Hyperforin Contributes to the Hepatic CYP3A-Inducing Effect of Hypericum perforatum Extract in the Mouse

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This study in mice investigated whether hyperforin accounts for the inductive effects on cytochrome P4503A enzymes of St. John’s wort extracts (SJW; Hypericum perforatum), one of the most popular herbal preparations because of its alleged activity in mild to moderate depression. A hydroalcoholic extract containing 4.5% hyperforin was given at a dose of 300 mg/kg, bis in die (b.i.d.), for 4 and 12 days. Hyperforin, its main phloroglucinol component, was given as dicyclohexylammonium (DCHA) salt (18.1 mg/kg, b.i.d.) on the basis of its content in the extract, to ensure comparable exposure to hyperforin. The extract increased hepatic erythromycin-N-demethylase (ERND) activity, which is cytochrome P450 enzyme (CYP) 3A-dependent, about 2.2-fold after 4 days of dosing, with only slightly greater effect after 12 days (2.8 times controls). Hyperforin too increased ERND activity within 4 days, much to the same extent as the extract (1.8 times the activity of controls), suggesting that it behaves qualitatively and quantitatively like the extract as regards induction of CYP3A activity. This effect was confirmed by Western blot analysis of hepatic CYP3A expression. Exposure to hyperforin at the end of the 4-day treatment was still similar to that with SJW extract, although it was variable and lower than after the first dose in both cases, further suggesting that hyperforin plays a key role in CYP3A induction by the SJW extract in the mouse. Standardization of the extracts based on the hyperforin content can be proposed for further evaluation of their potential action on first-pass metabolism and clearance of coadministered CYP3A substrates.

Key Words: hyperforin; Saint John’s wort; CYP3A; drug blood level; drug mechanism; drug metabolism.

Extracts of St. John’s wort (SJW) (Hypericum perforatum) are most popular herbal preparations, mainly because of their alleged activity in treating mild to moderate depression (Gaster and Holroyd, 2001; Laakmann et al., 2002; Linde et al., 1996). They appear to be implicated in a number of interactions with conventional drugs (Ioannides, 2002), possibly because repeated dosing for extended periods is needed to elicit induction to the full (Bray et al., 2002b). SJW extracts increased a CYP3A-dependent activity, in rodents given 140–280 mg/kg, ip, for 21 days (Bray et al., 2002b) and 1000 mg/kg orally for 14 days (Durr et al., 2000). This prompted us to further examine the role of hyperforin in the induction of CYP3A-dependent drug metabolism by SJW in rodents.

In this study, we further evaluated in the mouse the effects of short- and long-term oral administration of hydroalcoholic extract of SJW on the level and activity of CYP3A, compared with a stable salt of hyperforin (dicyclohexylammonium; DCHA) given on the basis of the hyperforin content in the extract (4.5%). Exposure to hyperforin after repeated dosing with the two compounds at equivalent hyperforin doses was also compared so as to evaluate the contribution of this constituent to the inducing effect of the extract.

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MATERIALS AND METHODS

Animals. Male CD1 mice (22–25 g, 4–5 weeks old) were used (Charles River, Calco, Como, Italy). Animals were housed 6 per cage with controlled room temperature (23°C) under a 12-h light-dark cycle with standard diet and water ad libitum. Procedures involving animals and their care were conducted in conformity with the institutional guidelines that are in compliance with national (D.L. n. 116, G.U., Suppl. 40, 18 Febbraio 1992, Circolare No. 8, G.U., 14 Luglio 1994) and international laws and policies (EEC Council Directive 86/609, OJL 358, 1, Dec. 12, 1987; Guide for the Care and Use of Laboratory Animals, U.S. National Research Council, 1996).

Drugs and treatment. The hydroalcoholic extract of SJW (INDENA S.p.A., Milan, Italy) was given to mice (n = 6) at the dose of 300 mg/kg, mid-way between those of similar preparations in previous studies in rodents (Bray et al., 2002a,b; Durr et al., 2000). This dose was administered orally twice daily for 11 days, while on the twelfth day it was administered once in the morning. In another experiment, SJW extract was given twice daily for 3 days and once on the fourth day.

Hyperforin DCHA (INDENA) was given (n = 6/group) at a dose of 18.1 mg/kg, which approximated the hyperforin content in the extract used in this study (4.5%) (INDENA, data on file). In a few experiments, the dose of 36.2 mg/kg was also evaluated. These doses were given twice daily for 3 days and once on the fourth day.

The extract was suspended in 1.6% carboxymethylcellulose in water, and hyperforin DCHA in 4% Tween 80 in water. Both were freshly prepared immediately before use and administered in a volume of 0.2 ml/mouse.

A further group of mice received 5-pregnen-3β-ol-20-one-16α-carbonitrile (PCN) (50 mg/kg) intraperitoneally, daily for 4 days.

All groups of mice were fasted from 5:00 p.m. on the last day of treatment and killed 24 h after the last dose.

To compare exposure to hyperforin, a few mice were killed at various times after the first dose of SJW extract and hyperforin DCHA (morning of day 1) or after the last dose, in the morning of day 4. Blood was collected in heparinized tubes and centrifuged to separate plasma, which was stored at −20°C until hyperforin quantitation.

Analytical methods. Hyperforin was extracted from plasma by a solid-liquid extraction procedure and quantified by high-performance liquid chromatography (HPLC) with UV detection (272 nm), as recently described (Cervo et al., 2002). The lower limit of quantification of hyperforin in plasma was about 0.02 μg/ml using 1 ml. At this concentration, the coefficients of variation (CV) for the precision and reproducibility of the assay were generally between 15 to 20%, and higher concentrations gave CV below 10%.

Liver microsomes were prepared according to Kato and Takayanagi (1966) and stored at −70°C.

The activity of hepatic microsomal erythromycin N-demethylase (ERND) and microsomal protein content were measured according to Carelli et al. (1996) and Lowry et al. (1951) respectively.

For Western blot analysis, microsomal proteins (5 μg) were separated by 7.5% SDS–polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane, and incubated with a polyclonal antibody against rat CYP3A1/2 (XenoTech, Kansas City, KS) that binds mouse and human CYP3A isoforms (Warrington et al., 2000).

After washing with 1X Tris-buffered saline/0.05% Tween 20 and incubating again with 5% milk, membranes were reprobed with goat anti-rabbit antibody coupled to horseradish peroxidase (1:3000) (StressGen Biotechnologies Corp., Victoria, Canada). The membrane-bound antibody was visualized with the Enhanced Chemiluminescence Western-Blotting Detection System Kit (Amersham Pharmacia Biotech Italia, Milan, Italy). The intensity of the signal on the X-ray film was quantified with the AIS Image Analyzer ( Imaging Research, Inc., Ontario, Canada).

Data analysis. The area under the curve (AUC) over the sampling interval was determined by the conventional trapezoidal method. The peak plasma concentration (Cmax) and the time of its occurrence (tmax) were read directly from the plasma concentration-time data.

Differences between groups (vehicle-, test compound-, SJW-, and hyperforin-treated mice) were analyzed by the Student’s t-test and p values lower than 0.05 were considered statistically significant.

RESULTS

Figure 1A shows the plasma concentration-time curve of hyperforin after single oral doses of the SJW extract containing...
4.5% hyperforin and hyperforin DCHA. After the hydroalcoholic extract, hyperforin rapidly reached the systemic circulation, achieving mean C\text{max} within 60 min. With the DCHA salt, hyperforin levels peaked (30 min), but mean values were slightly lower than after the extract. Quantifiable levels of hyperforin were thereafter evident up to 4–6 h, although they were only just above the limit of detection of 0.1 nmol/ml (using 0.2–0.4 ml of plasma) in most mice. Because of these low and variable levels, the hyperforin AUC could not be determined with precision, but calculation of the partial AUC, indicated that exposure to hyperforin was approximately the same after these equivalent doses of hyperforin DCHA and SJW extracts (Table 1).

With the intention of identifying a convenient schedule of repeated treatment for comparative studies with hyperforin, we then further examined the ability of SJW extract to induce hepatic ERND activity, mainly CYP3A-dependent (Bornheim and Correia, 1990). Because hyperforin disappears rapidly from the systemic circulation in the mouse, the extract was given orally twice daily except on the last day of treatment. ERND increased to 2.2 times the activity of controls (p < 0.01 by Student’s t-test) after 4 days of treatment (with a total of seven doses) (Fig. 2A), and the effect was slightly greater after 12 days (2.8 times control, respectively: 2.77 ± 0.49 and 0.97 ± 0.010 nmol/min/mg of protein in SJW and vehicle-treated mice, p < 0.05 by Student’s t-test).

Other mice were given hyperforin DCHA orally, twice a day for a total of seven doses, and the hepatic ERND activity was compared with that of mice receiving SJW extract by the same schedule. Hyperforin DCHA too increased ERND activity although slightly less than the extract (1.8 times controls, p < 0.01 by Student’s t test) (Fig. 2A). Again, hyperforin plasma concentrations after the last dose showed little or no differences between the salt and the extract, with low and variable but similar C\text{max} occurring at approximately the same times as after the first dose. Exposure to hyperforin was slightly higher with the salt but in this case too accurate determination of hyperforin AUC was not possible because of the low and short-lasting plasma concentrations after either the salt or the extract (Fig. 1B). Plasma C\text{max} and AUC\text{t}, however, tended to be lower than after the first dose, the difference reaching significance for C\text{max} with the SJW extract (Table 1).

Mice apparently tolerated these treatments, as there were no real differences in body weight with either SJW extract or hyperforin DCHA, except for a 5–10% decrease on day 5, mostly caused by fasting, as a similar reduction was seen in vehicle-treated mice (data not shown).

Western blot analysis of hepatic microsomes was then done to investigate the effect of hyperforin on CYP3A. The polyclonal rabbit anti-rat CYP3A1/2 identified two bands with slightly different molecular weights in microsomes from control mice and those treated with hyperforin DCHA or PCN, a specific inducer of CYP3A (Fig. 3) as a positive control. The top and the bottom bands may represent CYP3A11 and CYP3A13, respectively (Warrington et al., 2000). The induction of both bands was more marked for PCN (about 300% and 200% of control, respectively) than for hyperforin DCHA (Figs. 2B and 3A,B). The effect of hyperforin DCHA on CYP 3A proteins was clearly dose-dependent, since doubling the dose (from 18.1 to 36.2 mg/kg for seven doses) increased induction of the top band (from 148% to 210% of the control level) and of the bottom band (from 147% to 180% of the control level) (Fig. 3). The stronger inductive effect of this higher dose of hyperforin DCHA was also clear from the more marked increase of ERND activity (3.33 times control) (Fig. 2A).

Good agreement between ERND activity and CYP3A levels was shown by densitometric analysis of microsomes from mice given the SJW extract (300 mg/kg for 4 days), since both bands (respectively 216% and 155% of the control level for the top and the bottom band, Fig. 2B) were slightly higher than after the 18.1 mg/kg hyperforin DCHA, as suggested by the induction of ERND activity previously described (Fig. 2A).

**DISCUSSION**

As in previous studies in mice (Bray et al., 2002b), administration of an SJW extract (300 mg/kg twice daily) containing 4.5% of hyperforin led to a doubling of ERND activity. However, this effect was already significant after 4 days, whereas daily doses of 140–280 mg/kg for 4–14 days did not alter this CYP3A-dependent activity, which rose only after 21 days of dosing (Bray et al., 2002a,b). Similarly, in the rat, 300 mg/kg daily of SJW extract for 10 days did not alter the catalytic activity of any of the CYP examined (Noldner and Chatterjee, 2001), but 1000 mg/kg daily for 14 days induced the expression of hepatic CYP3A1/2 (Durr et al., 2000). It thus appears that the extent of induction of CYP3A in rodents depends on the daily intake of SJW and the duration of its administration.

It is still not clear whether this is also true for the inductive effect on human CYP3A4, since clinical studies have used different doses and schedules of supplementation. However, short-term ingestion (8 days or less) of 900 mg/day did not

**TABLE 1**

<table>
<thead>
<tr>
<th>Compound (mg/kg)</th>
<th>Dose</th>
<th>C\text{max} (μM)</th>
<th>AUC\text{t} (nmol/ml/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hyperforin DCHA (18.1)</td>
<td>First</td>
<td>0.84 ± 0.61</td>
<td>111</td>
</tr>
<tr>
<td></td>
<td>Last</td>
<td>0.38 ± 0.61</td>
<td>43</td>
</tr>
<tr>
<td>SJW extract (300)</td>
<td>First</td>
<td>1.19 ± 0.54</td>
<td>110</td>
</tr>
<tr>
<td></td>
<td>Last</td>
<td>0.40 ± 0.47*</td>
<td>24</td>
</tr>
</tbody>
</table>

*Note. Hyperforin DCHA was suspended in 4% Tween 80 in water, and SJW in 1.6% carboxymethylcellulose in water.

\*Calculated up to 6 h after the first dose, up to 2 h after the last dose.

\p < 0.01 vs. first dose (Student’s t-test).
alter CYP3A4 activity (Ereshefsky et al., 1999; Markowitz et al., 2000), whereas longer intake (14 days) of this and a higher dose (1800 mg) was associated with a significant increase, as evaluated by the 6β-hydrocortisol/cortisol ratio (Bauer et al., 2002; Roby et al., 2000). Given for 3 days at 300 mg/day ter in die (t.i.d.) SJW extracts did not alter the disposition of alprazolam (Markowitz et al., 2000), but longer intake markedly lowered blood/plasma concentrations and the efficacy of other CYP3A4 substrates, including indinavir, nevirapine, amitriptyline and oral contraceptives (Ioannides, 2002).

Hyperforin too, roughly doubled ERND activity and CYP3A content when given for 4 days, on the basis of its content in the extract, and the effect was clearly dose-dependent. This and the fact that 10–20 mg/kg of hyperforin once daily for 4–10 days did not have inducing effects in rats or mice (Bray et al., 2002a; Noldner and Chatterjee, 2001) suggests that the active component behaves qualitatively and quantitatively like the extract as regards the inductive effect of this subfamily of CYP. It remains to be established whether this is also true for enzymes other than CYP3A. CYP2E1 activity and expression were induced in mice (Bray et al., 2002b) and men (Gurley et al., 2002) after long-term SJW administration. Modest induction of CYP1A2 and CYP2D6 activities was also seen in man after SJW supplementation at 900 mg/day daily for 28 days (Gurley et al., 2002; Nebel et al., 1999).

Interestingly, mean plasma Cmax and partial AUC of hyperforin after the DCHA salt were similar (day 1) or only slightly lower (day 4) than after the hydroalcoholic extract, albeit with wide variability regardless of the hyperforin “formulation” and day of administration. These results further suggest that hyperforin plays a key role in the induction of CYP3A by this extract in the mouse.

Mean plasma Cmax of hyperforin concentration averaged 1 μM after the first day and approximately half this at the end of the study, after either the salt or the extract. Although most studies of the effects of SJW extracts on CYP-dependent drug metabolism provide no data about exposure to this active constituent, these concentrations are close to those found in pharmacokinetic studies in human volunteers (about 0.5 μM) given an extract containing 5% hyperforin (300 mg t.i.d. for 8 days) (Biber et al., 1998). Exposure of human hepatocytes to 1 μM hyperforin led to a marked increase in CYP3A4 mRNA levels and it was suggested that this induction depended on the activation of the PXR receptor (Moore et al., 2000). It is therefore likely that in mice too hyperforin concentrations in the μM range are high enough to induce CYP3A through binding to PXR. However, this must be viewed with caution as the human and mouse homologs of this receptor differ in terms of their ligand binding properties (Kliewer and Willson, 2002; Lehman et al., 1998).

As mentioned, exposure to hyperforin tended to be lower after the last dose than the first one. In the human study mentioned above (Biber et al., 1998), mean Cmax of hyperforin also decreased, whereas total body clearance increased by
about 30% during repeated dosing. This did not occur after daily supplementation with an extract containing only 0.5% hyperforin (Biber et al., 1998). Thus, the hyperforin metabolism may involve oxidative, possibly CYP3A-mediated, reactions, which may be auto-induced by repeated high doses. Although this needs to be confirmed in specific metabolic studies, it is already known that hyperforin acts as a potent, competitive inhibitor of CYP3A in vitro in microsomal preparations, providing further evidence of its direct interaction with this enzyme (Budzinski et al., 2000; Carson et al., 2000; Obach, 2000).

Specific investigations are also required to see whether hyperforin is a substrate of P-glycoprotein, since concomitant induction of this transporter in the intestine by SJW (Durr et al., 2000) may limit its absorption in man and rodents.

In conclusion, this study found that hyperforin induces the activity and expression of CYP3A proteins in the mouse, as do SJW extracts, in rodents and man, when given at appropriate doses for long enough periods. This and the fact that exposure to hyperforin in the mouse is similar to that with an extract containing an equivalent amount of hyperforin suggest that this component is involved in the effect of this extract on CYP3A in the mouse. Obviously, these results cannot be extrapolated to all SJW products available on the market. This poses a problem in assessing the inducing capacity of a SJW extract, because of species-specific effects of the SJW extract and xenobiotics in general on CYP3A gene expression (Gibson et al., 2002; Kliewer and Willson, 2002; Lehmann et al., 1998). Species differences are also evident in hyperforin pharmacokinetics, with exposure to hyperforin being higher in man, despite lower SJW daily intake (Biber et al., 1998).

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REFERENCES

