4'-Hydroxylation of Flurbiprofen by Rat Liver Microsomes in Fasting and Feeding Conditions

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We examined the 4'-hydroxylation of flurbiprofen in rat hepatocytes and liver microsomes in order to know whether the metabolism of flurbiprofen is changed on its administration to experimental animals after overnight fasting, because starvation and fasting change both the composition of cytochrome P450s (CYPs) and metabolic activity. CYPs involved in the hydroxylation were determined by various CYP inhibitors and inhibitory antibodies against rat CYP2C11 and CYP2E1 using the microsomes in fasting and feeding. The results provided a possibility that the 4'-hydroxylation might be regulated by CYP2C11, but not by CYP2E1, at fasting rather than feeding.

Key words flurbiprofen; 4'-hydroxyflurbiprofen; nonsteroidal anti-inflammatory drug (NSAID); fasting; cytochrome P450s (CYPs)

Flurbiprofen (FP) is a nonsteroidal anti-inflammatory drug (NSAID) of the 2-arylpropionic acid class. Although it possesses a chiral center, both R(-)- and S(+)-enantiomers may possess analgesic activity, and all FP-preparations to date are marketed as the racemate. The enantiomers exhibit differences in both the level of protein binding and metabolism.1) The former difference was observed using ultracentrifugation2) but not using equilibrium dialysis.3,4) The latter difference was observed in diastereomeric glucuronides, but the enantiomers were indiscernibly metabolized by oxidation via the cytochrome P450 system (CYP), followed by glucuronidation.5) The major oxidative metabolites were 4'-hydroxy and 3',4'-dioxgenated metabolites (Fig. 1),5) and it was known that 4'-hydroxy-flurbiprofen (4'-HOFP) was transformed from FP involving in CYP 2C9 and not CYPs 1A2, 2C8, 2E1, or 3A4 in human.5) Very little is known of the metabolic capabilities by CYP isoforms in rat liver microsomes.

Since drugs are sometimes administered to experimental animals after overnight fasting, it is necessary to know whether the metabolism of FP is changed after the fasting. Starvation and fasting change both the composition of cytochrome P450s and the metabolic activity of rat hepatic microsomes, inducing CYPs 2B1, 2E1, and reducing CYPs 1A2, 2C8, 2E1, or 3A4 in rats.6) Very little is known of the metabolic capabilities by CYP isoforms in rat liver microsomes.

MATERIALS AND METHODS

Chemicals Aniline, cimetidine, disulfiram, flurbiprofen, p-aminophenol, quinine, testosterone, and trocleandomycin were purchased from Wako Pure Chemicals (Tokyo). Diclofenac, furafylline, glucose-6-phosphate and SKF-525A were from Sigma Chemical Co. Kanamycin was from Meiji Seika (Tokyo), and anti-rat CYP2C11 serum and anti-rat CYP2E1 serum were from Dai-ichi Pure Chemicals Co. (Tokyo). G-6-P-D, β-NADP, and β-NADPH were from Oriental Fern. Co. (Tokyo).

Treatment of Animals Male Wistar rats (250—300 g) were obtained from Charles River Laboratories, Inc. (Japan). The animals were housed in cages under controlled conditions of temperature and humidity, and were subjected to a 12-h light/dark cycle. The rats were fed Oriental laboratory diets for the feeding condition and were fasted for 24 h for the fasting condition. They had free access to water under both conditions.

Preparation of Rat Liver Microsomes The animals were killed by decapitation, and individual liver microsomes were prepared by a general method: The liver was homogenized with 0.25 M sucrose (9 vol.) to give a supernatant by centrifugation at 10000×g for 10 min and then 9000×g for 20 min, then the supernatant was centrifuged at 105000×g for 60 min to give a pellet. The microsomal pellet was suspended in 0.1 M potassium phosphate buffer (pH 7.4) and stored at −30°C until use.

Microsomal Protein Assay Microsomal protein concentration was determined by the method of Bradford11) with Bio-Rad Protein Assay Kit. Absorbance was measured at 595 nm with a Hitachi Model U-2001 spectrophotometer.

Determination of Total Cytochrome P450 Content Total microsomal cytochrome P450 content was determined from a sodium di-thionite-reduced carbon monoxide difference spectrum using a molar extinction coefficient of between 450 and 490 nm.12) The liver microsomal pellet suspension was adjusted to be 1.0 mg/ml by dilution with a solution of 20% glycerol, 1 mM EDTA, 0.1 M potassium phosphate buffer (pH 7.25) and 0.2% Emulgen 913 (Kao Co. Ltd., Tokyo), and the diluted suspension was divided into a sample and reference. The sample was subjected to a carbon monoxide gas for 1 min, and the sample and reference both were treated with di-thionite to give a different spectrum OD\textsubscript{590...490}.

Preparation and Incubation of the Hepatocytes Hepatocytes were isolated from adult male Wistar rats (body weight 250—300 g) according to the procedure described by Shimaoka et al.13) Briefly, the liver was first perfused in situ through the portal vein with 200 ml (30 ml/min) of Ca-+-free Hanks’ solution containing 5 mM EGTA at 37°C for a few
minerals, followed by perfusion with 400 ml (30 ml/min) of 0.05% collagenase solution. The perfused liver was excised and dispersed in cold Eagle’s minimum essential medium (MEM), and the resulting cell suspension was filtered through a double layer of gauze. The liver cells were separated into two fractions, parenchymal hepatocytes and non-parenchymal cells (NPC), by differential centrifugation at 50 × g for 1 min. The precipitated cells were washed three times with MEM to be used as hepatocytes. Cell viability was determined by the Trypan blue dye exclusion method. The cells were plated at a density of 1 × 10^5 cell/0.2 ml/cm² in wells of Falcon culture dishes (60 × 15 mm) coated with collagen (Nitta Gelatin Co., Osaka) in 1 ml of William’s E (WE) medium supplemented with 5% calf serum. After incubation for 3 h for cell attachment, the medium and dead cells were removed. To the attached cells were added 1 ml of fresh medium supplemented with 10⁻⁹ M insulin, 10⁻⁹ M dexamethasone, and 30 mg/l kanamycin, and this mixture was incubated for 12 h to use in the experiment. After removal of the insulin, dexamethasone and kanamycin, followed by washing with WE medium, the cells were incubated with FP (25, 50, 100, 200 μM) in WE (total volume 4 ml) at 37 °C under 95% O₂–5% CO₂ for 3, 6, 12 and 24 h. The incubation was stopped by the addition of HCl. Metabolites were extracted with ether to determine the incubation conditions from the production of 4′-HOFP.

**Characterization of the Cytochrome P450s Involved in 4′-Hydroxylation of FP**

The reaction mixtures contained 0.1 m potassium phosphate buffer (pH 7.4), various inhibitors (0.1, 1, 10, 50, 100, 500, 1000 μM), an NADPH-generating system (0.33 mM β-NADPH, 8 mM G-6-P, 0.4 unit/ml G-6-P dehydrogenase, and 6 mM MgCl₂), and 250 μg microsomal protein in a final volume of 250 μl. The mixture was incubated at 37 °C for various times (0, 5, 10, 20, 40, 80 min), and the reaction was stopped by the addition of HCl. Metabolites were extracted with ether to determine the incubation conditions from the production of 4′-HOFP.

**Inhibition of FP Metabolism by Anti-cytochrome P450 Antibodies**

Inhibitory antisera against rat CYP2C11 and CYP2E1 (0, 10, 20, 50 μl) were incubated with rat hepatic microsomes (250 μg) at room temperature for 30 min, as suggested by the manufacturer, after dilution with goat serum to a final volume of 50 μl. To the mixture were added 0.1 mM EDTA, 200 μM FP, and a NADPH-generating system to a final total volume of 250 μl, followed by incubation at 37 °C for 20 min. The reaction was stopped with HCl and extracted.

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*Fig. 1. Biotransformation of Flurbiprofen Proposed by Risdall et al.*

* Denotes chiral center. When flurbiprofen (FP) was administered to rats and the metabolites were hydrolyzed with HCl, 4′-HOFP was in the urine (59.1%) and feces (14.9%), 3′,4′-DOFP: 8.0% and 52.4%, and 3′,4′-HMFP: 8.5% and 2.4%, and FP: 1.7% and 3.3%, respectively. Therefore, the sum % of their urinary and fecal metabolites was 74% (59.1 + 14.9) for 4′-HOFP and 71.3% (8.0 + 52.4 + 8.5 + 2.4%) for 3′,4′-DOFP and 3′,4′-HMFP. The ratios of 4′-HOFP to 3′,4′-DOFP and 3′,4′-HMFP were 74%: 71.3%, corresponding to about 1:1. In human urine, Risdall et al. found FP: 23.2%; 4′-HOFP: 14.9%, 4′-DOFP: 3.9%, 4′-methoxy-flurbiprofen, 3′,4′-HMFP: 24.6%, corresponding to a ratio of 1:1. 3′,4′-DOFP: 3′,4′-dihydroxy-flurbiprofen, 3′,4′-HMFP: 3′-hydroxy, 4′-methoxy-flurbiprofen.
with ether. The activities of cytochrome P450s were assayed using the peak area of 4'-HOFP in the ethereal metabolites on HPLC.

The anti-rat CYP2C11 serum (10 μl, 85.5 mg protein/ml) used has inhibitory activities of greater than 90% for CYP2C11 and about 15% for CYP2B2 involved in testosterone 16α-hydroxylation (the manufacturer's report). The anti-rat CYP2E1 serum (50 μl, 85.5 mg protein/ml) used has an inhibitory activity of 50% for CYP2E1 involved in aniline hydroxylation, according to the manufacturer's report.

**HPLC Analysis of 4'-HOFP**

The above incubation mixture was adjusted to be acidic with HCl and then hydrolyzed to non-glucuronides, according to the method by Risdall et al.5) To the mixture was added an internal standard, 10 μl of diclofenac (500 μM), and then ether (6 ml), and it was mixed for another 10 min. The ethereal material was dried under N2 gas and dissolved into a solution (200 it was mixed for another 10 min. The ethereal material was centrifuged at 600 g for 5 min to give a supernatant. The supernatant (30 μl) was subjected to HPLC analysis on a Shimadzu HPLC system Model CLASS-VP equipped with an LC-10AT pump, SPD-10AVP detector, SIL-10AXL autosampler, CTO10A open column CLASS-VP equipped with an LC-10AT pump, SPD-10A VP detector, SIL-10AXL autosampler, CTO10A open column and DGU-4A degasser. The following HPLC conditions were mainly used. The mobile phase was a solution of acetonitrile : triethylamine : acetic acid : water (50 : 0.65 : 15.0 mm I.D., Tosoh, Japan). The flow rate was 1 ml/min, and the column eluent was monitored with a UV detector at 280 nm.

**Activity of Aniline 4-Hydroxylase in the Microsomes from 24 h-Fasting Rats**

The microsomes from 24 h fasting rats were incubated with 2 mM aniline and 0.1 mM EDTA in 0.1 M phosphate buffer (pH 7.4), according to the known method.14) The mixture was incubated at 37 °C for 15 min and then an NADPH-generating system was added to adjust it to a total volume of 0.5 ml. The reaction mixture was incubated at 37 °C for 20 min, and the reaction was stopped by the addition of 0.5 ml of 10% trichloroacetic acid, then centrifuged at 600×g for 5 min. To the supernatant (0.75 ml) were added 10% Na2CO3 (0.5 ml) and 0.2× NaOH (0.75 ml) which included 2% phenol. The mixture was incubated at 37 °C for 30 min, and the activity of aniline 4-hydroxylase (the CYP2E1-dependent activity) was determined from the absorption at 630 nm compared with that from its standard curves.

**Analysis of Data**

The cytochrome P450 (CYP)-mediated activities in the presence of inhibitors are expressed as a percent of the corresponding control values, in the presence of methanol alone. The significance of the difference between the means of two treatment groups was evaluated by a two-tailed independent Student's t test. Statistical analyses were not performed on the data obtained from the microsomal pooled experiments. However, the microsomal experiments (n=5) were performed synchronistically.

**Spectrometric Examination of FP and 4'-HOFP**

1H-Nuclear magnetic resonance (1H-NMR) spectra were measured in CDCl3, with Me6Si as an internal standard, and chemical shifts, determined on a JEOL JNM-GX 270 Fourier transform NMR spectrometer, are given in δ value (ppm).

UV spectra were measured in methanol using a Shimadzu UV spectrometer (Model UV 2200).

Mass spectra (MS) were measured using a Hewlett Packard 580 SERIES-II gas chromatograph equipped with MSD 5971. The gas chromatography (GC) was carried out with a 1.5 m×0.25 mm i.D. and a 0.25 μm cross linked methylsilicone fused silica TC-1 (GL Sciences Inc., Tokyo, Japan) under the following condition: injection port temperature at 180 °C (splitless mode), helium as the carrier gas (4 psi head pressure) and oven temperature at 80 °C for 0.5 min following injection, then programmed to increase to 280 °C at a rate of 15 °C/min.

### RESULTS

**Transformation of FP to 4'-HOFP by the Rat Hepatocytes, and Identification of 4'-HOFP**

The hepatocytes were incubated with FP (25, 50, 100, 200 μM) in WE (total volume 4 ml) at 37 °C under 95% O2–5% CO2 for 3, 6, 12 and 24 h. The incubation mixture was hydrolyzed with HCl and extracted with ether. The ethereal materials were isolated by HPLC and gave a metabolite. The metabolite was identified at a retention-time (RT) of 3.3—3.4 min in dose-dependent and time-dependent manners, showing a proportional decrease by the additional dose-dependence of a nonspecific inhibitor, SKF-525A, accompanied by a decrease in FP at a RT of 7.2 min. The metabolite with RT 3.3—3.4 min was collected by repeated incubation experiments with FP (200 μM) for 24 h and its ultraviolet (UV) spectra showed an absorption maximum (λmax at 258 nm in methanol), similar to the λmax at 260 nm of 4'-HOFP reported by Risdall et al.5) but different from the UV spectra (λmax at 251 nm) of FP. The structure of the metabolite was inferred to be 4'-HOFP from the UV spectra, MS and 1H-NMR spectra compared with those of FP. The MS spectra of 4'-HOFP appeared at m/z 260 (parent ion: M+), m/z 245 (M+ – CH3) and m/z 232 (M+ – CO2). The 1H-NMR spectra (4'-HOFP) (δ CDCl3) showed signals at 1.56 ppm (CH3, δ, J = 7.17 Hz), 3.8 ppm (CH, δ, J = 7.17 Hz), 6.9 ppm (C2-H, δ), 7.14 ppm (C3-H, δ), 7.16 ppm (C4-H, δ), 7.35 ppm (C5-H, δ), and 7.43 ppm (C6-H, δ), but there was no signal for C7-H of FP. The NMR spectra of FP showed signals at 1.57 (CH3, δ, J = 7.35 Hz), 3.8 (CH, δ, J = 7.17 Hz), 7.15 (C3-H, δ), 7.18 (C4-H, δ), 7.35 (C5-H, δ), 7.36 (C6-H, δ), 7.44 (C7-H, δ), and 7.53 ppm (C8-H, δ).

The 4'-hydroxylation of FP by the hepatocytes was in reverse proportion to a decrease in FP level using at a dose of 200 μM until 24 h (Fig. 2). The levels of FP and 4'-HOFP were determined by relative intensities of the peak area at RT 7.2 and 3.3—3.4 min against that of the internal standard on HPLC, and represented the equivalent mole of FP.

**Characterization of the CYP Involved in the 4'-Hydroxylation of FP in the Liver Microsomes**

The in vitro 4'-hydroxylation of FP using rat liver microsomes was investigated in the presence of an NADPH-generating system. The activity of CYPs for 4'-hydroxylation was determined as an equivalent mole of FP by the relative intensity of the peak area at RT 3.3—3.4 min to that of the internal standard on HPLC.

The hydroxylation was increased in both a dose- and incubation-time dependent manner. The increasing curves...
showed linearity up to 200 μM FP and 20 min. After 20 min, the curves showed a very slow increase. Consequently, the effects of inhibitors on the 4′-hydroxylation of FP were investigated under this condition.

Nonenzymatic 4′-hydroxylation was not observed in the incubation medium. The hydroxylation activity was not detected without an NADPH-generating system and the reaction was not influenced when the incubation mixtures without an NADPH-generating system were preincubated at 37 °C for 15 min to denature flavin-containing monooxygenase (FMO). These results suggested the possibility that CYPs were involved in the 4′-hydroxylation in the liver microsomes with an NADPH-generating system.

The effects of CYP inhibitors on the 4′-hydroxylation were examined to estimate this possibility, and the results are shown in Fig. 3. The activity of CYPs for 4′-hydroxylation was determined as described above. The inhibitory activities were expressed as a ratio to the control activity without inhibitors. Inhibition was not observed by furafylline, a specific inhibitor of CYP1A2,15,16) up to 100 μM or by troleandomycin, a selective inhibitor of CYP3A, up to 100 μM.17) However, of other inhibitors at 100 μM, cimetidine decreased the hydroxylation to 82%, quinine to 75% and testosterone to 50%. Disulfiram at 1 and 10 μM decreased it to 85 and 27%, respectively.

4′-Hydroxylation of FP by the Microsomes from Fasting and Feeding Rats It is known3-10) that starvation and fasting induces the elevation of CYP 2E1 and a reduction of CYPs 1A2 and 2C11. Changes in the CYPs were examined. As shown in Table 1, the total CYP content decreased to 61.4% of the control at fasting and Feeding Rats, respectively. An inhibition of 4′-hydroxylation in the liver microsomes from fasting rats was observed using antibodies against rat CYP 2C11 and 2E1.

Fig. 2. Metabolism of Flurbiprofen (FP) by Rat Hepatocytes

The hepatocytes (1×10⁷) were incubated with 200 μM FP in WE at 37 °C under 95% O₂–5% CO₂ for 3, 6, 12 and 24 h. The incubation mixture was extracted with ether after being acidified with HCl. 4′-Hydroxy-flurbiprofen (4′-HOFP) in the ethereal materials was isolated by HPLC. The levels of FP and 4′-HOFP were determined by relative intensities of the peak at RT 7.2 and 3.3—3.4 min, respectively, against that of the internal standard on HPLC, and represented the equivalent mole of FP. FP: Δ; 4′-HOFP: □.

Table 1. Aniline 4-Hydroxylation at Feeding and Fasting

<table>
<thead>
<tr>
<th></th>
<th>Aniline 4-hydroxylase activity</th>
<th>Aniline 4-hydroxylase activity</th>
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<tbody>
<tr>
<td></td>
<td>P450 (nmol/mg)</td>
<td>Aniline 4-hydroxylase activity</td>
</tr>
<tr>
<td></td>
<td>(nmol/min/mg protein)</td>
<td>(nmol/min/mg protein)</td>
</tr>
<tr>
<td>Feeding</td>
<td>0.79</td>
<td>0.66</td>
</tr>
<tr>
<td>Fasting</td>
<td>0.69</td>
<td>0.73</td>
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</table>

Table 2. Michaelis–Menten Parameters for 4′-Hydroxy-flurbiprofen Formation by Male Rat Liver Microsomes

<table>
<thead>
<tr>
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<th>Kₘ (μM)</th>
<th>Vₘ₉ (nmol/min/mg protein)</th>
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<tr>
<td>Feeding</td>
<td>83.5</td>
<td>3.1</td>
</tr>
<tr>
<td>Fasting</td>
<td>61.4</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Microsomes from Fasting and Feeding Rats The results of inhibitors for CYP isoforms are shown in Fig. 4 and Table 3. The inhibitory activities were expressed as a ratio to the control activity without inhibitors: the transformation to 4′-HOFP underwent rather stronger inhibition by disulfiram in the fasting condition in comparison to feeding, weaker inhibition by SKF-525A in fasting against feeding, similar inhibitions by furafylline, quinine, testosterone and troleandomycin in fasting and feeding. The inhibition by cimetidine was not observed up to 1000 μM in fasting (Fig. 4), although cimetidine inhibited the hydroxylation at 100 μM in feeding about 18% (Fig. 3).

Effects of Inhibitory Antibodies of Rat CYPs 2C11 and 2E1 on the 4′-Hydroxylation by the Microsomes from Fasting and Feeding Rats To examine the effects of disulfiram for CYP2E1 and testosterone for CYP2C11, the inhibitory reaction was studied using antibodies against rat CYP2C11 and 2E1. The results are shown in Fig. 5, in which...
the anti-rat CYP2C11, but not the anti-CYP2E1, inhibited the 4'-hydroxylation in a strongly dose-dependent manner in fasting, but weakly in feeding. The anti-rat CYP2E1 increased levels of 4'-HOFP dose-dependently in both fasting and feeding, though it was higher with fasting. This suggested that the 4'-hydroxylation might be conducted mainly by CYP2C11, and that the anti-rat CYP2E1 might exhibit stronger inhibition to the further oxidation of 4'-HOFP during fasting in comparison to feeding.

Table 3. Values of Inhibitors on Metabolism of Flurbiprofen in Rat Microsomes

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>IC_{50} (µM)</th>
<th>Feeding</th>
<th>Fasting</th>
</tr>
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<tbody>
<tr>
<td>Furafylline</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td>Cimetidine</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td>Quinine</td>
<td>236.06</td>
<td>383.26</td>
<td></td>
</tr>
<tr>
<td>Disulfiram</td>
<td>3.68</td>
<td>1.41</td>
<td></td>
</tr>
<tr>
<td>Troleandomycin</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td></td>
</tr>
<tr>
<td>SKF-525A</td>
<td>34.52</td>
<td>54.8</td>
<td></td>
</tr>
<tr>
<td>Testosterone</td>
<td>207.83</td>
<td>&gt;1000</td>
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</table>
Volvement of CYPs 1A2, 2C8, 2E1, and 3A4. However, the hydroxylation of both enantiomers showed a lack of inversion seemed to occur in the animal models and in rat human. FP appeared to be negligible enantiomer than the some of the animal models after overnight fasting, it was necessary to determine the involvement of CYPs in the fasting and feeding was not well understood.

To identify the CYP isozymes involved in the 4'-hydroxylation of FP prior to determining the involvement of CYPs in the hydroxylation in fasting, the effect of specific inhibitors of CYPs on this reaction was examined (Fig. 3). The reaction was inhibited strongly and dose-dependently by disulfiram, SKF-525A and testosterone, and weakly by cimetidine and quinine, at 100 μM. Disulfiram is a specific inhibitor of CYP2E1,31 and SKF-525A is an inhibitor for the participation of P450 monooxygenase in the oxidation. Testosterone is hydroxylated at various locations by CYP2C11 for 2α- and 16α-orientations,24,25 CYP3A1/2 for 2β- and 6β-ones,26,27 CYP2A1 for 7α-ones28,29 and CYP2B1/2 for 16β-ones30 in rat hepatic microsomes. Cimetidine is an inhibitor for CYP2C6 and CYP2C11 but not for CYPs 1A1, 2B and 3A,30 and quinine is a selective inhibitor for CYP2D6, CYP2D131 and CYP3A2,31 but not for CYPs 2C11 and 2E1.32 Therefore, these results indicate that 4'-hydroxylation under the feeding condition was catalyzed by CYP2E1 and by another, probably CYP2C11, CYP2D and/or CYP3A, but not by CYP1A2.

4'-Hydroxylation during fasting was reduced when compared to that in feeding (Table 2). To determine why the hydroxylation was reduced by any CYPs in fasting, the activities of CYPs by inhibitors were compared during fasting and feeding (Fig. 4, Table 3). The 4'-hydroxylation in fasting was inhibited weakly by SKF-525A and testosterone against the feeding, and similarly by furafylline, quinine and troleandomycin, but strongly by disulfiram. Cimetidine did not inhibit at a dose of up to 1000 μM in fasting, in spite of inhibition at 100 μM in feeding, indicating that the hydroxylation in fasting was not carried out by CYP2C11, which mainly caused a decrease by fasting. Therefore, these inhibitory data suggested that the 4'-hydroxylation might be attributed primarily to CYP2E1, and to CYP2C11, 2A1, 2B1/2 and 3A1/2 concomitantly. The suggestion was expandable to prompt a hypothesis that the hydroxylation might be performed by the CYP induced by fasting. This hypothesis was supported by 1) reports that starvation and fasting induce an elevation of CYP2E1 and reductions of CYPs 2C11 and 1A2, and 2) the result that the total CYP content is reduced and CYP2E1-dependent activity (aniline-4-hydroxylase) is elevated by fasting (Table 1), although the liver weight decreased up to 60% by 24 h fasting, but was called into question by the result that the K_m and V_max for the 4'-hydroxylation were reduced by fasting (Table 2), suggesting that a decrease in the metabolism of FP to 4'-HOFP might be attributed to the involvement in CYP2C11 depleted by, and CYP2E1 induced by, fasting.

This indicates a discrepancy between two different phenomena: 4'-hydroxylation was inhibited by disulfiram, a specific inhibitor of CYP2E1,31 but the hydroxylation was seemingly more strongly inhibited by anti-rat CYP2E1 in fasting in comparison to feeding. The anti-rat CYP2C11 strongly inhibited the 4'-hydroxylation in fasting, and weakly in feeding, but the anti-rat CYP2E1 led to 2-fold increases in the levels of 4'-HOFP (Fig. 5). If the 2-fold increase was discussed on the assumption that FP was transformed first to 4'-HOFP and then to 3',4'-dioxygenated metabolites (3',4'-DOFP, 3',4'-HMFP) at the ratio 1:1 in the liver only, as described in the caption of Fig. 1, the 2-fold 4'-HOFP would

DISCUSSION

Fig. 5. Effects of the Antisera on the 4'-Hydroxylation of FP in the Microsomes of Feeding and Fasting Rats

Microsomes (250 μg) from the fasting or feeding rats were incubated with antibody (0, 10, 20, 50 μl) for 30 min at room temperature after dilution with goat serum to a final volume of 50 μl. To the mixture were added 0.1 mM EDTA, 200 μM FP, and a NADPH-generating system, to produce a final total volume of 250 μl, which was incubated at 37°C for 20 min. The cytochrome P450s were assayed as an equivalent mole of FP from the peak area at R 3.3—3.4 min on HPLC. The antibodies used were anti-rat CYP2C11 serum and anti-rat CYP2E1 serum. Control activities for 4'-hydroxylation of FP were 0.33 ± 0.05 and 0.21 ± 0.06 nmol/min/mg protein in the microsomes from the feeding and fasting rats, respectively. Activities by the anti-sera are expressed as a ratio relative to the respective control activity: (A) Effects of anti-rat CYP2C11 serum and (B) effects of anti-rat CYP2E1 serum.

FP is a chiral NSAID of the 2-arylpropionic acid class and behaves no difference in terms of metabolism, except for glucuronidation, between both R- and S-enantiomers. FP is extensively metabolized via hydroxylation and methylation, and is then eliminated as acetylglucuronide, all of which retain the chiral center. The major oxidative metabolites in human and rats are 4'-hydroxy-(4'-HOFP), 3',4'-dihydroxy-(3',4'-DOFP), and 3'-hydroxy-4'-methoxy-flurbiprofen (3',4'-HMFP) (Fig. 1). The glucuronidation was faster for the R-enantiomer than the S-form by liver microsomes of rats and human.18 FP appeared to be negligible R to S enantiomer inversion in human after oral administration.19 However, the inversion seemed to occur in the animal models20 and in rat hepatic microsomes in the presence of either CoASH and ATP or NADPH.21 The S-(+)-FP 4'-hydroxylation was determined with CYP 2C9 in human liver microsomes22 and the hydroxylation of both enantiomers showed a lack of involvement of CYPs 1A2, 2C8, 2E1, and 3A4.6 However, since the racemic drug was sometimes administered to experimental animals after overnight fasting, it was necessary to know whether the metabolism of FP was changed after the fasting, because of changes both in the composition of cytochrome P450s and in the metabolic activity of rat hepatic microsomes.7—10 The involvement of CYPs in the fasting and feeding was not well understood.
be conceivable in that 4'-HOFP might not undergo further oxidative metabolism by the anti-rat CYP2E1. Consequently, there remains a question of whether disulfiram was the specific inhibitor of CYP2E1 in this study. Martini et al.\(^1\) proposed that the inhibition by disulfiram resulted from it being a target for multiple CYPs, providing a basis for significant drug interaction involving CYPs other than CYP2E1, for example 2C11 and 3A2 with rat liver NADPH-supplemented microsomes. Therefore, the discrepancy might be resolved by the explanation that disulfiram was an inhibitor of CYP2C11 instead of CYP2E1. Starvation and fasting induced CYPs 2E1, 3A2, 2B and 4A, and reduced CYPs 2C11 and 1A2 in the metabolic activity of rat hepatic microsomes. 7\(^{-10}\),33\(^)\) Starvation increased the microsomal \(\alpha\) and \(\beta\) oxidation of fatty acids in rat hepatocytes 35,36\(^)\) and \(\omega\)-oxidation by CYP4A1 in the microsomes. 7\(^)\) Elevation of CYP 2E1 was involved in the 4\(-\)hydroxylation of fatty acids. 6,38\(^)\) These suggested the possibility of an effect of fasting on the metabolism of drugs and xenobiotics can be changed by the induction and/or reduction of CYPs. The CYP 2C11, which was reduced by fasting,\(^7\)^-\(^10\),33\(^)\) was involved in the 4\'-hydroxylation, according to the results of the CYP-inhibitory examinations, but the CYPs 3A and 2B induced by fasting\(^7\)^-\(^10\),33\(^)\) were not involved (Fig. 4). The CYP2E1 did not seem to be involved directly in the 4\'-hydroxylation, and 4\'-HOFP levels increased greater in fasting than in feeding by anti-rat CYP2E1. Taken together, these results suggested that the 4\'-hydroxylation might be conducted by CYP2C11 and CYPs other than CYP2E1 in fasting. There remains a question of whether CYP2E1 may contribute to the further oxidation of 4\'-HOFP from an increase in 4\'-HOFP level by the inhibition of CYP2E1 due to anti-rat CYP2E1 (Fig. 5).

In conclusion, the 4\'-hydroxylation of FP might be regulated directly by CYP2C11 and other CYPs, probably a CYP2D dependent activity for quinine, but not by CYP1A2 and CYP2E1, although CYP2C11 and CYP2E1 were reduced and induced by fasting, respectively. Therefore, the present study would be of value in judging the 4\'-hydroxylation of FP by CYPs, exhibiting its stronger pharmacological effects from a decrease in the oxidative metabolism of FP by CYP2C11 on administration after overnight fasting.

Further studies are needed to determine whether CYP2E1 contributes to further oxidation of 4\'-HOFP.

REFERENCES