Comparative Effects of Methylglyoxal-Bis (Guanylhydrazone), Phenformin, and Insulin upon the Metabolism of Glucose and Acetate by Rat Epididymal Fat Pads *in Vitro*

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SUMMARY

Methylglyoxal-bis (guanylhydrazone) (CH_3-G) increased the total uptake of glucose by epididymal fat pads of rats *in vitro* under aerobic, but not anaerobic, conditions. The following effects of CH_3-G upon glycolytic pathways of adipose cells were found: (1) increased incorporation of radioactivity from glucose-^{14}C into CO_2 and lipid; (2) decreased incorporation of radioactivity from glucose-^{14}C into glycogen; (3) increased incorporation of radioactivity from glucose-^{14}C into released lactate; (4) decreased incorporation of radioactivity from acetate-1-^{14}C into CO_2 and lipid.

Under the same experimental conditions, CH_3-G had no effect upon the rate of incorporation of radioactivity from glucose-^{14}C into CO_2 and lipid by epididymal fat pads of mice.

The effects of phenformin upon glycolytic pathways of epididymal fat pads of rats *in vitro* were similar in some respects to CH_3-G, but differed from the later in depressing incorporation of radioactivity from glucose-^{14}C into CO_2 and lipid. In addition, total glucose uptake was depressed by phenformin at the concentrations used.

The effects of CH_3-G and phenformin differed from those of insulin with regard to the incorporation of radioactivity from glucose-^{14}C into glycogen and incorporation from acetate-1-^{14}C into CO_2 and lipid. In addition, the effects of insulin were abolished in the presence of nethalide, whereas the effects of CH_3-G were not. Insulin stimulated total uptake of glucose by both rat and mouse epididymal fat pads *in vitro*, but CH_3-G had no effect upon the latter.

INTRODUCTION

Methylglyoxal-bis (guanylhydrazone) (CH_3-G) (Fig. 1) possesses antileukemic (1, 2) and trypanocidal activity (3), but is associated with toxic hypoglycemic effects in various species including man, rabbit, and rat (1, 2), but not the mouse (3). Precisely how this guanidine derivative causes the development of hypoglycemia in susceptible species remains uncertain. However, it has been suggested that the ability of CH_3-G to inhibit oxidative phosphorylation and glycogen synthesis in liver cells may be largely responsible for initiation of the effects observed (2).

This report describes observed effects of CH_3-G upon the carbohydrate metabolism of epididymal adipose tissues of the rat and mouse *in vitro*, tissues known to respond consistently to the action of insulin. In addition, the response of these tissues to CH_3-G is compared to that of phenformin, a clinically useful hypoglycemia-inducing guanidine derivative (Fig. 1).

MATERIALS AND METHODS

Albino male Wistar rats weighing between 150 and 170 g and Ha/ICR male albino mice between 19 and 22 g maintained on Purina Laboratory Chow and water ad

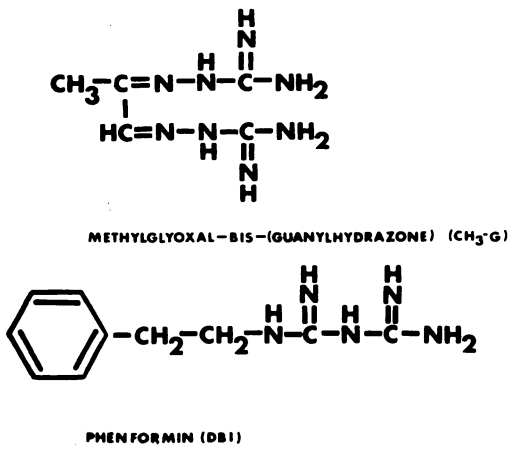

FIG. 1. *Structures of two drugs that cause hypoglycemia*

libitum were used in this study. All radioactive substances were obtained from the New England Nuclear Corporation, Boston, Massachusetts. CH_3-G was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Phenformin hydrochloride was obtained from the U.S. Vitamin and Pharmaceutical Corporation through the courtesy of Dr. H. S. Sadow. Glucagon-free insulin (Lot No. PJ-4609) was obtained from the Lilly Research Laboratories, Indianapolis, Indiana, through the courtesy of Dr. O. K. Behrens.

The animals were sacrificed by cervical dislocation and the fat pads were quickly removed, weighed, and placed in prepared incubation flasks containing 5 ml of Krebs-Ringer bicarbonate buffer (4). Labeled glucose and acetate were present in a concentration of 12 mmoles per liter with a specific activity of 1 mC per 12 mmoles. Incubations were carried out for 2 hr at 37° in a Dubnoff shaking incubator (90 oscillations per minute).

Unless otherwise specified, determination of radioactivity was by liquid scintillation counting (Packard Tri-Carb Liquid Scintillation Spectrometer, model 3214) of 0.2-ml aliquots dissolved in a 10 ml toluene:absolute ethanol (14:5) mixture containing 0.23% of 2,5-diphenyloxazole (PPO) (5). Counting efficiency of ^{14}C was 87%. Samples were checked for quenching by addition of an internal standard.

Liberated $^{14}CO_2$ was absorbed in 0.5 ml of Hyamine hydroxide 10 × (Packard) in a removable center well of the incubation flask. At the completion of incubation, 1 ml of 4 N HCl was added to the buffer solution following removal of the fat pads, and the flasks were allowed to stand for an additional 30 min to ensure complete CO_2 absorption. The vessel containing the Hyamine and absorbed ^{14}C was then placed in a counting vial, and 10 ml of the above scintillation mixture was added followed by a thorough shaking and removal of the vessel prior to counting.

Lactate released into the medium was separated by column chromatography using a Dowex-acetate resin prepared according to the directions of Kunin and Krane (6). Lactate was eluted with 3 N acetic acid from the ion-exchange column (0.8 × 5 cm) and the radioactivity of an aliquot was determined.

The lipid fraction was extracted by chloroform–methanol (2:1) followed by a salt wash, a technique originally described by Folch *et al.* (7). The specific procedure used here for determination of ^{14}C-lipid was essentially that reported by Fain *et al.* (8), except that isotonic saline was used instead of water to remove nonlipid radioactive substances.

Free glycogen was precipitated with ethanol from the supernatant following homogenization and centrifugation of the fat-free residue (obtained by drying the chloroform–methanol extracted tissue) with 10% trichloroacetic acid. In some experiments the fat-free residue was dissolved in hot 30% KOH and glycogen was then precipitated with ethanol. Nonradioactive glycogen was added to the supernatant (or KOH hydrolyzate) as a carrier to assist precipitation by ethanol. The extracted glycogen was then washed with absolute ethanol and centrifuged, followed by removal of the ethanol and hydrolysis in 2 ml of 2 N H_2SO_4 (boiling water bath, 1 hr). After cooling, appropriate aliquots were removed and counted.

All experiments were performed using paired fat pads, one pad serving as the control and the other for drug treatment.

TABLE 1

Comparative effects of CH_3-G, phenformin, and insulin upon incorporation of radioactivity derived from glucose-U-^{14}C into CO_2, lipid, glycogen and released lactate by rat epididymal fat pads

Experimental conditions and methods used in obtaining the fractions are described under Materials and Methods Radioactivity is expressed as CPM/g of tissue wet weight. All incubation flasks were gassed with 100% oxygen prior to incubation except those values designated by (N_2); these were gassed for 5 minutes with 100% nitrogen prior to incubation.

CPM ^{14}C INCORPORATED PER g TISSUE

	CO_2		LIPID		GLYCOGEN		LACTATE		TOTAL
	MEAN	DIFFERENCE[a] FROM CONTROL	MEAN	DIFFERENCE[a] FROM CONTROL	MEAN	DIFFERENCE[a] FROM CONTROL	MEAN	DIFFERENCE[a] FROM CONTROL	MEAN
CONTROL	66,100 (N_2;11,000)		107,500 (N_2;55,100)		2,400		20,400		196,400
10^{-4}M CH_3-G	92,700 (N_2;10,700)	+26,600±6,200 (N_2;-300±200)[b]	132,800 (N_2;53,500)	+25,300±6,500 (N_2;-1,600±800)[b]	1,900	-450±100	28,000	+7,600±1,600	255,400
$3x10^{-4}$M CH_3-G	143,800	+77,700±25,600	234,100	+126,600±24,500					
10^{-4}M PHENFORMIN	42,300	-23,800±5,700	50,000	-57,500±7,500	1,200	-520±60	29,200	+8,800±1,600	122,700
1mU/ml INSULIN	313,100	+247,000±11,500	390,200	+282,700±14,100	7,600	+5,200±1,100	25,000	+4,600±1,900	735,900

[a]Mean ±standard error of the difference between the control and drug treated. All mean values are significantly ($p < .05$) different from control values except those designated by b.

[b]Designates that mean value is not significantly different from control value. $P > .05$ in six replications.

Since all experiments were paired experiments, the statistical analysis of the differences between control and drug treated indicates the statistical significance of the drug effect (9).

Glucose uptake was not determined directly, due to the difficulty in accurately measuring small changes in glucose concentration in the medium (10). Instead glucose uptake was approximated by calculating the sum of radioactivity incorporated into CO_2, lipid, glycogen, and released lactate. These fractions account for nearly all the total glucose uptake (11).

RESULTS

CH_3-G increased the incorporation of radioactivity derived from glucose-^{14}C into CO_2 and lipid by the rat epididymal fat pad (Table 1). Such stimulation was not observed when mouse epididymal fat pads were used (Table 2), even when CH_3-G was present in concentrations as high as 4×10^{-3} M. By contrast, insulin stimulated the incorporation of radioactivity derived from glucose-^{14}C into CO_2 and lipid by both the rat (Table 1) and mouse fat pads (Table 2).

The above incubations were performed under 100% oxygen. When identical incubations were performed under relatively anoxic conditions (the incubation flasks were gassed with nitrogen for 5 min), the ability of CH_3-G to stimulate the incorporation of radioactivity derived from glucose-^{14}C into CO_2 and lipid was abolished (Table 1).

Phenformin (10^{-4} M) inhibited the incorporation of radioactivity derived from glucose-^{14}C into both CO_2 and lipid (Table 1), in contrast to the stimulatory potency of an equimolar concentration of CH_3-G. Such inhibition by phenformin was previously reported by Wick *et al.* (12).

Both CH_3-G and phenformin significantly reduced the incorporation of radioactivity from glucose-^{14}C into glycogen by the rat fat pad, while insulin stimulated such incorporation (Table 1).

CH_3-G, phenformin, and insulin all stimulated incorporation of radioactivity from glucose-^{14}C into released lactate (Table 1). Such stimulation by phenformin has been previously observed in the rat diaphragm preparation by Williams *et al.* (13; see also 14), and Winegrad and Renold (9) reported that insulin stimulated lactate formation by the rat fat pad *in vitro*.

Total glucose uptake, approximated by calculating the sum of radioactivity incorporated into CO_2 and lipid (Table 1), glycogen (Table 1) and released lactate (Table 1) was increased by CH_3-G.

As seen in Table 3, nethalide (10^{-3} M) abolished the insulin-mediated stimulation of incorporation of radioactivity derived from glucose-^{14}C into CO_2 and lipid of the

TABLE 2

Comparison of effects of CH_3-G and insulin upon radioactivity (cpm) incorporated into CO_2 and lipid derived from glucose-U-^{14}C by mouse epididymal fat pads in vitro

Experimental conditions were identical with those used in Table 1 except that mouse epididymal fat pads were used instead of rat epididymal fat pads.

Sample	Radioactivity (cpm) incorporated into CO_2		Radioactivity (cpm) incorporated into lipid	
	Mean (cpm/g)	Difference from control[a]	Mean (cpm/g)	Difference from control[a]
Control	50,700	—	101,400	—
4×10^{-3} M CH_3-G	46,700	−6,000 ± 3,200[b]	104,500	+3,100 ± 2,000[b]
Insulin, 1 mU/ml	154,500	+103,800 ± 26,000	249,500	+148,100 ± 40,000

[a] Mean ± standard error of the difference between the control and drug treated. All mean values are significantly ($p < 0.05$) different from control values except those designated by *b*.

[b] The mean value is not significantly different from control value. $P > 0.05$ in six replications.

TABLE 3

Comparison of effects of CH_3-G and insulin upon radioactivity (cpm) incorporated into CO_2 and lipid derived from glucose-U-^{14}C by rat epididymal fat pads in vitro in the presence of nethalide (10^{-3} M)

Experimental conditions were identical with those used in Table 1 except that nethalide (10^{-3} M) was present where indicated below.

Sample	Radioactivity (cpm) incorporated into CO_2		Radioactivity (cpm) incorporated into lipid	
	Mean (cpm/g)	Difference from control[a]	Mean (cpm/g)	Difference from control[a]
Control + nethalide	45,000	—	75,000	—
10^{-4} M CH_3-G + nethalide	50,300	+5,300 ± 1,000	83,400	+8,400 ± 3,200
Insulin, 1 mU/ml	236,700	+191,700 ± 16,100	350,000	+266,600 ± 32,000
Insulin, 1 mU/ml + nethalide	44,400	−600 ± 300[b]	70,100	−4,900 ± 2,100[b]

[a] Mean ± standard error of the difference between the control and drug treated. All mean values are significantly ($p < 0.05$) different from control values except those designated by *b*.

[b] The mean value is not significantly different from control. $P > 0.05$ in six replications.

rat epididymal fat pad, a finding which is consistent with that reported by Bewsher *et al.* (14). In the presence of nethalide (10^{-3} M), the ability of CH_3-G to stimulate incorporation of radioactivity derived from glucose-^{14}C into CO_2 and lipid was reduced (Table 3).

Since CH_3-G stimulated the incorporation of radioactivity derived from glucose-^{14}C into CO_2 and lipid, while equimolar amounts of phenformin inhibited such incorporation, it was possible that this difference might reflect different effects of CH_3-G and phenformin upon the tricarboxylic acid cycle. Consequently, the effects of CH_3-G, phenformin and insulin upon incorporation of radioactivity from acetate-^{14}C into CO_2 and lipid were compared both in the presence and absence of nonradioactive glucose (Tables 4 and 5).

TABLE 4

Comparison of the effects of CH_3-G, phenformin, and insulin upon radioactivity (cpm) incorporated into CO_2 and lipid derived from acetate-1-^{14}C in the absence of glucose

Paired rat epididymal fat pads were used in these experiments. Incubations were carried out for 2 hours with 5 ml of Krebs-Ringer bicarbonate buffer present and acetate-1-^{14}C at a concentration of 12 mmoles per liter with a specific activity of 1 mC per 12 mmoles. Radioactivity is expressed as cpm/g of tissue wet weight. CO_2 and lipid fractions are obtained as described in the text.

Sample	Radioactivity (cpm) incorporated into CO_2		Radioactivity (cpm) incorporated into lipid	
	Mean (cpm/g)	Difference from control[a]	Mean (cpm/g)	Difference from control[a]
Control	56,400	—	17,000	—
10^{-4} M CH_3-G	41,000	−15,400 ± 4,100	10,800	−6,200 ± 2,000
10^{-4} M Phenformin	36,100	−20,300 ± 6,000	12,400	−4,600 ± 3,000[b]
Insulin, 1 mU/ml	62,600	+6,200 ± 1,000	25,200	+8,200 ± 5,000[b]

[a] Mean ± standard error of the difference between the control and drug treated. All mean values are significantly ($p < 0.05$) different from control values except those designated by *b*.

[b] The mean value is not significantly different from control value. $P > 0.05$ in six replications.

TABLE 5

Comparison of the effects of CH_3-G, phenformin, and insulin upon radioactivity (cpm) incorporated into CO_2 and lipid derived from acetate-1-^{14}C in the presence of nonradioactive glucose

Experimental conditions were identical with those described in Table 4 except that nonradioactive glucose was present in the incubation solution in a concentration of 12 mmoles per liter.

Sample	Radioactivity (cpm) incorporated into CO_2		Radioactivity (cpm) incorporated into lipid	
	Mean (cpm/g)	Difference from control[a]	Mean (cpm/g)	Difference from control[a]
Control	43,700	—	60,000	—
10^{-4} M CH_3-G	40,700	−3,000 ± 6,900[b]	30,400	−29,600 ± 7,400
10^{-4} M Phenformin	43,300	−400 ± 600[b]	30,300	−29,700 ± 2,700
Insulin, 1 mU/ml	41,600	−2,100 ± 2,000[b]	269,000	+209,000 ± 43,000

[a] Mean ± standard error of the difference between the control and drug treated. All mean values are significantly ($p < 0.05$) different from control values except those designated by *b*.

[b] Designates that the mean value is not significantly different from control value. $P > 0.05$ in six replications.

In the absence of glucose (Table 4), CH_3-G and phenformin depressed incorporation of radioactivity from acetate-1-^{14}C into CO_2 while insulin stimulated this incorporation. CH_3-G also depressed incorporation of radioactivity from acetate-1-^{14}C into lipid while both phenformin and insulin were without significant effect.

In the presence of glucose (Table 5), CH_3-G, phenformin, and insulin were without effect on the incorporation of radioactivity from acetate-1-^{14}C into CO_2. CH_3-G and phenformin, however, depressed the incorporation of radioactivity from acetate-1-^{14}C into lipid, whereas insulin greatly stimulated this incorporation.

DISCUSSION

In several species including the rat and man, CH_3-G induces an initial hyperglycemic response which is followed by a profound irreversible hypoglycemia associated with depletion of liver glycogen and occasional hepatic necrosis (2). It has been suggested that the ability of CH_3-G to inhibit liver glycogen synthesis and hepatocellular oxidative phosphorylation might be responsible in part for the hypoglycemia observed. Results reported in this paper show that CH_3-G can stimulate peripheral uptake of glucose by the rat epididymal fat pad *in vitro*. If this and other peripheral tissues respond in the same way *in vivo*, such increased glucose uptake caused by CH_3-G may contribute to the observed hypoglycemia.

The finding that the stimulatory effect of CH_3-G upon glucose uptake by the rat fat pad was abolished under relatively anoxic conditions (Table 1), and that this agent inhibited incorporation of radioactivity from acetate-1-^{14}C into CO_2 under aerobic conditions (Table 4), suggests that CH_3-G might be interfering with oxidative metabolism. One possibility would be the inhibition of oxidative phosphorylation, as has been found in rat liver mitochondria (2). Such inhibition could cause a compensatory increase in glucose uptake and phosphorylative glycolysis via the Embden-Meyerhof pathway. That CH_3-G depresses the total functional capacity of the tricarboxylic acid cycle of adipose cells is suggested by its ability to increase the amount of released lactate. It is interesting that an increased blood lactate concentration was reported following administration of CH_3-G to the rabbit (2).

The ability of CH_3-G to depress incorporation of radioactivity from glucose-^{14}C

into glycogen by the rat fat pad *in vitro* could also be responsible for its observed effects upon hepatic glycogen levels *in vivo*. In the CH_3-G-treated hypoglycemic rabbit, for example, the liver glycogen concentration is sometimes as low as 0.02% although the muscle glycogen is not changed (2). The decreased glycogen levels in the rat epididymal fat pad may result from either a blockage of liver glycogen synthesis, increased glycogenolysis, or increased channeling of glucose into the glycolytic pathway.

The stimulation by CH_3-G of incorporation of radioactivity from glucose-^{14}C into CO_2 and lipid is of considerable interest. Since the incorporation of radioactivity from acetate-1-^{14}C into both CO_2 and lipid was depressed, the increased incorporation from glucose-^{14}C into lipid probably occurred at some point in glycolysis before the production of acetate. The increased incorporation into CO_2 may be derived from increased pentose-shunt activity and/or increased pyruvate oxidation (11). The observed increased incorporation into lipid could also be related to increased pentose pathway activity, with concomitant increased production of reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$), required for lipid synthesis (15). Alternatively, increased incorporation of radioactivity into lipid could also result from increased glycerol production from glucose-^{14}C.

The mode of action of the clinically useful oral hypoglycemic agent phenformin remains uncertain. Phenformin is known to produce a hypoglycemia in alloxanized rats (16) and in the diabetic patient. In normal human subjects this drug causes an increase in body glucose turnover, but hypoglycemia is not produced (17).

Effects of phenformin that have been reported using mainly muscle and liver slices *in vitro* include an increase in glucose uptake (18), decrease in glycogen stores (19), inhibition of various tricarboxylic acid cycle enzymes (12, 19), inhibition of glucose oxidation (12), stimulation of CO_2 production (20), and increased production of lactate (18).

Various hypotheses have been advanced to explain the hypoglycemic action of phenformin. Williams *et al.* (13) proposed that it may be due in part to the ability of the drug to increase anaerobic glycolysis by peripheral tissues. Daweke and Bach (21) have suggested that a biguanide, N_1-*n*-butylbiguanide (structurally very similar to phenformin), exerts an "insulin-sparing effect." Wick *et al.* (12) have presented evidence suggesting that phenformin inhibits the electron transport system associated with succinic oxidase, and they proposed that such action in hepatic cells could lead to increased glycolysis and lactate formation, with subsequent lowering of blood sugar in the diabetic patient.

On the other hand, Ungar *et al.* (19) found that phenformin inhibited oxidative phosphorylation by mitochondrial preparations, but doubted that this effect was related to the hypoglycemia observed *in vivo*. They found no correlation between activity *in vitro* and hypoglycemia *in vivo* in a series of biguanide compounds.

Our results indicate that the activity of phenformin (10^{-4} M) *in vitro* is inconsistent with its activity *in vivo* if other peripheral tissues respond in a manner similar to the rat fat pad. In fact, glucose uptake by the fat pad *in vitro* depressed in the presence of phenformin.

The effects of CH_3-G seem similar in many respects to those of phenformin at equimolar concentrations. Both drugs depressed incorporation of radioactivity into glycogen, stimulated incorporation into released lactate and had similar effects on acetate metabolism in the presence and absence of nonradioactive glucose. The opposite effects of CH_3-G and phenformin upon incorporation of radioactivity from glucose-^{14}C into CO_2 and lipid is of considerable interest. The ability of phenformin to inhibit incorporation of radioactivity from glucose-^{14}C into CO_2 and lipid seems inconsistent with the hypoglycemic effect of this drug *in vivo*. It is possible that the effects of phenformin *in vivo* are not due to the drug itself, but rather to one of its biotransformation products (22). This remains to be determined. The apparently

anomalous behavior of phenformin *in vitro* might also be due to concentration differences; for example, it was found by Daweke and Bach (21) that N_1-*n*-butylbiguanide inhibits glucose oxidation at concentrations above 1×10^{-4} M; at lower concentrations (6×10^{-6} M to 3×10^{-5} M), and in the presence of human serum, it increases glucose oxidation.

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