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MODE OF ACTION OF HYPOGLYCEMIC AGENTS—IV

CONTROL OF THE HYPOGLYCEMIC ACTIVITY OF PHENETHYLBIGUANIDE IN RATS AND GUINEA-PIGS*

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Abstract—Phenethylbiguanide (PEBG) is a more active hypoglycemic agent in guinea-pigs than in rats. The rat metabolizes the compound to the hypoglycemically inactive *p*-hydroxyphenethylbiguanide derivative to a greater extent than the guinea-pig. To determine if *para*-hydroxylation is the controlling factor in the differential response of rats and guinea-pigs to phenethylbiguanide, guinea-pigs were treated with 3,4-benzpyrene to induce microsomal hydroxylation. Phenethylbiguanide lowered blood glucose to 73 per cent of control in these guinea-pigs as compared to 45 per cent of control in those receiving only phenethylbiguanide. Rats given phenethylbiguanide and 2,4-dichloro-6-phenylphenoxyethyl-diethylamine HBr (Lilly (18947) to inhibit microsomal hydroxylation had blood glucose levels 65 per cent of control compared to 90 per cent of control for those receiving only phenethylbiguanide. Isolated perfused rat livers produced glucose from lactate at only 46 per cent of the rate of controls when phenethylbiguanide and Lilly 18947 were added to the perfusate as compared to 95 per cent of control when only phenethylbiguanide was added. The presence of Lilly 18947 in the perfusate had no effect on glucose production. It inhibited the *para*-hydroxylation of phenethylbiguanide. In the perfused rat liver system, Lilly 18947 had no effect on the inhibition of the rate of glucose production from lactate by the non-metabolized hypoglycemic biguanide, 1,1-dimethylbiguanide. Also, the *para*-substituted compound, *p*-fluorophenethylbiguanide, was a more effective inhibitor of gluconeogenesis in the perfused system than phenethylbiguanide. The data are consistent with the hypothesis that the hydroxylation of phenethylbiguanide is a major controlling factor in the differential response of rats and guinea-pigs to this drug.

PEBG is a hypoglycemic agent in diabetic humans[1,2] and several species of normal and diabetic laboratory animals.[3,4] In laboratory animals, it has been demonstrated that PEBG inhibits glucose production from common gluconeogenic compounds both *in vivo* and *in vitro*.[5–7] There is, however, a pronounced difference in the degree of

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The abbreviations used are: PEBG, phenethylbiguanide; *p*-hydroxy-PEBG, *p*-hydroxyphenethylbiguanide; *p*-fluoro-PEBG, *p*-fluorophenethylbiguanide; [$^{14}C_{2,4}$]-PEBG, [$^{14}C_{2,4}$]phenethylbiguanide; DMBG, 1,1-dimethylbiguanide; and Lilly 18947, 2,4-dichloro-6-phenylphenoxyethyldiethylamine hydrobromide.

hypoglycemic response to the compound among species.[3,6] For example, the rat exhibits a minimal hypoglycemic response to PEBG whereas the guinea-pig is highly responsive. It is also known that the rat effectively metabolizes PEBG to *p*-hydroxy-PEBG and the glucuronide conjugate of *p*-hydroxy-PEBG and that this metabolism is limited in the guinea-pig.[8–10]

p-Hydroxy-PEBG is not an active hypoglycemic agent. In one report *p*-hydroxy-PEBG was shown to be only about one-half as effective as PEBG as a hypoglycemic agent in the rat.[11] Another investigation has shown *p*-hydroxy-PEBG to be ineffective in lowering the blood sugar in mice under conditions where PEBG is effective.[8] *Para*-hydroxy-PEBG, unlike PEBG, does not inhibit active sugar transport, a phenomenon that is possibly related to PEBG inhibition of intestinal glucose absorption.[12] In the perfused guinea-pig liver, 0·16 mM *p*-hydroxy-PEBG has no effect on the rate of glucose production from fructose under conditions where 0·08 mM PEBG inhibits this rate to 65 per cent of control rate.* The data presented in this report indicate that the metabolism of PEBG to *p*-hydroxy-PEBG is the controlling factor in the differential hypoglycemic response of rats and guinea-pigs to PEBG.

EXPERIMENTAL

Animals. Male Sprague–Dawley rats were maintained on Purina Laboratory Chow *ad lib.* Male guinea-pigs, obtained from Marvin O'Brien, Madison, and Milan C. Mecklenburg, Janesville, Wis., were fed Wayne Guinea-Pig Diet *ad lib.*

Isolated liver perfusions. Male Sprague–Dawley rats, weighing 290–310 g were fasted 24 hr prior to use. The livers were perfused as described previously from this laboratory.[13] The perfusate consisted of 20% (v/v) washed bovine erythrocytes[14] in a Krebs–Ringer bicarbonate solution containing 3% (w/v) fatty acid-free bovine albumin (Research Products Division, Miles Laboratories, Inc.).

Analysis. Blood glucose and perfusate glucose concentrations were determined by the glucose oxidase method (Boehringer–Mannheim Corp.). Tissue samples for biguanide analysis were quick-frozen on tongs cooled in liquid N_2 and were homogenized in 0·9 per cent NaCl. Perfusate aliquots for biguanide analysis and homogenized tissue samples were treated with 4 N *n*-butanol for 30 min at 25°. Neutralized perchloric acid extracts of the butanol-treated samples were used for chromatography. Ascending paper chromatography was carried out at room temperature on Whatman No. 1 paper in *n*-butanol saturated with 3 M aqueous ammonia. In this system PEBG and *p*-hydroxy-PEBG had R_f values of 0·48 and 0·18 respectively. In chromatographed perfusate extracts, biguanide compounds more polar than *p*-hydroxy-PEBG were present in only insignificant amounts; however, in tissue extracts, no clear distinction could be made between *p*-hydroxy-PEBG and more polar radioactive biguanides, as judged by their chromatographic behavior. These compounds together with *p*-hydroxy-PEBG are referred to as polar metabolites of PEBG. Chromatographically separated biguanides were eluted from the paper with water for analysis, or the paper was cut into strips and placed directly into vials for determination of radioactivity. Isotope counting was done with a Packard liquid scintillation spectrometer, model 3310, in Bray's solution[15] for eluted samples or in a toluene-based scintillation fluid[16] for paper strips. Prior to counting in Bray's

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solution, untreated tissue and perfusate samples were solubilized with Packard Hydroxide of Hyamine 10-X or TS-I solubilizer (Research Products International Corp.) and decolorized with benzoyl peroxide. Bile was placed directly in Bray's solution for counting. Concentrations of the biguanides were estimated by the absorbance method described by Beckmann and Hübner[17] and by radioactivity using [$^{14}C_{2,4}$]-PEBG. Estimated by the absorbance method, recovery of added PEBG after extraction and chromatographic separation averaged 46 per cent, and that for *p*-hydroxy-PEBG averaged 61 per cent. Estimation of biguanide concentrations by the two methods gave essentially identical results.

Materials. PEBG hydrochloride and [$^{14}C_{2,4}$]-PEBG hydrochloride were obtained from U. S. V. Pharmaceutical Co. or purchased from Aldrich Chemical Company. *p*-Hydroxy-PEBG hydrochloride was the generous gift of Dr. Arne N. Wick, San Diego State College. Lilly 18947 was supplied by Eli Lilly & Co. and *p*-fluoro-PEBG hydrochloride was a gift from Dr. Jack Mills of the Lilly Research Laboratories. DMBG hydrochloride was purchased from Chemicals Procurement Labs., Inc. L-Lactate was purchased from Schwarz/Mann and sodium pyruvate from Sigma Chemical Co. USP corn oil was obtained from Magnus, Mabee & Reynard, Inc. 3,4-Benzpyrene was purchased from Aldrich Chemical Co. All other compounds used were of reagent grade. All biguanides and sodium pyruvate were placed in solution just prior to use.

RESULTS

Experiments in vivo

Guinea-pigs. Experiments were designed to determine if the hypoglycemic response of guinea-pigs to PEBG could be decreased by pretreating the animals with 3,4-benzpyrene, an inducer of microsomal hydroxylation.[18] A single dose of 3,4-benzpyrene, given 24 hr prior to PEBG injection, was observed to decrease significantly the response of fasted guinea-pigs to the blood glucose lowering effects of PEBG (Table 1). Administration of 3,4-benzpyrene alone had no effect on blood glucose levels.

TABLE 1. EFFECT OF 3,4-BENZPYRENE PRETREATMENT ON THE RESPONSE OF GUINEA-PIGS TO PEBG HYPOGLYCEMIA*

Pretreatment	Injection	Blood glucose† (mg/100 ml)	Blood glucose (% of control)
Corn oil	NaCl	120 ± 8 (5)	100
Corn oil	PEBG	54 ± 7 (4)‡	45
3,4-Benzpyrene	NaCl	122 ± 4 (5) §	102
3,4-Benzpyrene	PEBG	88 ± 17 (5)‖,¶	73

* Male American guinea-pigs (280–380 g) were injected intraperitoneally with either 25 mg/kg body wt of 3,4-benzpyrene in corn oil or corn oil alone, and food was removed. Twenty-four hr later, the fasted animals were injected intraperitoneally with either 20 mg/kg body wt of PEBG in 0·9% NaCl or NaCl alone. Two hr later, blood was collected by decapitation[19] and blood glucose levels were determined as described in Methods.

† The numbers given are the average ± S. D. (number of animals).

‡ $P < 0.01$ vs. corn oil control.

§ $P > 0.05$ vs. corn oil control.

‖ $P < 0.01$ vs. 3,4-benzpyrene control.

¶ $P < 0.01$ vs. corn oil plus PEBG.

Rats. Experiments were designed to determine if the hypoglycemic response of rats to PEBG could be increased by using Lilly 18947, a known inhibitor of hepatic microsomal hydroxylation.[20] Table 2 shows that the simultaneous administration of Lilly 18947 and PEBG was more effective in lowering blood glucose levels than administration of PEBG alone. Administration of Lilly 18947 alone had no effect on blood glucose levels.

Table 2. Effect of Lilly 18947 on the response of rats to PEBG hypoglycemia*

Injection		Blood glucose† (mg/100 ml)	Blood glucose (% of control)
1	2		
NaCl	NaCl	78 ± 5 (3)	100
NaCl	PEBG	70 ± 3 (4) ‡	90
Lilly 18947	NaCl	74 ± 4 (4)§	95
Lilly 18947	PEBG	51 ± 14 (4)‖,¶	65

* Male Sprague–Dawley rats (130–140 g) were fasted for 24 hr prior to injection. Each rat was injected intraperitoneally twice in rapid succession, first with the compound listed in injection 1 column and then with the compound listed in injection 2 column. Two hr later, blood was collected by decapitation[19] and the blood glucose was determined as described in Methods. The injection doses were PEBG, 100 mg/kg body wt in 0·9% NaCl, and Lilly 18947, 20 mg/kg body wt in 0·9% NaCl.

† The numbers given are average ± S. D. (number of animals).

‡ $P = 0{\cdot}05$ vs. NaCl control.

§ $P > 0{\cdot}05$ vs. NaCl control.

‖ $P < 0{\cdot}05$ vs. Lilly 18947 plus NaCl.

¶ $P < 0{\cdot}05$ vs. NaCl plus PEBG.

Isolated, perfused rat liver experiments

PEBG and DMBG dose response. Figure 1 shows the effect of increasing concentrations of PEBG and DMBG on the inhibition of glucose production from a mixture of 10 mM L-lactate and 1 mM pyruvate in perfused rat liver. It is apparent that, at sufficiently high concentrations, both PEBG (1·24 mM) and DMBG (2·48 mM) are effective in reducing the rate of glucose production from lactate. It has been reported that PEBG increases glucose production in man.[21] It can be seen in Fig. 1 that there may be a small stimulation of glucose production at low concentrations of both PEBG and DMBG. Investigation of this phenomenon has not been pursued in the present system.

Effect of Lilly 18947 on PEBG inhibition of gluconeogenesis. Table 3 illustrates that PEBG at a concentration (0·62 mM) that does not inhibit gluconeogenesis in perfused rat liver will inhibit glucose production from lactate plus pyruvate when added in combination with Lilly 18947. Lilly 18947 itself has no effect on glucose production in the perfused system.

Effect of para-substitution on PEBG. As shown in Table 3, *p*-fluoro-PEBG at a concentration of 0·62 mM inhibits the rate of glucose production to the same extent as either 1·24 mM PEBG or 0·62 mM PEBG plus Lilly 18947. In a single paired experiment the polar metabolite of PEBG, *p*-hydroxy-PEBG, had no effect on the rate of glucose production in the perfused system alone or in combination with Lilly 18947. The rates of glucose production from 10 mM L-lactate plus 1 mM pyruvate

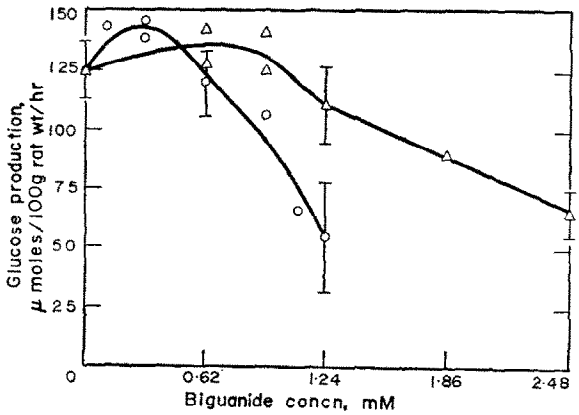

FIG. 1. Dose–response curves for PEBG (○) and DMBG (△) in isolated perfused rat liver. The vertical bars represent standard deviations of three or more perfusions. The perfusions were conducted as follows. The isolated livers were first perfused with 20 ml of perfusate and the perfusate was discarded. The livers were then connected to a recirculating system containing 100 ml of perfusate and either PEBG or DMBG was added to give the concentrations indicated. Sixty min after the addition of biguanide, substrate was added (final concentration 10 mM L-lactate plus 1 mM pyruvate) and the perfusion continued for an additional 30 min. Glucose concentrations were determined at timed intervals during the perfusion. Glucose production was linear for the 30 min after substrate addition and the rates of glucose production shown were calculated from the glucose appearing in the perfusate over this 30-min period.

were 108 μmoles/100 g rat wt/hr in the presence of 0·62 mM *p*-hydroxy-PEBG and 112 μmoles/100 g rat wt/hr with 0·62 mM *p*-hydroxy-PEBG plus 0·18 mM Lilly 18947.

Effect of Lilly 18947 on DMBG inhibition of gluconeogenesis. The hypoglycemic biguanide DMBG is not metabolized by the rat,[9] and its effect on gluconeogenesis from lactate plus pyruvate is not affected by the presence of Lilly 18947 (Table 3).

TABLE 3. EFFECT OF BIGUANIDES ALONE AND IN COMBINATION WITH LILLY 18947 ON GLUCOSE PRODUCTION IN PERFUSED RAT LIVER*

Additions†	Glucose produced‡ (μmoles/100 g body wt/hr)
None	125 ± 12 (17)
Lilly 18947	113 ± 9 (3)§
1·24 mM PEBG	54 ± 22 (11)‖
0·62 mM PEBG	119 ± 13 (7)§
0·62 mM PEBG + Lilly 18947	56 ± 13 (12)‖
0·62 mM *p*-fluoro-PEBG	62 ± 13 (4)‖
2·48 mM DMBG	66 ± 10 (3)‖
1·24 mM DMBG	111 ± 18 (3)§
1·24 mM DMBG + Lilly 18947	120 ± 4 (3)§

* Perfusion conditions are the same as described in Fig. 1.

† Additions were made at the start of each perfusion. Concentrations given are those in the perfusate at the time of addition. Lilly 18947 was added to give a concentration of 0·18 mM.

‡ The numbers given are average ± S. D. (number of perfusions).

§ $P > 0.05$ vs. control perfusion.

‖ $P < 0.01$ vs. control perfusion.

Distribution of PEBG and its metabolites in the perfusion system. The concentration of PEBG, *p*-hydroxy-PEBG and other polar metabolites of PEBG in the liver and the perfusate after 90 min of perfusion are shown in Table 4. All of the radioactivity added as [$^{14}C_{2,4}$]-PEBG was accounted for at the end of the perfusion in the perfusate, liver and bile. No radioactivity was found in the CO_2. In control perfusions 5–13 per cent of the radioactivity added as [$^{14}C_{2,4}$]-PEBG was recovered in the bile compared to 1 per cent when Lilly 18947 was also present. It can be seen from Table 4 that the presence of Lilly 18947 decreases the total amount of biguanide present in the liver and increases the amount present in the perfusate. It is especially important to note that the ratios of PEBG to PEBG metabolites are dramatically influenced by Lilly 18947. In control experiments, the concentration of the polar metabolites of PEBG is about three times greater than that of PEBG in the perfusate and approximately seven times greater in the liver. When the microsomal hydroxylation inhibitor is present, however, the ratios are reversed and the concentration of PEBG is about ten times greater than its metabolites in the perfusate and approximately six times greater in the liver.

Table 4. Concentrations of PEBG and PEBG metabolites in perfused rat liver and perfusate*

Additions	Perfusate concn†		Liver concn			
			PEBG (μmoles/g wet wt tissue)		Polar metabolites‡ (μmoles/g wet wt tissue)	
	PEBG (mM)	*p*-hydroxy-PEBG (mM)	Exp. 1	Exp. 2	Exp. 1	Exp. 2
0·62 mM PEBG	0·04 ± 0·03 (4)	0·13 ± 0·03 (4)	0·47	1·22	4·26	4·50
0·62 mM PEBG + 0·18 mM Lilly 18947	0·31 ± 0·06 (4)§	0·03 ± 0·03 (4)§	3·57	3·43	0·46	0·97

* The perfusion conditions are the same as described for Fig. 1 and Table 3. After 90 min of perfusion, liver tissue for analysis was quick-frozen in tongs cooled in liquid N_2, and samples of perfusate, bile and trapped CO_2 were taken for analysis. The specific radioactivity of the [$^{14}C_{2,4}$]-PEBG used was $8{\cdot}0 \times 10^6$ dis/min/μmole.

† Numbers given are average ± S. D. (number of perfusions). For each set of perfusion conditions, the concentrations of the compounds were estimated by radioactivity for two of the perfusions and by the absorbance method for two of the perfusions. See Methods.

‡ "Polar metabolites" represent the combined concentration of *p*-hydroxy-PEBG plus radioactive biguanide compounds more polar than *p*-hydroxy-PEBG. See Methods.

§ $P < 0{\cdot}01$ vs. 0·62 mM PEBG addition.

DISCUSSION

As discussed earlier in this report, the guinea-pig is very responsive to the hypoglycemic action of PEBG but exhibits only limited ability to hydroxylate PEBG. In contrast, the rat, which actively metabolizes PEBG to hypoglycemically inactive *p*-hydroxy-PEBG and the subsequent glucuronide conjugate, responds to PEBG only at elevated doses. In view of these facts it seemed reasonable to postulate that hydroxylation of PEBG to *p*-hydroxy-PEBG is a major factor responsible for the differential hypoglycemic response of rats and guinea-pigs to this drug. The evidence presented in the present report is consistent with this explanation.

The observation that fasted guinea-pigs were made less responsive to the hypoglycemic action of PEBG when pretreated with an inducer of microsomal hydroxylation (Table 1) is interpreted to indicate that under these conditions induction of the metabolism of PEBG to *p*-hydroxy-PEBG decreased the effective concentration of PEBG to levels below that of the non-pretreated animals.

The observation that fasted rats (Table 2) and perfused livers from fasted rats (Table 3) are more responsive to PEBG in the presence of Lilly 18947 is interpreted to indicate that in the presence of the microsomal hydroxylation inhibitor a higher concentration of PEBG is maintained than in its absence. The results shown in Table 4 clearly indicate that Lilly 18947 very effectively inhibits the hydroxylation of PEBG.

In order to rule out the possibility of an additive effect of biguanide plus hydroxylation inhibitor directly on the process of glucose production, the experiments were conducted with the hypolgycemic biguanide, DMBG. Since DMBG is excreted unchanged when administered to rats,[9] its effectiveness as an inhibitor of gluconeogenesis in perfused rat liver should not be altered by an inhibitor of microsomal drug-metabolizing enzymes. Table 3 indicates that Lilly 18947 indeed has no effect on DMBG inhibition of the rate of glucose production when tested under conditions comparable to those where Lilly 18947 is effective in increasing PEBG inhibition of gluconeogenesis (see Fig. 1 and Table 3).

Additional evidence that the inhibition of PEBG hydroxylation alone results in increased effectiveness of PEBG in the rat is seen in the experiments with *p*-fluoro-PEBG. Since it is known that microsomal *p*-hydroxylation of aromatic compounds is inhibited by *p*-halo-substitution,[22] the observation that it takes only $\frac{1}{2}$ the concentration of *p*-fluoro-PEBG to be as effective as a given concentration of PEBG in inhibiting gluconeogenesis is interpreted to indicate that *p*-fluoro-PEBG is metabolized at a much slower rate than PEBG (Table 3). Hence, *p*-fluoro-PEBG remains at an inhibitory concentration under conditions where the concentration of unsubstituted PEBG is decreased by hydroxylation to ineffective levels.

The experimental results presented and discussed in this paper are consistent with the hypothesis that the conversion of PEBG to *p*-hydroxy-PEBG is the controlling factor in the differential response of rats and guinea-pigs to the hypoglycemic action of PEBG.

In man, PEBG inhibits gluconeogenesis induced by fasting.[22] It is possible that inhibition of hepatic glucose output by PEBG is an important factor in PEBG-induced blood glucose lowering in diabetic humans. The experimental conditions established in the present studies have allowed the authors to investigate effectively the inhibitory mode of action of PEBG on gluconeogenesis in the isolated perfused rat liver.[24]

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