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THE DOSE-DEPENDENT TOXIC EFFECTS OF PHENFORMIN IN THE RAT

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Abstract—Determination of the blood lactate and glucose concentrations in the normal rat after increasing doses of phenformin (50–150 mg/kg, i.p) shows that the hyperlactataemic and hypoglycaemic effects of the drug are dose-related, and suggests the existence of a critical dose in the rat of 120–135 mg/kg. At this critical dose a marked increase in blood lactate and decrease in blood glucose is seen without a correspondingly large increase in plasma phenformin concentration. The major metabolite of phenformin in the rat, 4-hydroxyphenformin, did not affect blood glucose or lactate levels. The toxic effects of phenformin at high doses may represent saturation of the capacity of the liver to extract and metabolize the drug. These findings may be especially relevant to the predisposition to lactic acidosis of patients with renal and/or hepatic insufficiency. Sodium dichloroacetate (300 mg/kg/hr), infused intravenously, was effective in preventing hyperlactataemia and enhanced the hypoglycaemia associated with phenformin.

Despite the large amount of data on the effects of biguanides both *in vivo* and *in vitro*, the mechanism by which hypoglycaemia is produced is not clearly understood. A very important aspect is the relationship between the mode of action of the biguanides and lactate metabolism. There is no doubt, from clinical and experimental evidence, that lactate metabolism is influenced by the biguanides, and that in special circumstances, when lactate metabolism is disturbed, the drugs can exert a toxic action.

Although a slight elevation of blood lactate is common in patients treated with phenformin (N^1-phenethylbiguanide) [1–3], a significant increase is unlikely to occur except in cases where some other factor is present, such as poor renal function, liver disease, reduced circulatory efficiency, severe infection or acute alcoholosis [4–10].

The biochemical effects of phenformin such as glycogen depletion [11, 12], inhibition of oxidative phosphorylation [13] or reduced hepatic gluconeogenesis [14, 15] have been observed only with concentrations of the drug higher than those measured in the plasma of patients after therapeutic doses. Therefore, the hypoglycaemia and hyperlactataemia produced in animals by high doses of phenformin is a toxic effect that may be produced by a mechanism which is different from that responsible for its antidiabetic action.

The objectives of the present study were to investigate the dose-response effects of phenformin on blood glucose and lactate concentrations in the rat, in parallel with the determination of plasma phenformin concentrations. In addition, the effects of 4-hydroxyphenformin, the major metabolite of phenformin in the rat, on blood glucose and lactate concentrations were investigated. The results obtained are discussed in relation to the current data available on the metabolism and disposition of phenformin and its relevance to the toxic effects of the drug. The effects of sodium dichloroacetate (DCA) on phenformin-induced hypoglycaemia and hyperlactataemia were also studied in the light of recent reports that DCA can prevent [16] or reverse [17] the lactic acidosis caused by high doses of the drug.

MATERIALS AND METHODS

Chemicals. Lithium lactate, lactate dehydrogenase and NAD were obtained from Sigma (London) Chemical Co. Ltd., Poole, Dorset, U.K. Guaiacum, peroxidase and glucose oxidase (Fermcozyme 653AM) reagents were purchased from Hughes & Hughes (Enzymes) Ltd., Romford, Essex, U.K. Dichloroacetic acid (reagent grade) was supplied by Fisons, Loughborough, Leics., U.K., and was converted to the sodium salt by neutralizing the aqueous solution with sodium hydroxide and freeze-drying. The solid was pulverized and stored in an amber-glass bottle until used. Phenformin hydrochloride and 4-hydroxyphenformin dihydrochloride were kindly donated by Sterling Winthrop Research Laboratories, Fawdon, Newcastle-upon-Tyne, U.K. All other chemicals were purchased from common laboratory suppliers and, unless otherwise specified, were of reagent grade.

Materials. Neutral glass collection tubes (2 ml) were obtained from Glass Wholesale Supplies Ltd., London, U.K. Autoanalyser cups (1 ml) with concave base (FO7/c) pretreated with fluoride–oxalate were purchased from Stayne Laboratories Ltd., Marlow, Bucks., U.K.

Administration of dose. Phenformin hydrochloride (50–150 mg/kg) or 4-hydroxyphenformin dihydrochloride (150 or 183 mg/kg, the molar equivalent of 120 mg/kg and 150 mg/kg phenformin hydrochloride, respectively) in saline were administered as single i.p. injections.

Effect of phenformin on the blood concentration of lactate and glucose. Male, Wistar albino rats (200–

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300 g) were anaesthetized with sodium pentobarbital (60 mg/kg) and blood samples collected from the tail vein at 0.5 hr intervals over a period of 4 hr after phenformin or 4-hydroxyphenformin administration. The end of the tail was cleaned to prevent surface contamination of the blood and the first sample discarded as tissue damage and venous stasis may cause an elevation of blood lactate concentration. Blood samples were collected into fluoride–oxalate–heparin-treated Autoanalyser cups and appropriate aliquots taken for the determination of lactate and glucose. All animals were killed before recovery from anaesthesia.

In experiments incorporating sodium dichloracetate (DCA) the right femoral vein was catheterized with a butterfly infusion catheter and DCA (50 mg/ml isotonic saline, pH 7.4) administered as boluses at 10-min intervals (300 mg/kg/hr; [18]) commencing 1 hr before the administration of phenformin or saline.

Comparison between plasma phenformin concentration and the hyperlactataemic and hypoglycaemic responses. The experiment was performed over a period of 3 days on groups of 3 rats (200–300 g) per dose group with the doses randomized for each day. Saline injected control rats were examined at the beginning and end of each day's experiment to determine any changes in resting levels of lactate and glucose. Blood samples from the tail vein were taken from the pentobarbital-anaesthetized rats immediately before and 2.5 hr after dosing with phenformin (50–150 mg/kg) i.p. for the determination of lactate and glucose. A larger sample of blood (2.0–3.0 ml), taken immediately by cardiac puncture, was extracted and derivatized with trifluoroacetic anhydride (Sterling Winthrop Research Laboratories, personal communication, 1974) and analysed for phenformin essentially by the g.l.c. method of Matin *et al.* [19].

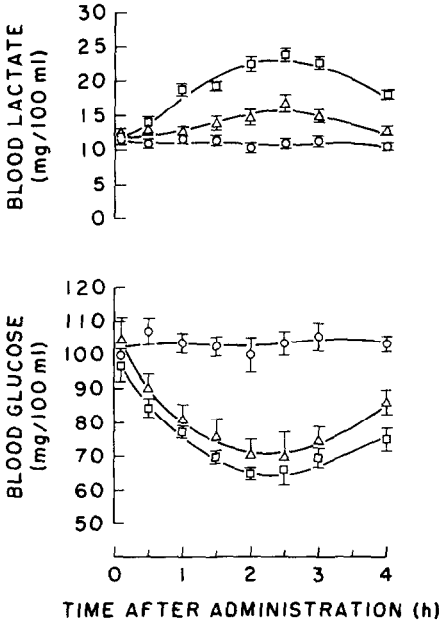

Fig. 1. Concentrations of lactate and glucose in rat blood following the single i.p. administration of phenformin. Phenformin hydrochloride was administered in saline (0.5 ml) at the following doses: △, 75 mg/kg; □, 120 mg/kg; ○, saline control. Points represent the means of three to nine experiments ± S.E.M. represented by vertical bars.

Determination of lactate and glucose. Lactate concentrations were determined by the method of Hohorst (see Henry *et al.* [20]) modified for small blood samples (0.2 ml). Blood glucose concentrations were determined by the glucose oxidase method of Marks *et al.* [21] using 0.1 ml samples of heparinized blood.

Analysis of data. Significant differences between the blood lactate or glucose concentrations in the different groups were determined using a modified form of Student's *t*-test for small samples; where variances in the population were shown by a variance-ratio test to be statistically unequal, a second modified form of *t*-test was used [22]. The level of significance chosen was $P < 0.05$.

RESULTS

The maximum effect of phenformin upon blood lactate and glucose concentrations occurred at 2.0–2.5 hr after administration (see Fig. 1). After a single 75 mg/kg dose of phenformin, lactate concentrations were increased from a resting value of 12 mg/100 ml (1.3 mM) to 17 mg/100 ml (1.9 mM) at 2.5 hr and glucose concentrations decreased from 100 mg/100 ml (5.6 mM) to 70 mg/100 ml (3.9 mM). After a single dose of 120 mg/kg phenformin blood lactate concentrations were twice those in control animals (24 mg/100 mg, 2.7 mM) at 2.5 hr while glucose concentrations fell to 65 mg/100 ml (3.6 mM).

The results of the dose–response studies with phenformin are shown in Fig. 2. The curves were fitted by computer using a non-linear least-squares method. Lactate levels in the rat after a 50 mg/kg dose of phenformin were not significantly different from those in control animals, but after doses of 75, 90 and 105 mg/kg small but significant increases up to 40 per cent were measured compared to controls. At doses of 120–150 mg/kg lactate levels increased 100–225 per cent. The hypoglycaemic response followed a related pattern, although it should be noted that a dose of 50 mg/kg produced a 20 per cent decrease in blood glucose concentrations without increasing lactate levels. Glucose concentrations after doses of 75–120 mg/kg showed a fairly uniform reduction to 65–70 per cent of resting levels, while 135 and 150 mg/kg doses produced a sharp fall to 40 per cent of resting levels. Plasma phenformin concentrations increased with increasing doses and ranged from about 0.2 μg/ml at 50 mg/kg to 2.7 μg/ml at 150 mg/kg.

The effects of DCA infusion on blood lactate and glucose concentrations are shown in Fig. 3. DCA produced a significant 40 per cent reduction in blood lactate levels to 5.6 mg/100 ml (0.6 mM) after 1 hr. Subsequent administration of phenformin (120 mg/kg) caused a 30 per cent increase in lactate concentrations in 2–2.5 hr, but the levels did not exceed normal resting concentrations. In the absence of DCA the same dose of phenformin produced a 100 per cent increase over normal resting concentrations. DCA has a slight but significant effect on resting

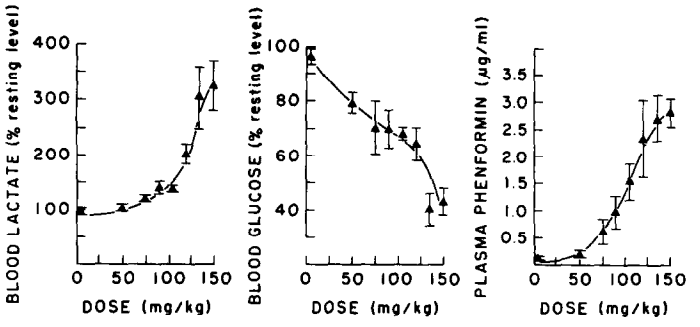

Fig. 2. Effects of dose on blood concentrations of lactate and glucose and plasma concentration of phenformin after i.p. administration of phenformin to rats. Phenformin hydrochloride was administered in saline (0.5 ml). Blood samples were taken 2.5 hr after dosing. Points represent the means of three experiments ± S.E.M. represented by vertical bars.

blood glucose concentrations in the rat. Throughout the 5 hr period of the experiment in the DCA-treated saline injected animals the mean blood glucose concentration (90 mg/100 ml, 5.0 mM) was significantly lower than that of rats studied for 4 hr after administration of saline alone (103 mg/100 ml, 5.7 mM). Administration of phenformin (120 mg/kg) to DCA-infused rats resulted in a severe hypoglycaemia reaching a maximum effect at 2 hr, at which time blood glucose levels were reduced to 30 per cent of normal levels (27 mg/100 ml, 1.5 mM). In phenformin treated animals not receiving DCA blood glucose levels fell to 65–70 per cent of resting levels at 2 hr. The blood glucose levels in rats receiving phenformin and DCA were significantly lower than those in rats receiving phenformin alone at all times after 0.5 hr.

Following a single i.p. administration of 4-hydroxyphenformin at doses equivalent to 120–150 mg phenformin/kg, blood lactate levels were unchanged (see Fig. 4). The molar equivalent doses of phenformin produced 100–225 per cent increases in blood lactate by 2.5 hr (see Figs. 1 and 2). At the higher dose of 4-hydroxyphenformin no changes in blood glucose concentrations were detected, while the equivalent dose of phenformin (150 mg/kg) produced a 55–60 per cent reduction in blood glucose levels by 2.5 hr (see Fig. 2).

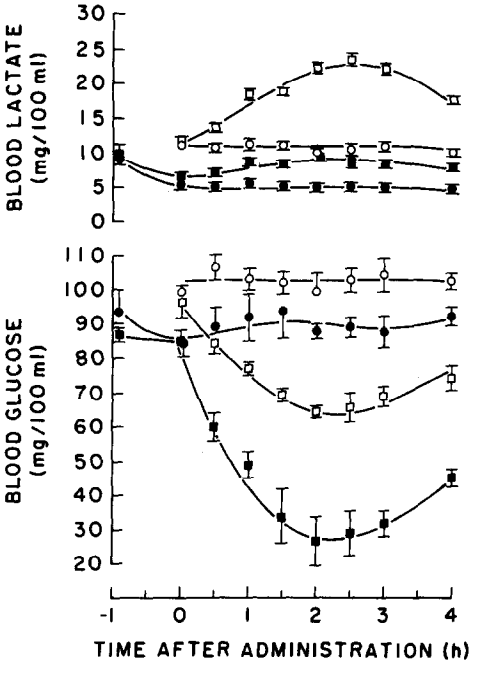

Fig. 3. Blood concentrations of lactate and glucose in the rat following a single i.p. administration of phenformin alone, or in conjunction with an i.v. infusion of dichloroacetate. Phenformin hydrochloride (120 mg/kg) was administered in saline (0.5 ml); sodium dichloroacetate (300 mg/kg/hr) in saline, pH 7.4, was infused intravenously (1.2–1.4 ml/hr) commencing 1 hr before administration of phenformin or saline. Treatments are indicated as follows: □, phenformin alone; ■, phenformin plus dichloroacetate; ○, saline alone; ●, saline plus dichloroacetate. Points represent the means of three to six experiments ± S.E.M. represented by vertical bars.

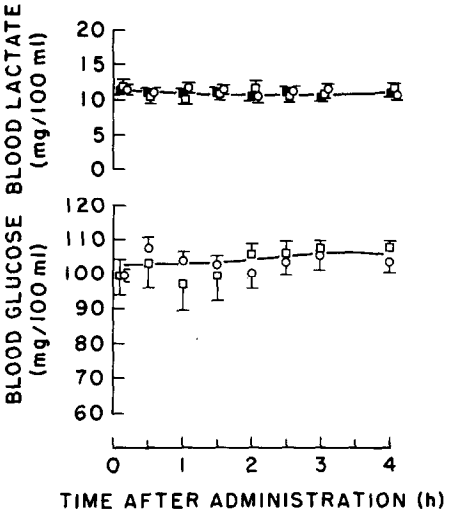

Fig. 4. Blood concentrations of lactate and glucose in the rat following the single i.p. administration of 4-hydroxyphenformin. 4-Hydroxyphenformin dihydrochloride was administered in saline (0.5 ml) at the following doses: ■, 150 mg/kg; □, 185 mg/kg; ○, saline control. Points represent the means of four to eight experiments ± S.E.M. represented by vertical bars.

DISCUSSION

In the normal rat the hyperlactataemic and hypoglycaemic effects of phenformin were dose-related (see Figs. 1 and 2). The maximum effect with these parameters occurred at around 2.5 hr. The time–effect curves (Fig. 1) suggest that these effects would have returned to normal by about 6 hr. The dose–effect relationships in Fig. 2 suggest the existence of a critical dose of phenformin in the normal rat of between 120 and 135 mg/kg (i.p.), at which point blood lactate concentrations rise sharply by 100–225 per cent of normal levels and glucose concentrations fall to 40 per cent of resting levels. The plasma phenformin concentration 2.5 hr after doses of 120–135 mg/kg was 2–3 μg/ml ($1.0–1.5 \times 10^{-5}$ M). These plasma concentrations are considerably lower than the drug concentrations used in most *in vitro* experiments demonstrating glycogen depletion and respiratory inhibition (10^{-3}–10^{-4} M; [11]), inhibition of enzymes of the Krebs cycle (2×10^{-3} M; [13, 23]) and inhibition of hepatic gluconeogenesis (4×10^{-4} M to 10^{-3} M; [14, 15]). There is, however, some dispute as to the concentration of phenformin required to produce some of these inhibitory effects. Thus, while Söling [12] has reported that no evidence of reduced oxidative phosphorylation is seen in rat livers perfused *in vitro* with biguanides at 2.5×10^{-5} M, Woods and Alberti [24] reported that 10^{-5} M phenformin caused a 45 per cent inhibition of gluconeogenesis in rat liver. It remains possible, therefore, that the rapid onset of hyperlactatemia and hypoglycaemia as seen in Fig. 2 indicates the point at which inhibition of oxidative phosphorylation and gluconeogenesis become evident.

The slight but significant reduction in blood glucose levels in DCA-treated rats is in contrast with previous reports that the compound lowers blood glucose concentrations only in diabetic or starved rats [25–27]. Previously, however, DCA has been administered either p.o. or i.p., while in the present studies the compound was given i.v. This may have resulted in a greater inhibition of the peripheral release of gluconeogenic precursors by DCA, an effect which contributes to its hypoglycaemic action [18, 28, 29].

DCA was effective in controlling phenformin-associated hyperlactataemia and enhanced the hypoglycaemic response to the drug (see Fig. 3). Similar findings have been reported using SKF 525A-treated [30] and starved rats [17]. A combination of DCA and a reduced dose of phenformin may be valuable, therefore, in the treatment of some forms of diabetes and may significantly reduce the hazard of lactic acidosis. However, more extensive biochemical and toxicological studies are required to determine the therapeutic potential of the combined treatment. The risks of ketosis are increased [16, 17], but this may cause less difficulty in ketoacidosis-resistant diabetes.

The pharmacological activity of phenformin is enhanced in animals treated with inhibitors of the microsomal mixed-function oxidase system [17, 30], suggesting that this compound is metabolized to a less active form. The major metabolite of phenformin in the rat is 4-hydroxyphenformin which, together with its glucuronic acid conjugate, accounts for more than half the radioactivity eliminated in the 24 hr urine (35–40 per cent of the dose) after a 100 mg/kg i.p. dose of [2′-^{14}C] phenformin [31]. 4-Hydroxyphenformin had no effect on blood lactate or glucose concentrations in the rat at doses equivalent to 120 or 150 mg phenformin/kg (see Fig. 4). However, this compound was not devoid of biological activity, as at a higher dose (305 mg/kg—equivalent on a molar basis to 250 mg/kg phenformin) it was lethal to all of the five rats used. Beckmann [32] reported that oral administration of 4-hydroxyphenformin (200–400 mg/kg) does not produce hypoglycaemia in the mouse and Heuclin *et al.* [8] found no change in blood lactate levels in rats with surgically-induced renal insufficiency after administration of 4-hydroxyphenformin (unspecified dose) for 8 days. In contrast, Wick *et al.* [33] have reported that the 4-hydroxy metabolite possesses half the hypoglycaemic activity of phenformin in the rat, but no details of this study were given.

It appears, therefore, that in the rat hydroxylation at the 4-position effectively inactivates phenformin. In the rat, after oral administration of a low dose of [2′-^{14}C] phenformin (7 mg/kg), almost 90 per cent of the 24 hr urinary radioactivity (45 per cent of the dose) is eliminated as 4-hydroxyphenformin (free and conjugated with glucuronic acid) and only small amounts of phenformin (3 per cent of the dose) are eliminated unchanged [31]. After a higher dose (100 mg/kg, p.o.) about 24 per cent of the 24 hr urinary radioactivity (12 per cent of the dose) is attributable to the parent compound [31]. These data suggest that a first-pass elimination process occurs in the rat and that at high doses of phenformin the hepatic capacity to extract and/or metabolize the drug may be saturated. The saturation of such a system may lead to dose-dependent disposition kinetics [34] and could explain the relatively constant increase in plasma phenformin concentrations with each dose increment above 50 mg/kg (see Fig. 2). Thus, high doses of phenformin may lead to the appearance of large amounts of unchanged drug in the peripheral circulation and the onset of the conditions such as respiratory inhibition and inhibition of hepatic gluconeogenesis which lead to lactic acidosis. These findings support the current view that lactic acidosis only occurs in the presence of abnormally high plasma levels of phenformin [35, 36] and may explain the predisposition of patients treated with phenformin to lactic acidosis when renal and/or hepatic dysfunction is present.

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