Biochemical Pharmacology, Vol. 29, pp. 987–992.

0006-2952/80/0401-0987 $02.00/0

A SUBCELLULAR STUDY OF THE CLOFIBRATE-INDUCED INCREASE IN COENZYME A CONCENTRATION IN RAT LIVER

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(*Received* 26 *July* 1979; *accepted* 6 *November* 1979)

Abstract—When clofibrate [ethyl 2-(4-chlorophenoxy)-2-methylpropionate] was administered subcutaneously to rats (600 mg/kg per day for 3 days), the concentration of CoA and its acyl derivatives in the liver increased 2.5-fold. Forty-eight per cent of the total cellular CoA in the clofibrate-treated rat liver and 51 per cent in the control liver was found in the mitochondrial fraction. In order to study the intermediates of CoA synthesis, clofibrate-treated rats were injected with [^{3}H]pantothenate intracardially and killed after 30 min, 1 or 2 hr for determination of the incorporation of radioactivity into CoA and its precursors. The incorporation of pantothenate into CoA after 2 hr was 5.9-fold in the liver and 4.5-fold in the liver mitochondrial fraction as compared with the control values. Measurement of the pantothenate concentration and radioactivity in clofibrate-treated and control rat liver showed that the higher incorporation of [^{3}H]pantothenate into CoA in clofibrate-treated rat liver cannot be the result of a higher specific radioactivity of pantothenate. It is therefore evident that clofibrate affects the CoA concentration by increasing the rate of synthesis, although the rate of CoA degradation is simultaneously decreased, as has been shown previously [9]. The present results indicate that clofibrate increases the total hepatic CoA concentration without affecting the intracellular compartmentation of CoA. The clofibrate-induced increase in the rate of CoA synthesis does not result in differences in the compartmentation of the intermediates of CoA synthesis.

Clofibrate [ethyl-2(4-chlorophenoxy)-2-methylpropionate], a hypolipidaemic drug, has been found to cause enlargement of the liver [1, 2], proliferation of peroxisomes [3] and mitochondria [4], changes in enzyme activities [3, 5], ATP/ADP ratio [6] and metabolite concentrations and a marked increase in hepatic CoA concentration [7–10]. The total cellular CoA concentration is obviously under physiological regulation, as it is affected by both food deprivation [11–13] and hormones [14–15]. Although there are previous reports on the subcellular localization of CoA under the effect of hormones and fasting [15, 16], both the mechanism itself and the subcellular localization of the clofibrate-induced increase have remained unknown.

The results of the present examination of the compartmentation of the clofibrate-induced increase in CoA concentration in rat liver show that about half of the total cellular CoA is located in the mitochondrial fraction in the control liver, while 3-day clofibrate treatment results in a 2.5-fold increase in the CoA concentration without affecting its intracellular compartmentation. The rate of CoA biosynthesis during clofibrate treatment is also re-evaluated here by measuring the pantothenic acid concentration and its specific radioactivity and the incorporation of radioactive pantothenate into 4′-phosphopantetheine and CoA.

EXPERIMENTAL

Materials

Bacto-Pantothenate Medium USP and Bacto-Pantothenate Culture Agar USP were purchased from Difco Laboratories, Detroit, MI, U.S.A. CoA, as the trilithium salt (grade 1) and the nucleotides were obtained from Boehringer GmbH, Mannheim, Germany, and clofibrate from Orion Pharmaceuticals, Helsinki, Finland. Dithiothreitol, rotenone, phosphotransacetylase and nucleotide pyrophosphatase were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; DEAE-cellulose (DE-52. pre-swollen, microgranular) from Whatman, Springfield Mill, Maidstone, Kent, U.K. [G-^{3}H]CoA (sp. radioact. 984 Ci/mole) and D-[3(*n*)-^{3}H]pantothenic acid, sodium salt (sp. radioact. 36.6 Ci/mmole) were obtained from NEN Chemicals GmbH, Dreieichenhain, Germany, and standard reagents from E. Merck AG, Darmstadt, Germany. 2-Oxoglutarate dehydrogenase (EC 1.2.4.2) was purified by the method of Sanadi [17].

Treatment of animals

Male Sprague–Dawley rats from the Department's own stocks were used. They were housed in an automatically illuminated room where the lights were on from 7:00 a.m. to 7:00 p.m. daily. One group was treated with daily subcutaneous injections of clofibrate, 600 mg/kg body wt per day for 3 successive days, and another group served as the controls. Both groups had access *ad lib.* to water and a pelleted diet (Astra-Ewos Ab, Södertälje, Sweden). At the end of the experiments the rats weighed 294–425 g, with no difference in body weight between the clofibrate-treated and normal rats.

All experiments were begun between 8:30 a.m. and 9:30 a.m. The rats in both groups were anaesthetized with diethyl ether, and 5 μCi of [^{3}H]-pan-

tothenate [adjusted to sp. radioact. of 1333 Ci/mole with calcium D(+)-pantothenate]. The corresponding amount (3.8 nmole) of non-radioactive calcium pantothenate was administered intracardially in 0.1 ml of 0.15 M NaCl per 100 g wt to animals in which the pantothenic acid concentration only was measured.

Sampling and tissue preparation

The rat was anaesthetized with diethyl ether 30, 60 or 120 min after injection of the radioactive pantothenate, and a tissue sample was obtained from the liver by the freeze-clamp technique for the determination of liver pantothenate concentration and the radioactivity of CoA and its precursors. These samples were stored under liquid nitrogen. Another sample was taken for the preparation of a tissue homogenate and the isolation of mitochondrial fraction.

Homogenization. From the rest of the liver, 6 g were removed, chilled, minced and homogenized in 30 ml of 0.225 M mannitol–0.075 M sucrose–0.05 mM EDTA adjusted to pH 7.4 with 1 M Tris (MSE-buffer) in a motor-driven glass–Teflon homogenizer. A 10-ml aliquot was removed into a tube cooled in liquid nitrogen and stored at −70° for the determination of CoA, total protein and marker enzymes in the tissue homogenate, and the remainder was used for the preparation of mitochondrial fraction.

Isolation of mitochondrial fraction. This was carried out principally according to Schneider [18]. The homogenate was centrifuged at 700 *g* for 7 min. The pellet was then discarded and the supernatant fraction was divided into two 10-ml portions and the mitochondrial fraction centrifuged at 8500 *g* for 10 min. A 5-ml aliquot was taken from the supernatant fraction for the assay of glutamate dehydrogenase. One of the 10-ml pellets was homogenized in MSE buffer at a protein concentration of 45 mg/ml and stored at −70° for the same determinations as in the tissue homogenate. The other mitochondrial pellet, also corresponding to 2 g of original liver, was immediately prepared for the separation of CoA and its precursors by column chromatography. All homogenizations were carried out in an ice-bath, and all centrifugation in a refrigerated Sorvall RC-5 Superspeed centrifuge.

Separation of CoA and its precursors

About 1 g of the freeze-clamped liver tissue was pulverized in a mortar under liquid nitrogen and homogenized in 4 ml of 750 μM HCl, containing 650 nmoles of carrier CoA, and then processed according to Nakamura *et al.* [19]. The mitochondrial pellet was treated similarly. The samples were diluted with cold distilled water to a measured conductivity value of 1/5 that of 3 mM HCl [20] before application to the column. The intermediates of CoA synthesis were separated by DEAE–cellulose column chromatography, and the radioactive peaks identified as described earlier [9]. Recovery of the [^{3}H]pantothenic acid added to the liver homogenate was 100 per cent and that of [^{3}H]CoA 88 per cent.

Analytical procedures

Radioactivity. The radioactivity of the fractions from DEAE-cellulose and paper chromatography was determined in Bray's scintillation solution [21] using a Wallac liquid scintillation counter.

Determination of concentrations of CoA, acetyl-CoA and long-chain fatty acyl-CoA. An aliquot of rapidly thawed tissue homogenate (approximately 0.2–0.3 g of tissue) was rapidly mixed with 8% (v/v) $HClO_4$ to a final concentration of 6% (v/v) and prepared for CoA and acetyl-CoA determination as described earlier [22]. The mitochondrial sample was diluted with cold MSE buffer to 6–8 mg protein/ml and homogenized in an equal volume of 12% (v/v) $HClO_4$ [23] and prepared in a manner similar to that used for the tissue homogenate for the determination of CoA and acetyl-CoA. Free-CoA was assayed by the method of Garland [24], and acetyl-CoA was determined in the same assay by a subsequent addition of phosphotransacetylase [25]. Long-chain fatty acyl-CoA was determined as described earlier [22] in the $HClO_4$-insoluble fraction of the tissue and mitochondrial fraction. Calculation of the mitochondrial and cytosolic concentration of CoA and its metabolites is based on a water content of 0.8 μl/mg protein in the mitochondrial compartment [26] and a cytosolic space in the liver tissue of 0.4 ml/g wet wt [27].

Estimation of cytochrome aa_3. Cytochrome aa_3 was assayed essentially as described by Hallman *et al.* [28] by recording the change in A_{605}-A_{630} due to cytochrome reduction with a dual wavelength spectrophotometer (Aminco DW-2). The concentration of mitochondrial protein in the tissue was calculated from the cytochrome aa_3 content in the tissue homogenate and in the isolated mitochondrial fraction [29] using $\varepsilon_{605-630} = 24{,}000$ l. mol^{-1}cm^{-1} for reduced cytochrome aa_3 [30].

Assay of glutamate dehydrogenase (EC 1.4.1.2). Triton X-100 to a 0.1% (v/v) concentration [31] was added to samples of total homogenate, cytosol and mitochondrial fraction in MSE buffer, and the suspensions were sonicated for 1 min in a Branson Europa Model D-50 ultrasonic cleaner (Sonogen) to release the full activity of the enzyme. The assays were then carried out according to Schmidt [32].

Determination of protein. Protein was measured by the biuret method [33] corrected for turbidity by the cyanide method [34]. Bovine serum albumin was used as standard.

Determination of pantothenate concentration. About 300 mg of frozen liver tissue was powdered in a mortar under liquid nitrogen and homogenized in 5.4 ml of 8% PCA–40% ethanol + 0.12 ml of 1 M dithiothreitol [15]. The homogenate was centrifuged at 8500 *g* for 20 min, neutralized with 3.75 M K_2CO_3 containing 0.5 M triethanolamine hydrochloride and centrifuged. The supernatant solution was heated for 1 min in a boiling water bath and the pantothenate concentration was assayed in duplicate determinations using *Lactobacillus plantarum* [35]. After 21 hr of bacterial growth at 37° in the pantothenate assay medium, the turbidity of the culture was measured spectrophotometrically at 600 nm.

Statistical analysis of the results. The statistical

Table 1. Effect of clofibrate on coenzyme A concentration and compartmentation in rat liver*

Subcellular fraction and source	Free CoA	Acetyl-CoA	Long-chain fatty acyl-CoA (nmoles/g wet wt)	Total CoA	Number of determinations
Clofibrate					
Total homogenate	341 ± 20‡	36 ± 4 n.s.	32 ± 2†	409 ± 22‡	12
Mitochondrial in tissue	158 ± 19‡	21 ± 3 n.s.	18 ± 2 n.s.	197 ± 16‡	12
Control					
Total homogenate	119 ± 8	30 ± 2	18 ± 5	167 ± 11	12
Mitochondrial in tissue	53 ± 4	19 ± 2	14 ± 5	86 ± 3	14
			(nmoles/mg protein)		
Isolated mitochondrial fraction					
Clofibrate	1.83 ± 0.17‡	0.24 ± 0.08 n.s.	0.21 ± 0.01 n.s.	2.29 ± 0.11‡	12
Control	0.69 ± 0.08	0.25 ± 0.07	0.19 ± 0.01	1.13 ± 0.08	14

* Clofibrate was injected subcutaneously for three days at a dosage of 600 mg/kg body wt per day. CoA and its derivatives were extracted from total homogenate and mitochondrial fraction and assayed as described under Experimental section. The results are the means ± S.E.M.

† $P < 0.05$ compared with the control rats.

‡ $P < 0.001$ compared with the control rats.

significance of the results was calculated using the Student's *t*-test.

RESULTS

Concentration of CoA and its derivatives in rat liver and liver mitochondrial fraction. Treatment with clofibrate for 3 days results in a 2.5-fold increase in the concentration of CoA and its derivatives in rat liver (Table 1). This increase is mainly in free CoA, although the concentrations of acetyl-CoA and long-chain fatty acyl-CoA were also a little higher in the liver tissue and liver mitochondrial fraction of the clofibrate-treated rats. Clofibrate treatment did not change the subcellular distribution of free CoA, but reduced the mitochondrial portion of acetyl-CoA from 63 to 58 per cent of total acetyl-CoA and that of acyl-CoA from 78 to 56 per cent of total acyl-CoA. Clofibrate treatment caused an increase in the protein content of the mitochondrial fraction from 76.3 to 86.3 mg/g wet wt ($P < 0.05$).

Cytosolic and mitochondrial concentrations of CoA and its derivates in control and clofibrate-treated rat livers. The cytosolic and mitochondrial concentrations of CoA and its derivatives, calculated from the values presented in Table 1, are shown in Table 2. Clofibrate-treatment resulted in a 2.6-fold increase in the cytosolic and 2.0-fold increase in the mitochondrial total CoA concentration, concomitantly with a 2.8-fold increase in cytosolic and a 2.7-fold increase in mitochondrial free CoA concentration. Measurement of the glutamate dehydrogenase activity showed that only 3.7 per cent of the mitochondria were broken or small enough to remain in the supernatant fraction.

Temporal pattern of the incorporation of [^{3}H]pantothenate into CoA and its precursors in rat liver and liver mitochondrial fraction. Figure 1 shows the temporal pattern of incorporation of the label into the pantothenic acid derivatives in rat liver and liver mitochondrial fraction after an intracardial injection of [^{3}H]pantothenate. The radioactivity incorporated into 4′-phosphopantetheine (in d.p.m./g wet wt) within 2 hr is 4.1-fold in the liver and 5.0-fold in the liver mitochondrial fraction of the clofibrate-treated rats compared with the control values.

Table 2. Cytosolic and mitochondrial concentration of CoA in clofibrate-treated and control rat liver*

Subcellular fraction and source	Concentration (mM)			
	Free CoA	Acetyl-CoA	Long-chain fatty acyl-CoA	Total CoA
Clofibrate				
Mitochondria	2.286 ± 0.161‡	0.307 ± 0.060 n.s.	0.260 ± 0.019 n.s.	2.853 ± 0.133‡
Cytosol	0.459 ± 0.037‡	0.039 ± 0.019 n.s.	0.034 ± 0.007†	0.532 ± 0.038‡
Control				
Mitochondria	0.856 ± 0.090	0.325 ± 0.058	0.232 ± 0.020	1.413 ± 0.094
Cytosol	0.165 ± 0.021	0.026 ± 0.010	0.012 ± 0.005	0.203 ± 0.034

* Results are expressed as means ± S.E.M.

† $P < 0.01$ compared to control rats.

‡ $P < 0.001$ compared to control rats.

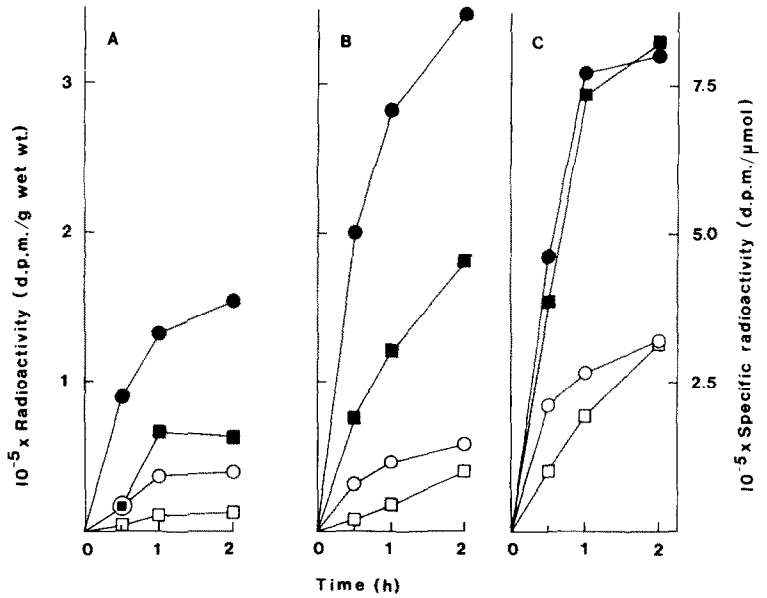

Fig. 1. Temporal pattern of the fate of labelled pantothenate in CoA biosynthesis in control and clofibrate-treated rat liver and liver mitochondrial fraction. Clofibrate treatment consisted of injections of clofibrate 600 mg/kg body wt for three successive days. Then rats were injected intracardially with 5 μCi of [^{3}H]pantothenate (sp. radioact. 1333 Ci/mole) per 100 g body wt and liver samples were taken 30, 60 or 120 min after this injection. The radioactivity determined in 4′-phosphopantetheine (a) and CoA (b) is in d.p.m./g wet wt, and that of CoA (c) in d.p.m./μmole: ●, radioactivity in tissue, clofibrate-treated rats; ■, radioactivity in mitochondrial fraction, clofibrate-treated rats; ○, radioactivity in tissue, control rats; □, radioactivity in mitochondrial fraction, control rats.

Figure 1b represents the radioactivity incorporated into CoA in 2 hr, this being 5.9-fold in the total liver of the clofibrate-treated rats and 4.5-fold in the mitochondrial fraction, respectively, compared with the control values. In terms of specific radioactivity (Fig. 1c), these ratios are 2.5 in the total liver and 2.6 in the mitochondrial fraction.

Precursor content in control and clofibrate-treated rat liver. Measurement of the pantothenate concentration and radioactivity in the clofibrate-treated and control rat liver showed that the higher incorporation of [^{3}H]pantothenate into CoA in clofibrate-treated rat liver cannot be due to a higher specific radioactivity of pantothenate (Table 3), as the specific radioactivity was only 7 per cent higher in the clofibrate-treated rat livers than in the controls. The rate of conversion of pantothenic acid to CoA (CoA radioactivity divided by specific radioactivity of pantothenic acid) was about 6.1 times higher in the clofibrate-treated rat livers than in the controls, and the ratio of the specific radioactivity of CoA to that of pantothenic acid is 2.6 in the clofibrate group compared with control values.

DISCUSSION

Previous descriptions of the effects of clofibrate on the CoA concentration in various tissues [8, 9, 36]

Table 3. Uptake of [^{3}H]pantothenate by liver *in vivo**

Treatment	Time (hr)	Pantothenate (nmoles/g wet wt)	%†	Specific radioactivity of pantothenate (d.p.m./nmoles)	%†
Clofibrate	0.5	5.27 ± 1.03 n.s. (N = 4)	92	10,490 (14,144; 6836) (N = 2)	138
	1.0	4.55 ± 0.31 n.s. (N = 3)	89	9770 ± 1558 (N = 3)	100
	2.0	6.17 (6.93; 5.41) (N = 2)	90	5052 (5616; 4488) (N = 2)	83
Control	0.5	5.70 ± 0.43 (N = 3)		7603 (9813; 5393) (N = 2)	
	1.0	5.10 ± 0.86 (N = 4)		9817 (10,203; 9431) (N = 2)	
	2.0	6.82 (7.75; 5.89) (N = 2)		6108 (6883; 5333) (N = 2)	

* Clofibrate and pantothenic acid were injected as described under Experimental. The pantothenic acid concentration was assayed from the liver samples microbiologically (see Experimental). The values are the means ± S.E.M. for 2 to 4 rat liver samples in each group, n.s. = not significant compared with the control rats.

† $\% = \frac{\text{clofibrate} \times 100}{\text{control}}$

leave several points undocumented, the two most important of which were the absolute rate of CoA synthesis and the subcellular distribution of the newly synthesized CoA in light of the known effects of clofibrate on the hepatic content of mitochondria [5] and peroxisomes [3].

It has been reported that an increase occurs in label incorporation from [^{3}H]pantothenate into CoA and its precursors, but when calculated from the turnover and concentration of CoA, the synthesis rate of CoA remains unchanged [9]. This discrepancy has been explained by a possible difference in precursor-specific radioactivity, probably due to a lower pantothenate concentration in the livers of the clofibrate-treated rats. The present results show, however, that this is not the case. The hepatic pantothenate concentration is not affected by clofibrate treatment, and the specific radioactivity of pantothenate is the same in the control and clofibrate-treated animals during an experiment on pantothenate incorporation into CoA. The findings are therefore difficult to reconcile with the previous interpretation, according to which the effect was solely due to the inhibition of CoA degradation [9]. The synthesis rate of CoA, as calculated from the incorporation of the label into CoA and from the specific radioactivity of pantothenate, is higher than the rate calculated from the apparent turnover of CoA. This suggests that label recirculation occurs, which may be a reason for the discrepancy between the data obtained by these two methods. The data cannot be explained by a change in the size of the CoA pool during the one-week CoA turnover experiment. Allowing for experimental error, the hepatic CoA concentration was constant after the fourth day of clofibrate treatment, the day on which the CoA turnover experiment began (Voltti, Savolainen and Jauhonen, unpublished observations).

The subcellular distribution of CoA, and also that of the newly synthesized CoA (as determined by label incorporation) was the same in the clofibrate-treated and control animals. The enzymes catalyzing the initial steps of CoA synthesis are located in the cytosol [16, 37], even though those catalyzing the final steps are distributed in both the mitochondria and the cytosol [38, 39]. Since CoA does not easily penetrate the mitochondrial membrane, an increase in CoA synthesis by enzymes in the cytosol, or an even distribution, should lead to a relative increase in the cytosolic portion of the CoA in the absence of any efficient system for transport into the mitochondria. The reason for the absence of any effect corresponding to the observed increase in the rate of CoA synthesis on its subcellular distribution is not apparent from the present data. Clofibrate induces a proliferation of peroxisomes [3]. The conventional mitochondrial fraction contains peroxisomes, and the catalase heme concentration of this fraction increases by 30 per cent under comparable conditions [40], indicating an increased contamination of this fraction by peroxisomes. The increase in the CoA concentration of the mitochondrial fraction was much greater, indicating that the changes observed in the CoA concentration and distribution cannot be explained by changes in the peroxisomal population. The conventional methods for estimating the tissue content of mitochondria are nevertheless prone to interference from the peroxisomes, as pointed out above.

It has been suggested that the cytosolic carnitine/CoA ratio partially determines the fate of fatty acids taken up by the cell [41]. In the present experiments the cytosolic free CoA concentration in the liver of the clofibrate-treated rats was 2.8 times that of the control rats, and the cytosolic long-chain fatty acyl-CoA concentration was similarly 2.8 times the control value. This is in accord with the view that it is the cytosolic free CoA concentration that regulates fatty acid activation in the cytosol. Somehow this increased precursor availability does not result in an increased rate of fatty acid esterification; on the contrary, opposite results have been reported [42, 43]. The hepatic carnitine concentration has also been reported to increase during clofibrate treatment [44], although no quantitative data have been published. It could be that the cytosolic carnitine concentration increases proportionally or even more, so that the oxidative branch of fatty acid metabolism is favoured under the influence of clofibrate.

Addendum—Since the submission of this manuscript, Skrede and Halvorsen [45] have published data showing increased activity of some CoA-synthesizing enzymes and increased rates of CoA synthesis in liver extracts from clofibrate-treated rats. They also found a reduction in the specific activity of acid phosphatase and nucleotide pyrophosphatase in the mitochondrial fraction, with a concomitant increase in the CoA-degrading capacity of the particle-free supernatant fraction. As CoA is preferentially located in the mitochondria, a decrease in the mitochondrial degradation capacity may lead to a decrease in the degradation of CoA.

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