Clofibrate-Induced Increase in Coenzyme A Concentration in Rat Tissues

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1. When clofibrate [ethyl 2-(4-chlorophenoxy)-2-methylpropionate] was administered subcutaneously to rats (600mg/kg per day for 5 days), the concentration of CoA and its acyl derivatives increased in several tissues. The increase in total CoA was 3.2-fold in the liver, 1.8-fold in the kidney, 2.7-fold in the heart and 2.4-fold in skeletal muscle. 2. To study the mechanism of this phenomenon, clofibrate-treated rats were injected with [³H]pantothenate intracardially and killed after 15 min, 30 min, 1 and 2 h and 1, 3, 5 and 7 days for the determination of the incorporation of radioactivity into CoA and its precursors. Incorporation into CoA after 2 h was 6.2-fold in the liver as compared with the control values and 4.6-fold in the kidneys. 3. The disappearance of the label from CoA was very slow compared with the rate of incorporation; it exhibited exponential kinetics, and was slower in the livers of the clofibrate-treated rats ($t_{\frac{1}{2}}$ 18.2 days) than in the controls ($t_{\frac{1}{2}}$ 5.6 days). 4. The rate of CoA degradation, calculated from the calculated rate constants of the apparent first-order kinetics of the disappearance of the label and from the CoA pool sizes, was approximately the same in the clofibrate-treated animals (11.5 pmol/min per g), and the controls (11.6 pmol/min per g). 5. These rates of CoA degradation indicate that the effect of clofibrate on CoA concentration may be mainly due to inhibition of the enzymes of CoA degradation, although recycling of the label cannot be excluded. The increase in the rate of pantothenate incorporation into CoA suggests that clofibrate also increases the synthesis of CoA.

CoA is an important cofactor in intermediary metabolism. The concentration of free CoA has only been demonstrated positively to function as a metabolic regulator in a few instances (see e.g. Randle *et al.*, 1966). Various studies (Akerboom & Zuurendonk, 1974; Soboll *et al.*, 1976; Idell-Wenger *et al.*, 1978) have revealed that most of the CoA and its derivatives occurs in the mitochondria and only a minimal portion is located in the cytosol. The cytosolic concentration is in the micromolar range (1–5 μ M) (Soboll *et al.*, 1976). Thus the concentration of free CoA may regulate the activity of some enzymes in the extramitochondrial compartment [e.g. acyl-CoA synthetase, which has a K_m value of 8–24 μ M for CoA (Oram *et al.*, 1975)]. The variation in tissue CoA content in various nutritional and hormonal conditions (Guynn *et al.*, 1972) similarly suggests a possible regulatory role for the CoA concentration.

It has been shown that clofibrate [ethyl 2-(4-chlorophenoxy)-2-methylpropionate], a hypolipid-aemic drug, causes a multiple increase in the hepatic CoA concentration (Miyazawa *et al.*, 1975; Savolainen *et al.*, 1977b). Since clofibrate is also known to increase the rate of oxidation of fatty acids in perfused liver and liver mitochondria (Hassinen & Kähönen, 1974; Mackerer, 1977), changes in the CoA

concentration may also be involved in the latter effect. The changes in CoA concentration were of such a magnitude that the effects of clofibrate could probably be used as a tool for studying the regulation of the total cellular CoA concentration. In the present case an effort was made to distinguish between the clofibrate effects on CoA synthesis and degradation.

Experimental

Materials

DEAE-cellulose (DE-52, pre-swollen, microgranular), was purchased from Whatman, Springfield Mill, Maidstone, Kent, U.K. CoA as the trithium salt (grade I) and NAD⁺ were obtained from Boehringer G.m.b.H., Mannheim, Germany, and phosphotransacetylase, nucleotide pyrophosphatase and dithiothreitol from Sigma Chemical Co., St. Louis, MO, U.S.A. Clofibrate was obtained from Orion Pharmaceuticals, Helsinki, Finland, calcium D(+)-pantothenate for biochemical purposes from E. Merck A.G., Darmstadt, Germany, [³H]CoA (sp. radioactivity 984 Ci/mol) and D-[3(n)-³H]pantothenic acid (sodium salt; sp. radioactivity 36.6 Ci/mmol) were from NEN Chemicals G.m.b.H., Dreieichenhain, Germany.

2-Oxoglutarate dehydrogenase (EC 1.2.4.2) was purified by the method of Sanadi (1969).

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 07:00 to 19:00h daily. One group was treated with daily subcutaneous injections of clofibrate, 600mg/kg body wt. per day for 3 successive days, and the other 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 210–390g, with no difference in body weight between the clofibrate-treated and normal rats.

All experiments were begun between 08:30 and 09:30h. In studies on the biosynthesis of CoA the rats in both groups were anaesthetized with diethyl ether and 5 μ Ci of [3 H]pantothenic acid [adjusted to sp. radioactivity of 1333Ci/mol with calcium D(+)-pantothenate] was administered intracardially in 0.1 ml of 0.15M-NaCl per 100g body wt. They were then anaesthetized again with diethyl ether 15, 30, 60 or 120min later, and tissue samples from the liver and kidney were obtained by the freeze-clamp technique. The samples were stored under liquid nitrogen until used. Blood samples were drawn from the inferior vena cava into heparinized ice-chilled glass tubes.

In the experiments on the degradation of CoA, labelled pantothenic acid was injected intracardially as described above, and 3h later the rats were injected intraperitoneally with 0.1ml of 3.75mM-calcium D(+)-pantothenate per 100g body wt. Tissue samples from the liver and kidney were taken by the freeze-clamp technique after 1, 3, 5 or 7 days.

In the experiments on the concentrations of CoA and its acetyl and long-chain fatty acyl derivatives (Table 1), the rats were given daily subcutaneous injections of clofibrate (600mg/kg body wt.) for 5 successive days. Between 08:30 and 09:30h on the sixth day they were anaesthetized with diethyl ether, and samples were taken from the skeletal muscle (m. soleus), liver, kidney and heart by the freeze-clamp technique. At the same time blood samples were drawn from the abdominal aorta into heparinized ice-chilled glass tubes and deproteinized within 15s by mixing with an equal volume of 10% (w/v) HClO₄.

Preparation of tissue samples for column chromatography

About 1g of liver or kidney tissues was pulverized in a mortar under liquid nitrogen. The samples were homogenized in 4ml of 750 μ M-HCl containing 650nmol of carrier CoA in a motor-driven glass/Teflon homogenizer. The homogenate was immedi-

ately put into a boiling-water bath for 5min, cooled and centrifuged at 8500g for 20min at 4°C; 0.5ml of 1M-Tris, adjusted to pH8.0 with 1M-HCl, and 0.5ml of 0.2M-dithiothreitol were then added to the supernatant solution (Nakamura *et al.*, 1972). The samples were stirred for 20min at 37°C and then cooled to 4°C and applied to the column.

DEAE-cellulose column chromatography

DEAE-cellulose was sequentially pretreated with 1M-NaOH and 1M-HCl and washed with water until the pH was 6.5. A portion (5ml) of the sample was applied to the column (1cm $\times$ 12cm), followed by 5ml of 1mM-dithiothreitol. The column was then eluted with a linear 400ml gradient of 0–75mM-LiCl in 3mM-HCl/1mM-dithiothreitol (Moffatt & Khorana, 1961; Larrabee *et al.*, 1965). The flow rate was 4ml/min, and 4.3ml fractions were collected.

Analytical procedures

Radioactivity. The radioactivity in the fractions from DEAE-cellulose and paper chromatography was determined in Bray's (1960) scintillation solution with a Wallac liquid-scintillation counter.

Tissue blood (haemoglobin) content. To correct the observed radioactivity of tissue pantothenic acid for interference due to the radioactivity in the blood, the blood content of the tissues was determined by measuring the haemoglobin concentrations in the tissues. Tissue samples were homogenized in 9vol. of 30mM-potassium phosphate buffer, pH7.4, in a glass homogenizer fitted with a Teflon pestle. A 0.1ml portion of the tissue homogenate was added to 1.0ml of aerated reaction mixture containing 27 μ mol of potassium phosphate, pH7.4, 1 μ mol of succinate and 1 μ mol of KCN. The absorbance difference at the wavelength pair 576–557nm was monitored in an Aminco DW-2 dual-wavelength spectrophotometer and the absorbance difference increment observed after the addition of few crystals of Na₂S₂O₄ was recorded and used for the calculation of the haemoglobin content. The amount of blood in the tissues was calculated from the haemoglobin content of the tissue sample and that in a suitably diluted blood sample from the same rat, assuming that the haematocrit value for the blood in the liver is the same as in the peripheral blood. This method gave somewhat lower values than those obtained by methods using ¹²⁵I-labelled albumin (Abrams & Cooper, 1976).

Determination of concentrations of CoA, acetyl-CoA and long-chain fatty acyl-CoA. Tissue samples for CoA and acetyl-CoA determinations were prepared as described earlier (Savolainen *et al.*, 1977a). Free CoA was assayed by the method of Garland (1964), and acetyl-CoA was determined in the same assay by a subsequent addition of phosphotransacetylase (Tubbs & Garland, 1969). Long-chain fatty acyl-CoA

was determined in the HClO₄-insoluble fraction of the tissue as described earlier (Savolainen *et al.*, 1977a).

Miscellaneous procedures. Thiol groups were determined as described by Ellman (1959), total phosphorus by the method of Chen *et al.* (1956), and

4'-phospho[³H]pantetheine was prepared from [³H]-CoA by using nucleotide pyrophosphatase (EC 3.6.1.9) (Loewen, 1977). Pantothenate and 4'-phosphopantetheine were separated by ascending paper chromatography at 4°C in a solvent containing butan-1-ol/acetic acid/water (5:2:3, by vol.) essen-

Table 1. *Effect of clofibrate treatment on the concentrations of CoA and its derivatives in certain rat tissues*
Clofibrate was injected subcutaneously for 5 days at a dosage of 600 mg/kg body wt. Tissue samples were obtained by the freeze-clamp method. CoA and its derivatives were extracted and assayed as described in the Experimental section. The results are means ± S.E.M. for four rats in each group. **P* < 0.05, ***P* < 0.01 and ****P* < 0.001 compared with values in control rats. Abbreviations: n.m., not measurable; n.s., not significant.

Tissue	Treatment	Metabolite concentrations (nmol/g wet weight)			
		CoA	Acetyl-CoA	Long-chain fatty acyl-CoA	Total CoA
Liver	—	78 ± 4	58 ± 3	30 ± 5	166 ± 7
	Clofibrate	386 ± 14***	95 ± 12*	56 ± 6**	537 ± 19***
Kidney	—	43 ± 4	23 ± 2	5 ± 2	71 ± 6
	Clofibrate	90 ± 2***	32 ± 6 n.s.	6 ± 1 n.s.	128 ± 10**
Heart	—	60 ± 4	21 ± 4	7 ± 1	88 ± 7
	Clofibrate	213 ± 23***	17 ± 5 n.s.	4 ± 5 n.s.	234 ± 26**
Skeletal muscle (m. soleus)	—	7.4 ± 0.4	1.2 ± 0.3	n.m.	8.6 ± 0.6
	Clofibrate	19.3 ± 1.5***	1.6 ± 0.4 n.s.	n.m.	20.9 ± 2.2**

Fig. 1. *DEAE-cellulose column chromatography of CoA and its precursors extracted from rat liver 0.5h after intracardial injection of [³H]pantothenate*

Rats were injected intracardially with 5 μCi of [³H]pantothenate (sp. radioactivity 1333 Ci/mol) per 100 g body wt., and liver samples taken 30 min later by the freeze-clamp technique. CoA and its precursors were extracted and chromatographed as described in the Experimental section. The first (I) and second (II) peaks were identified as pantothenate, the third (III) as 4'-phosphopantetheine and the fourth (IV) as CoA (see the Results section). ●, Clofibrate-treated rat; ○, control rat.

tially as described by Nakamura *et al.* (1972). The paper was cut into 5 mm strips for the determination of radioactivity by liquid-scintillation counting.

Statistical analysis of the results. The statistical significance of the results was calculated by Student's *t* test.

Results

Concentration of CoA and its derivatives in different tissues

Table 1 shows that clofibrate treatment results in a 3.2-fold increase in the concentration of CoA and its derivatives (acetyl-CoA and long-chain fatty acyl-CoA esters) in rat liver, and corresponding increases of 1.8-fold, 2.7-fold and 2.4-fold in the kidney, heart and skeletal muscle respectively. The increase occurs mainly in the free CoA content, although the concentrations of acetyl-CoA and long-chain fatty acyl-CoA were also higher in the livers. No measurable quantities of free CoA (more than 0.2 nmol/ml) were observed in the blood samples from either the clofibrate-treated or control rats.

Incorporation of [^3H]pantothenate into CoA and its precursors

Identification of the radioactive metabolites. Fig. 1 shows a typical column chromatogram for pantothenic acid derivatives in rat liver 30 min after an intracardial injection of [^3H]pantothenate. The first and second peaks were identified as pantothenate by comparison with the elution characteristics of authentic [^3H]pantothenate. The third peak contained SH groups and phosphorus, and its elution characteristics were identical with those of 4'-phosphopantetheine. Since the separation of peaks II and III was incomplete, the contents of these combined peaks were further analysed by paper chromatography, which separated out pantothenate and 4'-phosphopantetheine (Fig. 2). Peak IV eluted in the column chromatography (Fig. 1) was identified as CoA by its reactivity in the enzymic CoA assay and by reference to the elution pattern of authentic [^3H]CoA.

Temporal pattern of label incorporation. Fig. 3 shows the temporal pattern of incorporation of the label into the pantothenic acid derivatives in the rat liver and kidney after an intracardial injection of [^3H]pantothenate. In both tissues the radioactivity in

Fig. 2. Separation of the components of peaks II and III from column chromatography by paper chromatography. DEAE-cellulose column chromatography fractions containing peaks II and III were pooled, freeze-dried and dissolved in 1 ml of water; 0.1 ml was applied to Whatman no. 1 paper and ascending paper chromatography carried out in a solvent containing butan-1-ol/acetic acid/water (5:2:3, by vol.). Shaded areas above the chromatogram show the migration of CoA, 4'-phosphopantetheine and pantothenic acid standards. ●, Clofibrate-treated rat; ○, control rat.

Fig. 3. Temporal pattern of the fate of labelled pantothenate in CoA biosynthesis

Experimental conditions were as in Fig. 1, except that the liver and kidney samples were taken 15, 30, 60 or 120min after the injection of [³H]pantothenate. The radioactivity determined in pantothenate, 4'-phosphopantothenate and CoA is shown for the liver in (a)–(c) respectively, and for the kidney in (d)–(f). Each point represents the average of two separate experiments, the vertical bar representing the range, except at 15min, where the result is from one rat in each group. ●, Radioactivity (d.p.m./g wet wt.), clofibrate-treated rats; ○, radioactivity (d.p.m./g wet wt.), control rats; ▲, specific radioactivity (d.p.m./μmol), clofibrate-treated rats; △, specific radioactivity (d.p.m./μmol), control rats.

pantothenic acid decreases sharply after 1 h (Figs. 3a and 3d), whereas that of 4'-phosphopantetheine, an intermediate of CoA synthesis, increases markedly during the first hour and continues to rise slowly up to 2 h in the liver (Fig. 3b). In the kidney the increase ceases after 30min, after which the radioactivity in 4'-phosphopantetheine assumes an almost constant value (Fig. 3e). The radioactivity incorporated into 4'-phosphopantetheine (in d.p.m./g wet weight) within 2 h is 5.5-fold in the liver and 3.8-fold in the kidney of the clofibrate-treated rats compared with that in the control values.

Figs. 3(c) and 3(f) represent the radioactivity incorporated into CoA over 2 h in the liver and kidney respectively, that in the clofibrate-treated rats being

6.2-fold in the liver and 4.6-fold in the kidney compared with that in the controls. In terms of specific radioactivity these ratios are smaller, 2.0 in the liver and 3.8 in the kidney.

The labelled pantothenate was taken up into tissues rapidly, so that at 30min the blood radioactivity per unit volume was only 22 and 7% of that in the liver and 4 and 4.5% of that in the kidneys in the control and clofibrate-treated rats respectively. Thus there can be said to have been little distortion of the determination of tissue pantothenate by intravascular pantothenate.

CoA turnover

The disappearance of the label from CoA is very slow compared with the rate of incorporation. Fig. 4

Fig. 4. Disappearance of the label from hepatic and renal CoA

Rats were injected intracardially with $5\mu\text{Ci}$ of $[^3\text{H}]$ pantothenate (sp. radioactivity 1333 Ci/mol) per 100 g body wt. at zero time and with 375 nmol of calcium D(+)-pantothenate 3 h later. Liver and kidney samples were taken $1, 3, 5$ or 7 days after the last injection. CoA and its precursors were separated by DEAE-column chromatography as described in the Experimental section. The lines were fitted to the experimental data by least-squares regression analysis. (a) Liver; (b) kidney. ●, Clofibrate-treated rats; ○, control rats.

shows the least-squares regression lines for log(specific radioactivity of CoA) versus time. The lines represent half-lives of 18.2 days ($r = 0.88$, $n = 5$, $P < 0.05$) and 5.6 days ($r = 0.91$, $n = 6$, $P < 0.01$) in the liver and 6.9 days ($r = 0.85$, $n = 5$, $P < 0.1$) and 4.2 days ($r = 0.96$, $n = 5$, $P < 0.01$) in the kidney for the clofibrate-treated and control rats respectively.

The disappearance of the label is exponential obeying the equation:

$$a = a_0 \cdot e^{-kt}$$

where a_0 is the initial specific radioactivity, a is the specific radioactivity at time t , and k is the first-order velocity constant of the assumed relation $da/dt = -ka$. The turnover of CoA can be calculated from the tissue concentration of CoA and the constant k . In these experiments on the CoA turnover, clofibrate injections were continued for the 7 -day period of liver sampling. The total CoA concentration of the liver remained constant during this period. The constants k obtained here were 0.264×10^{-4} and $0.859 \times 10^{-4} \text{ min}^{-1}$ in the liver and 0.697×10^{-4} and $1.15 \times 10^{-4} \text{ min}^{-1}$ in the kidneys of the clofibrate-treated and control rats respectively. The turnover rate of CoA in the liver was therefore $11.5 \text{ pmol/min per g}$ in the clofibrate-treated rats and $11.6 \text{ pmol/min per g}$ in the control rats, and that in the kidney 11.3 and $12.2 \text{ pmol/min per g}$ respectively.

Discussion

The present results can be interpreted as indicating that the cellular CoA concentration is effectively regulated and that this regulation can be influenced by a hypolipidaemic drug, with the phenomenon observed perhaps partly explaining the pharmacological effects of the drug.

There are also previous reports that other hypolipidaemic drugs cause similar effects. 2-Methyl 2-[4-(1,2,3,4-tetrahydro-1-naphthyl)phenoxy]propionic acid, for instance, is known to increase the hepatic content of CoA derivatives (Schacht & Granzer, 1970).

In the present case, the results are equivocal in that they apparently demonstrate an increase in the rate of CoA synthesis and a decrease in its rate of degradation. One should bear in mind, however, that owing to the rapid disappearance of pantothenic acid from the blood and tissues, it was not possible to measure the specific radioactivity of the precursor of the pathway of CoA synthesis. The surprising agreement between the results on CoA turnover in the clofibrate-treated and control rats, however, suggests that it is more likely that under these experimental conditions the CoA concentration was regulated by the rate of CoA degradation.

The total CoA concentration is obviously under

physiological regulation, as it is affected by food deprivation (Guynn *et al.*, 1972) and insulin deficiency (Smith *et al.*, 1978). Although there are previous reports on the rate of pantothenate incorporation into CoA, there are no previous studies on the turnover of CoA. The turnover results fit very well with the assumption that the rate of CoA degradation is a first-order reaction, and the synthesis a zero-order reaction, so that they yield exactly similar synthesis rates in the control and clofibrate-treated rats. This result is intriguing, however, as the free CoA concentration is higher in the clofibrate-treated rats, which should result in a proportionally increased rate of CoA degradation. The results can only be reconciled by proposing an enzyme inhibition somewhere in the CoA-degradation pathway. The increased rate of incorporation of label in the clofibrate-treated rats could be due to a higher precursor specific radioactivity, or to an effect of a larger pool size combined with label recycling. Similarly, the contribution of label recycling to the turnover results cannot be excluded when considering these results. The kinetic properties of the CoA-degradation enzymes are not known in detail, but at least the aminohydrolase involved, namely pantethinase, is subject to inhibition by taurine (Duprè *et al.*, 1970). The effects of clofibrate on the individual enzymes are not known.

There are several connections between the effects of clofibrate reported in the present paper and its effects on hepatic metabolism and structure. Results have been obtained that suggest that the total hepatic mitochondrial mass increases by more than 100% (Gear *et al.*, 1974) concomitantly with a 40% increase in the liver weight (Hawkins *et al.*, 1974), which means that the hepatic concentration of mitochondria increases. On the other hand, mitochondrial protein synthesis has been reported to diminish and the activity of mitochondrial proteinases to be inhibited (Gear *et al.*, 1974) under the conditions. The results have been interpreted as showing that the mitochondrial mass could be regulated by changes in the degradation of nascent mitochondrial proteins. The hepatic concentration of mitochondria increases maximally by 40% [calculated from the data of Gear *et al.* (1974) and Hawkins *et al.* (1974)] compared with a 300% increase in the CoA concentration (Table 1). The latter fact cannot then be explained by an increase in the mitochondrial population and a preferential location of CoA derivatives in the mitochondria. If the effects of clofibrate on the turnover of CoA are due to its effect on degradation, there is an analogy between its effects on the biogenesis of mitochondria and on the CoA concentration.

It is also known that clofibrate induces a proliferation of peroxisomes (Hess *et al.*, 1965) in the liver. Moreover, kidney cells also contain peroxisomes. It remains to be established whether a portion of the

newly synthesized CoA is located in the hepatic or renal peroxisomes.

It has been observed both in isolated mitochondria and in perfused rat livers that clofibrate increases the rate of fatty acid oxidation (Hassinen & Kähönen, 1974; Mackerer, 1977), and it is commonly accepted that the rate-limiting step in the oxidation of fatty acids is the synthesis of acyl-carnitine by CoA-carnitine acyltransferase, the activity of which increases during clofibrate treatment (Daae & Aas, 1973). The role of the concentration of free CoA in the regulation of fatty acid oxidation is not known exactly, but it has been postulated that the cytosolic carnitine/CoA ratio may determine the fate of fatty acids in the oxidative or esterification pathway, at least in the heart (Oram *et al.*, 1975), and it has been calculated that the cytosolic free CoA concentration is probably below the K_m of fatty acid activation (Idell-Wenger *et al.*, 1978). Fatty acid activation may therefore be regulated by the CoA concentration. It may be concluded from the above that the effects of clofibrate on the CoA concentration could explain the increased uptake and activation of fatty acids in tissues, but not necessarily the increased oxidation of fatty acids.

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