THE REGIOSELECTIVE HYDROXYLATION OF LAURIC ACID BY RAINBOW TROUT (ONCORHYNCHUS MYKISS) CYTOCHROME P450S

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ABSTRACT:

We have reexamined the hydroxylation of [1-14C]-lauric acid by trout liver microsomes and reconstituted trout P450s using a new HPLC system that gave an improved separation of hydroxylauric acids. Under these conditions, hepatic microsomes from yearling juvenile trout were shown to form \( \omega \), \( \omega^1 \), \( \omega^2 \), \( \omega^3 \), \( \omega^4 \), \( \omega^5 \), and \( \omega^6 \)-OH lauric acid oxidation products (12-OH, 11-OH, 10-OH, 9-OH, 8-OH, 7-OH, and 6-OH lauric acid, respectively) as verified by GC/MS analysis. Microsomes from male and female juvenile trout liver formed (\( \omega \)-1)-OH lauric acid as the major metabolite (23–29% of total radioactivity) and no major differences were observed between males and females. By contrast, liver microsomes from 3-year-old sexually mature trout had substantially lower lauric acid hydroxylase activity than juvenile microsomes and produced small quantities of only the \( \omega \)-1, \( \omega \)-2, and \( \omega \)-6-hydroxylation products. Moreover, microsomes from sexually mature female trout had markedly lower lauric acid hydroxylase activity than those from the sexually mature male trout. Rat liver microsomes were quite catalytically active but formed mostly the \( \omega \)- and \( \omega^1 \)-1 lauric acid oxidation products. Lauric acid metabolism also was analyzed in reconstituted systems containing purified juvenile trout LMC1 (CYP2M1) and LMC2 (CYP2K1) and with hepatic microsomes from juvenile trout in the presence of rabbit polyclonal antibodies raised against the two purified trout P450s. CYP2M1 catalyzed the \( \omega \)-6-hydroxylation of lauric acid while the trout CYP2K1 form produces mainly \( \omega \)-1-OH lauric acid together with a smaller quantity of the \( \omega \)-2-hydroxylation product. All of the trout and rat radiometric lauric acid metabolism results were confirmed by direct mass spectrometric analysis of derivatized lauric and dicarboxylic acid mixtures. Use of direct mass spectrometric analysis for the hydroxylated lauric acids offers considerable advantages since the method did not require use of a radioactive fatty acid, completely separated all of the lauric acid hydroxylation products, confirmed identification of each metabolite, and was more sensitive than the radiometric analysis method.

Saturated and unsaturated fatty acids have two major functions in fish and other animals. Esterified to phosphoglycerides they are integral components of membrane lipids and also function as the animal’s major energy stores in the form of triglycerides (1). In addition, fatty acids, notably arachidonic acid, can be metabolized by cytochrome P450s and other enzymes to form various biologically active compounds such as prostaglandins, leukotrienes, hydroxyeicosatetraenoic acids (HETEs), and cis-epoxyeicosatrienoic acids (EETs) (2–5).

Fatty acid oxidation generally proceeds via stepwise mitochondrial or peroxisomal \( \beta \)-oxidation from the carboxyl end of the chain, two carbons at a time, to yield acetyl CoA which in turn generates ATP (1, 6). In addition, hydroxylation of the terminal methyl group or the penultimate methylene group of the fatty acids also can occur (2, 3). Such reactions, termed \( \omega \)- and \( \omega \)-1 hydroxylation, respectively, are catalyzed by cytochrome P450 enzymes and lead to the formation of the corresponding primary or secondary alcohols (7, 8). Further oxidation of the hydroxylated fatty acids then can take place. The \( \omega \)- and \( \omega \)-1-hydroxylation products, for example, can be converted to dicarboxylic acids. The resulting long-chain dicarboxylic acids are subsequently shortened by \( \beta \)-oxidation, and excreted as \( \text{C}_6 \)–\( \text{C}_{10} \) dicarboxylic acids in the urine (6).

Cytochrome P450s in the endoplasmic reticulum of liver, kidney, and other mammalian tissues thus catalyze the conversion of fatty acids such as laurate (\( \text{C}_{12} \)), palmitate (\( \text{C}_{16} \)), and stearate (\( \text{C}_{18} \)) to the corresponding \( \omega \)- and \( \omega \)-1-hydroxylation products. Lauric acid metabolism primarily occurs in the \( \omega \)-position (2, 3, 9–12) but production of \( \omega \)-1 and \( \omega \)-2 hydroxylation products also are observed (11, 13). \( \omega \)-Hydroxylation reactions in mammals are associated with members of the CYP4A gene family (9,11), while \( \omega \)-1-hydroxylation products are formed by mammalian CYP1A1, CYP1A2, CYP2A1, CYP2C2, CYP2C6, CYP2C9, CYP2C11, and CYP2E1 isofoms (3, 10, 14, 15).

Oxidation of saturated fatty acids to hydroxylated metabolites also occurs in fishes (16–19). Fish preferentially use lipids rather than carbohydrates as energy sources (20) and, as a result, tend to be hyperlipidemic with much higher plasma fatty acid levels compared with mammals (21). Our previous research had established that rainbow trout hepatic microsomes metabolize fatty acids such as lauric acid (dodecanoic acid) primarily at the \( \omega \)- and \( \omega \)-1-positions to form 12-OH and 11-OH lauric acid, respectively (16, 17). In addition, purified trout P450 LMC1 (17), recently cloned and now designated CYP2M1 (22), also was shown to catalyze the \( \omega \)-hydroxylation of...
lauric acid. Similarly, trout P450 LMC2 (17), previously cloned and now named CYP2PK1 (23), formed the (α-1)-hydroxylation product. A recent report by Lemaire et al., (19), however, indicates that liver microsomes from sea bass hydroxylate lauric acid at several other subterminal positions in addition to producing the α- and (α-1)-
hydroxylation products. Therefore, we have reexamined the hydroxy-
lation of [1-14C]-lauric acid and nonradioactive lauric acid by trout
liver microsomes and reconstituted trout P450s, using both a new
HPLC system that gives an improved separation of hydroxylauric
acids and by direct mass spectrometry of derivatized lauric acid
microsomal incubation mixtures. As a result, we discovered that fatty
acid oxidation by trout liver microsomes was considerably more
complex than we had previously believed, as reflected by the observed
conversion of lauric acid to α-, (α-1)-, (α-2)-, (α-3)-, (α-4)-,
(α-5)-, and (α-6)-OH lauric acid metabolites. We also have found that
the direct GC/MS analysis of a derivatized lauric acid incubation extract
gave comparable results with those obtained with the radiometric
assay, albeit with somewhat greater sensitivity. Using both analytical
techniques we also have determined that in reconstituted systems
CYP2M1 catalyzed only the (α-6)-hydroxylation of lauric acid while
CYP2PK1 formed both (α-1)-OH lauric acid (major) together with
(α-2)-OH lauric acid (minor) oxidation products. In addition, marked
differences were observed in the microsomal oxidation of lauric acid
between sexually mature male and female trout. A preliminary report
of this work was presented earlier (24).

Materials and Methods

Experimental Animals. Rainbow trout (Oncorhynchus mykiss) of the Mt.
Shasta strain were obtained from the Oregon State University Marine/Fresh-
water Biomedical Sciences aquatic facility. Microsomes were prepared by
differential centrifugation as described previously by Miranda et al. (25) from
livers of male and female juvenile (14–15 months) and post-spawning,
sexually mature (3-year-old) fish. Microsomes from the livers of young (100–120
g) male Sprague-Dawley rats were similarly prepared.

Chemicals. [1-14C]-Lauric acid (dodecanoic acid; 40 mCi/mmole) was pur-
chased from ICN Pharmaceuticals Inc. (Costa Mesa, CA). Trout P450 LMC1
(CYP2M1) and P450 LMC2 (CYP2PK1) were isolated and purified as previ-
ously described (25). Rabbit polyclonal antibodies to these proteins were raised
in rabbits as reported (25).

Lauric Acid Hydroxylation Assays. Oxidation of lauric acid by rat
and trout liver microsomes was assayed essentially as described by Salhab et al.
(18). Mixtures containing 0.5 mg microsomal protein, 50 mM Tris-HCl buffer
(pH 7.4), 200 μM [1-14C]-lauric acid, and 1 mM NADPH, in a total volume
of 0.5 ml, were incubated for 1 hr at 30°C for trout microsomes or 30 min at
37°C for rat microsomes. Controls contained no NADPH. Reactions were
terminated by the addition of 0.1 ml 10% H2SO4 and extracted twice with 0.5 ml
diethyl ether. The ether extracts were taken to dryness under a stream of N2
and methylated using diazomethane (1 hr at room temperature). The dried samples
were then dissolved in 25 μl dry pyridine (Pierce Chemical Co., Rockford, IL), 25 μl of N.O.
bis(trimethylsilyl)trifluoroacetamide (BSTFA, Regisil, Regis Technologies
Inc., Morton Grove, IL) added and the samples heated for 1 hr at 60°C.

HPLC Metabolite Identification by GC-MS. The derivatized peak
samples were analyzed on a Finnigan 4023 quadrupole GC-MS. The instru-
ment has a 4500 source, Galaxy 2000 data system, and a Varian 3400 gas
chromatograph. The samples were chromatographed using splitless injection
onto a 30-m SE-54 silica capillary column with 0.25 mm ID. The column was
operated with a helium linear velocity of 40 cm/sec and was programmed from
100–150°C at 20°C/min, then heated to 250°C at 4°C/min. Ionization was by
electron impact (EI) with 70 eV electrons with a source temperature of 140°C

Direct GC-MS of Lauric Acid Metabolism Mixtures. In other experi-
ments, the microsomal metabolism of lauric acid was stopped by the addition
of 0.1 ml 10% H2SO4 and the incubation mixtures then extracted twice with 4
ml diethyl ether. The ether extracts were taken to dryness under a stream of N2
and dissolved in CH3CN:H2O:acetic acid as described above. A 50 μl aliquot
of the sample was then removed for HPLC analysis. The other 50 μl was taken
to dryness under a stream of N2, redissolved in a small volume of diethyl ether,
and methylated using diazomethane (1 hr at room temperature). The dried
samples were then dissolved in 25 μl dry pyridine, 25 μl of BSTFA added,
and the samples heated for 1 hr at 60°C. The derivatized reaction mixture then was
subjected to GCMS electron impact analysis as described.

Results

Microosomal Metabolism of Lauric Acid. Multiple radioactive
peaks were detected when extracts from incubations of [1-14C]-lauric
acid with juvenile trout hepatic microsomes were examined by HPLC
using the improved HPLC conditions of Lemaire et al. (19) (fig. 1A).
The various metabolites were identified as the terminal methyl and
several subterminal hydroxylation products of lauric acid after the
radioactive peaks were collected from a 10-fold scale-up of the
incubation mixture derivatized and examined via GC-MS (see below).
The hydroxylated lauric acid metabolites formed by the juvenile trout
microsomes (fig. 1A) thus were found to be eluted in the order: (α-1),
(α-2), (α-3), (α + α + 4-), (α-5), and (α-6)-OH lauric acid, respec-
tively, followed by the unoxidized parent lauric acid. The presence of
both α- and (α-4)-OH lauric acid in peak 4 (fig. 1A) was established
by the mass spectrometric analysis of the derivatized metabolites in
the eluted peak. By contrast, as has been reported (13), only the
radioactive α- and (α-1)-OH laurate metabolites (fig. 1B) were de-
tected after incubation of rat liver microsomes with [1-14C]-lauric
acid.

Only minor differences in metabolite production were observed between hepatic microsomes from juvenile male and female trout
(table 1). As with the sea bass (19) and our previous reports (16,17),

P450 with the microsomes for 20 min at room temperature before proceeding
with the usual analysis method. Controls contained IgG from untreated rabbits
(pre-immune).

HPLC of Lauric Acid and its Hydroxylated Metabolites. HPLC of
the hydroxylauric acids was performed as described by Lemaire et al. (19)
with slight modification. Samples were analyzed on a Beckman HPLC equipped
with a 421 controller, two 100A pumps, and a 5 μm Zorbax ODS column (0.46 x
25 cm) (Mac-Mod Analytical Inc., Chadds Ford, PA) using CH3CN and
water each containing 0.2% acetic acid, as the mobile phases, a flow rate of 1.5
ml/min, and detection by a Packard Flo-One A-100 radioactivity flow monitor.
The mobile phase concentration was 25% CH3CN for 35 min followed by a 2
min linear gradient to 80% CH3CN to elute the parent compound. At 45 min,
the CH3CN was returned to 25% over 2 min. The column was allowed to
equilibrate 23 min before the next injection.

Collection of Metabolites for Identification by GC-MS. Scaled-up (10-
fold) incubations of juvenile trout liver microsomes with lauric acid were
performed as described above. Eluent corresponding to each radioactive
metabolite peak was collected and an aliquot reanalyzed by HPLC to verify peak
collection. The samples were then taken to dryness on a rotary evaporator,
redissolved in a small volume of diethyl ether, and methylated using dia-
zomethane (1 hr at room temperature). The dried samples were then dissolved in
25 μl dry pyridine (Pierce Chemical Co., Rockford, IL), 25 μl of N.O.
bis(trimethylsilyl)trifluoroacetamide (BSTFA, Regisil, Regis Technologies
Inc., Morton Grove, IL) added and the samples heated for 1 hr at 60°C.

HPLC of Lauric Acid and its Hydroxylated Metabolites.
v-1)-OH lauric acid was the predominate metabolite (28.8% and 23.5% of total radioactivity) formed by pooled hepatic microsomes from juvenile male and female trout, respectively. While significant (3.0 to 8.5% of total radioactivity) conversion of lauric acid to various in-chain hydroxylation products was detected (fig. 1A; table 1), v-1)-OH lauric acid was formed in only minor amounts by the trout microsomes. Similar results were obtained in an experiment employing individual analyses of hepatic microsomes from four male juvenile trout (table 2).

The microsomal oxidation of lauric acid in sexually mature trout was substantially diminished compared to the juvenile animals with only 2–10% of the lauric acid being oxidized when incubated under the standard assay conditions (table 1). Microsomes from the sexually mature male fish produced (v-1)-, (v-2)-, and (v-6)-OH lauric acid metabolites whereas microsomes from mature females gave only small quantities of the (v-1)-, (v-3)-, and (v-6)-hydroxylation products (fig. 2; table 1).

GC/MS Analyses. Electron impact GC/MS analysis was used to identify the radioactive metabolite peaks recovered from the microsomal metabolism of [1-14C]-lauric acid (fig. 1; table 1). Mass spectra of the methyl ester trimethylsilyl ether derivatives of the various hydroxylauric acid metabolites gave typical (19, 27, 28) fragmentation patterns associated with monohydroxylated fatty acids, including m/z 287 [M-15]+, m/z 271 [M-31]+, m/z 255 [M-47]+, m/z 159 ([CH3CO2CH2O]HI[CH3]2]+, and m/z 73 [CH3Si]+. Typical mass spectra for the recovered (v-1)- and (v-6)-lauric acid oxidation products are shown in fig. 3.

Extracts from trout microsomal incubation mixtures also were

**TABLE 1**

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>Retention Time (min)</th>
<th>Metabolite Identity</th>
<th>% of Total Radioactivitya</th>
<th>Juvenile Trout</th>
<th>Mature Trout</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>1</td>
<td>29.5</td>
<td>(v-1)</td>
<td>28.8</td>
<td>6.5</td>
<td>0.4</td>
</tr>
<tr>
<td>2</td>
<td>31.5</td>
<td>(v-2)</td>
<td>8.5</td>
<td>0.6</td>
<td>ND</td>
</tr>
<tr>
<td>3</td>
<td>33.8</td>
<td>(v-3)</td>
<td>4.2</td>
<td>0.6</td>
<td>ND</td>
</tr>
<tr>
<td>4</td>
<td>37.4</td>
<td>(v + v-4)</td>
<td>0.5</td>
<td>0.2</td>
<td>ND</td>
</tr>
<tr>
<td>5</td>
<td>38.3</td>
<td>(v-5)</td>
<td>4.9</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>6</td>
<td>40.3</td>
<td>(v-6)</td>
<td>5.1</td>
<td>3.1</td>
<td>0.8</td>
</tr>
<tr>
<td>7</td>
<td>45.2</td>
<td>lauric acid</td>
<td>47.4</td>
<td>90.0</td>
<td>98.4</td>
</tr>
</tbody>
</table>

*Mean of duplicate determinations on a pooled liver sample from 50 juvenile and four sexually mature male and female trout.

ND, not detected.

(v-1)-OH lauric acid was the predominate metabolite (28.8% and 23.5% of total radioactivity) formed by pooled hepatic microsomes from juvenile male and female trout, respectively. While significant (3.0 to 8.5% of total radioactivity) conversion of lauric acid to various in-chain hydroxylation products was detected (fig. 1A; table 1), (v-1)-OH lauric acid was formed in only minor amounts by the trout microsomes. Similar results were obtained in an experiment employing individual analyses of hepatic microsomes from four male juvenile trout (table 2).

The microsomal oxidation of lauric acid in sexually mature trout was substantially diminished compared to the juvenile animals with only 2–10% of the lauric acid being oxidized when incubated under the standard assay conditions (table 1). Microsomes from the sexually mature male fish produced (v-1)-, (v-2)-, and (v-6)-OH lauric acid metabolites whereas microsomes from mature females gave only small quantities of the (v-1)-, (v-3)-, and (v-6)-hydroxylation products (fig. 2; table 1).

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Extracts from trout microsomal incubation mixtures also were
methylated, trimethylsilylated, and analyzed directly by GC/MS. This method was quite convenient and appeared to be more sensitive than the HPLC radiometric assay when performed on the same samples. The peaks of the derivatized lauric acid and its metabolites were well separated upon GC/MS analysis, eluted in the following order, and showed characteristic peaks at the indicated m/z and (relative intensity %): lauric acid [m/z 74 (100), m/z 87 (47), m/z 171 (6), m/z 214 (3)]; (ω-6)-OH lauric acid (6-OH dodecanoic acid [m/z 187 (100), m/z 217 (88)]); (ω-5)-OH lauric acid (7-OH dodecanoic acid [m/z 173 (100), m/z 231 (58)]); (ω-4)-OH lauric acid (8-OH dodecanoic acid

**TABLE 2**

Mass spectrometric and radiometric determination of lauric acid hydroxylation

<table>
<thead>
<tr>
<th>HPLC Peak No.</th>
<th>Identity</th>
<th>% of Total Radioactivity of or of Total Ion Current</th>
<th>Trout Radioactivity</th>
<th>GC/MS Radioactivity</th>
<th>Rat Radioactivity</th>
<th>GC/MS Radioactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>12-OH (ω)</td>
<td>1.0 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.2 ± 0.2</td>
<td>14.0 ± 1.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>20.3 ± 2.7</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>11-OH (ω-1)</td>
<td>16.4 ± 3.9</td>
<td>26.0 ± 4.6</td>
<td>10.6 ± 1.7</td>
<td>17.6 ± 2.2</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>10-OH (ω-2)</td>
<td>4.4 ± 1.5</td>
<td>5.6 ± 1.4</td>
<td>0.5 ± 0.4</td>
<td>0.7 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>9-OH (ω-3)</td>
<td>1.3 ± 0.8</td>
<td>2.7 ± 1.6</td>
<td>ND&lt;sup&gt;c&lt;/sup&gt;</td>
<td>tr&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>8-OH (ω-4)</td>
<td>μ</td>
<td>0.4 ± 0.3</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>7-OH (ω-5)</td>
<td>0.1 ± 0.2</td>
<td>0.3 ± 0.2</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>6-OH (ω-6)</td>
<td>2.7 ± 1.9</td>
<td>4.3 ± 2.6</td>
<td>tr&lt;sup&gt;c&lt;/sup&gt;</td>
<td>tr&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>lauric acid</td>
<td>73.6 ± 3.6</td>
<td>59.9 ± 4.3</td>
<td>74.6 ± 2.6</td>
<td>61.5 ± 3.0</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean ± S.D. for four male animals.

<sup>b</sup> The radioactive data includes the contribution of both 8-OH and 12-OH lauric acid as these two metabolites are not resolved in the HPLC separation.

<sup>c</sup> ND, not detected; tr, trace amount detected but too low to quantitate.

**FIG. 2.** HPLC profile of [1-<sup>14</sup>C]lauric acid metabolites formed by liver microsomes from sexually mature male and female trout.
lauric acid (11-OH dodecanoic acid [m/z 118 (100), m/z 258 (7)]) and (ω-2)-OH lauric acid (0.5% of total radioactivity) and traces of the (ω-3)- and (ω-6)-hydroxylation products (table 2).

An estimation of the relative distribution of metabolites based on peak areas using the assumption that all of the hydroxylated lauric acid metabolites were ionized with similar efficiency gave generally good agreement with the results obtained from the radiometric analysis (table 2). Based on both the mass spectrometry and radioactivity analysis, no evidence was obtained for the conversion of lauric acid to epoxides, diols, or metabolites other than monohydroxylation products.

**Immunoinhibition Studies.** To help establish the identity of the cytochrome P450s responsible for the oxidation of lauric acid by trout liver microsomes, we carried out immunoinhibition experiments using rabbit polyclonal antibodies raised against purified trout CYP2M1 protein (LMC1) and CYP2K1 protein (LMC2) (25) which were preincubated with trout liver microsomes. Upon addition of anti-LMC1 IgG, the oxidation of lauric acid to its 6-hydroxylation (ω-6) product was almost completely obliterated compared with the controls (table 3). These results suggest that CYP2M1 is responsible for most if not all of the (ω-6)-oxidation of lauric acid catalyzed by trout hepatic microsomes. Preincubation of the anti-LMC2 IgG with trout liver microsomes, however, resulted in only a 47 and 63% reduction in the formation of (ω-1)- and (ω-2)-OH lauric acids, respectively (table 3).

Nevertheless, these results confirm that CYP2K1 is likely a major contributor to the formation of both of these lauric acid oxidation products in the trout.

**Studies with Purified Trout Cytochrome P450s.** Further evidence for the contribution of specific trout P450 isozymes to the regioselectivity of lauric acid metabolism in the trout was provided from incubations employing reconstituted trout P450s. When trout CYP2M1 protein (LMC1) was used for metabolism experiments with [1-14C]-lauric acid, only (ω-6)-OH lauric acid was produced (fig. 5). Similar incubations of the reconstituted trout CYP2K1 protein (LMC2), however, gave both (ω-1)- and (ω-2)-OH lauric acid metabolites in a ratio of 6 to 1 (fig. 5). The results of the studies with the purified trout P450s, therefore, are in good agreement with the findings of the antibody inhibition experiments (table 3).

**Discussion**

The formation of lauric acid hydroxylation products was linear with respect to time under the incubation conditions used (data not shown). Juvenile trout had high hepatic lauric acid hydroxylase activities since trout liver microsomes incubated at 30°C gave similar yields of hydroxylated lauric acid metabolites compared with those found when rat liver microsomes were incubated at 37°C (table 2). Upon examination of the radiometric (fig. 1B and table 1) and mass spectrometric results (table 2), as shown previously (7, 9, 12), male rat liver microsomes were found to oxidize the model saturated fatty acid, lauric acid, primarily (14.0% of total radioactivity) at the ω-position. However, a significant amount of the (ω-1)-hydroxylation products (10.6% of total radioactivity) also was generated together with other (ω-2)- and (ω-3)- OH lauric acid (0.5% of total radioactivity) and traces of the (ω-6)-hydroxylation products. The formation of (ω-1)-, (ω-2)-, and (ω-6)-OH metabolites of lauric acid by male Fisher rat liver microsomes has been described (13).

In contrast, trout microsomes mainly gave (ω-1)-OH lauric acid (16.4% of total radioactivity) as shown previously (16–19), but ω-6-hydroxylation was a very minor (1.0%) pathway (fig. 1A and table 2). Juvenile male trout liver microsomes also catalyzed formation of a number of other subterminal lauric acid hydroxylation products. These included the (ω-2) (4.4% of total radioactivity), (ω-3) (1.3% of total radioactivity), (ω-4) (trace), (ω-5) (0.1% of total radioactivity), and (ω-6)-OH lauric acid (2.7% of total radioactivity) as verified by GC/MS analysis. No major differences were seen between pooled male and female juvenile trout (table 1), but substantial differences were seen in the regiospecific lauric acid catalytic activity among individual animals (table 2). Previously, sea bass liver microsomes were shown (19) to oxidize lauric acid mainly to (ω-1)-OH lauric acid (44% of total metabolites) plus smaller quantities of the (ω-2)-, (ω-3)-, and (ω-4)-hydroxylation products. Lauric acid hydroxylations catalyzed by trout microsomes seemed to be catalyzed primarily by cytochrome P450 isoforms since the production of lauric acid metabolites during microsomal incubations was dependent on the presence of NADPH and O2 (data not shown).

In addition, the hydroxylations were almost completely eliminated by addition of P450 inhibitors such as ellipticine and α-naphthoflavone.

Lauric acid hydroxylase activity of sexually mature trout was substantially less than that found in juvenile fish (fig. 2; table 1). Only 10% and 1.6% of the lauric acid substrate, respectively, was oxidized by hepatic microsomes from sexually mature male and female trout.
while oxidation by microsomes from juvenile males and females amounted to 52.6% and 45.3%, respectively. Microsomes from mature trout also gave a less complex mixture of hydroxylation products, with males yielding only the \((\omega - 1)\), \((\omega - 2)\), and \((\omega - 6)\)-OH lauric acids and females giving the \((\omega - 1)\), \((\omega - 3)\), and \((\omega - 6)\) metabolites. The reduced generation of \((\omega - 1)\) and \((\omega - 2)\) lauric acid hydroxylation products seen with microsomes from sexually mature trout is likely a reflection of the remarkable differential sexual expression of CYP2K1 between mature males and females noted previously (16, 26). Higher P450 metabolism in male animals is well documented for mammalian species (29–33). Studies conducted in rats and other mammals also show a considerably greater lauric acid oxidation to occur with hepatic or renal microsomes from untreated male animals and in males pretreated with peroxisome proliferators or other chemicals compared to that found in females (30–33). Sexual differences in lauric acid hydroxylation between male and female rats is thought to arise from tissue differences in CYP4A isozyme concentrations (30). However, no CYP4A form has yet been reported for any fish species (34).

Although the HPLC method of Lemare et al. (19) was an improvement over the old method of separating lauric acid and its hydroxylation products, the GC/MS analysis also used in this study was superior in several aspects. The two procedures used gave similar results with the GC/MS method showing somewhat increased sensitivity (table 2). GC/MS analysis had the advantage of not requiring the use of radioisotopes as well as the solvents and scintillation cocktails necessary for the radiometric HPLC analysis. Additionally, the GC/MS analyses took less time than the HPLC runs (22 min versus 70 min). More importantly, all of the metabolites were resolved by the GC (fig. 4) and each metabolite’s identity could be verified by its mass spectra on any particular run (fig. 3).
Immunoinhibition experiments demonstrated that trout LMC1 (CYP2M1) was responsible for most if not all of the (ω-6)-hydroxylation of lauric acid catalyzed by liver microsomes from juvenile trout (table 3). Antibodies against LMC2 (CYP2K1), however, only reduced by 47 and 63%, respectively, the microsomal (ω-1) and (ω-2) hydroxylation of lauric acid. These results indicate that while LMC2 (CYP2K1) catalyzes much of the fatty acid (ω-1) and (ω-2) oxidation in trout, they suggest that other P450 isoforms also contribute to this regiospecific fatty acid metabolism. Detection of more than one source of fatty acid (ω-1)-oxidation in the trout is also consistent with the relative lack of P450 isoform specificity for such (ω-1)-oxidations seen in mammals (3, 10, 14, 15). Antibodies against other purified constitutive trout P450s (LMC3, LMC4, and LMC5 (23)) failed to alter the relative distribution of lauric acid hydroxylation products formed by juvenile trout liver microsomes (data not shown).

Further evidence for the contribution of specific trout P450 isozymes to the regioselectivity of lauric acid metabolism in the trout was provided from incubations employing reconstituted trout P450s. When trout P450 LMC1, recently cloned, sequenced and expressed, and now named CYP2M1 (25), was used for metabolism experiments with [1-14C]-lauric acid, only (ω-6)-OH lauric acid was produced (fig. 5). Similar incubations of the reconstituted trout LMC2, previously cloned and named CYP2K1 (26), however, demonstrated the formation of both (ω-1)- and (ω-2)-OH lauric acid metabolites in a ratio of 6 to 1 (fig. 5). The results of the studies with the purified trout P450s, therefore, are in good agreement with the findings of the antibody inhibition experiments (table 2).

Preliminary results with longer chain saturated fatty acids indicate that trout microsomes also hydroxylate these substrates, although at much reduced conversion rates compared with lauric acid, the preferred fatty acid substrate. Since fatty acid metabolism seems to be quite important for fishes (20, 21), this may help explain the greater diversity of lauric acid metabolites formed by trout hepatic microsomes compared with those produced by mammals.

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3 Erkoc et al., manuscript in preparation.

References

LAURIC ACID HYDROXYLATION BY RAINBOW TROUT P450s


