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DECREASED INCORPORATION OF GLUCOSE INTO LIPIDS AND INCREASED LACTATE PRODUCTION BY ADIPOSE TISSUE AFTER LONG-TERM TREATMENT OF RATS WITH D-FENFLURAMINE

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Abstract—Male rats were treated with ten daily doses of 10 mg of D-fenfluramine/kg. Body weight decreased after days 1 and 2, but thereafter the weight gain paralleled that of the control rats. After the tenth injection there were decreases in the weights of the epididymal fat pads, their fat content, and the average size of the adipocytes after collaginase digestion. The rate of glucose uptake by incubated pieces of adipose tissue was maintained after D-fenfluramine treatment, and the production of lactate increased. The incorporation of glucose into fatty acids by adipose tissue pieces decreased by 65–74% after treatment with D-fenfluramine. This effect was not reversed by adding insulin or phenylisopropyladenosine to the incubations. D-Fenfluramine also decreased the incorporation of glucose into glyceride-glycerol, but this effect was less pronounced than that for fatty acid synthesis. Direct addition of D-fenfluramine to the incubation inhibited lipid synthesis from [^{14}C]glucose but only at drug concentrations above 1 mM. It is concluded that the treatment of rats with D-fenfluramine modifies the metabolic balance of adipose tissue so as to direct glucose metabolism away from lipid synthesis and towards lactate production. This could be a significant mechanism in the overall loss of adipose tissue mass caused by the administration of D-fenfluramine.

Fenfluramine was introduced originally as an anorectic agent, and it is widely used in the treatment of obesity. It has also become evident that fenfluramine has important metabolic effects that may be unrelated to its action in decreasing food intake. These include anti-hyperglycemic and hypolipidemic effects and an improvement in insulin sensitivity which would also be of value in the treatment of obesity [1, 2].

The present experiments were intended to investigate the effects of a prolonged treatment of rats with D-fenfluramine on adipose tissue metabolism. Adipose tissue was removed from the rats after the drug treatment and then incubated for periods of up to 24 hr so that the metabolism of glucose could be evaluated. The results show that treatment of the rats with D-fenfluramine decreased the synthesis of fatty acids and triacylglycerols and diverted the glucose into lactate production.

MATERIALS AND METHODS

Male Wistar rats weighing 150–170 g were placed in grid-bottomed cages (two rats per cage) and were maintained on 41B diet for 7 days. They were then changed to the corn oil diet for a subsequent 7 days. This diet provided 48% of its energy from fat, 19% from protein and 33% from carbohydrate [3]. Its effects on the growth rates of the rats, body composition, the concentrations of some metabolites in blood and brain, and the activities of some enzymes in liver, heart and adipose tissue have already been described [3–6]. The rats were then treated for 10 days with daily doses of 10 mg of D-fenfluramine/kg, and control rats were given equivalent volumes of sterile 0.16 M NaCl [7, 8]. The rats were killed 2 hr after the last injection, and adipose tissue was removed.

Determination of adipocyte size and the fat content of adipose tissue. The method is based on that of Jamdar *et al.* [9]. Samples of adipose tissue were cut into small pieces and incubated at 37° for 1 hr in Medium 199 that contained 1 mg of collagenase/ml under an atmosphere of air/CO_2 (95:5, v/v). The suspension was then strained into a warm tube, and any retained tissue was gently stirred. The layer of adipocytes that formed in the top of the combined filtrates was then sampled and placed on the counting chamber of a microscope. The diameters of at least 100 cells for each rat were recorded, and the volume of the cells was calculated assuming the cells to be spherical.

Fat pads were extracted with chloroform [10], and the weight of fat was determined after removal of the solvent.

Incubation of adipose tissue. Small pieces of adipose tissue (about 100 mg in total) were incubated in

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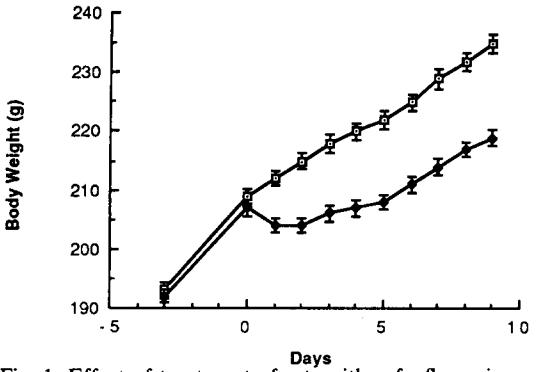

Fig. 1. Effect of treatment of rats with D-fenfluramine on body weight. Rats were fed on the corn oil diet and then treated daily with 10 mg of D-fenfluramine/kg beginning on day 0. Results for the D-fenfluramine-treated rats (◆) and the controls (⊡) are expressed as means ± SE for 68 rat in each group.

2 ml of Medium 199 that contained 5.5 mM glucose, Earle's salts, L-glutamine, 25 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), pH 7.4, 20 g of fatty acid poor bovine serum albumin/L, 100 mg of streptomycin/L, 60 mg of penicillin/L and 10 mg of neomycin/L [10, 11]. The medium was equilibrated with air/CO_2 (95:5, v/v). Other additions and the time of incubations are given in the legends to the tables and figures.

The concentrations of glucose at the end of the incubation were measured by using a commercial glucose kit (Boehringer) and a microtitration plate reader. Lactate was determined by using the following reaction mixture: 200 mM Tris/HCl, pH 8.2, 0.18 units/ml of diaphorase, 55 units/ml of lactate dehydrogenase, 0.4 mg of [1-(4,5-dimethylthiazole-2-yl)-3,5 diphenyltetrazolium bromide/ml, 1.6 mM NAD and 2 μl of Triton X-100/ml. The formation of the formazan was determined with the microtitration plate reader at 600 nm, and lactate concentrations were calculated by using standards prepared in Medium 199.

Conversions of glucose into lipids and CO_2. These were determined by adding 0.25 μCi/ml of [U-^{14}C]glucose to the incubations. The incorporation into lipids over 2 hr (which gave initial reaction rates) was determined after their extraction into chloroform [10] in the presence of 0.1 M HCl. The top phase of the extract was removed, and the chloroform phase was washed with a synthetic top phase. A sample of the bottom phase was dried with a stream of N_2 and then dissolved in heptane. A part of this was used directly to measure the total ^{14}C incorporated into lipid, whereas the remainder was again dried under N_2. To this was added 1 ml of 6 M KOH in 75% (v/v) ethanol containing 0.02% thymol blue as indicator. The mixture was incubated at 50° for 4 hr to saponify the lipids. The mixture was acidified with HCl until the thymol blue turned pink and fatty acids were extracted into heptane. Samples of heptane and the aqueous phase were used to determine the incorporation of [^{14}C]glucose into the fatty acid and glycerol portions of the triacylglycerol.

The conversion of [^{14}C]glucose to $^{14}CO_2$ was determined over a 2-hr period by incubation in plastic bottles that were closed with a Subaseal. HCl was injected to stop the incubation, and the $^{14}CO_2$ was collected in hyamine hydroxide solution. A sample of this solution was then taken for the determination of $^{14}CO_2$ by scintillation counting.

RESULTS

Effects of D-fenfluramine on weight gain, food intake and adipocyte size. Daily treatment of the rats with 10 mg of D-fenfluramine/kg resulted in a decrease in body weight ($P < 0.005$) after days 1 and 2 (Fig. 1), as demonstrated previously [7, 8]. Thereafter, the rate of body weight gain was restored to that of the control rats. This coincided with the increased food intake of the D-fenfluramine-treated rats which was not significantly different from that of the control rats after day 4 of treatment [7, 8]. The rats used in the present studies were therefore treated over 10 days with D-fenfluramine so that they were in a stable condition as regards food intake and weight gain. At this time there was a highly significant

Table 1. Effect of chronic treatment of rats with D-fenfluramine on the weight of the epididymal fat pads and the size of the adipocytes

	Control	Fenfluramine treated
Fat pad weight (g)	2.23 ± 0.44 (40)	1.65 ± 0.42 (40)
		$P < 0.001$
Fat (g) in fat pad	1.46 ± 0.21 (4)	0.91 ± 0.10 (4)
		$P < 0.025$
$\frac{\text{Fat pad weight}}{\text{Body weight}} \times 100$	1.18 ± 0.1 (22)	0.87 ± 0.07 (22)
		$P < 0.001$
Mean cell volume (pl)	154 ± 38 (32)	90 ± 24 (32)
		$P < 0.001$

Rats were fed the corn oil diet and treated for 10 days with 10 mg of D-fenfluramine/kg. Results are means ± SE for the number of rats indicated in parentheses. The P values show the significance of the differences between control and treated animals.

Table 2. Effects of chronic treatment of rats with D-fenfluramine on the uptake of glucose and production of lactate by epididymal fat pads

Additions	Glucose uptake (μmol/pair of pads)		Lactate production (μmol/pair of pads)	
	Control	Fenfluramine treated	Control	Fenfluramine treated
1. None	92 ± 15 (8)	112 ± 45 (8)	65 ± 12 (8)	101 ± 44 (8) $P < 0.025$
2. Insulin (0.17 nM)	106 ± 11 (4)	116 ± 13 (4)	52 ± 13 (4)	111 ± 24 (4) $P < 0.005$
3. Insulin (1.7 nM)	172 ± 18 (4)	157 ± 24 (4)	66 ± 14 (4)	89 ± 32 (4)
4. DEX	35 ± 8 (8)	57 ± 37 (8) $P < 0.05$	21 ± 14 (8)	65 ± 44 (8) $P < 0.01$
5. DEX + insulin (0.17 nM)	53 ± 7 (4)	67 ± 19 (4)	8 ± 1 (4)	66 ± 50 (4) $P < 0.05$
6. DEX + insulin (1.7 nM)	167 ± 31 (4)	158 ± 18 (4)	42 ± 18 (4)	65 ± 29 (4)
7. PIA	110 ± 26 (8)	126 ± 33 (8)	60 ± 12 (8)	104 ± 35 (8) $P < 0.005$
8. PIA + insulin (0.17 nM)	114 ± 8 (4)	116 ± 18 (4)	54 ± 10 (4)	111 ± 32 (4) $P < 0.01$
9. PIA + insulin (1.7 nM)	188 ± 32 (4)	181 ± 26 (4)	71 ± 20 (4)	99 ± 38 (4)
10. PIA + DEX + insulin (0.17 nM)	75 ± 12 (4)	94 ± 26 (4)	12 ± 9 (4)	102 ± 53 (4) $P < 0.01$
11. PIA + DEX + insulin (1.7 nM)	187 ± 37 (4)	143 ± 27 (4)	52 ± 10 (4)	75 ± 25 (4)

Rats were fed the corn oil diet and treated for 10 days with 10 mg of D-fenfluramine/kg. Samples of adipose tissue were incubated for 24 hr [6] with 10 nM dexamethasone (DEX), 1 μM phenylisopropyladenosine (PIA) or the concentration of insulin as indicated. The results are means ± SD for the number of rats given in parentheses. The P values show the significance of the difference between control and fenfluramine-treated rats under the incubation conditions as indicated.

decrease in the weight of the two epididymal fat pads when expressed in either absolute terms or relative to the body weight (Table 1). The fat content of the pads was decreased by about 37%. There was also a 42% decrease in the mean cell volume of the adipocytes recovered after collaginase digestion. This procedure underestimates the numbers of very large adipocytes [12] which are fragile, and which should be more abundant in the control rats. It may also underestimate the numbers of very small adipocytes which should predominate in the treated rats since these cells may not float. Consequently, the adipocyte volumes given in Table 1 may only express a minimum difference between the control and treated rats.

Effects of D-fenfluramine on the uptake of glucose and the production of lactate by adipose tissues. Adipose tissue from control and D-fenfluramine-treated rats was incubated with various combinations of insulin, dexamethasone and phenylisopropyladenosine. The rates of glucose uptake and lactate production were then measured (Table 2). As expected, insulin stimulated glucose uptake whereas dexamethasone decreased it [11]. These actions were mutually antagonistic, and the higher concentration of insulin reversed the dexamethasone effect. Phenylisopropyladenosine was used to counteract effects on the N_i complex of adenylate cyclase and so decrease cyclic AMP concentrations in the adipose tissue. It therefore exerts an insulin-like effect. The only significant difference in the uptake of glucose between the control and treated animals in all of these incubation conditions was the increase obtained in tissue from the D-fenfluramine-treated rats in the presence of dexamethasone.

Lactate production appeared to be consistently higher in adipose tissue taken from D-fenfluramine-treated rats (Table 2). This reached the level of statistical significance in all incubations that contained the lower concentrations of insulin (0.17 nM). Furthermore, lactate production was higher in tissue from the D-fenfluramine-treated rats when these were incubated with dexamethasone alone, or with 1 μM phenylisopropyladenosine and 0.17 nM insulin.

Effects of fenfluramine treatment on the conversion of [^{14}C]glucose to lipids and CO_2 by adipose tissue. The incorporation of glucose into total lipid, fatty acid and glyceride-glycerol was determined after a 2-hr incubation in the presence or absence of a saturating concentration (17 nM) of insulin. The latter addition stimulated lipid synthesis as expected (Table 3). Prior treatment of the rats with D-fenfluramine decreased the incorporation of [^{14}C]glucose into triacylglycerol in the presence or absence of insulin. The effects were most pronounced in decreasing the synthesis of fatty acids rather than the incorporation into the glycerol moiety of the triacylglycerol. D-Fenfluramine treatment, however, did decrease significantly the latter incorporation in the absence of insulin, but not in its

Table 3. Effects of chronic treatment of rats with D-fenfluramine on the incorporation of [U-^{14}C]glucose into triacylglycerol by epididymal fat pads

	Incorporation of [U-^{14}C]glucose (nmol/pair of fat pads per hr)			
	Insulin absent		Insulin present (17 nM)	
Incorporation into:	Control	Fenfluramine treated	Control	Fenfluramine treated
Total lipid	722 ± 394 (16)	420 ± 184 (16)	1237 ± 402 (12)	876 ± 319 (12)
	$P < 0.01$		$P < 0.05$	
Fatty acid	280 ± 194 (16)	99 ± 70 (16)	614 ± 264 (12)	292 ± 171 (12)
	$P < 0.001$		$P < 0.002$	
Glycerol moiety	398 ± 136 (16)	260 ± 82 (16)	622 ± 139 (12)	592 ± 151 (12)
	$P < 0.002$			

Rats were fed the corn oil diet and treated for 10 days with 10 mg of D-fenfluramine/kg. Samples of the epididymal fat pads were incubated to measure the rates of [^{14}C]glucose incorporation into lipid. Results are means ± SD for the numbers of rats indicated in parentheses. The P values show the significance of the differences between control and treated animals.

Table 4. Effects of chronic treatment of rats with D-fenfluramine on the incorporation of [U-^{14}C]glucose into fatty acid by epididymal fat pads

	Incorporation (nmol/hr per pair of pads)	
Additions	Control	Fenfluramine treated
1. None	334 ± 153	87 ± 27*
2. Insulin (0.17 nM)	620 ± 219	169 ± 100†
3. Dexamethasone (10 nM)	353 ± 53	81 ± 38‡
4. Insulin (0.17 nM) + dexamethasone (10 nM)	867 ± 225	191 ± 129§
5. Phenylisopropyladenosine (1 μM)	616 ± 269	133 ± 75*
6. Phenylisopropyladenosine (1 μM) + insulin (0.17 nM)	943 ± 449	180 ± 114*
7. Phenylisopropyladenosine (1 μM) + dexamethasone (10 nM)	506 ± 76	128 ± 66‡
8. Phenylisopropyladenosine (1 μM) + insulin (0.17 nM) + dexamethasone (10 nM)	743 ± 117	215 ± 153

Rats were fed corn oil diet and treated for 10 days with 10 mg of D-fenfluramine/kg. Samples of the epididymal fat pad were incubated for 2 hr to measure the rates of glucose incorporation into lipid. Results are means ± SD for four rats in each group.

*–§ Significantly different from control rats: *$P < 0.02$; †$P < 0.01$; ‡$P < 0.001$; and §$P < 0.002$.

presence. These effects of fenfluramine have been expressed per pair of fat pads since this is the functional organ in the rat. However, similar significant results are also obtained if the results are expressed per g of tissue.

Further work determined whether the decrease in fatty acid synthesis produced by fenfluramine might also result from an inability of low concentrations of insulin to stimulate lipid synthesis. Addition of 0.17 nM insulin also stimulated fatty acid synthesis (Table 4). However, this exaggerated the differences between the control and the D-fenfluramine-treated rats. The presence of dexamethasone in these short-term incubations (2 hr) had no significant effects on the differences between the control and the fenfluramine-treated rats, although it appeared to facilitate the action of insulin in stimulating fatty acid synthesis (Table 4) as expected [10].

Treatment of the adipose tissue samples with adenosine deaminase failed to produce significant effects on fatty acid synthesis (results not shown). This indicates that an accumulation of adenosine in the incubations for the control rats was not responsible for their higher rates of fatty acid synthesis. Furthermore, addition of phenylisopropyladenosine failed to restore the rate of fatty acid synthesis in adipose tissue of treated rats in the presence or absence of insulin (Table 4).

D-Fenfluramine was also added directly to the incubations to see whether this would mimic the effects seen after treatment *in vivo*. Concentrations of D-fenfluramine at 1 mM stimulated fatty acid synthesis ($P < 0.05$) and inhibited it ($P < 0.025$) at 5 mM (Fig. 2).

The oxidation of [^{14}C]glucose to $^{14}CO_2$ in the presence or absence of insulin appeared to be decreased by 32 and 35%, respectively, in the adipose tissue from eight D-fenfluramine-treated rats compared to eight controls, but this did not reach the level of statistical significance.

DISCUSSION

The rats that were used in the present paper were

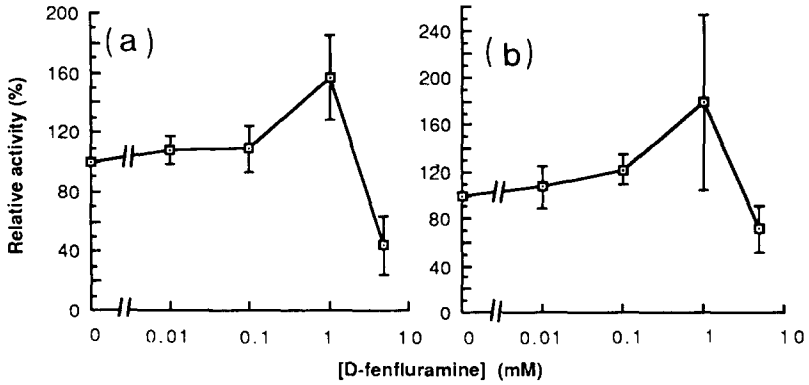

Fig. 2. Effect of direct addition of D-fenfluramine to incubations of adipose tissue pieces on the incorporation of [U-^{14}C]glucose into lipids. The results show the effects of various concentrations of D-fenfluramine on the relative rates of incorporation of [U-^{14}C]glucose into the fatty acid (a) and glyceride-glycerol moieties (b) of triacylglycerol. The incubations were performed in the presence of 1 mM oleate. Results are means ± SD from three independent experiments. They are expressed relative to the activities obtained in incubations where D-fenfluramine was absent. The absolute incorporations were 0.43 ± 0.38 and 0.77 ± 0.19 nmol of glucose incorporated/hr per mg of tissue into fatty acids and glyceride-glycerol respectively. Similar effects of D-fenfluramine were obtained in parallel experiments where oleate was omitted from the incubations.

fed a corn oil diet which contained about 48% of its energy as fat [3] to produce an exaggerated stress response [4, 7, 8, 13]. This is seen particularly in terms of the release of corticosterone after feeding a test meal of fructose [4]. The fat content of this diet is not dissimilar from the diets cf obese human beings in many developed countries. The same dietary model was previously used to study the effects of D-fenfluramine [7, 8] and benfluorex [13]. It was concluded that these compounds counteracted the increased stress reaction in terms of corticosterone release and the increased concentrations of circulating fatty acid and glycerol [7, 8, 13]. This same system was used therefore to study the effects of D-fenfluramine on glucose metabolism.

The most striking result from the present work was the decrease of about 65% in fatty acid synthesis that persisted through the isolation of the fat pads of the rats treated with D-fenfluramine. A decrease of 46% ($P < 0.025$) in the rate of fatty acid synthesis was also obtained after D-fenfluramine treatment when the rats were fed on a standard laboratory diet rich in carbohydrate (results not shown). These results confirm that the animals did not become tolerant to the metabolic action of D-fenfluramine [7, 8]. Previous work had indicated that treating obese patients with fenfluramine decreased the rate of fatty acid synthesis in adipose tissue biopsies, but the results did not reach the level of statistical significance [14]. The decreased fatty acid synthesis that was observed after treatment *in vivo* was unlikely to have been caused by a direct action of D-fenfluramine. Inhibitions were only obtained when more than 1 mM D-fenfluramine was added to the incubations (Fig. 2; Refs 1, 15, 16). It is doubtful whether adipose tissue *in vivo* could be exposed to such high concentrations of D-fenfluramine, or its metabolites.

D-Fenfluramine treatment had relatively less effect on fatty acid esterification than on fatty acid synthesis (Table 3). The lower rates of lipid synthesis may reflect the decreased size of the adipocytes of the fenfluramine-treated rats (Table 1). For example, the rates of lipogenesis [17] and glycerolipid synthesis [9] can increase with increasing adipocyte size. However, this relationship does not always occur. The recovery in the capacity of adipocytes from starved rats to synthesize glycerolipids occurs before there is an increase in cell size [18]. This restored capacity to synthesize fatty acids and glycerolipids after starvation takes place within hours of refeeding and it often exceeds the rates obtained before starvation [18–21]. It is therefore unlikely that the anorexia seen during days 1–2 [7, 8, 22] could have been solely responsible for the decreased lipid synthesis that was observed in adipose tissue of rats treated with D-fenfluramine (Tables 3 and 4). These rats had been eating quantities of food that were not significantly different from the controls for the 6 days prior to the experiment [7, 8], and the incubations were supplemented with glucose and in some cases insulin. Furthermore, the rate of glucose incorporation into triacylglycerol was 40% lower in adipose tissue from adult female rats treated with D-fenfluramine compared to pair fed controls (A. Al-Sieni, L. Hopkins, R. Inman and D. N. Brindley, unpublished work). These assays were performed in the presence of 5.5 mM glucose and 0.17 nM insulin. It is concluded, therefore, that D-fenfluramine has persistent effects of its own in addition to any residual effects resulting from the initial decrease in food intake.

The decreased lipid synthesis in adipose tissue of treated rats could also have resulted from an altered endocrine balance that was induced by D-fenfluramine. Chronic treatment with D-fenfluramine can improve insulin sensitivity and decrease the action of stress hormones, particularly glucocorticoids, in regulating metabolism [2, 7, 8, 23]. The maintenance of glucose uptake and increased lactate production in fat pads from the treated rats despite

a decrease in fat pad size is compatible with this prediction. We have also measured glucose uptake and lactate production by pieces of parametrial fat obtained from adult female rats (A. Al-Sieni, L. Hopkins, R. Inman and D. N. Brindley, unpublished work). These results also confirmed that fenfluramine treatment increases both glucose uptake and lactate production.

The higher rates of lactate production from the adipose tissue of D-fenfluramine-treated rats were observed in incubations containing suboptimum (0.17 mM) insulin rather than with 1.7 nM insulin (Table 2, row 2). This pattern was also observed when dexamethasone or phenylisopropyladenosine was present (Table 2; rows 5, 8 and 10). Incubation with dexamethasone inhibited lactate production (Table 2, row 4; [11]). This effect was also seen in the presence of phenylisopropyladenosine and 0.17 nM insulin (Table 2, row 10) for the control rats. However, there appeared to be no inhibition of lactate output under these conditions with the tissue from the D-fenfluramine-treated rats. The combined results from Table 2, therefore, indicate that the adipose tissue of D-fenfluramine-treated rats is more sensitive to low concentrations of insulin and less sensitive to the effects of glucocorticoids with respect to glucose uptake and lactate production.

The control of fatty acid synthesis cannot be equated to insulin sensitivity as can the uptake of glucose. Low rates of fatty acid synthesis for the fenfluramine-treated rats occurred at the same time as relatively high rates of glucose uptake (Table 2), and fatty acid synthesis could not be returned to control levels by adding saturating concentrations of insulin (Table 3) or phenylisopropyladenosine (Table 4). Incubation of adipose tissue with glucocorticoids for 24–48 hr decreased glucose uptake and lactate production and antagonized the effect of insulin (Table 2; Refs 10, 11, 24, 25). It can also decrease the rate of fatty acid synthesis, but the combination of glucocorticoids with insulin leads to a further enhancement of fatty acid synthesis (Table 4; Refs 10, 26). Glucocorticoids also increase the activity of lipoprotein lipase when they are added with insulin [27]. Thus, insulin and glucocorticoids together increase the availability of fatty acids for triaclyglycerol synthesis and thereby promote energy storage. This may partly explain the excessive deposition of triaclyglycerol in adipose tissue that is observed in Cushing's disease. Also, the concentrations of insulin and cortisol increase in human beings after the consumption of meals [28–30], and this probably promotes energy storage.

Chronically, D-fenfluramine decreases fatty acid synthesis in adipose tissue by diverting the flux of glucose into lactate production and away from energy storage. The lactate is probably liberated into the blood since chronic treatment of adult female rats with D-fenfluramine increased circulating lactate concentrations by about 25% (A. Al-Sieni, L. Hopkins, R. Inman and D. N. Brindley, unpublished work). However, fenfluramine treatment does not produce a lactic acidosis [31, 32]. Lactate is a metabolite that seems to be preferred to glucose by the liver [33–35], and it is probably metabolized mainly by this organ. The production of lactate by muscle during exercise is well-known. It now seems likely that glucose is metabolized, to a significant extent, to lactate by adipose tissue (Table 2; Refs 11, 36, 37) and this may be particularly important postprandially.

It may be significant in this respect that treatment of rats with D-fenfluramine increases the glycogen content in the liver [38, 39]. Thus, the action of fenfluramine in diverting glucose metabolism away from lipid deposition and towards lactate production could be an important aspect of its action in treating obesity and in decreasing adipose tissue mass.

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