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Evidence of a Coupled Mechanism Between Monoamine Oxidase and Peroxidase in the Metabolism of Tyramine by Rat Intestinal Mitochondria

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ABSTRACT. The relationship between monoamine oxidase (EC 1.4.3.4; MAO) and peroxidase (EC 1.11.1.7; POD) in the metabolism of tyramine was investigated using the crude mitochondrial fraction of rat intestine. When tyramine was incubated with mitochondria, the formation of the peroxidase-catalysed oxidation product, 2,2′-dihydroxy-5,5′-bis(ethylamino)diphenyl (dityramine), identified by mass spectrometric analysis, was monitored spectrophotometrically. After an initial lag time, the formation rate of dityramine was linear up to 2 hr, amounting to 17 nmol $\times$ hr^{-1} $\times$ mg $protein^{-1}$. A similar value was found for the oxidative deamination of tyramine catalysed by intestinal MAO. Either 10^{-3} M clorgyline or 10^{-3} M NaCN suppressed this reaction by completely inhibiting MAO or POD, respectively. In the former case, however, addition of H_2O_2 to the incubation mixture promptly started the reaction. Selective inhibition of MAO-A and MAO-B was achieved with 3×10^{-7} M clorgyline and 3×10^{-7} M deprenyl, respectively, and the formation rate of dityramine decreased in a corresponding manner. Preincubation with histamine or spermidine reduced the lag time without affecting the steady-state reaction rate. Higher levels of dityramine were also detected *in vivo* in rat intestine after oral administration of tyramine. These results indicate that the peroxidase-dependent metabolism of tyramine in the gut may be driven by H_2O_2 produced by MAO activities and that MAO-A is mainly responsible for this process, as well as for the oxidative deamination of tyramine. BIOCHEM PHARMACOL 55;1:37–43, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. amine oxidases; peroxidase; tyramine; intestine; hydrogen peroxide

Dietary tyramine, which is present in a number of foods and beverages [1–3], is oxidatively deaminated by monoamine oxidase (EC 1.4.3.4; MAO).§ The hypertensive crisis (cheese effect) that can occur as a result of tyramine ingestion in subjects treated with MAO inhibitors [1–5] is due to the uptake of this amine by peripheral nerves which release stored noradrenaline. The effects of tyramine are not observed under normal circumstances, except if large amounts are ingested, although there may be considerable individual variation in this response [4]. This has been interpreted as indicating that MAO plays a major role in the efficient presystemic metabolic disposition of this amine. Intestinal MAO-A has been shown to be mainly responsible for the metabolism of ingested tyramine, and inhibition of this form of the enzyme gives a pronounced cheese effect [3–7]. In a previous study [8], an alternative pathway of tyramine metabolism involving intestinal peroxidase activity was investigated. In the presence of H_2O_2, peroxidase (EC 1.11.1.7, POD) was shown to catalyse the oxidative ring-coupling of some phenol derivatives in a reaction which involves the formation of radical intermediates [9, 10]. Furthermore, purified rat intestinal POD catalyses the oxidation of tyramine via a ring-coupling reaction, forming the corresponding dimer 2,2′-dihydroxy-5,5′-bis(ethylamino)diphenyl (dityramine) [8]. This suggests that intestinal POD contributes to the first-pass metabolism of tyramine.

The activity of POD *in vivo* depends on an adequate supply of H_2O_2. Several metabolic processes may contribute to this supply and one such contributor may be MAO itself, since H_2O_2 is a product of the oxidative deamination of amines. Although some of the intestinal POD activity could arise from eosinophil infiltration [10], endogenous POD activity has also been detected in rat intestine epithelial cells [11] which contain MAO [6, 12]. Furthermore, subcellular fractionation studies have shown that the crude mitochondrial fraction of intestinal homogenate is significantly enriched in both these enzyme activities [13,

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§ *Abbreviations:* BHA, butylated hydroxyanisole; MAO, monoamine oxidase; POD, peroxidase; SSAO, semicarbazide sensitive amine oxidase.

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14]. These findings suggest that the two enzymes cooperate in the metabolism of endogenous and exogenous compounds. Intestine has been shown to contain an amine oxidase activity that is inhibited by semicarbazide (semicarbazide sensitive amine oxidase, EC 1.4.3.6.; SSAO) and a histaminase activity also belonging to the class EC 1.4.3.6. [15–20]. Since H_2O_2 is a product of the catalytic activity of this enzyme, it is conceivable that it supplies the substrate for POD-catalysed reactions. This report describes the relationships between amine oxidases and POD in the ring-coupling oxidative metabolism of tyramine by crude mitochondrial fractions of rat intestine and provides evidence that this metabolic pathway also operates *in vivo*.

MATERIALS AND METHODS

Chemicals

All the reagents were supplied by Sigma except the radiolabelled compounds, which were supplied by Amersham.

Dityramine Synthesis

Acetic anhydride (3 mmol) was slowly added to 200 mL of 1 M Na_2CO_3 containing tyramine (3 mmol) at room temperature. The acetylated product, *N*-acetyltyramine, was precipitated by adding HCl up to pH 3, then filtered and dissolved in 200 mL 0.1 M NaOH. The solution pH was then adjusted to pH 8. Horseradish peroxidase (2 mg) and H_2O_2 (1.5 mmol) were added and incubated at 37° for 24 hr. After concentration in a rotatory evaporator, di-(*N*-acetyl)tyramine was dissolved in hot methanol, dried in rotatory evaporator and crystallised in acetone. Di-(*N*-acetyl)tyramine was subsequently purified in a silica gel column (35 × 2 cm) preequilibrated with ethylacetate and ethanol (80:20, v/v). The compound was eluted with the same solution and the UV-fluorescent fractions, containing di-(*N*-acetyl)tyramine, were collected, concentrated and recrystallised in acetone. The precipitate was hydrolysed under reflux with 6 M HCl at 80° for 24 hr. After concentration, dityramine was dissolved in the minimum amount of methanol and recrystallised three times in ethyl-ether. The resulting dityramine, a brown hygroscopic powder, was analysed by elemental analysis, NMR, and mass spectroscopy. The mass spectrum was determined with a VG 70S spectrometer (VG Analytical) after direct injection with a probe for solid samples. The mass spectrum was recorded in electron ionization mode at 80 eV, 200 μamp trap current, 8000 V accelerating voltage, using a DEC micro PDP 11/83 computer at a scan rate 2 sec/decade at 1000 M/ΔM mass resolution (5% valley definition) over the 30–500 mass range. Fluorimetric characterization of dityramine was also performed using a Shimadzu RF-5000 spectrophotofluorimeter (Shimadzu Europa GmbH). The fluorescence spectra showed a pH-dependent intensity, with a maximum in emission at pH 8.6. Under our experimental conditions (Tris-HCl buffer, pH 8.2, 25°, at 315 nm excitation wavelength and slit widths of 3 nm), the spectra presented a large emission band (350–500 nm) with a maximum at 400 nm. The minimum amount of dityramine detectable was 0.30 ± 0.06 nmol (total volume 2.5 mL, $n = 5$), and the fluorescence intensity was linear up to 100 nmol with a correlation coefficient 0.996.

In vitro *Formation of Dityramine by Rat Intestinal Mitochondria*

Crude intestinal mitochondrial fractions from male Wistar rats (200–250 g) were prepared by homogenisation 1:10 (w:v) in 100 mM Tris-HCl buffer, pH 7.5 containing 0.25 M sucrose, followed by differential centrifugation [21]. Protein content was determined by the Bradford method using BSA as standard [22].

POD activity was assayed using guaiacol as substrate [10]; it was completely inhibited by 1 mM NaCN added to the assay mixture. MAO activity was assayed spectrophotometrically by the method of Weissbach et al. [23] using kynuramine as substrate and monitoring the 4-hydroxyquinoline formation rate at 328 nm. In another series of experiments, MAO-A and MAO-B activities were determined radiometrically using radiolabelled [^{3}H]serotonin, [^{14}C]phenylethylamine and [^{14}C]tyramine [24] as substrates. Selective inhibition of either MAO-A or MAO-B was achieved by incubating the mitochondrial fractions or whole homogenates with 3×10^{-7} M clorgyline or 3×10^{-7} M deprenyl, respectively, for 30 min and subsequently washing out by centrifugation [24]. The dityramine formation rate at 37° in 100 mM Tris-HCl buffer, pH 7.5, in presence of 0.6 mM tyramine was monitored spectrophotometrically at 318 nm, as described previously [8]. In preliminary experiments, the reaction mixture containing dityramine was dried, dissolved in methanol and subsequently purified by thin layer chromatography (TLC) analysis on silica gel plates (20 × 20 cm, 0.5-mm thick, Merck), using *n*-propanol-25% NH_3 (7:3 v/v) as eluent. The spot corresponding to dityramine was scraped off, dissolved in methanol and analysed by mass spectroscopy as described above.

In vivo *Formation of Dityramine in Rat Gut*

Male Wistar rats (230–280 g) were fed the day before the experiment with 5% glucose solution and received by gavage 200 mg/kg tyramine in physiological solution (5 mL/kg body weight); 90 min later, rats underwent CO_2 euthanasia and ilea were excised, weighed and homogenised in 0.154 M KCl (1:5, w/v). One mL 5% $ZnSO_4$ and 1 ml $Ba(OH)_2$ saturated solution were added to 1 mL homogenate in order to precipitate the proteins. After centrifugation at 3000 × g for 15 min, 1 mL of the supernatant was mixed with 1.5 mL 1 M Tris-HCl buffer, pH 8.2. Dityramine was measured spectrofluorimetrically at 315 and 400 nm as excitation and emission wavelengths, respectively. Standard curves were obtained by adding different amounts of dityramine (0.070–27 μg) to control

TABLE 1. Kinetic parameters and contributions of different intestinal enzymes in the amine oxidase-dependent metabolism of tyramine

	K_m (mM)	V_{max} (pmol/min/mg)	V_{max}/K_m	% Contribution[a]
MAO-A + MAO-B	169 ± 26	496 ± 29	2.93 ± 0.37	85.3 ± 5.6
MAO A	101 ± 18	293 ± 17	2.90 ± 0.39	60.5 ± 7.4
MAO B	395 ± 26	244 ± 7	0.62 ± 0.01	25.4 ± 4.1
SSAO	114 ± 22	75 ± 4	0.66 ± 0.01	15.5 ± 2.1

[a] The contribution of amine oxidases present in rat intestine was calculated from the clorgyline (10^{-10}–10^{-3} M) and semicarbazide (10^{-3} M) inhibition curves by measuring residual activity towards [^{14}C]tyramine as substrate (100 μM). These results are the mean of three experiments ± SEM.

intestine homogenates which did not show fluorescence detectable in the range 350–500 nm emission at 315 mn excitation wavelength.

Statistical Analysis

Data are presented as means ± SEM; n is the number of independent experiments. Statistical analysis of the differences within means was performed by Student's *t*-test. *P* values < 0.05 were considered significant.

RESULTS

In vitro *Tyramine Metabolism by Rat Intestinal Mitochondria*

As shown in Table 1, MAO-A present in the intestine was responsible for *ca.* 60% of the total oxidative deamination of tyramine, while MAO-B contributed only 25%. The remaining 15% was resistant to clorgyline but was inhibited by semicarbazide in a dose-dependent manner (Fig. 1). The biphasic curve obtained in the presence of different concentrations of clorgyline is consistent with tyramine being a substrate for both forms of MAO, as reported elsewhere [24]. The predominant role of MAO-A in tyramine metabolism is confirmed by the highest catalytic efficacy expressed as V_{max}/K_m ratio, which was approximately 5 times greater than the values for either MAO-B or SSAO activities (Table 1).

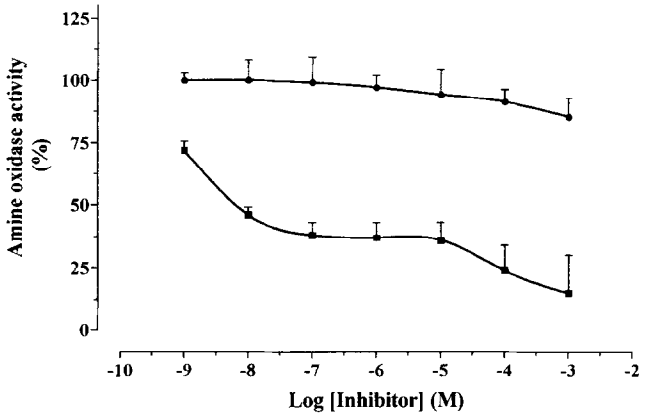

FIG. 1. Inhibition of rat intestinal tyramine oxidative deamination by clorgyline (■——■) and semicarbazide (●——●). The enzyme preparation was preincubated with the indicated inhibitor concentrations for 30 min at 37°. Residual activity towards 100 μM tyramine as substrate was measured. Values are the mean of three separate experiments. Bars indicate SEM.

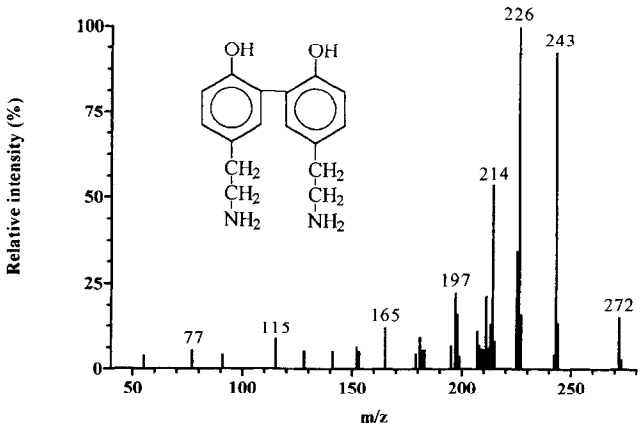

FIG. 2. Electron impact mass spectrum of dityramine formed from tyramine metabolism by rat intestinal mitochondria. The mass spectrum was determined in electron ionisation mode at 80 eV, 200 μamp trap current, 8000 V accelerating voltage at 1000 M/ΔM mass resolution (5% valley definition). The ions (m/z) were: 272 [M^+]; 243 [$M^+ - CH_2NH$]; 226 [$243^+ - OH$]; 214 [$243^+ - CH_2NH$]; 197 [$214^+ - OH$].

Incubation of rat gut mitochondria with 0.6 mM tyramine resulted in a spectral change characterised by an increase in absorbance which was maximum at 318 nm (data not shown). This spectrum was similar to that observed in a previous study [8] where tyramine was incubated in the presence of H_2O_2 and POD purified from rat intestine. The tyramine concentration used in these experiments (0.6 mM) was higher than the Km value of either intestinal MAO (see Table 1) or POD [8]. The formation rate of H_2O_2 was therefore the only limiting factor of the reaction.

Dityramine formation was confirmed by TLC and mass spectrometric analysis of the incubation mixture (Fig. 2). After an initial lag phase of *ca.* 20 min, the formation rate of dityramine was linear up to 2 hr, as shown in Fig. 3, and amounted to 17 nmol × hr^{-1} × mg $protein^{-1}$, in line with the rate of the oxidative deamination of tyramine by rat and human intestine [6]. The former reaction was suppressed by 10^{-3} M clorgyline, which inhibited kynuramine oxidation by more than 80% and by 10^{-3} M NaCN, which blocked guaiacol oxidation. The addition of H_2O_2 to the mixture containing clorgyline induced a rapid formation of dityramine (Fig. 3), but had no effect in the samples containing NaCN (data not shown). However, the presence of NaCN in the reaction mixture did not inhibit MAO activity, determined with kynuramine as a substrate for both A and B forms of the enzyme (Table 2).

Interestingly, when dityramine formation was assayed in

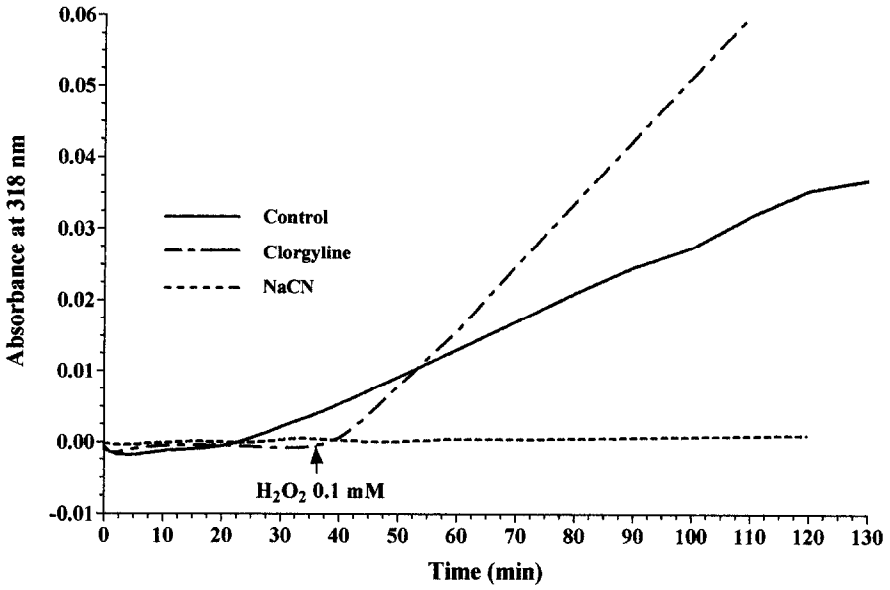

FIG. 3. Time-course of dityramine formation by rat intestinal mitochondrial fractions. The mitochondrial fractions were preincubated with clorgyline or NaCN for 30 min at 37°. Six hundred μM tyramine was added at zero time. The control sample was incubated with the same volume of buffer. H_2O_2 was added to the clorgyline-treated sample at the time indicated by the arrow. Other experimental details are given in the text. The curves shown are representative of five replicate determinations which differed from each other by less than 10% at any given time point.

the presence of 3×10^{-7} M clorgyline, a concentration which selectively inhibits MAO-A, the inhibition amounted to *ca.* 60%, whereas in the presence of 3×10^{-7} M deprenyl, which selectively inhibits MAO-B, it was only 20% (Table 2).

To assess whether other amine-oxidising enzymes promoted the formation of hydrogen peroxide for use by intestinal POD, crude mitochondria were preincubated with 0.6 mM histamine or 0.6 mM spermidine for 30 min before the addition of tyramine. As shown in Fig. 4, both histamine and spermidine reduced the lag time of the reaction, but had little effect on the steady-state rate of dityramine formation. The lag time was reduced 4- and 2-fold by preincubation with histamine and spermidine, respectively, suggesting that diamine oxidase and polyamine oxidase enzymes, which are both present in rat intestine [12, 19–20], supplied H_2O_2 for dityramine formation (Table 3).

In vivo *Formation of Dityramine in the Rat Gut*

The *in vivo* formation of dityramine in rat gut was checked by analysing the amount of dityramine present in the gut wall 90 min after tyramine ingestion. This time was selected because it is when the hypertensive effect takes place after tyramine administration in rats pretreated with MAO inhibitors [25], thus indicating the passage of tyramine from the gut into general circulation. Furthermore, a previous experiment performed on rats showed that 0.5–2 hr after the oral administration of butylated hydroxyanisole (BHA), its POD-dependent metabolite, di-BHA, reached maximum concentration in intestine as well as in plasma [26].

The amount of dityramine found in treated animals was 11.628 ± 2.84 μg $\times$ g^{-1} wet tissue (range 4.434–19.050; $n = 5$), significatively different ($P < 0.01$) from that observed in control rats (0.705 ± 0.230 μg $\times$ g^{-1} wet tissue; range 0–1.410; $n = 5$). The highest amount of dityramine detected in the whole small intestine (*ca.* 10 g wet tissue) was more than 100 μg, which corresponds to approximately 0.2% of the tyramine administered. The intestine of some control animals contained trace amounts of dityramine which were less than 7% of the values observed in treated rats.

Dityramine formation was confirmed by TLC and mass spectrometric analysis. Both the R*f* values and the mass

TABLE 2. Effects of MAO and POD inhibitors on the oxidative ring-coupling of tyramine: Evidence of dependence of POD metabolism on MAO activity

	Formation rate[a]		
	POD (Tetraguaiacol)	MAO (4-Hydroxyquinoline)	MAO & POD (Dityramine)
Control	92.2 ± 37.4 (100)	21.3 ± 4.5 (100)	17.4 ± 3.5 (100)
$+3.10^{-7}$ M Clorgyline	—	—	6.8 ± 1.0 (39)
$+3.10^{-7}$ M Deprenyl	—	—	14.0 ± 1.4 (80)
$+1.10^{-3}$ M Clorgyline	86.2 ± 20.8 (93)	4.0 ± 0.8 (19)	0 (0)
$+1.10^{-3}$ M NaCN	0 (0)	20.9 ± 2.1 (98)	0 (0)

[a] Formation rate of the different metabolic products was calculated in nmol $\times$ min^{-1} $\times$ mg protein $^{-1}$ when guaiacol was used and in nmol $\times$ hr^{-1} $\times$ mg $protein^{-1}$ when kynuramine and tyramine were added. The substrate concentrations were 6.6 mM for guaiacol and 0.1 mM and 0.6 mM for kynuramine and tyramine, respectively. Values represent the means ± SEM from five different mitochondrial preparations. Residual enzyme activity expressed as a percent of control conditions is shown in brackets.

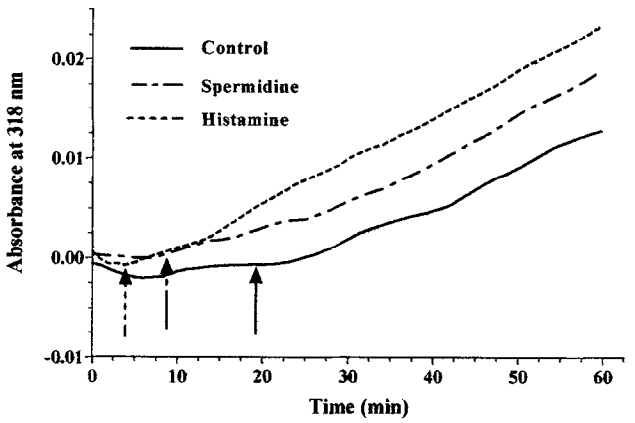

FIG. 4. Time-course of dityramine formation by rat intestinal mitochondrial fractions after preincubation with histamine or spermidine. The mitochondrial fractions were incubated for 30 min at 37° with 600 μM histamine or 600 μM spermidine. Six hundred μM tyramine was added at zero time. Experimental conditions are given in the text. Arrows indicate the end of lag phase. The curves shown are representative of five replicate determinations which differed from each other by less than 10% at any given time point.

spectrum corresponded to those observed with authentic dityramine and with dityramine formed in *in vitro* experiments as reported in Fig. 2.

DISCUSSION

Previous *in vitro* experiments have indicated that intestinal POD could make a substantial contribution to tyramine metabolism [8] by catalysing a ring-coupling reaction with H_2O_2 as cosubstrate. Several metabolic processes such as amine oxidase enzymes supply H_2O_2. The present results demonstrate the coupling between MAO and other amine oxidase enzymes and POD in the peroxidatic oxidation of tyramine by mitochondrial fractions from rat intestine, a metabolic pathway also shown to operate *in vivo* in the rat.

The oxidative deamination of tyramine by amine oxidase enzymes was determined by inhibition experiments with clorgyline (10^{-9}–10^{-3} M), a specific inhibitor of FAD-dependent amine oxidase (MAO-A and MAO-B) [27], and semicarbazide (10^{-9}–10^{-3} M), a specific inhibitor of carbonyl-dependent amine oxidase (SSAO) [15]. The results showed that MAO-A is mainly responsible for the oxidative deamination of tyramine, thus confirming a previous observation that 15% of the deaminating activity is resistant to clorgyline inhibition and due to SSAO activity [6, 16, 28].

Our data, however, showed that 10^{-3} M semicarbazide did not inhibit dityramine formation, whereas 10^{-3} M clorgyline abolished it. MAO is an intracellular enzyme, associated with outer mitochondrial membrane [29], whereas at least a portion of tissue-bound SSAO activity is associated with the plasma membrane and may be oriented on its external surface. Furthermore, within tissues SSAO activity is associated with the blood vessels supplying smooth muscles [15–16, 30]. These differences in the localization of MAO and SSAO, and hence the site of H_2O_2 generation, may account for the absence of semicarbazide effect on dityramine formation in the present study. Moreover, the observation that the steady-state rate did not increase significantly in the presence of histamine or spermidine while treatment of mitochondrial fractions with 1 mM semicarbazide prevented the reduction of the lag time caused by histamine, indicates that although other enzymes might contribute to the supply of H_2O_2 for the POD reaction, MAO-A dependent production of H_2O_2 is sufficient to give the maximal rate of POD-catalysed dityramine formation. These results suggest that the coupling of POD reaction to MAO activity is of some relevance for the metabolism of endo- and xenobiotics. Thus, for example, MAO reaction could also supply H_2O_2 for the POD-catalysed ring-coupling of BHA, which leads to the accumulation of di-BHA in human tissues, as shown in a recent study by this laboratory [31]. The involvement of MAO as a source of H_2O_2 for peroxidative reactions is also consistent with the results of Seregy et al. [32–33], who demonstrated that the stimulation of arachidonic acid metabolism by catecholamines in brain homogenates was due at least in part to the H_2O_2 generated by MAO activity.

The present study also provides evidence that rat intestine transforms ingested tyramine into dityramine *in vivo*, which indicates that POD present in gut wall is involved in the metabolism of this amine. The fact that dityramine was also detected in the rats under control conditions could be due to endogenous tyramine formed by bacteria in the gut.

TABLE 3. Effects of histamine and spermidine on dityramine formation from tyramine by rat intestinal mitochondrial fractions

Addition	Lag Phase (min)	Activity ($A_{318} \times min^{-1} \times 10^4$)
None	18.0 ± 2.9	3.3 ± 0.3
Histamine	4.3 ± 1.6[a]	5.3 ± 1.1 NS
Spermidine	8.3 ± 1.0[a]	3.8 ± 1.3 NS
Semicarbazide + Histamine	12.3 ± 1.0 NS	3.4 ± 0.2 NS

0.6 mM histamine, 0.6 mM spermidine or 1 mM semicarbazide were added 30 min before 0.6 mM tyramine. Values represent means ± SEM from three different experiments.

[a] $P < 0.05$; NS, not significant (Student's *t*-test).

Further experiments are required to clarify the *in vivo* relevance of MAO activity towards the efficiency of the POD-catalysed metabolism of tyramine. If this process is shown to operate *in vivo* in human gut, it nevertheless does not seem to proceed with sufficient efficiency to prevent the "cheese reaction" occurring in the case of MAO-A inhibition. However, the accumulation of dityramine itself, which is more hydrophobic than tyramine [8], could have toxic consequences. Furthermore, the interactive relations between MAO and POD could also play an important role in the toxicity of other sympathomimetic amines such as 5-hydroxytryptamine, which is known to be metabolized by POD to toxic intermediates [34].

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