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EFFECTS OF FENFLURAMINE ON HEPATIC INTERMEDIARY METABOLISM

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Abstract—Effects of fenfluramine on hepatic intermediary metabolism have been studied using the isolated hepatocyte system.

Fenfluramine inhibits the formation of glucose from lactate plus pyruvate and from alanine as well as the production of ketone bodies from added oleate. The latter observation suggests that inhibition of gluconeogenesis may result from a decrease in the level of mitochondrial acetyl-CoA.

Fatty acid synthesis by hepatocytes is slightly stimulated by fenfluramine, perhaps as a consequence of the increase in cystosolic reducing equivalents, observed as an increase in the ratio of lactate/pyruvate.

In the course of our studies on the hormonal regulation of hepatic lipogenesis we became interested in finding new inhibitors of intermediary metabolism. If it can be shown that their action is specific, such compounds may be potent tools in studies on metabolic regulation.

Fenfluramine, a racemic mixture of the *d*- and *l*-forms of 1-(3-trifluoromethylphenyl)-2-[*N*-ethylamino]propane, is currently being used as a drug in the treatment of obesity and diabetes. The compound has been implicated as an agent affecting carbohydrate and lipid metabolism [1–5]. The biochemical bases for its actions are not clear.

The present experiments were designed to further elucidate the effects of fenfluramine on hepatic carbohydrate and lipid metabolism. Attention has been focused on the acute effects of the drug since, if the drug has potential as a tool in biochemical studies, it will be of most interest in short-term manipulation of metabolism.

Parts of this study have been presented in preliminary form [6].

MATERIALS AND METHODS

Isolation and incubation of hepatocytes. Hepatocytes were isolated from male Wistar rats (225–250 g) which had free access to water and were meal-fed a stock pelleted diet between 4 a.m. and 7 a.m. by an automatic feeding machine. The animals were sacrificed at 9 a.m. Liver cells were isolated according to Seglen [7]. Assessment of cell viability and incubation of hepatocytes were carried out as described previously [8].

Glucose and lipid synthesis. The rate of gluconeogenesis was determined using hepatocytes from 24-hr starved rats incubated for 60 min with gluconeogenic substrates in the absence or presence of fenfluramine. Incubations were terminated with $HClO_4$ (final concentration 0.5 M) and glucose production was assayed by the GOD–Perid method.

To monitor rates of fatty acid synthesis ^3H_2O (0.3 mCi/ml) and 10 mM glucose were added to the cell suspension isolated from fed rats. Incubations were terminated after 60 min by addition of $HClO_4$ (final concentration 0.5 M). Fatty acids were extracted according to Harris [9] and Kates [10].

Cellular uptake of fatty acids. To study the uptake of fatty acids cells from fed rats were incubated with 1 mM [1-^{14}C]oleate. After 15 min cells were separated from the medium by spinning through silicone oil as described before [11]. Fatty acid uptake was taken as total radioactivity associated with the cells after centrifugation through the organic layer. The uptake was linear for at least 15 min both in the absence and in the presence of fenfluramine.

Esterification of fatty acids. Cells from fed rats were incubated for 1 hr with 2 mM [1-^{14}C]oleate. Lipids in aliquots of the cell suspension were extracted according to Sundler *et al.* [12] and subjected to thin-layer chromatography. Neutral lipids were separated on silica gel G using petroleum ether (b.p. 40–60°)-diethylether-acetic acid (80 : 20 : 2; v/v) as developing solvent. The silica, containing the triacylglycerols, was scraped from the plate and counted for its radioactivy.

Krebs-cycle activity. In order to detect possible effects of fenfluramine on the Krebs cycle, the oxidation of [2-^{14}C]pyruvate was monitored. Hepatocytes from fed rats were preincubated for 45 min in the absence or presence of various concentrations of the drug. Following this preincubation, samples were removed from the incubation flasks for the determination of pyruvate and 1 μCi [2-^{14}C]pyruvate was added to the cell suspension. After 5 min of continued incubation 0.25 ml 6 N HCl was added to the incubations and the $^{14}CO_2$ released was trapped in 0.1 ml 2 N KOH absorbed on filter paper in center wells suspended from serum stoppers. For calculating the oxidation of pyruvate the $^{14}CO_2$ released from [2-^{14}C]pyruvate was corrected for differences in pyruvate levels due to drug treatment.

Ketogenesis. Rates of ketogenesis from endogenous as well as from added fatty acids were estimated

using hepatocytes from fed donor rats. Cell suspensions, incubated in the absence or presence of fenfluramine, were quenched with $HClO_4$ (final concentration 0.5 M) and ketone bodies were measured in neutralized aliquots.

Assay of oxygen consumption. The rate of oxygen consumption was followed with a Clark oxygen electrode in a closed reaction vessel. The incubation medium (2 ml) contained 3–4.5 mg of mitochondrial protein or 4–5 mg of cellular protein. In the studies with mitochondria the final concentration of ADP, when added, was 0.15 mM.

Assay of metabolites. Metabolite assays were conducted on KOH-neutralized $HClO_4$ extracts of hepatocytes according to the methods of Hohorst [13] for pyruvate and lactate, Williamson *et al.* [14] for acetoacetate and D-3-hydroxybutyrate and Hassid and Abraham [15] for glycogen.

Sources of materials. Radioactive compounds were obtained from the Radiochemical Centre, Amersham; collagenase type I and bovine serum albumin were purchased from Sigma; other enzymes, GOD–Perid kits and biochemicals were from Boehringer; most other chemicals were from Baker; fenfluramine was a gift from Servier, Paris.

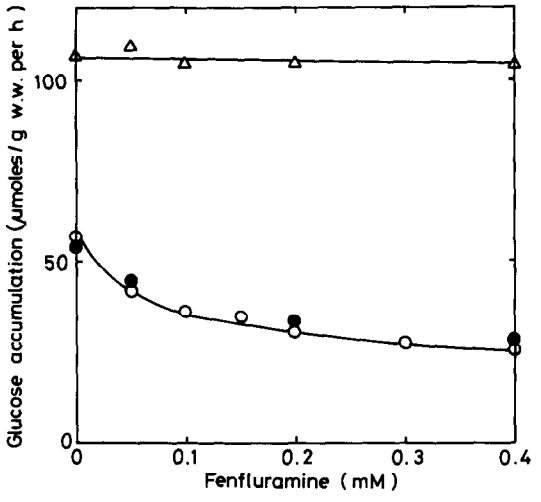

Fig. 1. Effect of fenfluramine on gluconeogenesis. Glucose formation from 10 mM lactate plus 1.5 mM pyruvate (○), from 10 mM alanine (●) or from 10 mM dihydroxyacetone (△) by hepatocytes from 24-hr starved rats was monitored by measuring net glucose production.

RESULTS

Effect of fenfluramine on gluconeogenesis. Gluconeogenesis from lactate plus pyruvate and from alanine by hepatocytes isolated from 24-hr starved rats is progressively inhibited by increasing concentrations of fenfluramine (Fig. 1). Fifty percent inhibition of gluconeogenesis required about 0.30 mM fenfluramine both with lactate plus pyruvate and with alanine as the gluconeogenic precursor. Gluconeogenesis from dihydroxyacetone, on the other hand, was not affected by addition of fenfluramine (Fig. 1).

Effect of fenfluramine on fatty acid metabolism. In order to explain the inhibitory effect of fenfluramine on gluconeogenesis two possibilities were considered. First, gluconeogenesis is an energy-requiring process. Fenfluramine may interfere with the synthesis of glucose by limiting the energy supply. Therefore, the effect of fenfluramine on oxidative phosphorylation in isolated rat-liver mitochondria and in intact rat hepatocytes was studied. The results (not shown) indicate that oxidative phosphorylation is not significantly affected by the drug. This notion is derived from the observations that following drug addition state-4 respiration in isolated rat-liver mitochondria is unaffected (no uncoupling) and respiration in intact cells is unchanged (no effect on respiratory chain or phosphorylation). This conclusion is further substantiated by the observation that fatty acid synthesis, which is also an energy-requiring process, is not inhibited by fenfluramine (*cf.* Table 3). Secondly, the rate of gluconeogenesis is dependent among other things on the level of acetyl-CoA, an allosteric activator of pyruvate carboxylase which is a key enzyme in gluconeogenesis. Addition of fenfluramine may result in a lower acetyl-CoA level. Therefore, the following aspects of fatty acid metabolism were measured in suspensions of hepatocytes incubated in the absence or presence of fenfluramine: (i) uptake of fatty acids by hepatocytes, (ii) esterification of fatty acids, (iii) Krebs-cycle activity, and (iv) ketogenesis. Of these parameters only ketogenesis is negatively affected by drug treatment (Table 1), supporting the suggestion of a diminution of acetyl-CoA levels in mitochondria. Ketogenesis from a medium-chain fatty acid like octanoate, on the other hand, is not affected by the presence of fenfluramine (data not shown). The suggestion that

Table 1. Effect of fenfluramine on fatty acid metabolism in hepatocytes isolated from fed rats

Fenfluramine (mM)	Krebs-cycle activity*	Esterification†	Ketogenesis‡	Uptake of fatty acids§
0	15.5	47.3	234.6	34.4
0.05	14.5	40.7	210.0	34.1
0.20	14.8	51.7	179.9	33.8
0.40	16.0	42.7	134.3	34.0

* nmoles pyruvate oxidized/mg protein per hr.
† nmoles fatty acid esterified/mg protein per hr.
‡ nmoles ketone bodies formed/mg protein per hr in the presence of 2 mM oleate.
§ nmoles fatty acid taken up/mg protein per hr.

Table 2. Effect of fatty acid on fenfluramine-inhibited gluconeogenesis

Oleate (mM)	Glucose synthesis (μmoles/g w.w. per hr) Control	0.4 mM Fenfluramine	% Inhibition
0	63.0	30.2	52.1
0.05	66.9	33.2	50.4
0.10	70.4	37.5	46.7
0.20	71.6	40.3	43.7
0.50	72.8	50.9	30.1

inhibition of gluconeogenesis is due to a decrease in the mitochondrial acetyl-CoA concentration is supported by the observation that increasing fatty acid concentrations gradually diminish the inhibition of gluconeogenesis by fenfluramine (Table 2).

Effect of fenfluramine on fatty acid synthesis monitored with ^3H_2O. Bicarbonate-buffered suspensions of hepatocytes were incubated in the presence of 10 mM glucose as the sole exogenous carbon source and of fenfluramine at various concentrations. The incorporation of ^3H_2O into fatty acids was determined over the 0–60 min incubation period. The ^3H_2O method is considered the most reliable method available to assess rates of fatty acid synthesis [16]. Table 3 documents that the effect of fenfluramine on fatty acid synthesis is slightly stimulatory.

Effect of fenfluramaine on metabolite levels of hepatocytes. The rate of *de novo* fatty acid synthesis by isolated rat hepatocytes is strongly influenced by the flux of glucose carbon through the glycolytic pathway [9]. For this reason, the effect of fenfluramine on the levels of glucose and glycogen and on the rate of glycolysis was investigated. The decrease of cellular glycogen is not affected to any appreciable extent by the presence of the drug (Table 4). In the presence of fenfluramine the accumulation of glucose in the cell suspension is slightly lowered, indicating an increased utilization of glucose. However, the increase in the accumulation of lactate plus pyruvate and in the rate of fatty acid synthesis in the presence of fenfluramine is in balance with the decrease in the accumulation of ketone bodies (Table 4). Therefore, fenfluramine does not affect the flux of glucose carbon through the glycolytic sequence.

Table 4. Effects of fenfluramine on metabolite levels

Metabolite	$t = 0$	$t = 60$ Control	$t = 60$ Fenfluramine
Glycogen*	320	285	285
Glucose*	235	253	246
Lactate†	82	114	147
Pyruvate†	17	41	32
Acetoacetate†	11	61	28
D-3-hydroxybutyrate†	9	16	11

* μg/mg protein.
† nmoles/mg protein.
Hepatocytes were incubated for 60 min with 10 mM glucose in the absence or presence of 0.4 mM fenfluramine. Data are expressed as means of results obtained with two preparations of hepatocytes.

DISCUSSION

In short-term incubations (up to 1 hr), fenfluramine provokes a change in the cytosolic redox state of isolated hepatocytes. The presence of this drug results in an increase of the lactate level and a decrease in the concentration of pyruvate. This increased reduction level in the cytosol (lactate/pyruvate ratio) may be responsible for the slight enhancement of lipogenesis.

Fenfluramine inhibits gluconeogenesis and ketogenesis in isolated hepatocytes. The rate of glucose formation is strongly dependent on the concentration of mitochondrial acetyl-CoA, which is a positive allosteric effector of pyruvate carboxylase, a key enzyme in gluconeogenesis. It is suggested that the inhibition of gluconeogenesis by fenfluramine is the result of a decrease in the mitochondrial acetyl-CoA concentration, which in turn might be the consequence of an inhibition of fatty acid oxidation (ketogenesis) by the drug. This suggestion is supported by the observation that increasing fatty acid concentrations gradually diminish the inhibition of gluconeogenesis by fenfluramine. Furthermore, gluconeogenesis from dihydroxyacetone, which enters the gluconeogenic sequence beyond pyruvate carboxylase, is unaffected by the presence of fenfluramine. This indicates that the site of fenfluramine action is at the early stages of gluconeogenesis.

Table 3. Effect of fenfluramine on fatty acid synthesis by hepatocytes isolated from fed rats. Incorporation of ^3H_2O into fatty acids

Fenfluramine (mM)	Fatty acids (μmoles acetyl units/g w.w. per hr)	Stimulation (%)
0	9.8 ± 0.2	
0.05	10.5 ± 1.0	7.7
0.10	10.6 ± 0.2	8.2
0.15	11.2 ± 0.3	14.3
0.20	11.7 ± 0.2	19.4
0.30	11.4 ± 0.3	16.3
0.40	11.5 ± 0.1	17.3

For their oxidation long-chain fatty acids like oleate are activated mainly extramitochondrially [17]. Since the mitochondrial inner-membrane is impermeable for CoA-esters the activated fatty acids are converted into the corresponding carnitine esters by carnitine acyltransferase [18]. Medium-chain fatty acids, however, are activated mitochondrially and do not use the carnitine acyltransferase system [19]. Taken together with an inhibition of ketogenesis from long-chain fatty acids the lack of effect on the oxidation of medium-chain fatty acids suggests a site of drug action at the level of carnitine acyltransferase.

Fenfluramine mimics in many ways the short-term actions of insulin on the metabolic processes in liver. Insulin addition to hepatocytes changes the metabolism within the hepatocyte. The consequences of insulin addition include an activation of lipogenesis [20], an increase in cytosolic redox state [20], and an inhibition of ketogenesis [21] and gluconeogenesis [22]. These are the same effects that are observed with fenfluramine. The fact that fenfluramine acts at least in part to influence the same processes of hepatic metabolism in the same way as insulin substantiates its use as a suitable antidiabetic agent.

In vivo experiments have shown that fenfluramine is metabolized in the body [23]. It is not known yet whether the parent compound, fenfluramine, or (a) metabolite(s) are responsible for the actions of fenfluramine on the metabolism of the hepatocytes. Details concerning the active principle of this drug await further investigation.

In conclusion, this paper describes an inhibition of gluconeogenesis and of ketogenesis in isolated hepatocytes incubated with fenfluramine. It is suggested that the inhibition of gluconeogenesis is the result of a decrease in the mitochondrial acetyl-CoA concentration, which in turn might be the consequence of an inhibition of fatty acid oxidation possibly at the level of carnitine acyltransferase.

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