Regioselective O-Demethylation of Scoparone: Differentiation between Rat Liver Cytochrome P-450 Isozymes

Dieter Müller-Enoch, Eva Büttgen, and Antje Nonnenmacher

Abteilung Physiologische Chemie, Universität Ulm, Oberer Eselsberg, D-7900 Ulm, Bundesrepublik Deutschland

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The ratios of the scoparone O-demethylation products scopoletin to isoscopoletin were determined for reconstituted complexes of NADPH-P-450 reductase and each of four P-450 isozymes in a 2:1 molar ratio with a 1:1 mixture of [7-O-methyl-^{14}C]- and [6-O-methyl-^{14}C]-scoparone as substrate. The two phenobarbital inducible forms P-450$_{PB\text{-}B}$ and P-450$_{PB\text{-}D}$ have a 1:0.8 ± 0.05 scopoletin to isoscopoletin ratio, and the two β-naphthoflavone inducible forms P-450$_{\beta NF\text{-}B}$ and P-450$_{\beta NF/ISF\text{-}G}$ have ratios of 1:4.4 ± 0.1 and 1:3.8 ± 0.1, respectively. The scoparone-O-demethylation activities of the reconstituted preformed complexes of the four P-450 isozymes are given.

Introduction

The regioselective O-demethylation of 6,7-dimethoxycoumarin (scoparone) to 6-hydroxy-7-methoxycoumarin (isoscopoletin) and 7-hydroxy-6-methoxycoumarin (scopoletin) has been reported to vary significantly with the state of induction of the cytochrome P-450 dependent monooxygenase system in rat liver microsomes [1] and in living mice [2].

In this study we used purified rat liver P-450 isozymes to show that the reported alteration of the scopoletin:isoscopoletin ratio and the increase in the scoparone O-demethylation activity [1] are due to a change in the P-450 isozyme pattern, mediated by an induction of the phenobarbital- or polycyclic hydrocarbon-inducible P-450 isozymes.

Materials and Methods

Materials

7-Hydroxy-6-methoxycoumarin (scopoletin) and 6-hydroxy-7-methoxycoumarin (isoscopoletin) were purchased from C. Roth (Karlsruhe, Germany). 6,7-Dimethoxycoumarin (scoparone), [7-O-methyl-^{14}C]6,7-dimethoxycoumarin (spec. act. 51.1 μCi/mmol) and [6-O-methyl-^{14}C]6,7-dimethoxycoumarin (spec. act. 51.1 μCi/mmol) were prepared as described previously [3]. All materials used for the purification procedure of the P-450 isozymes and the NADPH-cytochrome-P-450 reductase used in this study are described by Guengerich *et al.* [4].

Abbreviations: P-450, liver microsomal cytochrome P-450; naming of individual P-450 isozymes is adapted from Guengerich *et al.* [4]; PB, phenobarbital; βNF, β-naphthoflavone (5,6-benzoflavone); ISF, isosafrole; 3-MC, 3-methylcholanthrene.

Reprint requests to Dr. Müller-Enoch.

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Purification of enzymes

Liver P-450$_{PB\text{-}B}$ (apparent monomeric M_r = 50,000), P-450$_{PB\text{-}D}$ (M_r = 50,000), and NADPH-cytochrome P-450 reductase (M_r = 74,000) were purified to electrophoretic homogeneity from phenobarbital-treated male Sprague-Dawley rats using procedures described by Guengerich *et al.* [4]. The same reference describes the purification of the P-450$_{\beta NF\text{-}B}$ (M_r = 54,000) and the P-450$_{\beta NF/ISF\text{-}G}$ (M_r = 51,000) from β-naphthoflavone-treated rats.

General assays

Protein concentration were estimated using the general method of Lowry *et al.* [5]. The cytochrome P-450 content was assayed according to Omura and Sato [6]. The NADPH-cytochrome P-450 reductase activity was determined according to Yasukochi and Masters [7]. The reconstitution of the P-450 monooxygenase systems consisting of one of the four P-450 isozymes (P-450$_{PB\text{-}B}$, P-450$_{PB\text{-}D}$, P-450$_{\beta NF\text{-}B}$ and P-450$_{\beta NF/ISF\text{-}G}$) with the NADPH-cytochrome P-450 reductase was performed as described by Müller-Enoch *et al.* [8]: The formation of the catalytically active P-450:NADPH-P-450 reductase complexes of each of the four P-450 isozymes were made by preincubating the P-450 isozymes and the reductase at concentrations of 6 and 12 μM, respectively, in small test tubes in final volumes of 50 μl of 0.1 M Tris-HCl

buffer (pH 7.6) including 20% glycerol for 1 h at 23 °C. Following these preincubations, aliquots of these preformed P-450: NADPH-P-450 reductase complexes were added to test tubes containing substrate and NADPH to start the reactions.

The ratios of the scoparone O-demethylation products scopoletin: isoscopoletin were estimated with aliquots (100 µl) of the preformed complexes. The assay media (total volume of 300 µl of 0.1 M Tris-HCl buffer (pH 7.6) including 20% glycerol) contained 0.1 µmol of the two radio isomers of [6-O-methyl-^{14}C]- and [7-O-methyl-^{14}C]-scoparone ($1.12 \cdot 10^4$ dpm), 3 µmol $MgCl_2$, and 0.4 µmol NADPH. The reaction was started by adding the preformed complexes and performed at 35 °C for 10 min. The analysis and the ratios of the radioactive demethylation products scopoletin to isoscopoletin were performed as previously described [1].

The scoparona O-demethylation activities of the preformed complexes of NADPH-P-450 reductase and the various P-450 isozymes were estimated according to Müller-Enoch *et al.* [1] using the continuous fluorimetric assay. In all cases the initial rate (1–3 min) of the scopoletin formation at 35 °C was followed after starting the reaction by adding aliquots (10 µl) of the preformed complexes. The reaction mixtures (total volume of 200 µl of 0.1 M Tris-HCl buffer (pH 7.6) including 20% (v/v) glycerol) contained 80 nmol scoparone, 1 µmol $MgCl_2$, 0.1 µmol NADPH and 0.060 nmol P-450 in the preformed complexes.

Results and Discussion

Preformed complexes of various purified P-450 isozymes with NADPH-P-450 reductase are reported by Müller-Enoch *et al.* [8] to have maximum P-450 supported monooxygenase activities in the absence of phospholipids, when the P-450:NADPH-P-450 reductase complex was prepared by preincubation of the enzymes at 23 °C for 60 min at a molar ratio of P-450 to reductase of 1:2, respectively, with a P-450 concentration greater than 5 µM.

Aliquots of these preformed complexes were utilized to estimate the ratio of the scoparone O-demethylation products scopoletin : isoscopoletin for various purified P-450 isozymes. The results, presented in Table I, show that the ratio differs significantly with the cytochrome P-450 isozymes. That of the two phenobarbital-inducible forms P-450$_{PB-B}$ and P-450$_{PB-D}$ is about 4–5 times higher than that obtained from the two β-naphtho-flavone-inducible forms P-450$_{\beta NF-B}$ and P-450$_{\beta NF/ISF-G}$. Thus the PB-inducible P-450 isozymes favor the 7-O-demethylation to produce more scopoletin than isoscopoletin, whereas the βNF-inducible P-450 isozymes is selective for the 6-O-demethylation to form 4-times more isoscopoletin than scopoletin.

Table I. Ratios of the scoparone O-demethylation products scopoletin : isoscopoletin obtained with reconstituted complexes of NADPH-P-450 reductase and various P-450 isozymes in a 2:1 molar ratio with a 1:1 mixture of [7-O-methyl-^{14}C]- and [6-O-methyl-^{14}C]-scoparone as substrate. Assay methods are described under "Materials and Methods". Each ratio represents the mean ± SE for four different estimations.

P-450 isozyme	Ratio scopoletin : isoscopoletin
P-450$_{PB-B}$	1:0.8 ± 0.05
P-450$_{PB-D}$	1:0.8 ± 0.05
P-450$_{\beta NF-B}$	1:4.4 ± 0.1
P-450$_{\beta NF/ISF-G}$	1:3.8 ± 0.1

These results explain the reported scopoletin:isoscopoletin ratios [1] obtained with microsomal fractions from PB-treated rats (1:0.6) and that of microsomes from benzo(a)pyrene or 3-MC-induced rats (1:2.5), which have a 4-fold lower ratio than microsomal fractions from PB-treated rats. Since phenobarbital induces mainly P-450$_{PB-B}$ and P-450$_{PB-D}$, and 3-methylcholanthrene or other polycyclic hydrocarbons induce to a similar extent P-450$_{\beta NF-B}$ and P-450$_{\beta NF/ISF-G}$ [9], one can conclude that the reported significant shift in the ratio of the scoparone O-demethylation products scopoletin to isoscopoletin, following PB- or 3-MC-administration, reflects the change of the corresponding P-450 isozyme pattern.

Inducers have often been classified as PB-like or 3-MC-like. If one considers induction of P-450$_{\beta NF-B}$ and P-450$_{\beta NF/ISF-G}$ as a 3-MC-like response and the induction of the P-450$_{PB-B}$ and P-450$_{PB-D}$ as a PB-like response, one can conclude that scoparone is a good substrate to decide whether a xenobiotic causes a phenobarbital-like (scopoletin/isoscopoletin ratio 1:0.59 ± 0.01) or a 3-methylcholanthrene-like (ratio 1:2.5 ± 0.1) induction in rat liver microsomes [1].

The scoparone O-demethylation activities given in Table II were calculated as the sum of the scoparone-7-O-demethylation (scopoletin formation), meas-

Table II. Scoparone O-demethylation activities of preformed complexes of NADPH-P-450 reductase and various P-450 isozymes at a molar ratio of 2:1, respectively, at a concentration of P-450 greater than 5 μM. The scoparone O-demethylation activities were calculated as the sum of the scoparone 7-O-demethylation, measured direct fluorimetrically as described [1], and the scoparone 6-O-demethylation, using the scopoletin : isoscopoletin ratios given in Table I. Results are expressed as means of triplicate measurements.

P-450 isozyme	Rate	
	Scoparone 7-O-demethylation [nmol scopoletin formed × min^{-1} × nmol P-450^{-1}]	Scoparone O-demethylation [nmol scoparone demethylated × min^{-1} × nmol P-450^{-1}]
$P\text{-}450_{PB\text{-}B}$	6.9	12.5
$P\text{-}450_{PB\text{-}D}$	0.005	0.01
$P\text{-}450_{\beta NF\text{-}B}$	0.09	0.5
$P\text{-}450_{\beta NF/ISF\text{-}G}$	0.46	2.2

ured direct fluorimetrically as described [1], and the scoparone 6-O-demethylation (isoscopoletin formation) using the scopoletin : isoscopoletin ratios given in Table I.

Comparisons of the scoparone O-demethylation activities (Table II) among the purified P-450 isozymes show that the $P\text{-}450_{PB\text{-}B}$ and $P\text{-}450_{\beta NF/ISF\text{-}G}$ have the highest demethylation rates. This reflects the observed [1] several-fold increase of the scoparone-O-demethylation activities mediated by phenobarbital or by polycyclic aromatic hydrocarbons as a true enzyme induction, accompanied by a significant shift of the ratio of the scoparone O-demethylation products scopoletin to isoscopoletin.

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