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5-Hydroxytryptamine is biotransformed by CYP2C9, 2C19 and 2B6 to hydroxylamine, which is converted into nitric oxide

[1]Caroline Fradette, [2]Nobuharu Yamaguchi & *,[1]Patrick du Souich

[1]Department of Pharmacology, Faculty of Medicine, University of Montréal, P.O. Box 6128, Stat. Centre-Ville, Montréal, Québec, Canada H3C 3J7 and [2]Faculty of Pharmacy, University of Montréal, P.O. Box 6128, Stat. Centre-Ville, Montréal, Québec, Canada H3C 3J7

1 There is circumstantial evidence suggesting that 5-hydroxytryptamine (5-HT) could be biotransformed by enzymatic systems other than monoamino oxidase A, and that the isoforms of cytochrome *P*450 may be a source of nitric oxide. This study aimed to assess whether cytochrome *P*450 contributes to 5-HT biotransformation, and to provide evidence that 5-HT metabolism generates nitric oxide.

2 Addition of 5-HT to cultured hepatocytes yielded 5-hydroxyindol acetic acid, a formation modulated by cytochrome *P*450 enzyme inducers and inhibitors. Recombinant human CYP2B6, 2C9 and 2C19 biotransformed 5-HT in 5-hydroxyindol acetic acid, but not CYP1A2, 2D6 or 3A4.

3 Cultured hepatocytes with 5-HT generated nitric oxide, the amount of which was altered by cytochrome *P*450 enzyme inducers and inhibitors. In the presence of CYP2B6, 2C9 and 2C19, 5-HT relaxed precontracted isolated aortic rings, with or without endothelium, an effect prevented by the addition of methylene blue and an inhibitor of catalase, but not by myoglobin. In the absence of catalase, hydroxylamine was always assayed as a byproduct of 5-HT metabolism.

4 In conclusion, CYP2B6, 2C9 and 2C19 biotransform 5-HT, yielding hydroxylamine, which is converted to nitric oxide in the presence of catalase.
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Keywords: Cytochrome *P*450; 5-hydroxytryptamine; nitric oxide; CYP2B6; CYP2C9; CYP2C19; hydroxylamine; hepatocytes

Abbreviations: 3-AT, 3-amino-1,2,4-triazole; EDHF, endothelium-derived hyperpolarizing factor; EETs, epoxyeicosatrienoic acids; H_2O_2, hydrogen peroxide; HETE, hydroxyeicosatetranoic acid; diHETEs, dihydroxyeicosatetraenoic acids; 5-HIAA, 5-hydroxyindole acetic acid; 5-HT, 5-hydroxytryptamine; KTZ, ketoconazole; L-Arg, L-arginine; L-NAME, *N*,*G*-nitro-L-arginine methyl ester; MAO A, monoamine oxidase A; $NO^{\bullet}$, nitric oxide; NOS2, nitric oxide synthase 2; OME, omeprazole; PB, phenobarbital; PE, phenylephrine; PEITC, β-phenylethyl isothiocyanate; RIF, rifampicin; SNP, sodium nitroprusside

Introduction

There is clear evidence that the isoforms of the cytochrome *P*450 (P450) superfamily are implicated in the formation of vasoactive substances, primarily through the biotransformation of arachidonic acid (Capdevila *et al*., 1981). Enzymes of the CYP4A, 4B and 4F subfamilies catalyse the ω-hydroxylation of fatty acids, and enzymes of the CYP1A, 2B, 2C, 2D, 2E, 2J, 3A and 4A subfamilies catalyse the formation of epoxyeicosatrienoic acids (EETs) (Roman, 2002). An endothelium-derived hyperpolarizing factor (EDHF), the 11,12-EET is a metabolite of arachidonic acid synthesized by CYP2C8/34 (Fisslthaler *et al*., 1999). Enzymes of *P*450 have also been implicated in the formation of nitric oxide ($NO^{\bullet}$), particularly the CYP3A subfamily (Boucher *et al*., 1992).

5-Hydroxytryptamine (5-HT) is an effector on various types of tissue receptors. Contraction of blood vessels to 5-HT is mediated by smooth muscle $5\text{-HT}_{1B/1D}$ and/or 5-HT_{2A} receptors (Saxena *et al*., 1998). On the other hand, 5-HT is a vasodilator. The vasodilatation elicited by 5-HT is mediated by endothelial 5-HT receptors (Glusa & Pertz, 2000) and smooth muscle 5-HT receptors that appear coupled to endothelial cells to release $NO^{\bullet}$ (Mylecharane, 1990; Bruning *et al*., 1993). However, the role of $NO^{\bullet}$ remains questionable because 5-HT-induced relaxation is not inhibited by rubbing the endothelium or by *N*,*G*-nitro-L-arginine, a nitric oxide synthase inhibitor (Tsuru *et al*., 1998).

5-HT is believed to be primarily metabolized by monoamine oxidase A (MAO A), which deaminates 5-HT, yielding 5-hydroxyindole acetaldehyde that is converted to 5-hydroxyindole acetic acid (5-HIAA) by an aldehyde dehydrogenase. However, there is indirect data suggesting that 5-HT may also be biotransformed by other enzymatic systems. Metyrapone and ketoconazole (KTZ) show an antidepressant profile (Murphy, 1997; Healy *et al*., 1999), possibly associated with enhanced postsynaptic response to 5-HT (Kennett *et al*., 1985). Moreover, in rats, metyrapone treatment increases 5-HT and its metabolites in the brain (Leret *et al*., 1998). Since metyrapone (Hildebrandt, 1972) and KTZ (Lomaestro & Piatek, 1998) are known *P*450 enzyme inhibitors, we speculated that, besides MAO A, 5-HT is biotransformed by enzymes of *P*450. Supporting such hypothesis, it has been

*Author for correspondence; E-mail: patrick.du.souich@umontreal.ca
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shown *in vitro* that 5-HT inhibits the oxidase activity of CYP1A2, CYP2C9 and CYP3A4 (Gervasini *et al*., 2001). On the other hand, to explain why rubbing the endothelium and *N*,*G*-nitro-L-arginine do not prevent 5-HT relaxation (Tsuru *et al*., 1998), we hypothesized that deamination of 5-HT by P450 generates $NO^{\bullet}$. By combining experiments conducted in hepatocytes, human recombinant *P*450 isoforms and in aortic rings, we demonstrate that 5-HT is biotransformed by CYP2B6, CYP2C9 and CYP2C19 to hydroxylamine, which in the presence of catalase generates nitric oxide.

Methods

Biotransformation of 5-HT by P*450 isoforms*

Animals and hepatocyte collection Male New Zealand rabbits (1.8–2.2 kg, Charles Rivers, St-Constant, Québec, Canada) were maintained on Purina Laboratory Chow and water *ad libitum* for at least 7 days before any experimental work was undertaken. All the experiments were conducted according to the Canadian Council on Animal Care guidelines for the use of laboratory animals. Hepatocytes were isolated according to the two-step liver perfusion method of Seglen (1976), with minor modifications (El-Kadi *et al*., 1997). Viability was assessed by trypan blue exclusion to ensure that it was greater than 90%. Cell concentration was adjusted to $1 \times 10^6\,ml^{-1}$.

Experimental protocols Hepatocytes were incubated for 72 h with cytochrome *P*450 enzyme inducers, rifampicin (RIF, 5–25 μM) and phenobarbital (PB, 50–200 μM), and enzyme inhibitors, such as KTZ (10–50 μM), omeprazole (OME, 10–50 μM) and β-phenylethyl isothiocyanate (PEITC, 10–50 μM), with the nitric oxide synthase inhibitor L-NAME (0.1–1 mM), and with the MAO A inhibitor chlorgyline (10–50 μM). At 4 h before ending the incubation period, for example, at 68 h, L-arginine (L-Arg, 0.1–1 mM) and 5-HT (10–125 μM) were added. Finally, following the 72 h incubation period, 5-HT biotransformation, protein expression of CYP1A1/1A2, CYP2B6, CYP2C9/2C19, CYP3A6 and nitric oxide synthase 2 (NOS2), as well as $NO^{\bullet}$ concentration were documented.

Human recombinant *P*450 isoforms CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 were incubated with 5-HT (10–125 μM) and the *P*450 inhibitors in the presence of an NADPH regenerating system, as described elsewhere (Projean *et al*., 2003), for 55 min, and 5-HT, 5-HIAA, $NO^{\bullet}$ and hydroxylamine were assayed.

Western blot analysis The amount of proteins in hepatocytes was measured in cell lysates (Lowry *et al*., 1951). For Western blot analysis, 40 μg of cell lysate was separated by SDS–polyacrylamide gel electrophoresis, as described elsewhere (Fradette *et al*., 2002). CYP1A1 and 1A2 were detected with a polyclonal anti-rabbit CYP1A1 (Oxford Biochemical Research, Oxford, MI, U.S.A.) and visualized with an alkaline phosphatase-conjugated secondary antibody, using nitro blue tetrazolium as substrate. Specific antibodies against CYP3A6 (Oxford Biochemical Research, Oxford, MI, U.S.A.), CYP2B6, CYP2C9/2C19 (Research Diagnostics, Inc., Flanders, NJ, U.S.A.) and NOS2 (BD Transduction Laboratories, Mississauga, Canada) were used and identified with a secondary antibody conjugated with horseradish peroxidase enzyme, and visualized by autoradiography. All antibodies had crossreactivity for rabbit proteins. Western blot results were normalized with a protein sample to allow for comparisons between measurements. Intensity of the bands was quantified with the software Un-Scan-It-Gel (Silk Scientific Inc., Orem, UT, U.S.A.), and are presented in arbitrary units.

5-HT and 5-HIAA assay 5-HT and its metabolite 5-HIAA were assayed in cells and recombinant *P*450 isoform supernatants by high-performance liquid chromatography with an electrochemical detector, as described by Yamaguchi (1993).

$NO^{\bullet}$ and hydroxylamine assays $NO^{\bullet}$ was determined by converting nitrate into nitrite in the culture media, and total nitrite was assayed spectrophotometrically using the Griess reaction (Grisham *et al*., 1996).

Hydroxylamine could only be assayed in the absence of catalase, for example, in experiments conducted with recombinant CYP2B6, CYP2C9 and CYP2C19, *via* a transglutaminase activity assay (Folk & Chung, 1985). The assay is based on the fact that transglutaminase catalyses the formation of hydroxamate from CBZ-glutaminylglycine and hydroxylamine at pH 6.0. The formed hydroxamate was measured spectrophotometrically at 525 nm.

Vasorelaxant activity of 5-HT byproduct

Functional studies in isolated aortic rings Functional studies with aortic rings were conducted as described elsewhere (Thollon *et al*., 2002). Rings of thoracic aorta of Sprague–Dawley rats of approximately 2.5 mm in length were mounted under $2 \times g$ resting tension in the chamber bath. After equilibration, to test their ability to contract, rings were initially contracted with 100 nM phenylephrine (PE), followed by the addition of 1 μM acetylcholine, to test the presence of the endothelium. After re-equilibration, 50 μM of ketanserin (5-HT receptor antagonist) was added, and 10 or 100 nM PE was used to generate a sustained tension in vessels without and with endothelium, respectively. Thereafter, 10–125 μM of 5-HT was added into the chamber bath. All experiments were conducted in the presence or absence of recombinant CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 (25 pM). Each experiment included a control relaxation with 1 μM sodium nitroprusside (SNP), an $NO^{\bullet}$ donor.

To further characterize the nature of the relaxing agent, aortic rings were incubated with OME (50 μM), methylene blue (30 μM), myoglobin (50 μM) and 3-amino-1,2,4-triazole (3-AT, 50 mM). Tissues were precontracted with 5 nM of PE. Since pretreatment with 3-AT depresses PE-induced tension by $32.2 \pm 2.4\%$ ($n = 6$), PE concentration was 30 nM.

Drugs and chemicals All reagents, enzymes and substrates were purchased from Sigma Chemicals (Sigma, St Louis, MO, U.S.A.). Recombinant human *P*450 CYP1A2, CYP3A4, CYP2D6, CYP2C9, CYP2C19 and CYP2B6 were purchased from Gentest (BD Biosciences Company, Woburn, MA, U.S.A.).

Calculation of the maximal effect and statistical analysis The predicted maximal concentration of $NO^{\bullet}$ (C_{max}) generated by 5-HT was estimated from the dose–response curves by using the E_{max} model with a subroutine written in

FORTRAN for the computer program WinNonlin (Scientific Consulting Inc., Apex, NC, U.S.A.). All results are presented as mean ± s.e. Comparison of results from the various experimental groups and their corresponding controls was carried out by a one-way analysis of variance (ANOVA), followed by the Newman–Keuls *post hoc* test. Differences were considered significant when $P<0.05$.

Results

Following 24 h incubation, hepatocytes from control rabbits expressed CYP1A1, CYP1A2, CYP2B6, CYP2C9, CYP2C19 and CYP3A6, as well as NOS2. After 72 h of incubation, NOS2 expression was almost undetectable. The time of incubation did not affect the expression of *P*450 isoforms (Figure 1a). Therefore, all the experiments reported herein were conducted with hepatocytes incubated for 72 h, for example, in the absence of NOS2. Incubation of hepatocytes for 72 h with the enzyme inducer RIF increased the expression of CYP2C9 from 0.79 ± 0.12 to 0.98 ± 0.15 ($P<0.05$), that of CYP2C19 from 0.95 ± 0.15 to 1.88 ± 0.15 ($P<0.05$), and that of CYP2B6 from 0.55 ± 0.09 to 0.84 ± 0.08 ($P<0.05$); PB, another enzyme inducer, increased the expression of CYP2C9 from 0.79 ± 0.12 to 1.59 ± 0.11 ($P<0.05$), that of CYP2C19 from 0.95 ± 0.15 to 2.25 ± 0.21 ($P<0.05$), and that of CYP2B6 from 0.55 ± 0.09 to 2.35 ± 0.11 ($P<0.05$). Neither RIF nor PB affected NOS2 expression (Figure 1b).

Biotransformation of 5-HT

Rabbit's hepatocytes In the supernatant of cultured hepatocytes, baseline concentrations of 5-HT and 5-HIAA were 0.045 ± 0.005 and 0.120 ± 0.007 μM, respectively ($n=8$). The enzyme inducers RIF and PB dose dependently increased the production of 5-HIAA by 118 and 72% ($P<0.05$), respectively, while 5-HT was decreased to an undetectable level by both inducers ($n=8$). On the other hand, KTZ, an unspecific *P*450 inhibitor, OME, an inhibitor of CYP2C9 and 2C19, and PEITC, an inhibitor of CYP2B6, reduced baseline concentrations of 5-HIAA by 42, 75 and 58% ($P<0.05$, $n=8$), respectively, whereas the concentrations of 5-HT increased by 167, 390 and 227% ($P<0.05$, $n=8$), respectively. Chlorgyline, a specific inhibitor of MAO A, dose-dependently decreased 5-HIAA concentrations up to 15% baseline values.

In hepatocytes incubated with 5-HT (0–125 μM), there was a dose-dependent increase in 5-HIAA production (Figure 2). The enzyme inducers RIF and PB dose dependently increased the production of 5-HIAA by around 50% ($P<0.05$, $n=8$), and slightly reduced 5-HT. On the other hand, KTZ, OME and PEITC reduced the concentrations of 5-HIAA by 10–30% and increased 5-HT concentrations by 10–50%. Finally, in hepatocytes incubated with increasing concentrations of 5-HT, chlorgyline reduced the formation of 5-HIAA by 79% (Figure 2).

Human recombinant P*450 isoforms* Incubation of 5-HT (0–125 μM) with human recombinant CYP2C9, CYP2C19 and CYP2B6 dose-dependently increased the concentrations of 5-HIAA to plateau at around 3.5 μM, with 5-HT concentrations of 100 μM or higher. Human recombinant CYP1A2, CYP3A4 and CYP2D6 did not biotransform 5-HT to 5-HIAA. KTZ reduced the formation of 5-HIAA in CYP2C9, CYP2C19 and CYP2B6 by 54, 52 and 55% ($P<0.05$, $n=8$), whereas OME and PEITC impeded 5-HIAA production almost completely (Figure 3).

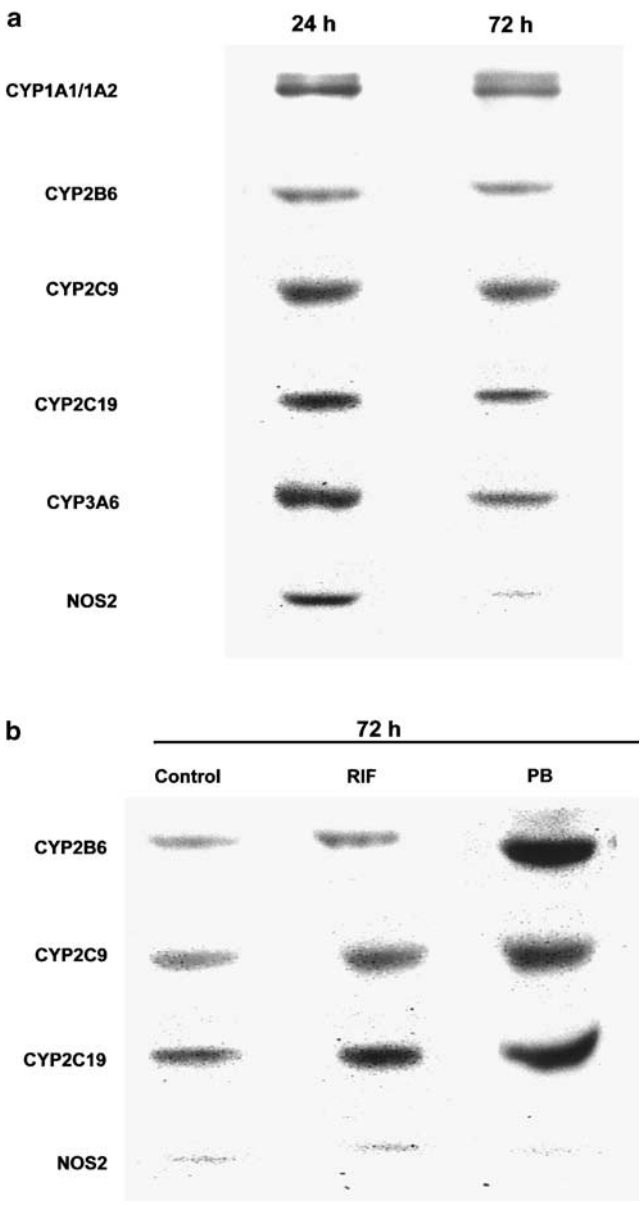

Figure 1 (a) Immunoblot analysis of CYP1A1/1A2, CYP2B6, CYP2C9, CYP2C19, CYP3A6 and NOS2 in rabbit's hepatocytes incubated for 24 and 72 h. (b) Immunoblot analysis of CYP2B6, CYP2C9, CYP2C19 and NOS2 in rabbit's hepatocytes following 72 h incubation with RIF (10 μM) and PB (125 μM).

$NO^{\bullet}$ production

The mean baseline $NO^{\bullet}$ concentration in 72 h hepatocytes was 0.28 ± 0.02 μM. L-Arg and L-NAME did not affect baseline $NO^{\bullet}$. However, $NO^{\bullet}$ concentrations were dose-dependently decreased by KTZ, OME and PEITC up to 32, 18 and 23% baseline values, respectively ($P<0.05$, $n=8$). In addition, chlorgyline reduced $NO^{\bullet}$ concentrations by 21%. On the other hand, PB enhanced baseline $NO^{\bullet}$ concentrations

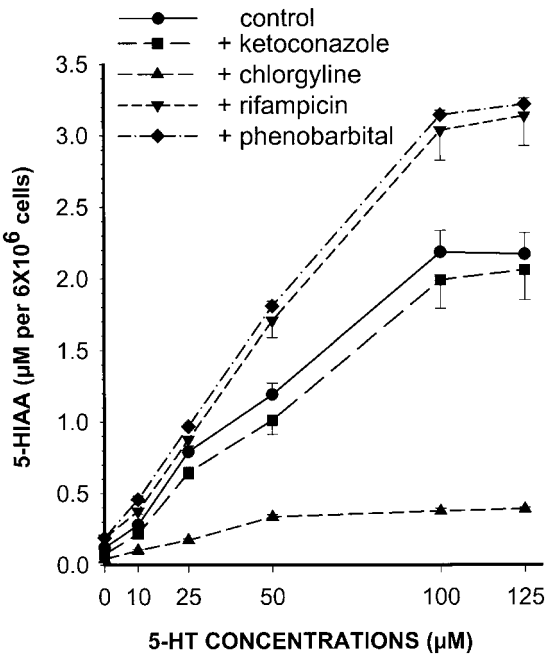

Figure 2 5-HIAA concentrations following 4 h of incubation of increasing concentrations of 5-HT with control hepatocytes and hepatocytes exposed for 72 h to KTZ (50 μM), chlorgyline (25 μM), RIF (10 μM) and PB (125 μM). Each point is mean $\pm$ s.e. of $n=8$.

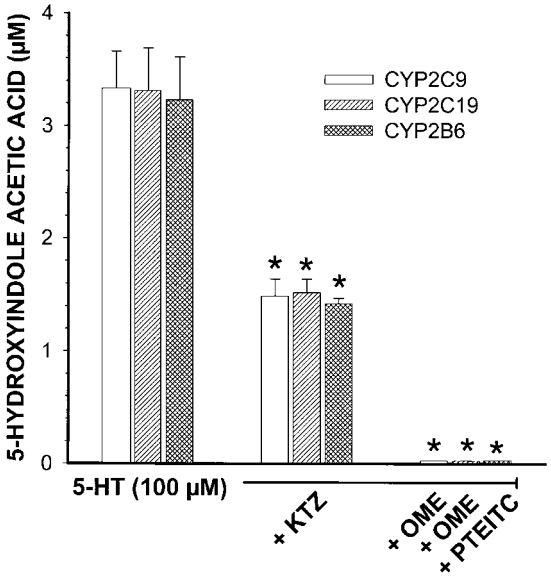

Figure 3 Formation of 5-HIAA by human recombinant CYP2B6, CYP2C9 and CYP2C19 after incubation with 5-HT (100 μM) alone or in the presence of KTZ (50 μM), OME (50 μM) and PEITC (50 μM). Each point is mean $\pm$ s.e. of $n=8$.

dose-dependently to a maximum increase of 316% ($P<0.05$, $n=4$). The predicted maximal effect or efficacy on baseline NO$^\bullet$ concentrations (C_{max}) of these enzyme inhibitors and inducers is depicted in Figure 4a.

In hepatocytes, 5-HT increased the concentration of NO$^\bullet$ dose-dependently, and predicted C_{max} reached 0.6 μM. L-Arg and L-NAME did not modify the NO$^\bullet$ concentrations or predicted C_{max} in hepatocytes incubated with 5-HT. On the other hand, chlorgyline, KTZ, OME and PEITC reduced the

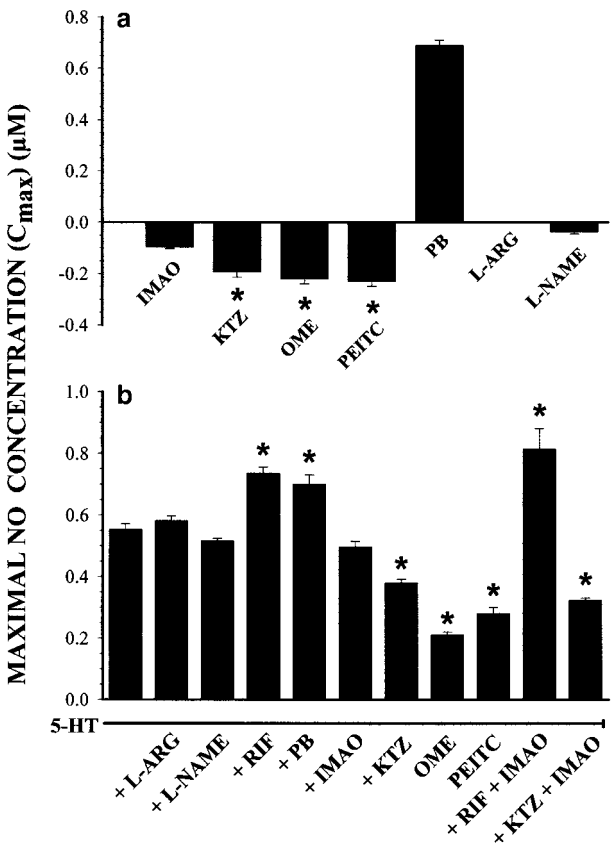

Figure 4 (a) Predicted maximal concentrations of baseline NO$^\bullet$ (C_{max}) in hepatocytes incubated for 72 h with chlorgyline (IMAO, 10–50 μM), KTZ (10–50 μM), OME (10–50 μM), β-phenylethyl-isothiocyanate (PEITC, 10–50 μM), PB (50–200 μM), L-Arg (0.1–1 mM) and L-NAME (0.1–1 mM) added to the incubation media at 68 h. (b) Predicted NO$^\bullet$ C_{max} in hepatocytes incubated for 72 h with L-ARG (0.5 mM), L-NAME (0.5 mM), RIF (10 μM), PB (125 μM), IMAO (25 μM), KTZ (25 μM), OME (50 μM) and PEITC (50 μM) in the presence of 5-HT (10–125 μM) added at 68 h. Vertical bars are mean $\pm$ s.e. of $n=4$.

production of NO$^\bullet$ induced by 5-HT, as well as predicted C_{max} of NO$^\bullet$. Finally, the enzyme inducers RIF and PB increased the concentrations of NO$^\bullet$ generated by 5-HT, as well as predicted C_{max} of NO$^\bullet$ (Figure 4b).

Dynamic studies

Vasorelaxant effect of 5-HT metabolite Preliminary experiments confirmed that, in rat aorta rings with endothelium, 50 μM of 5-HT produced a spontaneous contraction of 197 $\pm$ 17% ($n=6$). However, when 5-HT (50 μM) was incubated in the presence of 50 μM ketanserin, the precontracted aorta ring showed a discrete relaxation of 11 $\pm$ 0.89% ($P=0.07$; $n=6$) (data not shown). Interestingly, in the presence of ketanserin, the mild relaxation elicited by 5-HT was totally abolished in endothelium-denuded aorta rings (relaxation of 0%) ($n=6$) (data not shown). Chlorgyline (25 μM) did not modify the ability of 5-HT to induce a relaxation of the precontracted rat aorta ring (data not shown).

In rat aorta rings with endothelium precontracted by PE (1.6 $\pm$ 0.1 g), 50 μM of 5-HT produced a powerful relaxation in

the presence of human recombinant CYP2C9, CYP2C19 and CYP2B6, reaching a maximal relaxation of around 95±8.6% ($n=6$). The relaxation generated by 5-HT in the presence of CYP2C9, CYP2C19 and CYP2B6 was abolished by 50 μM of OME ($n=3$). The presence of CYP1A2, CYP3A4 or CYP2D6 did not induce any relaxation. In endothelium-denuded segments, a lower concentration of PE (10 nM) was required to induce a tension similar to that produced in endothelium-containing segments ($1.55\pm0.12\times g$), and a greater concentration of 5-HT (75 μM) was necessary to produce maximal relaxations of around 95±11% ($n=6$) (Figure 5a). The addition of CYP1A2, CYP3A4 or CYP2D6 did not produce relaxation. SNP induced a 100% relaxation of precontracted aorta rings with and without endothelium ($n=6$).

Characterization of the mechanism underlying the vasorelaxation induced by 5-HT Myoglobin ($NO^{\bullet}$ scavenger) added to the medium containing CYP2C9, CYP2C19 and CYP2B6 abolished SNP-induced relaxation of PE precontracted aorta rings. In contrast, myoglobin did not affect the vasorelaxation elicited by 5-HT ($n=6$) (Figure 5b). On the other hand, the relaxation induced by both SNP and 5-HT was abolished by methylene blue, an inhibitor of soluble guanylate cyclase (Figure 5c). These results suggested that the vasorelaxant factor produced by 5-HT is not $NO^{\bullet}$, but rather a byproduct that has to be converted to $NO^{\bullet}$ in the smooth muscle.

Assuming that the product resulting from 5-HT deamination by CYP2C9, CYP2C19 and CYP2B6 was hydroxylamine (NH_2OH), which is not scavenged by myoglobin (Taira *et al*., 1997), we hypothesized that hydroxylamine is transformed to $NO^{\bullet}$ by catalase in the presence of hydrogen peroxide (H_2O_2). Pretreatment of the aortic rings with the catalase inhibitor 3-AT (Mian & Martin, 1995) almost completely abolished 5-HT-induced relaxations of PE precontracted endothelium-denuded rings, and that independently of the *P*450 isoforms present, for example, CYP2C9, CYP2C19 and CYP2B6 ($n=6$). As expected, 3-AT did not affect SNP-induced relaxation (Figure 5d).

When 5-HT was incubated with recombinant human *P*450 isoforms, $NO^{\bullet}$ was not detected. Assuming that the product generated by *P*450 isoforms was hydroxylamine, recombinant human CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 were incubated with catalase (1000 U ml^{-1}) and H_2O_2 (1 mM), in the presence of a NADPH regenerating system and several concentrations of 5-HT. Under these

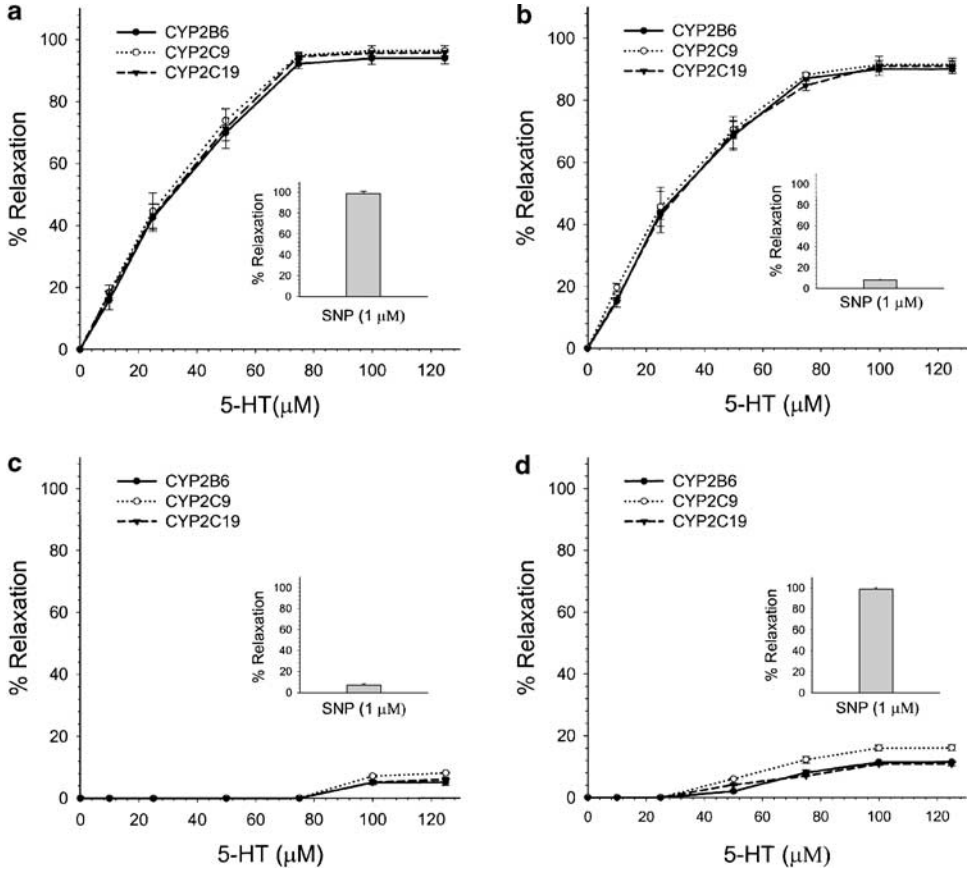

Figure 5 (a) Concentration–response curves of the relaxation induced by 5-HT in the presence of CYP2B6, CYP2C9 and CYP2C19 in endothelium-denuded aortic rings precontracted with PE (100 nM). The inside histogram represents the relaxation elicited by SNP. (b) Effect of myoglobin (50 μM) on concentration–response curves of 5-HT- and SNP-induced relaxation in the presence of CYP2B6, CYP2C9 and CYP2C19 in endothelium-denuded rings precontracted with PE (5 mM). (c) Effect of methylene blue (30 μM) on concentration–response curves of 5-HT- and SNP-induced relaxation in the presence of CYP2B6, CYP2C9 and CYP2C19 in endothelium-denuded rings precontracted with PE (5 nM). (d) Effect of the catalase inhibitor 3-AT (50 mM), on concentration–response curves of curves of 5-HT- and SNP-induced relaxation in the presence of CYP2B6, CYP2C9 and CYP2C19 in endothelium-denuded rings precontracted with PE (5 nM). Each point is the mean±s.e. of $n=6$.

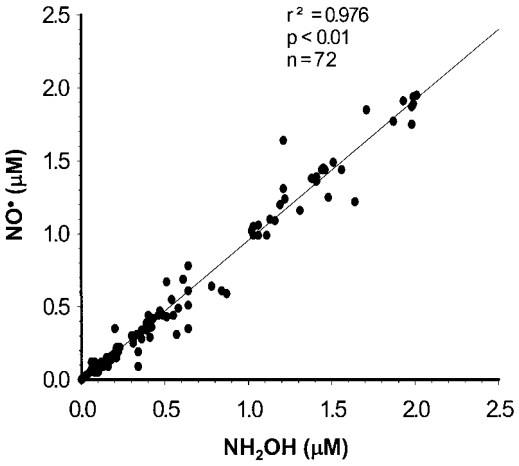

Figure 6 Concentration of $NO^{\bullet}$ generated by the biotransformation of 5-HT by CYP2B6, CYP2C9 and CYP2C19 in the presence of catalase, as a function of hydroxylamine concentration produced by CYP2B6, CYP2C9 and CYP2C19 in the absence of catalase.

experimental conditions, 5-HT dose-dependently generated $NO^{\bullet}$. With CYP2C9, predicted C_{max} of $NO^{\bullet}$ was $2.04 \pm 0.07\,\mu M$ ($n=7$), significantly greater ($P<0.05$) than the predicted C_{max} for CYP2C19 and CYP2B6, for example, 1.48 ± 0.04 and $1.30 \pm 0.10\,\mu M$, respectively ($n=7$). In the presence of catalase and H_2O_2, CYP1A2, CYP2D6 and CYP3A4 did not generate $NO^{\bullet}$ (data not shown). In the presence of KTZ, the production of $NO^{\bullet}$ and predicted C_{max} was reduced to 0.496 ± 0.030, 0.230 ± 0.014 and $0.132 \pm 0.016\,\mu M$ for CYP2C9, CYP2C19 and CYP2B6, respectively ($n=7$, $P<0.05$). On the other hand, OME and PEITC totally prevented the production of $NO^{\bullet}$ by CYP2C9, CYP2C19 and CYP2B6.

To confirm that the byproduct generated by *P*450 isoforms was hydroxylamine, recombinant human CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 were incubated without catalase and H_2O_2, in the presence of the NADPH regenerating system, and several concentrations of 5-HT. Under these experimental conditions, hydroxylamine was generated dose-dependently only by CYP2B6, CYP2C9 and CYP2C19, and KTZ reduced its production, while OME and PEITC totally abrogated hydroxylamine production. There was a close relationship between the $NO^{\bullet}$ produced by CYP2B6, CYP2C9 and CYP2C19 in the presence of 5-HT and catalase, and the concentration of hydroxylamine assayed in the absence of catalase (Figure 6).

Discussion

The above investigations provide evidence that *P*450 isoforms CYP2C9, CYP2C19 and CYP2B6 biotransform 5-HT generating 5-HIAA and NH_2OH, which is converted to $NO^{\bullet}$ by catalase. It has always been assumed that 5-HT is exclusively biotransformed by MAO A; however, the present results demonstrate that several isoforms of the P450 contribute to 5-HT degradation. Incubation of hepatocytes with an IMAO A, chlorgyline, shows that *P*450 contributes with around 20% of the 5-HIAA produced. However, in the presence of *P*450 inducers, RIF and PB, the contribution of *P*450 to 5-HIAA formation is similar to that of MAO A. Can 5-HT biotransformation by *P*450 occur in the central nervous system? Circumstantial evidence supports this eventuality. Firstly, CYP2C9, CYP2C19 and CYP2B6 are expressed in different regions of the brain (Miksys & Tyndale, 2002), and, secondly, *P*450 inhibitors metyrapone and KTZ increase 5-HT concentrations in the brain (Kennett *et al*., 1985; Murphy, 1997; Leret *et al*., 1998; Healy *et al*., 1999). Moreover, phenytoin, a drug biotransformed by CYP2C9 and CYP2C19 (Giancarlo *et al*., 2001), increases 5-HT in the brain without enhancing its synthesis (Chadwick *et al*., 1978; Pratt *et al*., 1985), suggesting that phenytoin competitively inhibits 5-HT biotransformation.

The biotransformation of 5-HT by *P*450 yields hydroxylamine, which in the presence of catalase and H_2O_2 generates $NO^{\bullet}$. In hepatocytes incubated for 72 h, NOS2 was barely detectable, and the amount of $NO^{\bullet}$ was independent of L-Arg and L-NAME, supporting that the source of $NO^{\bullet}$ was primarily the *P*450. Moreover, specific inhibitors of CYP2B6, 2C9 and 2C19 reduced baseline $NO^{\bullet}$ by around 80%. The source of the 20% remaining $NO^{\bullet}$ is probably by the MAO A pathway. The experiments conducted with isolated aortic rings provide evidence that the relaxation elicited by 5-HT depends upon the formation of $NO^{\bullet}$. The relaxation is not due to hydroxylamine, because inhibition of catalase prevents the 5-HT-induced relaxation as well as the formation of $NO^{\bullet}$. The fact that methylene blue, an inhibitor of soluble guanylate cyclase (Gruetter *et al*., 1979), prevents the aortic relaxation triggered by SNP, a $NO^{\bullet}$ donor (Cellek *et al*., 1996), as well as that elicited by 5-HT in the presence of *P*450 isoforms, further supports that $NO^{\bullet}$ is responsible for the vasorelaxation by activating soluble guanylate cyclase (Murad, 1994).

In hepatocytes incubated for 72 h, the maximal efficacy to generate $NO^{\bullet}$ from 5-HT, as illustrated by the predicted C_{max}, was $0.55 \pm 0.02\,\mu M$. As a reference, in hepatocytes incubated for 24 h where NOS2 was expressed, the predicted C_{max} of $NO^{\bullet}$ was $0.95 \pm 0.04\,\mu M$ in the presence of increasing doses of L-arg. The difference in $NO^{\bullet}$ production between the two sources *P*450 and NOS2 was reduced when hepatocytes were exposed to *P*450 enzyme inducers. More than 15 years ago, the group of Moncada reported that EDRF was $NO^{\bullet}$ which was generated from L-arg by NOS (Palmer *et al*., 1987). The present study is evidence that $NO^{\bullet}$ can also be generated from 5-HT by CYP2C9, CYP2C19 and CYP2B6 in the presence of catalase. The actual results confirm the hypothesis raised by Mansuy and Boucher, who proposed that cytochrome *P*450 isoforms might be good candidates to catalyse oxidations similar to those performed by NOS, because of the great analogy between these two classes of heme-thiolate proteins (Morgan *et al*., 2001).

At the present time, there seems to be a fairly clear consensus that at least four distinct receptor types are involved in the endothelium-dependent relaxant effect of 5-HT. Relaxation to 5-HT is mediated *via* activation of smooth muscle 5-HT_7 receptors (Hoyer *et al*., 2002) and 5-HT_4 receptors (Prins *et al*., 1999). In addition, it has been proposed that endothelial 5-HT_{2B} receptors mediate vascular relaxation by the release of $NO^{\bullet}$ and increase in cyclic GMP in smooth muscle cells (Glusa & Pertz, 2000). Moreover, 5-HT counteracts smooth muscle contraction by stimulating sodium influx and hyperpolarizing the membrane, an effect mediated by 5-HT2A (Rhoden *et al*., 2000). On the other hand, several reports have shown that 5-HT triggers the relaxation of

smooth muscle cells by mechanisms independent of 5-HT receptors, such as the relaxation induced in isolated guinea-pig gallbladder strips and in the rat anococcygeus muscle (Emre *et al*., 2000; Emre-Aydingoz *et al*., 2001). The present results add another mechanism to explain the receptor-independent smooth muscle relaxation elicited by 5-HT. It is noteworthy that, in the presence of ketanserin, 5-HT produced a slight relaxation ($P=0.07$) of precontracted aorta rings, an effect completely abolished in denuded aorta rings, suggesting that endothelial cytochrome *P*450 biotransformed 5-HT. Supporting this hypothesis, CYP2C and 2B are expressed in the endothelial cells of arteries (Fisslthaler *et al*., 2000; Earley *et al*., 2003).

The fact that myoglobin, an $NO^{\bullet}$ scavenger (Taira *et al*., 1997), hinders SNP- but not 5-HT-induced relaxation, and that incubation of 5-HT with recombinant CYP2C9, 2C19 and 2B6 does not generate $NO^{\bullet}$, even if 5-HIAA is produced dose-dependently, is further proof that 5-HT biotransformation does not generate $NO^{\bullet}$ directly. We speculate that the deamination of 5-HT generates ammonia, which is spontaneously transformed into water and hydroxylamine in the presence of H_2O_2. Hydroxylamine is a well-known vasodilator in endothelium-denuded arterial segments, *via* its decomposition to $NO^{\bullet}$ (DeMaster *et al*., 1989). In agreement with the actual results, the relaxation of smooth muscle induced by hydroxylamine is endothelium-independent, requires catalase to be transformed to $NO^{\bullet}$ (Craven *et al*., 1979), and the relaxation is blocked by methylene blue (Gruetter *et al*., 1979). Further supporting that 5-HT-induced vasorelaxation is mediated by a catalase-dependent by-product is the fact that addition of 5-HT, catalase and H_2O_2 to the incubation media with recombinant human CYP2C9, 2C19 and 2B6 generated 5-HIAA and $NO^{\bullet}$.

*P*450 is heavily implicated in the metabolism of arachidonic acid generating EETs, dihydroxyeicosatetraenoic acids (di-HETEs) and hydroxyeicosatetranoic acid (HETE) derivatives with an important role as paracrine factors and second messengers in the regulation of vascular function. EETs are potent vasodilators, and CYP2C9 contributes to 50% of their production (Roman, 2002). The present report adds to the known role of CYP2C9, 2C19 and 2B6 in the formation of vasoactive substrates. Moreover, since $NO^{\bullet}$ modulates the release of neurotransmitters, such as acetylcholine, catecholamines, excitatory and inhibitory amino acids, 5-HT, histamine and adenosine (Prast & Philippu, 2001), the present results raise several questions, primarily which is the role of the cytochrome *P*450 expression and activity in diseases such as depression and stress (McLeod *et al*., 2001), aggressive behaviour (Chiavegatto *et al*., 2001), hyperphagia (Yamada *et al*., 2000), hypertension (de Wardener, 2001), learning and memory (Yamada *et al*., 1995) and cephalalgia (D'Andrea, 1999).

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