METABOLISM OF SAMERIDINE TO MONOCARBOXYLATED PRODUCTS BY HEPATOCYTES ISOLATED FROM THE MALE RAT

ANNA-KARIN SOHLENIUS-STERNBECK, HANS VON EULER CHELPIN, ACHIM ORZECHOWSKI, AND MAGNUS M. HALLDIN

Department of Preclinical Development, AstraZeneca R&D Södertälje, Södertälje, Sweden

(Received November 4, 1999; accepted February 22, 2000)

This paper is available online at http://www.dmd.org

ABSTRACT:
The metabolism of sameridine (LPB) (an amide-type local anesthetic-analgescic agent with a hexyl side chain) to carboxylic acid derivatives by isolated male rat hepatocytes was studied using gradient reversed-phase HPLC and mass spectrometry. Incubation of sameridine with hepatocytes resulted in the formation of numerous different metabolites. Two carboxylic acids, i.e., the C6 and C4 carboxylated derivatives of sameridine (LPB-6'-oic acid and LPB-4'-oic acid), were found to be produced from the intermediate ω-hydroxy metabolite (6'-hydroxy-LPB). Shortening of the aliphatic chain in LPB-6'-oic acid by two carbon atoms resulted in LPB-4'-oic acid. However, incubation of rat hepatocytes with 5'-hydroxy-LPB [the (ω-1)-hydroxy derivative of sameridine] did not give rise to any carboxylated derivative. Addition of SKF525A inhibited the metabolism of sameridine by rat hepatocytes, indicating that the initial step is catalyzed by cytochrome P450. Furthermore, the metabolism of sameridine to LPB-4'-oic acid was enhanced in hepatocytes isolated from rats treated with clotibenate, an up-regulator of peroxisomal fatty acid β-oxidation and of microsomal cytochrome P450 4A. L-Carnitine (which increases the rate of mitochondrial fatty acid β-oxidation) had no effect on the level of LPB-4'-oic acid produced by isolated rat hepatocytes. The metabolism of 6'-hydroxy-LPB to LPB-6'-oic acid was inhibited almost completely by 4-methylpyrazole, an inhibitor of alcohol dehydrogenase. Considered together, our findings suggest that cytochrome P450 4A4, cytosolic dehydrogenases, and the enzymes involved in peroxisomal fatty acid β-oxidation catalyze the metabolism of sameridine to LPB-4'-oic acid.

Sameridine (LPB)1 (see Fig. 1) exhibits both local anesthetic and analgesic properties (Westman et al., 1998). In vivo studies have revealed that sameridine is metabolized in the rat to approximately 30 different metabolites, of which 25 have so far been identified (Elofsson et al., 1996). The major metabolic pathways involved are oxidation of the hexyl side chain to subsequently produce alcohols, ketones, and carboxylic acids. Two carboxylic acids, the C6 and C4 carboxylic acid derivatives of sameridine, have been detected in the urine of male rats treated with this substance. Amide dealkylations are also acid derivatives of sameridine, have been detected in the urine of two carboxylic acids, the C5 and C4 carboxylic acids. However, incubation of rat hepatocytes with 5'-hydroxy-LPB [the (ω-1)-hydroxy derivative of sameridine] did not give rise to any carboxylated derivative. Addition of SKF525A inhibited the metabolism of sameridine by rat hepatocytes, indicating that the initial step is catalyzed by cytochrome P450. Furthermore, the metabolism of sameridine to LPB-4'-oic acid was enhanced in hepatocytes isolated from rats treated with clotibenate, an up-regulator of peroxisomal fatty acid β-oxidation and of microsomal cytochrome P450 4A. L-Carnitine (which increases the rate of mitochondrial fatty acid β-oxidation) had no effect on the level of LPB-4'-oic acid produced by isolated rat hepatocytes. The metabolism of 6'-hydroxy-LPB to LPB-6'-oic acid was inhibited almost completely by 4-methylpyrazole, an inhibitor of alcohol dehydrogenase. Considered together, our findings suggest that cytochrome P450 4A4, cytosolic dehydrogenases, and the enzymes involved in peroxisomal fatty acid β-oxidation catalyze the metabolism of sameridine to LPB-4'-oic acid.

1 Abbreviations used are: sameridine (LPB), N-ethyl-1-hexyl-N-methyl-4-phenyl-4-piperidine-carboxamide hydrochloride; CYP, cytochrome P450; LC, liquid chromatography; MS, mass spectrometry; SKF525A, proadifen hydrochloride; TFA, trifluoroacetic acid.

Send reprint requests to: Anna-Karin Sohlenius-Sternbeck, Department of Preclinical Development, AstraZeneca R&D Södertälje, S-151 85 Södertälje, Sweden. E-mail: Anna-Karin.Sternbeck@astrazeneca.com

1990). Recently, it has been proposed that the fatty acid anilide CI-976 is metabolized by rat hepatocytes to carboxylic acid derivatives through reactions involving cytochrome P450 4A, cytosolic dehydrogenases, and subsequent oxidation by the enzymes catalyzing peroxisomal fatty acid β-oxidation (Sinz et al., 1997).

The cytochrome P450 4A subfamily catalyzes ω- and (ω-1)-hydroxylation of fatty acids, and derivatives thereof, including prostaglandins, leukotrienes, and arachidonic acid (Gibson, 1989; Hardwick, 1991). Further oxidation of the ω-hydroxylated intermediates to yield ω-carboxylic acids is presumably carried out by cytosolic alcohol and aldehyde dehydrogenases. Carboxylic fatty acids can subsequently undergo β-oxidation in either peroxisomes and/or mitochondria.

Before a fatty acid can enter any of these β-oxidation pathways, it must be activated enzymatically to an acyl-CoA thioester. Several isoforms of acyl-CoA synthetase, exhibiting different substrate specificities and subcellular distributions, have been described (Bromfan et al., 1984; Vamecq et al., 1985; Singh and Poulos, 1988).

To a certain extent, peroxisomes and mitochondria can β-oxidize the same fatty acid substrates (Hyrb and Hogg, 1979; Vamecq and Draye, 1987). For example, medium- and long-chain fatty acids (C10 through C16) can be oxidized in both of these compartments (Hyrb and Hogg, 1979). However, peroxisomes are also able to oxidize unusual and/or bulky fatty acids that are unable to enter the mitochondrial matrix (Yamada et al., 1987). Indeed, it has been found that the peroxisomal β-oxidation pathway can use certain xenobiotic acyl-CoA compounds as substrates (Yamada et al., 1984, 1986; Gatt et al., 1988). It has even been suggested that the primary function of peroxisomal β-oxidation is detoxication (Osmundsen et al., 1991).
This study was designed to elucidate the metabolic pathways involved in the formation of monocarboxylic acid derivatives from sameridine. We have used hepatocytes isolated from male rats because the metabolism of sameridine in this animal is well characterized and leads to the excretion of carboxylated derivatives in the urine (Elofsson et al., 1996). Treatment of rats with clofibrate and other hypolipidemic compounds results in a dramatic up-regulation of peroxisomal fatty acid β-oxidation (Lazarow and de Duve, 1976) and in potent induction of microsomal cytochrome P450 4A (Orton and Guzelian, 1980). The liver was perfused in situ at 37°C with a calcium-free buffer (pH 7.2) containing 0.5 mM EGTA, followed by perfusion with Williams’ E medium containing collagenease type XI (0.16 mg/ml). These solutions and all culture media were supplemented with penicillin G (100 U/ml). After release from the liver, the hepatocytes were suspended in Williams’ E medium, filtered, washed by centrifugation, and resuspended in fresh culture medium. The viability (as determined by Trypan blue exclusion) was routinely 85 to 95%.

Preparation and Incubation of Microsomes. Livers from control and clofibrate-treated rats were homogenized individually at 440 rpm in ice-cold 0.25 M sucrose using four up-and-down strokes of a Potter-Elvehjem homogenizer. More sucrose was then added to obtain a 20% (w/v) homogenate, which was subsequently centrifuged at 10,000 × g for 20 min. The resulting supernatant was further centrifuged at 105,000 × g for 60 min, and the pellet obtained (designated the microsomal fraction) was washed once by suspension in 0.15 M Tris-HCl (pH 8.0) and was centrifuged again. Protein was determined by the procedure of Lowry et al. (1951). Rat liver microsomes thus prepared were incubated (1 mg of protein/ml) with 250 μM M sameridine and 1 mM NADPH, and/or SKF525A; 5 mM 4-methylpyrazole; and 1 mM L-carnitine.

Analysis of the Metabolites of Sameridine. Each incubation involving hepatocytes was terminated by the addition of 125 μl of 6 M KOH. Following a brief centrifugation, the pH of the supernatant obtained was adjusted to 6.0. Solid-phase extraction on a cation-exchange column (SCX, Mega Bond Elut; Varian, Harbor City, CA) was then performed as follows: After application of the sample (20 ml), the SCX column was washed with 5 ml of water. The sample was subsequently eluted with 25% aqueous ammonium hydroxide/methanol (1:4), dried under a stream of nitrogen, redissolved in 3.3 mM acetic acid/ammonium formate, pH 2.0, and thereafter analyzed by gradient reversed-phase HPLC (Elofsson et al., 1996).

Two HPLC columns (Lichrocart 250-4 and 125-4, Superspher 60 RP Select B, 4 μm; Merck, Darmstadt, Germany) were coupled in series, and the flow rate was maintained at 0.8 ml/minute. The buffers used were 3.3 and 5.5 mM acetic acid/ammonium formate, pH 2.0, and the mobile phases were: (A) the 3.3 mM buffer or (B) the 5.5 mM buffer/acetonitrile (60:40 v/v). During the first 44 min of the run, the mobile phase was stepped up from 0 to 82.5% B and, thereafter, 100% B was used for the remainder of the run, i.e., 60 min. The absorption of the eluant at 208 nm was monitored using a UV-Vis detector (Spectra-Physics, Stockholm, Sweden). For determination of the metabolites produced by microsomes, the supernatants obtained after termination of the experiments were also run on the columns.

**Materials and Methods**

**Chemicals.** Williams' E medium, collagenase type XI, penicillin G, Trypan blue, EGTA, 4-methylpyrazole, L-carnitine, clofibrate, Trizma base, NADPH (Sigma, St. Louis, MO); SKF525A (Research Biochemicals Incorporated, Natick, MA); acetonitrile, formic acid, trifluoroacetic acid (TFA), and dimethyl sulfoxide (Merck, Darmstadt, Germany); ammonium formate (The British Drug Houses Ltd., Poole, England); and Ultima liquid scintillation cocktail (Packard Instrument Co., Downers Grove, IL) were purchased from the sources indicated.

Sameridine (Fig. 1) and [14C]sameridine [24 mCi/mmol (0.9 GBq/mmol), labeled in the benzylic position and with a radiochemical purity of >99%], 6'-hydroxy-LPB, 5'-hydroxy-LPB, LPB-5'-oic acid, LPB-4'-oic acid, 5'-keto-LPB, and 5'-hydroxy-LPB were all synthesized at AstraZeneca R&D, Söder	älje, Sweden (Elofsson et al., 1996) (Fig. 1). All of these and the other chemicals used were of a purity of >97%.

**Animals.** Male Sprague-Dawley rats (B&K Universal AB, Sollentuna, Sweden) weighing 170 to 180 g were maintained under a 12-h light/dark cycle in steel cages with free access to commercial food pellets (Lactamin, Stockholm, Sweden).

**Clofibrate Treatment.** Commercial food pellets were ground into a powder. Clofibrate (0.5 g) was dissolved in 20 ml of acetone and then mixed with 100 g of powdered food. This chow was dried in a ventilated hood until the smell of acetone was no longer detectable (12–24 h). Treated animals received this clofibrate-containing diet for 10 days, a regimen that results in maximal up-regulation of hepatic peroxisomal fatty acid β-oxidation in the male Sprague-Dawley rat (Cai et al., 1996).

**Isolation of Hepatocytes.** Hepatocytes were isolated from the male Sprague-Dawley rats using a collagenase perfusion technique, modified from the procedure described by Bissell and Guzelian (1980). The liver was perfused in situ at 37°C with a calcium-free buffer (pH 7.2) containing 0.5 mM EGTA, followed by perfusion with Williams' E medium containing collagenase type XI (0.16 mg/ml). After release from the liver, the hepatocytes were suspended in Williams’ E medium, filtered, washed by centrifugation, and resuspended in fresh culture medium. The viability (as determined by Trypan blue exclusion) was routinely 85 to 95%.

**Hepatocyte Incubations.** Cells were diluted in medium so as to obtain 1 × 10^6 cells/ml. For each incubation, 20 × 10^5 cells were used, and all incubations were performed in a shaking water bath at 37°C under 95% O_2, 5% CO_2. The final concentrations of the substances added to the medium were as follows: 10 μM LPB, 6'-hydroxy-LPB, LPB-6'-oic acid, 5'-hydroxy-LPB, and/or SKF525A; 5 μM 4-methylpyrazole; and 1 mM L-carnitine.

To obtain more quantitative information concerning sameridine metabolism in isolated rat hepatocytes, [14C]sameridine (2.25 mCi/mmol) was used. Sameridine and its metabolites were dissolved in 50 mM Tris-HCl, pH 7.4, before addition to the medium. 4-Methylpyrazole was added to the medium as a solution in dimethyl sulfoxide. In this latter case, the same amount of dimethyl sulfoxide (the vehicle) was added, giving a final concentration of 0.1%. No sign of toxicity (cell death) was observed in response to any of the treatments.
reaction with KOH and subsequent centrifugation (as above), were subjected directly to reversed-phase HPLC.

For radiochemical analysis, a radiometric FLO-ONE/Beta A-500 detector (Radiomatic Instrument & Chemical Co., Tampa, FL) was installed in series after the UV detector. One volume of mobile phase was mixed with three volumes of liquid scintillation fluid for counting.

The carboxylic acids LPB-6'-oic acid, LPB-5'-oic acid, and LPB-4'-oic acid, as well as many additional metabolites of sameridine, are eluted after 30 to 40 min using the gradient HPLC system described above. To separate the carboxylic acids from the bulk of other metabolites, an isocratic HPLC at pH 7.0 was developed. The columns, flow rate, and period of analysis were the same as for the gradient system, but the mobile phase consisted of 5.5 mM ammonium formate (pH 7.0)/acetonitrile (60:40 v/v). In this system, synthetic LPB-6'-oic acid, LPB-5'-oic acid, and LPB-4'-oic acid standards elute after 8 to 12 min and are separated from other metabolites of sameridine. However, this system is not suitable for the separation of the metabolites other than the carboxylic acids, because metabolites eluting at times later than 20 min are poorly resolved.

Mass Spectrometry. Liquid chromatography-(LC)-mass spectrometry (MS) experiments were carried out using a Quattro II triple quadrupole mass spectrometer (Micromass Ltd., Manchester, UK) with an electrospray ion source and a cross-flow counterelectrode. The source temperature was 90°C, and the cone voltage was 30 V. The HPLC system (HP 1100) comprised a binary pump and an autosampler (Hewlett-Packard, Palo Alto, CA). A Symmetry C8 column (1.0 × 150 mm; Waters Corporation, Milford, MA) and a 10 × 1.0 mm C8 Opti-guard column (Alltech Associates, Deerfield, IL) were used with a flow rate of 40 μl/min. The mobile phases were (A) 0.3 ml trifluorooacetic acid (TFA)/liter of water, and (B) 0.03% (v/v) TFA in water/acetonitrile (50:50 v/v). A gradient going from 10 to 85% B in 48 min was applied.

In confirmatory LC-MS-MS experiments, the collision gas pressure was 1.6 μbar and the collision energy 30 eV. Multiple reaction monitoring acquisitions were made in which a transition involving loss of the complete amide group had no effect on the level of LPB-4'-oic acid, LPB-6'-oic acid, and LPB-5'-oic acid standards elute after 8 to 12 min and are separated from other metabolites of sameridine. However, this system is not suitable for the separation of the metabolites other than the carboxylic acids, because metabolites eluting at times later than 20 min are poorly resolved.

Statistical Analysis. Analysis of statistical significance, where appropriate, was performed using Student’s t test.

Results

Metabolism of Sameridine by Hepatocytes Isolated from Male Rats. The metabolism of sameridine by isolated rat hepatocytes was studied using gradient reversed-phase HPLC and LC-MS. It was shown that many metabolites (approximately 20–30) were formed after incubation with sameridine. Figure 2A shows the HPLC radiochromatogram of sameridine metabolites obtained after incubation of these cells with 10 μM [14C]sameridine for 1 h. Certain metabolites were poorly resolved in this system, because they demonstrated similar retention times. For example, 6'-hydroxy-LPB was eluted at exactly the same time as was 5'-hydroxy-LPB.

The metabolism of sameridine by hepatocytes isolated from rats treated with clofibrate was enhanced (Fig. 2B, Table 1) compared with the corresponding metabolism by control hepatocytes (Fig. 2A, Table 1). After only a 15-min incubation with hepatocytes from clofibrate-treated animals, [14C]sameridine was almost completely metabolized, whereas about 60% of the [14C]sameridine remained unmetabolized by control hepatocytes at this same time. After 2 h, the metabolism of sameridine by hepatocytes from untreated rats was also almost complete.

Figure 2 shows that a peak eluted at the same time as the reference substances 6'-hydroxy-LPB and 5'-hydroxy-LPB [i.e., the ω- and (ω-1)-hydroxy derivatives of sameridine] is relatively much lower in hepatocytes isolated from clofibrate-treated rats than in control hepatocytes. This difference is observed as early as after 15 min of incubation (not shown). The reference substances 6'-hydroxy-LPB and 5'-hydroxy-LPB could not be resolved from one other by LC-MS in acid solution.
Metabolism of 6'-Hydroxy-LPB by Hepatocytes Isolated from Male Rats. Analysis of the products formed upon incubation of isolated rat hepatocytes with 10 \( \mu M \) 6'-hydroxy-LPB for 2 h by LC-MS demonstrated two major metabolites, LPB-4'-oic acid and LPB-6'-oic acid (i.e., the \( C_4 \) and \( C_6 \) carboxylic acid derivatives of sameridine). In addition, the amide dealkylation products of LPB-4'-oic acid and LPB-6'-oic acid were easily identified by LC-MS.

Figure 3A shows the HPLC chromatogram of metabolites formed from 6'-hydroxy-LPB. Three independent experiments gave a mean ratio of 1.1 for the formation of LPB-4'-oic acid compared with LPB-6'-oic acid (as determined from the UV absorbance). In the case of hepatocytes isolated from rats treated with clofibrate, the corresponding ratio was 3.6 (chromatogram not shown). Carboxylic acids with fewer than four carbon atoms in the alkyl chain were not observed in any of the incubations performed in this study.

In another experiment, isolated rat hepatocytes were preincubated with 5 \( \mu M \) 4-methylpyrazole for 10 min before the addition of 10 \( \mu M \) 6'-hydroxy-LPB and further incubation for 30 min. This resulted in potent inhibition of the metabolism of 6'-hydroxy-LPB to LPB-6'-oic acid. After incubation for 30 min, the relative amount of 6'-hydroxy-LPB remaining was significantly higher in hepatocytes treated with 4-methylpyrazole than in control hepatocytes (as determined from the UV absorbance) (Fig. 4). In contrast, the metabolism of 6'-hydroxy-LPB was not affected by 10 \( \mu M \) SKF525A (not shown).

**Metabolism of LPB-6'-oic Acid by Hepatocytes Isolated from Male Rats.** The metabolism of LPB-6'-oic acid by isolated rat hepatocytes was studied using gradient reversed-phase HPLC and LC-MS. In Fig. 3B, the HPLC chromatogram of metabolites formed upon incubation of isolated control hepatocytes with 10 \( \mu M \) LPB-6'-oic acid for 2 h is shown. One major metabolite, identified as LPB-4'-oic acid (i.e., the \( C_4 \) side-chain-carboxylated derivative of sameridine), was observed.

Comparison of Fig. 3, A and B, reveals that the formation of LPB-4'-oic acid was much lower upon incubation with LPB-6'-oic acid than upon incubation with 6'-hydroxy-LPB. The UV absorbance of LPB-4'-oic acid after incubation with 10 \( \mu M \) LPB-6'-oic acid was 20% of the corresponding absorbance after incubation with 6'-hydroxy-LPB. Incubation of hepatocytes isolated from clofibrate-treated rats with 10 \( \mu M \) LPB-6'-oic acid for 2 h resulted in approximately the same amount of LPB-4'-oic acid (chromatogram not shown) as was formed by hepatocytes from untreated animals (Fig. 3B).

**Metabolism of 5'-Hydroxy-LPB by Hepatocytes Isolated from Male Rats.** The HPLC chromatogram of the products formed upon incubation of isolated rat hepatocytes with 10 \( \mu M \) 5'-hydroxy-LPB for 2 h is presented in Fig. 3C. The major metabolite was identified as 5'-keto-LPB, i.e., the (\( \omega-1 \))-keto derivative of sameridine, by MS. No carboxylic acid metabolites of sameridine could be detected in this case.

**Metabolism of Sameridine by Liver Microsomes Prepared from Male Rats.** Analysis of the products formed upon incubation of liver microsomes with 250 \( \mu M \) sameridine for 30 min by reversed-phase HPLC demonstrated the presence of one or more major products, eluting at the same time as the reference substances 5'-hydroxy-LPB and 6'-hydroxy-LPB (chromatogram not shown). The ratio between the UV absorbance of this peak and that of the peak for sameridine was 0.32 for microsomes from control rats and 0.45 for microsomes from clofibrate-treated animals. A minor metabolite was identified as 5'-keto-LPB.
Rat hepatocytes were incubated for 30 min with 10 μM 6'-hydroxy-LPB in the presence or absence of 5 μM 4-methylpyrazole (4-MP). The samples were analyzed using reversed-phase HPLC using a mobile phase with a pH of 2.0 (for further details, see Materials and Methods), and the metabolites were detected on the basis of UV absorbance. The relative amount of each compound is expressed as the percentage of the total amount of 6'-hydroxy-LPB + LPB-6'-oic acid + LPB-4'-oic acid (i.e., 100%). Data are the mean ± S.D. of three independent experiments. **P < .01 compared with the control group. □, control; ■, 4-MP.

**Discussion**

Here we have investigated the metabolism of sameridine (LPB), an amide-type local anesthetic-analgesic agent with a hexyl side chain, to carboxylic acid derivatives. Incubation of rat hepatocytes with this compound results in the formation of many metabolites. Interestingly, hepatocytes metabolize sameridine to several products that are not formed by liver microsomes. This finding illustrates the significance of hepatocytes as an in vitro system for studying the metabolism of drugs by enzymes other than cytochromes P450.

Although the ω- and (ω-1)-hydroxy derivatives of sameridine (i.e., 6'-hydroxy-LPB and 5'-hydroxy-LPB) cannot be separated from each other using HPLC or LC-MS, by incubating hepatocytes with each of these synthetic metabolites alone, we could clearly demonstrate that the C6 and C4 carboxylated derivatives of sameridine (i.e., LPB-6'-oic acid and LPB-4'-oic acid) originate from 6'-hydroxy-LPB. LPB-4'-oic acid is formed via a 2-carbon, chain-shortening of LPB-6'-oic acid.

Preincubation of hepatocytes with SKF525A, an inhibitor of cytochrome P450, resulted in potent inhibition of the metabolism of sameridine (Table 1). This finding supports the hypothesis that the first step in the metabolism of sameridine is catalyzed by cytochrome P450. The metabolism of 6'-hydroxy-LPB was not affected by SKF525A, which indicates that this metabolism is not mediated by cytochrome P450. However, the metabolism of 6'-hydroxy-LPB to LPB-6'-oic acid was inhibited by 4-methylpyrazole, indicating the involvement of alcohol dehydrogenase in this conversion.

Treatment of rats with clofibrate results in up-regulation of the enzymes catalyzing peroxisomal fatty acid β-oxidation (Lazarow and deDuve, 1976) and of microsomal ω-hydroxylation of fatty acids (i.e., cytochrome P450 4A) (Orton and Parker, 1982; Sharma et al., 1988). In male rat liver, three forms of cytochrome P450 4A, CYP4A1, -4A2, and -4A3, have been characterized (Kimura et al., 1989; Sundseth and Waxman, 1992). All three of these isoenzymes are induced by clofibrate (Sundseth and Waxman, 1992).

The metabolism of sameridine is increased in hepatocytes isolated from rats treated with clofibrate (Fig. 2B). Moreover, the rate of LPB-4'-oic acid production from sameridine by these cells is also increased. These results indicate that cytochrome P450 4A and peroxisomal β-oxidation are involved in the metabolism of sameridine to LPB-4'-oic acid.

To stimulate mitochondrial fatty acid β-oxidation in hepatocytes or in isolated subfractions, l-carnitine is generally added to the incubation medium (Yamada et al., 1987; Christensen et al., 1991). Here, incubation with l-carnitine had no effect on the formation of LPB-4'-oic acid from sameridine by isolated rat hepatocytes. These observations suggest that peroxisomal β-oxidation, rather than mitochondrial β-oxidation, is involved in the formation of LPB-4'-oic acid from LPB-6'-oic acid.

With hepatocytes from clofibrate-treated rats, the profile of sameridine metabolites obtained was somewhat different from the profile obtained using control hepatocytes. Apparently, 6'-hydroxy-LPB and/or 5'-hydroxy-LPB accumulate at lower levels in the hepatocytes isolated from animals treated with clofibrate. Because it is difficult to separate the ω-hydroxy derivative (6'-hydroxy-LPB) from the (ω-1)-hydroxy metabolite (5'-hydroxy-LPB) by HPLC or MS, it was not possible to definitely confirm this hypothesis. However, it can be speculated that the lower concentration of 6'-hydroxy-LPB in hepatocytes from clofibrate-treated rats is a consequence of their increased capacity for peroxisomal β-oxidation (assuming that peroxisomal β-oxidation is rate-limiting).

With 6'-hydroxy-LPB as substrate, the ratio of LPB-4'-oic acid formed to LPB-6'-oic acid formed was approximately 3.3-fold higher.

**Fig. 4. Effect of 4-methylpyrazole on the metabolism of 6'-hydroxy-LPB by isolated hepatocytes.**

**Fig. 5. Proposed metabolic pathway for the formation of LPB-4'-oic acid from sameridine by rat hepatocytes.**
with hepatocytes from clofibrate-treated animals than with control hepatocytes. Thus, it appears likely that peroxisomal $\beta$-oxidation is involved in the conversion of LPB-6'-oic acid to LPB-4'-oic acid.

On the other hand, when hepatocytes from clofibrate-treated rats were incubated with LPB-6'-oic acid, no increase in the formation of LPB-4'-oic acid was observed. One explanation for this finding might be that the rate-limiting step in this case is movement of LPB-6'-oic acid across the plasma membrane, rather than peroxisomal $\beta$-oxidation. The rate of uptake of LPB-6'-oic acid by cells may be low, due to the carboxylic group present. The hypothesis that the uptake of LPB-6'-oic acid into the cells was rate-limiting is strengthened by the observation that the formation of LPB-4'-oic acid from 6'-hydroxy-LPB is much more rapid than from LPB-6'-oic acid (Fig. 3, A and B).

In our system using isolated rat hepatocytes, both LPB-4'-oic acid and LPB-6'-oic acid were formed upon incubation with sameridine. Although LPB-6'-oic acid is not detected in the urine of rats to which sameridine has been administered (Elofsson et al., 1996), there is no reason to believe that the mechanism underlying the formation of LPB-4'-oic acid in vivo differs from the corresponding mechanism in vitro. It is highly likely that LPB-6'-oic acid is also formed in rat liver in vivo, where it subsequently undergoes $\beta$-oxidation to yield LPB-4'-oic acid.

There are other examples of xenobiotic fatty acids, which are $\beta$-oxidized in the peroxisomes (Yamada et al., 1984, 1986; Gatt et al., 1988; Sinz et al., 1997). Because various endogenous fatty acids and bulky exogenous fatty acids can be metabolized by peroxisomal $\beta$-oxidation, this pathway must have a very broad substrate specificity. This conclusion is strengthened by the demonstration that at least three different forms of acyl-CoA oxidase (the enzyme that catalyzes the first and rate-limiting steps in peroxisomal $\beta$-oxidation) are present in rat liver peroxisomes (Scherpers et al., 1990; Van Veldhoven et al., 1992). In addition, several forms of acyl-CoA synthetase (the enzyme involved in the activation of fatty acids to CoA thioesters) are expressed in rat liver (Bronfman et al., 1984; Vamecq et al., 1985; Singh and Poulois, 1988). One round of $\beta$-oxidation of a carboxylic acid results in the removal of 2 carbon atoms. However, certain metabolites of some xenobiotic carboxylic acids are formed by the removal of an odd number of carbon atoms. For example, the major metabolites of 4'-hydroxy-$\Delta^1$-tetrahydrocannabinol (the $\omega$-1 derivative of $\Delta^1$-tetrahydrocannabinol) in mouse liver are two carboxylated derivatives with chains that are either one or two carbon atoms shorter than they are in parent drug (Harvey, 1990). It has been suggested that the removal of an odd number of carbon atoms could involve ($\omega$-1)- or ($\omega$-2)-hydroxy intermediates and/or $\alpha$-oxidation (Harvey, 1989; 1990; Sinz et al., 1997).

Because in vivo studies have revealed that a carboxylic acid metabolite with a side chain containing five carbon atoms (LPB-5'-oic acid) is excreted in the urine of rats after sameridine administration (Elofsson et al., 1996), we incubated isolated rat hepatocytes here with 5'-hydroxy-LPB (the ($\omega$-1)-hydroxy derivative of sameridine). However, incubation with neither sameridine nor 5'-hydroxy-LPB, 6'-hydroxy-LPB, or LPB-6'-oic acid results in any detectable formation of LPB-5'-oic acid. This finding may indicate that extrahepatic metabolism is involved in the formation of LPB-5'-oic acid in the rat.

Considered together, the results presented here suggest that cytochrome P450 4A, alcohol dehydrogenases, and peroxisomal $\beta$-oxidation are involved in the metabolism of sameridine to LPB-4'-oic acid by isolated rat hepatocytes (Fig. 5). Recently, Sinz and coworkers (Sinz et al., 1997) have demonstrated that the alkyl side chain of a fatty acid anilide, CI-976, is metabolized via this same pathway. It appears likely that this pathway may also be involved in the metabolism of other drugs possessing alkyl side chains.

Acknowledgments. We thank Pr. Joseph W. DePierre at the Unit for Biochemical Toxicology, Stockholm University, for correcting the language.

References


Harvey DJ and Hogg JM (1979) Chain length specificities of peroxisomal and mitochondrial $\beta$-oxidation in rat liver. Biochem Biophys Res Commun 177:1200–1206.


