Platelet Monoamine Oxidase Kinetics, Alcoholism Subtypes and Cigarette Smoking

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Key Words
Platelets · Monoamine oxidase · Alcoholism subtypes · Serotonin · Cigarette smoking · Alcohol withdrawal

Abstract
In trying to dissociate the effect of alcohol and tobacco use on platelet monoamine oxidase-B (MAO-B) activity, we compared the enzyme kinetics in controls (n = 66) and alcohol-dependent patients (n = 81), subdivided according to the severity of both, alcohol and tobacco use. Platelet MAO-B kinetics was measured spectrophotofluorimetrically in chronic alcohol intoxication and after 3 weeks abstinence. In alcoholic patients, an increased Michaelis-Menten constant (16%, p < 0.01) was shown, notwithstanding smoking status. Maximal velocity did not differ between patients and controls when adjusted for smoking. In cigarette smokers, a highly significant dose-dependent reduction of platelet MAO velocity (40%, p < 0.001) was demonstrated, with a similar degree of reduction in patients and controls. Tobacco use itself had no influence on MAO affinity. No differences were shown between subtype 1 and 2 alcoholics, or between the day of admission and the 21st day of abstinence. In conclusion, it seems that both, alcohol and tobacco consumption, may contribute to the lowering of overall platelet MAO-B activity. The effect of alcohol is small, due to interference with substrate binding, and not alteration of catalytic activity. In contrast, the effect of cigarette smoking is pronounced and relates to the dose-dependent reduction of platelet MAO velocity, with no influence on its affinity.

Introduction
Monoamine oxidase (MAO), a mitochondrial enzyme that catalyzes the oxidative deamination of physiologically active biogenic amines, including catecholamines and serotonin, has an important regulatory role in brain neurotransmission. Numerous studies have evidenced its contribution to complex human behavior and etiology of psychiatric and neurologic disorders [1–4].

MAO is abundantly present in the brain and peripheral tissues. From its two isoenzymes, A and B, differing in substrate preference, inhibitor specificity and expression pattern [5] only MAO-B is found in human platelets. Platelet MAO is encoded by the same gene as its central counterpart [6], which may help to explain notable biochemical and pharmacological similarities between MAO-B in platelets and in serotonergic (5HT) neurons of the brain, where the B subtype is predominantly found.
Owing to easy accessibility of human blood, platelet MAO has long been investigated as a potential peripheral indicator of brain function [3–5, 7]. Reported associations of low platelet MAO activity and several psychiatric/behavioral disorders, point to the possibility that this parameter may serve as a trait (genetic) or state (disease)-dependent marker in biological psychiatry. Recent studies indicating that its reduced activity does not directly predispose individuals to development of a psychiatric disorder, but is related to specific personality traits, which, in turn, represent a vulnerability factor for a disorder [2–4], give a support to an early so-called ‘vulnerability hypothesis’ for platelet MAO [8].

Since the mid-1970s a large number of reports, although not all, has associated low platelet MAO activity and alcohol abuse, especially in type 2 alcoholics [7, 9–12]. According Cloninger type 1/ type 2 concept [13], type 2 alcoholics have a strong genetic load, early age of onset of alcohol problems and are characterized by more psychiatric disturbances and social complications than type 1 alcoholics. Association between low platelet MAO and 5HT-related personality measures, such as sensation seeking and impulsivity, repeatedly shown in type 2 alcohol-dependent patients [13–15], suggests their brain 5HT system could be more extensively impaired.

In spite of early evidence that cigarette smoking has a direct inhibitory effect on the platelet MAO activity [16], most studies have not controlled for tobacco use. Findings of dramatically reduced MAO activity in brains and peripheral organs of smokers [17, 18], obtained by positron emission tomography, seriously questioned the significance of most previous platelet MAO research in alcoholism. Namely, the majority of alcoholics are also heavy smokers [19], so adjustment for smoking is imperative. During the past years, a number of studies have reinvestigated association between platelet MAO and tobacco and alcohol use but their relationship remained controversial. Several studies which have controlled for smoking, demonstrated no relation between low platelet MAO and alcoholism [11, 20, 21], while in other reports, differences in either activity or MAO-B protein between alcoholics and controls persisted after smoking was taken into account [22–24]. Experimental studies in a primate model supported a relation between activity of platelet MAO and type 2 alcoholism [25]. Oreland et al. [26] concluded that smoking can explain only part of the association between platelet MAO and alcoholism and emphasized the need for further longitudinal investigations. Surprisingly, there are still studies on platelet MAO where information of the patients’ smoking status was not reported [27, 28].

The aim of the present study was to further reevaluate the relationship between platelet MAO activity, cigarette smoking and alcohol dependence. Specific aims were (1) to explore to which extent the inhibitory effect of cigarette smoke contributes to the association (if any) between subtypes of alcoholics and platelet MAO; (2) to relate kinetic parameters determining MAO activity to severity of both, alcohol and tobacco use; (3) to compare platelet MAO activity in acute intoxication and after withdrawal from alcohol. In addition, several potentially confounding methodological issues are evaluated.

### Table 1. Descriptive information on the sample

<table>
<thead>
<tr>
<th>Subject type</th>
<th>Smoking (cig./day)</th>
<th>Subjects</th>
<th>Age, years (range)</th>
<th>Comorbidity</th>
<th>Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>AD</td>
<td>PTSD</td>
</tr>
<tr>
<td>Type 1</td>
<td>0</td>
<td>11</td>
<td>49 (38–65)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>20 (19 ± 3)</td>
<td>18</td>
<td>46 (29–55)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>40 (39 ± 14)</td>
<td>13</td>
<td>47 (34–65)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>Type 2</td>
<td>0</td>
<td>6</td>
<td>32 (22–43)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>20 (18 ± 5)</td>
<td>23</td>
<td>32 (18–49)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>40 (39 ± 8)</td>
<td>10</td>
<td>31 (22–41)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>46</td>
<td>42 (22–61)</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>20 (17 ± 5)</td>
<td>20</td>
<td>39 (21–54)</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Smoking status assigned as 0, 20 and 40 cigarettes per day (mean ± SD). Number of patients diagnosed with comorbid disorder and on psychiatric therapy is indicated. AD = Anxiety/depression; PTSD = posttraumatic stress disorder; PD = personality disorder; BD = benzodiazepines; SSRI = selective serotonin reuptake inhibitors; T = tianeptine; NL = neuroleptics.
Methods

Subjects
A total of 81 male patients were recruited from inpatients of the Department of Psychiatry, Sestre Milosrdnice University Hospital, Zagreb, over a period of 12 months (2005/2006). All patients had a full physical examination and routine hematological and biochemical analyses [29]. The diagnosis of alcohol dependence and comorbidity was assessed by the structured clinical interview based on DSM-IV criteria [30] performed independently by 2 experienced psychiatrists. Each patient had to meet at least three of the seven criteria for alcohol dependence. Additionally, patients were evaluated whether they fulfilled the criteria for type 1 or type 2 alcoholism [13]. For inclusion in the type 2 group, patient's subjective alcohol problems had to start before 21 years of age and reporting of at least two instances of social complications related to alcoholism before that age was required.

In order to ensure reliable statistical comparison between groups, similar number of type 1 (n = 42) and type 2 patients (n = 39) were recruited. Smoking status was registered as number of cigarettes smoked per day: of the total sample, 21% were nonsmokers, 51% smoked approximately a pack daily and 28% were heavy smokers (mostly two packs daily). They were allowed to continue their regular smoking habits during withdrawal. Comorbid disorders as well as psychiatric medications among subgroups are shown in Table 1. Our earlier study demonstrated no effect of benzodiazepines and 5HT reuptake inhibitors on platelet MAO activity [31], while neuroleptics and tricyclic antidepressants may act as weak MAO inhibitors [5, 32].

For the control group, 66 healthy males were recruited from blood donors at the National Institute for Transfusion Medicine. All were ascertained not to be alcohol- or drug-dependent by 2 experienced psychiatrists. Each patient had to meet at least three of the seven criteria for alcohol dependence. Additionally, patients were evaluated whether they fulfilled the criteria for type 1 or type 2 alcoholism [13]. For inclusion in the type 2 group, patient's subjective alcohol problems had to start before 21 years of age and reporting of at least two instances of social complications related to alcoholism before that age was required.

In order to ensure reliable statistical comparison between groups, similar number of type 1 (n = 42) and type 2 patients (n = 39) were recruited. Smoking status was registered as number of cigarettes smoked per day: of the total sample, 21% were nonsmokers, 51% smoked approximately a pack daily and 28% were heavy smokers (mostly two packs daily). They were allowed to continue their regular smoking habits during withdrawal. Comorbid disorders as well as psychiatric medications among subgroups are shown in Table 1. Our earlier study demonstrated no effect of benzodiazepines and 5HT reuptake inhibitors on platelet MAO activity [31], while neuroleptics and tricyclic antidepressants may act as weak MAO inhibitors [5, 32].

For the control group, 66 healthy males were recruited from blood donors at the National Institute for Transfusion Medicine. All were ascertained not to be alcohol- or drug-dependent by questionnaire. Additional exclusion criteria were neurological, psychiatric or major medical illness.

The study was approved by the Ethics Committee of the Sestre Milosrdnice University Hospital, and carried out in accordance with the Declaration of Helsinki. Written informed consent was obtained from all participants after a complete description of the study.

Laboratory Procedures
Blood samples were collected between 8 and 9 a.m. by venipuncture in ACD-containing tubes at the day of admission to the hospital and after 3 weeks of abstinence. Platelet-rich plasma (PRP) was obtained by centrifuging anticoagulated blood in syringes specifically prepared for this purpose [33]. Aliquots of PRP were separated for platelet and leukocyte counting and for biochemical measuring.

Kinetics of platelet MAO was assayed with kynuramine as a substrate (with six final concentrations of 1–32 μM) by a method described previously [30]. Michaelis-Menten constant (Km) and maximal velocity (Vmax) were calculated by Eadie-Hofstee plots and expressed as μM concentration for Km and nmol product per platelet number or per platelet mass for Vmax. To minimize the influence of day-to-day variations, determinations were always performed simultaneously on samples of both types of patients and controls, all subgrouped according to smoking habits.

Intra-assay coefficients of variation were 3.5% for Vmax and 8% for Km, calculated on assayed replicates of different aliquots of the same PRP sample. The coefficients of variation for the inter-assay precision were 6% for Vmax and 12% for Km.

Statistics
Results are shown as means ± standard deviation (SD) or standard error of the mean (SEM). Data were analyzed by using GraphPad Prism and GraphPad Instat software, version 3 (GraphPad, San Diego, Calif., USA) with the level of significance set at p < 0.05. The normality assumption was examined by the Kolmogorov-Smirnov test. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by post hoc Tukey’s test or two-way ANOVA followed by Bonferroni test where appropriate. Since the controls do not comprise the very heavy smokers, data were analyzed firstly among three groups (control, A-type 1, A-type 2) with two levels of smoking (0cig, 20cig), referred to as 1 × 2 ANOVA in the text, and then between two groups (A-type 1, A-type 2) with three smoking levels (0cig, 20cig, A-40cig), referred to as 2 × 3 ANOVA. Comparison of values at admission and after abstinence was performed by a paired t test. Parameters not normally distributed (leukocyte contamination of PRP samples) were compared by the nonparametric Kruskal-Wallis test. Relationship between parameters was analyzed using Pearson’s correlation test.

Results
Typical saturation curve for deamination of kynuramine by human platelet MAO-B is shown in figure 1. Correlation coefficients (r) of values transformed according to the Eadie-Hofstee method were all in the range of 0.93–0.99.

There were no differences in kinetic parameters of platelet MAO measured at the admission to the hospital and after 21 days of abstinence, notwithstanding alcohol
typology or smoking status (paired t test, NS for all subgroups). Therefore, for all further analyses mean values of these two measurements, performed 3 weeks apart, were used.

Comparison of MAO velocity expressed per unit number of platelets (see fig. 4 and 5) and per platelet hematocrit (plateletcrit; not shown), which measures total volume of platelets (platelet mass) in given samples, gave correlation coefficients of 0.93 in controls, 0.91 in type 1 and 0.82 in type 2 alcoholics (all p < 0.001).

In all six subgroups of patients, K_m values tended to be higher than in control groups (fig. 2). Statistical analysis revealed no overall effects of alcohol or smoking for the K_m, although the effect of alcohol approached significance [3 × 2 ANOVA: alcohol, F(2, 118) = 2.795, p = 0.0652; smoking, F(1, 118) = 0.0856, p = 0.770; interaction, F(2, 118) = 0.0111, p = 0.989]. Indeed, further analysis performed on groups not subdivided according smoking habits (fig. 3) revealed a significantly lower MAO affinity in alcoholic patients of both subtypes in comparison to control [p < 0.001 and p < 0.05, type 1 and 2, respectively; Tukey’s test following significant one-way ANOVA: F(2, 144) = 5.763, p = 0.0039].

As can be seen in figure 4, V_max did not differ between control and patient groups when adjusted for smoking. However, the catalytic activity of platelet MAO in smokers was markedly reduced (up to 40%) in comparison with the matched group of nonsmokers studied simultaneously. This reduction was similar in controls and alcoholic patients of both subtypes and depended on the severity of smoking [3 × 2 ANOVA for V_max: smoking, F(1, 118) = 9.81, p = 0.0022; alcohol, F(2, 118) = 1.525, p = 0.222; interaction, F(2, 118) = 0.43, p = 0.649; 2 × 3 ANOVA for V_max: smoking, F(2, 75) = 9.54, p = 0.0022, alcohol, F(1, 75) = 1.93, p = 0.168; interaction, F(2, 75) = 0.558, p = 0.574]. Post-hoc tests showed significant differences between subgroups of smokers and the respective controls (p < 0.05 or less, Bonferroni test).

Cumulative effect of alcohol and tobacco use on platelet MAO was demonstrated by calculating the ratio of V_max/K_m (fig. 5), which represents a measure of overall enzyme efficiency. Results thus indicate that both alcohol consumption and cigarette smoking may contribute to the lowering of MAO activity. The effect of alcohol is small (approximately 10%; see A-0cig in fig. 5) and due to interference with substrate binding only, while the effect of cigarette smoking is massive (up to 40%) and relates to dose-dependent reduction in MAO velocity [p < 0.001, smokers versus nonsmokers; Tukey’s post-hoc test following significant one-way ANOVA: F(3, 119) = 11.36, p < 0.0001].

**Discussion**

MAO is suggested to be involved in the mechanisms of addiction, due, among others, to the findings of decreased activity of this enzyme in platelets of alcohol and
tobacco users. Because of high concomitant tobacco use by alcoholics [19], it is difficult to separately analyze the relationship between platelet MAO, smoking and alcoholism. In trying to dissociate the effect of alcohol and tobacco use on MAO activity, animals that had never been in contact with both compounds were used [25], or the effect of either smoking or alcohol on MAO activity was eliminated by the use of multivariate statistical analysis [23]. In the present work, we have studied kinetic parameters of platelet MAO in alcohol-dependent patients, subdivided according to both, smoking habits and severity of alcoholism. Having in mind a large variability of platelet MAO activity caused by a number of endogenous (sex, age, hormones, genetic variability), exogenous (medication, smoking, neurotoxins) and also methodological factors, we tried to control for them as much as possible. Thus, only males not older than 65 years were recruited and subjects of the study groups were matched as close as possible for psychiatric comorbidity and medications. Measuring full kinetics of platelet MAO allowed us to compare the enzyme affinity across groups, in addition to usually measured enzyme velocity. Finally, because MAO activity was shown to differ between platelets of different size and density [34], we relate its velocity to both, platelet number and platelet mass. The key findings of this study are: (1) alcohol use is associated with slight reduction in platelet MAO affinity, without any effect on its velocity; (2) cigarette smoking is associated with pronounced, dose-dependent reduction in MAO velocity, without any effect on the affinity; (3) platelet MAO kinetics did not differ between alcohol subtypes 1 and 2, adjusted for severity of smoking; (4) withdrawal from alcohol did not influence platelet MAO activity.

While the inhibitory effect of cigarette smoking on platelet MAO velocity has been observed previously [16, 21, 23], there are no data relating to platelet MAO affinity in smokers. It is not clear which tobacco constituents are responsible for the inhibition of MAO velocity, but a few candidates have been isolated from tobacco leaves/cigarette smoke that act as competitive or mixed-type MAO inhibitors in vitro [35, 36]. According to our results, it seems that in vivo cigarette smoking alters only Vmax, while substrate binding remains unaffected.

A clear association between the amount of cigarettes smoked per day and decrease in MAO-B Vmax (fig. 4, 5) confirms the pharmacological nature of the inhibition and is in line with other studies [21, 23], although there are also dissonant reports [37]. Smoking of only few cigarettes daily has no measurable effect on platelet MAO velocity (not shown). It should be noted that a degree of MAO inhibition in platelets of heavy smokers (~40%; fig. 4) corresponds to the reported level of enzyme reduction in the living brain and peripheral organs (33–46% [17, 18]) of such individuals. We can argue that MAO-B

Fig. 4. V_max of platelet MAO in control group and alcoholic patients (A) of type 1 and type 2, subdivided according to their smoking status (0, 20 or 40 cigarettes per day). Mean ± SEM; number of subjects given in parentheses. * p < 0.05 A-type 1: 0cig versus 20cig and A-type 2: 0cig versus 40cig; ** p < 0.01 control: 0cig versus 20cig; *** p < 0.001 A-type 1: 0cig versus 40cig; Bonferroni test following significant two-way ANOVA.

Fig. 5. Index of platelet MAO efficiency (V_max/K_m) in alcoholic patients (A) subdivided according to their smoking status (0, 20 or 40 cigarettes per day). Values are expressed as percent of activity in nonsmoking control (2.9 ± 0.95 = 100%); number of subjects given in parentheses. * p < 0.05 A-40cig versus A-0cig; *** p < 0.001 A-20cig versus control, A-40cig versus control, Tukey’s test following significant one-way ANOVA.
activity in platelets parallels changes in its central counterpart, confirming thus the usefulness of platelets as peripheral indicators of pharmacological inhibition of central MAO-B [38].

We were not able to find association between velocity of platelet MAO and alcohol consumption; however, interference with substrate binding was demonstrated. Increased $K_m$ values were present in both subtypes of alcohol-dependent patients (fig. 3), probably due to ethanol-induced conformational changes of enzyme molecule. Competitive type of inhibition of platelet MAO has been reported in vitro, in different tissue preparations, at 25 mM ethanol [39] and this concentration can be achieved in the plasma of alcoholics. The mode of interaction of ethanol and platelet MAO is rarely encountered in the literature and, to our knowledge, this is the first report on the relationship between platelet MAO affinity and alcohol abuse.

By calculating the index of MAO efficiency ($V_{\text{max}}/K_m$; fig. 5), it was shown that decrease in MAO affinity in alcoholics (interference of alcohol with substrate binding) may combine with decrease in its velocity (enzyme inhibition by compounds present in tobacco smoke) to potentiate MAO inhibition in alcohol-dependent heavy smokers. If it happens also in the brain, it may lead to greater disturbances in the level of neurotransmitters involved in reward reinforcement that lead to addiction (e.g. dopamine). It can be speculated that this phenomenon underlies the reported increase in cigarette smoking as a result of sustained drinking in heavy alcoholics.

Regarding the temporal pattern of platelet MAO velocity after withdrawal from alcohol, either an increase or decrease, transient or permanent, was shown. Having in mind a number of factors that could influence MAO activity, such large interstudy variability may reflect certain methodological biases. For example, an increase in MAO activity a week after withdrawal (in fact – a return to the normal values) reported by Coccini et al. [40] probably due to ethanol-induced conformational changes of enzyme molecule. Competitive type of inhibition of platelet MAO has been reported in vitro, in different tissue preparations, at 25 mM ethanol [39] and this concentration can be achieved in the plasma of alcoholics. The mode of interaction of ethanol and platelet MAO is rarely encountered in the literature and, to our knowledge, this is the first report on the relationship between platelet MAO affinity and alcohol abuse.

Similarly, there are mixed results regarding platelet MAO activity and alcohol subtypes: either no differences [11, 20] or a higher reduction in its activity in type 2 alcoholics was shown [22, 42, 43], the later results probably due to the fact that heavy smoking is more frequent in type 2 alcoholics. Our results show no differences in platelet MAO activity between type 1 and type 2 alcoholics when adjusted for severity of smoking. This is in accordance with similar prolactin response to fenfluramine (used as an index of central serotonergic transmission) between controls and type 1 and type 2 alcoholics, in contrast to reduced prolactin response in cigarette smokers in comparison to nonsmokers [44]. By the use of same approach, it was shown that cigarette-smoking alcoholics have reduced central 5HT neurotransmission in comparison to nonsmoking alcoholics [45]. This means that the influence of tobacco use must be taken into consideration also in the evaluation of alterations in central serotonergic transmission.

Finally, we would like to draw attention to some methodological issues, which may contribute to the variability of the literature results. Namely, in our study, the same procedure for platelet isolation resulted in a significantly higher number of lymphocytes in PRP samples of alcoholic patients than in control subjects (1.03 ± 0.97 versus 0.59 ± 0.72, respectively, p = 0.0012, Kruskal-Wallis test). Higher contamination of platelet sample by leukocytes means higher contribution of their protein to the total protein mass – a warning that relating MAO activity to protein mass, used sometimes in the literature, may not give reliable results. Measuring of platelet MAO activity as a function of the platelet count was previously reported to be more reliable than the platelet protein method [46] and, according to our results, this seems to be even more important in pathophysiological studies.

The problems that we have encountered in this study were the ongoing therapy and comorbidity, which are characteristic for alcoholism [47]. Subjects with these characteristics were dispersed similarly throughout subgroups, so the results should not be confounded by these factors. Reduced $V_{\text{max}}$ of platelet MAO in patients with PTSD and depressive/anxiety disorders has been reported previously, but, as in studies on alcohol, cigarette smoking was rarely controlled, meaning that much of this work should be revisited. Recently, it was suggested that also coffee may contain compounds acting as com-

Platelet MAO, Alcoholism and Smoking


143
petitive and reversible MAO inhibitors [48]. An uneven distribution of nonsmokers in our control and patient groups should also be considered as a potential confounding factor. However, we believe that in our experimental design this difference in distribution may influence the degree of statistical significance between group means (e.g. compare type 1 and type 2 patients in fig. 5), but not the essence of findings.

In summary, our study demonstrated that both alcohol dependence and tobacco use might contribute to the lowering of platelet MAO-B activity, with the influence of smoking being much stronger. The decrease in MAO velocity is entirely due to inhibition by constituents of cigarette smoke, while alcohol use is responsible for the decrease in enzyme affinity. Measuring of MAO full kinetics should be considered in further studies since this may give better insight into the relationship between its activity and addiction.

Acknowledgement

This paper is part of the project ‘Sero-tergic Mechanisms in Alcoholism’ supported by the Croatian Ministry of Science, Education and Sports.

References


