The Mechanism of Action of Hypoglycin on Long-Chain Fatty Acid Oxidation

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SUMMARY

Intravenous administration of hypoglycin (L-α-amino-β-methylenecyclopropanepropionic acid) to mice resulted in hypoglycemia, which was preceded by a decrease in palmitate oxidation by myocardial homogenates from treated animals. The homogenates from treated animals carried out normal rates of glucose and hexanoate oxidation, but demonstrated depressed levels of palmityl-CoA:carnitine acyltransferase activity. The addition of carnitine to the myocardial homogenates restored both the depressed palmitate oxidation and palmityl-CoA:carnitine acyltransferase activity to normal levels. The administration of carnitine to hypoglycin-treated animals prevented the decreases in myocardial palmitate oxidation, palmityl-CoA:carnitine acyltransferase activity, and the hypoglycemia.

INTRODUCTION

"Vomiting sickness" is a disease which occurs in Jamaica and has been associated with the ingestion of the unripe fruit of the tropical plant *Blighia sapida* (ackee fruit) (1). The illness is characterized by pernicious vomiting and hypoglycemic convulsions and coma (2). Hypoglycin (L-α-amino-β-methylenecyclopropanepropionic acid) an amino acid isolated from the ackee fruit has been shown to produce the toxic illness in laboratory animals (3–5). Studies from the laboratories of Chen (5), De Renzo (6, 7), and Holt (8) suggested that hypoglycin impaired the utilization of lipids. Holt and Benedict ascertained that in the intact rat hypoglycin decreased the oxidation of palmitate-1-^{14}C, whereas that of octanoate-1-^{14}C was unchanged (9). Holt, Holt, and Böhm found that in the hypoglycin-treated rat a shift in substrate utilization occurred which resulted in a decreased use of fatty acids and an augmented use of carbohydrate (10). These investigators postulated that the hypoglycemic effect of the toxin was the result of a relative increase of glucose utilization in face of an impairment of fatty acid oxidation.

The effects of hypoglycin are not demonstrable *in vitro;* this led to the suggestion that a metabolite of hypoglycin was the active hypoglycemic compound (11). Holt and his co-workers recently found that enzyme systems in the liver convert hypoglycin to methylenecyclopropanepyruvic acid via transamination, and subsequently to methylenecyclopropaneacetic acid via oxidative decarboxylation (12, 13). It was shown that this degradation product was the active compound, which could effect a depression of long-chain fatty acid oxidation and acetoacetate formation in liver homogenates and mitochondria (10, 13).

The depressive effect of hypoglycin on palmitate oxidation and its failure to affect octanoate oxidation suggested that the toxin could be inhibiting the carnitine-dependent oxidation of long-chain fatty acids. The role of carnitine in long-chain fatty acid oxidation has been attributed to the formation of long-chain fatty acylcarnitines, which in contrast to the acyl-CoA derivatives, can penetrate to mitochondrial sites of fatty

acid oxidation (14). Acylcarnitine formation from acyl-CoA is catalyzed by the long-chain acyl-CoA:carnitine acyltransferase (15). Shepherd *et al.* have shown that this is the rate-limiting enzyme in long-chain fatty acid oxidation (16).

In this communication data are presented which show that the administration of hypoglycin to mice resulted in a fall in palmitate oxidation by myocardial homogenates, which preceded a decrease in plasma glucose. The depressed fatty acid oxidation rate was associated with a decrease in myocardial palmityl-CoA:carnitine acyltransferase activity. The addition of (—)-carnitine to myocardial homogenates of hypoglycin-treated mice restored both fatty acid oxidation rates and palmityl-CoA:carnitine acyltransferase activity to normal levels. The administration of (—)-carnitine to hypoglycin-treated mice prevented the depression of palmitate oxidation and palmityl-CoA:carnitine acyltransferase activity, and the hypoglycemia.

METHODS

White male mice weighing 20–30 g were used. All animals were offered a standard diet consisting of 50% Purina rabbit chow and 50% oats. Food was withdrawn from the animals 18 hr prior to and during the course of the experimental procedures. The treated mice received 15 mg of hypoglycin in 0.5 ml normal saline by tail vein, whereas the control animals received only saline. At the end of the desired period of time (90 min except where indicated) the animals were killed by a blow on the head. The entire heart was immediately removed, the great vessels were trimmed off at their origin, and the cardiac cavities were opened and freed of blood. The hearts were homogenized in 5.0 ml of calcium-free Krebs-Ringer phosphate buffer, pH 7.4 for use in assays. Protein concentration was measured by the biuret method (17). After completion of the biuret reaction the solution was filtered through a layer of Celite in a Büchner-type funnel to remove lipids which produce turbidity of the solution. The results obtained by this procedure agreed with those obtained by Kjeldahl nitrogen determination (18).

The concentration of free fatty acids (FFA) in the myocardium was determined by the method of Dole as modified by Trout *et al.* (19).

Assays of palmitate, hexanoate, and glucase oxidations were carried out as previously described (20). Since the myocardial concentrations of FFA in the treated and control animals were not the same, the endogenous pools of FFA in the hearts were ascertained before the addition of radioactive palmitate. By this means the specific activities of the substrate pools were determined at the beginning of the incubation period. In the treated group the FFA were significantly higher (mean 2.9 μmoles per gram of protein, SE 0.4) than in the control group (mean 1.3, SE 0.3) ($p < 0.01$) at 90 min after hypoglycin administration. The FFA in the hearts of the treated animals were also elevated over those of the controls at 30, 60, 180, 210, and 270 min after hypoglycin administration. These differences in endogenous FFA and the subsequent differences in specific activity of the substrate resulting from the addition of 0.1 μmole of labeled palmitate to both the treated and control incubations were taken into account in the calculations of the rates of long-chain fatty acid oxidation. In these calculations complete mixing of the endogenous FFA and the added radioactive palmitate was assumed.

In glucose oxidation studies the amount of radioactive substrate added was over 20-fold greater than the endogenous levels, and no corrections were made for differences in specific activities.

Assays of palmityl-CoA:carnitine acyltransferase was carried out on myocardial homogenates by a modification of the method by Norum (21). Our assay procedure was based on the rate of release of tritiated carnitine from synthetic radioactive palmitylcarnitine-^{3}H in the presence of myocardial homogenate and coenzyme A. Reaction rates were linear for up to 7–10 min. Our assays were carried out for 2.5 and 5 min for each reaction. This assay has the advantage of not requiring the addition of carnitine as does the procedure of Norum. The reactions were halted by the addition of perchloric acid to a final concentration

of 0.7 M, which resulted in a complete precipitation of the proteins, residual palmitylcarnitine-^{3}H and the product palmityl-CoA. The enzymatically liberated labeled carnitine-^{3}H remained in the supernatant. Samples (0.2 ml) of the supernatant were placed in 15 ml of a phosphor solution consisting of 10 ml of toluene containing 2,5-diphenyloxazole (4 g/l) and 1,4-bis-2-(5-phenyloxazolyl)benzene (100 mg/l), and 5 ml of Triton X-100. Radioactivity was determined with a Packard Tri-Carb liquid scintillation spectrometer.

The incorporation of palmitate-1-^{14}C into myocardial triglycerides was quantitated by extraction of the lipids of the myocardial homogenates into chloroform–methanol (2:1 v/v) and then separating the lipid extracts into various classes by thin layer chromatography on silica gel according to the procedure of Skipski *et al.* (22). Neutral plates were developed in hexane–diethyl ether–acetic acid (85:15:1). The bands were visualized by exposure to iodine vapors, and the triglyceride band was then scraped off the plate with a spatula and placed in vials for scintillation counting. Ten milliliters of toluene containing 2,5-diphenyloxazole (4 g/l), 1,4-bis-2′-(5′-phenyloxazolyl)benzene (100 mg/l), and 4% Cabosil thixotropic gel powder was used as the phosphor solution. This procedure yielded an 85–90% recovery from the plated extract.

Plasma glucose determinations were carried out on tail vein samples by the procedure of Saifer and Gerstenfeld using glucose oxidase (23). The synthesis of palmitylcarnitine was carried out by the procedure of Brendel and Bressler (24).

Palmitate-1-^{14}C, hexanoate-1-^{14}C, glucose-U-^{14}C were obtained from New England Nuclear Corp., Boston, Massachusetts. (±)-Carnitine and (—)-carnitine were obtained from R and D Suppliers, Durham, North Carolina.

Hypoglycin (L-α-amino-β-methylenecyclopropanepropionic acid was a gift of the Abbott Company, North Chicago, Illinois.

RESULTS

Hypoglycin and Plasma Glucose

The intravenous administration of 15 mg of hypoglycin to mice resulted in decreases in plasma glucose that were apparent at 1 hr and maximal by 90 min. The hypoglycemia persisted for over 3 hr. A rise in

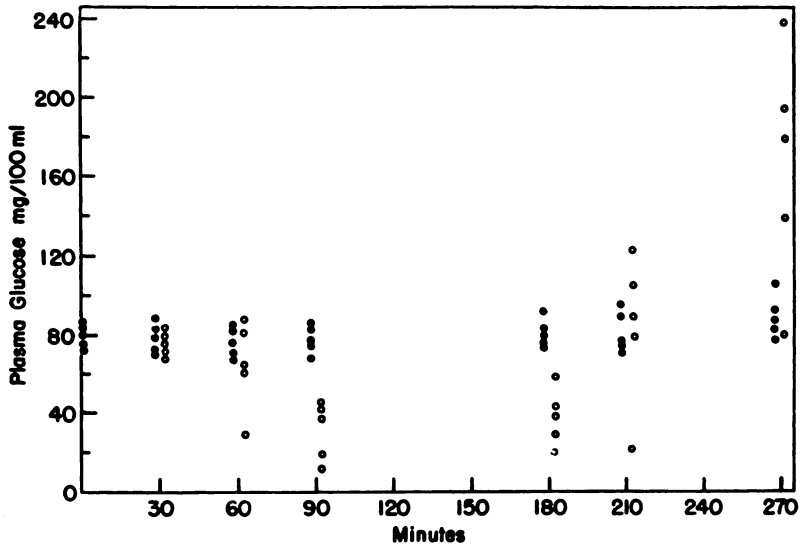

FIG. 1. *The effect of hypoglycin on plasma glucose*

Mice were divided into two groups. At zero time, one group received 15 mg of hypoglycin in 0.5 ml of normal saline by tail vein (○). The other group received only saline (●). Plasma glucoses were determined at the times indicated.

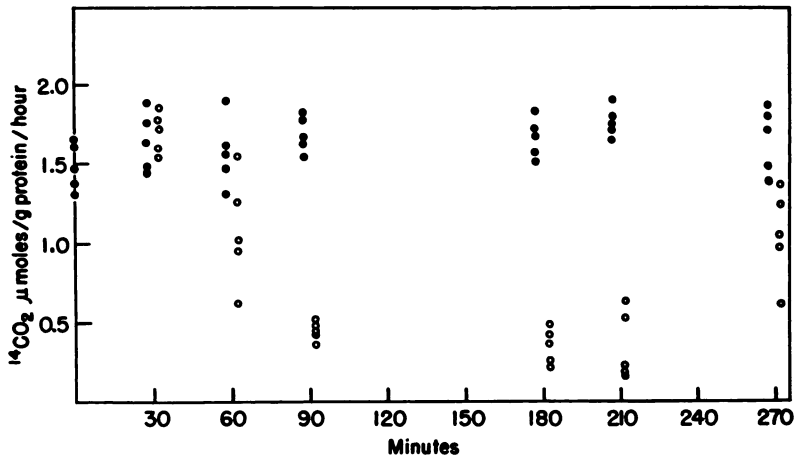

FIG. 2. *The effect of hypoglycin on palmitate oxidation*

Mice were divided into two groups and treated as described in Fig. 1 legend. At the end of the times indicated, hypoglycin-treated (○) and control animals (●) were sacrificed. The hearts were removed and used for assay of palmitate oxidation.

Each reaction mixture contained 8–12 mg of myocardial homogenate in 1 ml of calcium-free Krebs-Ringer phosphate buffer, pH 7.4 and 0.1 μmole palmitate-1-^{14}C (184,000 cpm). Final reaction volume was 1.1 ml. Incubations were carried out at 35° for 30 minutes.

plasma glucose was discernible at 3½ hr, and by 4½ hr had attained normal to hyperglycemic levels. The hyperglycemic overshoot was characteristic of the recovery phase. A typical study of the effect of hypoglycin on plasma glucose is shown in Fig. 1. Each point represents an individual animal which was used for the determination of plasma glucose and then sacrificed and the heart used for *in vitro* studies.

Hypoglycin and Palmitate Oxidation

Myocardial homogenates from hypoglycin-treated animals showed an impairment of long-chain fatty acid oxidation at 1 hr. The decreased palmitate oxidation persisted for over 3½ hr. At 4½ hr, at a time when the plasma glucose had manifested a hyperglycemic overshoot (cf. Fig. 1), the palmitate oxidation had not yet returned to normal. These data are shown in Fig. 2.

The impairment in fatty acid oxidation preceded the hypoglycemia. At 1 hr after the intravenous administration of hypoglycin the fall in plasma glucose was variable, whereas the impairment of palmitate oxidation was usually obtained. It was also found that the phase of recovery from the toxin at around 4½ hr was characterized by normoglycemia to hyperglycemia in association with palmitate oxidation rates which had not yet returned to normal (cf. Fig. 2).

Hypoglycin and the Oxidation of Palmitate, Hexanoate, and Glucose

Myocardial homogenates prepared from hypoglycin treated mice 90 min after the administration of the toxin showed a decreased rate of palmitate oxidation, and normal rates of oxidation of both hexanoate and glucose (Table 1).

Hypoglycin and the Incorporation of Palmitate into Triglycerides

The effect of hypoglycin on the incorporation of palmitate-1-^{14}C into triglycerides of myocardial homogenates is shown in Table 2. The homogenates from the treated animals incorporated twice as much of the labeled fatty acid into triglycerides as did the controls. The triglyceride content of the hearts of the treated animals was also increased over that of the control group (mean ± standard deviation, treated 6.7±, control, 2.8 ± 0.4 mg/g wet weight, $p < 0.01$).

TABLE 1

Effect of hypoglycin on the myocardial oxidation of glucose-U-^{14}C, hexanoate-1-^{14}C, and palmitate-1-^{14}C[a]

Each reaction mixture contained 8–12 mg of myocardial homogenate in 0.7 ml of calcium-free Krebs-Ringer phosphate buffer, pH 7.4 and either 10 μmoles of D-glucose-U-^{14}C (440,000 cpm), 25 μmoles of hexanoate-1-^{14}C (270,000 cpm) or 0.1 μmole of palmitate-1-^{14}C (230,000 cpm). Final reaction volumes were 0.9 ml. Incubations were at 32° for 30 minutes.

	$^{14}CO_2$ (μmoles/g protein per hour)		
Substrate	Hypoglycin		Control
Glucose-U-^{14}C	5.2		5.0
SD	1.4		1.8
p		>0.5	
Hexanoic-1-^{14}C	5.9		5.5
SD	1.3		0.6
p		>0.5	
Palmitate-1-^{14}C	0.32		1.35
SD	0.03		0.28
p		<0.01	

[a] Each group contained 5 animals.

Effect of Carnitine on Hypoglycin Inhibition of Palmitate Oxidation

In view of the depressed rate of palmitate oxidation and the normal rate of hexanoate oxidation in myocardial homogenates from hypoglycin-treated animals, it was suggested that the toxin only impairs fatty acid oxidation which is influenced by carnitine (15, 25). The effect of added carnitine on palmitate-1-^{14}C oxidation by myocardial homogenates from hypoglycin-treated animals and controls is shown in Table 3. The addition of carnitine to the control homogenates resulted in a stimulation of palmitate oxidation which was less than 2-fold, whereas the addition of carnitine to the homogenates from toxin-treated animals resulted in an 8-fold stimulation and a restoration of palmitate oxidation to the same levels as those of the carnitine-containing controls.

TABLE 2

Effect of hypoglycin on the incorporation of palmitate-1-^{14}C into myocardial triglycerides[a]

Each reaction mixture contained 8–21 mg of myocardial homogenate protein in 1 ml of calcium-free Krebs-Ringer phosphate buffer, pH 7.4, and 0.1 μmole of palmitate-1-^{14}C (270,000 cpm). Final reaction volumes were 1.1 ml. Incubations were at 35° for 30 minutes.

	Palmitate-1-^{14}C incorporation into triglycerides (μmoles/g protein per hour)		
	Hypoglycin		Control
Mean	0.510		0.248
SD	0.110		0.080
p		<0.025	

[a] Each group contained 5 animals.

TABLE 3

Effect of carnitine on hypoglycin-induced inhibition of palmitate oxidation[a]

Each reaction mixture contained 8–12 mg o myocardial homogenate in 1 ml of calcium-free Krebs-Ringer phosphate buffer, pH 7.4, 0.1 μmole of palmitate-1-^{14}C (130,000 cpm), and 2 μmoles of (−)-carnitine where indicated. Final reaction volumes were 1.3 ml. Incubations were at 35° for 30 minutes.

	$^{14}CO_2$ (μmoles/g protein per hour)		
Addition	Hypoglycin		Control
None	0.23		1.28
SD	0.17		0.21
p		<0.01	
Carnitine	1.90		1.85
SD	0.18		0.30
p		>0.5	

[a] Each group contained 5 animals.

Effect of Hypoglycin and in Vitro Carnitine on Palmityl-CoA:Carnitine Acyltransferase Activity

Palmityl-CoA:carnitine acyltransferase activity was depressed in myocardial homogenates from toxin-treated mice. The addition of exogenous carnitine to the assay of the control homogenates resulted in less than a 2-fold stimulation of activity, whereas, the addition of carnitine to the toxin-treated homogenates resulted in a 4-

TABLE 4

Effects of hypoglycin and in vitro carnitine on palmityl-CoA: carnitine acyltransferase

Each reaction mixture contained 1.8–2.5 mg of myocardial homogenate, 25 μmoles of Tris-HCl buffer, pH 7.4, 5 μmoles of reduced glutathione, 0.5 μmole of (±)-palmitylcarnitine-^{3}H (125,000 cpm), 0.25 μmole of CoA, and the indicated amounts of (−)-carnitine. Final reaction volumes were 0.7 ml. Incubations were at 28° for 5 minutes.

Reaction	Carnitine-^{3}H released (μmoles/g protein per minute)
Control	0.25
Control + 0.25 μmole of carnitine	0.36
Hypoglycin	0.06
Hypoglycin + 0.25 μmole of carnitine	0.24
Hypoglycin + 0.12 μmole of carnitine	0.28

fold stimulation of activity restoring it to normal levels. An experiment representative of four which were done is shown in Table 4. Lesser degrees of stimulation were found with lower concentrations of carnitine.

Effect of Administration of Hypoglycin and Carnitine of Palmityl-CoA:Carnitine Acyltransferase

The effect of carnitine administration on the myocardial palmityl-CoA:carnitine acyltransferase activity of hypoglycin-treated mice was investigated. The data of Table 5 show that carnitine prevented the loss of enzyme activity in the homogenates from toxin-treated animals.

TABLE 5

Effects of hypoglycin and in vivo carnitine on palmityl-CoA: carnitine acyltransferase

The animals were all given 15 mg of intravenous hypoglycin at the start of the experiment. Intraperitoneal administration of 6 mg of (−)-carnitine was also carried out at the start of the experiment and again at 1 hour. The animals were used for assay at 90 minutes after the start of the experimental procedures.

Each reaction mixture contained 1.3–2.7 mg of myocardial homogenate, 25 μmoles of Tris-HCl buffer, pH 7.4, 5 μmoles of reduced glutathione, 0.5 μmole of (±)-palmitylcarnitine-^{3}H (125,000 cpm), 0.25 μmole of CoA. Final reaction volumes were 0.8 ml. Incubations were at 28° for 2.5 and 5 minute intervals of time.

Group	Carnitine-^{3}H released (μmoles/g protein per minute)		
Control	0.24	0.22	0.26
Control + carnitine	0.26	0.30	0.32
Hypoglycin	0.05	0.09	0.11
Hypoglycin + carnitine	0.17	0.25	0.33

Effect of Carnitine Administration on Hypoglycin-Induced Hypoglycemia

Holt, Holt, and Böhm have suggested that the hypoglycemic effect of the toxin is due to an increased use of carbohydrate, and subsequent depletion of glycogen stores (10). Because of the stimulating effect of carnitine on fatty acid oxidation we investigated the effect of *in vivo* administration of carnitine on hypoglycin-induced hypoglycemia and depression of fatty acid oxidation. Mice were given the same treatment as described for Table 5. Carnitine

TABLE 6

Effect of carnitine on the plasma glucose and on myocardial fatty acid oxidation in the hypoglycin-treated mouse

Fatty acid oxidations were carried out as described in Table 2. Plasma glucoses were done on cardiac blood at time of sacrifice of the animals, 90 minutes after intravenous administration of hypoglycin and/or intraperitoneal administration of carnitine (saline controls).

Experiment	Plasma glucose (mg/100 ml)	$^{14}CO_2$ (μmoles/g protein per hour)		
Controls	84,79,66	1.58	1.72	1.67
Carnitine treated	70,74,88	1.84	1.96	1.78
Hypoglycin treated	13,28,33	0.29	0.40	0.18
Hypoglycin and carnitine treated	62,64,51	1.21	1.12	1.40

administration to toxin-treated mice largely prevented both the decrease of myocardial palmitate oxidations, bringing them to near normal, and the development of symptomatic or marked hypoglycemia. These data are shown in Table 6.

DISCUSSION

The content of hypoglycin is greatest in the immature seeds. Because seed maturity is delayed in the winter, the toxic illness occurs most often at this time in the economically deprived people whose diets are substandard (1). Malnutrition which results in lowered liver glycogen could enhance the susceptibility to hypoglycemia in these people. A number of investigators have contributed to the elucidation of the chemical structure of hypoglycin (26–29), and in recent years, the mechanism of action of the toxin has been studied (30). In this communication we have shown that the hypoglycin-induced impairment of long-chain fatty acid oxidation precedes the hypoglycemia, and is consistent with the hypothesis of Holt that the decrease in fatty acid oxidation results in an augmented use of carbohydrate and subsequent hypoglycemia (10, 13).

We undertook a study of the reactions in long-chain fatty acid oxidation in the heart to elucidate the site of action of hypoglycin. The increased conversion of palmitate-1-^{14}C to triglycerides, and the increased quantity of triglyceride in the hearts of toxin-treated animals attest to the integrity of the long-chain fatty acyl thiokinase activity. The normal oxidation of hexanoate-1-^{14}C and glucose-U-^{14}C prove the intactness of glycolysis, β-oxidation and the tricarboxylic acid cycle.

The inhibitory effect of hypoglycin on the long-chain acyl-CoA:carnitine acyltransferase would account for the depressed long-chain fatty acid oxidation and the increased levels of myocardial triglyceride. The mechanism of action of methylenecyclopropaneacetic acid on the acyl-CoA:carnitine acyltransferase has not yet been elucidated, but the inhibition is a readily reversible one as shown by the restoration of both long-chain fatty acid oxidation and the acyl-CoA:carnitine acyltransferase activity to normal by carnitine. Studies on the mechanism of action of the inhibition are in progress.

Carnitine administration to hypoglycin-treated mice served to restore or prevent the fall in long-chain fatty acid oxidation and prevent the hypoglycemia. The possible use of carnitine as an adjunct to glucose therapy is feasible; it has already been used in man at levels of 10–30 mg/kg without toxicity (31).

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