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THE MECHANISM OF ACTION OF PHENFORMIN IN STARVED RATS

PETER F. EVANS, LAURENCE J. KING and DENNIS V. PARKE
Department of Biochemistry, University of Surrey, Guildford, Surrey, GU2 5XH, England
and
GEORGE MARGETTS and WILLIAM E. JONES
Sterling Winthrop, Research and Development, Sterling Winthrop House, Surbiton, Surrey, KT6 4PH, England

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Abstract—The ability of phenformin to lower the blood glucose concentration after an intraperitoneal glucose load, with a concomitant increase in blood lactate concentration, indicated that the drug was increasing the rate of anaerobic glycolysis. The results of experiments in which glucose and gluconeogenic precursors were given to starved rats were explained by a hypothesis for the mechanism of action of phenformin involving inhibition of certain NAD^+-dependent dehydrogenases. Substrates with NAD^+-linked oxidations could be discriminated from those, like succinate, with FAD-linked oxidations, and succinate may be of use in the treatment of clinical lacticacidosis caused by biguanide drugs.

The mechanism of biochemical action of phenformin remains uncertain even after many years of investigation, although it is generally agreed that the hypoglycaemia and hyperlactataemia it causes are closely related. It was initially proposed that the fall in blood glucose concentration was due to increased peripheral anaerobic glycolysis secondary to an inhibition of aerobic cell respiration [1, 2]. Virtually all the evidence implicating inhibition of respiration as the underlying mechanism of action of biguanides has, however, been derived from studies carried out *in vitro* and the role of inhibition of respiration in the whole animal is not clear. Other mechanisms of hypoglycaemic action have, therefore, been proposed for the biguanides, including inhibition of gluconeogenesis [3, 5], inhibition of intestinal glucose absorption [6, 7] and inhibition of fatty acid oxidation [8].

The objective of the present study was to determine the effects of phenformin on the ability of rats *in vivo* to utilise glucose and gluconeogenic precursors. Changes in blood glucose concentrations after a parenteral glucose load would indicate changes in the disposition of glucose in the animal, while differences caused by phenformin in blood concentrations after administration of a gluconeogenic load would indicate alterations in the ability of the animal to synthesise glucose from that precursor. Blood lactate concentrations were measured to give an indication of the extent of anaerobic glycolysis. Starved rats were used so that initial blood glucose concentrations were low and the rate of gluconeogenesis was high [9].

MATERIALS AND METHODS

Chemicals

Lithium lactate, lactate dehydrogenase, NAD^+, sodium malate, disodium succinate and 2-oxoglutaric acid were obtained from Sigma (London) Chemical Co. Ltd., Poole, Dorset, England. Before use, 2-oxoglutaric acid solution (1 M) was brought to pH 6 using 2 M sodium hydroxide. Guaiacum, peroxidase and glucose oxidase (Fermcozyme 652 AM) reagents were purchased from Hughes and Hughes (Enzymes) Ltd., Romford, Essex, England. Sterling Winthrop Research Laboratories, Alnwick, Northumberland, England donated phenformin hydrochloride. Sodium pentobarbitone solution (Sagatal) was supplied by May and Baker Ltd., Dagenham, Essex, England. All other chemicals were obtained from British Drug Houses Ltd., Poole, Dorset, England or Sigma and were generally of analar grade.

Experimental animals and collection of blood samples

Male, Wistar albino rats (135–280 g) were starved for 18 hr but allowed water *ad libitum*. Before collection of blood samples, animals were anaesthetised with sodium pentobarbitone (60 mg/kg, i.p.) and about 0.2 hr later the tip of the tail was removed with a scalpel blade. The volume of blood required (0.05 ml for glucose, 0.1 ml for lactate) was collected in a blood pipette, immediately expelled into the appropriate deproteinising solution and centrifuged. The first few drops of blood from each bleed were discarded to avoid elevated lactate concentrations due to tissue damage and venous stasis. Blood flow to the tail was encouraged by keeping the animal warm under a lamp. Further blood samples were collected in a similar way at half-hourly intervals for the duration of the experiment. All animals were killed before recovery from anaesthesia.

Effect of phenformin on blood glucose and lactate concentrations following an intraperitoneal glucose load

Starved rats were given phenformin (0.25 M in 0.15 M saline, 0.50 mmol/kg, i.p.) or a similar vol-

ume of saline. After 0.8 hr the animals were anaesthetised with sodium pentobarbitone, a resting blood sample taken and at 1 hr an intraperitoneal load of glucose (20 mM in distilled water, 13.3 mmol/kg) administered. In an experiment using fluoride to inhibit glycolysis, sodium fluoride (0.25 M in 0.15 M saline, 0.50 mmol/kg, i.p.) was administered at the same time as the phenformin or saline, 1 hr before the load of glucose. In both experiments blood samples for glucose and lactate determinations were taken at hourly intervals for 3 hr after administration of glucose.

Effects of gluconeogenic precursors on phenformin-induced hypoglycaemia and hyperlactataemia

Starved rats were given phenformin or saline and anaesthetised, as described above, and 1 hr later a gluconeogenic precursor (1 M in distilled water or saline, 2 mmol/kg, i.p.) or saline was administered. Blood for glucose and lactate determinations was collected from the tail at intervals for 3 hr. In a further experiment, disodium succinate (1 M in distilled water, 2 mmol/kg, i.p.) was administered at the same time as the phenformin or saline. After 0.8 hr the rats were anaesthetised and blood collected at intervals over 3 hr.

Determinations of glucose and lactate

Blood glucose concentration was measured using the glucose oxidase method of Morley *et al.* [10]. Blood L-lactate concentration was determined by the method of Hohorst [11] modified for small blood volumes.

Deproteinised blood samples were stored at 4° and assayed within 24 hr of collection.

Analysis of data

The statistical significance of differences found in group mean values was assessed using Student's *t*-test. Where a variance-ratio test (F-test) indicated that the variances of the group were statistically unequal, a modified form of the *t*-test (the *d*-test) was used [12]. The level of significance chosen was $P < 0.05$.

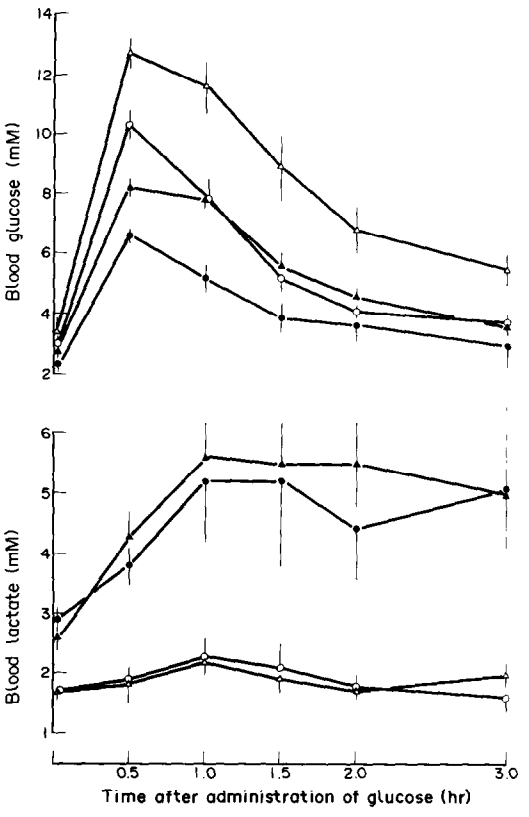

Fig. 1. Blood glucose and lactate concentrations in starved, glucose-loaded rats following intraperitoneal administration of phenformin with or without sodium fluoride. Treatments are indicated as follows: ○, saline; ●, phenformin plus saline; △, fluoride plus saline; ▲, fluoride plus phenformin. Points represent the means of 3–5 experiments ± S.E.M. represented by vertical bars.

RESULTS

Pretreatment of starved rats with phenformin significantly decreased the mean peak blood glucose concentration following an intraperitoneal glucose load, from the control value of 10.3 to 6.6 mM (see Fig. 1). The phenformin-dosed rats showed a marked rise in blood lactate concentrations. Comparison of areas between the curves for control and phenformin-dosed animals for blood glucose and blood lactate indicated that all of the excess lactate circulating in rats receiving phenformin could be accounted for by 86% of the decrease in blood glucose over the duration of the experiment.

Administration of sodium fluoride to starved rats increased blood glucose concentrations following an intraperitoneal glucose load, the peak blood glucose concentration being significantly higher than that in animals receiving only saline (see Fig. 1). Phenformin in combination with fluoride reduced glucose concentrations when compared with animals receiving fluoride and saline. The ratios of the areas under the glucose curves, using the initial blood glucose concentrations as the lower boundaries, were fluoride 1.82, fluoride with phenformin 1.03 and phenformin 0.66, compared with 1.00 for saline. The administration of fluoride did not alter either the hyperlactataemia produced by phenformin or the basal concentration of blood lactate in saline-treated control animals.

When phenformin was administered before the pentobarbitone anaesthetic to starved rats, it caused a marked hypoglycaemia, blood glucose concentrations continuing to fall throughout the 3 hr of the experiment (see Fig. 2). The blood lactate concentrations of these animals were raised (see Fig. 3). Thus the action of phenformin in starved, initially-unanaesthetised rats was to cause pronounced hypoglycaemia and hyperlactataemia, and in further experiments various gluconeogenic compounds were given in an attempt to prevent these effects. All of these compounds were apparently gluconeogenic as they increased the blood glucose concentrations of control animals to a maximum at 0.5 hr (see Fig. 2). In all phenformin-treated animals the blood glucose concentration at the zero time point, the time of

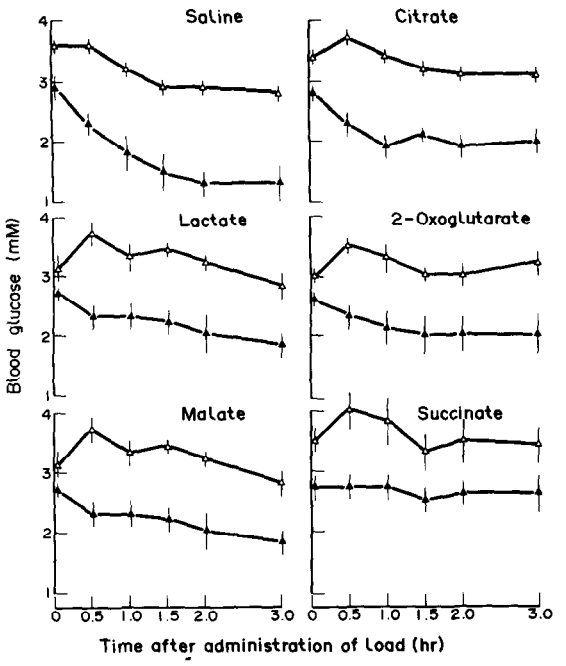

Fig. 2. Blood glucose concentrations in starved rats following intraperitoneal administration of phenformin and a gluconeogenic load. Treatments, given 1 hr before administration of the load, are indicated as follows: △, saline; ▲, phenformin. Points represent the mean of 4–7 experiments ± S.E.M. represented by vertical bars.

administration of the gluconeogenic load, was decreased due to the action of phenformin which had been dosed 1 hr earlier. The blood lactate concentration at the zero time point is raised for the same reason (see Fig. 3). Succinate and malate prevented the development of the marked hypoglycaemia normally caused by phenformin while lactate, 2-oxoglutarate and citrate had less effect on this action of phenformin. Succinate also prevented any significant increase in the phenformin-induced hyperlactataemia beyond that established at the zero time point. In contrast, with lactate, citrate, 2-oxoglutarate and to a lesser extent malate the increase in the blood lactate concentration above the initial level was similar to or greater than that found in animals receiving phenformin and saline. The ratios of the areas under the lactate curves, using the lactate concentrations at zero time as the lower boundaries, were compared over the 3 hr experimental period for animals receiving phenformin. The values were lactate 0.90, citrate 1.80, 2-oxoglutarate 1.12, succinate 0.27 and malate 0.59 compared with saline controls as 1.00.

Administration of succinate at the same time as phenformin (Fig. 4) significantly decreased both the hypoglycaemic and hyperlactataemic effects of the biguanide. The blood lactate concentrations in rats receiving phenformin and succinate were only slightly higher than control levels and were significantly lower than those in animals receiving phenformin and saline for the full duration of the experiment.

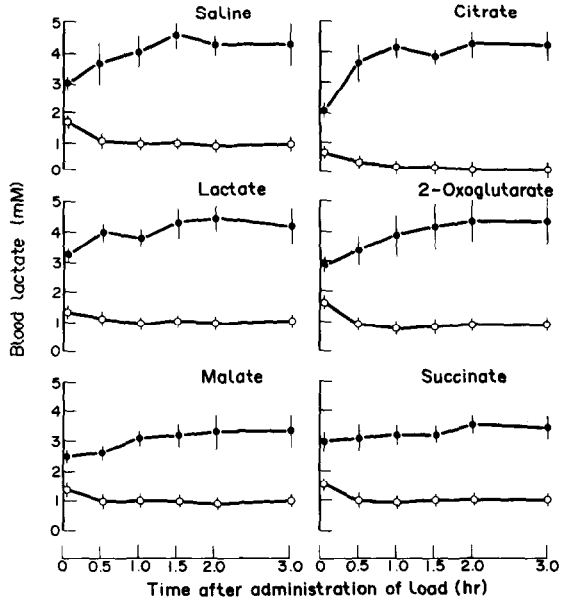

Fig. 3. Blood lactate concentrations in starved rats following intraperitoneal administration of phenformin and a gluconeogenic load. Treatments, given 1 hr before administration of the load, are indicated as follows: ○, saline; ●, phenformin. Points represent the mean of 4–7 experiments ± S.E.M. represented by vertical bars.

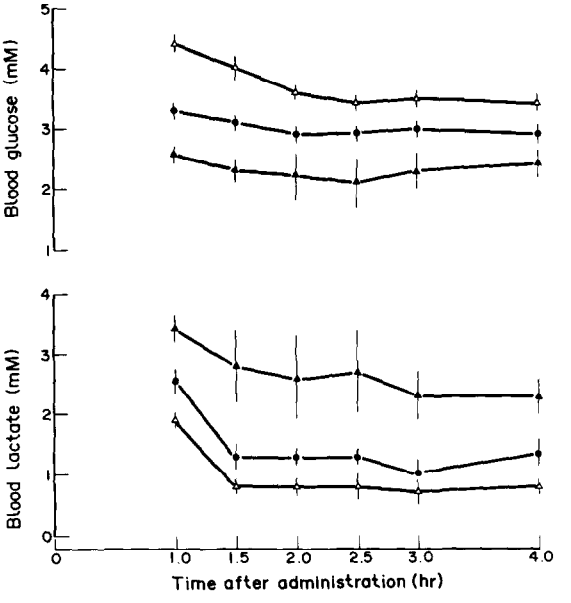

Fig. 4. Blood glucose and lactate concentrations in starved rats following administration of phenformin and sodium succinate. Treatments are indicated as follows: ▲, saline plus succinate; ●, phenformin plus saline; △, phenformin plus succinate. Points represent the means of 3–6 experiments ± S.E.M. shown by vertical bars.

DISCUSSION

Only a proportion of an intraperitoneal glucose load could be measured in the blood of starved, phenformin-treated rats compared with the saline-treated controls. The increased blood lactate concentration observed suggests that phenformin caused increased uptake and glycolytic conversion of glucose by the peripheral tissues. The ability of phenformin to decrease blood glucose concentrations in starved rats receiving sodium fluoride, which causes a partial inhibition of the glycolytic enzyme enolase [13], supports the view that the biguanide is causing an increased flux through glycolysis. These results are in contrast with previous reports that biguanides reduce blood glucose concentrations after the administration of oral but not intraperitoneal nor intravenous loads of glucose to animals [14, 15] and humans [16], but are consistent with effects found *in vitro* using isolated hemidiaphragms from starved rats at concentrations of 0.1–2.5 mM phenformin [1, 2, 17]. Studies in human maturity-onset diabetics have also suggested that phenformin accelerates anaerobic glycolysis [18, 19].

A number of gluconeogenic compounds were tested for their ability to counteract the hypoglycaemic and hyperlactataemic actions of phenformin in starved rats. The results suggest that most substrates which are metabolised in their first nucleotide-requiring reaction by NAD^+-dependent enzymes, namely lactate, citrate and 2-oxoglutarate, are unable to prevent either the hypoglycaemic or hyperlactataemic effects of phenformin. These results are in agreement with the effect of lactate reported in phenformin-treated fed rats [20] and are consistent with the observations that phenformin inhibited (a) oxidative phosphorylation in guinea pig liver mitochondria with malate or glutamate [21] and (b) the oxidation of pyruvate, citrate, 2-oxoglutarate and fumarate in tissue homogenates from several species [22]. It is possible therefore that phenformin prevents gluconeogenesis both from these precursors and the cicrulating lactate by inhibiting mitochondrial NADH dehydrogenase, thus reducing both the supply of ATP from oxidative phosphorylation and the production of oxaloacetate.

In our experiments, malate did produce some alleviation of both the hypoglycaemic and hyperlactataemic effects of phenformin, in contrast to the other NAD^+-dependent substrates. Malate may have given rise to glucose through the action of extra-mitochondrial malate dehydrogenase and phosphoenolpyruvate carboxykinase, however it is more difficult to envisage how the hyperlactataemic effect of phenformin was ameliorated without the production of energy from mitochondria. This result suggests that the action of phenformin may result in inhibition of mitochondrial NAD^+-dependent dehydrogenases other than malate dehydrogenase, rather than inhibition of NADH dehydrogenase. Thus the inhibition of pyruvate, isocitrate and 2-oxoglutarate dehydrogenases in some way by phenformin may result in inadequate energy production from oxidative phosphorylation with a consequent increase in anaerobic glycolysis and lactate production in an attempt to meet the energy demands of the tissues.

Of the gluconeogenic precursors tested, succinate was the most successful in combating both the hypoglycaemic and hyperlactataemic effects of phenformin (see Figs. 2–4). This suggests that succinate was able to produce energy in the mitochondria from both the flavin-dependent succinate dehydrogenase and malate dehydrogenase and yield oxaloacetate for gluconeogenesis. It is possible that other compounds which give rise to $FADH_2$ within the mitochondria could reverse the metabolic effects of phenformin and this may explain the reported effect of glyceryl tripalmitate in this respect [23].

Mortality in phenformin-induced lacticacidosis is about 50% [24]. Treatment usually involves alkalinisation with bicarbonate, the use of dichloroacetate having been curtailed due to its toxicity [25] and mutagenicity [26]. The results of the present study indicate that sodium succinate should be investigated with regard to reversing clinical lacticacidosis following biguanide therapy. Since it could be given as an intravenous infusion, it would rapidly reach the peripheral tissues and its toxicity is likely to be extremely low. By allowing the production of increased amounts of mitochondrial ATP, it should decrease the rate of anaerobic glycolysis thus allowing the blood glucose concentration and pH to rise and the blood lactate concentration to fall.

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