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STIMULATION OF FATTY ACID UTILIZATION BY SODIUM CLOFIBRATE IN RAT AND MONKEY HEPATOCYTES

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Abstract—The acute effects of sodium clofibrate (NaCPIB) on the metabolism of [1-^{14}C]palmitate, [1-^{14}C]octanoate, [1-^{14}C]butyrate, and [2-^{3}H]glycerol by freshly isolated hepatocytes were tested to explore its mechanism of action. Labeled long-, medium-, and short-chain fatty acids were incorporated into all the major lipid classes and were oxidized to $^{14}CO_2$ by the liver cells. The partitioning of labeled fatty acids from lipogenic towards oxidative pathways was inversely related to fatty acid chain length. [1-^{14}C]Palmitate was incorporated mainly into cellular triglycerides and phospholipids; [1-^{14}C]octanoate, mainly into triglycerides and free cholesterol; and [1-^{14}C]butyrate, mainly into free cholesterol and phospholipids of the cells. NaCPIB (1–3 mM) rapidly stimulated the esterification of labeled palmitate or glycerol to triglycerides, but drug levels greater than 5 mM were inhibitory to esterification. NaCPIB (1 mM) increased the oxidation of [1-^{14}C]palmitate to $^{14}CO_2$ by either rat or monkey hepatocytes and enhanced the release of labeled lipids from [2-^{3}H]glycerol-prelabeled cells into the extracellular medium. Acclerated [1-^{14}C]octanoate incorporation into glycerolipids and sterols and increased [1-^{14}C]octanoate conversion to $^{14}CO_2$ were observed in rat liver cells incubated with 1 mM NaCPIB. In contrast, the same drug level stimulated the oxidation of [1-^{14}C]butyrate to $^{14}CO_2$ but greatly diminished its incorporation into hepatocellular sterols or glycerolipids. These results indicate that (a) NaCPIB acutely alters hepatic ultilization of fatty acids by actions at diverse loci; (b) these metabolic alterations vary with fatty acid chain length; and (c) these effects are probably due to rapid changes in biochemical regulatory mechanism and/or in substrate channelling within the cells. These data further suggest that the early hypolipidemic effect of the drug in rats and primates may be related to an enhanced hepatic oxidation of long-chain fatty acids, but cannot be attributed simply to a reduction in their esterification to complex lipids.

The mechanism of action of clofibrate (ethyl α,*p*-chlorophenoxyisobutyrate) is unknown despite a host of prior studies in whole animals and in isolated tissues. Since this agent is usually administered chronically for therapeutic purposes, most investigations involving humans or animal models have been undertaken after clofibrate has been given for at least several days. Under these circumstances, stimulation of the activities of several hepatic enzymes [1–3] and proliferation of hepatic mitochondria and peroxisomes [4, 5] have been observed. Chronic intake of clofibrate has produced dramatic changes in lipid metabolism in the liver [6–8], heart [9], the adipose tissues [10, 11], and in skeletal muscle [12]. Differing conclusions from various laboratories concerning the mechanism of its hypolipidemic effect may be due to use of divergent tissue preparations, diverse assay systems, and varying experimental conditions. Clofibrate or its sodium salt has been administered to animals under a variety of conditions in which the dose, duration, route of drug entry, and feeding state of the animals have differed.

The present investigation was aimed at obviating some of these variables by examination of the early metabolic actions of the sodium salt of this agent when added *in vitro* to suspensions of liver cells freshly isolated from rats or squirrel monkeys. The water-soluble, sodium salt of the drug was used in this study since the clofibrate anion is found in plasma after ingestion of the ethyl ester [13]. Problems of inter-animal variation were minimized in our metabolic experiments by the use of aliquots of hepatocytes prepared from one liver at a time. Since these cells retain active biosynthetic mechanisms and intact subcellular architecture [14, 15], data derived from incubations of hepatocytes with levels of drug usually attained during therapy are likely to relate to early metabolic changes that occur in animals given clofibrate.

The utilization of labeled fatty acids or glycerol by dispersed hepatocytes was examined to delineate some of the initial biochemical effects produced by sodium clofibrate and to determine clues to its mode of action. The results obtained clearly demonstrate that sodium clofibrate produces very rapid, diverse, and probably direct alterations of hepatic lipid metabolism, and they suggest a very early, complex

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sequence of metabolic events that precedes its chronic effects.

MATERIALS AND METHODS

White, male Sprague–Dawley rats (200–250 g), fed Purina Laboratory Chow, and male squirrel monkeys (*Saimiri sciureus*, weighing 400–600 g) fed Purina Monkey Chow plus fruit, were used. [2-^{3}H]Glycerol (200 mCi/mmole), [1-^{14}C]palmitic acid (58.2 mCi/mmole), sodium [1-^{14}C]octanoate (25 mCi/mmole), sodium [1-^{14}C]butyrate (14.9 mCi/mmole), and Aquasol were obtained from the New England Nuclear Corp., Boston, MA. Type II collagenase was from the Worthington Biochemical Corp., Freehold, NJ; Fraction V fatty acid-poor bovine serum albumin (BSA) was from Miles Products, Elkhart, IN; and sodium clofibrate was from Ayerst Laboratories, New York, NY. The sources of the other reagents and products have been detailed previously [14, 16]. Stock solutions of [1-^{14}C]-palmitate (0.22 mM)-BSA (0.66 mM) were prepared by the addition of warm solutions of BSA in saline to the sodium salts of palmitic acid, as described before [17]. Since sodium octanoate and sodium butyrate are soluble in water, these labeled fatty acids were added to incubations without prior conversion to BSA complexes.

Preparation of cells and cell incubations. The livers were perfused by the non-recirculating method previously described [14]. The liver was first cleared of blood with 0.15 M NaCl (15–25 ml) and then with 25 ml of Ca^{2+}- and HCO_3^--free Hanks solution (pH 7.4), followed by perfusion with 50 ml of this solution modified to contain Worthington Type II collagenase (120 units/ml). After filtration, gentle centrifugation and cell washing, a batch of packed hepatocytes was obtained which was suspended in 40 vol. of HCO_3^--free Hanks solution containing 1.25 mM $CaCl_2$ and used for metabolic incubation studies. At least 80% of the cells excluded the vital stain trypan blue (0.25%). Dispersed hepatocytes were incubated in 4-ml aliquots under air at 37° in 25-ml plastic Erlenmeyer flasks in a rotary water-bath shaker at about 120 rotations/min. Each flask contained penicillin (50 units/ml) and streptomycin (50 μg/ml).

Isolation of labeled lipids. After incubations with labeled glycerol, palmitate, or octanoate, labeled lipids were extracted from the hepatocytes by the method of Dole and Meinertz [18]. The flasks were placed in crushed ice, and their contents were individually transferred to 12-ml conical centrifuge tubes. After centrifugation at 100 *g* for 5 min, the supernatant medium was aspirated down to the 0.5 ml level and discarded. The cell pellets were vigorously shaken with 5 ml of isopropanol–heptane (4:1, v/v) to extract the labeled lipids. After separation into two phases by the addition of heptane (3 ml) and H_2O (2 ml) and discarding of the bottom phase, the upper phase was washed free of labeled precursor with 50% ethanolic NaOH [19]. The neutral lipids, mainly triglycerides and cholesterol esters, were recovered in the washed upper phase. The washing procedure resulted in the complete removal of fatty acids or glycerol and the removal of greater than 80% of the phospholipids, free cholesterol, and mono- and diglycerides from the upper phase. Samples of the upper phase which contained esterified lipids were then transferred to glass scintillation vials and dried under a hair dryer. After the addition of 10 ml of scintillation fluid [0.5% 2,5-diphenyloxazole (PPO), 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene (POPOP) in toluene], the samples were counted in a Packard Tri-Carb scintillation counter with an efficiency of 82% for ^{14}C and 42% for ^{3}H.

When [1-^{14}C]butyrate was the labeled precursor or whenever the isolated lipids were to be separated and recovered by thin-layer chromatography, the lipids were first extracted from the cell pellets with chloroform–methanol (2:1, v/v) by the method of Folch *et al.* [20] and washed free of labeled precursor with upper phase. This procedure resulted in greater than 95% recovery in the bottom phase of all the major lipid classes including long-chain fatty acids, free and esterified cholesterol, phospholipids and glycerides. Samples of the bottom phase containing washed labeled lipids were dried and then dissolved in 10 ml of toluene scintillation fluid and were counted as above. [1-^{14}C]Butyrate was removed completely by the washing steps, but only up to 80% of [1-^{14}C]octanoate could be removed from the bottom phase by this method. Therefore, unless the radioactive fatty acids were later to be removed by thin-layer chromatography, neutral lipids labeled after incubations with [1-^{14}C]palmitate or [1-^{14}C]octanoate were isolated with isopropanol–heptane.

Isolation of $^{14}CO_2$. For studies of fatty acid oxidation, 25-ml Erlenmeyer glass flasks with center wells containing 0.5 ml of hydroxide of Hyamine were used. After incubation, $^{14}CO_2$ was released from cell suspensions by the addition of 0.5 ml of 10 N H_2SO_4. The liberated $^{14}CO_2$ was collected in the glass center wells after a subsequent 30-min incubation. When [1-^{14}C]palmitate was the labeled precursor, the acidified suspensions were incubated at 37°. When [1-^{14}C]octanoate or [1-^{14}C]butyrate were used, the acidified mixture was incubated at 4° because of the volatility of these fatty acids at 37°. The center wells were removed, placed in glass scintillation vials containing 10 ml of Aquasol, and then counted.

The significance of differences in labeled substrate utilization between control and sodium clofibrate-supplemented incubations was determined with the Student's *t*-test with data obtained from several batches of hepatocytes.

RESULTS

Effect of sodium clofibrate on [1-^{14}C]*palmitate esterification.* Incubation of cells with sodium clofibrate resulted in a stimulation of labeled palmitate incorporation into the esterified lipids of either rat or monkey hepatocytes (Fig. 1). With rat hepatocytes, the degree of stimulation was about 35% at 0.1 mM levels of the drug and was maximal at levels of 1–3 mM which caused a 60–110% rise in the esterification of labeled palmitate. Sodium clofibrate produced a similar response when added to dispersed

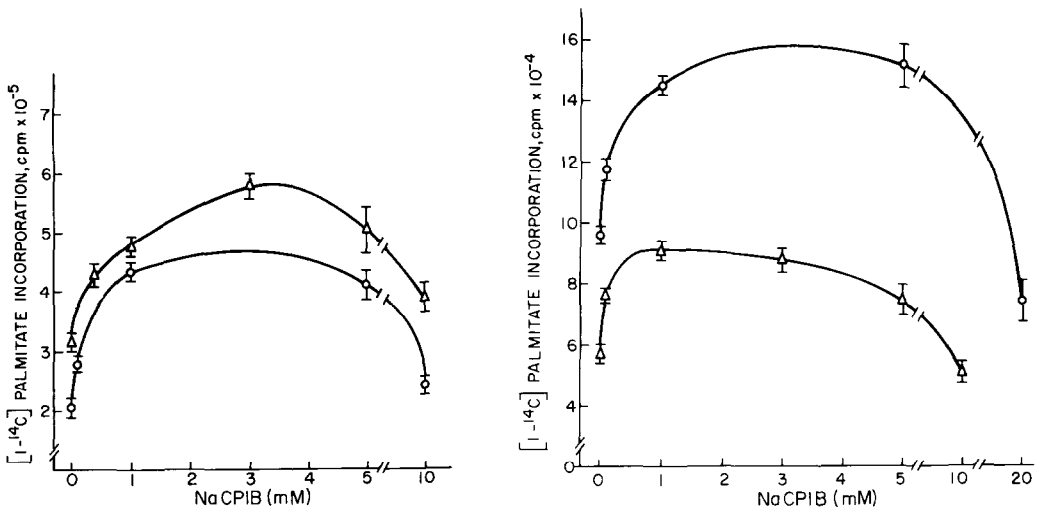

Fig. 1. Effect of increasing concentrations of NaCPIB on [1-^{14}C]palmitate incorporation into esterified lipids by liver cells isolated from rats (left panel) or monkeys (right panel). Duplicate aliquots of hepatocytes were preincubated in 25-ml Erlenmeyer flasks with various levels of NaCPIB for 30 min. [1-^{14}C]Palmitate (2.75 μg) and unlabeled palmitate (25.6 μg) were then added as albumin complexes, and incubation was continued for an additional 30 min. Labeled cellular lipids were extracted with isopropanol–hepatane (4:1; v/v), washed, and counted as described in Materials and Methods. Two different experiments are shown for both rat and monkey hepatocytes in which the experimental conditions were the same, but the cells were isolated from two different animals. Each point represents the cpm in lipids per 10^7 cells ± S.E.M. of duplicate samples.

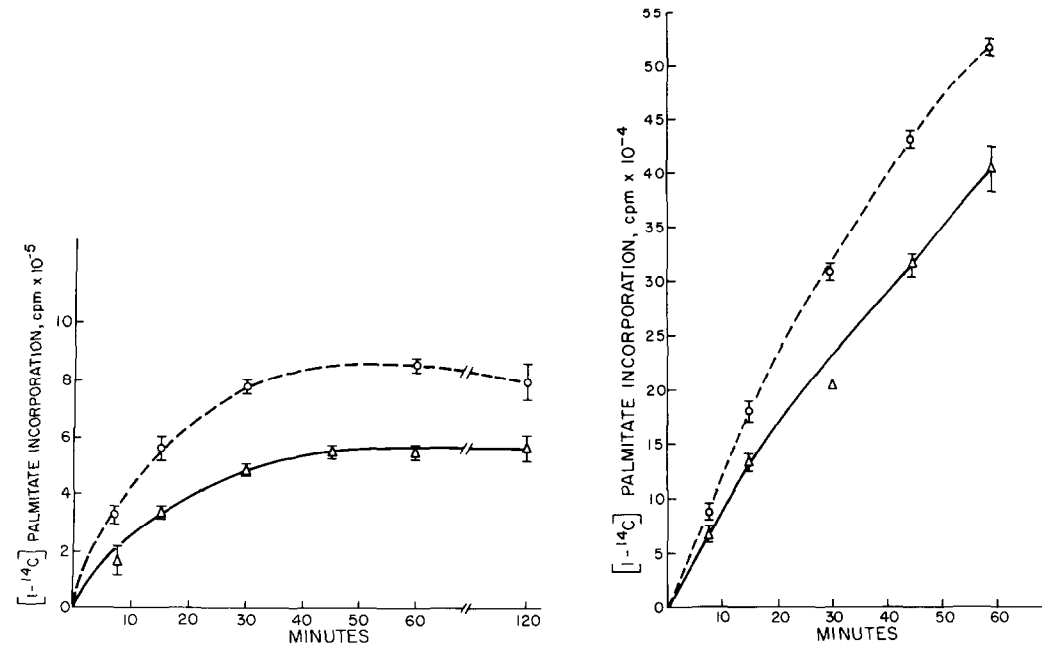

Fig. 2. Time course of incorporation of [1-^{14}C]palmitate into esterified lipids by rat (left panel) and monkey (right panel) hepatocytes in the absence (△) and in the presence (○) of 1 mM NaCPIB. Duplicate aliquots of hepatocytes were incubated for various time periods with [1-^{14}C]palmitate (2.75 μg) and unlabeled palmitate (25.6 μg) added as albumin complexes. Lipids were extracted and counted as in Fig. 1. Each point represents the cpm in lipids per 10^7 cells ± S.E.M. of duplicate samples.

monkey hepatocytes with 50–80% increases observed at concentrations of 1–3 mM (Fig. 1). At drug levels above 5 mM, stimulation of palmitate esterification was not found with liver cells from either species and, at levels of sodium clofibrate above 10 mM, the incorporation of labeled palmitate fell to below control values. Thus, at 1–3 mM levels of sodium clofibrate which approximate therapeutic plasma concentrations [21, 22], the stimulation of fatty acid esterification caused by the drug was maximal in liver cells from both species. No significant differences in the percentage of hepatocytes that excluded the trypan blue dye were found upon comparison of cell incubations exposed to drug levels ranging from 0.1 to 10 mM for 30 min.

The onset of the rise in esterification due to sodium clofibrate was rapid with both rat and monkey hepatocytes as shown in representative time courses (Fig. 2). After 60 min of incubation of rat liver cells with 1 mM sodium clofibrate, the incorporation of [1-^{14}C]palmitate was increased to 1.6 times the mean control value. With the monkey cells, the esterification of labeled palmitate in the presence of the drug was 1.4 times the control value after 60 min of incubation. In nine separate experiments with rat hepatocytes and four with monkey hepatocytes in which duplicate aliquots of cells were incubated with [1-^{14}C]palmitate for 30 min following a 30-min preincubation with 1 mM sodium clofibrate, a highly significant enhancement of fatty acid esterification was observed compared to control incubations. The rates of stimulation (mean ± S.E.M.) due to the drug were 72 ± 8% ($N = 9$, $P < 0.001$) with rat hepatocytes and 46 ± 7% ($N = 4$, $P < 0.05$) with monkey hepatocytes.

Effect of sodium clofibrate on [1-^{14}C]*palmitate oxidation to* $^{14}CO_2$. Sodium clofibrate (1 mM) stimulated [1-^{14}C]palmitate oxidation to $^{14}CO_2$ by both rat and monkey liver cells. The oxidation of labeled palmitate by rat hepatocytes (Fig. 3) was increased at a constant rate over the course of 45 min of incubation. In six separate experiments after 30 min of incubation with label following a 30-min preincubation with sodium clofibrate, the rates of $^{14}CO_2$ production in the presence of the drug ranged from 130 to 365% of control values. The average stimulation (mean ± S.E.M.) was 80 ± 37% ($N = 6$, $P < 0.05$) by 30 min. A similar enhancement was observed in experiments with three batches of monkey hepatocytes in which the drug produced increases in fatty acid oxidation that were 130–170% of control values by 30 min after the start of incubation with label. Since the cellular conversion of [1-^{14}C]palmitate both to $^{14}CO_2$ and to labeled esterified lipids rose after exposure to sodium clofibrate, the increased esterification could not be attributed to an inhibition of fatty acid oxidation by the drug.

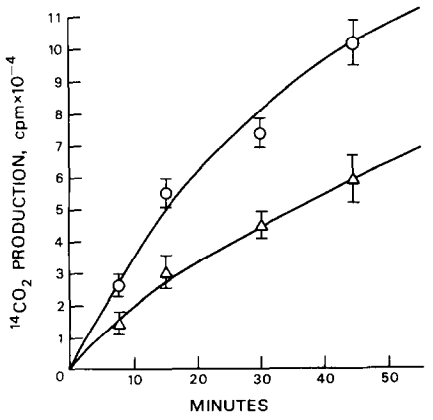

Fig. 3. Time course of [1-^{14}C]palmitate oxidation to $^{14}CO_2$ by rat hepatocytes in the absence (△) and in the presence (○) of NaCPIB (1 mM). Aliquots of rat hepatocytes were incubated for various time periods after the addition of labeled and unlabeled palmitate as in Fig. 1. After incubation, $^{14}CO_2$ was released from cells and medium after acidification and was counted as described in Materials and Methods. Each point represents the cpm in $^{14}CO_2$ per 10^7 cells ± S.E.M. of triplicate samples.

Effect of sodium clofibrate on esterified lipid formation from [2-^{3}H]*glycerol.* The actions of sodium clofibrate on lipogenesis were investigated further in studies using labeled glycerol as the substrate for esterification. As shown in Fig. 4, [2-^{3}H]glycerol was incorporated very rapidly into cellular lipids during incubation and reached a plateau by about 30 min. Preincubation with sodium clofibrate (1 mM) for 30 min enhanced the incorporation of labeled glycerol at each time point (Fig. 4), but this effect required the presence of unlabeled long-chain fatty acid in the incubation medium. The increase in [2-^{3}H]glycerol esterification was observed at 0.1 to 5 mM levels of sodium clofibrate, but was maximal at 1–3 mM levels. Similar to the findings with labeled palmitate, 10 mM sodium clofibrate inhibited [2-^{3}H]glycerol esterification by about 40%. The rise in [2-^{3}H]glycerol esterification after incubations of several batches of rat or monkey hepatocytes with sodium clofibrate (1 mM) was significant with cells from either species. The average stimulation (mean ± S.E.M.) of esterification was 42 ± 10% ($N = 5$, $P < 0.02$) with rat hepatocytes and 52 ± 11% ($N = 3$, $P < 0.05$) with monkey hepatocytes after 60 min of incubation with 1 mM levels of the drug.

Effect of sodium clofibrate on cellular triglyceride production. [1-^{14}C]Palmitate was incorporated primarily into triglycerides by rat or monkey hepatocytes with three-fourths of the labeled cellular lipids found in the triglyceride fraction with rat liver cells and about one-seventh found in the phospholipid fraction (Table 1). The increased hepatic esterification of labeled palmitate caused by sodium clofibrate represented mainly a stimulation of cellular triglyceride formation and, to a lesser extent, an increment in phospholipid synthesis. In the typical experiment shown, increased synthetic rates for cellular triglycerides and phospholipids produced by the drug were about 1.4 and 1.3-fold the control values respectively. A comparable pattern of enhanced [1-^{14}C]palmitate esterification into complex lipids was found when monkey hepatocytes were incubated with 1 mM sodium clofibrate. The increase in labeled cellular triglyceride did not result from a drug-induced inhibition of lipid secretion since sodium clofibrate actually augmented the export of

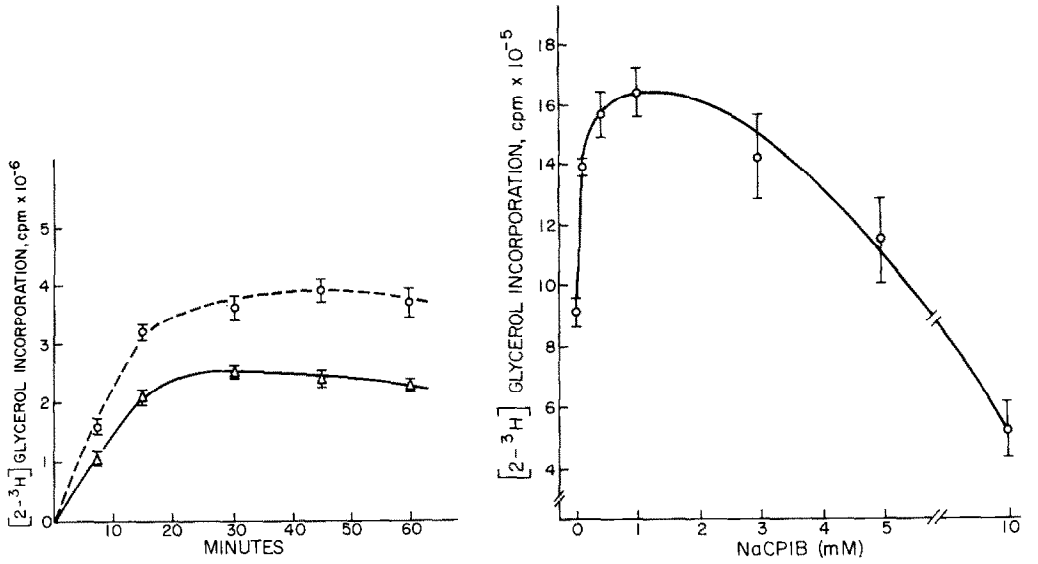

Fig. 4. Effect of NaCPIB on [2-^{3}H]glycerol incorporation into esterified lipids by rat hepatocytes. Two batches of rat liver cells were prepared to examine the time course (left panel) and the dose–response effects (right panel) of NaCPIB on [2-^{3}H]glycerol incorporation into cellular lipids. Duplicate aliquots were preincubated in the absence (△) and in the presence (○) of NaCPIB for 30 min and then were incubated with [2-^{3}H]glycerol (5 μCi) for various periods up to 60 min (left panel). With another batch, duplicate aliquots were preincubated for 30 min with increasing concentrations of NaCPIB (right panel) and then were incubated with [2-^{3}H]glycerol (5 μCi) for an additional 30 min. In each experiment, unlabeled palmitate (25.6 μg) as the albumin complex was added at the beginning of incubation with label. Cellular lipids were extracted with isopropanol–heptane (4:1, v/v), washed, and counted as described in Materials and Methods. Each point represents cpm in cellular lipids per 10^7 cells ± S.E.M. of duplicate samples.

Table 1. Distribution of incorporated [1-^{14}C]palmitate among lipid classes of rat hepatocytes*

	[1-^{14}C]Palmitate incorporation	
Lipid class	Control	NaCPIB (1 mM)
Phospholipids	8.6†	11.2
Monoglycerides	0.4	0.2
1,2-Diglycerides	0.4	0.4
Cholesterol	4.0	3.8
Triglycerides	46.2	72.0
Cholesterol esters	0.4	0.5

* Rat hepatocytes were preincubated for 30 min in the absence or in the presence of 1 mM sodium clofibrate. Then [1-^{14}C]palmitate and unlabeled palmitate were added to the suspended cells as in Fig. 1, and the incubation was continued for an additional 1 hr. Cellular lipids were extracted with chloroform–methanol (2:1, v/v) and washed as described in Materials and Methods. The lipid classes were separated by thin-layer chromatography on silica gel G plates using hexane–diethyl ether–glacial acetic acid (70:20:1, by vol.) as previously described [14]. The various lipid classes were identified using lipid standards. Individual scraped samples were placed in scintillation vials containing toluene scintillation fluid and then counted as described in Materials and Methods.

† Expressed in nmoles incorporated per 10^7 cells.

labeled lipids from rat liver cells into the extracellular medium during incubation as we reported previously [17]. In three separate experiments, 1 mM levels of the drug stimulated the export of labeled lipids from [2-^{3}H]glycerol-prelabeled cells into the medium by 2.7-, 1.8- and 2.0-fold after 1 hr of incubation.

Effect of sodium clofibrate on [1-^{14}C]*octanoate utilization.* Since sodium clofibrate can displace fatty acids from albumin binding sites [23] and since the partitioning of incoming fatty acids between the pathways of esterification and oxidation depends upon fatty acid chain length [24, 25], a series of experiments was carried out to explore the utilization of shorter-chain, water-soluble fatty acids by hepatocytes. Thus, the metabolism of [1-^{14}C]octanoate or of [1-^{14}C]butyrate was examined with several batches of rat hepatocytes incubated in albumin-free medium. As shown in Fig. 5, [1-^{14}C]octanoate was incorporated into lipids and oxidized to $^{14}CO_2$ at a very rapid rate by the cells, and sodium clofibrate (1 mM) stimulated the utilization of labeled octanoate by either pathway. In experiments with different batches of liver cells, the stimulation of cellular lipid synthesis from [1-^{14}C]octanoate with 1 mM sodium clofibrate was 33 ± 11% ($N = 7$, $P < 0.05$) while the mean increase in labeled octanoate oxidation to $^{14}CO_2$ was 22 ± 6% ($N = 4$, $P < 0.05$) after 60 min

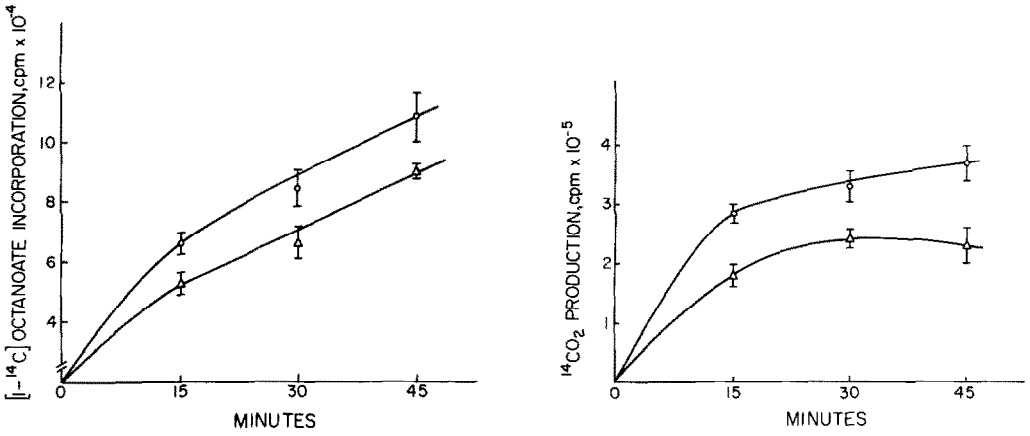

Fig. 5. Time course of incorporation of [1-^{14}C]octanoate into cellular lipids or oxidation of [1-^{14}C]octanoate to $^{14}CO_2$ by rat hepatocytes in the absence (△) or in the presence (○) of 1 mM NaCPIB. Aliquots of liver cells were preincubated with or without the drug for 30 min. Then [1-^{14}C]octanoate (1.7 μCi, 11.3 μg) was added to each flask and incubation was continued for up to 45 min. With some samples (left panel), esterified neutral lipids were extracted with isopropanol–heptane (4:1, v/v) and measured as described in Materials and Methods. With other samples (right panel), $^{14}CO_2$ was released after acidification and subsequent incubation as described in Materials and Methods. Each point represents the cpm in cellular lipids or in $^{14}CO_2$ per 10^7 cells ± S.E.M. of triplicate samples.

of incubation. In other experiments, the drug produced similar degrees of enhanced [1-^{14}C]octanoate utilization at concentrations that ranged from 0.4 to 2.5 mM.

Effect of sodium clofibrate on [1-^{14}C]*butyrate utilization.* In contrast to its enhancement of labeled palmitate or octanoate incorporation into cellular lipids, sodium clofibrate consistently inhibited lipid biosynthesis from [1-^{14}C]butyrate by rat hepatocytes. In the representative time course shown in Fig. 6, 1 mM sodium clofibrate produced about a 25% reduction in the rate of cellular lipogenesis from labeled butyrate by 30 min and a 35% inhibition by 45 min. In other experiments with three separate batches of liver cells, rates of lipogenesis from [1-^{14}C]butyrate were diminished by 62, 56, and 70% with 1, 3, and 4 mM levels, respectively, of the drug after 60 min of incubation. The dose–response relations of this effect with another batch of cells demonstrated a progressive inhibition of lipid synthesis from [1-^{14}C]butyrate that ranged from 21 to 62% as drug levels in the medium were raised from 0.5 to 2.5 mM.

Unlike its effect on [1-^{14}C]-incorporation into lipids, 1 mM sodium clofibrate actually increased the conversion of this labeled fatty acid to $^{14}CO_2$. The drug enhanced the oxidation of [1-^{14}C]butyrate to $^{14}CO_2$ by about one-third after 15, 30, and 45 min

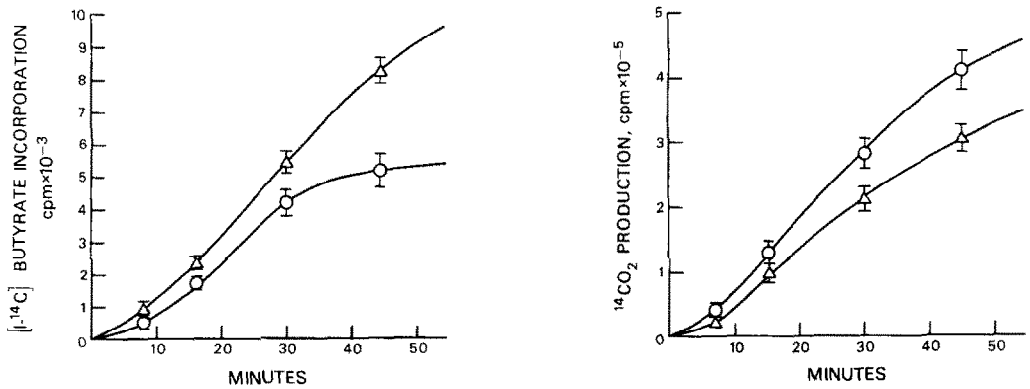

Fig. 6. Time course of incorporation of [1-^{14}C]butyrate into cellular lipids or oxidation of [1-^{14}C]butyrate to $^{14}CO_2$ by rat hepatocytes in the absence (△) or in the presence (○) of 1 mM NaCPIB. Aliquots of liver cells were preincubated with or without the drug for 30 min. Then [1-^{14}C]butyrate (2.5 μCi, 19 μg) was added to each flask and incubation was continued for up to 45 min. With some samples (left panel), cellular lipids were extracted with chloroform–methanol (2:1, v/v) and measured as described in Materials and Methods. With other samples (right panel), $^{14}CO_2$ was released after acidification and subsequent incubation as described in Materials and Methods. Each point represents the cpm in cellular lipids or in $^{14}CO_2$ per 10^7 cells ± S.E.M. of triplicate samples.

of incubation with label (Fig. 6). In a dose–response experiment, the drug-induced stimulation of cellular $^{14}CO_2$ production from [1-^{14}C]butyrate rose from 24 to 79% by 30 min as levels of the drug were raised from 0.1 to 2.5 mM. In two additional experiments, labeled butyrate oxidation to $^{14}CO_2$ was enhanced by 62 and 70% after 1-hr incubations of cells with 2 mM sodium clofibrate.

Effect of sodium clofibrate on distribution of label among specific lipids. Since the effects of sodium clofibrate on cellular lipogenesis from labeled octanoate and from labeled butyrate were found to be strikingly divergent, it was important to identify the specific lipid classes that were synthesized at altered rates during these incubations. Thus, the incorporation of [1-^{14}C]octanoate into the major lipid classes after incubation of liver cells in the absence and presence of the drug was measured after chromatographic separation of the extracted cellular lipids (Table 2). With [1-^{14}C]octanoate as the biosynthetic precursor, over half the labeled lipid was found in triglycerides, about one-third in free cholesterol, and about 5% in phospholipids. The quantities of label recovered in the other lipid classes were minor. However, the extracted lipids of cells that had been incubated with [1-^{14}C]octanoate and with 1 mM sodium clofibrate showed major increases in radioactivity in the triglyceride, free cholesterol, and phospholipid fractions that exceeded the control values by 2.5-, 1.3- and 2.7-fold respectively. Thus, the drug greatly stimulated the biosynthesis of both sterols and esterified lipids using a fatty acid of medium chain length as the labeled precursor.

Unlike palmitate and octanoate, at least half of the [1-^{14}C]butyrate incorporated into lipids in control incubations was found in the unesterified cholesterol fraction; about one-fourth was identified in phospholipids and one-eighth in triglycerides (Table 3). However, upon incubation of cells with [1-^{14}C]butyrate and 1 mM sodium clofibrate, a dramatic change in the distribution of labeled cellular lipids and a striking inhibition of lipogenesis were observed when compared to control incubations. Sodium clofibrate produced reductions of cellular label incorporation into the lipid classes. Thus, the radioactivity found in the cellular free cholesterol, phospholipid, and triglyceride fractions after incubation with the drug amounted, respectively, to only 25, 50, and 70% of the control values. These data strongly suggest that labeled octanoate and labeled butyrate were metabolized by hepatocytes by strikingly dissimilar pathways or by pathways that differed in their mode of regulation.

Table 2. Distribution of incorporated [1-^{14}C]octanoate among lipid classes of rat hepatocytes*

Lipid class	[1-^{14}C]Octanoate incorporation	
	Control	NaCPIB (1 mM)
Phospholipids	2.9†	7.1
Monoglycerides	0.8	1.4
1,2-Diglycerides	0.6	1.1
Cholesterol	16.9	22.7
Triglycerides	25.6	69.8
Cholesterol esters	0.7	0.6

* Rat hepatocytes were preincubated for 30 min in the absence or in the presence of 1 mM sodium clofibrate. Then [1-^{14}C]octanoate was added and the incubation was continued for an additional 1 hr. Labeled lipids were extracted from the cells by chloroform–methanol (2:1, v/v), separated by thin-layer chromatography, and counted as in Table 1.

† Expressed in nmoles incorporated per 10^7 cells.

Table 3. Distribution of incorporated [1-^{14}C]butyrate among lipid lasses of rat hepatocytes*

Lipid class	[1-^{14}C]Butyrate incorporation	
	Control	NaCPIB (1 mM)
Phospholipids	6.0†	3.0
Monoglycerides	0.9	1.1
1,2-Diglycerides	0.5	0.3
Cholesterol	12.8	3.0
Fatty acids	1.0	0.7
Triglycerides	3.0	2.1
Cholesterol esters	0.1	0.3

* Rat hepatocytes were preincubated for 30 min in the absence or in the presence of 1 mM sodium clofibrate. Then [1-^{14}C]butyrate was added, and the incubation was continued for an additional 1 hr. Labeled lipids were extracted from the cells by chloroform–methanol (2:1, v/v), separated by thin-layer chromatography, and counted as in Table 1.

† Expressed in nmoles $\times 10^2$ of [1-^{14}C]butyrate incorporated per 10^7 cells.

DISCUSSION

Chronic administration of clofibrate to animals has produced a variety of metabolic alterations in the liver as documented by a large number of reports. While general agreement exists that clofibrate inhibits the hepatic synthesis of sterols and fatty acids [6, 7, 13], dissimilar data about its effects on fatty acid utilization have been reported. Clofibrate treatment of rats has variously been found to increase [5, 26], to decrease [27], or to have little effect on [28] the oxidation of long-chain fatty acids by liver mitochondria. With intact hepatocytes prepared from clofibrate-treated rats, the oxidation of long-chain fatty acids to CO_2 was stimulated by prior intake of the drug [4, 8]. These increases in CO_2 production have been accompanied by either a decreased [8] or an unaltered [4] rate of fatty acid esterification due to the drug. With hepatic homogenates prepared from clofibrate-treated rats, glycerolipid synthesis was reduced compared to control preparations [21, 29]. However, Gould *et al.* [6] described a stimulation of hepatic synthesis but a diminished export of triglycerides with liver slices and *in vivo* and proposed that administration of the drug interfered with lipoprotein assembly. With perfused livers from clofibrate-fed rats, both an increased uptake without a change in esterification rate [30] and an unchanged uptake with an increased esterification [31] of labeled palmitate have been

observed compared to control livers. Secretion rates of triglycerides by perfused rat livers have been described as diminished [31, 32] and accompanied by either a rise [32] or a fall [31] in hepatic fatty acid oxidation following pretreatment with the drug.

The above alterations in hepatic fatty acid utilization occurred after daily intake of clofibrate and could be due to induction of lipid-metabolizing enzymes [1–5], or to drug-induced increases in hepatic levels of carnitine [4, 26], Coenzyme A [33, 34], fatty acyl CoA thioesters [34], or fatty acid binding protein [30, 34]. However, these metabolic changes required prolonged administration of the drug and cannot account for the rapid effects that we observed. Because of contradictory data derived from studies with clofibrate-fed rats, the present study was devised to measure the earliest changes in fatty acid and glycerol utilization by intact hepatocytes incubated with clofibrate anion. Despite some variation in biochemical activity among the hepatocyte batches used that probably reflects differences in proteases retained from different collagenase lots [35, 36], our observations were quite consistent with this liver preparation. Though preliminary work suggested an enhanced metabolism of labeled palmitate by hepatocytes incubated with sodium clofibrate [17, 37], the current study clearly defines these observations and demonstrates that altered patterns of fatty acid oxidation and esterification produced by this agent vary dramatically with the chain length of the fatty acid utilized.

Palmitate, a common component of human dietary triglyceride, was converted to glycerolipids and to CO_2, and its utilization by monkey and rat hepatocytes was similarly increased by sodium clofibrate. Incorporation of labeled palmitate or glycerol into esterified lipids by liver cells was biphasic with maximal esterification rates reached at drug levels attained during therapy [21, 22]. Though plasma membrane damage at drug concentrations over 5 mM could not be detected by trypan blue staining, an adverse effect on cellular metabolism at these higher levels cannot be excluded, and the biphasic responses obtained may relate to the toxicity reported with excessive oral intake of clofibrate [12]. An analogous biphasic effect of clofibrate on state 4 respiration was found upon its addition to intact rat mitochondria in the presence of 3-hydroxybutyrate or succinate [38]. While some studies of labeled triglyceride turnover in the clofibrate-treated rat [39] or human [40] have suggested a decline in hepatic triglyceride synthesis and secretion, treatment with this agent has also led to an early rise in very low density lipoprotein (VLDL) production in rats [41] and to an undiminished output of splanchnic triglycerides in hyperlipidemic human subjects [22]. Furthermore, we previously observed a stimulation of esterified lipid secretion by [2-^{3}H]glycerol-prelabeled hepatocytes perifused in a column apparatus upon addition of sodium clofibrate to the medium [15].

The divergent actions of sodium clofibrate on the synthesis of specific lipid classes and on the partitioning of the fatty acids of differing chain length between synthetic and oxidative pathways as observed in the present study suggest direct effects of the drug upon hepatocellular metabolic processes. The cellular oxidation of long-, medium-, or short-chain fatty acids to $^{14}CO_2$ was consistently enhanced by sodium clofibrate, but the partitioning toward oxidation in control incubations varied inversely with fatty acid chain length. Since palmitate transport into mitochondria is regulated by specific carnitine acyltransferases [24], activation of this enzyme mechanism by the drug [12] may account for observed increases in labeled palmitate oxidation. Although sodium clofibrate can complete with long-chain fatty acids for binding sites on BSA [23], such competition cannot explain our observations with shorter chain fatty acids which were added to cells suspended in BSA-free medium. Thus, the rapid increases detected in labeled fatty acid utilization and in labeled triglyceride secretion by cells exposed to sodium clofibrate preclude causation by a simple fatty acid-displacement mechanism. These alterations in hepatic lipid metabolism probably resulted from a stimulation of fatty acid-metabolizing enzymes or from an intracellular redirection of substrate channelling caused by the drug.

The augumented incorporation of palmitate or of octanoate into cellular phospholipids and glycerides may reflect an increased availability of lipogenic substrates in the cytosol or a stimulation of extramitochondrial fatty acid-activating or -esterifying enzymes due to the drug, but it cannot be attributed to an inhibition of fatty acid oxidation. The accelerated biosynthesis of cellular lipids from labeled octanoate found in drug-supplemented incubations may be consequent to enhanced mitochondrial oxidation of this substrate to acetyl CoA [42]. The increased acetyl CoA formed may then be incorporated into sterols and into glycerolipids in the extramitochondrial compartment. Alternatively, acetoacetyl CoA may be generated in the mitochondria from octanoate, transferred to the cytosol, and then used directly as a substrate for enhanced sterol or complex lipid formation [43].

Unlike palmitate or octanoate, butyrate itself is not esterified to hepatic glycerolipids and its fate in liver is mainly mitochondrial oxidation [25]. The different labeling pattern of specific hepatocyte lipids from [1-^{14}C]butyrate and the inhibition of its incorporation by sodium clofibrate provide additional evidence that the liver utilizes short-chain fatty acids by mechanisms unlike those used to metabolize fatty acids of longer chain length. [1-^{14}C]Butyrate may be incorporated into sterols or longer chain fatty acids either prior to [44] or after its cleavage to acetyl CoA fragments. The predominant labeling of cellular sterol versus nonsterol lipids from [1-^{14}C]butyrate and the striking inhibition of sterol synthesis by sodium clofibrate found closely paralleled the results obtained when [1-^{14}C]acetate was used as precursor [16]. In contrast, sodium clofibrate greatly enhanced the cellular oxidation of labeled butyrate but not of labeled acetate to $^{14}CO_2$ [16]. The decline in [1-^{14}C]butyrate incorporation into cellular lipids and the rise in its oxidation to $^{14}CO_2$ may stem from a further diversion of this substrate from lipogenic to oxidative pathways consequent to an inhibition of lipogenic enzymes by the drug.

Thus, our data convincingly demonstrate that

short-term incubations of dispersed hepatocytes with sodium clofibrate produce diverse, but consistent perturbations in the hepatic utilization of glycerol and of fatty acids that are strikingly dependent upon chain length. Since clofibrate has been shown to improve the disposal of circulating triglycerides [22] and to stimulate the activities of postheparin triglyceride lipases in human plasma [45], the increases observed in fatty acid metabolism by liver cells exposed to the drug may in some way relate to an enhanced capacity to remove triglyceride-rich lipoproteins from the circulation. In all likelihood, these dramatic changes in patterns of fatty acid utilization represent acute, direct actions of sodium clofibrate on hepatocellular biochemical processes. Such actions of the clofibrate anion may simulate changes that occur *in vivo* after intake of the drug and suggest that the hypolipidemic action of clofibrate is not mediated simply by a reduction in hepatic formation or secretion of esterified lipids. Further studies of the early metabolic changes brought about by clofibrate should clarify understanding of the mechanism of its plasma lipid-lowering effect.

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