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The stereospecific inhibition of endogenous triacylglycerol synthesis by fenoprofen in rat isolated adipocytes and hepatocytes

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Most of the 2-arylpropionate non-steroidal anti-inflammatory agents such as fenoprofen, ibuprofen and ketoprofen are used clinically as racemates, although anti-inflammatory activity is thought to reside primarily with the *S*-enantiomers [1]. The *R*-enantiomers were, until recently, thought to be inactive, or at best, to act as prodrugs for the active *S*-enantiomer formed via stereospecific chiral inversion [2, 3], involving formation of a highly reactive *R*-2-arylpropionyl–CoA thioester intermediate [4–6]. Fatty acyl–CoA thioesters are the endogenous substrates for triacylglycerol synthesis and, consistent with the stereospecific formation of *R*-2-arylpropionyl–CoA thioesters, fenoprofen [7] and ibuprofen [8] undergo stereospecific incorporation into rat triacylglycerols, forming hybrid fenoprofen– and ibuprofen–triacylglycerols.

Racemic mixtures of various 2-arylpropionates have been shown to inhibit cholesterogenesis and fatty acid synthesis *in vitro*, and also to decrease serum triacylglycerol and cholesterol levels *in vivo* in rats [9]. The hypolipidaemic activity of the 2-arylpropionates appears to be correlated with their capacity to form hybrid triacylglycerols [9], and in this paper we now report the effects of *R*- and *S*-fen-

oprofen on endogenous triacylglycerol synthesis in the rat isolated hepatocytes and adipocytes of our previous study [7].

Methods

Using additional cells that had been obtained during a previous study of fenoprofen–triacylglycerol synthesis [7] we subsequently measured endogenous triacylglycerol synthesis by isolated adipocytes and hepatocytes incubated with *R*- or *S*-fenoprofen and [^{3}H]glycerol.

Cells had been isolated from adult male Hooded Wistar rats (N = 6), and studied using the incubation conditions as previously described [7]. Briefly, the conditions were as follows. Isolated adipocytes (10^5 viable cells) were incubated in duplicate for 30 min in plastic scintillation vials with either *R*- or *S*-fenoprofen to give final concentrations in the range of 0–500 μM, and 50 μCi [^{3}H]glycerol giving a final concentration of 14.8 mM. Isolated hepatocytes (4×10^5 viable cells) were incubated in duplicate for 15 min in glass Erlenmyer flasks with either *R*- or *S*-fenoprofen to give final concentrations in the range of 0–5000 μM, and 10 μCi [^{3}H]glycerol giving a final concentration of 1.6 mM. All incubations were carried out at 37° in a shaking water bath in an atmosphere of 5% CO_2/95% O_2. Quantitation of endogenous [^{3}H]triacylglycerol was carried out using a specific method capable of resolving endogenous and fenoprofen–triacyclglycerols as two separate classes [10].

Statistical analysis of differences in the synthesis of endogenous triacylglycerol by isolated hepatocytes and adipocytes incubated with varying concentrations of *R*- or *S*-fenoprofen was carried out using a one-way analysis of variance and Tukey's HDS test in the case of homogeneous populations, or by using a Kruskall–Wallis one-way analysis of variance by ranks and distribution free multiple comparisons in the case of heterogeneous populations [11]. Homogeneity of a sample population was determined using Bartlett's test for homogeneity of variance [11].

Results

In adipocytes, the mean (SE) synthesis of endogenous triacylglycerol in the absence of fenoprofen was 27.8 (2.2) pmol/hr/10^6 viable cells. At all fenoprofen concentration up to 30 μM, only *R*-fenoprofen significantly inhibited ($P < 0.05$) endogenous triacylglycerol synthesis to approximately 38% of control values (Fig. 1). At 500 μM endogenous triacylglycerol synthesis was inhibited to 12% and 42% of control values by *R*- and *S*- fenoprofen respectively (Fig. 1). The inhibition at 500 μM was also accompanied by a dramatic decrease in total triacylglycerol synthesis and fenoprofen–triacylglycerol synthesis [7], and is therefore thought to be due to fenoprofen toxicity. At the lower concentrations there was no evidence of fenoprofen toxicity as determined by total triacylglycerol synthesis.

In hepatocytes, the mean (SE) endogenous triacylglycerol synthesis in the absence of fenoprofen was 257 (20) pmol/hr/10^6 viable cells. Endogenous triacylglycerol synthesis was inhibited by both *R*- and *S*-fenoprofen to approximately 40–50% of control values (Fig. 2B). This inhibition was stereoselective with *R*-fenoprofen significantly inhibiting ($P < 0.05$) endogenous triacylglycerol synthesis at concentrations of 1 μM or greater (Fig. 2A), compared to 100 μM or greater for *S*-fenoprofen (Fig. 2B). There was no evidence of fenoprofen toxicity in the hepatocyte incubations as assessed by total triacylglycerol synthesis and the trypan blue exclusion method.

Discussion

During the last decade there has been growing evidence that xenobiotic carboxylic acids are able to mimic endogenous fatty acids and thereby alter, or act as substrates in the pathways of lipid synthesis and metabolism [12, 13]. Like endogenous fatty acids, xenobiotic carboxylic acids are thought to require activation to CoA thioesters before they can enter the pathways of lipid synthesis and metabolism [14]. The 2-arylpropionic acids are an interesting family of drugs since, although they are administered as racemates, only the "therapeutically inactive" *R*-enantiomers are metabolized to CoA thioester intermediates, as evidenced by their stereospecific chiral inversion [2, 3], lipid incorporation [7, 8], and more direct studies of CoA thioester formation [4–6].

In both adipocytes and hepatocytes, at clinically relevant concentrations [7], we have shown that only *R*-fenoprofen inhibits endogenous triacylglycerol synthesis to approximately 40–50% of control values (Figs 1 and 2A). In hepatocytes, at the high concentration range (Fig. 2B), *S*-fenoprofen also inhibits endogenous triacylglycerol synthesis, however, because of the small contamination of *S*-fenoprofen with its *R*-enantiomer (approximately 2–3%), the observed inhibition of endogenous triacylglycerol syntheis is consistent with the presence of the *R*-enantiomer. The high fenoprofen concentration range is approximately 100-fold greater than the unbound fenoprofen concentrations usually achieved clinically in humans [7] and is therefore not considered pharmacologically relevant.

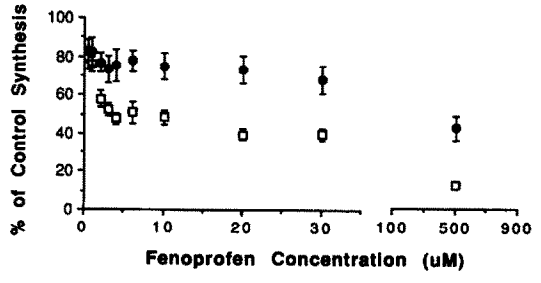

Fig. 1. The effects of (□) *R*- and (●) *S*-fenoprofen on endogenous triacylglycerol synthesis in isolated rat adipocytes. Results are expressed as mean (±SE) of six preparations.

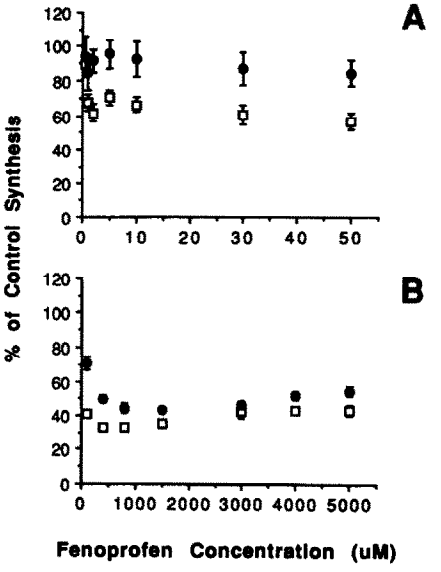

Fig. 2. The effects of (A) low and (B) high concentrations of (□) *R*- and (●) *S*-fenoprofen on endogenous triacylglycerol synthesis in isolated rat hepatocytes. Results are expressed as mean (±SE) of six preparations.

Endogenous fatty acids are mutually competitive inhibitors of each other's activation to CoA thioesters [15], and *R*-fenoprofen is a competitive inhibitor of the activation of palmitate to palmitoyl-CoA with a K_i of 15 μM whereas *S*-fenoprofen displays partial mixed inhibition with a K_i of 70 μM [16]. The stereospecific (or highly stereoselective) inhibition of endogenous triacylglycerol synthesis demonstrated in this study may therefore result from competitive inhibition of the activation of endogenous fatty acids. However, it is also possible that fenoprofen–CoA and endogenous fatty acyl-CoAs compete with each other for esterification with glycerol. In both adipocytes and hepatocytes, inhibition of endogenous triacylglycerol synthesis appears to mirror fenoprofen–triacyglycerol synthesis [7], and this observation also supports a mechanism of competitive inhibition. In hepatocytes there is a component of endogenous triacylglycerol synthesis which cannot be inhibited even by high concentrations of fenoprofen, suggesting the presence of a separate process involved in the formation of endogenous lipids which is independent of the high and low affinity processes described for fenoprofen–triacylglycerol synthesis [7].

Fears and Richards [9] have reported that racemic mixtures of various 2-arylpropionic acids are capable of decreasing serum triacylglycerol and cholesterol levels *in vivo* in rats, and also of inhibiting cholesterogenesis and fatty acid synthesis *in vitro*. The hypolipidaemic activity of the 2-arylpropionic acids appears to be correlated with their capacity to form hybrid triacylglycerols [9] and may therefore also be correlated with their ability to form 2-arylpropionyl–CoAs. Xenobiotic acyl–CoAs may have a role in the hypolipidaemic actions of a variety of drugs. McCune and Harris [17] demonstrated that the CoA ester of the hypolipidaemic agent 5-(tetradecyloxy)-2-furoic acid is an effective inhibitor of acetyl–CoA carboxylase, the rate limiting enzyme in fatty acid synthesis. 4-(4′-chlorobenzyloxy)benzoyl-CoA, a metabolite of the hypolipidaemic agent KCD-232, was shown to inhibit fatty acid synthesis in a cell-free enzyme system from rat liver whereas the parent acid had no effect [18]. The hypolipidaemic actions of benzoic acid, *p*-tert-butylbenzoic acid and the structurally related hypolipidaemic agent SC-33459 have also been associated with reduced CoA and acetyl CoA levels, and increased medium chain acyl–CoA levels, presumably reflecting the synthesis of xenobiotic-CoAs, in rat isolated hepatocytes [19]. More recently the hypolipidaemic agents clofibric acid, nafenopin and ciprofibrate were all shown to form CoA thioesters [20].

In conclusion, whilst the *S*-enantiomers of the 2-arylpropionates appear to be responsible for their anti-inflammatory activity, the stereospecific inhibition of endogenous triacylglycerol synthesis by *R*-fenoprofen described in this study, and previous work showing stereospecific lipid incorporation and CoA thioester formation by the *R*-2-arylpropionates, clearly demonstrate that the *R*-enantiomers are not inactive components of the racemic preparations used clinically. They possess fatty acid-like properties and can therefore act as substrates and modulators of the pathways of lipid synthesis and metabolism. In view of the stereospecificity in the fatty acid-like behaviour of the 2-arylpropionates, these agents may become useful tools with which to investigate the mechanisms of action of hypolipidaemic drugs. This study further supports the use of enantiomerically pure 2-arylpropionate preparations to obtain more specific anti-inflammatory effects.

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BENEDETTA C. SALLUSTIO*†
KATHLEEN M. KNIGHTS‡
PETER J. MEFFIN✝

*Department of Clinical Pharmacology
The Queen Elizabeth Hospital
Woodville South
5011 Australia
‡Department of Clinical Pharmacology
The Flinders University of South Australia
Bedford Park 5042
Australia

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† To whom correspondence should be addressed at: Department of Clinical Pharmacology, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South, 5011, S.A., Australia.
✝ Deceased.

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AY9944 inhibits early activation of phosphatidylinositol metabolism in concanavalin A-stimulated lymphocytes

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AY9944, an agent which blocks cholesterol synthesis [1], can inhibit blastic transformation of lymphocytes in a cholesterol-free medium [2]. The inhibition does not occur if plasma low density lipoproteins (LDL) are added to the medium. Since LDL provide exogenous cholesterol to cells, it was thought that AY9944 inhibits blastic transformation by means of a blockade of cholesterol synthesis.

Nevertheless, in a plasma-containing medium, despite the presence of exogenous cholesterol, an excess of AY9944 can inhibit blastic transformation [3, 4]. Thus, another mechanism, irrespective of cholesterol synthesis, could be involved. We have hypothesized that AY9944, which is an amphiphilic substance with strong affinity for the phospholipid moiety of biological membranes [5], inhibits blastic transformation of lymphocytes by means of an effect on the plasma membrane of cells, during the first stages of the mitogen's action [4]. In order to check this point, we studied the effect of AY9944 on the early activation of phosphatidylinositol (PI) metabolism by lectins ("PI response") [6]. We observed that AY9944 inhibits the activation of PI metabolism during the first hours of the action of concanavalin A on lymphocytes, and that it blocks cell entry into G1.

Materials and Methods

Preparation of lymphocytes. Human peripheral lymphocytes were prepared by Böyum's method [7], under precise conditions previously described [8].

Cell cultivation. Cells were incubated in RPMI medium, supplemented with 20 mM HEPES buffer, 0.3 g of L-glutamine, 300,000 I.U. of penicillin and 0.3 g of streptomycin per liter, and with 10% autologous plasma. The incubation was performed at 37°, in a 5% CO_2 atmosphere. When present, AY9944 (Ayerst Lab., U.S.A.) was used at a concentration of 10, 20 or 40 μM.

[^{32}P]*Phosphate incorporation into phospholipids.* Four million cells were incubated in 1.5 mL of medium (final volume), in glass tubes (in which subsequent direct extraction of phospholipids was possible). Cells were first preincubated for 48 hr, with or without AY9944. Then, they received 300 μCi (15 μg) of [^{32}P]phosphate (CEA, France) per tube, and simultaneously, when stimulated, 100 μg of concanavalin A (the Sigma Chemical Co., Poole, U.K.). We verified that this dose of concanavalin A (66 μg/mL) corresponds, in our experimental conditions (4 million cells per tube), to the optimal rate of [^{3}H]thymidine incorporation into DNA (unpublished results).

For pulse experiments, 5 hr after the onset of incorporation, the incubation was stopped by the addition of 10 mL of methanol–chloroform (1/1, v/v). Phospholipids were extracted, separated and counted for radioactivity as previously described [8].

For pulse-chase experiments, 5 hr after the onset of incorporation, cells were centrifuged, washed with RPMI and re-suspended in 1.5 mL of the supernatant from ^{32}P-free cultures, which were performed simultaneously. The incubation was then carried on for a further 19 hr-period and stopped as described above.

Cell viability. The percentage of dead cells was evaluated, after 48 hr-preincubation with or without AY9944, by the method of trypan blue exclusion.

[^{3}H]*Thymidine incorporation into DNA.* Four hundred thousand cells were incubated per tube, in 150 μL of medium, with 10 μg of concanavalin A. Forty-eight hours after the onset of incubation, each tube received 2 μCi of [^{3}H-methyl]thymidine (CEA). Two hours later, the incorporation was stopped by the addition of 1 mL of a 25% solution of trichloracetic acid (TCA). After 15 min, the TCA-insoluble material was centrifuged, washed four times with TCA and directly transferred into scintillation phials, where it was dried and its radioactivity counted.

Results and Discussion

Table 1 indicates that, as previously described [2–4], AY9944 inhibits [^{3}H]thymidine incorporation into cell

Table 1. Effect of AY9944 on [^{3}H]thymidine incorporation into DNA and on cell viability

AY9944 (μM)	0	10	20	40
[^{3}H]Thymidine incorporation*	100†	37 (±7)	8 (±2)	—
Cell mortality‡	3 (±1)	2 (±1)	7 (±1)	16 (±3)

* Rate of [^{3}H]thymidine incorporation (2 hr-pulses), evaluated after 48 hr-incubation with concanavalin A, and with or without AY9944. Percentage of the value obtained in the absence of AY9944.

† This corresponds to 8480 cpm/10^5 cells (±1270).

‡ Percentage of dead cells evaluated after 48 hr-preincubation of unstimulated lymphocytes, with or without AY9944.

Average of six experiments (±SD).