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THE INFLUENCE OF CLOFIBRATE ON LIPID TURNOVER IN MOUSE TISSUES

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The double isotope ratios of individual lipid fractions in major tissues of mice were determined before and after treatment with the peroxisome proliferator, clofibrate. Several significant alterations in these ratios were caused by this treatment, with the triglycerides of liver, heart and adipose tissue being indicated as the most responsive fractions. These data establish that clofibrate perturbs lipid metabolism in all the major tissues of this animal, and indicate that this response is not solely due to an increase in the number and oxidative capacity of peroxisomes. Instead, the increased metabolic flux with clofibrate treatment appears to be multivalent in causation, and associated with the interactions with thyroxine metabolism and increased activity of several non-peroxisomal enzymes involved in lipid metabolism, in addition to peroxisomal influences.

One of the most significant findings in recent research on lipid metabolism has been the demonstration of the β-oxidation capacity of peroxisomes in mammalian tissues (1). For many years, fatty acid oxidation had been considered to be the exclusive preserve of the mitochondrial compartment of cells, but it is now clear that the peroxisomes in liver are capable of playing an important role in this major pathway of lipid degradation (2). Various workers have shown that up to half the fatty acids in liver may be catabolized via the peroxisome, and that the enzymes of this pathway have a specificity towards the longer fatty acid chains and are induced by treatments with hypolipidemic agents such as clofibrate (chlorphenoxyisobutyric acid ethyl ester) (3-6).

Several significant questions remain unanswered, however, in relation to the ramifications of the peroxisomal influence of lipid metabolism. While liver is known to be the major location of these organelles in mammalian tissues, for example, appreciable distributions do occur in other tissues. Neither the contribution of these extrahepatic peroxisomes nor the nature of the interactions between the major sites of lipid metabolism in response

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to peroxisomal proliferation have yet been defined, though, as most previous studies have been restricted to isolated tissue preparations rather than a consideration of metabolism in the whole animal.

The present studies are aimed at meeting some of these deficiencies in our present understanding of the interactions between hypolipidemic agents and peroxisomal lipid metabolism, by investigating the effect of clofibrate on the turnover of the principal lipid classes in all the major tissues of the mouse.

MATERIALS AND METHODS

All lipid standards used for thin-layer chromatography were obtained from Sigma Company, (St. Louis, Missouri, U.S.A.). Silica gel G was obtained from Merck, Darmstadt, Germany. [2(n)-^{3}H]-glycerol, [2-^{14}C]-glycerol, and ACS scintillant were purchased from the Radiochemical Centre Ltd, Amersham. Chlorophenoxyisobutyric acid (clofibrate) was a gift from ICI Australia, Ltd. Mice were obtained locally. All other chemicals and solvents were analytical grade.

Mature female mice (Quackenbush strain) were placed on a clofibrate diet (0.35% in food pellets ad libitum) for 14 days. Labelled glycerol was injected intraperitoneally in a volume of 0.5 ml of 0.9% sodium chloride. On the first day 10 µCi of [2-^{14}C]-glycerol were administered to the mice, followed by 15 µCi of [2(n)-^{3}H]-glycerol seventy two hours later. Three hours after the latter injection, the mice were killed and the tissues quickly removed and snap-frozen in liquid nitrogen.

Tissue lipids were extracted using the method of Folch, Lees and Sloane Stanley (7), and stored at -20°C under nitrogen. For separation of neutral lipids, extracts were chromatographed on thin layer Silica G plates (20 cm x 20 cm) in a solvent consisting of hexane - diethyl ether - acetic acid (80:20:1). Polar lipids were separated by developing plates in a solvent mixture consisting of chloroform - methanol - ammonia (65:25:2).

After chromatography was complete, plates were air dried and sprayed with rhodamine B (0.05% in ethanol) to enable visualization of the various lipid components, relative to lipid standards run concurrently.

The lipid bands were then scraped off the plates and counted directly for radioactive labelling. Where both the quantity of lipid separated and the radioactive labelling of each lipid class were being determined, two equal extracts were developed separately on one silica gel plate, and only one chromatogram was stained with Rhodamine B, the other being used for quantification of lipid in the separate lipid classes. Total tissue lipid extract or lipids separated by thin-layer chromatography were quantitated by the method of Amenta (8). This involved oxidizing the lipid with 0.25% potassium dichromate in 36N sulphuric acid, and measuring the change in absorbance at 350 nm, relative to lipid standards.

The lipid classes which had been detected on Silica gel with Rhodamine B were scraped off and added to scintillation vials. To each vial was then added 1 ml of water and the slurry mixed rapidly. Finally 10 ml of scintallant was added. For total tissue lipid extracts, aliquots were added directly to 10 ml of scintillation cocktail. Samples were counted on a Beckman LS-250 scintillation counter, using the automatic quenching correction mode set up with relevant quenched standards. The channels chosen were the standard ^{14}C

isoset (range 300-490) and a narrow tritium window (0-200). Counting was normally to 5% error or less, and the double isotope ratios were calculated by dividing ^{3}H counts per minute by ^{14}C counts per minute for each sample.

RESULTS AND DISCUSSION

The results for the analyses of double isotope ratios in the tissue lipids of normal animals and those treated with clofibrate are listed in Table 1. It is apparent from these data that this administration of clofibrate has caused significant alterations to many of the double isotope ratios; and these changes are discernible not only in the liver, but also in all the other tissues analysed. Furthermore, a general similarity of response is discernible within these analyses in relation to the direction of these effects and their localization within individual lipid classes. In liver, for example, a marked increase in the double isotope ratio is discernible in both the triglyceride and neutral lipid fractions; and

TABLE I. The Effect of Clofibrate on the Double Isotope Ratios of Mouse Tissue Lipids.[a]

Lipid Fraction	Liver	Kidney	Heart	Adipose Tissue	Carcass
Total Lipid	28.8±5.8 (23.2±0.1)	6.6±0.6 (6.0±0.6)	6.9±2.0 (5.5±0.6)	2.5±0.4 (0.8±0.1)	1.9±0.5 (0.5±0.2)
Triglyceride	48.6±4.9* (23.2±4.2)	4.5±0.6* (2.0±1.4)	15.8±3.7* (4.9±1.3)	1.5±0.1* 0.3±0.1	0.9±0.3* (0.2±0.1)
Total Phospholipid	23.8±4.1 (19.8±1.3)	7.0±0.9 (6.7±0.8)	5.1±1.5 (5.2±0.7)	10.5±2.3 (7.9±0.9)	6.3±0.4 (5.5±0.6)
Phosphatidyl Ethanolamine	26.2±0.2 (26.2±0.2)	2.5±0.2 (3.2±0.3)	2.1±0.9 (2.9±0.7)	4.9±2.1 (4.4±0.4)	3.2±0.8 (2.8±0.6)
Phosphatidyl Choline	30.0±2.7* (24.5±0.4)	12.8±1.3 (12.4±2.0)	6.5±2.4 (5.3±0.6)	13.0±5.6 (10.6±1.1)	8.9±1.1 (7.6±1.3)
Phosphatidyl Serine and Inositol	9.6±1.0* (6.5±0.9)	3.3±0.8 (2.1±0.4)	2.8±0.8 (2.7±0.7)	6.9±3.0 (3.1±0.5)	4.4±0.2 (3.5±0.7)
Neutral Lipid	43.1±6.9* (21.7±0.1)	2.5±0.3* (1.6±0.5)	15.9±4.0* (6.2±0.7)	1.6±0.2* (0.4±0.1)	0.8±0.2* (0.2±0.1)

[a] - For experimental conditions see Materials and Methods. Double isotope data are given as the means of three separate analyses ± S.E.M. Values for normal control animals are given in brackets. An asterisk indicates a significant divergence from normal ($p > 0.05$).

increases in these two fractions are also observable in kidney, heart, depot lipid and carcass. Next to liver, heart and adipose tissue are indicated as the most responsive of these tissues.

In addition to this commonality of response, it may be noted that significant increases in the double isotope ratio were also observed in the phosphatidyl choline, phosphatidyl inositol and phosphatidyl serine fractions of liver, and the total lipids of adipose tissue and carcass.

The purpose of establishing the comparative magnitudes of the double isotope ratios in this investigation was to clarify the nature of the influence of clofibrate on lipid turnover in the different tissues and lipid classes of this mammal. A similar methodology has been extensively used previously in determinations of the relative rates of synthesis and degradation of a range of biological molecules (8, 13, 14).

Overall, these results serve to establish that clofibrate perturbs lipid metabolism not only in the liver but in all the major tissues of this experimental animal. The localization of the response, and the specific radioactivities of the individual lipid fractions are consistent with an increased degree of fatty acid oxidation in liver and heart, especially, and an associated mobilization of the acylglycerols in adipose tissue and depot fat. Increased utilization of fatty acids from liver phospholipids is also indicated by these results.

Clofibrate has been in clinical use for a number of years as a potent hypolipidemic agent, but the mechanisms by which this drug exerts its physiological effect have not so far been conclusively delineated. The three main theories for the mechanism of action of this drug relate to its role in peroxisome proliferation, involvement in thyroxine effects, and the stimulation of enzyme activities in key areas of lipid metabolism (2).

On the basis of an increase of the number of peroxisomes in tissues along with their content of enzymes involved in β-oxidation (1, 2, 10), the contribution of clofibrate to the metabolic changes observed in these

studies is readily recognizable. Such an increase would be expected to lead to higher turnover of liver triglycerides, for example. It is perhaps surprising, though, that kidney did not participate to a greater extent in these perturbations, when it is commonly recognized as one of the major tissue sources of peroxisomes; and peroxisomal increases alone do not appear to provide a sufficient explanation for all the other observed effects.

Part of the mode of action of clofibrate may be due to its interaction with thyroxine. Early observations demonstrated that clofibrate displaced thyroxine from its serum binding proteins with the net result of a hyperthyroid effect in liver, and a hypothyroid effect in the rest of the body (11, 12), and several other lines of evidence support this theory. On this basis, increased oxygen consumption in the liver resulting from clofibrate treatment would be expected to be accompanied by decreased fatty acid oxidation in non-hepatic tissues, especially heart and skeletal muscle, and hence allow an increased mobilization of lipid from these tissue sources to the liver. In consequence, the thyroxine involvement may well help to explain why triglyceride turnover was greater in heart than kidney with clofibrate treatment.

The other main category of hypothesis on the mode of action of clofibrate seeks to implicate the many key enzymes in the overall metabolism of cholestorol and triglycerides which are influenced by this compound (2). Thus, in addition to catalase and the enzymes of β-oxidation in the peroxisome, carnitine acetyl transferase, NADH oxidation and α-glycerophosphate oxidase have been shown to be increased by clofibrate treatment, whereas acetyl CoA carboxylase and β-hydroxy-β-methylglutaryl CoA synthetase are decreased in activity. Overall, these changes may be expected to favour degradation of fatty acids in the liver, and result in lowered fatty acid and cholesterol synthesis.

From an inspection of this body of evidence, then, it is apparent that clofibrate should be considered as exerting a multivalent effect on inter-

mediary lipid metabolism. The overall impression is one of an influence of lipid metabolism via an increased metabolic flux, but with a preference, to degradation and mobilization of triglycerides in most tissues. Peroxisome proliferation is an obvious and important element in the influence of this reagent, but the interaction with thyroxine metabolism and the augmentation of several non-peroxisomal enzymes are also indicated as playing an important role in the overall metabolic effects which are induced in several major tissues by treatment with this drug *in vivo*.

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