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Naturally-occurring tetrahydro-β-carboline alkaloids derived from tryptophan are oxidized to bioactive β-carboline alkaloids by heme peroxidases

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ABSTRACT

β-Carbolines are indole alkaloids that occur in plants, foods, and endogenously in mammals and humans, and which exhibit potent biological, psychopharmacological and toxicological activities. They form from naturally-occurring tetrahydro-β-carboline alkaloids arising from tryptophan by still unknown way and mechanism. Results in this research show that heme peroxidases catalyzed the oxidation of tetrahydro-β-carbolines (*i.e.* 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid and 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid) into aromatic β-carbolines (*i.e.* norharman and harman, respectively). This oxidation followed a typical catalytic cycle of peroxidases through redox intermediates I, II, and ferric enzyme. Both, plant peroxidases (horseradish peroxidase, HRP) and mammalian peroxidases (myeloperoxidase, MPO and lactoperoxidase, LPO) catalyzed the oxidation in an efficient manner as determined by kinetic parameters (V_{MAX} and K_M). Oxidation of tetrahydro-β-carbolines was inhibited by peroxidase inhibitors such as sodium azide, ascorbic acid, hydroxylamine and excess of H_2O_2. The formation of aromatic β-carbolines by heme peroxidases can help to explain the presence and activity of these compounds in biological systems.

1. Introduction

Heme-containing peroxidases use hydrogen peroxide (H_2O_2) as electron acceptor to catalyze oxidative reactions in biosynthesis and metabolism of biological molecules [1]. In that reaction, native ferric enzyme reacts with one equivalent of H_2O_2 under a two electron process being oxidized to redox intermediate compound I (Fe(IV) = $O^{+\cdot}$). Compound I is reduced back directly to native ferric enzyme by a two electron process (*e.g.* the oxidation of halides by mammalian peroxidases), or instead by alternative substrates (AH) via intermediate compound II ($PorFe^{IV}$ = O) through two successive one-electron reductions [2]. Compounds I and II oxidize substrates (AH) to give radicals ($AH^{\cdot}$) that evolve to stable products. Heme peroxidases are widely distributed in nature and include plant peroxidases (*e.g.* horseradish peroxidase, HRP) and mammalian peroxidases (*e.g.* myeloperoxidase, MPO; lactoperoxidase, LPO, eosinophil peroxidase, EPO). Peroxidases play a protective role against oxidative damage and in response toward wounding or stress in plants. In mammals, peroxidases catalyze the oxidation of halides (*e.g.* Cl^-) to hypohalous acids being cytotoxic against microorganisms [1] and participate in inflammatory pathologies [3,4]. Mammalian peroxidases have been found in brain where they can oxidize dopamine to dopaminochrome exerting cytotoxicity [5,6] and are involved in the bioactivation of protoxins and procarcinogens [7–9].

β-Carbolines are naturally-occurring bioactive alkaloids originally detected in plants and which also appear in mammalian fluids and tissues, including the human brain [10–14]. In addition, β-carbolines occur in foods and cigarette smoke, suggesting human uptake and exposure to these compounds [10,15–17]. They exhibit an array of biological, psychopharmacological and toxicological activities, including antitumor, antimicrobial, antimalarial, anti-inflammatory, vasorelaxant, antioxidant, neuroactive, psychoactive or neurotoxic actions [10,11,18–20]. Biosynthetically, β-carboline alkaloids arise from the amino acid tryptophan which affords the nitrogen atoms and carbon skeleton. Thus, an indolethylamino acid or amine derivative reacts with an aldehyde or α-keto acid under an enzymatic or chemical reaction (Pictet–Spengler) to give an intermediate Schiff base that cyclizates to give tetrahydro-β-carbolines [21] (Fig. 1). Subsequently, tetrahydro-β-carboline molecules are oxidized to β-carbolines. However, an enzymatic step able to accomplish the latter bioconversion is currently unknown and lacking. This research reports the biotransformation

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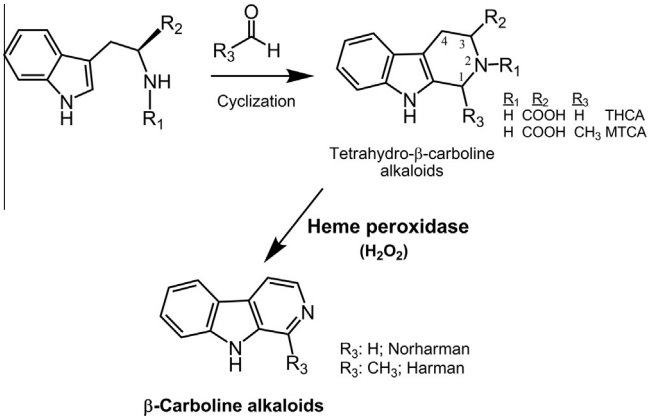

Fig. 1. Biosynthesis of naturally-occurring β-carboline alkaloids in biological systems. The oxidation step from tetrahydro-β-carbolines to aromatic β-carboline alkaloids can be accomplished by heme peroxidases occurring in plants and mammals as suggested here.

of tetrahydro-β-carboline-3-carboxylic acids, which are naturally-occurring alkaloids derived from tryptophan, into aromatic β-carbolines as catalyzed by heme peroxidases arising from both mammalian and plant origin. This metabolic conversion contributes to rationalize the biological presence and activity of β-carbolines (*e.g.* norharman and harman) found in biological fluids and tissues and the possible implications are discussed.

2. Materials and methods

2.1. Chemicals and enzymes

Horseradish peroxidase (HRP) (type II) and bovine milk lactoperoxidase (LPO) were obtained from Sigma; human myeloperoxidase (MPO) from polymorphonuclear leucocytes was obtained from Calbiochem (Merck); human cytochrome P450s Supermix expressing CYP1A2, CYP2C8, CYP2C9, CYP2C19, CYP2D6 and CYP3A4 enzymes was obtained from Gentest Co (BD) (Woburn, MA, USA); catalase from bovine liver and glutathione peroxidase from bovine erythrocytes were from Sigma. All enzymes were prepared in phosphate buffer and used as supplied. Norharman (9*H*-pyrido[3,4-*b*]indole), Harman (1-methyl-9*H*-pyrido[3,4-*b*]indole), (1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) and 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THCA) were obtained from Sigma. Hydrogen peroxide was obtained from a 30% solution (Scharlau Chemical) and diluted to desired concentration.

2.2. Enzymatic metabolism

Fresh solutions of peroxidases were prepared to make: 0.32 μM HRP, 0.178 μM LPO or 0.0126 μM MPO in 50 mM phosphate buffer, pH 7 (0.5 ml final volume for MPO or 1 ml for HRP or LPO) containing in separate, 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THCA) or 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (MTCA) in a range from 0 to 2 mM. The reaction was initiated by addition of H_2O_2 solution (5–500 μM, final concentration), and the samples incubated at 37 °C for 40 min. Following addition of $HClO_4$ + methanol (1/1) (50 or 100 μl, 10% v/v), the tubes were centrifuged at 10,000 rpm, 10 min, and the reaction products were analyzed by HPLC and HPLC-MS. Selected concentrations of H_2O_2 used for kinetic studies with tetrahydro-β-carbolines were as follows: 100 μM for HRP/THCA, 50 μM for HRP/MTCA; 10 μM for LPO/THCA; 25 μM for LPO/MTCA, and 25 μM for THCA/MPO and MTCA/MPO. Incubations were at least in duplicate and control reactions were carried out in absence of enzyme, H_2O_2 or tetrahydro-β-carbolines, and in presence of catalase (0.05–0.2 mg prot./ml). Reactions were also carried out in presence of 1 mM ascorbic acid, 1 mM sodium azide, 1 mM hydroxylamine or excess H_2O_2 (2 mM) as inhibitors of peroxidase and substrate (250 μM). On the other hand, cytochrome P450 and catalase (heme proteins), and glutathione peroxidase were incubated with tetrahydro-β-carbolines to assess possible oxidation, as follows: (a) 0.2 ml phosphate buffer 50 mM, pH 7.0, containing a mixture of cytochrome P450s (Supermix) (20 pmol P450) and THCA and/or MTCA (250 μM), with or without NADPH (1 mM) and H_2O_2 (50–500 μM); (b) 1 ml phosphate buffer 50 mM, pH 7, containing catalase (0.05–0.2 mg prot./ml), THCA or MTCA (250 μM) and H_2O_2 (100–500 μM); (c) 1 ml phosphate buffer 50 mM (pH 7), containing glutathione peroxidase (9.6 μg prot./ml), H_2O_2 (50 μM) and MTCA or THCA (500 μM). The mixtures were incubated (37 °C, 25 min), the reaction stopped with 10 % v/v $HClO_4$/methanol (1:1), centrifuged and analyzed by RP-HPLC-DAD.

2.3. Spectral measurements

UV–vis spectra of enzymatic incubations were obtained at room T in a Beckman-Coulter DU 800 spectrophotometer. Concentration of peroxidases (HRP, LPO, MPO) was determined by absorbance using the extinction coefficients of the respective Soret bands (403, 412, 430 nm) [22]: ε_{403} (HRP) = 102 mM^{-1} cm^{-1}, ε_{412} (LPO) = 112 mM^{-1} cm^{-1}, and ε_{430} (MPO) = 91 mM^{-1} cm^{-1}. H_2O_2 concentration was calculated at 240 nm (ε = 39.4 M^{-1} cm^{-1}).

2.4. RP-HPLC and HPLC-MS (electrospray ionization)

The chromatographic analysis of incubation media was performed by RP-HPLC coupled to diode array (DAD) and fluorescence detectors and by HPLC-MS (electrospray) [9,16]. A 150 × 3.9 mm, 4 μm, Nova-pak C18 column (Waters, Milford, MA) was used for separation. Conditions were: 50 mM ammonium phosphate buffer (pH 3) (buffer A) and 20% A in acetonitrile (buffer B). Gradient from 100% A to 32% B in 8 min and 90% B at 10 min; flow rate 1 ml/min; the column temperature, 40 °C and injection volume 20 μl. Absorbance detection was 254 nm for β-carbolines and 280 nm for tetrahydro-β-carbolines, and concentration was determined from calibration curves of area vs concentration of standards. Analysis by HPLC-MS was carried out by using a Hewlett–Packard 1100 HPLC-mass spectrometer working under ESI positive ionization mode. Chromatographic separation was done with a 2.1 × 150 mm Zorbax SB C18 column (Agilent technologies) using 0.5% formic acid (eluent A) and 0.5% formic acid in acetonitrile (eluent B). The gradient was 0–80% B in 30 min. The flow rate was 0.3 ml/min and the column temperature, 40 °C. The drying gas temperature was 350 °C, flow of 11 l/min, nebulizer pressure of 55 psi, capilar voltage of 4000 V and fragmentator of 100 V. Acquisition was from 50 to 700 u.

2.5. Kinetic studies

Peroxidase-catalyzed oxidations were studied as a function of the concentration of substrates and the apparent K_M and V_{MAX} were determined from non-linear regression fitting to Michaelis–Menten curves (GraphPad prism). Peroxidases have not true V_{MAX} and k_{cat}, and a pseudo-first order rate constant k_4 corresponding to the global oxidation leading to β-carbolines was calculated from K_M^{app} and k_{cat} as k_{cat}/K_M^{app} [23]. The reaction rate (v) was determined as the amount of β-carboline formed as a function of time and concentration of enzyme.

3. Results and discussion

Incubation of tetrahydro-β-carboline-3-carboxylic acids (*i.e.* THCA and MTCA) with heme peroxidases (HRP, LPO or MPO) and H_2O_2 gave oxidation products that were identified as the corresponding aromatic β-carbolines by HPLC-DAD (λ_{max} at 248, 302 and 365–370 nm), fluorescence spectra (λ_{max} for emission at around 435–445 nm for exc. 300/254 nm) and co-elution with authentic standards (Fig. 2). These results were confirmed by HPLC-MS analysis (positive ESI). Thus, incubation of THCA with peroxidases gave norharman (*m/z* 169, $(M+H)^+$) whereas MTCA gave harman (*m/z* 183 ($M+H^+$)). Formation of β-carbolines increased with incubation time and peroxidase concentration and it was negligible in absence of enzyme, H_2O_2 or in presence of catalase (0.2 mg/ml).

The time-course of peroxidase-catalyzed reaction was followed by spectrophotometry (Fig. 3). Changes in the Soret bands were monitored to follow the formation of peroxidase compounds I and II. Following addition of H_2O_2 to HRP (403 nm), compound I was formed as detected by a lower intensity Soret band (418 nm) (Fig. 3A). The addition of MTCA (an electron donor) to H_2O_2-HRP formed compound II (more intense Soret band at 420 nm) that later disappeared to recover the native ferric enzyme (403 nm). This process was accompanied by the presence of a new spectral band that corresponded to formation of harman (1-methyl-β-carboline) (350–365 nm). Similar patterns were observed for LPO and MPO although compound I was not easily detected for these enzymes [22]. Thus, following addition of MTCA to H_2O_2-LPO, LPO-compound II was formed (Soret band red-shifted from 412 to 430 nm) and simultaneously, MTCA oxidized to harman (band at 356 nm) (Fig. 3B). Then, LPO-compound II disappeared and native enzyme recovered (Soret band at 412 nm). Similar spectral features were obtained for MPO with native enzyme (430 nm) giving rise to compound II (456 nm) while MTCA oxidized to harman (result not shown). Inserts in Fig. 3 illustrate spectral changes in characteristic absorption maxima. Thus, after mixing LPO, H_2O_2 and MTCA, compound II formed (430 nm) and remained for several minutes while native enzyme decreased (412 nm) and MTCA oxidized to harman (356 nm). Formation of β-carboline decreased or stopped when native enzyme (412 nm) recovered. These results indicate that oxidation of tetrahydro-β-carbolines by HRP, LPO or MPO followed a typical catalytic cycle of peroxidases through compounds I and II. Other heme enzymes such as catalase or cytochrome P-450, and glutathione peroxidase (non-heme peroxidase) were unable to accomplish this oxidation, suggesting it is heme peroxidase-dependent.

Reaction rates were determined as a function of the concentration of substrates. They reached a maximum at relative low concentrations of H_2O_2 ($\leqslant$100 μM) with inhibition detected at higher concentrations. In Fig. 4 are the curves corresponding to the formation of norharman by HRP, LPO and MPO as a function of the concentration of tetrahydro-β-carboline (similar curves obtained for harman). These curves were used to calculate the apparent kinetic parameters K_M, V_{MAX} and k_{cat} (Table 1). A pseudo-first order rate constant (k_4) was calculated from K_M and k_{cat} that reflects the global oxidation process of substrates by peroxidases [9,23]. The three peroxidases catalyzed the oxidation of THCA and MTCA in an efficient manner. The values of K_M^{app} were relatively similar for THCA and MTCA and the three enzymes. V_{MAX}^{app} and turnover (k_{cat}) were higher for the oxidation of THCA than MTCA, suggesting that THCA was a better substrate. The k_4 was also higher for the oxidation of THCA than MTCA. Turnover (k_{cat}) and k_4 were higher for LPO and MPO, suggesting that they were better catalysts of tetrahydro-β-carboline oxidation than HRP. Finally, the formation of norharman or harman by peroxidases was strongly inhibited (from 65% to 100%) in presence of peroxidase inhibitors such as sodium azide, hydroxylamine, ascorbic acid and excess of H_2O_2, with hydroxylamine and ascorbic acid being the best inhibitors (Fig. 4D).

The results described above have shown that heme peroxidases efficiently catalyze the metabolic conversion of naturally-occurring tetrahydro-β-carboline-3-carboxylic acids (THCA and MTCA) alkaloids into aromatic β-carbolines. This conversion was higher for mammalian peroxidases (MPO and LPO) than for plant peroxidase (HRP). Tetrahydro-β-carbolines were good substrates and electron donors of peroxidase-redox intermediates and their oxidation followed a typical catalytic cycle of peroxidases through compounds I, II and native enzyme. Peroxidases oxidize indoles by a one-electron oxidation to give nitrogen centered radicals (*i.e.* indolyl cation or neutral radical) [1,22,24–27] that evolve to indole-ring cleavage products. Indoles and tetrahydro-β-carbolines also form indolyl

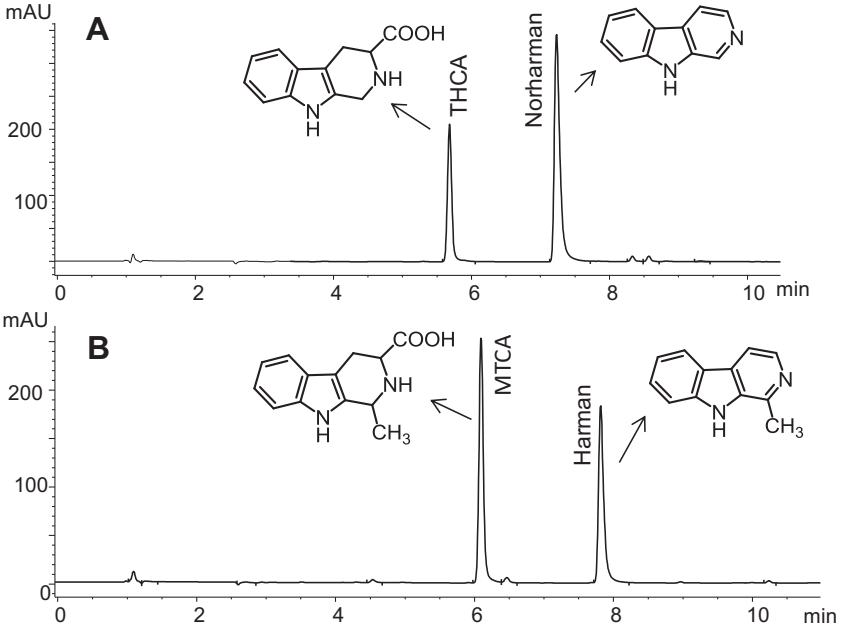

Fig. 2. RP-HPLC chromatograms (absorbance at 254 nm) of incubation media containing tetrahydro-β-carbolines and heme peroxidases: (A) THCA (250 μM) and HRP (0.32 μM) and (B) MTCA (250 μM) and LPO (0.178 μM). Incubation and chromatographic conditions as indicated in Section 2.

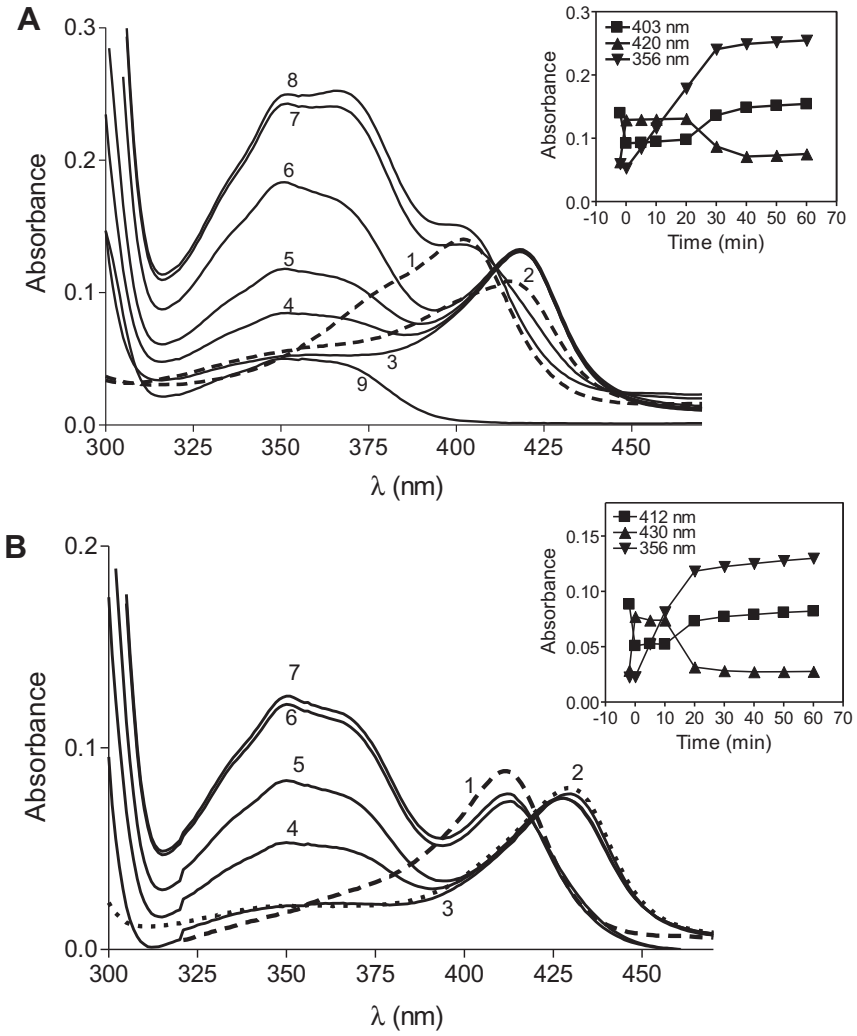

Fig. 3. Time-course of peroxidase reaction followed by spectrophotometry. (A) MTCA (250 μM) oxidation with HRP (1.28 μM): HRP (1), HRP + H_2O_2, 50 μM (2), HRP + H_2O_2 + MTCA initial (3), and after 5 min (4), 10 min (5), 20 min (6), 30 min (7), 40 min (8); spectrum of harman (1-methyl-β-carboline) (9). (B) MTCA (250 μM) oxidation with LPO (0.9 μM): LPO (1), LPO + H_2O_2, 25 μM (2), LPO + H_2O_2 + MTCA initial (3), and after 5 min (4), 10 min (5), 20 min (6), and 30 min (7). Inserts illustrate spectral changes in Soret bands of HRP and LPO and formation of β-carbolines (356 nm).

radicals when acting as radical scavengers [28–30]. Therefore, oxidation of tetrahydro-β-carbolines by heme peroxidases suggests a stepwise oxidation in which peroxidases catalyze the initial one-electron oxidation to a tetrahydro-β-carboline nitrogen radical (*e.g.* indolyl radical) which is further oxidized to afford the aromatic β-carboline accompanied by decarboxylation [29,31,32]. Peroxidases use hydrogen peroxide (H_2O_2) that is a major reactive oxygen species (ROS) involved in oxidative stress [7,8,33]. As THCA and MTCA are substrates of peroxidases, they may exert antioxidant actions because they are good electron donors of peroxidase redox intermediates and consume H_2O_2 during enzymatic oxidation. These compounds are also radical scavengers as reported elsewhere [29,31].

Tetrahydro-β-carbolines and β-carbolines (*i.e.* pyridoindole alkaloids) are widely distributed in plants and marine organisms. They exert many biological activities including antitumor, antimicrobial, antimalarial, anti-inflammatory, neuroactive and psychoactive actions [10,19]. Most of these activities often relay on the aromatic β-carboline (pyrido[3,4-*b*]indole) ring-unit. Their biosynthesis starts with indolethylamines or indolethylamino acids that cyclizate to tetrahydro-β-carboline alkaloids (Fig. 1). Then, tetrahydro-β-carbolines experiment an oxidation step to afford β-carbolines. Results in this research highlight the basis for heme peroxidases as key catalysts for this oxidation in nature. Indeed, peroxidases abound in plants and fungi, take part in the metabolism, extracellular defenses and stress [7] and are involved in key steps within metabolic pathways of indole alkaloids [34,35] linked to oxidative stress [36].

Mammalian peroxidases (*e.g.* MPO, EPO and LPO) abound in neutrophils, eosinophils and secretory cells of human exocrine glands. They take part in inflammatory processes and oxidize halides to oxidants (*e.g.* hypochlorous acid) that help to kill microorganisms [37–39]. Peroxidases are involved in diseases and are current targets for inhibitory drugs [4–6,25,26,40,41]. MPO occurs at sites of neuroinflammation and increases in Alzheimer's disease [4,42] while its ablation or inhibition mitigates neurodegeneration [40,43–45]. Oxidation of physiological donors and xenobiotics by peroxidases often results in biological and toxic effects [3,7,8]. Tetrahydro-β-carboline-3-carboxylic acids and their β-carboline products occur in human biological tissues and fluids, and humans are exposed to these compounds through the diet and environment (*e.g.* smoking) [10,15,18,29]. Then, human peroxidases could perform the oxidation of tetrahydro-β-carboline-3-carboxylic acids (THCA and MTCA) which are relatively abundant in biological

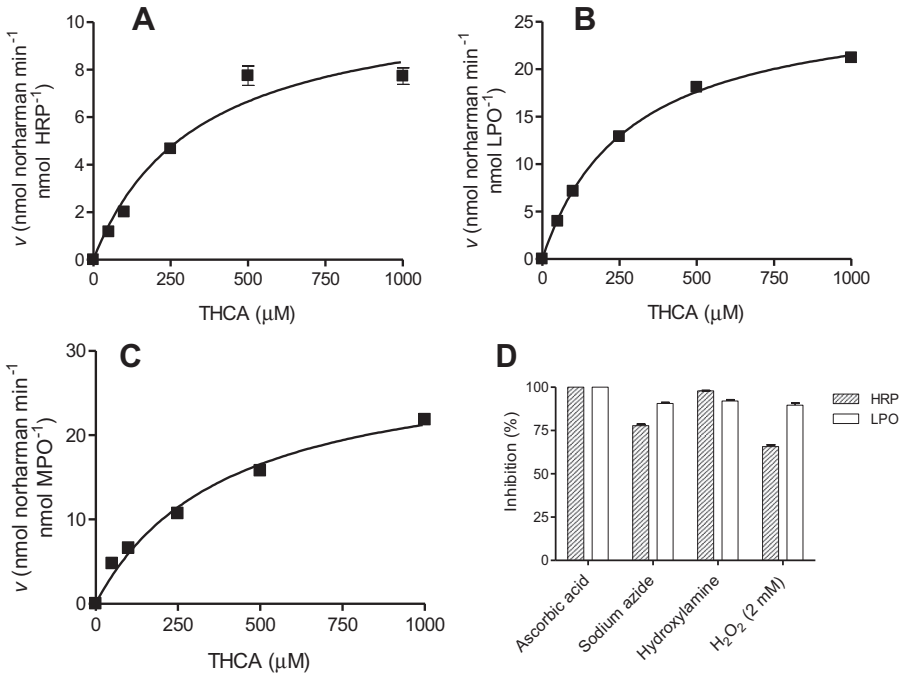

Fig. 4. Formation of the β-carboline norharman (nmoles/min/nmol prot.) from THCA by (A) HRP (0.32 μM) and H_2O_2 (100 μM); (B) LPO (0.178 μM) and H_2O_2 (10 μM); and (C) MPO (0.0126 μM) and H_2O_2 (25 μM); (D) inhibition (%) of oxidation of THCA (250 μM) by HRP or LPO in presence of ascorbic acid (1 mM), sodium azide (1 mM), hydroxylamine (1 mM), and excess of H_2O_2 (2 mM). Results are from duplicates.

Table 1
Apparent kinetic parameters for the oxidation of tetrahydro-β-carboline-3-carboxylic acids by heme peroxidases.

	Substrate	V_{MAX}^{app} (nmol β-carboline/min nmol prot.)	K_M^{app} (μM)	k_{cat} (min^{-1})	k_4 (ml $nmol^{-1}$ min^{-1})
HRP	THCA	11.1 ± 1.1	335.0 ± 81.3	11.1	0.033
	MTCA	9.6 ± 0.5	393.5 ± 63.2	9.6	0.024
LPO	THCA	27.5 ± 0.5	279.6 ± 14.0	27.5	0.098
	MTCA	8.8 ± 0.2	254.1 ± 22.4	8.8	0.034
MPO	THCA	29.7 ± 1.9	397.3 ± 58.8	29.7	0.075
	MTCA	11.9 ± 0.7	243.2 ± 40.6	11.9	0.049

k_4 is calculated from k_{cat}/K_M^{app} [23]; the β-carboline products are norharman from THCA (1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid) and harman from MTCA (1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid). Incubations and amount of enzymes and H_2O_2 are described in Section 2.

systems resulting in an increase of aromatic β-carbolines (norharman and harman) that exhibit singular biological actions. Aromatic β-carbolines bind to brain receptors and inhibit monoamine oxidase (MAO) exerting psychopharmacological and antidepressant actions [11,16,46]. On the other hand, aromatic β-carbolines are involved in toxicological processes [47–49]. They accumulate in the substantia nigra [12,50] and could be bioactivated to neurotoxic β-carbolinium cations [9,18,49,51,52]. Indeed, a higher presence of aromatic β-carbolines and their β-carbolinium cations have been found in parkinsonian patients [13,53].

In summary, this research have shown that mammalian (MPO and LPO) and plant (HRP) heme peroxidases efficiently catalyze the biotransformation of the naturally-occurring tetrahydro-β-carboline-3-carboxylic acid alkaloids derived from tryptophan into aromatic β-carboline alkaloids. This oxidation followed a typical catalytic cycle of heme peroxidases. Tetrahydro-β-carbolines and heme peroxidases abound in nature, including the human body. Then, oxidation of tetrahydro-β-carbolines by heme peroxidases can help to explain the formation and presence of β-carboline alkaloids in biological systems. This oxidation might have implications as β-carbolines (*e.g.* norharman and harman) exhibit potent biological, pharmacological and toxicological actions.

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