Oxidation of celecoxib by polymorphic cytochrome P450 2C9 and alcohol dehydrogenase

Mia Sandberg,[1] Ümit Yasar,[1] Patrik Strömberg,[2] Jan-Olov Höög[2] & Erik Eliasson[1]

[1]*Division of Clinical Pharmacology, Huddinge University Hospital, Karolinska Institutet, Stockholm and* [2]*Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden*

Aims Celecoxib is a novel selective cyclooxygenase-2 inhibitor, which is subject to extensive hepatic metabolism. The aims of the present *in vitro* investigation were 1) to compare the rate of celecoxib hydroxylation by different genetic variants of cytochrome P450 2C9 (CYP2C9), and 2) to identify the enzyme(s) involved in the formation of the major metabolite carboxycelecoxib.

Methods Hydroxycelecoxib formation was studied in human liver microsomes from 35 genotyped livers, as well as in yeast microsomes with recombinant expression of different P450 variants. Carboxycelecoxib formation was studied in liver microsomes incubated in the absence or presence of liver cytosol. The metabolites were identified and quantified by h.p.l.c. In addition, hydroxycelecoxib oxidation by different variants of recombinant human alcohol dehydrogenase (ADH1–3) was analysed by spectrophotometric monitoring of NADH generation from NAD^+.

Results The intrinsic clearance of celecoxib hydroxylation was significantly lower for yeast-expressed CYP2C9.3 (0.14 ml min^{-1} $nmol^{-1}$ enzyme) compared with CYP2C9.1 (0.44 ml min^{-1} $nmol^{-1}$ enzyme). In human liver microsomes, a significant 2-fold decrease in the rate of hydroxycelecoxib formation was evident in *CYP2C9*1/*3* samples compared with *CYP2C9*1/*1* samples. There was also a marked reduction (up to 5.3 times) of hydroxycelecoxib formation in a liver sample genotyped as *CYP2C9*3/*3*. However, the *CYP2C9*2* samples did not differ significantly from *CYP2C9*1* in any of the systems studied. Inhibition experiments with sulphaphenazole (SPZ) or triacetyloleandomycin indicated that celecoxib hydroxylation in human liver microsomes was mainly dependent on CYP2C9 and not CYP3A4. The further oxidation of hydroxycelecoxib to carboxycelecoxib was completely dependent on liver cytosol and NAD^+. Additional experiments showed that ADH1 and ADH2 catalysed this reaction *in vitro* with apparent K_m values of 42 μM and 10 μM, respectively, whereas ADH3 showed no activity.

Conclusions The results confirm that CYP2C9 is the major enzyme for celecoxib hydroxylation *in vitro* and further indicate that the *CYP2C9*3* allelic variant is associated with markedly slower metabolism. Furthermore, it was shown for the first time that carboxycelecoxib formation is dependent on cytosolic alcohol dehydrogenase, presumably ADH1 and/or ADH2.

Keywords: cytochrome P450, genetic polymorphism, COX-2-inhibitors, drug metabolism, adverse drug reactions, alcohol dehydrogenase

Correspondence: Erik Eliasson, MD, PhD, Karolinska Institutet, Department of Medical Laboratory Sciences and Technology, Division of Clinical Pharmacology, Huddinge University Hospital, SE-141 86 Stockholm, Sweden. Fax: + 468 58581070; Tel: + 468 58587899; E-mail: erik.eliasson@labtek.ki.se

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Introduction

Most nonsteroidal anti-inflammatory drugs (NSAIDs) inhibit cyclooxygenases (COX). These enzymes appear in at least two distinct forms [1], where COX-2, in contrast to COX-1, is inducible and involved in the formation of prostanoids produced during pain, inflammation and

fever. It is believed that common gastrointestinal side-effects associated with NSAIDs are explained by inhibition of COX-1, a constitutively expressed enzyme that generates prostaglandins involved in gastric cytoprotection [2].

Celecoxib is a potent selective COX-2 inhibitor used in the treatment of rheumatoid and ostheoarthritis [3]. Celecoxib is metabolized in the liver by methyl hydroxylation, followed by further oxidation to carboxycelecoxib, which is the major metabolite found in blood. Carboxycelecoxib is subject to glucuronic acid conjugation followed by bile excretion [4]. It was recently reported that cytochrome P450 2C9 (CYP2C9) appears to be the major P450 enzyme involved in the initial methyl hydroxylation of celecoxib [5].

CYP2C9 is a genetically polymorphic cytochrome P450 enzyme that is responsible for the metabolism of many widely used drugs, such as warfarin, tolbutamide, phenytoin, losartan and many NSAIDs [6]. At present, six different allelic variants have been reported (http://www.imm.ki.se/CYPalleles). In Caucasians, the frequency of the two most important allele variants, *CYP2C9*2* and *CYP2C9*3*, has ranges 8–12.5% and 3–8.5%, respectively, but lower frequencies have been reported for other ethnic groups [6, 7]. The different alleles code for enzymes with different amino acid composition, and the functional significance of this polymorphism has been studied both *in vitro* and *in vivo*. For several CYP2C9 substrates, metabolism by the CYP2C9.3 enzyme variant is markedly reduced compared to the enzyme encoded by *CYP2C9*1* [6, 8, 9]. This has clinical implications for CYP2C9 drugs with narrow therapeutic windows, such as warfarin and phenytoin, where drug accumulation due to slow metabolism will increase the risk of severe adverse reactions [10–12]. The primary objective of the present study was to assess the impact of CYP2C9 polymorphism on celecoxib oxidation *in vitro*, and to characterize further the enzymatic steps involved in the formation of carboxycelecoxib.

Methods

Chemicals

Celecoxib, hydroxycelecoxib (OH-celecoxib) and carboxycelecoxib (COOH-celecoxib) were kindly provided by Searle (St Louis, MO, USA). Triacetyloleandomycin (TAO) and sulphaphenazole (SPZ) as well as NADPH, $NADP^+$, NAD^+, glucose-6-phosphate and glucose-6-phosphate dehydrogenase were obtained from Sigma. Acetonitrile, methanol, ortho-phosphoric acid and potassium phosphate were purchased from Merck.

Preparation of yeast microsomes

Yeast microsomes expressing either CYP2C9.1, CYP2C9.2, or CYP2C9.3 originated from previous site directed mutagenesis experiments where variant cDNAs were cloned into a pYeDP60 (V60) yeast expression vector, followed by expression in the *Saccaromyces cerevisiae* strain W(R) that overexpresses yeast reductase. Yeast microsomes were prepared and evaluated for P450 expression as described [9]. All different yeast preparations expressed similar levels of P450 apoprotein and spectrally determined cytochrome P450 holoenzyme (148, 141, 114 and 130 pmol of cytochrome P450 mg^{-1} protein for CYP2C9.1, CYP2C9.2, CYP2C9.3 and CYP2C19.1, respectively), as well as cytochrome P450 reductase [9]. Two different preparations of yeast microsomes were used in the experiments.

Preparation of human liver microsomes

Liver microsomes were prepared essentially as described [13] from 35 different human livers belonging to a donor liver biobank established at the Division of Clinical Pharmacology, Huddinge University Hospital, after approval from the hospital Ethics Committee. Each liver was genotyped for the *CYP2C9*1, CYP2C9*2* and *CYP2C9*3* alleles [7]. During preparation of microsomes, the cytosolic protein fraction was isolated as the supernatant resulting from the first ultracentrifugation. This supernatant was mixed with glycerol (final concentration 13.7 mM), sucrose (280 mM), dithiothreitol (2.25 mM) and EDTA (0.1 mM) before storage at −70 °C. The microsomal pellet was washed, again ultracentrifuged and finally resuspended in potassium phosphate buffer (50 mM), pH 7.4, with storage at −70 °C.

Expression and purification of ADHs

Human ADH1C2 (previously class I, $\gamma_2\gamma_2$), ADH2 (class II, $\pi\pi$) [14] and ADH3 (class III, $\chi\chi$) [15] were expressed in *E. coli* and purified in two steps with DEAE cellulose (Whatman) and either AMP sepharose or blue sepharose (Amersham Biosciences), as described earlier [16]. Proteins were concentrated on 30K cut-off filters in either centrifugal cells (centriplus YM-30, Millipore) or in a stirred ultrafiltration cell (Amicon/Millipore). Purity was analysed by SDS/polyacrylamide gel electrophoresis and protein concentrations were determined with the Bio-Rad protein assay, standardized with bovine serum albumin.

Celecoxib metabolism

The kinetic constants (V_{max} and K_m) of OH-celecoxib formation were determined in both human liver

Table 1 V_{max} (nmol nmol^{-1} enzyme min^{-1}), apparent K_m (μM) and intrinsic clearance (V_{max}/K_m, ml min^{-1} nmol^{-1} enzyme) for *CYP2C9*1, CYP2C9*2* and *CYP2C9*3* expressed in two different preparations of yeast. Number of independent experiments = 3, data expressed as mean ±s.d., $^{\S}P < 0.05$, compared with 2C9.1, $\#P < 0.05$, compared with 2C9.2

CYP form	V_{max}	K_m	V_{max}/K_m
CYP2C9*1	2.1 ± 0.38	5.1 ± 1.4	0.44 ± 0.18
CYP2C9*2	2.1 ± 0.32	5.9 ± 1.5	0.38 ± 0.16
CYP2C9*3	1.2 ± 0.50§#	11 ± 5.4	0.14 ± 0.12§

microsomes and yeast microsomes using celecoxib concentrations ranging from 0.05 μM to 100 μM. In subsequent experiments, the rate of OH-celecoxib formation was compared in human liver microsomes with different *CYP2C9* genotypes, using either 1 or 20 μM of celecoxib. These concentrations were chosen according to the K_m values obtained in yeast (Table 1) and also considering the relevant *in vivo* concentrations of celecoxib, i.e. around 1 μM. Moreover, the apparent K_m in a sample of human liver microsomes, genotyped as *CYP2C9*1/*1* was found to be very similar to the yeast system (7 μM), reaching saturation at 20 μM.

Microsomes, corresponding to 0.8 mg of protein in 50 mM potassium phosphate buffer, pH 7.4, were preincubated with celecoxib for 4 min at 37 °C in a shaking water bath. Reactions were started by the addition of NADPH (0.5 mM) and incubation time was 10 min. All incubations were carried out in the linear range of product formation, with respect to both time and protein concentration. The volume of the final incubation medium was 0.5 ml. Celecoxib was dissolved in pure acetonitrile and the final concentration of solvent in the incubation was 0.25% (v/v). This concentration of acetonitrile was found to inhibit the formation of OH-celecoxib by no more than 10 %. Pure acetonitrile (250 μl) was added to stop the reactions and the samples were immediately put on ice. Samples were centrifuged at 14 000 *g* for 5 min and the resulting supernatant was subjected to h.p.l.c. analysis. All experiments were performed in duplicate.

In order to assess the role of CYP2C9 and CYP3A4 in celecoxib hydroxylation, samples were pretreated with either sulphaphenazole (SPZ, 10 μM; [18, 19]) or triacetyloleandomycin (TAO, 50 μM; [20]). TAO and SPZ were both dissolved in methanol, which was evaporated to dryness before addition of the incubation mixture. The samples were preincubated with NADPH (0.5 mM) for 4 min at 37 °C in a shaking water bath, followed by the addition of celecoxib. The remaining part of the procedure was performed as described above for the samples without inhibitors.

Further oxidation of OH-celecoxib to COOH-celecoxib was investigated under experimental conditions similar to those described above, with the following modifications. Microsomal samples were incubated in the presence or absence of cytosol (3 mg of protein), or in samples with cytosol only, and by inclusion of either celecoxib (20 μM) or OH-celecoxib (1–500 μM) as substrate. An NADPH-regenerating system (0.5 mM $NADP^+$, 4.6 mg ml^{-1} glucose-6-phosphate (G6P), 40 U ml^{-1} glucose-6-phosphate dehydrogenase) was used for incubations with celecoxib as longer incubation times were required. The dependence of COOH-celecoxib formation on the cofactor NAD^+ (0.5 mM) was also evaluated.

H.p.l.c. analysis

Separation of OH-celecoxib and COOH-celecoxib was achieved on a Zorbax SP-phenyl column (250 × 4.6 mm) attached to a precolumn, using a mobile phase of acetonitrile/0.05 mM phosphoric acid, pH 2.5 (44/56, v/v) with a flow rate of 1.7 ml min^{-1} [5]. COOH-celecoxib, OH-celecoxib and celecoxib eluted at 13.5, 15.5 and 19.4 min, respectively, in a total run time of 23 min. A u.v. detector (Gilson 118, WI, USA) with the wavelength set at 254 nm, was used. A standard curve between 0.025 and 2 μM was established. The coefficient of variation (CV) for the analysis of OH-celecoxib was 6.6% at 1.0 μM. The lower limit of quantification for OH-celecoxib was 250 nM, as determined from our standard curve, covering the range from 20 nM to 5 μM of OH-celecoxib.

ADH enzyme assays

Activity was determined with a Hitachi U-3000 spectrophotometer by monitoring the production of NADH, i.e. the formation of aldehyde metabolite, at 340 nm using a NADH absorption coefficient (ε) of 6220 M^{-1} cm^{-1}. Steady-state enzyme kinetics were performed at 25 °C in 0.1 M glycine/NaOH, pH 10.0 or 0.1 M sodium phosphate, pH 7.5 with 2.4 mM NAD^+ [14]. The protein concentration was 0.87 mg ml^{-1}. OH-celecoxib (in a final concentration range of 1.56 μM to 100 μM) was dissolved in acetonitrile giving a final solvent concentration of 2% in the photometric cell. A weighted nonlinear-regression analysis program (SigmaPlot 2001 for Windows; SPSS) was used to calculate the kinetic parameters for the ADH assays. The turnover numbers (k_{cat}) were calculated per subunit (40 kDa) and standard errors were less than 10% for k_{cat} values and less than 15% for apparent K_m values.

Statistical analysis

One-way ANOVA test was applied to all groups and when $P \leq 0.05$ the Student's *t*-test was performed, comparing the formation rates of OH-celecoxib between the different genotype groups. Significance was assumed when $P < 0.05$. The power of the study was calculated using dstplan 4.2, 2000, Brown B.W., Houston, TX (http://odin.mdass.tmc.edu/anonftp/page_2.html(DSTPLAN). Apparent K_m and V_{max} values for CYP-mediated metabolism were obtained by applying Michaelis-Menten kinetic model, using GraphPad Prism 3 [5].

Inhibition of OH-celecoxib formation by TAO and SPZ were expressed as a percent age of the formation rate obtained in control samples without inhibitor.

Results

In initial experiments, the kinetics of celecoxib hydroxylation were compared in the different variants of CYP2C9 expressed in yeast, over a substrate concentration range of 0.05 to 100 μM. Intrinsic clearance (V_{max}/K_m) was 3.1 times lower for CYP2C9.3 compared with CYP2C9.1, resulting from an approximately equal effect on both apparent K_m and V_{max} (Table 1). Moreover, the reduction in V_{max} was significant comparing CYP2C9.3 to either CYP2C9.1 or CYP2C9.2. However, no significant difference between CYP2C9.1 and CYP2C9.2 was found. The hydroxylation of celecoxib by a structurally related forms of cytochrome P450, CYP2C19.1, was also studied in yeast microsomes. It was found that intrinsic clearance by CYP2C19.1 was 5.4-fold lower than by CYP2C9.1 and this was mainly due to a higher apparent K_m (39 ± 11 μM) for CYP2C19.1.

The formation rate of OH-celecoxib was compared in 35 different samples of genotyped human liver microsomes (Table 2) using either 1 or 20 μM of celecoxib (see Methods). In the only liver sample expressing *CYP2C9*3/*3*, the formation rate of OH-celecoxib was 5.3 times lower than in *CYP2C9*1/*1* liver samples ($n = 14$) at 1 μM celecoxib, and 3.3 times lower at 20 μM. A significant decrease in the rate of celecoxib hydroxylation was found between *CYP2C9*1/*1* and *CYP2C9*1/*3* liver samples. In *CYP2C9*3* heterozygous samples, hydroxylation was 2.2 and 1.8 times lower at 1 and 20 μM celecoxib, respectively. The statistical power of this comparison was 99%. There was no significant difference in the hydroxylation rate between the samples expressing one or two *CYP2C9*2* alleles and those genotyped as *CYP2C9*1/*1*. Apoprotein and spectrally determined P450 contents were not significantly different in the various genotype groups (Table 2).

Pre-treatment of human liver microsomes with the CYP2C9-specific inhibitor sulphaphenazole (SPZ) caused a relatively pronounced inhibition of celecoxib hydroxylation at both 1 and 20 μM of substrate (mean (± s.d.) decrease 68% ± 14 and 60% ± 19, respectively) whereas pretreatment with triacetyloleandomycin (TAO), a CYP3A4-specific inhibitor, inhibited celecoxib hydroxylation to a smaller extent (24% ± 16 and 22% ± 13, respectively). SPZ-inhibition of celecoxib hydroxylation was markedly lower in the only liver sample homozygous for *CYP2C9*3* (mean inhibition of 25%), whereas the inhibition by TAO was in the same range as for other microsomal samples. A possible differential effect of SPZ on the activities of the different CYP2C9 enzyme variants was tested in yeast microsomes expressing either *CYP2C9*1*, **2*, or **3*. However, no difference regarding the extent of SPZ inhibition (ranging from 60% to 82% for the three different variants) of celecoxib hydroxylation was evident.

In order to study the formation of carboxycelecoxib, microsomal incubations were initially carried out using OH-celecoxib as substrate. Since no COOH-celecoxib formation was detected, even at high concentrations of OH-celecoxib (up to 500 μM), liver cytosol was added to the incubation medium. This caused a marked increase in the rate of COOH-celecoxib formation, and it was

Table 2 The formation rate (nmol min^{-1} mg^{-1} protein) of OH-celecoxib at 1 and 20 μM celecoxib concentration in human liver microsomes, as well as total spectral P450 (pmol mg^{-1} protein) and apoprotein content in arbitrary units. n = number of individual liver samples, data expressed as mean ± s.d., $^{\S}P < 0.05$ compared with 2C9*1/*1, $\#P < 0.01$ compared with 2C9*1/*2.

		Formation rate of OH-celecoxib			
Genotype	n	*1 μM celecoxib*	*20 μM celecoxib*	*Total P 450*	*CYP2C9 apoprotein*
*CYP2C9*1/*1*	14	0.033 ± 0.012	0.26 ± 0.089	399 ± 201	7.2 ± 4.6
*CYP2C9*1/*2*	6	0.030 ± 0.015	0.24 ± 0.070	454 ± 99	6.9 ± 3.3
*CYP2C9*1/*3*	8	0.0150 ± 0065§#	0.15 ± 0.14§	255 ± 105	4.8 ± 2.5
*CYP2C9*2/*3*	2	0.037; 0.016	0.30; 0.18	594 ± 71	6.3; 5.3
*CYP2C9*2/*2*	4	0.021 ± 0.011	0.16 ± 0.12	515 ± 356	4.1 ± 0.7
*CYP2C9*3/*3*	1	0.0061	0.079	516	3.1

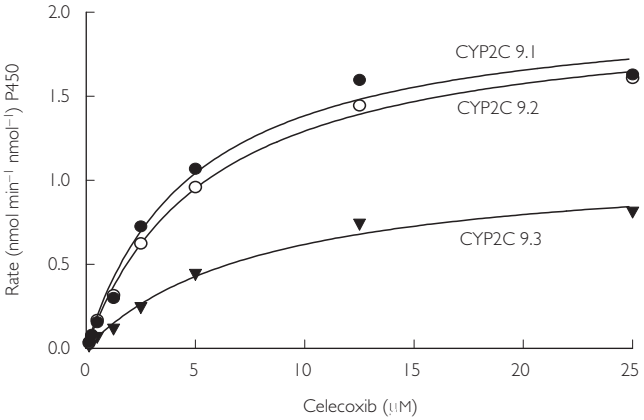

Figure 1 Kinetic plots for the formation of OH-celecoxib from celecoxib in yeast expressing CYP2C9.1, CYP2C9.2 and CYP2C9.3. Data presented as mean of three different incubations for each variant.

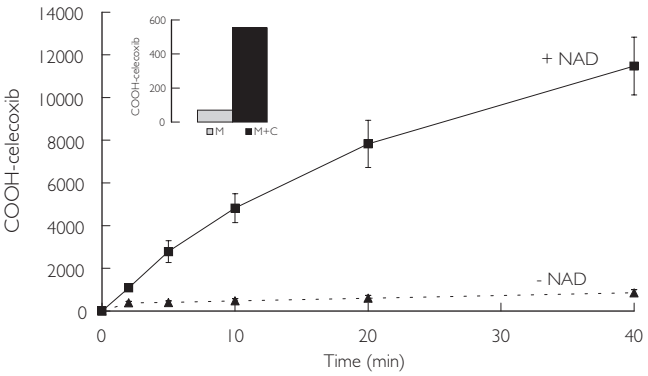

Figure 2 Formation of COOH-celecoxib (pmol min^{-1} mg^{-1} protein) from OH-celecoxib (500 μM) in human liver cytosol alone and in the presence (■) or absence (▲) of NAD^+. Data are presented as mean ± range of two different cytosol preparations. Inserted bar diagram shows the formation of COOH-celecoxib (pmol min^{-1} mg^{-1} protein) in microsomes after 120 min in the absence or presence of cytosol.

evident that the oxidation of OH-celecoxib to COOH-celecoxib only required cytosol together with NAD^+ as cofactor (see Figure 2). The formation of COOH-celecoxib from celecoxib (20 μM) was also studied. COOH-celecoxib was not detected when celecoxib was incubated with cytosol alone. Furthermore, almost no COOH-celecoxib formation was observed in incubations where only microsomes were present. In agreement with the results from experiments with OH-celecoxib, oxidation of celecoxib to COOH-celecoxib was clearly dependent on the addition of cytosol to microsomes (see insert in Figure 1). In samples containing both microsomes and cytosol, a considerable lag time (about 30 min) was evident in the formation of the carboxy-metabolite compared with that of OH-celecoxib (data not shown).

Testing the hypothesis that ADH was the cytosolic enzyme responsible for oxidation of OH-celecoxib, purified enzymes (ADH1C2, ADH2 and ADH3) were used for further kinetic analysis. No activity was observed for human ADH3, whereas OH-celecoxib was readily oxidized by both ADH1 and ADH2 (Figure 3). Under standard alkaline ADH assay conditions (see Methods), the apparent K_m value obtained for ADH2 was 10 μM and for ADH1 42 μM. The turnover numbers (k_{cat}) also differed between the two enzyme variants and were lower for ADH1 (9 min^{-1}) as compared with ADH2 (25 min^{-1}). This resulted in a catalytic efficiency (k_{cat}/K_m) of 2.4 min^{-1} μM^{-1} and 0.21 min^{-1} μM^{-1} for ADH2 and ADH1, respectively. However, lowering of the pH to 7.5 decreased the velocity rate 3 times for ADH1 and 6 times for ADH2 when measuring the oxidation of OH-celecoxib at 100 μM.

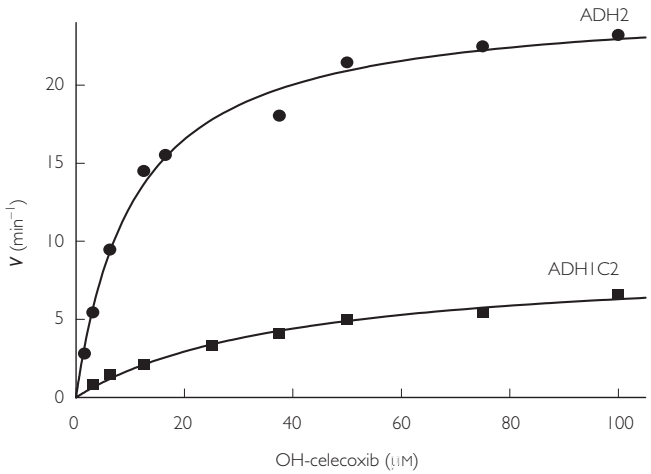

Figure 3 Kinetics of OH-celecoxib oxidation by human ADH1C2 and ADH2. The reactions were performed using *E. coli*-expressed enzymes under standard alkaline conditions at 25 °C in the presence of NAD^+ (see Methods).

Discussion

The rate of hydroxylation of celecoxib by CYP2C9.3 was clearly lower than that by CYP2C9.1 and CYP2C9.2. This was also evident in a relatively large sample of livers from heterozygous *CYP2C9*1/*3*-individuals. Tang *et al.* recently reported similar results, but our experiments showed a greater difference in apparent K_m between CYP2C9.1 and CYP2C9.3, and could not confirm a lower intrinsic clearance reported previously for CYP2C9.2 [21]. These discrepancies might relate to the different expression systems used for the

kinetic analysis. Our data confirm that CYP2C9 is the main catalyst of celecoxib hydroxylation [5]. Inhibition by the selective CYP3A4-inhbitor TAO was clearly less than that by SPZ, which selectively inhibits CYP2C9. The latter was observed in all livers tested and was not dependent on *CYP2C9* genotype. If CYP3A4 was involved in the hydroxylation of celecoxib to any major extent, greater inhibition by TAO in the liver samples homo- or heterozygous for *CYP2C9***3* would be expected. However, a possible contribution by additional CYP enzymes is clearly possible, as indicated by the lack of complete inhibition (100%) by TAO and SPZ. Another candidate would be CYP2C19, even though it has a higher apparent K_m for this reaction than CYP2C9 (Table 1). CYP2C8, which has a high sequence homology with CYP2C9, presumably has no major involvement because of no obvious correlation between celecoxib and taxol, a selective CYP2C8 substrate, metabolism [5].

The two-step oxidation of celecoxib to COOH-celecoxib was clearly dependent on cytosol and NAD^+. The results strongly indicate that after an initial CYP2C9-dependent hydroxylation, cytosolic enzyme(s) are responsible for further metabolism. We could not find any major differences in the rate of oxidation of OH-celecoxib to COOH-celecoxib using cytosols from different liver samples. However, under conditions where celecoxib was incubated with a mixture of microsomes and cytosol, COOH-celecoxib formation seemed to correlate with *CYP2C9* genotype and the formation rate of OH-celecoxib ($r = 0.79$).

The results from experiments with liver cytosol indicated that ADH was involved in the formation of COOH-celecoxib. This was confirmed using recombinantly expressed ADH1 and ADH2 enzymes (Figure 3). The latter showed a higher catalytic efficiency, but since ADH1 is expressed at higher levels in the liver than ADH2, it is difficult to predict which one is more important *in vivo*. The enzymology becomes even more complex considering that there are different isoforms of ADH1, and also genetic polymorphisms of both ADH1 and ADH2 [22. 23]. The enzyme involved in the ultimate oxidation of the ADH product to COOH-celecoxib has not yet been identified, but could be aldehyde dehydrogenase. Such a mechanism is similar to that described for tolbutamide, another CYP2C9 substrate [24].

Our findings are of potential clinical importance. Since celecoxib specifically inhibits COX-2 ($IC_{50} = 0.04$ μM for COX-2, compared with $IC_{50} = 15$ μM for COX-1) [25], an increase in its plasma concentration in patients expressing *CYP2C9***3* allelic variants might be expected. However, it seems possible that other CYP enzymes may partially compensate for reduced CYP2C9 activity. Polymorphisms in ADH could further contribute to variable concentrations of celecoxib metabolites [22, 23]. Clinical studies are needed to clarify a possible relationship between celecoxib-induced adverse reactions and the polymorphic enzymes involved in its metabolism.

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