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THE STEREOSPECIFIC INCORPORATION OF FENOPROFEN INTO RAT HEPATOCYTE AND ADIPOCYTE TRIACYLGLYCEROLS

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Abstract—The formation of triacylglycerols containing fenoprofen was studied in rat isolated adipocytes and hepatocytes incubated with [^{3}H]glycerol and *R* or *S* fenoprofen. In both hepatocytes and adipocytes there was a high-affinity enzymatic process for the synthesis of triacylglycerol containing fenoprofen which was stereospecific for the *R* enantiomer. The apparent K_m values for *R* fenoprofen were 1.0 μM in adipocytes and 2.8 μM in hepatocytes. These results are consistent with the proposed stereospecific formation of *R*-2-arylpropionyl-CoA thioesters resulting in the stereospecific formation of *R*-triacylglycerol at clinically relevant unbound fenoprofen concentrations. In isolated hepatocytes, but not adipocytes, a second low-affinity enzymatic process for the synthesis of triacylglycerol containing fenoprofen was also observed. However, this process (K_m = 3780 μM) occurred at concentrations much higher than those found in man with usual doses.

Non-steroidal anti-inflammatory drugs such as fenoprofen, ibuprofen and ketoprofen are 2-arylpropionic acid derivatives which exist as *R* and *S* enantiomers. The anti-inflammatory activity, as determined by *in vitro* cyclooxygenase inhibition, is thought to reside almost exclusively with the *S* enantiomers [1]. However, with the exception of naproxen, all of these compounds are administered to man as the racemates. Most of the 2-arylproprionates have been shown to undergo stereospecific metabolic chiral inversion of the *R* distomers to the *S* eutomers [2–4], a process which is thought to take place via the stereospecific formation of *R*-2-arylpropionyl-CoA thioester intermediates [5, 6].

The formation of xenobiotic acyl-CoA intermediates gives rise to alterations in lipid metabolism such as hypolipidemia, the formation of hybrid fatty acids, hybrid triacylglycerols and sterol esters [7]. A number of xenobiotic carboxylic acids have been shown to substitute for endogenous fatty acids and become incorporated into hybrid triacylglycerols via their acyl-CoA intermediates [8–11]. In particular, racemic preparations of the 2-arylpropionic acids ketoprofen, ibuprofen and fenoprofen were shown to form hybrid triacylglycerols containing the 2-arylpropioic acid moiety [8]. The proposed stereospecific formation of *R*-2-arylpropionyl-CoAs implies that the formation of hybrid triacylglycerols containing 2-arylpropionates should be stereospecific for the *R*-enantiomers. The stereospecificity of this process has recently been demonstrated *in vivo* with ibuprofen in the rat [12].

The present studies were undertaken with fenoprofen because racemic fenoprofen has the highest degree of hybrid triacylglycerol formation of the 2-arylpropionates examined [8] and isolated hepatocytes and adipocytes were used as these are the cells primarily responsible for the synthesis and storage of triacylglycerols.

MATERIALS AND METHODS

Chemicals. The *R* and *S* enantiomers of fenoprofen were obtained by resolution of racemic fenoprofen as described by Hayball and Meffin [3]. They were determined to be 98.1% (*R*) and 97.4% (*S*) pure using an enantiospecific HPLC assay [13]. [1(3)^{3}H]glycerol (3.0 Ci/mole) was purchased from Amersham Australia Pty. Ltd (Sydney, Australia). [^{3}H]glycerol was diluted with either Krebs–Henseleit buffer or Waymouth's medium to final concentrations in the incubation media of 14.8 mM and 1.6 mM for adipocytes and hepatocytes respectively. Synthetic triacylglycerol containing fenoprofen (1,2-dipalmitoyl-3-(2-(3-phenoxyphenyl)propanoyl)glycerol) was synthesised from 1,2-dipalmitoyl-*sn*-glycerol purchased from Sigma Chemical Co. as previously described [14].

Preparation of isolated adipocytes and hepatocytes. Adipocytes were isolated from the epididymal fat pads of adult male Hooded Wistar rats (250–300 g) according to the procedure of Rodbell [15] using Krebs–Henseleit buffer (pH 7.4) which did not contain bovine serum albumin. Cells were counted and viabilities assessed using the trypan blue exclusion method. They were finally resuspended in Krebs–Henseleit buffer to give a concentration of 10^5 cells/ml. The fat pads of two adult animals were pooled in order to provide enough cells for each series of incubations. The average yield for an adipocyte preparation was 2.74×10^6 cells.

Rat isolated hepatocytes were prepared from adult male Hooded Wistar rats (200–250 g) according to

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the method of Seglen [16]. Cells were counted and viabilities assessed using the trypan blue exclusion method. Only preparations with viabilities greater than 85% were used and the cells were finally resuspended in Waymouth's medium (pH 7.4) to give a concentration of 2×10^5 viable cells/ml. The average yield of hepatocytes from each liver was 1.37×10^8 viable cells.

Synthesis of triacylglycerols containing fenoprofen by adipocytes and hepatocytes. The synthesis of triacylglycerols containing fenoprofen by isolated adipocytes was examined in six adipocyte preparations using the following incubation conditions. Isolated adipocytes (1 ml containing 10^5 cells) were transferred into 20 ml plastic scintilation vials and allowed to equilibrate for 15 min in a shaking water bath at 37° in an atmosphere of 5% CO_2/95% O_2. Either *R*- or *S*-fenoprofen was then added to each vial in 20 μl of dimethyl sulphoxide to give final concentrations in the range of 0–500 μM. After 10 min, 100 μl of Krebs–Henseleit buffer containing 50 μCi of [^{3}H]glycerol was added. Incubations were carried out in duplicate for a further 30 min in a shaking water bath at 37° in the presence of 5% CO_2/95% O_2. The final incubation volume was 1.12 ml. The reaction was stopped by transferring 500 μl of the incubation mixture into a 5 ml culture tube containing 3 ml of ice-cold diethyl ether which was then briefly vortexed.

The synthesis of triacylglycerols containing fenoprofen by isolated hepatocytes was examined in six rats using incubation conditions similar to those described for adipocytes. Incubations were carried out in 25 ml glass Erlenmyer flasks with 4×10^5 viable cells in 2 ml of Waymouth's medium. *R*- or *S*-fenoprofen was added to give final concentrations of 0–5000 μM and 100 μl of Waymouth's medium containing 10 μCi of [^{3}H]glycerol was added after 2 min. Incubations were carried out in duplicate for a further 15 min as described for adipocytes and the reaction was stopped by transferring 500 μl of the incubation mixture into a 5 ml culture tube containing 3 ml of ice-cold diethyl ether, which was then briefly vortexed and frozen using a dry ice/acetone bath. The final incubation volume was 2.12 ml.

The synthesis of triacylglycerols containing fenoprofen had previously been determined to be linear with respect to incubation time and number of cells in both adipocytes and hepatocytes using the incubation conditions described above.

Analytical methods. Quantitation of the [^{3}H]triacylglycerol containing fenoprofen synthesised during the incubations was carried out using a specific HPLC method which is described in detail elsewhere [14]. Briefly, the method consists of extracting the lipids into diethyl ether with [^{14}C]tripalmitin as internal standard, separating triacylglycerols from more polar endogenous lipids using a silica cartridge and separating endogenous triacylglycerols and triacylglycerols containing fenoprofen by HPLC and quantitation by scintillation counting [14]. For the 18 chromatographic runs carried out during these studies, the mean (SE) coefficients of variation of quality control samples for endogenous triacylglycerols and triacylglycerols containing fenoprofen were 2.7% (0.4) and 2.4% (0.4) respectively. Samples were extracted on the same day as the incubation period and HPLC analysis was carried out on the following day.

Kinetic analysis in order to obtain the K_m* and V_{max} of each individual data set for the synthesis of triacylglycerol containing fenoprofen was carried out using MK Model [17], an extended least squares regression modelling programme. Equations modelling a single enzyme or two enzymes acting on the substrate were fitted to the data as appropriate.

RESULTS

Synthesis of triacylglycerols containing fenoprofen by adipocytes

As shown in Fig. 1, there was saturable synthesis of triacylglycerol containing fenoprofen consistent with Michaelis–Menten kinetics from *R*-fenoprofen but there was no evidence of saturable synthesis from *S*-fenoprofen. for the six adipocyte preparations studied the mean (SE) composite apparent K_m and V_{max} for the synthesis of triacylglycerol containing fenoprofen from *R*-fenoprofen were 1.0 (0.1) μM

* Abbreviations used: K_m, Michaelis constant; V_{max}, maximum velocity.

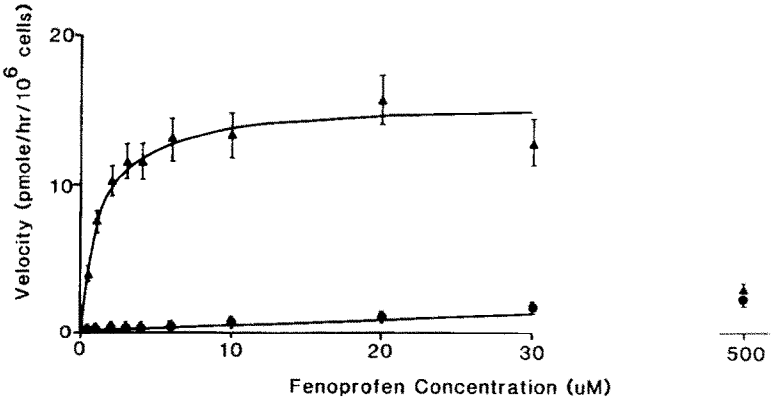

Fig. 1. The mean (SE) synthesis of triacylglycerol containing fenoprofen by rat isolated adipocytes incubated in the presence of 50 μCi of [^{3}H]glycerol and 0–500 μM *R*(▲) or *S*(●)fenoprofen. The reaction velocity is expressed in terms of pmoles of [^{3}H]glycerol incorporated into triacylglycerol containing fenoprofen per hour per 10^6 viable adipocytes.

and 15.0 (2.4) pmol/hr/10^6 cells respectively. At both *R* and *S* fenoprofen concentrations of 500 μM there was a large decrease in the synthesis of triacylglycerol containing fenoprofen (Fig. 1) and also of endogenous triacylglycerol from a mean (SE) of 9.4 (1.1) to 1.8 (0.1) pmole/hr/10^6 cells for cells incubated in the presence of *R* fenoprofen and from 12.9 (1.3) to 7.0 (1.0) pmole/hr/10^6 cells for cells incubated in the presence of *S* fenoprofen. This is thought to be due to fenoprofen toxicity, however, at the low fenoprofen concentrations there was no evidence of toxicity as determined by the cells' total capacity to synthesise triacylglycerol.

Synthesis of triacylglycerol containing fenoprofen by hepatocytes

At the low concentrations of fenoprofen (<50 μM) there was saturable synthesis of triacylglycerol containing fenoprofen only from the *R*-enantiomer (Fig. 2A) the mean (SE) composite apparent K_m and V_{max} for this process being 2.8 (0.8) μM and 34.3 (5.7) pmole/hr/10^6 viable cells respectively. At the higher fenoprofen concentrations (100–5000 μM) there appeared to be a second enzymatic process for the synthesis of triacylglycerol containing fenoprofen for both the *R* and *S* enantiomers (Fig. 2B). The mean (SE) composite apparent K_m and V_{max} for the *R*-enantiomer were 3780 (2000) μM and 113 (38) pmole/hr/10^6 viable cells respectively, for the *S*-enantiomer the values were 2490 (760) μM and 65 (19) pmole/hr/10^6 viable cells respectively. In the hepatocyte preparations there was no evidence of fenoprofen toxicity over the full concentration range as measured by total triacylglycerol synthesis and the trypan blue exclusion method using the incubation conditions described in the Materials and Methods section.

DISCUSSION

The synthesis of triacylglycerols containing fenoprofen from [^{3}H]glycerol and fenoprofen by isolated cells involves multiple enzymatic steps including activation of the fenoprofen to the CoA thioester and its subsequent stepwise esterification with glycerol. The K_m values obtained in this study, therefore, are composite apparent K_m values of a multi-enzyme system. In enzyme systems with several successive steps, if a rectangular hyperbolic

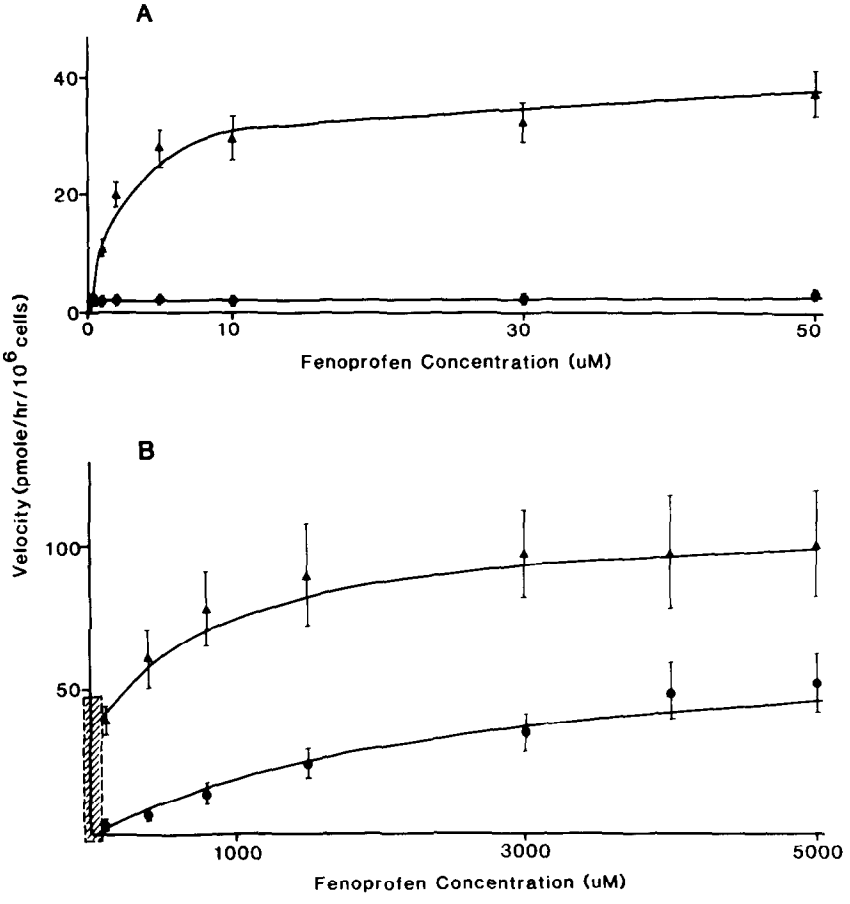

Fig. 2. The mean (SE) synthesis of triacylglyerol containing fenoprofen by rat isolated hepatocytes incubated in the presence of 10 μCi of [^{3}H]glycerol and (A) 0–50 μM *R*(▲) or *S*(●) fenoprofen; (B) 100–5000 μM *R*(▲) or *S*(●) fenoprofen. Figure 2A is an enlargement for the hatched area in Fig. 2B. The reaction velocities are expressed in terms of pmoles of [^{3}H]glycerol incorporated into triacylglycerol containing fenoprofen per hour per 10^6 viable hepatocytes.

relationship between velocity and substrate concentration occurs then the data obey the single-substrate Michaelis equation and the composite apparent K_m reflects the rate-limiting step in the overall process [18]. In adipocytes we have described a high affinity enzymatic process for the synthesis of triacylglycerols from *R*-fenoprofen whilst in hepatocytes there appear to be both a high affinity and a lower affinity enzymatic process. From the data obtained in this study it is unclear which step in the formation of triacylglycerols containing fenoprofen is rate limiting. It is of interest, however, that the composite apparent K_m values obtained in both hepatocytes and adipocytes for the synthesis of triacylglycerol containing fenoprofen at the low concentrations of *R* fenoprofen are similar to the K_m values for the synthesis of acyl-CoAs of the endogenous fatty acids palmitate and oleate [19]. In addition the rate-limiting step in the formation of hybrid triacylglycerols containing the xenobiotic 3-phenoxybenzoic acid appears to be the synthesis of the CoA thioester [11].

The synthesis of triacylglycerols containing fenoprofen by the low affinity enzymatic process described in hepatocytes occurred at unbound concentrations of fenoprofen which were at least 100-fold greater than those achieved with usual doses and it is therefore not considered to be pharmacologically relevant. Synthesis took place from both *R*- and *S*-fenoprofen although the rate of synthesis was higher for the *R* enantiomer. Taking into account the 2.6% contamination of the *S*-fenoprofen with *R*-fenoprofen, the synthesis of triacylglycerol containing fenoprofen observed from the 100–5000 μM *S*-fenoprofen may predominantly have been due to the action of the high affinity process on the small contaminating amounts of *R*-fenoprofen. If this conjecture is correct, then the low affinity process may also be stereospecific for the *R* enantiomer (Fig. 2B). It was not possible to determine whether this second enzymatic process was also present in adipocytes because of the observed fenoprofen toxicity at concentrations of 500 μM or greater.

The usual dose of racemic fenoprofen for the treatment of rheumatoid arthritis in adults is 600 mg, four times daily up to a maximum of 3 g a day [20]. A single 600 mg oral dose gives rise to maximum plasma concentrations in the range of 165–250 μM [18]. Taking into account the high degree of fenoprofen protein binding (99%) [21] the maximum unbound plasma fenoprofen concentrations resulting from a single dose should be within the range of 1–2.5 μM. In both hepatocytes and adipocytes the apparent K_m values for the high affinity synthesis of triacylglycerols containing *R*-fenoprofen were within the range of unbound fenoprofen concentrations achieved with usual doses in man. This high affinity process was stereospecific for *R*-fenoprofen which is consistent with the proposed stereospecific formation of *R*-2-arylpropionyl-CoA intermediates [5, 6] and supports the hypothesis that only the *R*-enantiomers of the 2-arylpropionic acids are capable of becoming incorporated into hybrid triacylglycerols. The generality of the stereospecific formation of triacylglycerol containing 2-arylpropionic acids is also supported by the recent *in vivo* study in which only *R*-ibuprofen was incorporated into rat triacylglycerols [12]. Assuming similar characteristics in man to those found in rat, the formation of triacylglycerol containing fenoprofen should be stereospecific for the *R* enantiomer at the concentrations achieved clinically.

Using the acidic hypolipidemic agent BRL-10894, Fears *et al.* [8] reported that 48 hr following a single dose, 70% of the dose was found in rat adipose tissue as hybrid triacylglycerol and following dosing for 8 days at 5 g/kg of BRL-10894, 30% of the lipid in rat adipose tissue was hybrid triacylglycerol containing the BRL-10894 moiety. Both racemic fenoprofen and ibuprofen have been reported to have *in vitro* rates of hybrid triacylglycerol synthesis comparable to those of endogenous fatty acids [22]. The low apparent K_m values obtained in this study for *R* fenoprofen and the work of Fears *et al.* [8] demonstrating that the *in vitro* rate of racemic fenoprofen incorporation into triacylglycerols was 140% that of BRL-10894, further support the hypothesis that *R*-fenoprofen may behave similarly to endogenous fatty acids and become readily incoporated into hybrid triacylglycerols *in vivo*.

The pharmacological and toxicological consequences of the formation of hybrid triacylglycerols are unknown. These hybrid lipids may represent long-lived tissue residues of the 2-arylpropionic acids and may themselves possess biological activity or exert activity via the release of the 2-arylpropionic acid moiety. Fears and Richards [22] reported that racemic mixtures of various 2-arylpropionates were capable of inhibiting cholesterogenesis and fatty acid synthesis *in vitro* and, that there was a correlation between their capacity to form hybrid triacylglycerols and their hypolipidemic actions. By using enantiomerically pure preparations it may be possible to achieve more specific pharmacological actions based on the anti-inflammatory activity of the *S*-enantiomers or on the ability of the *R*-enantiomers to modify lipid metabolism.

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