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ORIGINAL ARTICLE

Inhibition of monoamine oxidase activity by cannabinoids

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Abstract Brain monoamines are involved in many of the same processes affected by neuropsychiatric disorders and psychotropic drugs, including cannabinoids. This study investigated in vitro effects of cannabinoids on the activity of monoamine oxidase (MAO), the enzyme responsible for metabolism of monoamine neurotransmitters and affecting brain development and function. The effects of the phytocannabinoid Δ^9-tetrahydrocannabinol (THC), the endocannabinoid anandamide (*N*-arachidonoylethanolamide [AEA]), and the synthetic cannabinoid receptor agonist WIN 55,212-2 (WIN) on the activity of MAO were measured in a crude mitochondrial fraction isolated from pig brain cortex. Monoamine oxidase activity was inhibited by the cannabinoids; however, higher half maximal inhibitory concentrations (IC_{50}) of cannabinoids were required compared to the known MAO inhibitor iproniazid. The IC_{50} was 24.7 μmol/l for THC, 751 μmol/l for AEA, and 17.9 μmol/l for WIN when serotonin was used as substrate (MAO-A), and 22.6 μmol/l for THC, 1,668 μmol/l for AEA, and 21.2 μmol/l for WIN when phenylethylamine was used as substrate (MAO-B). The inhibition of MAOs by THC was noncompetitive. *N*-Arachidonoylethanolamide was a competitive inhibitor of MAO-A and a noncompetitive inhibitor of MAO-B. WIN was a noncompetitive inhibitor of MAO-A and an uncompetitive inhibitor of MAO-B. Monoamine oxidase activity is affected by cannabinoids at relatively high drug concentrations, and this effect is inhibitory. Decrease of MAO activity may play a role in some effects of cannabinoids on serotonergic, noradrenergic, and dopaminergic neurotransmission.

Keywords Anandamide · Enzyme inhibition · Monoamine oxidase · Tetrahydrocannabinol · WIN 55,212-2

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Introduction

The enzyme monoamine oxidase (MAO; EC 1.4.3.4) catalyzes inactivation of monoamines, including neurotransmitters, such as dopamine, norepinephrine, and serotonin (5-hydroxytryptamine [5-HT]). Monoamine oxidase exists in two isoforms (Bach et al. 1988). The type A (MAO-A) metabolizes serotonin and is sensitive to inhibition by low concentrations of clorgyline, whereas the type B (MAO-B) prefers benzylamine or 2-phenylethylamine (PEA, also 2-phenethylamine) as substrate and is sensitive to inhibition by low concentrations of *l*-deprenyl (selegiline). Tyramine, tryptamine, dopamine, norepinephrine, and epinephrine are equally well oxidized by both isoforms of MAO (Youdim et al. 2006). High levels of both forms are found in the brain. Monoamine oxidase is tightly associated with the mitochondrial outer membrane (Youdim et al. 2006; Youdim and Bakhle 2006), and structures of active sites cavities in MAO-A and MAO-B were recognized (Edmondson et al. 2009). Hydrogen peroxide produced by MAO-catalyzed reactions (Pizzinat et al. 1999) has sufficient deleterious reactivity to account for associated health-related problems.

Monoamine oxidases have an important role in brain development and function, and MAO inhibitors have a range of potential therapeutic uses (Ramsay and Gravestock 2003). The first MAO inhibitory antidepressant was iproniazid; it is an irreversible and nonselective MAO

inhibitor (Fagervall and Ross 1986). Generally, MAO-A and nonselective MAO inhibitors seem to be effective in the treatment of patients with depression, panic disorder, and other anxiety disorders who have failed first-line treatments (Stahl and Felker 2008). Selective inhibitors of MAO-B may be efficacious in treating Parkinson's disease (Horstink et al. 2006). Monoamine neurotransmitters, and consequently MAO activity, are involved in many of the same processes affected by stress, various mental disorders, and neurochemical and behavioural effects of some psychotropic drugs, including cannabinoids. Cannabinoids form a group of substances originally found in the cannabis plant, but the term *cannabinoid* refers to any substance that is specifically recognized by the endocannabinoid system in the body (Howlett et al. 2002; Lambert and Fowler 2005). The endocannabinoid system consists of cannabinoid receptors, endogenous ligands, and several proteins responsible for their synthesis and degradation. Currently, there are three general types of cannabinoids: herbal cannabinoids (e.g., Δ^9-tetrahydrocannabinol [THC], cannabinol, cannabidiol), endogenous cannabinoids (e.g., anandamide, 2-arachidonoylglycerol), and synthetic cannabinoids (e.g., WIN 55,212-2, CP 55,940) (Fišar 2009).

Endocannabinoids mediate retrograde signalling from postsynaptic neurons to presynaptic ones (Kano et al. 2009). It is likely that by this mechanism, endocannabinoids play an important role in both the regulation of food intake and energy metabolism (Viveros et al. 2008), in response to chronic stress (Hill et al. 2005), and in the etiology of certain neuropsychiatric disorders such as anxiety, depression, and schizophrenia (Viveros et al. 2007; Hill and Gorzalka 2009).

An in vivo effect of the MAO inhibitor tranylcypromine on cannabinoid receptor binding and endocannabinoid contents has been recently described (Hill et al. 2008). These data suggest that the endocannabinoid system is altered by chronic antidepressant treatment; i.e., monoaminergic neurotransmission can regulate the endocannabinoid system. Conversely, MAO activity was proposed as a biochemical marker for drug dependence, including cannabinoid abuse (Faraj et al. 1994).

Understanding the biochemical targets of cannabinoids includes not only CB_1 and CB_2 receptors but also downstream targets that are regulated by chronic drug administration (Rubino et al. 2007; Moranta et al. 2004, 2009) and receptor-independent effects (Howlett et al. 1989; Tahir and Zimmerman 1991; Steffens and Feuerstein 2004). It was shown that endocannabinoids, phytocannabinoids, and synthetic cannabinoid receptor agonists are able to cause changes in integrated mitochondrial function, directly, in the absence of cannabinoid receptors (Athanasiou et al. 2007). There are pieces of evidence for functional neural interactions between the cannabinoid and dopamine receptor systems (Laviolette and Grace 2006); disturbances in this neural circuitry may be concerned in addiction, schizophrenia, or Parkinson's disease. Cannabinoids modulate the firing of monoaminergic neurons and release of dopamine (Cheer et al. 2007), norepinephrine (Mendiguren and Pineda 2006), and serotonin (Sagredo et al. 2006). The interest in the interaction of cannabinoids with the serotonin system was renewed in the context of the role of serotonergic neurons in mediating cannabinoid effects such as antiemesis, hypothermia, analgesia, sleep, and appetite stimulation. Moreover, cannabinoid receptors could play a role in the regulation of the serotonin transporter activity (Kenney et al. 1999). Serotonin uptake was inhibited at higher doses of THC, anandamide (*N*-arachidonoylethanolamide [AEA]), or WIN 55,212-2 (WIN); the inhibition was noncompetitive (Velenovská and Fišar 2007). Several lines of evidence suggest that the endocannabinoid system plays a role in the regulation of mood or anxiety (Rubino et al. 2007), as well as in the pathogenesis and treatment of depression and other stress-related disorders (Pacher et al. 2006; Viveros et al. 2007; Mangieri and Piomelli 2007; Jans et al. 2007; Gorzalka et al. 2008; Hill and Gorzalka 2009; Hill et al. 2009) accompanied by disturbed serotonergic and noradrenergic neurotransmission. So, it is not surprising that the endocannabinoid system is altered by chronic treatment with antidepressants, including serotonin and/or norepinephrine reuptake inhibitors and MAO inhibitors (Hill et al. 2008).

Incomplete data exist on the effect of cannabinoids on monoaminergic neurotransmission, and the extent to which the endocannabinoid system is modulated by monoamines is not well understood. The acute administration of the cannabinoid receptor agonists, THC, and WIN induced differential effects on the synthesis of monoamine neurotransmitters (Moranta et al. 2004). Little is known on the regulation of dopamine, norepinephrine, and serotonin catabolism in cannabinoid addiction. Early studies found different effects of cannabinoids on MAO activity; THC elicits inhibitory (Mazor et al. 1982), stimulatory (Banerjee et al. 1975; Chakrabarty et al. 1976; Banerji et al. 1977; Gawienowski et al. 1982), or no effects (Clarke and Jandhyala 1977; Schurr and Rigor 1984). There is insufficient information about different mechanisms that underlie the coupling of the cannabinoid system with monoaminergic systems in the brain; so, the study of the changes of MAO activity triggered by cannabinoids could contribute to the understanding of the mechanism of the pharmacological effects of cannabinoids and their interactions with other drugs.

The present study was designed to analyze effects of the phytocannabinoid THC, the endocannabinoid AEA, and the synthetic cannabinoid receptor agonist WIN on MAO activity, since we hypothesize that cannabinoids can

modulate monoaminergic neurotransmission in the brain not only by the activation of presynaptic CB_1 receptors but also by their direct effect on the MAO activity. The in vitro effects of these cannabinoids on MAO activities in mitochondria isolated from pig brains were examined. Brain mitochondria were assayed for MAO-A and MAO-B using serotonin and PEA as substrates. Finally, we studied changes in kinetic parameters of MAO activity in the presence of different concentrations of cannabinoids to determine the mechanism of inhibition.

Materials and methods

Brain mitochondrial MAO preparation for inhibitory and kinetic experiments

Fresh pig brains were obtained from the slaughterhouse and immediately placed to ice-cold buffered sucrose (0.32 mol/l sucrose, 4 mmol/l HEPES; pH 7.4). All subsequent procedures were performed at 0 to 4°C. Brain cortex was separated without cerebellum, brain stem, and most of the midbrain. The brain cortex was gently homogenized in ten volumes (w/v) of ice-cold 0.32 mol/l buffered sucrose supplemented with aprotinin (competitive serine protease inhibitor), by means of a homogenizer with Teflon piston. Mitochondria were prepared by a standard differential centrifugation method (Whittaker 1969). Briefly, the homogenate was centrifuged at 1,000*g* for 10 min to remove unbroken cells, nuclei, and cell debris. The supernatant was carefully decanted; the pellet was resuspended in buffered sucrose and centrifuged again under the same conditions. Supernatants were collected and recentrifuged at 10,000*g* for 15 min. The final pellet containing mitochondria was washed twice with buffered sucrose (10,000 g, 15 min), resuspended to a protein concentration of 20 to 40 mg/ml, and stored at -70°C until the assays. Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin as the standard.

Monoamine oxidase-A and MAO-B assays

Monoamine oxidase-A and MAO-B activities were determined radiochemically by a modification of the previously published method (Ekstedt 1976; Egashira et al. 1978, 1999; Ozaita et al. 1997) with either [^{14}C]5-HT (maximum concentration, 100 μmol/l) or [^{14}C]PEA (maximum concentration, 10 μmol/l) as substrates, respectively. At such concentrations, these amines have been shown to behave as specific substrates for the A and B isoforms of MAO, respectively (Fowler and Tipton 1981; Youdim et al. 2006).

Mitochondria were diluted in modified Krebs–Henseleit buffer without Ca^{2+} (KH solution; 118 mmol/l NaCl, 4.7 mmol/l KCl, 1.2 mmol/l KH_2PO_4, 1.2 mmol/l $MgSO_4 \cdot 7H_2O$, 25 mmol/l $NaHCO_3$, 11.1 mmol/l glucose; pH 7.4). The reaction mixture containing the crude MAO and various drug concentrations was preincubated in round-bottom glass tubes for 60 min at 37°C. Reaction was started by addition of radiolabeled substrate, and MAO activity was measured at 37°C for 30 min for MAO-A and for 1 min for MAO-B.

MAO-A activity was measured using [^{14}C]5-HT solution with specific activity of approximately 37 kBq/ml, which was prepared by mixing of [^{14}C]5-HT stock solution (5-HT binoxalate 5-[2-^{14}C], specific activity of 1.85 GBq/mmol, radioactive concentration of 3.7 MBq/ml, radiochemical purity greater than 99%; American Radiolabeled Chemicals, Inc., St. Louis, MO, USA) with KH buffer and unlabeled (cold) serotonin (5-HT hydrochloride; Sigma-Aldrich Co., St. Louis, MO, USA).

Monoamine oxidase-B activity was measured using [^{14}C]PEA solution with specific activity of approximately 37 kBq/ml, which was prepared by mixing of [^{14}C]PEA stock solution (PEA [ethyl-1-^{14}C] hydrochloride, specific activity of 2.03 GBq/mmol, radioactive concentration of 3.7 MBq/ml, radiochemical purity greater than 99%; American Radiolabeled Chemicals) with unlabeled PEA (PEA hydrochloride, Sigma-Aldrich Co.) solution in KH buffer.

The exact proportion of radioactively labeled compound and "cold" compound was 1:100 by volume. The final sample volume was 250 μl containing 200 μg of protein. The reaction was stopped by the addition of 250 μl of 2 N hydrochloric acid. The reaction products, i.e., the corresponding aldehydes of 5-HT and PEA after oxidative deamination, were extracted into benzene/ethyl acetate 1:1 (v/v), and the radioactivity of the extracts in the organic phase were measured by liquid scintillation counting (LS 6000IC; Beckman Instruments, Inc., Fullerton CA, USA). Both MAO assays were performed over times where product formation was shown to proceed linearly under the condition used so that the values obtained corresponded to the initial velocities of the enzyme-catalyzed reaction.

Δ^9-Tetrahydrocannabinol, AEA, and WIN 55,212-2 mesylate salt were purchased from Sigma-Aldrich Co. Since THC, AEA, and WIN are virtually insoluble in aqueous media and organic solvents of these drugs are effective inhibitors of MAO, the cannabinoids were sonicated in KH solution to obtain homogenous suspensions. Iproniazid (nonselective MAO inhibitor) was used in the form of phosphate salt; clorgyline (selective MAO-A inhibitor) and pargyline (selective MAO-B inhibitor) were used in the form of hydrochlorides (all purchased from Sigma-Aldrich Co.).

Inhibition of MAO-A and MAO-B activities

Dependence of MAO activity on drug concentration was measured at the final concentrations 0 to 3,000 μmol/l drug (at least seven concentrations). The effect of THC, AEA, WIN, and iproniazid was examined. Blank values were obtained by addition of the hydrochloric acid before the substrate was added.

Monoamine oxidase-A activity was measured using 16 μmol/l [^{14}C]5-HT solution. The final [^{14}C]5-HT concentration in samples was 3.2 μmol/l. Monoamine oxidase-B activity was measured using 50 μmol/l [^{14}C]PEA solution. The final [^{14}C]PEA concentration in samples was 10 μmol/l. The remaining MAO-A and MAO-B activities were expressed as portions of control basal activity and plotted as a function of the drug concentration.

Enzyme kinetics of MAO-A and MAO-B

Steady-state kinetic constants (K_m, Michaelis constant, and V_{max}, maximum rate) were determined from studies of the effects of substrate concentration on the initial reaction rate of MAO-A or MAO-B in the absence of drugs and in the presence of different concentrations of THC (10–100 μmol/l), AEA (100–1,000 μmol/l), or WIN (10–100 μmol/l).

Kinetic constants for MAO-A were assessed with seven different concentrations of [^{14}C]5-HT (5, 8, 10, 12.5, 25, 50, 100 μmol/l) using aliquots of 250 μmol/l [^{14}C]5-HT solution. Blank values were obtained by the addition of 100 μmol/l clorgyline before the substrate was added.

Similarly, kinetic constants for MAO-B were determined with seven different concentrations of [^{14}C]PEA (0.5, 0.8, 1, 1.25, 2.5, 5, 10 μmol/l) using aliquots of 25 μmol/l [^{14}C]PEA solution. Blank values were obtained by the addition of 1 μmol/l pargyline before the substrate was added.

Data analysis

One way to measure the effect of an inhibitor is to measure enzyme rate at a single concentration of a substrate with varying concentrations of an inhibitor. Inhibition of MAO activity by drugs was analyzed using the four-parameter logistic function (SigmaPlot; Systat Software, Inc., Richmond, CA, USA), to establish the half maximal inhibitory concentration (IC_{50}) and Hill slope (coefficient). The Hill slope characterizes the slope of the curve at its midpoint, and it is used to determine the degree of cooperativity of the ligand binding to the enzyme. A coefficient of 1 indicates completely independent binding, numbers greater than one indicate positive cooperativity, while numbers less than one indicate negative cooperativity. IC_{50} represents the concentration of a drug that is required for 50% inhibition of enzymic reaction at a specific substrate concentration (median effective concentration). IC_{50} values are dependent on conditions under which they are measured; so, data obtained are valid for the existing enzyme concentration and the type of inhibition. Because the inhibitory potency is related to the dissociation constant of the enzyme–inhibitor complex (K_i, the reciprocal of the binding affinity of the inhibitor to the enzyme), this parameter can be a predictor of in vivo inhibitory potency. The analysis shows that K_i is equal to IC_{50} under conditions of either noncompetitive (when the affinity of inhibitor to the free enzyme and to the enzyme–substrate complex is the same) or uncompetitive (when inhibitor binds only to enzyme–substrate complex, at substrate concentration >> K_m) kinetics; however, if the inhibitor is a competitive inhibitor of monosubstrate reaction, K_i will be equal to $IC_{50}/(1+S/K_m)$, where S is substrate concentration (Cheng–Prusoff equation) (Cheng and Prusoff 1973).

Another experimental design is to measure enzyme rate at a variety of substrate concentrations in the presence and absence of an inhibitor. The MAO activity obeyed simple Michaelis–Menten kinetics with maximum rate V_{max} and Michaelis constant K_m (numerically equal to the substrate concentration required at rate of $V_{max}/2$). Different effects of the inhibitor on V_{max} and K_m result from its binding to the enzyme, to the enzyme–substrate complex, or to both. Reversible enzyme inhibitors can be classified as competitive, noncompetitive, uncompetitive, or mixed. We used nonlinear regression (SigmaPlot with Enzyme Kinetics Module) to calculate the parameters V_{max} and K_m and to determine the mechanisms of inhibition. The information criterion of Akaike (1974) with a second-order correction for small sample sizes (AICc) was used as a tool for model selection, i.e., as a measure of the goodness of fit of an estimated type of inhibition.

Statistical analysis

All data presented are expressed as the mean ± standard deviation (SD), and each value reflects the mean of at least five independent measurements. Results were analyzed by STATISTICA (data analysis software system, version 9.0; StatSoft, Inc., Tulsa, OK, USA).

Results

Cannabinoids inhibit MAO

The effects of cannabinoids (THC, AEA, or WIN) on MAO activity (i.e., potency and nature of the interaction) in pig brain mitochondria were assessed in comparison with iproniazid, a well-known potent irreversible MAO inhibitor.

As was to be expected, the treatment of mitochondria with iproniazid resulted in the decrease of MAOs activity at micromolar drug concentrations (Fig. 1). Because inhibitory potency is related to the K_i and K_i is related to IC_{50} (Cheng and Prusoff 1973), IC_{50} values were determined by analyzing dose–response curves of the MAO activity towards the cannabinoid concentration (Tables 1 and 2). It must be noted that iproniazid (and other irreversible inhibitors) displays time-dependent inhibition, and its potency cannot be correctly characterized by an IC_{50} value. The apparent IC_{50} for inhibition of MAO by iproniazid was 1.55±0.12 μmol/l for MAO-A and 1.21±0.06 μmol/l for MAO-B (mean ± SD, n=10).

Among the tested cannabinoids, THC was the most potent inhibitor of MAO-A. *N*-Arachidonoylethanolamide fully inhibited the MAO-A activity but with lower potency compared to THC. WIN was an inhibitor with equal potency as THC, whereas MAO-A was not fully inhibited even at high WIN concentrations (lower efficacy). The residual activities of MAO-A were about 4.6%, 30.6%, and 0.0% with THC, WIN, and AEA, respectively (Fig. 1a). Both THC and WIN displayed positive cooperativity of the binding to MAO-A; AEA showed noncooperativity (Table 1).

The effects of the same drugs also were evaluated on pig brain mitochondrial MAO-B activity using PEA as substrate. The THC was the most potent inhibitor of MAO-B. *N*-Arachidonoylethanolamide fully inhibited the MAO-B activity but with much lower potency compared to THC. WIN was an inhibitor with equal potency as THC, whereas MAO-B was not fully inhibited even at high WIN concentrations. The residual activities of MAO-B were about 11.1%, 42.2%, and 0.0% with THC, WIN, and AEA, respectively (Fig. 1b). Both AEA and WIN displayed noncooperativity of the binding to MAO-B; THC showed positive cooperativity (Table 2).

Kinetic parameters of MAO activity

Isothermal saturation curves for MAOs were measured following incubation of mitochondria with different concentrations of radiolabeled substrates ([^{14}C]5-HT, [^{14}C]PEA), and kinetic parameters of MAOs activity were calculated using nonlinear regression analysis (Table 3).

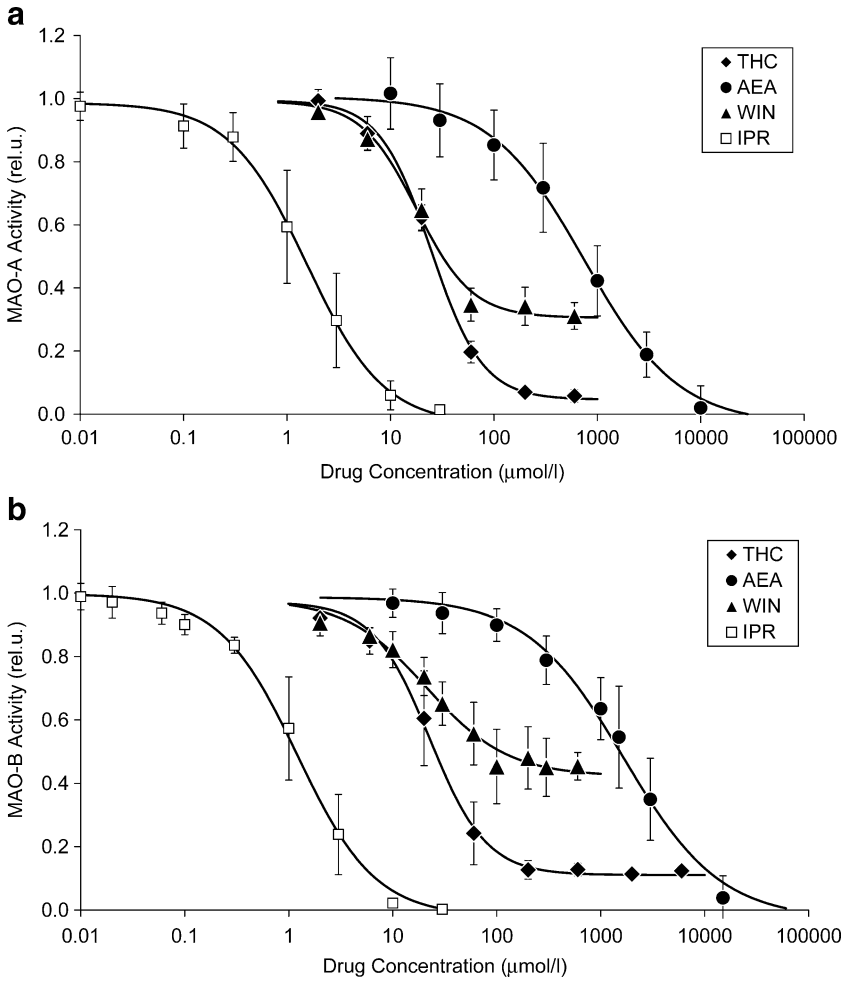

Fig. 1 Inhibition of basal MAO activity by cannabinoids in a brain crude mitochondrial fraction. Concentration–response curves are displayed as plots of the initial activity of MAO against the cannabinoid concentration. The samples were incubated with drugs at 37°C for 60 min, and the reaction was started **a** by the addition of 3.2 μmol/l [^{14}C]serotonin and **b** by the addition of 10 μmol/l [^{14}C]phenylethylamine. Following incubation at 37°C for 30 min (**a**) or for 1 min (**b**), the reaction was stopped by the addition of hydrochloric acid. The samples were measured in doublets, and blank values were deducted. Median effective concentrations (IC_{50}) were calculated using nonlinear regression analysis software (Tables 1 and 2). Values are means ± SD; the means were calculated at least from five independent measurements. Lines represent the best fitted curves using the four-parameter logistic function. ♦ THC; ● anandamide (AEA); ▲ WIN 55,212-2 (WIN); □ iproniazid (IPR)

Table 1 Inhibition of MAO-A activity in brain crude mitochondrial fraction

	IC_{50} (μmol/l)	Hill slope	Mechanism of inhibition	*n*
THC	24.7±1.3	1.75±0.15	Noncompetitive	5
Anandamide	751±99	0.95±0.10	Competitive	15
WIN 55,212-2	17.9±1.8	1.62±0.24	Noncompetitive	5

The samples were incubated with drugs at 37°C for 60 min, and the reaction was started by the addition of 3.2 μmol/l [^{14}C]serotonin; following incubation at 37°C for 30 min, the reaction was stopped by the addition of hydrochloric acid. The samples were measured in doublets and blank values were deducted

Values are means ± SD. IC_{50} drug concentration that is required for 50% inhibition of enzyme activity, Hill slope describes the cooperativity of the drug binding, *n* number of measurements

Kinetic parameters of MAO activity in pig brain crude mitochondrial fraction towards 5-HT as substrate demonstrated significantly lower V_{max}, higher K_m, and lower V_{max}/K_m compared to PEA as substrate. The ratio V_{max}/K_m can be considered as an efficiency criterion of an enzyme system; so, MAO efficiency was found much higher using PEA as substrate (MAO-B) compared to 5-HT as substrate (MAO-A).

Mechanism of MAO inhibition by cannabinoids

To determine the mechanism of MAO inhibition by the three types of cannabinoids, the effects of various concentrations of these drugs (10–100 μmol/l THC, 100–1,000 μmol/l AEA, 10–100 μmol/l WIN) on MAO kinetics were studied. The reversibility of the inhibition of MAO by drugs was proven using the dilution method (Ulus et al. 2000). A nonlinear approach is superior to classical methods of data analysis for enzyme inhibitor kinetics (Maurer and Fung 2000); however, graphic methods provide an important supplement that ought not to be neglected. So, we used nonlinear regression (SigmaPlot with Enzyme Kinetics Module) to calculate the parameters V_{max} and K_m and to determine the mechanism of inhibition. Enzyme kinetic equations for competitive, noncompetitive, uncompetitive, and mixed inhibition type were tested. The lowest AICc value in combination with some graphic processing of experimental data was used for selection of the best model.

When the kinetics of the interactions of cannabinoids with the enzyme were assessed, SigmaPlot calculation of MAO-A kinetics revealed that the interaction was competitive for AEA (i.e., apparent increases in K_m values with no changes in V_{max} values in the presence of the inhibitor and therefore decreased V_{max}/K_m ratios) and noncompetitive for THC or WIN (i.e., apparent decreases in V_{max} values with no changes in K_m values in the presence of the inhibitor and therefore decreased V_{max}/K_m ratios). For illustrative purposes Lineweaver–Burk plots (double reciprocal plots) of the MAO inhibition by cannabinoids using [^{14}C]5-HT as substrate are shown (Fig. 2a), which can visually distinguish competitive, noncompetitive, mixed, and uncompetitive inhibitors. Noncompetitive inhibition of MAO-A by THC or WIN means that the K_i (describing the affinity between THC or WIN and MAO-A in brain crude mitochondrial fraction) is equal to IC_{50} in the Table 1. *N*-Arachidonoylethanolamide is a competitive inhibitor of MAO-A and K_i value calculated using the Cheng–Prusoff equation equals 508 μmol/l.

The effects of the same cannabinoids also were evaluated on kinetic parameters of pig brain mitochondrial MAO-B activity. SigmaPlot calculation of MAO-B kinetics revealed that the interaction was uncompetitive for WIN (i.e., apparent decreases both in K_m and V_{max} values in the presence of the inhibitor and unchanged V_{max}/K_m ratios) and noncompetitive for THC or AEA. For illustrative purposes Lineweaver–Burk plots of the inhibition by cannabinoids using [^{14}C]PEA as substrate are shown

Table 2 Inhibition of MAO-B activity in brain crude mitochondrial fraction

	IC_{50} (μmol/l)	Hill slope	Mechanism of inhibition	*n*
THC	22.6±2.1	1.60±0.22	Noncompetitive	6
Anandamide	1,668±177	0.93±0.08	Noncompetitive	13
WIN 55,212-2	21.2±2.9	1.11±0.15	Uncompetitive	10

The samples were incubated with drugs at 37°C for 60 min, and the reaction was started by the addition of 10 μmol/l [^{14}C]phenylethylamine; following incubation at 37°C for 1 min, the reaction was stopped by the addition of hydrochloric acid. The samples were measured in doublets, and blank values were deducted

Values are means ± SD. IC_{50} drug concentration that is required for 50% inhibition of enzyme activity, Hill slope describes the cooperativity of the drug binding, *n* number of measurements

Table 3 Kinetic parameters of MAO activity in brain crude mitochondrial fraction towards serotonin (5-HT) or PEA as substrates

Substrate	V_{max} (pmol/[min · mg protein])	K_m (μmol/l)	V_{max}/K_m (10^{-6} · l/[min · mg protein])	*n*
[^{14}C]5-HT	24.8±5.3	6.7±2.3	3.89±0.68	15
[^{14}C]PEA	486±85	0.44±0.14	1,185±207	10

The reaction mixture was preincubated for 60 min at 37°C. Reaction was started by addition of radiolabeled substrate, and MAO activity was measured at 37°C for 30 min for [^{14}C]5-HT and for 1 min for [^{14}C]PEA. Kinetic constants for MAOs were assessed with seven different concentrations of substrate (5–100 μmol/l [^{14}C]5-HT; 0.5–10 μmol/l [^{14}C]PEA). Final protein concentration in samples was 800 μg/ml. Blank values were obtained by the addition of 100 μmol/l clorgyline, or 1 μmol/l pargyline before the substrate was added

Values are means ± SD. K_m Michaelis constant, V_{max} maximum reaction rate, *n* number of measurements

(Fig. 2b). Uncompetitive or noncompetitive inhibition of MAO-B by THC, AEA, or WIN means that their inhibition constants K_i equal their IC_{50} in Table 2.

Discussion

The current study demonstrates that cannabinoids can inhibit MAO activity. The inhibitory potency and mechanism of inhibition of THC, AEA, and WIN towards pig brain MAO-A and MAO-B was assessed. The results indicate that some effects of cannabinoids on brain functions could be related to a nonreceptor modulation of monoaminergic neurotransmission.

Under the experimental conditions of the present study, the use of the selective substrates 5-HT for MAO-A and PEA for MAO-B allowed the inhibitory effects and nature of the interactions of cannabinoids on the two MAO isoforms to be evaluated separately. Cannabinoids used in the present experiment were not specific MAO inhibitors; thus, it was necessary to use high concentrations of drugs to inhibit MAO activity in pig brain mitochondria. Inhibition of 5-HT-metabolizing (MAO-A) activity by THC, AEA, and WIN was very similar to the inhibition of PEA-metabolizing (MAO-B) activity. The inhibitory potency of THC and WIN towards MAOs was rather similar; however, WIN showed lower efficacy. The inhibitory potency of AEA towards MAOs was only moderate.

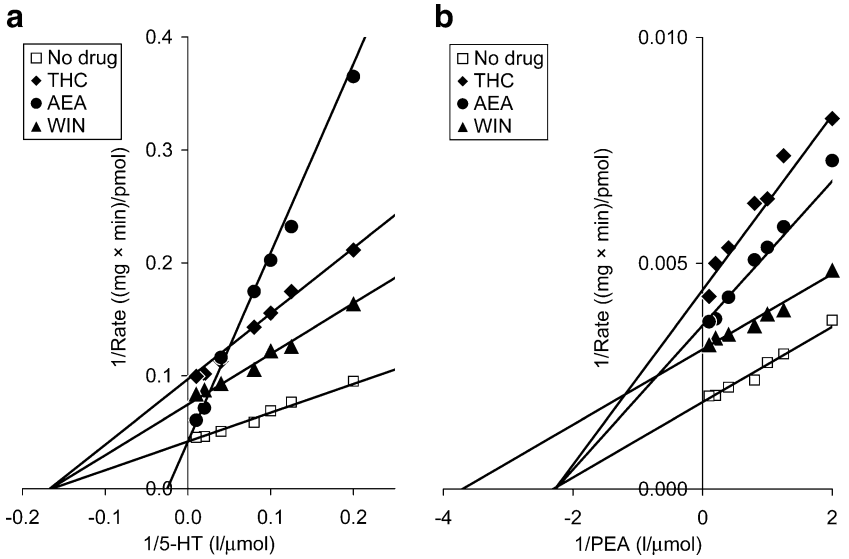

Fig. 2 Lineweaver–Burk plot of the MAO inhibition by cannabinoids in a brain crude mitochondrial fraction. The samples were incubated for 60 min at 37°C in the absence or presence of the drug. The reaction was started **a** by the addition of [^{14}C]serotonin in a final concentration of 5, 8, 10, 12.5, 25, 50, or 100 μmol/l, and samples containing no drug (□), 30 μmol/l THC (♦), 1,000 μmol/l anandamide (AEA, ●), or 30 μmol/l WIN 55,212-2 (WIN, ▲) were incubated for 30 min at 37°C; **b** by the addition of [^{14}C]phenylethylamine in a final concentration 0.5, 0.8, 1, 1.25, 2.5, 5, or 10 μmol/l, and samples containing no drug (□), 30 μmol/l THC (♦), 600 μmol/l AEA (●), or 10 μmol/l WIN (▲) were incubated for 1 min at 37°C. The rest of the assay was performed as described in the text. Each point represents the mean of duplicate determinations of one typical experiment. Bisectors were constructed on the basis of parameters of enzyme kinetics for the noncompetitive, competitive, or uncompetitive type of inhibition calculated using nonlinear regression with maximum rate V_{max}, Michaelis constant K_m, and the dissociation constant of the enzyme–inhibitor complex K_i: **a** V_{max}=24 pmol/(mg protein · min), K_m= 6.1 μmol/l, and K_i=24, 359, and 37 μmol/l for THC, AEA, and WIN, respectively; **b** V_{max}=516 pmol/(mg protein · min), K_m=0.43 μmol/l, and K_i=23, 690, and 17 μmol/l for THC, AEA, and WIN, respectively

In the present study, all three agonists of cannabinoid receptors inhibited MAO at high concentrations only. These concentrations were much higher than the physiologically active concentrations of cannabinoids in vivo. *N*-Arachidonoylethanolamide concentrations in selected brain regions were below 100 pmol/g (Felder et al. 1996; Bazinet et al. 2005), a plasma concentration of about 0.1 μmol/l of Δ^9-THC was described as sufficient for production of psychotropic effects (Cone and Huestis 1993; Naef et al. 2004), and WIN in nanomolar concentrations inhibits excitatory synaptic transmission via CB_1 receptors (Németh et al. 2008; Blair et al. 2009). However, the brain levels of drugs are a reflection not only on their plasma concentrations but also on their distribution between plasma and red blood cells (Fišar et al. 1996, 2006). For drugs such as cannabinoids, which are highly bound to fat tissues, lipid bilayers, and plasma proteins, the relative ratio of free to bound drug is important for establishing of brain levels (Reid and Bornheim 2001). Δ^9-Tetrahydrocannabinol concentrations in brain are higher than THC concentrations in blood (Mura et al. 2005). There is no selective distribution of THC in any one area of the brain (Layman and Milton 1971), but it was found that THC was accumulated preferentially in neurons (Monnet-Tschudi et al. 2008). The possibility exists that MAO activity is affected due to long-lasting action of lipophilic cannabinoids and their accumulation in mitochondrial membrane during chronic treatment.

Competitive inhibition of MAO-A by AEA would be consistent with AEA binding to active sites of the MAO-A, although the possibility that the compound binds to a distinct site and exerts its effects through a conformational change cannot be ruled out. It is possible to speculate why competitive inhibition of 5-HT-metabolizing activity by AEA, but not by THC or WIN, occurs. The AEA molecule features by much greater flexibility compared to THC or WIN. The four most common conformations of AEA are as follows: (1) extended conformation, (2) *U*-shaped conformation, (3) *J*-shaped conformation, and (4) helical conformation (Barnett-Norris et al. 1998). It has been confirmed that high affinity binding of AEA to CB_1 receptors depends on the great flexibility of the AEA acyl chain (Thomas et al. 1996; Tong et al. 1998). It is supposed that the flexibility of the AEA molecule facilitates its binding to the same active site as 5-HT on the MAO molecule.

Inhibitory and kinetic parameters calculated in this article result from total concentrations of cannabinoids added to crude brain mitochondrial fraction. The situation in various tissues in vivo may be different from our experimental conditions. This was the reason why the effects of cannabinoids on MAOs activity were compared with the effect of a well-known MAO inhibitor such as iproniazid. The results show that THC and WIN are much weaker MAOs inhibitors compared to iproniazid; however, they possess the same in vitro inhibitory potency compared to many antidepressants (Egashira et al. 1996, 1999; Gnerre et al. 2001) or antipsychotics (Suzuki et al. 1988).

In general, cannabinoids were found to be weak nonselective MAO inhibitors. The present study revealed THC and AEA are full inhibitors, although with rather low potency, of both MAO-A and MAO-B enzymes. *N*-Arachidonoylethanolamide was a weaker inhibitor of both MAO isoforms compared to THC. The cannabinoids showed little discrimination between inhibitions of the two MAO isoforms. The analysis showed that THC inhibits both MAO-A and MAO-B noncompetitively. *N*-Arachidonoylethanolamide seems to be a competitive inhibitor of MAO-A and a noncompetitive inhibitor of MAO-B; WIN shows noncompetitive inhibition of MAO-A and uncompetitive inhibition of MAO-B.

Although it is always difficult to extrapolate from in vitro studies to the clinical reality, the present results suggest that the examined cannabinoids might contribute to an inhibitory effect on MAO, and a systematic in vivo investigation of the cannabinoids' effect on MAO activity is needed. The exact physiological role of MAO inhibition by cannabinoids is not known; however, the effects of cannabinoids on MAO activity may contribute to non-receptor actions of cannabinoids participating in modulation of monoamine neurotransmission in the brain. It can be speculated that the inhibition of MAO by cannabinoids could contribute to the regulation of mood and emotions or to the interindividual differences in the drug response.

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