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Short Communication

KINETIC CHARACTERISTICS OF RAT LIVER PEROXISOMAL NAFENOPIN-CoA LIGASE

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Abstract—In this study we have demonstrated that rat hepatic peroxisomes catalyse the formation of nafenopin-CoA. The process is mediated by apparent high affinity (K_m 6.7 μM), low capacity (V_{max} 0.31 nmol/mg/min) and low affinity, high capacity isoforms. Palmitic acid (K_i 1.1 μM), R(-) ibuprofen (K_i 7.9 μM), ciprofibrate (K_i 60.2 μM) and clofibric acid (K_i 86.8 μM) competitively inhibited nafenopin-CoA formation catalysed by the apparent high affinity isoform. An antibody raised against the microsomal palmitoyl-CoA ligase inhibited the equivalent peroxisomal enzyme significantly ($P < 0.001$) but did not inhibit peroxisomal nafenopin-CoA ligase activity. These data suggest that nafenopin-CoA formation is catalysed by a peroxisomal CoA ligase which differs from the peroxisomal long chain fatty acid-CoA ligase in relation to its xenobiotic/antibody inhibitor profile and kinetic characteristics.

Key words: hepatic peroxisomes; nafenopin; nafenopin-CoA ligase; palmitic acid, long chain fatty acid-CoA ligase; xenobiotic carboxylic acids

The formation of acyl-CoAs occurs as an obligatory step in the metabolism of a variety of endogenous substrates such as fatty acids, icosanoid precursors, short branched chain aliphatic acids and bile acids. The enzymes catalysing these reations belong to a group of ATP dependent acid:CoA ligases which exhibit diverse intracellular localisation and tissue distribution. The hepatic mitochondrial medium chain and microsomal long chain fatty acid-CoA ligases activate not only endogenous substances but also xenobiotic carboxylic acids to their corresponding acyl-CoA thioesters [1, 2]. The long chain fatty acid-CoA ligases (EC 6.2.1.3), which are distributed between the smooth endoplasmic reticulum, the outer mitochondrial membrane and the peroxisomal membrane, have similar amino acid composition, immunological cross reactivity and kinetic characteristics suggesting that the activities reside in either the same or closely related proteins [3, 4].

In a recent study the existence of isoforms of rat hepatic microsomal long chain fatty acid-CoA ligase was demonstrated with activity of the high affinity form showing inhibition by various 2-arylpropionate enantiomers [5]. This enzyme in rats has also been implicated in the formation of the acyl-CoAs of 3-phenoxybenzoic acid [6], ciprofibrate, clofibric acid and nafenopin [2] as well as the R(-) enantiomers of ibuprofen [7] and fenoprofen [8]. Evidence is also emerging that, in addition to the microsomal long chain (palmitoyl)-CoA ligases, separate microsomal xenobiotic-CoA ligases exist [9, 10]. Similarly, in rat hepatic peroxisomes, multiplicity of long chain fatty acid-CoA ligases has been demonstrated [11]. The high affinity peroxisomal isoform was inhibited competitively by nafenopin and ciprofibrate whilst a number of other xenobiotic carboxylic acids were found to be non-inhibitory at all concentrations studied [11].

Administration of hypolipidaemic compounds such as nafenopin and ciprofibrate to rats induces hepatic peroxisomal proliferation and increases peroxisome associated enzyme activities [12]. This peroxisomal response to xenobiotics has been suggested to be mediated either by a peroxisome proliferator-activated receptor (PPAR‖) [13, 14] or through perturbations in lipid metabolism. Long term administration of nafenopin to mice and rats produces liver tumours [15, 16] and it has been suggested that the hepatocarcinogenicity is linked to the sustained increase in peroxisomal activity [15]. A striking feature of chemicals that cause proliferation of hepatic peroxisomes is the effect on lipid metabolism. Bronfman *et al.* [2] and others [12, 17] have suggested that the acyl-CoA esters of hypolipidaemic drugs play a central role in proliferation and that xenobiotic-CoA formation may initiate the adaptive responses.

To date, the relative contribution of peroxisomal 'long chain like' fatty acid-CoA ligases to the formation of xenobiotic-CoAs have not been fully elucidated. In light of recent evidence of multiplicity of hepatic microsomal CoA ligases, it is conceivable that peroxisomes may also possess distinct xenobiotic-CoA ligases. This has important implications because formation of xenobiotic-CoAs by peroxisomes has the potential to significantly increase acylation of limited CoA pools hence perturbing cellular integrity and decreasing peroxisomal β-oxidation of medium, long and very long chain fatty acids. In the present paper we report, for the first time, the kinetic characteristics of a rat peroxisomal nafenopin-CoA ligase.

Materials and Methods

Xenobiotic carboxylic acids were obtained from the following sources: R(-) ibuprofen (97.3% pure, Boots Co.,

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‖ Abbreviation: PPAR, peroxisome proliferator-activated receptor.

Nottingham, U.K.), nafenopin (Ciba-Geigy, Sydney, Australia), ciprofibrate (Sterling Drug Inc., Rensselaer, NY, U.S.A.) and clofibric acid (Sigma Chemical Co., St Louis, MO, U.S.A.). [^{14}C]-Palmitic acid (specific radioactivity 58 mCi/mmol) and [^{3}H]-nafenopin (specific radioactivity 27.8 Ci/mmol) were obtained from Amersham (Australia Pty Ltd), Nycodenz from BDH Chemicals (Melbourne, Australia) and Matrex Gel Red A from the Amicon Corporation (Danvers, MA, U.S.A.). All other chemicals were of the highest analytical purity available and purchased from various commercial sources.

Preparation of peroxisomes, enzyme assays and protein determination. Male outbred Sprague–Dawley rats (200–300 g) were used for all experimental work. Animals were housed under standard conditions of light and temperature, fed a commercial chow diet and water *ad lib.* Rats were fasted over night and the liver was removed from each animal (N = 5) under sodium pentobarbitone anaesthesia (60 mg/kg). Hepatic peroxisomes were prepared by Nycodenz gradient centrifugation (11), assayed for purity (> 93%) and stored at −70°. Activities of the marker enzymes, catalase (peroxisomal matrix), glucose-6-phosphatase (microsomes), glutamate dehydrogenase (mitochondrial matrix) and palmitoyl-CoA ligase were determined as stated previously [11]. Protein was determined according to the procedure of Lowry *et al.* [18].

Synthesis, purification and identification of nafenopin-CoA. Synthesis of nafenopin-CoA was carried out as described previously for the synthesis of fatty acyl-CoAs [19]. A reaction mixture (12.5 mL) consisting of $MgCl_2$ (30 mM), ATP (10 mM), dithiothreitol (5 mM), CoA (2 mM), Tris-HCl (pH 8.0, 0.1 M) and nafenopin (2 mM) was recycled through a chromatography column (1 × 10 cm) at room temperature for 4 hr. Samples (100 μL) were collected at various times and analysed by HPLC for nafenopin and nafenopin-CoA. HPLC separations were performed using a μBondapak C18 column (30 × 0.4 cm), a variable wavelength detector (model 480) and pump (model 501, Waters Associates, Milford, MA, U.S.A.). Detection was at 254 nm and the mobile phase of 55% (v/v) methanol/40 mM KH_2PO_4 (pH 5.5) was pumped at 1.5 mL/min. Using these conditions the retention times of nafenopin and nafenopin-CoA were 9 min 15 sec and 16 min 10 sec, respectively. The column eluents corresponding to the nafenopin-CoA peaks were collected, pooled and evaporated to dryness under nitrogen. To remove phosphate salts the sample was reconstituted in 50% (v/v) methanol/water and rechromatographed using a mobile phase of 50% (v/v) methanol/water. The nafenopin-CoA peak eluents were collected, pooled, evaporated to dryness under nitrogen and the residue stored desiccated at −70°. The identity and purity of nafenopin-CoA was established by TLC (isobutanol/acetic acid/H_2O, 5:2:3, single spot R_f = 0.61 [20] and ultraviolet spectral analysis, while definitive evidence for the 1:1 covalent nafenopin-CoA complex was provided by positive and negative FAB mass spectrometry. The mass spectra of the complex were measured on a VG ZAB-2SEQ mass spectrometer in a glycerol/thioglycerol (1:1) matrix using a beam of 30 kV caesium ions. The positive FAB spectrum showed a series of potassium-containing molecular ions of the complex (1059 Da) at *m/z* 1136, 1174 and 1212, corresponding to $(M + 2K - H)^+$, $(M + 3K - 2H)^+$ and $(M + 4K - 3H)^+$ while in its negative FAB spectrum, molecular anions representing $(M + K - 2H)^-$, $(M + 2K - 3H)^-$ and $(M + 3K - 4H)^-$ were present at *m/z* 1096, 1134 and 1172. The dominance of the potassium adduct ions was due to the presence of KH_2PO_4 in the buffer used for HPLC.

In vitro *assay of nafenopin-CoA ligase activity.* The assay system used for determining palmitoyl-CoA ligase activity was adapted for assessing formation of nafenopin-CoA. Palmitic acid was replaced by [^{3}H]nafenopin (0.1–1000 μM, specific activity 2.25 mCi/mmol) and the reaction terminated by the addition of 0.1 M H_2SO_4 (50 μL). Preliminary studies established the linearity of nafenopin-CoA formation with respect to duration of incubation (180 min) and protein concentration (100 μg/mL). [^{3}H]Nafenopin and nafenopin-CoA were quantified by HPLC using the conditions described above. The column eluent was collected at 1 min intervals, scintillant added and the samples counted using a Beckman LS 501 scintillation counter. Recovery of the radiolabel was consistently 99–100% and the retention windows for both nafenopin and nafenopin-CoA were set using the authentic compounds. The reproducibility (coefficient of variation) of the assay both inter and intra day was 5.5% and 3%, respectively. Inhibition of nafenopin-CoA ligase activity was studied using 1, 2.5 and 5 μM [^{3}H]-nafenopin and either palmitic acid (0–5 μM), R(-) ibuprofen (0–10 μM), ciprofibrate or clofibric acid (0–100 μM).

Immunochemical procedures. A polyconal antibody raised in rabbits to rat hepatic microsomal palmitoyl-CoA ligase [21] was obtained from the Medical University of South Carolina. The antibody received in ammonium sulphate was dialysed (16 hr) against PBS (pH 7.4) and assayed for protein content [18]. Inhibition of peroxisomal palmitoyl-CoA ligase activity was studied at antibody:protein ratios up to and including 20:1 whilst inhibition of nafenopin-CoA ligase was studied at a ratio of 20:1 only. Preliminary studies established that binding of the antibody was not compromised using either pre- or co-incubated conditions and all subsequent studies involved co-incubating substrate and antibody.

Peroxisomal protein was electrophoresed according to the procedure of Laemmli [22] as modified by McManus *et al.* [23]. Proteins were transferred and subjected to Western blot analysis with minor modifications, i.e. the nitrocellulose filter was blocked for a period of 5 hr and incubated with the primary antibody (150 μg) for 16 hr [23].

Data analyses. Data from the inhibition studies were analysed using the method of Dixon [24] and the Modified Gram–Schmidt Algorithm (CoStat, CoHort Software, Berkley, CA, U.S.A.). Values for K_m and V_{max} were determined using MKMODEL [25]. Statistical significance of the data from the antibody studies was assessed using a paired Student's *t*-test with a level of significance of at least $P < 0.05$.

Results and Discussion

Characterisation of nafenopin-CoA ligase. The activity of peroxisomal nafenopin-CoA ligase was assessed over a concentration range of 0.1–1000 μM nafenopin. Eadie–Hofstee plots of the data from five individual rats clearly exhibited biphasic kinetics, corresponding to high affinity, low capacity and low affinity, high capacity isoforms. This type of kinetic profile has been reported in previous studies investigating the multiplicity of microsomal [5, 9] and peroxisomal palmitoyl-CoA ligases [11] and microsomal nafenopin-CoA ligases [10].

The apparent K_{m1} and V_{max1} values for the high affinity nafenopin-CoA ligase were 6.7 ± 3.1 μM and 0.31 ± 0.09 nmol/mg/min (mean ± SD, N = 5), respectively. Although there were clear indications of a low affinity isoform with an apparent K_{m2} in the order of 260 μM and V_{max2} approximating 2100 nmol/mg/min, the data were not modelled because of evidence of aberrant kinetics at concentrations of nafenopin exceeding 100 μM. This problem has been encountered previously in studies investigating formation of the CoA esters of R(-) flurbiprofen [9], nafenopin and clofibric acid [26] and may be explained by formation of micelles at high substrate concentrations.

In relation to substrate activation, the K_{m1} for nafenopin was 3-fold greater than that observed for palmitic acid (i.e. 2.3 μM) for the peroxisomal high affinity palmitoyl-CoA ligase [11] whilst V_{max1} differed 64-fold between the two peroxisomal ligases indicating differences in intrinsic clearance via acyl-CoA ester formation. This clearly could

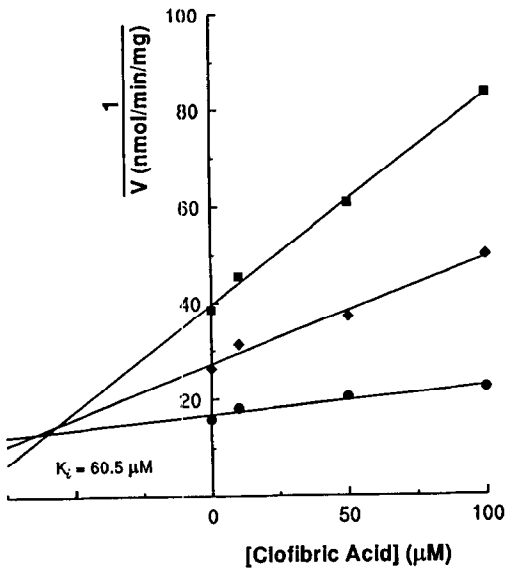

Fig. 1. Inhibition of the high affinity peroxisomal nafenopin-CoA ligase by clofibric acid (0–100 μM). The concentrations of nafenopin used were 1 (■), 2.5 (◆) and 5 μM (●).

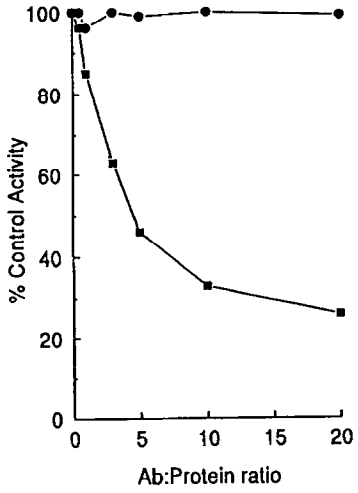

Fig. 2. Palmitoyl-CoA ligase activity was assessed using 4 μM palmitic acid in the presence of either pre-immune serum (●) or the antibody to palmitoyl-CoA ligase (■).

give rise to a situation in which these metabolic pathways would compete for the limited pool CoA. Under those circumstances relative rates of hydrolysis of the resulting acyl-CoAs would assume a degree of importance in maintaining cellular homeostasis.

Effects of xenobiotic inhibitor probes. The apparent high affinity peroxisomal nafenopin-CoA ligase was inhibited competitively by palmitic acid (K_i 1.14 ± 0.67 μM), R(-) ibuprofen (K_i 7.92 ± 4.05 μM), ciprofibrate (K_i 60.2 ± 35.2 μM) and clofibric acid (Fig. 1, K_i 86.8 ± 27.2 μM, mean ± SD, N = 5). Interestingly R(-) ibuprofen was a potent inhibitor at concentrations which are achieved following normal dosing regimes in humans. This is in contrast to the high affinity peroxisomal palmitoyl-CoA ligase which is inhibited by R(-) ibuprofen (K_i 460 μM) but at concentrations unlikely to be physiologically relevant [11].

In contrast to palmitic acid and ibuprofen, when ciprofibrate and clofibric acid were studied as inhibitors, a degree of variability was observed with the data, namely ciprofibrate K_i range 22–120 μM and clofibric acid K_i range 56–134 μM. Although the predominant form of inhibition observed was competitive, one rat in the case of ciprofibrate and two rats in the case of clofibric acid exhibited mixed inhibition kinetics. A profile of mixed inhibition implies that there are two or more sites on the enzyme with binding at one site influencing the affinity of the ligand at the other site [5]. Under these circumstances, fitting of the data to various models may represent an over-simplification of reality and in fact the kinetics of inhibition by ciprofibrate and clofibric acid are considerably more complex. These data are in accord with Amigo *et al.* [27] who have previously demonstrated mixed inhibition kinetics with ciprofibrate using rat hepatic microsomes. Despite the kinetic complexity, ciprofibrate inhibits both nafenopin and palmitoyl-CoA formation [11] and thus may be considered an alternative substrate for both peroxisomal ligases. At present the role of ciprofibroyl-CoA *per se* in the induction of peroxisomal proliferation remains to be investigated.

Clofibric acid is a potent peroxisomal proliferative agent and peroxisomes are known to mediate clofibroyl-CoA formation [17]. A previous studies [11] has however discounted a role for the peroxisomal palmitoyl-CoA ligase in that process. In this study clofibric acid inhibited the peroxisomal nafenopin-CoA ligase and thus may function as an alternative substrate. Current studies are investigating the role of the nafenopin-CoA ligase in the formation of clofibroyl-CoA.

Xenobiotics are commonly used as probes to differentiate isoforms of drug metabolising enzymes [28]. In that context, nafenopin is an inhibitor of palmitoyl-CoA formation in peroxisomes (K_i 390 μM) but also functions as a substrate (K_m 6.7 μM) for a peroxisomal nafenopin-CoA ligase. For nafenopin the respective K_i and K_m values differ 60-fold between the ligases, whilst in relation to palmitic acid, its K_m in relation to palmitoyl-CoA formation and K_i with regard to inhibition of nafenopin-CoA formation are similar, i.e. 2.3 and 1.1 μM, respectively. Collectively data from this and other studies suggest that palmitic acid is not an isoform specific probe. In contrast, when screening with xenobiotics, it is apparent that the peroxisomal palmitoyl-CoA and nafenopin-CoA ligases differ both in their xenobiotic inhibitor profiles and kinetic characteristics.

Immunochemical studies. The antibody raised against hepatic microsomal palmitoyl-CoA ligase inhibited significantly peroxisomal acyl-CoA ligase activity for palmitic acid (Fig. 2, $P < 0.001$). Interestingly peroxisomal nafenopin-CoA ligase activity was not significantly inhibited at the same antibody:protein ratio, i.e. 20:1 (Fig. 3). When examined by Western blot analysis using two concentrations of peroxisomal protein, i.e. 50 and 100 μg, the antibody recognised several protein bands with the most intensely staining at a molecular weight of 75 kDa. The peroxisomal fraction also exhibited an array of weakly staining bands, once at 90 kDa and several diffuse bands clustered above and below the 75 kDa band. The cluster of bands around 75 kDa suggests the presence of closely related forms with molecular weights within the range reported for the hepatic microsomal palmitoyl-CoA ligase, i.e. 75–78 kDa [21, 29]. Although the polyclonal antibody used clearly cross reacted with more than one protein, it consistently inhibited peroxisomal palmitoyl-CoA ligase activity whilst showing no inhibitory activity towards

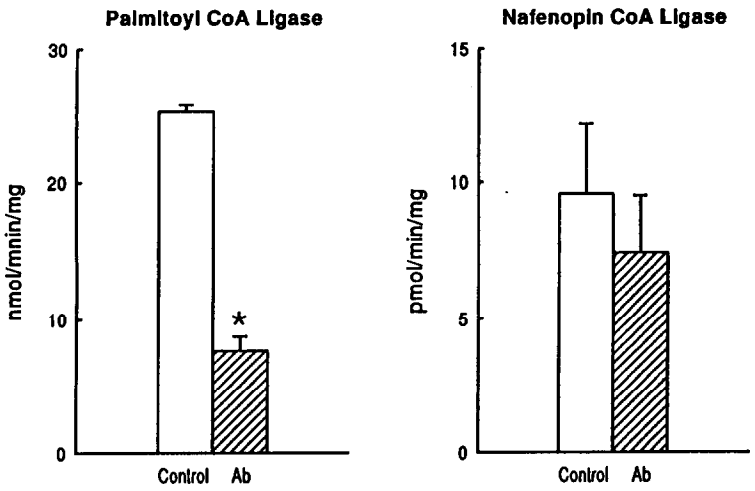

Fig. 3. Palmitoyl and nafenopin-CoA ligase activities were assessed in the presence of either pre-immune serum or the antibody to palmitoyl-CoA ligase using an antibody:protein ratio of 20:1. The data are presented as mean ± SD, N = 5 and statistical significance is denoted as $^*P < 0.001$.

nafenopin-CoA ligase. These results suggest that immuno-recognition of the active sites differs between the nafenopin and palmitoyl-CoA ligases. Using this antibody similar discrimination has been observed between brain microsomal palmitoyl-CoA ligase and arachidonoyl- and lignoceroyl-CoA ligase activities [21].

Nafenopin, ciprofibrate and clofibric acid are hypolipidaemics which share a common characteristic in that they are metabolised to acyl-CoA esters. These highly reactive intermediates have been shown to activate protein kinase C [30, 31], inhibit the peroxisomal fatty acyl-CoA-oxidising system [27], act as precursors in the formation of hybrid triacylglycerols [32] and perturb CoA homeostasis [33]. Undoubtedly xenobiotic-CoAs have the potential to profoundly influence cellular homeostasis. The results of this study demonstrate that rat hepatic peroxisomes contain at least one high affinity, low capacity enzyme which catalyses formation of the acyl CoA thioester of nafenopin and which has a different xenobiotic inhibitor profile to the peroxisomal palmitoyl-CoA ligase. An analogous situation has been observed using hepatic microsomes [10] and suggests that formation of nafenopin-CoA may occur at multiple intracellular sites. At present either a casual or causal role for nafenopin-CoA in peroxisomal proliferation and initiation of liver tumours remains to be investigated.

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