Biochemical Pharmacology, Vol. 31, No. 1, pp. 63–68, 1982.
Printed in Great Britain.

0006-2952/82/010063-06 $03.00/0

THE EFFECT OF 5-METHYLPYRAZOLE-3-CARBOXYLATE AND NICOTINIC ACID ON ABNORMALITIES OF CARBOHYDRATE METABOLISM IN ALLOXAN-DIABETIC RAT MUSCLE

A. McAllister,* J. P. Haddy, M. V. Sennitt and D. E. Thorne
Beecham Pharmaceuticals, Research Division, Biosciences Research Centre, Great Burgh, Yew Tree Bottom Road, Epsom, Surrey, U.K.

(*Received* 7 *April* 1981; *accepted* 29 *June* 1981)

Abstract—The administration of the antilipolytic agents sodium nicotinate (1 mmole/kg i.p.) or sodium 5-methylpyrazole-3-carboxylate (0.5 or 1.0 mmole/kg i.p.) to alloxan-diabetic rats produced a significant reduction in the plasma concentration of free fatty acids and a slight reduction in blood glucose concentration. The concentrations in the freeze-clamped heart of citrate, acetyl CoA, glucose-6-phosphate and fructose-6-phosphate were increased in untreated alloxan-diabetic rats relative to normoglycaemic controls. Treatment of alloxan-diabetic rats with the antilipolytic agents or insulin (60 U/kg i.p.) lowered these increased concentrations of metabolites in the heart. Treatment of the diabetic rats with the antilipolytic agents also produced an increase in the activity of pyruvate dehydrogenase in heart, but only treatment with 5-methylpyrazole-3-carboxylate had a significant effect on the activity of the enzyme in freeze-clamped soleus muscle.

There is considerable evidence that in the heart of the alloxan-diabetic rat the activities of the enzymes hexokinase (EC 2.7.1.1), phosphofructokinase (EC 2.7.1.11), and pyruvate dehydrogenase (EC 1.2.4.1) are low [1–4]. These reduced enzyme activities are responsible for the reduction in the rate of glucose utilisation and oxidation by isolated hearts from alloxan-diabetic rats. In other experiments, it has been shown that the activities of these enzymes are also inhibited in hearts from normal rats by perfusion *in vitro* with medium containing long chain fatty acids or ketone bodies [3–7]. Hence it has been suggested that a high rate of fatty acid oxidation in heart and skeletal muscle leads to an inhibition of glucose utilisation [2]. A high rate of fatty acid oxidation produces an increase in the concentration of citrate, glucose-6-phosphate and acetyl-CoA and it is these metabolites that regulate the activities of hexokinase, phosphofructokinase and pyruvate dehydrogenase.

In the alloxan-diabetic rat there is a high plasma concentration of non-esterified fatty acids [8, 9]. This arises because of the rapid rate of lipolysis in adipose tissue; a process normally inhibited by insulin [10]. Thus it has been suggested [2] that the increased circulating concentrations of fatty acids in alloxan-diabetic rats and the consequent high rate of fatty acid oxidation in diabetic heart muscle may be responsible for the inhibiting effects on carbohydrate metabolism. If this suggestion is correct, it would be expected that reduction in the circulating fatty acid concentrations might reverse these metabolic changes. However Randle *et al.* [2] showed that acute reduction of the plasma free fatty acids concentration by insulin failed to reverse the abnormalities of glucose metabolism of cardiac muscle after a brief perfusion *in vitro*. They proposed, therefore, that another source of free fatty acids, namely tissue triglycerides, served to maintian heart concentrations of citrate, acetyl-CoA and glucose-6-phosphate when plasma free fatty acids concentrations were lowered.

In the present paper, we report on the effects of acute lowering of the plasma concentration of free fatty acids using insulin and the antilipolytic agents nicotinate and 5-methylpyrazole-3-carboxylate on the concentrations of citrate, acetyl-CoA, glucose-6-phosphate and fructose-6-phosphate in the hearts of alloxan-diabetic and normal rats *in vivo*. We have also examined the effects of these agents on pyruvate dehydrogenase activity in heart muscle *in vivo*.

Effects of free fatty acids and ketone bodies on diaphragm muscle similar to those summarised above were shown by Randle *et al.* [6]. However, studies using the perfused rat hindquarter showed no effects of ketone bodies or palmitate on glucose uptake [11, 12]. These negative findings may have been the result of inadequate oxygenation, since in preparations using rejuvenated red cells [13], and in isolated soleus muscle [14], effects similar to those seen in heart and diaphragm muscle are seen. We have therefore examined the effects of lowering free fatty acid concentrations using nicotinate and 5-methylpyrazole-3-carboxylate on the activity of pyruvate dehydrogenase in soleus muscle *in vivo*.

MATERIALS AND METHODS

Materials

Enzymes, co-enzymes and other biochemicals were purchased from Sigma (London) Chemical Co.,

* Present address: Biochemistry Department, Glaxo Group Research, Ware, Herts SG12 0DJ, U.K.

Poole, U.K., with the following exceptions: fermocozyme (glucose oxidase reagent) was obtained from Hughes and Hughes Ltd., Romford, U.K. and oxaloacetic acid and citrate synthase were obtained from the Boehringer Corporation Ltd., London, U.K. 5-Methylpyrazole-3-carboxylic acid was a gift from Dr. M. Tauscher, Johann A. Wulfing, Gronau, West Germany. Nicotinic acid was obtained from Aldrich Chemical Co. Ltd., Gillingham, U.K.

CFY albino male rats (remote Sprague–Dawley) weighing 250–300 g were obtained from Anglia Laboratory Animals, Alconbury, U.K. and were maintained on an Oxoid pasteurised rat breeder diet (H.C. Styles, Bewdley, U.K.).

Methods

Alloxan-diabetes were induced, 48 hr prior to the experiment, by intravenous injection of alloxan monohydrate (60 mg/kg) in physiological saline. Only rats that had a blood glucose concentration greater than 20 mmoles/l were used for the experiment. Thirty min prior to the intraperitoneal administration of insulin or anti-lipolytic agents, food was removed and a blood sample (20 μl) obtained from the tail of the unanaesthetised, unrestrained rats. The blood sample was diluted with 0.58 ml 0.02% (w/v) sodium fluoride containing 4000 U/l heparin for analysis of blood glucose.

Six hours after injection of insulin or antilipolytic agents, another blood sample was obtained from the tail and then the rats were killed by decapitation using a Luckman animal guillotine (Luckman Ltd., Burgess Hill, U.K.). Two ml of blood was collected in tubes containing 100 U of heparin, centrifuged within 1 hr and the plasma stored at −20° until assay for free fatty acid. Immediately following decapitation, a second cut was made in the thoracic region using the guillotine in order to expose the heart, which was then freeze-clamped, with tongs cooled in liquid nitrogen [15]. The hearts were freeze-clamped 4–6 sec after decapitation. Soleus muscles were rapidly dissected and freeze-clamped within 10 sec of decapitation. Either the heart or the soleus muscle was obtained from each animal. Tissues were stored in liquid nitrogen until assay of metabolites and enzymes. Muscle tissue was ground at the temperature of liquid nitrogen in 150 mm × 25 mm pyrex tubes using a close fitting case-hardened stainless steel pestle with a knurled end, driven by a TriR stirrer motor (model K41 from TriR Instruments, Rockville, NY) and the powdered muscle was extracted for metabolites at 0° with 5% (w/v) perchloric acid (2 ml/g powdered tissue) using a close fitting power driven Teflon pestle. The extracts were centrifuged (1800 *g* min) at room temperature and the supernatant neutralised with saturated $KHCO_3$ and $KClO_4$ removed by centrifugation. Powdered muscle was extracted for enzymes with 2 ml/g of 100 mM potassium phosphate buffer pH 7.0 containing 10 mM sodium pyruvate, 5 mM EDTA and 5 mM 2-mercaptoethanol [16, 17]. Extracts were centrifuged for 2 min using a Beckman Microfuge B (Beckman Instruments, Hitchin, U.K.) and the supernatants stored at 0° for up to 2 hr before assay for pyruvate dehydrogenase and at −20° for 24 hr before assay for citrate synthase.

Glucose was measured using a Technicon Auto analyzer MK1 system [18]. Free fatty acid was extracted from plasma [19] and assayed by the method of Kashket [20] using a Technicon Auto analyzer MK1. Citrate [21], and glucose-6-phosphate and fructose-6-phosphate [22], were assayed spectrophotometrically. Acetyl-CoA was assayed by the method of Chase [23] after removal of coenzyme A by incubating sample for 1 hr at 30° in 100 mM triethanolamine buffer pH 6.5 containing 0.05% (w/v) hydrogen peroxide. Excess hydrogen peroxide was then removed by the addition of 5 units/ml catalase. Control experiments (not reported in this paper) indicated that acetyl CoA was not affected by this procedure. Pyruvate dehydrogenase (EC 1.2.4.1) was assayed spectrophotometrically by coupling with arylamine acetyltransferase (EC 2.3.1.5) [24]. Citrate synthase (EC 4.1.3.7) was assayed by a modification of the method of Srere *et al.* [25] as described by Coore *et al.* [24]. All enzyme activities are expressed as μmoles substrate converted/min/g wet weight of muscle. In order to compensate for any incomplete extraction of enzymes, the pyruvate dehydrogenase activity is also calculated as the pyruvate dehydrogenase:citrate synthase ratio; since the activity of citrate synthase, being a constitutive enzyme, is unlikely to be affected by the treatments used. Citrate synthase has been shown to be an exclusively mitochondrial enzyme in rat heart muscle [26].

RESULTS

In diabetic rats, the administration of the antilipolytic agents 5-methylpyrazole-3-carboxylate (0.5 and 1.0 mmole/kg i.p.) and nicotinate (1 mmole/kg i.p.) produced a significant reduction in the concentration of plasma free fatty acids (Table 1). Blood glucose concentrations were reduced slightly by treatment with the antilipolytic agents and to a much greater extent by insulin (60 U/kg i.p.). In non-diabetic rats, nicotinate did not affect either free fatty acid or blood glucose concentration. The higher dose of 5-methyl-pyrazole-3-carboxylate produced a small reduction in both blood glucose and plasma free fatty acid concentrations (Table 2).

Alloxan-diabetes in the rat resulted in an increase in the concentration in the heart of citrate, acetyl CoA, glucose-6-phosphate and fructose-6-phosphate (Tables 1 and 2). Adenosine triphosphate, adenosine diphosphate, and adenosine monophosphate concentrations were not affected by the diabetic state or by dosing with antilipolytic agents (results not shown).

In diabetic rats, treatment with the antilipolytic agents or insulin lowered the concentration in heart muscle of citrate, acetyl CoA, glucose-6-phosphate and fructose-6-phosphate towards the values found in non-diabetic rats (Table 1). With the exception of acetyl CoA, neither compound produced any profound alteration in these metabolite conditions in the heart of normal rats.

The hearts and soleus muscle from alloxan-diabetic rats showed a low activity of the pyruvate

Table 1. Effects of 5-methylpyrazole-3-carboxylate, nicotinate and insulin on plasma free fatty acid, blood glucose and tissue metabolite concentrations in freeze-clamped hearts from diabetic rats

	Concentrations (μmoles/g wet wt)					
	Citrate	Acetyl CoA	Glucose-6-phosphate	Fructose-6-phosphate	Plasma free fatty acid	Per cent fall in blood glucose
Control	0.954 ± 0.078	0.0170 ± 0.0020	0.574 ± 0.068	0.123 ± 0.014	1.30 ± 0.07	12.8%
5-Methylpyrazole-3-carboxylate (1 mmole/kg)	0.536 ± 0.083†	0.00840 ± 0.0021†	0.241 ± 0.039†	0.0750 ± 0.011*	0.86 ± 0.13†	14.9%
5-Methylpyrazole-3-carboxylate (0.5 mmole/kg)	0.292 ± 0.057‡	0.0100 ± 0.0022*	0.204 ± 0.026‡	0.0420 ± 0.0090‡	0.78 ± 0.10‡	36.1%†
Nicotinate (1 mmole/kg)	0.260 ± 0.032‡	0.00625 ± 0.015†	0.137 ± 0.020‡	0.0660 ± 0.0090†	0.75 ± 0.12‡	30.8%†
Nicotinate (0.5 mmole/kg)	0.534 ± 0.11†	0.0166 ± 0.0044	0.249 ± 0.031‡	0.0540 ± 0.0080‡	1.20 ± 0.17	26.6%
Insulin (60 U/kg)	0.253 ± 0.034‡	—	0.184 ± 0.035‡	0.0321 ± 0.086‡	—	80.4%‡

The rats were made diabetic by treatment with alloxan (60 mg/kg) 48 hr prior to the experiment. All substances were administered i.p. Six hours after dosing the animals were killed by decapitation and the hearts removed and freeze-clamped using tongs cooled in liquid nitrogen. Grinding and extraction of the frozen muscle and assays of metabolites were performed as described in the experimental section. Values are means ± S.E.M. for 18 animals in the control group and 9 in the others.

* $P < 0.05$ against control.

† $P < 0.01$ against control.

‡ $P < 0.001$ against control.

Table 2. Effect of 5-methylpyrazole-3-carboxylate and nicotinate on plasma, free fatty acid, blood glucose and metabolite concentrations in freeze-clamped hearts from normal rats

	Concentrations (μmoles/g wet wt)				Plasma free fatty acid (mmoles/l)	Blood glucose (mmoles/l)
	Citrate	Acetyl-CoA	Glucose-6-phosphate	Fructose-6-phosphate		
Control	0.312 ± 0.023	0.00154 ± 0.00042	0.231 ± 0.017	0.0483 ± 0.0035	0.60 ± 0.02	5.45 ± 0.09
5-Methylpyrazole-3-carboxylate (1 mmole/kg)	0.196 ± 0.024†	0.000425 ± 0.00010	0.179 ± 0.028	0.0374 ± 0.0058	0.46 ± 0.02*	4.90 ± 0.13*
5-Methylpyrazole-3-carboxylate (0.5 mmole/kg)	0.238 ± 0.031	0.00128 ± 0.00070	0.245 ± 0.033	0.0440 ± 0.0060	0.56 ± 0.03	5.27 ± 0.15
Nicotinate (1 mmole/kg)	0.388 ± 0.042	0.000198 ± 0.00010*	0.286 ± 0.034	0.0701 ± 0.0080†	0.63 ± 0.05	5.42 ± 0.16
Nicotinate (0.5 mmole/kg)	0.303 ± 0.36	0.000603 ± 0.00030	0.246 ± 0.0050	0.0439 ± 0.0050	0.60 ± 0.04	5.39 ± 0.14

All substances were administered intraperitoneally. Six hours after dosing the animals were killed by decapitation and the hearts removed and freeze-clamped using tongs cooled in liquid nitrogen. Grinding and extraction of the frozen muscle and assays of metabolites were performed as described in the experimental section. Values are means ± S.E.M. for 18 animals in the control group and 9 in the others.

* $P < 0.005$ against control.

† $P < 0.01$ against control.

Table 3. Effects of 5-methylpyrazole-3-carboxylate, nicotine and insulin on pyruvate dehydrogenase activities in freeze-clamped hearts and soleus muscle from diabetic rats

	Pyruvate dehydrogenase (μmoles substrate converted min/g wet wt)	Citrate synthase (μmoles substrate converted min/g wet wt)	Pyruvate dehydrogenase: citrate synthase ratio ($\times 10^3$)	Free fatty acids (mmoles/l)
Heart muscle				
Control	0.08 ± 0.01	92.3 ± 7.0	0.946 ± 0.18	1.30 ± 0.07
5-Methylpyrazole-carboxylate (1 mmole/kg)	0.969 ± 0.48*	88.2 ± 3.4	9.91 ± 4.4*	0.86 ± 0.13†
Nicotinate (1 mmole/kg)	1.21 ± 0.67*	89.6 ± 6.4	11.6 ± 6.4*	0.75 ± 0.12‡
Insulin (60 U/kg)	0.877 ± 0.26†	77.7 ± 9.9	11.3 ± 3.3‡	—
Soleus muscle				
Control	0.0278 ± 0.005	26.28 ± 3.57	1.6 ± 0.35	1.41 ± 0.08
5-Methylpyrazole-carboxylate (0.5 mmole/kg)	0.112 ± 0.079*	27.33 ± 7.7	12.96 ± 8.5*	0.72 ± 0.09‡
Nicotinate (2.0 mmole/kg)	0.0435 ± 0.014	20.44 ± 1.5	8.41 ± 6.16	0.76 ± 0.12‡
Insulin (60 U/kg)	0.0424 ± 0.01	19.10 ± 2.2	2.69 ± 0.71	0.66 ± 0.06‡

The rats were made diabetic by treatment with alloxan (60 mg/kg) 48 hr prior to the experiment. All drugs were administered intraperitoneally and the rats killed by decapitation 4 hr later. Hearts or soleus muscle were rapidly removed and freeze-clamped using tongs cooled in liquid nitrogen. Frozen powdered muscle was homogenised in extraction buffer and assayed for pyruvate dehydrogenase and citrate synthesis as described. Results are expressed as the means ± S.E.M.

* Significantly different from controls, $P < 0.05$.

† Significantly different from controls, $P < 0.01$.

‡ Significantly different from controls, $P < 0.001$.

dehydrogenase and a low pyruvate dehydrogenase: citrate synthase ratio (Table 3). Treatment with the antilipolytic agents or insulin produced up to a 12-fold increase in the pyruvate dehydrogenase: citrate synthase ratio in heart. However, only treatment with 5-methylpyrazole-3-carboxylate had a significant effect on pyruvate dehydrogenase activity in soleus muscle (Table 3).

DISCUSSION

The antilipolytic agents lowered plasma FFA when they were being rapidly mobilised from adipose tissue, i.e. in alloxan-diabetes, but not in the normal fed rats. Concomitant with the reduction in plasma FFA, the concentrations in heart of citrate, acetyl-CoA, and hexose monophosphate decreased. These effects of the antilipolytic agents were mimicked by insulin. The activity of pyruvate dehydrogenase was increased by 5-methylpyrazole-3-carboxylate in heart and soleus muscle, and by nicotinate and insulin in heart only. The antilipolytic agents were without consistent effects in normal fed rats.

In contrast to the findings of Randle *et al.* [2] we have shown insulin to be capable of reversing acutely the abnormalities of diabetic heart muscle. These effects are not due to hypoglycaemia, since the 4 hr blood glucose concentration was 7.41 ± 3.0 mmoles/litre. Similar effects on heart metabolites are seen with the antilipolytic agents. The reasons for the difference between our *in vivo* results and the previous results of Randle *et al.* [2] are not clear. The antilipolytic agents and insulin may be inhibiting lipolysis in muscle in addition to adipose tissue. However insulin [27] and nicotinate [28] are reported not to do so, and we have found no effect of 5-methylpyrazole-3-carboxylate on glycerol output of the isolated perfused hearts of alloxan-diabetic rats (unpublished observations). The indirect effects of antilipolytic agents on muscle may be related either to effects on plasma FFA or to the large falls in blood ketone body concentrations also observed (results not shown). Ohlen *et al.* [29] have shown that insulin but not nicotinate can increase the activity of pyruvate dehydrogenase in alloxan-diabetic rat hearts. This discrepancy may be related to the period of exposure to nicotinate: Ohlen *et al.* measured pyruvate dehydrogenase activity only 2 hr after dosing, compared with 6 hr in the case of our experiments. The effect of the antilipolytic agents on pyruvate dehydrogenase may involve a direct effect on, for example, pyruvate dehydrogenase kinase, which is inhibited by a number of halogenated fatty acids [30] or pyruvate dehydrogenase phosphate phosphatase. If effects of the antilipolytic agents on pyruvate dehydrogenase activity in soleus muscle are mediated by the fall in plasma FFA concentrations, this is additional evidence for the operation of the glucose fatty acid cycle in skeletal muscle.

Acknowledgement—We thank Mr. T. Elliot for skilled technical assistance.

REFERENCES

1. C. R. Park, H. E. Morgan, M. J. Henderson, D. M. Regen, E. Cadenas and R. L. Post, *Recent Prog. Horm. Res.* **17**, 493 (1961).
2. P. J. Randle, P. B. Garland, C. N. Hales, E. A. Newsholme, R. M. Denton and C. I. Pogson, *Recent Prog. Horm. Res.* **22**, 1 (1966).
3. O. H. Wieland, H. Von Funcke and G. Loffler, *FEBS Lett.* **15**, 295 (1971).
4. A. L. Kerbey, P. J. Randle, R. H. Cooper, S. Whitehouse, H. T. Pask and R. M. Denton, *Biochem. J.* **154**, 327 (1976).
5. E. A. Newsholme and P. J. Randle, *Biochem. J.* **93**, 641 (1964).
6. P. J. Randle, E. A. Newsholme and P. B. Garland, *Biochem. J.* **93**, 652 (1964).
7. O. H. Wieland, E. A. Siess, F. H. Schulze-Wethmar, H. Von Funcke and B. Winton, *Archs Biochem. Biophys* **143**, 593 (1971).
8. J. D. Schnatz and R. H. Williams, *Diabetes* **12**, 174 (1963).
9. D. F. Steiner, V. Rauda and R. H. Williams, *Endocrinology* **68**, 809 (1961).
10. R. L. Jungas and E. G. Ball, *Biochemistry* **2**, 383 (1963).
11. F. Reimer, G. Loffler, G. Hennig and O. H. Wieland, *Hoppe-Seylers J. Physiol. Chem.* **356**, 1055 (1975).
12. M. Berger, S. A. Hagg, M. N. Goodman and N. B. Ruderman, *Biochem. J.* **158**, 191 (1976).
13. M. J. Rennie and J. O. Holloszy, *Biochem. J.* **168**, 611 (1977).
14. E. Z. Maizels, N. B. Ruderman, M. N. Goodman and D. Lan, *Biochem. J.* **162**, 557 (1977).
15. A. Wollenberger, O. Ristau and G. Schoffa, *Pflugers Arch.* **270**, 399 (1960).
16. Whitehouse and R. J. Randle, *Biochem. J.* **134**, 651 (1973).
17. A. L. Kerbey, P. J. Randle, R. H. Cooper, S. Whitehouse, H. T. Pask and R. M. Denton, *Biochem. J.* **154**, 327 (1976).
18. P. Trinder, *Ann. Clin. Biochem.* **6**, 24 (1969).
19. V. P. Dale and H. Meinertz, *J. biol. Chem.* **235**, 2595 (1960).
20. S. Kashket, *Analyt. Biochem.* **41**, 166 (1971).
21. H. Moellering and W. Gruber, *Analyt. Biochem.* **17**, 369 (1966).
22. H. J. Hohort, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), pp. 134–138. Academic Press, New York (1963).
23. J. F. A. Chase, *Biochem. J.* **104**, 503 (1967).
24. H. G. Coore, R. M. Denton, B. R. Martin and P. J. Randle, *Biochem. J.* **125**, 115 (1971).
25. P. A. Srere, H. Brazil and J. Gonen, *Acta. Chem. scand.* **17**, S 129 (1963).
26. D. Pette, in *Regulation of Metabolic Processes in Mitochondria* (Eds J. M. Tager, S. Papa, E. Quagliariello and E. C. Slater), p. 28. Elsevier (1966).
27. P. B. Garland and P. J. Randle, *Biochem. J.* **93**, 678 (1964).
28. P. J. Randle, *Nobel Symp.* **13**, 173 (1970).
29. J. Ohlen, E. A. Siess, G. Loffler and O. H. Wieland, *Diabetologia* **14**, 135 (1978).
30. S. Whitehouse, R. H. Cooper and P. J. Randle, *Biochem. J.* **141**, 761 (1974).