Hypolipidaemic drugs are activated to acyl-CoA esters in isolated rat hepatocytes

Detection of drug activation by human liver homogenates and by human platelets

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The formation of acyl-CoA esters of the hypolipidaemic peroxisome proliferators clofibric acid, ciprofibrate and nafenopin was studied in isolated rat hepatocytes. The concentration of ciprofibril-CoA in the liver of ciprofibrate-treated rats was in the range of 10–30 μM . The three drugs formed acyl-CoA esters when incubated with isolated hepatocytes. Their formation was saturable and reached a plateau after 30 min incubation. Maximal intracellular concentrations of ciprofibril-CoA and clofibril-CoA (100 μM and 55 μM respectively) were attained at 0.5 mM of the free drugs in the incubation medium, whereas for nafenopin-CoA, the maximal intracellular concentration (9 μM) was reached at 1 mM-nafenopin. At low concentrations of the hypolipidaemic compounds in the incubation medium a significant proportion of the total intracellular drug was present as its acyl-CoA ester (25–35 % for ciprofibrate). When isolated hepatocytes were incubated with a ciprofibrate concentration comparable with that observed in the blood of drug-treated rats (0.1 mM), ciprofibril-CoA attained an intracellular concentration similar to that previously observed in the liver of treated rats. The formation of ciprofibril-CoA by isolated rat hepatocytes was stimulated by the addition of carnitine and partially inhibited by the addition of palmitate. Further, it was shown that human liver homogenates synthesized ciprofibril-CoA at a rate similar to that observed for rat liver homogenates. Solubilized human platelets also formed ciprofibril-CoA, although at a rate two orders of magnitude lower than that of liver. The results support the view that acyl-CoA esters of hypolipidaemic peroxisome proliferators may be the pharmacologically active species of the drugs.

INTRODUCTION

Clofibric acid and associated hypolipidaemic drugs, as well as a number of other structurally unrelated compounds, are known to cause several metabolic effects in the liver [for a review, see Hawkins *et al.* (1987)], together with peroxisomal proliferation and hepatocellular carcinomas in rats (Reddy & Lalwani, 1983). Most of these compounds hold carboxylic acid groups in their structure. We have shown that three of them are activated to acyl-CoA esters by rat liver microsomes (Bronfman *et al.*, 1986). In the case of ciprofibrate, we presented evidence suggesting that long-chain-unspecific-acyl-CoA synthetase, which has a multiple subcellular localization (Krisans *et al.*, 1980; Mannaerts *et al.*, 1982; Bronfman *et al.*, 1984), might be responsible for its activation [the preceding paper (Amigo *et al.*, 1992)].

Other compounds, non-structurally related to clofibric acid, also undergo activation *in vitro* to acyl-CoA esters (Hertz & Bar-Tana, 1988; Aarsland *et al.*, 1990). Furthermore, chemically synthesized acyl-CoA esters of several of these drugs, one of them non-related structurally to clofibric acid, were shown to potentiate the activity of protein kinase C *in vitro* (Bronfman *et al.*, 1989; Orellana *et al.*, 1990). Protein kinase C is a key enzyme involved in a number of cellular processes, including receptor function, cellular differentiation and carcinogenesis (Nishizuka, 1986; Berridge, 1987).

The multiple and similar effects of hypolipidaemic peroxisome proliferators are in contrast with their heterogeneous chemical structure. We have previously proposed that their acyl-CoA esters might be their common pharmacological active species (Bronfman *et al.*, 1986). The presence of clofibril-CoA in the liver of clofibrate-related rats has been reported (Lygre *et al.*, 1986), whereas the acyl-CoA ester of ciprofibrate has been also detected in the liver of ciprofibrate-treated rats (Bronfman *et al.*, 1986, 1989).

The formation of these acyl-CoA esters *in vivo* has not been

characterized in detail. In the present study we have measured the hepatic concentration of ciprofibril-CoA in the liver of ciprofibrate-treated rats. We have compared the time course and concentration-dependence of ciprofibril-CoA, clofibril-CoA and nafenopin-CoA formation in isolated hepatocytes. The influence of other molecules potentially capable of altering their formation, such as carnitine and palmitic acid, was also determined. Results show that the three acyl-CoA esters are formed by isolated hepatocytes. By using ciprofibrate as a model compound it was found that carnitine enhanced the formation of ciprofibril-CoA, whereas palmitic acid partially inhibited it. In addition, it was shown that human liver homogenates and Triton X-100-solubilized human platelets were also capable of activating ciprofibrate.

EXPERIMENTAL

Materials

Ciprofibrate and Nafenopin were kindly provided by Sterling-Winthrop Research Institute, Rensselaer, NY, U.S.A., and CIBA-GEIGY, Basel, Switzerland, respectively. Clofibril acid, coenzymes, Triton X-100, substrates and chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A. H.p.l.c.-grade methanol was purchased from Merck, Darmstadt, Germany. Culture medium, foetal-calf serum and culture dishes were obtained from Flow Laboratories, Baar, Switzerland. 1-Bromodecane was from Janssen Chimica, Beerse, Belgium.

Animals

Sprague-Dawley rats, weighing 180–200 g, were used. They were maintained on a standard laboratory chow diet. For ciprofibrate treatment, rats were fed the same standard chow diet containing 50 mg of the drug per kg of chow.

Incubation of hepatocytes and cell cultures

Rat hepatocytes were isolated as described by Berry & Friend (1969). Viability of the cells was 85–90%. Incubation of hepatocytes with hypolipidaemic compounds was performed in Krebs–Henseleit buffer, containing 0.175 mM defatted BSA, in a total volume of 1 ml, at 37 °C. Stock solutions of ciprofibrate and clofibric acid were prepared (10 mM) in Krebs–Henseleit buffer. Nafenopin was prepared in the same buffer as a 2 mM-solution. Extensive sonication was required to obtain a clear solution of nafenopin. The incubation was started by the addition of cells (8–10 mg of protein) and stopped by decanting 0.8 ml of the incubated cells over 0.2 ml of 1-bromodecane in an Eppendorf (1.5 ml) tube. After centrifuging for 1 min in an Eppendorf centrifuge, the supernatant and the 1-bromodecane layer were carefully removed, and the cell pellet resuspended in 0.1 ml of 0.1% Triton X-100. After vigorous mixing, 0.4 ml of chloroform/methanol (2:1, v/v) was added, the suspension was mixed again and centrifuged for 1 min in an Eppendorf centrifuge. The water/methanol upper phase was decanted off and used directly for the determination of acyl-CoA esters of the drugs by h.p.l.c. Cell viability after 30 min incubation with 2 mM of the drugs was 80% or higher.

For cultures, freshly isolated hepatocytes were seeded at 5×10^6 cells/dish (6 cm diameter) as described by Bieri *et al.* (1984). At 4 h after seeding, the medium was replaced by one containing ciprofibrate. After 24 h the monolayers were washed with phosphate/saline buffer (0.01 M-KH₂PO₄/0.15 M-NaCl, pH 7.4) and then scraped off with a rubber policeman to a final volume of 500 μ l. Chloroform/methanol (2:1, v/v; 2 ml) was added and the tube was vigorously mixed. After decanting the phases by centrifugation, 0.5 ml of the methanol/water phase was evaporated under N₂ flow, and resuspended in 0.1 ml of water. Ciprofibroyl-CoA was determined in this fraction by h.p.l.c. Using chemically synthesized acyl-CoA esters of the drugs as internal standards (Bronfman *et al.*, 1989), we found that 90–95% of the acyl-CoA esters had been recovered in the methanol/water extract of isolated or cultured hepatocytes.

Extraction of ciprofibroyl-CoA from the liver of ciprofibrate-treated rats

The livers were homogenized in a Ultra-Turrax apparatus for 15 s in 50 mM-Tris/HCl, pH 8.0; 4 vol. of chloroform/methanol (2:1, v/v) per volume of homogenate were immediately added. After vigorous mixing, the suspension was centrifuged 10 min at 1800 rev./min (r_{av} , 17 cm) in a IEC-6000 centrifuge. The water/methanol upper phase was decanted off, and the chloroform phase was washed twice with 2 vol. of water/methanol (1:1, v/v). The upper phase and the washings were evaporated under N₂ flow and the remaining solution was filtered through a Sep-pak C₁₈ cartridge (Waters Associates, Milford, MA, U.S.A.). The cartridge was washed with 20 ml of water and the acyl-CoAs and the free acids were eluted with 10 ml of methanol. The methanol fraction was evaporated under N₂ flow, and the residue was resuspended in 100 μ l of water. Ciprofibroyl-CoA was analysed in this fraction by h.p.l.c. Adding authentic ciprofibroyl-CoA to the initial homogenate we found a recovery of 70–75% of the CoA ester.

Measurement of acyl-CoA esters of hypolipidaemic drugs by h.p.l.c.

H.p.l.c. separations were accomplished on a RP-18 (5 μ m) column (lichroCart 125-4; Merck, Darmstadt, Germany). The mobile phases were 0.5 M-KH₂PO₄ (pH 5.5)/water/methanol (4:11:10, by vol.) for ciprofibroyl-CoA; 0.5 M-KH₂PO₄

(pH 5.5)/water/methanol (1:4:5, by vol.) for clofibroyl-CoA; and 0.5 M-KH₂PO₄ (pH 5.5)/water/methanol (5:29:16, by vol.) for nafenopin. Under these conditions, retention times of ciprofibroyl-CoA, clofibroyl-CoA and nafenopin-CoA were 17, 10.7 and 16.2 min respectively. Ciprofibrate, clofibric acid and nafenopin were eluted at 6, 6.9 and 11.7 min. The mobile phase was pumped at a flow rate of 1.5 ml/min in all cases. Detection was at 254 nm. Identities of the acyl-CoA esters of the drugs were confirmed as previously described (Bronfman *et al.*, 1989).

Measurement of acyl-CoA synthetase activity in microsomes, liver homogenates and platelets

Rat liver microsomes were prepared as described elsewhere (Bronfman *et al.*, 1986). Human liver biopsies were obtained from patients undergoing surgery for uncomplicated gall-stone or gastroduodenal-ulcer disease. Informed consent from the patients was obtained by the procedures approved by the Ethics Committee of the Medical School of the Universidad Católica de Chile. Preparation of human liver homogenates was performed as previously described (Bronfman *et al.*, 1984). Platelets were prepared from freshly EDTA-anticoagulated human blood as described by Orellana *et al.* (1990). The rate of acyl-CoA synthetase activity, using hypolipidaemic drugs as substrates, and rat liver microsomes, total human liver homogenates, or platelets as source of enzyme, was measured in the presence of Triton X-100, as described for ciprofibroyl-CoA synthetase [the preceding paper (Amigo *et al.*, 1992)].

RESULTS

Determination of ciprofibroyl-CoA concentration in isolated and cultured rat liver hepatocytes and in the liver of ciprofibrate-treated rats

Fig. 1 shows the elution pattern of methanol/water extracts of isolated hepatocytes incubated in the presence or absence of ciprofibrate. Peaks with the same retention time of ciprofibrate and of ciprofibroyl-CoA were clearly detected in hepatocyte extracts incubated with ciprofibrate. Their identities were confirmed by analysis of their alkaline hydrolysis products (ciprofibroyl-CoA), as previously described (Bronfman *et al.*, 1986), and by u.v. spectra and t.l.c. in two different solvents (ciprofibrate). No peaks with the retention time of ciprofibroyl-CoA or ciprofibrate are observed in the chromatogram from control hepatocytes. Since before the extraction with methanol/water the hepatocytes were centrifuged through a bromodecane layer (see the Experimental section) in order to eliminate ciprofibrate or any amount of ciprofibroyl-CoA present in the incubation medium, the amount of ciprofibrate or ciprofibroyl-CoA detected was considered as the intracellular concentration. However, the intracellular localization of both compounds is not known, and some fraction of them may be absorbed to membranes. No ciprofibroyl-CoA was detected in the supernatant. Essentially identical results were observed with cultured hepatocytes.

Fig. 2 shows the elution pattern of methanol/water extracts prepared from the liver of a control rat and from that of a ciprofibrate-treated rat. Several new peaks appear in the treated rat liver chromatogram as compared with the control chromatogram. The peak with a retention time of 17 min was identified as ciprofibroyl-CoA, as described in the Experimental section. No attempt was made to identify the other peaks. They might correspond to short-chain-acyl-CoA derivatives, since their concentration is increased in the liver of ciprofibrate-treated rats (Bhuiyan *et al.*, 1988), or to other ciprofibrate metabolites, such as glucuronic acid or taurine conjugates, which have been

Fig. 1. H.p.l.c. chromatograms of a methanol/water extract of isolated hepatocytes and of ciprofibrate-incubated hepatocytes

Isolated hepatocytes were incubated in the absence (a) or presence (b) of 2 mM-ciprofibrate for 30 min, and partitioned as described in the Experimental section. In each case, 50 μ l of the methanol/water extract was injected. Detection was at 254 nm with 0.005 absorbance unit full scale, as indicated. The mobile phase was 0.5 M- KH_2PO_4 (pH 5.5)/water/methanol (4:11:10, by vol.), and was pumped at a flow rate of 1.5 ml/min. Peaks 2 and 3 were positively identified as ciprofibrate and ciprofibril-CoA respectively, as already described (Bronfman *et al.*, 1986).

detected as metabolites of the ciprofibrate-related drug, clofibrilic acid (Caldwell *et al.*, 1979).

The concentration of ciprofibril-CoA in the liver of ciprofibrate-treated rats, was 0.056 ± 0.022 and 0.068 ± 0.032 nmol/mg of protein in rats treated for 7 and 14 days respectively (mean $\pm$ S.D. for four rats). Peroxisomal fatty-acyl-CoA oxidase activity, a marker of peroxisomal proliferation, increased from 0.28 ± 0.02 units/g of liver to 2.6 ± 0.05 and 4.26 ± 0.80 units/g of liver after 7 and 14 days treatment respectively (mean $\pm$ S.D. for four rats).

On the crude approximation that 1 g of liver corresponds to 1 ml of intracellular water space, and to 200 mg of protein, it can be estimated from the above data that the liver concentration of ciprofibril-CoA ranges between 10 and 30 μ M and is probably higher, since only a fraction of the intracellular water space should be accessible to the acyl-CoA. Thus, in ciprofibrate-treated rats, a significant amount of the hepatocyte esterified CoA might be ciprofibril-CoA. Bhuiyan *et al.* (1988) have reported that total CoA increases from 124 ± 6.2 to 253 ± 13.7 nmol/g wet wt. in the liver of ciprofibrate-treated rats, whereas the short-chain-acyl-CoA fraction increases from 12.2 ± 2.5 to 35.2 ± 6.1 nmol/g wet wt. (i.e. from approx. 12 to 35 μ M). According to the ciprofibril-CoA solubility in acid media and to the fractionation techniques used by these authors, most of the ciprofibril-CoA should partition in their short-chain-acyl-CoA fraction, and the observed increase might, at least in part, be a consequence of ciprofibril-CoA formation.

The blood level of ciprofibrate in treated rats was not determined in the present work. However, under similar conditions, Edelson *et al.* (1979) have reported serum levels of 30 μ g of ciprofibrate/ml (0.1 mM). When isolated rat hepatocytes were

Fig. 2. H.p.l.c. chromatograms of a methanol/water extract of liver homogenate from a control and a ciprofibrate-treated rat

Liver homogenates from a control and a 14-day-ciprofibrate-treated rat were partitioned as described in the Experimental section, and the methanol fraction was analysed by h.p.l.c. (a) Chromatogram from control liver; (b) chromatogram from the liver of a ciprofibrate-treated rat. In each case, 10 μ l of the final extract was injected. Detection was at 254 nm with 0.02 absorbance unit full scale, as indicated. Chromatographic conditions were similar to those described in Fig. 1. Peak 6 was positively identified as ciprofibril-CoA, as described elsewhere (Bronfman *et al.*, 1986).

incubated with 0.1 mM-ciprofibrate for 30 min, the intracellular concentration of ciprofibril-CoA, determined as described in the Experimental section, was found to be 0.07 ± 0.056 nmol/mg of protein (mean $\pm$ S.D. for four independent experiments), which is in good agreement with the data from the liver of treated rats. Furthermore, it suggests that the plateau concentration of ciprofibril-CoA is reached in a very short time. In 24 h-cultured rat hepatocytes in the presence of 0.1 mM-ciprofibrate, ciprofibril-CoA concentration, determined as described in the Experimental section, was 0.16 ± 0.06 nmol/mg of protein (mean $\pm$ S.D. for three independent experiments).

Time course of ciprofibril-CoA formation in isolated hepatocytes: effect of palmitate and of carnitine

When isolated hepatocytes were incubated with ciprofibrate, the rate of ciprofibril-CoA formation was found to be maximal after 20–30 min incubation, either at high (1 mM) or low (0.1 mM) ciprofibrate concentrations. With ciprofibrate at 1 mM in the incubation medium (Fig. 3a), the addition of 1.5 mM-carnitine

Fig. 3. Time course of ciprofibroyl-CoA formation in isolated hepatocytes: effect of carnitine and palmitic acid

Isolated hepatocytes were incubated in the presence of 1 mM- (a) or 0.1 mM- (b) ciprofibrate, and the concentration of ciprofibroyl-CoA was measured as described in the Experimental section. A representative experiment out of three is presented. Incubations were performed in the presence of ciprofibrate (○), ciprofibrate plus 1.5 mM-carnitine (●) or ciprofibrate plus 1.5 mM-carnitine and 0.5 mM-palmitic acid (□).

showed no effect. The addition of palmitate (0.5 mM), in the presence of carnitine, partially inhibited the rate of ciprofibroyl-CoA formation, in agreement with previous observations suggesting that ciprofibrate and palmitate are activated by long-chain-acyl-CoA synthetase [the preceding paper (Amigo *et al.*, 1992)]. Surprisingly, with ciprofibrate at 0.1 mM in the incubation medium (Fig. 3b), the addition of 1.5 mM-carnitine increased both the rate of ciprofibroyl-CoA formation and its maximal concentration. Addition of palmitate (0.5 mM) in the presence of carnitine inhibited the carnitine-stimulating effect, but the rate of ciprofibroyl-CoA formation and its maximal concentration were still higher than that observed in the absence of carnitine and palmitate.

Several interesting points arise from this experiment. First we have previously shown evidence that the enzyme activating ciprofibrate is the same long-chain-acyl-CoA synthetase activating palmitate. Its K_m for ciprofibrate was found to be almost 30-fold higher than that for palmitate, and ciprofibroyl-CoA synthetase was strongly and competitively inhibited by palmitate [the preceding paper (Amigo *et al.*, 1992)]. These previous data obtained *in vitro* contrast with the relatively low inhibitory action of palmitate on ciprofibroyl-CoA formation by isolated hepatocytes, in the presence of carnitine, and suggest

Fig. 4. Time course of ciprofibroyl-CoA, clofibroyl-CoA and nafenopin-CoA formation in isolated hepatocytes

Isolated hepatocytes from a single rat were incubated with 0.5 mM-ciprofibrate, 0.5 mM-clofibric acid or 0.5 mM-nafenopin for the indicated time, and the intracellular concentration of the respective acyl-CoA ester of each drug was measured as described in the Experimental section. (a) Ciprofibrate (○) and clofibric acid (●); (b) nafenopin. Similar results were obtained in a second experiment.

that factors other than the relative affinity of acyl-CoA synthetase play a role in the activation of the drug. These factors might be related to the greater water solubility of ciprofibrate as compared with that of palmitate, or to competition for fatty-acid-binding proteins which are involved in the intracellular transport of fatty acids and their acyl-CoA esters (Glatz & van der Vusse, 1989). The inhibitory effect of palmitate was not much higher than that observed with palmitate plus carnitine (results not shown). Data obtained in the presence of carnitine are more relevant physiologically, since hepatocytes lose an important fraction of their intracellular carnitine during liver perfusion and hepatocyte isolation that is readily recovered after incubation with carnitine (Christiansen & Bremer, 1976).

Secondly, the activating effect of carnitine at low (0.1 mM) ciprofibrate concentrations might be explained because, in the presence of carnitine, the equilibrium between fatty-acyl-CoAs and fatty-acylcarnitines catalysed by carnitine acyltransferases is displaced to the formation of acylcarnitines, liberating CoA, which becomes available for ciprofibrate activation. Thus ciprofibrate might be effectively sequestering CoA, as has already been proposed by us (Bronfman *et al.*, 1986). Finally, the experiment of Fig. 3 suggests that the acylcarnitine ester of ciprofibrate is not formed, since, upon incubation of isolated hepatocytes with

ciprofibrate, ciprofibroyl-CoA reaches a maximal concentration which does not decrease upon incubation or by the addition of carnitine, suggesting no precursor-product relationship. Further, at a low concentration of the drug comparable with that obtained in the serum of ciprofibrate-treated rats (Edelson *et al.*, 1979), carnitine increases ciprofibroyl-CoA concentration. We have been unable to demonstrate the formation of ciprofibroyl-carnitine *in vitro* using total homogenates, subcellular fractions or commercially available carnitine acyltransferases from various sources, and ciprofibroyl-CoA as substrate (results not shown). Hertz & Bar-Tana (1987) reached a similar conclusion concerning the related drug bezafibrate.

Comparative time course and drug-concentration-dependence of hypolipidaemic drug acyl-CoA ester formation in isolated hepatocytes

In order to compare the fate of other hypolipidaemic drugs in isolated hepatocytes, the time course of clofibroyl-CoA and nafenopin-CoA formation were measured and compared with that of ciprofibroyl-CoA. One of these experiments is presented in Fig. 4. Clofibroyl-CoA and ciprofibroyl-CoA were formed at a similar rate and followed a similar pattern. However, the maximal concentration of clofibroyl-CoA was half that of ciprofibroyl-CoA, whereas that of nafenopin-CoA was almost 10-fold lower. Since we had previously reported that the rate of nafenopin-CoA formation *in vitro* is higher than that of clofibroyl-CoA (Bronfman *et al.*, 1986), we studied the concentration-dependence of the drug acyl-CoA ester formation after incubation with isolated hepatocytes for 30 min (Fig. 5). The formation of the acyl-CoA esters of the three hypolipidaemic compounds was saturable, agreeing with an enzyme-dependent process, whereas the apparent intracellular concentrations of the free drugs was not saturable up to the maximal concentration used, suggesting a simple diffusion mechanism of entry.

Maximal intracellular concentration of ciprofibroyl-CoA and of clofibroyl-CoA was already observed at 0.5 mM in the incubation medium, whereas for nafenopin-CoA only half of the maximal concentration was reached at 0.5 mM-nafenopin in the incubation medium. Further, the apparent intracellular concentration reached by nafenopin was lower than that of ciprofibrate or clofibrac acid. This difference might be related to the lower solubility of nafenopin or to a possible lower diffusion rate attributable to its more hydrophobic structure. However, on the assumption that the free cell-bound drug as determined in these experiments is approximately equivalent to its intracellular concentration, some additional conclusions may be drawn. In our experiments we have determined that 1 g wet wt. isolated hepatocytes corresponds to nearly 200 mg of protein. On this basis, with the data of Fig. 5, and on the assumption that 1 g of liver has a volume of 1 ml, we have estimated and plotted the intracellular concentration of each acyl-CoA ester of the drugs against the free-drug apparent intracellular concentration (Fig. 6). The maximal intracellular concentrations of the acyl-CoA esters of the drugs are roughly 100, 55 and 9 μM for ciprofibroyl-CoA, clofibroyl-CoA and nafenopin-CoA respectively. This maximal concentration is attained at the same apparent intracellular concentration of the free drug, nearly 300 μM , for ciprofibrate and nafenopin. For clofibrac acid a 2-fold higher apparent intracellular concentration (600 μM) is required for saturation.

On the other hand, the experiments reported in Figs. 5 and 6 show that low concentration of the drugs are far more effective in terms of the relative amount of the drug acyl-CoA ester formed. At 0.1 mM-ciprofibrate, about 25% of the total intracellular drug is esterified to CoA and increases to a maximum of 35% at 0.25 mM-drug. This percentage decreases to less than

Fig. 5. Intracellular concentration of the acyl-CoA ester of ciprofibrate, clofibrac acid and nafenopin, and of the free drugs, in isolated hepatocytes: dependence on the drug concentration in the incubation medium

Isolated hepatocytes from a single rat were incubated for 30 min at increasing concentrations of ciprofibrate (a), clofibrac acid (b) or nafenopin (c), and the concentrations of the acyl-CoA ester of the drug (○) and of the free drug (●) were measured as described in the Experimental section. Note the different scale for the acyl-CoA derivatives and for the free drugs. Essentially identical results were obtained in two other experiments for the relative amount of each one of the acyl-CoA derivatives of the drugs. However, the maximal concentration attained varied from 70 to 120% of the values reported in this experiment.

5% at 2 mM-ciprofibrate. At 0.1 mM-clofibrac acid, about 17% of the intracellular drug is esterified to CoA and decreases to less than 2% at 2 mM-drug. In contrast, at 0.1 mM-nafenopin, less than 3% of the intracellular drug is esterified to CoA and increases to a maximum of 5% at 0.5 mM-drug.

Time course of hypolipidaemic drug acyl-CoA ester formation by rat liver microsomes

Results presented above show that, in isolated hepatocytes, ciprofibroyl-CoA and clofibroyl-CoA concentrations are almost one order of magnitude higher than nafenopin-CoA concentration under the same experimental conditions. In previous studies of acyl-CoA ester formation from hypolipidaemic drugs by incubation with rat liver microsomes we found that nafenopin-

Fig. 6. Effect of the apparent intracellular concentration of free drugs on the formation of their acyl-CoA esters

Apparent intracellular concentrations of free drugs were calculated from data in Fig. 5 and plotted against the respective acyl-CoA ester concentration. ○, Ciprofibril-CoA; ●, nafenopin-CoA; □, clofibril-CoA. Note the different scale for nafenopin-CoA.

CoA was formed at a higher rate than clofibril acid (Bronfman *et al.*, 1986). To understand this difference, the time course of the drug-CoA formation catalysed by rat liver microsomes was re-examined. As shown in Fig. 7, the formation of ciprofibril-CoA is linear up to 60 min incubation, and proceeds at a rate of 3.2 nmol/min per mg of protein. Under the same experimental conditions, clofibril-CoA formation is not linear, and its initial velocity can be estimated to be 0.04 nmol/min per mg of protein, i.e. 80-fold lower than that of ciprofibril. Nafenopin-CoA formation is not linear either, and its initial velocity is 0.1 nmol/min per mg of protein, i.e. 30-fold lower than that of ciprofibril, but 2.5-fold higher than that of clofibril acid. These formation rates are better estimates than those previously reported (Bronfman *et al.*, 1986), since they were determined using authentic acyl-CoA esters of the drugs as standards, whereas previous data were calculated on the assumption that their absorption coefficients were the same as that of free CoA.

Activation of ciprofibrate by human liver homogenates and by human platelets

Since hypolipidaemic-drug effects are different in the rat and in humans (Hawkins *et al.*, 1987) we decided to find out whether human tissues would be also capable of activating hypolipidaemic drugs. By using ciprofibrate as a model compound it was found that human liver homogenates activated this drug at a rate of 1.07 and 0.73 nmol/min per mg of protein in two independent experiments. We had previously found comparable activity in rat liver homogenates [1.6 ± 0.39 nmol/min per mg of protein; the preceding paper (Amigo *et al.*, 1992)].

The activity of ciprofibril-CoA synthetase in Triton X-100-solubilized human platelets was two orders of magnitude lower than that of liver (6.9×10^{-3} and 8.0×10^{-3} nmol/min per mg of protein in two independent experiments). The time course of

Fig. 7. Time course of the formation of hypolipidaemic-drug acyl-CoA esters catalysed by rat liver microsomes

Rat liver microsomes were incubated with 3 mM-ciprofibrate (a), 3 mM-clofibril acid (b) or 3 mM-nafenopin (c), and the formation of the respective acyl-CoA ester was determined by h.p.l.c., as described in the Experimental section.

ciprofibril-CoA formation was linear for almost 60 min, both with human liver homogenates and human platelets.

DISCUSSION

The results presented here show that the acyl-CoA esters of three hypolipidaemic drugs are formed by isolated liver cells. In addition, we show that the liver concentration of ciprofibril-CoA in ciprofibrate-treated rats is similar to the one observed in isolated liver cells, and that a significant fraction of the cell-bound drug is present in the form of its acyl-CoA ester. These observations support the possibility that the acyl-CoA esters of hypolipidaemic peroxisome proliferators might be the pharmacological active species of these compounds, as previously suggested (Bronfman *et al.*, 1986). On the basis that carnitine addition stimulates rather than decreasing ciprofibril-CoA formation in isolated hepatocytes, it is proposed that ciprofibrate can effectively sequester CoA and that the carnitine ester derivative of the drug is probably not formed. Thus availability of CoA and alterations in the metabolism of its acyl esters might be in part responsible for the multiple effects that these drugs

produce in experimental animals. It is noteworthy that both the levels and relative composition of hepatic acyl-CoAs and acylcarnitines are dramatically altered in ciprofibrate-treated rats (Bhuiyan *et al.*, 1988). It is also shown that ciprofibroryl-CoA and clofibroryl-CoA are formed at a similar rate by isolated hepatocytes and reach intracellular concentrations of the same order of magnitude, whereas nafenopin-CoA has a lower intracellular concentration. However, as hypolipidaemic agent, ciprofibrate is by far the more active drug (Best & Duncan, 1970), and nafenopin is 5-fold more active than 'clofibrate' (Arnold *et al.*, 1979). Thus no apparent correlation exists between the concentration of the acyl-CoA esters of the drugs in isolated hepatocytes and their relative hypolipidaemic activity. The nature of the hydrophobic backbone might explain this effect. We have shown that the acyl-CoA esters of several of these compounds potentiate protein kinase C *in vitro* (Bronfman *et al.*, 1989). At comparable concentrations, nafenopin-CoA and ciprofibroryl-CoA are, respectively, 3–4-fold and 2–3-fold more active than clofibroryl-CoA in potentiating this enzyme activity. It is then possible that the concentration of the acyl-CoA ester required for a particular effect might vary from one drug to other, depending on their respective hydrophobic backbone. On the other hand, the extrapolation to drug-treated rats is complicated by the fact that the doses used are quite different. For obtaining similar metabolic changes in rat liver, we use respectively 50 mg, 1 g and 3 g per kg of chow of ciprofibrate, nafenopin or clofibric acid.

The relative rate of acyl-CoA ester formation of the drugs by liver microsomes was not found to correlate with their relative formation rate or maximal concentration in isolated hepatocytes. Although the rate of ciprofibroryl-CoA formation by rat liver microsomes is far higher than that of clofibroryl-CoA, their maximal concentration in isolated hepatocytes differs only by a factor of 2. In turn, nafenopin-CoA, which is formed by rat liver microsomes at a rate 2.5-fold higher than that of clofibroryl-CoA, showed in isolated hepatocytes a cell concentration almost 5-fold lower than that of clofibroryl-CoA. This observation might be attributed to the fact that the '*in vitro*' assay is performed in the presence of Triton X-100, under conditions where the differences in solubility between nafenopin and clofibric acid may not play a major role in activity. Nafenopin is only slightly soluble in the hepatocyte incubation media, and opalescent solutions are formed, whereas 10 mM solutions of ciprofibrate and clofibric acid in incubation media are easily prepared. On the other hand, the kinetic properties and relative affinities for the drugs of the Triton X-100-solubilized acyl-CoA synthetase might differ from those of the membrane-bound enzyme in the intact hepatocyte. Also, the possibility that the different drugs may be activated by separate enzymes cannot be ruled out. Free drug availability for acyl-CoA synthetase will also depend on the '*in vivo*' formation rate of other drug derivatives, as their glucuronide or taurine conjugates, which might vary for each drug. Different rates of hydrolysis by acyl-CoA hydrolases may also be of importance. Further kinetic studies *in vitro* and *in vivo* should be performed to elucidate these questions.

Acyl-CoA synthetase activity was demonstrated in human platelets using ciprofibrate as substrate. We have shown elsewhere that acyl-CoAs increase the phosphorylation of a protein kinase C substrate in human platelets (Orellana *et al.*, 1990). Whether acyl-CoA esters of hypolipidaemic compounds might have a

similar action, accounting for the various effects that hypolipidaemic drugs induce in these cells (Favis & Colman, 1978; Gantmacher, 1979), remains to be investigated.

Human liver homogenates were found to activate ciprofibrate at a rate comparable with that observed using rat liver homogenates. This shared activity would contradict the different effects of hypolipidaemic drugs on rat and man. For instance, the peroxisomal proliferation induced by these compounds seems to be restricted to rodents (Holloway *et al.*, 1982a,b). Further research on the metabolism and deposition of hypolipidaemic-drug acyl-CoA esters and other possible metabolites in humans and in the rat might help us to understand these differences.

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