PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 166, 241–248 (1981)

Fenfluramine Effects on Insulin Resistance and Lipogenesis in Genetically Obese (ob/ob) Mice (41053)

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Abstract. Treatment of 15-week-old obese and lean mice with the amphetamine derivative fenfluramine (15, 25, and 40 mg/kg body wt, ip) for 14 days did not reduce body weight, except in lean mice receiving 40 mg/kg. *In vivo* insulin sensitivity, measured as the ability of exogenous insulin (1 IU/kg body wt, ip) to lower blood glucose, was improved in obese mice receiving the two higher doses (25 and 40 mg/kg) of fenfluramine for 14 days, when compared to untreated obese mice. There was no change in insulin sensitivity between treated and untreated lean mice. This improvement, seen in obese mice which did not lose weight, demonstrates an effect of this drug distinct from its anorexigenic properties. On Day 21 of fenfluramine treatment, the *in vivo* rates of fatty acid synthesis were determined by injecting ^3H_2O ip and measuring the amount of ^3H incorporated into fatty acids over a 1-hr period. The rates of fatty acid synthesis per gram of liver and adipose tissue of obese and lean mice receiving 40 mg/kg fenfluramine were unchanged when compared to sham-injected controls. However, fat depletion was observed in epididymal fat pads of treated (40 mg/kg) lean mice, indicating either reduced lipid deposition or stimulated lipolysis in this group. *In vitro*, a therapeutic concentration (100 ng/ml) of fenfluramine increased insulin-stimulated conversion of [U-^{14}C]glucose to fatty acids but not CO_2 and total lipids in isolated adipocytes from lean but not obese mice. These results indicate that the reduction of insulin resistance in obese mice treated with fenfluramine does not influence rates of lipogenesis in liver and adipose tissue but may be associated with increased insulin sensitivity of other tissues such as muscle.

The genetically obese (ob/ob) mouse is characterized by hyperglycemia, hyperinsulinemia, and insulin resistance, and thus provides an interesting model of adult onset diabetes mellitus (1). While the insulin sensitivity of these mice is improved with food restriction and subsequent weight loss (2), this effect also is observed in alloxan-treated obese mice with no reduction in body weight (3), suggesting that improved insulin sensitivity is not dependent on the body weight of these animals.

The amphetamine derivative, fenfluramine, which has been used therapeutically as an anorexigenic agent in the treatment of human obesity, also is reported to elicit a number of metabolic effects, including increased glucose uptake by muscle (4), improved glucose tolerance in streptozotocin-diabetic rats (5), decreased plasma triglycerides (6), and increased plasma free fatty acids (7). It is reported that at a low dose this drug reduces hyperglycemia in obese mice without concomitant weight loss (8). We therefore postulated that fenfluramine may improve the insulin sensitivity of these mice and thus alleviate the abnormal characteristics of glucose metabolism in this diabetic syndrome. In addition, we investigated possible alterations in lipid metabolism in obese mice as a consequence of reduced insulin resistance.

Materials and Methods. Animals used in this study were male C57BL/6J-*ob* obese mice and their lean controls purchased from the Jackson Laboratory (Bar Harbor, Maine). They were housed singly in wire-bottom cages in a light- (12-hr light/dark cycle) and temperature (20 ± 2°)-controlled room and were fed *ad libitum* a stock diet containing a minimum of 19% protein, 7.5% fat, 2.5% fiber, and 52% nitrogen-free extract (diet 96W. Emory Morse Company, Guilford, Conn.).

In experiment 1, the *in vivo* effects of fenfluramine on the insulin sensitivity and rate of fatty acid synthesis in obese and lean mice were investigated. Obese mice and lean controls received either daily ip injec-

0037-9727/81/020241-08$01.00/0

tions of fenfluramine hydrochloride (A. H. Robins Company, Richmond, Va., 15, 25, or 40 mg/kg body wt) or drug diluent (0.9% sterile physiological saline) over a 14-day period beginning at 15 weeks of age. Body weight was recorded daily prior to each injection between 3:00 and 4:00 PM. On Days 0, 3, 6, 9, and 12 of treatment, the animals were anesthetized with 50% CO_2:50% O_2, and blood samples were collected from the orbital sinus in heparinized capillary tubes between 9:00 and 10:00 AM. Blood glucose concentrations were determined by the method of Fales (9) and plasma insulin concentrations by the method of Herbet *et al.* (10), using human insulin as a standard (Lot 615-1054B-230, generously supplied by Dr. Mary A. Root, Eli Lilly and Company, Indianapolis, Ind.).

In vivo insulin sensitivity was tested by measuring the ability of exogenous insulin to lower blood glucose. The animals were fasted for 16 hr after the injection of fenfluramine at 14 days and blood samples were collected from the tail using heparinized capillary tubes (zero-time samples) for the determination of blood glucose concentration. Then regular insulin (Eli Lilly, 1 IU/kg) was injected ip and blood samples collected at 30, 60, 120, and 180 min after the insulin injection.

Fenfluramine treatment was continued for 7 days in animals receiving 40 mg/kg and sham-injected controls. For determining the *in vivo* rate of fatty acid synthesis, 2–5 mCi ^3H_2O (specific activity 20–50 mCi/ml; New England Nuclear Corp., Boston, Mass.) was administered ip to each animal 16 hr after the final injection of fenfluramine, at which time water bottles were removed from the cages. After 1 hr, animals were anesthetized under diethyl ether anesthesia, and blood collected by cardiac puncture for the determination of plasma radioactivity, plasma insulin, and glucose concentration. The liver and epididymal fat pads were excised, rapidly frozen on solid CO_2, and weighed.

Fatty acids were extracted by first placing small (100–200 mg) portions of tissue in 5 ml 30% KOH and boiling until disrupted. Then 1 ml absolute ethanol was added and the samples were saponified at 90° for 2–3 hr. After saponification, 5 ml water was added and nonsaponifiable lipids were extracted in petroleum ether and discarded. Following acidification, fatty acids were extracted two times with 10 ml petroleum ether. The extracts were evaporated and dissolved in scintillant and radioactivity was counted in a liquid scintillation counter. The rate of fatty acid synthesis was calculated as follows:

$$\mu\text{mol } C_2 \text{ units/hr/g tissue} = \frac{\text{dpm } ^3\text{H in fatty acid/g tissue}}{\text{dpm } ^3\text{H in 1 } \mu\text{l plasma}} \times \frac{106}{1.36}.$$

This formula is that described by Hems *et al.* (11), except that a discrimination factor of 2.94 against ^3H was used for the mouse (12) rather than the value of 2.38 used for the rat (13). The molarity of water in plasma was assumed to be 53 *M*.

In experiment 2, the *in vitro* effect of fenfluramine on the insulin-stimulated glucose metabolism by adipose tissue of obese and lean mice was investigated. Fifteen-week-old ob/ob mice and lean controls were sacrificed by decapitation and their epididymal fat pads were removed. Adipocytes were isolated from whole tissue by the method of Rodbell (14). The washed fat cells were suspended in 5 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, containing 4% albumin (Armour Pharmaceuticals, Phoenix, Ariz.) and 5 m*M* glucose. For the measurement of basal glucose oxidation, and incorporation into total lipids and fatty acids, 0.5 ml of fat cell suspension was added in triplicate to plastic flasks with 0.5 ml Krebs–Ringer bicarbonate buffer containing 5 m*M* of [U-^{14}C]-glucose (0.2 μCi/flask, New England Nuclear Corp.) and incubated at 37° for 2 hr in a metabolic shaker. For the measurement of insulin-stimulated glucose metabolism, an insulin concentration of 500 μU/ml was used in the medium. To examine the *in vitro* effects of fenfluramine on basal and insulin-stimulated glucose metabolism, fenfluramine hydrochloride was added to the medium at a concentration of 100 ng/ml immediately before each experiment. This concentration was in accordance with pre-

viously reported steady state plasma concentrations of 40–120 ng/ml after repeated therapeutic administration (15). The flasks were gassed with 95% O_2:5% CO_2 for 30 sec and capped with a stopper equipped with a center well (Kontes Glass, Vineland, N.J.). After a 2-hr incubation, 0.3-ml Protosol tissue solubilizer (New England Nuclear Corp.) was added to the center well and 0.2 ml 10% $HClO_4$ to the medium, respectively. Carbon dioxide was collected during an additional 60-min incubation and the center wells were then transferred into liquid scintillation vials. The contents of the incubation flasks were extracted for total lipids according to the procedure of Folch *et al.* (16) and fatty acids extracted as described for experiment 1. The radioactivity of the CO_2, total lipids, and fatty acids was counted in a liquid scintillation counter. Blanks were run in parallel with each experiment by incubating cells in the presence of $HClO_4$ in order to correct the measured parameters for nonmetabolic ^{14}C labeling. The DNA content of 0.5-ml fat cell suspension was measured in duplicate (17) after removal of most of the infranatant buffer. [U-^{14}C]Glucose conversion to CO_2, total lipids, and fatty acids is expressed as nanomoles of glucose converted per milligram DNA per hour.

Statistical comparisons were made by Student's *t* test for independent samples comparing fenfluramine-treated animals (or adipocytes) to untreated animals (or adipocytes), and expressed as mean ± SE (18).

Results. Table I summarizes the effects at Day 12 of daily administration of 15, 25, and 40 mg/kg fenfluramine on the body weight, blood glucose, and plasma insulin concentrations of lean and obese mice. The typical high body weights and elevated blood glucose and plasma insulin concentrations were observed in obese control mice. Fenfluramine treatment had a statistically significant effect on reducing body weight only in lean mice receiving 40 mg/kg, an effect which was initially observed on Day 2 of treatment and which continued through Day 21. No significant

TABLE I. BODY WEIGHT, BLOOD GLUCOSE, AND PLASMA INSULIN OF LEAN AND OBESE MICE ON DAY 0 AND DAY 12 OF FENFLURAMINE TREATMENT[a]

Dose of fenfluramine (mg/kg)	Lean		Obese	
	Day 0	Day 12	Day 0	Day 12
		Body Weight (g)		
0	27.8 ± 0.6 (9)[b]	27.9 ± 0.6 (9)	54.6 ± 1.2 (8)	54.4 ± 1.3 (8)
15	27.8 ± 0.4 (8)	27.1 ± 0.4 (8)	53.1 ± 1.0 (8)	53.0 ± 0.6 (8)
25	27.0 ± 0.7 (8)	26.9 ± 0.8 (8)	51.3 ± 1.2 (8)	51.1 ± 1.5 (7)
40	27.1 ± 0.4 (8)	25.4 ± 0.4 (7)[c]	52.4 ± 0.9 (8)	53.0 ± 1.3 (7)
		Blood Glucose (mg/dl)		
0	71 ± 3 (9)	89 ± 3 (9)	314 ± 17 (8)	410 ± 18 (7)
15	73 ± 5 (8)	89 ± 4 (6)	336 ± 29 (7)	397 ± 24 (7)
25	70 ± 5 (7)	97 ± 5 (8)	359 ± 32 (7)	436 ± 28 (7)
40	75 ± 3 (7)	86 ± 9 (6)	314 ± 25 (8)	393 ± 26 (7)
		Plasma Insulin (μU/ml)		
0	24.2 ± 1.5 (9)	31.9 ± 4.2 (8)	73.2 ± 3.6 (7)	72.5 ± 7.9 (7)
15	27.3 ± 3.1 (7)	27.1 ± 5.6 (5)	88.5 ± 9.8 (5)	94.6 ± 15.7 (6)
25	26.2 ± 3.4 (6)	22.2 ± 3.2 (7)	93.1 ± 14.0 (8)	92.7 ± 12.7 (6)
40	23.6 ± 2.4 (8)	22.9 ± 4.1 (5)	81.9 ± 7.6 (6)	79.8 ± 11.3 (7)

[a] Blood was collected between 9:00 and 10:00 AM in heparinized capillary tubes from the orbital sinus. A portion of the blood was used to prepare a sodium–zinc filtrate for the determination of glucose concentration. The remainder of the blood was centrifuged and the plasma was collected and stored frozen until the insulin concentration was determined by radioimmunoassay using a human insulin standard.

[b] Mean ± SE; number in parentheses indicates number of determinations.

[c] $P < 0.05$ versus untreated control within genotype.

effects on blood glucose and plasma insulin concentrations were observed at the three doses in lean and obese mice. Similar observations to those made on Day 12 were made for body weight and blood glucose and plasma insulin concentrations of lean and obese mice at the three doses on Days 3, 6, and 9 (data not shown).

As the mean fasting blood glucose concentration did not differ significantly among the four groups of animals in each genotype, the blood glucose concentration following the insulin injection was expressed as a percentage of the preinsulin concentration (Fig. 1). While the blood glucose concentrations of lean control mice fell 30 min after the insulin injection, those of obese control mice rose, possibly due to the stress of the bleeding procedure, although this was kept at a minimum by collecting small (10–30 μl) volumes of blood from the tail. Lean mice under similar stress probably also released glucose into the circulation, but they were able to transport this glucose into tissues showing normal insulin sensitivity.

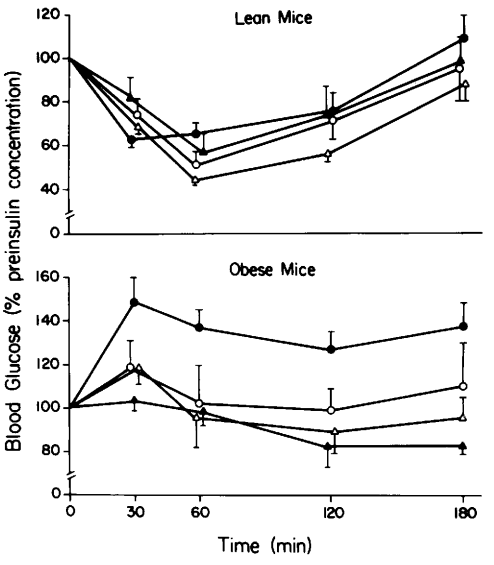

FIG. 1. Blood glucose response of lean and obese mice following ip injection of regular insulin (1 IU/kg). The mice were fasted 16 hr, blood samples collected, and sodium-zinc filtrates of the blood prepared for determination of glucose. ●, Control; ○, 15 mg/kg; △, 25 mg/kg; and ▲, 40 mg/kg fenfluramine. Each point is the mean ± SE for eight mice. Preinsulin (fasting) blood glucose concentrations did not differ significantly among the four groups of animals.

Fenfluramine treatment had a statistically significant effect on increasing insulin sensitivity of obese mice receiving 25 and 40 mg/kg beginning at 30 min after the injection of insulin when compared to control obese mice, while this effect was observed only in the 60-min time sample in lean mice receiving 25 mg/kg. The group of obese mice receiving 40 mg/kg showed a greater fall of blood glucose than those receiving 25 mg/kg, although both groups showed a delayed response to insulin as compared to lean mice.

Table II summarizes the effects of 40 mg/kg fenfluramine administered daily on the rate of fatty acid synthesis in liver and adipose tissue of lean and obese mice after 21 days of treatment. Typical elevated rates of fatty acid synthesis were observed in the liver of obese control mice, while in epididymal adipose tissue they were similar to those observed in lean mice when expressed per gram tissue. It has been reported that in obese mice, the rate of fatty acid synthesis in adipose tissue declines with age while the liver continues to synthesize fatty acids at an elevated rate (19). Although the rate of fatty acid synthesis per gram of tissue was not significantly different in liver and fat tissue of fenfluramine-treated lean and obese mice, the weight of the fat pads was significantly decreased in treated lean mice. Thus, the rate of fatty acid synthesis in this depot when expressed for whole tissue was significantly lower.

The results of experiment 2 are summarized in Table III. Glucose metabolism of isolated adipocytes is presented as nanomoles of glucose converted per milligram of DNA per hour of incubation in order to express the results in terms related to cell number rather than cell mass. Adipocytes from obese mice in the control medium showed reduced basal [U-^{14}C]glucose conversion to total lipids, CO_2, and fatty acids in comparison to those from lean mice. The addition of insulin to the control medium increased [U-^{14}C]glucose utilization significantly in adipocytes from lean mice when compared to their basal glucose metabo-

TABLE II. EFFECTS OF FENFLURAMINE ON THE *IN VIVO* SYNTHESIS OF FATTY ACIDS IN LIVER AND EPIDIDYMAL ADIPOSE TISSUE OF LEAN AND OBESE MICE[a]

Group	Tissue	Weight Tissue (g)	Fatty acid synthesis (μmole C_2 units/hr/g)	Fatty acid synthesis (μmole C_2 units/hr/tissue)
Lean				
Control	Liver	1.36 ± 0.03 (12)[b]	24.7 ± 3.0 (12)	33.2 ± 3.7 (12)
Fenfluramine	Liver	1.32 ± 0.04 (8)	22.9 ± 2.7 (8)	30.7 ± 3.9 (8)
Control	Fat	0.75 ± 0.03 (12)	1.7 ± 0.4 (12)	1.4 ± 0.3 (12)
Fenfluramine	Fat	0.44 ± 0.05 (8)[c]	1.1 ± 0.2 (8)	0.5 ± 0.1 (8)[b]
Obese				
Control	Liver	4.97 ± 0.17 (11)	74.4 ± 8.1 (11)	375 ± 49 (11)
Fenfluramine	Liver	4.95 ± 0.27 (10)	79.2 ± 17.3 (10)	397 ± 85 (10)
Control	Fat	3.16 ± 0.16 (11)	1.7 ± 0.4 (11)	5.4 ± 1.1 (11)
Fenfluramine	Fat	2.98 ± 0.10 (10)	1.8 ± 0.3 (10)	5.4 ± 1.0 (10)

[a] Lean and obese mice were administered 40 mg/kg fenfluramine·HCl ip or saline diluent for 21 days between 3:00 and 4:00 PM; ^3H_2O was administered between 8:00 and 9:00 AM. After 1 hr the amount of ^3H in fatty acid in liver and epididymal adipose tissue was determined. Other details are in the text.

[b] Values are mean ± SE; numbers in parentheses indicate number of determinations.

[c] $P < 0.05$ versus untreated control within genotype.

lism. Such an effect was not observed in adipocytes from obese mice.

The addition of 100 ng/ml fenfluramine hydrochloride to the medium did not significantly affect the basal glucose metabolism of both lean and obese adipocytes. In adipocytes from lean mice, fenfluramine increased insulin-stimulated conversion of [U-^{14}C]glucose to fatty acids significantly.

Discussion. Chronic fenfluramine administration reduced the insulin resistance of obese mice without reducing body weight and without increasing *in vivo* rates of lipogenesis in liver or adipose tissue. Our *in vitro* findings also showed that improved insulin sensitivity did not influence lipogenesis in obese mice.

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TABLE III. [U-^{14}C]GLUCOSE CONVERSION TO CO_2, TOTAL LIPIDS, AND FATTY ACIDS (nmole/mg DNA/hr)[a]

Group	Insulin	Control	Fenfluramine
		CO_2	
Lean	−	643 ± 62 (15)[b]	656 ± 68 (11)
	+	1280 ± 140 (15)[c]	1590 ± 170 (11)[c]
Obese	−	98 ± 19 (14)	123 ± 23 (12)
	+	107 ± 22 (14)	129 ± 20 (12)
		Total lipids	
Lean	−	2370 ± 250 (15)	1950 ± 200 (11)
	+	3450 ± 380 (15)[c]	3420 ± 410 (11)[c]
Obese	−	361 ± 52 (14)	264 ± 49 (12)
	+	410 ± 56 (14)	300 ± 40 (12)
		Fatty acids	
Lean	−	88 ± 20 (15)	128 ± 17 (11)
	+	142 ± 27 (15)[c]	253 ± 41 (11)[d]
Obese	−	8 ± 1 (14)	11 ± 2 (12)
	+	12 ± 4 (14)	14 ± 4 (12)

[a] [U-^{14}C]Glucose metabolism in isolated fat cells from 15-week-old lean and obese mice in the presence (+) and absence (−) of insulin (500 μU/ml) and in the presence and absence of fenfluramine (100 ng/ml). Fat cells were incubated at 37° in Krebs–Ringer bicarbonate buffer containing 4% albumin and 5 m*M* glucose for 2 hr.

[b] Mean ± SE; number in parentheses indicates number of determinations.

[c] $P < 0.05$ versus basal.

[d] $P < 0.05$ versus control.

showed that with a high oral dose (100 mg/kg/day), fenfluramine reduced food intake, body weight, and plasma glucose and insulin concentrations in obese mice while a lower dose (50 mg/kg/day) reduced only their plasma glucose concentrations (8). These observations and those of improved glucose tolerance without weight reduction in obese humans treated with fenfluramine (20) suggest that reduction of insulin resistance was a consequence of low dose treatment in obese mice when no reduction in body weight was observed.

In the present study, administration of the drug ip rather than orally in the drinking water as done in our previous study enabled us to control the doses with more precision. Three doses of fenfluramine were administered to provide various circulating concentrations of fenfluramine in lean and obese mice, since steady state is influenced both by body weight and storage in adipose tissue (21). Our observation of reduced body weight only in lean mice receiving the highest dose of fenfluramine is not in agreement with the report of Bizzi *et al.* (22), who observed that fenfluramine administered on the basis of total body weight to lean and gold-thioglucose (GTG) obese mice was more effective in reducing food intake in obese mice than in lean mice. This discrepancy may be due to metabolic differences between the genetically obese mouse and the GTG obese mouse, as demonstrated by Carr *et al.* (8), who report a greater reduction in body weight by a high oral dose of fenfluramine in GTG mice than in genetically obese mice. Moreover, since administration of the drug to rodents in the evening is necessary for the development of the pharmacological action at the time when they eat (23), differences in the diurnal eating patterns of lean and obese mice which have been reported (24) may also result in differences in the efficacy of the dose as administered.

Since improved insulin sensitivity is reported in food-restricted obese mice (2), our observations that the insulin sensitivity of obese mice treated with 25 and 40 mg/kg fenfluramine was significantly increased in spite of no change in body weight demonstrated that this effect of the drug was not mediated by its anorexigenic effect. Moreover, the possibility that this effect was mediated by the suppression of "stress-induced" hyperglycemia was ruled out in an experiment that showed no significant differences in percent of zero-time blood glucose or in actual blood glucose concentrations between fenfluramine-treated and untreated lean and obese mice 30, 60, 120, and 180 min after a saline injection without insulin (unpublished data). However, since basal blood glucose concentrations of obese mice were not reduced as reported by Carr *et al.* (8) at low doses of fenfluramine, insulin resistance was maintained to a significant extent. Further studies examining the effects of higher doses of fenfluramine in obese mice are needed in order to assess those parameters that are most sensitive to fenfluramine.

Our observations that 40 mg/kg fenfluramine reduced the insulin resistance in obese mice led us to investigate whether the drug had an effect on lipogenesis at this dose. It has been suggested (25, 26) that fenfluramine's effect on the depletion of fat stores *in vivo* is related to its inhibition of lipogenesis as demonstrated by decreased incorporation of [U-^{14}C]glucose into neutral lipid *in vitro* at high concentrations (268 μg/ml) of fenfluramine. In addition, Dannenburg and Kardian (27) have suggested that fenfluramine exerts a lipolytic effect which is mediated through the sympathomimetic release of catecholamines, since no effect on basal lipolysis is observed *in vitro*. Our findings indicate that although fenfluramine-treated obese mice showed increased sensitivity to insulin, their rates of *in vivo* fatty acid synthesis in liver and adipose tissue remained unaffected. Since fenfluramine increases glucose uptake in muscle both *in vivo* (28) and *in vitro* (4), it is possible to suggest that the observed improvement in insulin sensitivity in obese mice treated with fenfluramine may be associated with other tissues such as muscle rather than adipose tissue. In adipose tissue from lean treated mice, the rate of fatty acid synthesis for whole tissue was significantly reduced by fenfluramine,

suggesting either reduced lipogenesis per cell, or increased lipolysis. Depending upon the rate of lipolysis, reesterification may suppress the *de novo* synthesis of fatty acids (29). Similar effects of fenfluramine on fatty acid synthesis were not observed in liver of lean mice. These observations may be related to the relative tissue concentrations of fenfluramine, since adipose tissue has a greater capacity to sequester fenfluramine (22).

To further elucidate the primary effects of fenfluramine on insulin sensitivity with respect to lipogenesis, we investigated basal and insulin-stimulated glucose metabolism in isolated fat cells using therapeutic (100 ng/ml) concentrations of the drug. The observed increase in insulin-stimulated conversion of glucose to fatty acids but not to CO_2 and total lipids in adipocytes from lean mice may indicate reduced glycerol synthesis by fenfluramine. Glucose metabolism of adipocytes from obese mice was not altered intracellularly by fenfluramine in the same manner as those from lean mice and may indicate that higher plasma concentrations of the drug are required to elicit an effect.

The obese (ob/ob) mouse, with its characteristic obesity associated with hyperglycemia, hyperinsulinemia, and insulin resistance, has been used extensively as a model for human adult onset diabetes. These studies were designed to demonstrate whether the drug fenfluramine is beneficial in reducing the hyperglycemia and insulin resistance in this animal model without producing weight reduction, since such an effect would have therapeutic value in humans. Indeed, a reduction in insulin resistance was shown. Thus, fenfluramine may have a therapeutic role in the treatment of the obese diabetic patient, especially those who are refractory to the effects of diet alone.

This research was supported by USPHS National Institutes of Health Grants AM-02911 and AM-00106 and the Fund for Research and Teaching, Department of Nutrition, Harvard School of Public Health. The fenfluramine·HCl was a gift from Dr. Oscar Klioze of A. H. Robins Company, Richmond, Virginia. The technical assistance of Mr. Douglas Colwell and Mr. Olden Green is gratefully acknowledged.

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Received March 5, 1980. P.S.E.B.M. 1981, Vol. 166.