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BIOTRANSFORMATION OF SCOPARONE USED TO MONITOR CHANGES IN CYTOCHROME P450 ACTIVITIES IN PRIMARY HEPATOCYTE CULTURES DERIVED FROM RATS, HAMSTERS AND MONKEYS

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Abstract—The coumarin derivative scoparone is regioselectively demethylated yielding isoscopoletin and scopoletin. The ratio of the formation rates of these two metabolites (isoscopoletin/scopoletin; I/S ratio) is reported to mirror the contribution of several cytochrome P450 (P450) isoenzymes to the biotransformation of scoparine. The metabolism of scoparine has been studied in primary liver cell cultures of rats, hamsters, cynomolgus monkeys and in human liver cells. Rat hepatocytes appeared to metabolize scoparone 7 to 10 times slower than those of hamsters and monkeys. In hepatocyte monolayers of all three species the loss of P450 was paralleled by a decrease in total scoparone metabolism. In hamsters but not in rats, a decrease of the I/S ratio was found during primary culture of liver cells. A similar shift in the metabolic pattern of scoparine observed with the monkey hepatocytes was statistically not significant. Most likely, in hamster and possibly in monkey hepatocyte cultures the different P450s involved in scoparone metabolism decrease at unequal rates. In rat liver cells, however, the pattern of these P450 isoenzymes remains more or less unaltered. In contrast to liver cells from the other species, human hepatocytes did not secrete scopoletin in detectable amounts. Scoparone demethylation in humans may be qualitatively different from that in other mammals.

Primary hepatocyte cultures have proven to be a useful tool to study numerous biochemical phenomena. However, the use of this *in vitro* system in biotransformation and cytotoxicity studies is hampered because of the rapid loss of cytochrome P450 (P450‡) from liver cells in primary culture (for a comprehensive review see Ref. 1). Although many authors have studied this phenomenon the nature of the rapid decay of P450 has not been elucidated as yet, nor has it been shown how this loss can be prevented in a convenient or reproducible way that does not involve the use of P450 inducers or ligands inhibiting the enzyme's degradation [2].

The decrease of P450 in hepatocytes in primary culture may affect the biotransformation of xenobiotics quantitatively as well as qualitatively. For a number of compounds it has been demonstrated that the formation of the complete range of metabolites requires the activity of several P450 isoenzymes. Such complex biotransformations are observed, for instance, with testosterone, warfarin and benzo[*a*]pyrene [3–7]. Changes in relative activities of P450 isoenzymes during primary culture may therefore lead to a shift in the metabolic pattern of such compounds. Subsequently, this may result in toxicological properties of the compound of interest, deviating from that observed in freshly isolated cells or from that found in cells in the *in vivo* situation.

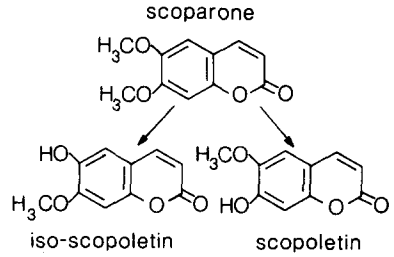

Fig. 1. Structural formulas of scoparone and its major metabolites.

Recently it has been proposed to use the biotransformation of scoparone (6,7-dimethoxycoumarin) as a means to study the P450 status of rats [8]. The regioselective demethylation of scoparone is catalysed by P450s, yielding scopoletin (6-methoxy-7-hydroxycoumarin) and isoscopoletin (7-methoxy-6-hydroxycoumarin; Fig. 1). In rats the ratio of the rates at which these two scoparone metabolites are formed (I/S ratio) is dependent on the contribution of at least four P450 isoenzymes namely P450IA1, IA2, IIB1 and IIB2 [9, 10]. The two representatives of the P450I family each demonstrate an I/S ratio of *ca.* 4, whereas the other two enzymes mentioned show a ratio of 0.8. The effects of *in vivo* treatment with several inducers of

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‡ Abbreviations: P450, cytochrome P450; PB, phenobarbital; 3MC, 3-methylcholanthrene; HBSS, Hank's Balanced Salt Solution; EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid.

P450 isoenzymes on the metabolic profile of scoparone have been well documented. In the rat, pretreatment with phenobarbital (PB) results in an increase in total scoparone demethylation and in a reduced I/S ratio (i.e. scopoletin becomes relatively more important). Administration of 3-methylcholanthrene (3MC) enhances the scoparone metabolism as well but this treatment favours the production of isoscopoletin thus resulting in a higher I/S ratio [8, 9]. Quantitative and qualitative changes in scoparone metabolic profile have also been reported after treatment of rats with benzo[*a*]pyrene, sulmazole and several polychlorobiphenyl congeners [8, 11, 12]. It is therefore suggested that the determination of this I/S ratio can be used to detect shifts in the P450 pattern and to identify a P450 inducer as "PB-like" or "3MC-like" [8].

The scoparone assay may be an attractive tool to study monooxygenase activities in primary hepatocyte cultures from rats as well as other mammals. As demonstrated in preliminary experiments [13], the monodemethylated products of scoparone can be detected not only in microsomal incubations but in primary hepatocyte cultures as well. We here report data obtained with primary cultures of rat, hamster, cynomolgus monkey and human liver cells.

MATERIALS AND METHODS

Animals and cell isolation. Male young-adult CPB/wu rats (180–200 g) and Syrian golden hamsters (80–100 g) were purchased from Harlan Sprague Dawley (Woudenberg, The Netherlands) and housed in makrolon cages. The animals had unlimited access to a standard rodent chow (Hope Farms, Woerden, The Netherlands) and tap water. Liver cells were prepared by the two step collagenase perfusion technique essentially described by Paine *et al.* [14]. The preparation of hamster hepatocytes required the addition of 0.25 mM EGTA (final concentration) to the first 100 mL perfusate (HBSS). During the perfusion of hamster livers the flow rate was 25 mL/min.

Cynomolgus monkeys (*Macaca fascicularis*) were bred at the National Institute of Public Health and Environmental Protection (RIVM, Bilthoven, The Netherlands). Young males (2 to 3 years old) served as donors for kidneys used in the production of poliomyelitis vaccin at this institute. Immediately following nephrectomy, the liver was perfused *in situ* via the portal vein with ice-cold saline by gravity flow. After most of the blood had been washed away, the liver (60–80 g) was removed, put on ice, transferred to our laboratories and connected to a perfusion apparatus within 30 min after hepatectomy. Monkey liver was flushed with 500 mL HBSS containing 0.25 mM EGTA followed by 1000 mL of HBSS without Ca^{2+} and Mg^{2+} at a flow rate of *ca.* 80 mL/min succeeded by a recirculating perfusion with collagenase (Boehringer, 50 mg/100 mL HBSS supplemented with 0.25 mM $CaCl_2$) for 30 min, during which the pH of the perfusate was maintained to approximately neutral values.

For all three species hepatocytes were obtained through a similar purification procedure consisting of repeated washing and centrifugation at 50–100 *g* of the crude cell suspensions using Waymouth MB 752/1 medium (Gibco). To prevent the formation of cell aggregates in the monkey hepatocyte suspensions during this procedure, the wash medium for monkey hepatocytes was supplemented with 1% bovine serum albumin (Sigma). Cell yields and viabilities (V) (rats: 600×10^6 cells, $V \geq 88\%$; hamsters: 250×10^6 cells, $V \geq 82\%$; monkeys: 470×10^6 cells, $V \geq 75\%$) were assessed using the trypan blue exclusion test.

Part of a human liver lobe (female patient, aged 39) was obtained from a local hospital after compliance with legal and ethical regulations. Hepatocytes were prepared by means of the perfusion technique as described by Rijntjes *et al.* [15]. The viability of these human cells was 76%.

Cell culture and incubations. Hepatocytes were cultured in plastic 9 cm dishes (Sterilin) at a density of 6×10^5 cells/mL in 10 mL Waymouth MB752/1 medium to which 0.1 mM hydrocortisone (Sigma), 1 μM insulin (Sigma) and 50 μg/mL gentamycine (Sigma) were added and 3% (rats and hamsters) or 5% (monkeys, human) newborn calf serum (Flow). Additionally, the media of hamster, monkey and human hepatocytes were supplemented with 4 mM $CaCl_2$ and 4 mM $MgCl_2$ [16]. Where appropriate, media were substituted after 24 hr for the same medium but omitting extra $CaCl_2$ or $MgCl_2$.

Incubation with scoparone started at 2, 24 or 48 hr after cell seeding. Media were replaced by media without serum and other additives. Scoparone was added as a solution in DMSO (final DMSO concentration 0.1%). After 1 hr of exposure, media were collected and stored at −20°. Cells were harvested with a rubber policeman in a phosphate/glycerol/Emulgen 913 buffer [17] and stored at −70° until further analysis.

Biochemical determinations. Media samples were treated with glucuronidase/aryl sulphatase for 56 hr after which the metabolites of scoparone were extracted twice with ether. Quantification of the coumarin derivatives was by reversed phase HPLC and detection at 430 nm as described [13].

After thawing, cell suspensions were homogenized using a glass homogenizer tube with Teflon pestle according to Potter–Elvehjem. With the fluorimetric method described by Hukkelhoven *et al.* [18], DNA levels were determined in 16-μL aliquots of cell suspensions, in a buffer consisting of 10 mM Tris–HCl buffer (pH 7.4) containing 10 mM EDTA and 100 mM NaCl. Before addition of this buffer and the assay reagents, nuclear proteins were digested with pronase E (Sigma, 0.1 units in 300 μL water) for 1 hr. A calibration curve was constructed using highly polymerized calf thymus DNA (Sigma).

Total P450 levels were determined in unexposed cells following the method of Rutten *et al.* [19] using the buffer in which the cells were originally harvested. In rat and hamster hepatocyte cultures the concentration of this haemoprotein was measured using the carbon monoxide difference spectrum (CO + dithionite versus dithionite). To avoid any possible interference of haemoglobin with the assay, in monkey hepatocytes the dithionite difference spectrum (CO + dithionite versus CO) had to be

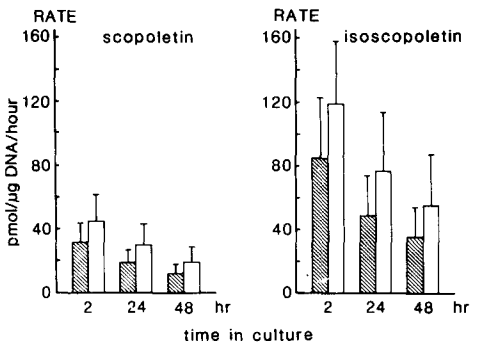

Fig. 2. Formation rates of scoparone metabolites in primary cultures of rat hepatocytes. Shaded bars: 50 µM scoparone, open bars: 100 µM scoparone. Data are means ± SD of five animals.

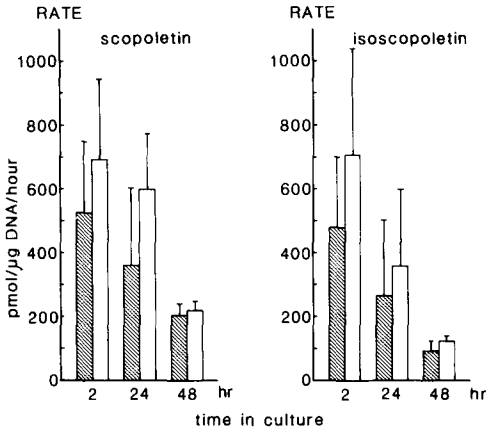

Fig. 3. Formation rates of scoparone metabolites in primary cultures of hamster hepatocytes. Shaded bars: 50 µM scoparone, open bars: 100 µM scoparone. Data are means ± SD of three animals.

used, owing to the presence of small numbers of erythrocytes.

Statistics. All experiments were carried out in triplicate and the numbers of animals used are specified with the figures and tables. Statistical analysis (one- or two-way analysis of variance combined with Student's *t*-test; critical value P = 0.05 for both tests) was performed on the individual mean values. Statistical inter-species comparison was not performed. Only statistically significant differences will be discussed unless stated otherwise.

RESULTS

Primary cultures of hepatocytes of rats, hamsters and cynomolgus monkeys metabolized scoparone to both monomethoxy-coumarin derivatives (Fig. 1). The formation rates of scopoletin and isoscopoletin are shown in Figs 2 to 4.

Shortly after isolation, rat hepatocytes metabolized scoparone (50 µM) to scopoletin at an average rate of approximately 30 pmol/hr/µg DNA (Fig. 2). The formation rate of isoscopoletin was *ca.* 85 pmol/hr/µg DNA. Biotransformation rates increased with higher substrate concentrations, but the metabolite ratio was not affected by this variable (Table 1). The formation of both scopoletin and isoscopoletin decreased with the time the cells were kept in culture. After 24 and 48 hr in culture, the total regioselective O-demethylation of scoparone was diminished by a factor 2 to 3 (Fig. 2) as compared with the conversion rates determined shortly after cell isolation. Culturing rat hepatocytes for prolonged periods of time (up to 48 hr) did not influence the scoparone metabolite ratio (Table 1).

Monolayer cultures of both hamster and monkey liver cells (Figs 3 and 4, respectively) displayed a higher metabolic activity towards scoparone than those of rat hepatocytes. Shortly after isolation, hamster and monkey hepatocytes secreted 10 times more scopoletin per unit of time than those of rats at both substrate concentrations tested (50 and 100 µM). For isoscopoletin formation the difference in metabolic rate amounted to about a factor of seven for both species as compared to the rat. As was found with rat cells, the overall rate of scoparone metabolism declined upon aging of the cultures with cells from hamsters as well as from monkeys. In

Table 1. Ratios of scoparone metabolites in media samples of primary hepatocyte cultures after exposure for 1 hr

Preincubation (hr)	Scoparone (µM)	Isoscopoletin/scopoletin ratio		
		Rat (5)	Hamster (3)	Monkey (4)
2	50	2.72 ± 0.50	0.99 ± 0.25	1.53 ± 0.39
	100	2.66 ± 0.25	1.08 ± 0.48	1.48 ± 0.40
24	50	2.54 ± 0.30	0.67 ± 0.18	1.46 ± 0.39
	100	2.49 ± 0.34	0.80 ± 0.21	1.39 ± 0.39
48	50	2.88 ± 0.65	0.50 ± 0.14	1.20 ± 0.42
	100	2.81 ± 0.38	0.58 ± 0.15	1.02 ± 0.32

The numbers of animals used are given in parentheses. Ratios are expressed as means ± SD

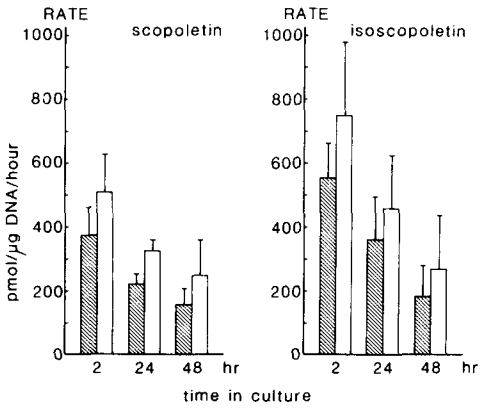

Fig. 4. Formation rates of scoparone metabolites in primary cultures of monkey hepatocytes. Shaded bars: 50 μM scoparone, open bars: 100 μM scoparone. Data are means ± SD of four animals.

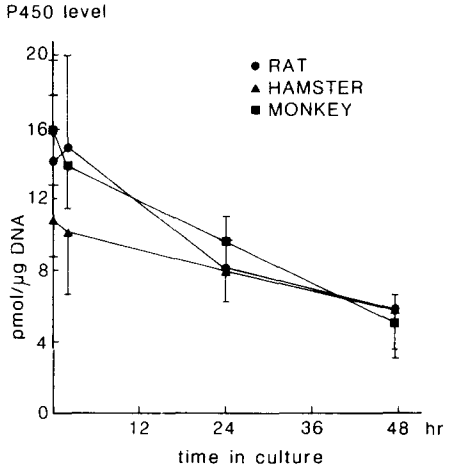

Fig. 5. Cytochrome P450 contents in freshly isolated and cultured hepatocytes. Data are means ± SD of five (rat, ●), three (hamster, ▲) or four (monkey, ■) animals.

hamsters this decline was accompanied by a time-dependent shift in the metabolite pattern. The scoparone metabolite ratio decreased from *ca.* unity in cultures tested after 2 hr of incubation to about 0.5 in cultures of 48 hr of age (Table 1). A similar decrease of the metabolite ratio was found with the monkey hepatocyte cultures, but here the effect of maintenance in culture was not statistically significant.

In primary hepatocyte cultures of all three species the total P450 levels declined time-dependently (Fig. 5). In rat and monkey hepatocytes the P450 levels in freshly isolated cells and 2-hr-old cultures amounted to approximately 15 pmol/μg DNA, decreasing to *ca.* 5 pmol/μg DNA during the next 48 hr. In hamster hepatocytes the initial level was slightly lower (*ca.* 11 pmol P450/μg DNA). The decrease in the hamster hepatocyte P450 concentration over the entire incubation period amounted *ca.* 45%, slightly less than in the liver cell cultures of the other two species. On the whole the declines in total cellular P450 were reflected in lower metabolic rates.

We also had the opportunity to investigate the scoparone biotransformation in one batch of human hepatocytes. Shortly after cell seeding, scoparone was metabolized to isoscopoletin at a rate of *ca.* 80 pmol/hr/μg DNA. This rate decreased steadily during the next 56 hr to approximately 25 pmol/hr/μg DNA. In contrast with the liver cells from the other species investigated the liver cells of this patient did not secrete detectable amounts of scopoletin after incubation with scoparone.

DISCUSSION

The results presented in this paper show that in rat, hamster, cynomolgus monkey and human primary hepatocyte cultures the metabolism of scoparone can be used to study P450-dependent biotransformation. The study of the metabolic conversion of this compound not only provides quantitative information about the monooxygenase activity; it is useful to monitor qualitative changes in P450 activity as well. Rats appeared to be comparatively slow metabolizers of scoparone. Both hamster and monkey hepatocytes demonstrated far greater biotransformation activity towards this coumarin derivative than rats.

It is of interest to note that in rat hepatocytes the metabolite ratio is not altered as a result of the aging of the cultured cells. In this aspect, hamster and cynomolgus monkey hepatocytes differ substantially from those obtained from the rat. In cells derived from the first two species, the scoparone metabolite ratio was decreased after 24 and 48 hr in culture though to a statistically not significant extent in the monkey liver cells. This indicates that in hamster and possibly in monkey hepatocytes at least two P450 isoenzymes are involved in the scoparone demethylation (similar to the scoparone metabolism in the rat [9] and that these enzymes follow the decline in total P450 at unequal rates. For the human liver cells it was impossible to determine a scoparone metabolite ratio, as these cells produced only isoscopoletin.

In the rat P450IA2 is the most active scoparone metabolizing enzyme known so far and this protein produces both isoscopoletin and scopoletin (ratio: 4). An orthologous enzyme is present in human liver [20], but apparently this enzyme lacks the ability to produce scopoletin. Interestingly, other important enzymes (P450IIB1/2)—which effectively produce scopoletine—and their respective genes seem to be present in the rat but not in other species [10]. Nevertheless, both hamsters and monkeys produced large amounts of scopoletin. The absence of scopoletin after biotransformation of scoparone in human hepatocyte cultures indicates that in humans the metabolic pattern of this substrate may be fundamentally different from that in other mammals. However, as only cells from one single patient could be tested, this needs further experimental support.

The observation that in rat hepatocytes the scoparone metabolite profile does not change during

cell culture is in full concordance with the results of an extensive study into the behaviour of a number of P450s in primary hepatocyte cultures [21]. In this study the hepatocyte P450 population has been described by immunochemical determination as well as by determination of the characteristic metabolic activities of these enzymes. Steward *et al.* [22] reported that in hepatocyte cultures form untreated rats some isoenzymes decreased more rapidly than others while in cells isolated from P450-inducer treated rats this effect was even more pronounced. However, these authors [22] determined the P450 pattern only using immunochemical techniques. Steward *et al.* [22] also found that the spectrally determined holocytochrome P450—which indicates enzymes with possible activity—decreased less rapidly than the immunochemical determined proteins. From these studies [21, 22] it can be concluded that changes in P450 protein patterns are apparently not reflected in alterations of the activity profile, *per se*.

Assuming that in liver cells from rats not treated with P450-inducers a number of different enzymes are responsible for the formation of the two monomethoxy metabolites of scoparone, it can be concluded that in rat hepatocytes (the activities of) these enzymes decline at a similar rate more or less equal to the rate of total P450-decay. Another explanation might be that a possible shift in the scoparone metabolism due to differences in rates of decline of specific P450 isoenzymes is compensated by complementary I/S ratios of other P450 isoenzymes.

In species other than the rat only limited data concerning the biotransformation of scoparone are available [13, 23, 24]. The study of Legrum *et al.* [24] demonstrated that in C_{57}BI mice treatments with various compounds affecting P450 activity, among which were PB and 3MC, resulted in changes in scoparone biotransformation differing from those reported for rats [8, 11]. Similar results have been reported for the hamster [25]. This indicates that for the study of changes in P450 activities, the scoparone assay described by Müller-Enoch and Greischel [8] is only of limited value. Although in the rat the role of P450IA1/2 and P450IIB1/2 in scoparone metabolism has been established [9] other forms, more abundant in cells from untreated animals [22], may be involved as well. A recent paper by Nakajima *et al.* [26] showed that the indicator activities EROD and PROD, which are commonly used to specifically determine the catalytic activities of P450IA1 and P450IIB1, respectively, do not provide information about these two enzymes when determined in untreated animals. Results from Witkamp, which will be published shortly, show that sex hormones strongly influence the total biotransformation rate of scoparone as well as the I/S ratio. This indicates that enzymes of the IIC subfamily might be involved in the metabolism of scoparone as well. Other studies [25] demonstrated that treatment of rats with dexamethasone, a known inducer of members of the P450IIIA subfamily, was largely uneffective which indicates that these enzymes probably do not contribute to scoparone metabolism to a major extent. Without further information on the enzymology of P450 isoenzymes in other species than the rat the interpretation of the observed changes in metabolite profiles in terms of "PB- or 3MC-like P450 status" (cf. Ref. 8) is impossible. However, the assay appears to be useful for detecting gross alterations in P450-dependent biotransformation activity.

In routine toxicity testing the choice of the experimental animal is of paramount importance. Biotransformation studies of the compounds of interest in *in vitro* systems derived from several species before planning a routine *in vivo* toxicity test will increase the value of such a test to a considerable extent. Preferably, biological systems of human origin should be incorporated in such species comparisons.

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