increases from 0.05 under basal conditions and rises to 0.35 on adrenaline stimulation; see Newsholme & Crabtree, 1976). Similarly, the increased rate of heat generation due to the increased rate of this cycle is also small; nonetheless, if, for example, catecholamines increase the rate of a number of similar substrate cycles simultaneously, the contribution to the thermogenic effect of these hormones could be substantial.

Received 15 March 1985/17 July 1985; accepted 29 July 1985

We thank Professor R. R. Porter, F.R.S., for his interest and encouragement, and Professor G. Lynis Dohm for assistance with the exercise experiments. R.A.J.C. was a recipient of a Science and Engineering Research Council co-operative award with Beecham Pharmaceuticals.

REFERENCES

Baldwin, K. M., Klinderfuss, G. H., Terjung, R. L., Mole, P. A. & Holloszy, J. O. (1972) Am. J. Physiol. **222**, 373–378

Brooks, B. J., Arch, J. R. S. & Newsholme, E. A. (1983) Biosci. Rep. **3**, 263–267

Challis, R. A. J., Espinal, J. & Newsholme, E. A. (1983) Biosci. Rep. **3**, 675–679

Challiss, R. A. J., Arch, J. R. S. & Newsholme, E. A. (1984*a*) Biochem. J. **221**, 153–160

Challiss, R. A. J., Crabtree, B. & Newsholme, E. A. (1984*b*) Biochem. J. **221**, 160–161

Challiss, R. A. J., Arch, J. R. S., Crabtree, B. & Newsholme, E. A. (1984*c*) Biochem. J. **223**, 849–853

Challiss, R. A. J., Arch, J. R. S. & Newsholme, E. A. (1985) Biochem. Soc. Trans. **13**, 269–270

Crabtree, B., Higgins, S. J. & Newsholme, E. A. (1972) Biochem. J. **130**, 391–396

Dohm, G. L., Tapscott, E. B., Barakat, H. A. & Kasperek, G. J. (1983) J. Appl. Physiol.: Respir. Environ. Exercise Physiol. **55**, 830–833

Gleeson, M., Brown, J. F., Waring, J. J. & Stock, M. J. (1982) Br. J. Nutr. **47**, 173–181

Hermansen, L., Grandmontagne, M., Maehlum, S. & Ingnes, I. (1984) Med. Sport Sci. **17**, 119–129

Hill, A. V., Long, C. N. H. & Lupton, H. (1924) Proc. R. Soc. London, Ser. B **97**, 84–138

Huang, M.-T. & Lardy, H. A. (1981) Arch. Biochem. Biophys. **209**, 41–51

Jung, R. T., Shetty, P. S. & James, W. P. T. (1980) Clin. Sci. **58**, 183–191

Malbon, C. C., Moreno, F. J., Cabelli, R. J. & Fain, J. N. (1978) J. Biol. Chem. **253**, 671–678

Newsholme, E. A. (1978) Biochem. Soc. Symp. **43**, 183–205

Newsholme, E. A. (1980) N. Engl. J. Med. **302**, 400–405

Newsholme, E. A. & Crabtree, B. (1973) Symp. Soc. Exp. Biol. **27**, 429–460

Newsholme, E. A. & Crabtree, B. (1976) Biochem. Soc. Symp. **41**, 61–109

Opie, L. H. & Newsholme, E. A. (1967) Biochem. J. **103**, 391–399

Steinberg, D. (1963) Biochem. Soc. Symp. **24**, 111–144

Williams, R. S. & Lefkowitz, R. J. (1983) in The Molecular Basis of Thyroid Hormone Action (Oppenheimer, J. H. & Samuels, H. H., eds.), pp. 325–351, Academic Press, New York

Zammit, V. A. & Newsholme, E. A. (1976) Biochem. J. **160**, 447–462