

Title: Monoamine oxidase and semicarbazide-sensitive amine oxidase kinetic analysis in mesenteric arteries of patients with type 2 diabetes

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Short title: MAO & SSAO kinetic analysis in arteries of diabetic patients

Summary

Monoamine oxidase (MAO, type A and B) and semicarbazide-sensitive amine oxidase (SSAO) metabolize biogenic amines, however, the impact of these enzymes in arteries from patients with type 2 diabetes patients remains poorly understood. We investigated the kinetic parameters of the enzymes to establish putative correlations with noradrenaline (NA) content and patient age in human mesenteric arteries from type 2 diabetic patients. The kinetic parameters were evaluated by radiochemical assay and NA content by high-performance liquid chromatography (HPLC).

The activity of MAO-A and SSAO in type 2 diabetic vascular tissues was significantly lower compared to the activity obtained in non-diabetic tissues. In the correlation between MAO-A (K_m) and NA content, we found a positive correlation for both the diabetic and non-diabetic group, but no correlation was established for patient age. In both groups, MAO-B (V_{max}) showed a negative correlation with age.

The results show that MAO-A and SSAO activities and NA content of type 2 diabetic tissues are lower compared to the non-diabetic tissues, while MAO-B activity remained unchanged. These remarks suggest that MAO-A and SSAO may play an important role in vascular tissue as well as in the vascular pathophysiology of type 2 diabetes.

Key Words: Human mesenteric arteries; monoamine oxidase; semicarbazide-sensitive amine oxidase; type 2 diabetes; noradrenaline content.

Introduction

Oxidative stress can be defined as a disturbance in the balance between the production of free radicals – such as superoxide anion, hydroxyl radicals and hydrogen peroxide – and antioxidant mechanisms (Ramakrishna and Jailkhani 2008). These pathways also include monoamine oxidases (A and B) and semicarbazide-sensitive amine oxidase (SSAO) enzymes which take part in pathological changes, such as cardiac diseases and diabetic complications (Bianchi *et al.* 2005, Obata 2006).

Monoamine oxidase (MAO, EC 1.4.3.4) catalyzes the oxidative deamination of biogenic amines (adrenaline, noradrenaline, serotonin, dopamine, tyramine and tryptamine) to the corresponding aldehyde, hydrogen peroxide (H_2O_2) and ammonia (Nagatsu 2004). *In vitro*, serotonin and noradrenaline (NA) are the preferential MAO-A substrates and the MAO-A enzyme is inhibited by clorgyline, whereas β -phenylethylamine is a substrate for MAO-B and selegiline as a selective MAO-B inhibitor. MAO (type A and B) functions include catabolism of exogenous amines, regulation of neurotransmitter levels and control of intracellular amine stores. Thus, it might be expected that their distribution in central nervous system and in other tissues is a reflection of these physiological roles (Billett 2004).

The semicarbazide-sensitive amine oxidase enzyme (SSAO, EC 1.4.3.6) catalyzes the oxidative deamination of primary amines, such as methylamine or aminoacetone, to produce the corresponding aldehyde (formaldehyde and methylglyoxal, respectively) and converts circulating aliphatic and aromatic amines (tyramine, tryptamine, histamine and dopamine) into the corresponding aldehyde, H_2O_2 and ammonia, but is relatively inactive for NA and serotonin (Elliott *et al.* 1989, Obata 2006). The SSAO enzyme is present in blood, associated with cell membranes, such as endothelial and smooth muscle cells of blood vessels, and is selectively inhibited by semicarbazide (del Mar Hernandez *et al.* 2005). This enzyme is also known as vascular adhesion protein-1 (VAP-1) and plays an important role in the adhesion and migration of leukocytes to sites of inflammation (Bour *et al.*, 2009).

The mesenteric innervation system plays an important role in controlling mesenteric capacitance, and there are some studies reporting MAO (type A and B) and SSAO activity in non-diabetic patients (Figueiredo *et al.* 1998, Birch *et al.* 2008). However, the impact of those enzymes in the inferior mesenteric arteries of patients with type 2 diabetes remains poorly understood. The present study aims to determine (K_m) and (V_{max}) for the three enzymes, as well as to determine the NA content in inferior mesenteric arteries, in order to establish correlations between MAO-A and MAO-B with NA content, and also between MAO enzymes and patient age. A putative correlation can help to understand the influence

of MAO and SSAO enzymes in the pathophysiological damage in the arterial walls from patients with type 2 diabetes.

Material and Methods

Study population

Segments of human inferior mesenteric arteries from 12 non-diabetic patients (average age: 62.2 ± 10.9 years old, 7 men and 5 women) and 8 patients with type 2 diabetes (average age: 63.1 ± 6.2 years old, 5 men and 3 women), obtained from patients submitted for colorectal surgery, were provided by the Surgery Department of the Portuguese Oncology Institute of Coimbra. The study was approved by local medical ethics committee following the rules from Declaration of Helsinki of the World Medical Association, and all participants provided informed consent. Only non-smokers participated in the study, and none of the participants suffered from thyroid dysfunction or liver disorders.

The population included in this study consisted of patients with sigmoid or rectum carcinoma who were not subject to neo-adjuvant therapeutics and who presented surgery as the primary option. The type of surgical intervention used depended on the location of the tumour and whether sigmoidectomy or anterior resection of the rectum was carried out. The non-diabetic patients were selected according to the following criteria: blood glucose concentrations < 110 mg/dL, not suffering from hypertension or renal disease (serum creatinine < 1.20 mg/dL). The group with type 2 diabetes was chosen with blood glucose concentrations > 110 mg/dL, with HbA_{1c} >6% and on treatment with an oral antidiabetic drug. Vessels of greatest calibre (namely the inferior mesenteric artery) were selected as study material; in order to avoid interference from pathological anatomy. Only macroscopically healthy vessels were used.

The arteries were placed in cold physiological saline solution and immediately transported to the laboratory where ±200 mg of each artery were homogenized 1:10 (w/v) with a concentric glass homogenizer in 10 mM sodium phosphate buffer (pH 7.4) at 4° C and the supernatant from the 600 g spin was taken and stored at – 80° C until further analysis.

Determination of MAO (A and B) and SSAO in the vascular tissues

MAO (A and B) and SSAO activities were determined by radiochemical methods using ³H-5-hydroxytryptamine creatinine sulphate (³H-5-HT), in a concentration range between 50–1,000 μM for MAO-A, ¹⁴C-β-phenylethylamine hydrochloride (¹⁴C-β-PEA), 5–160 μM for MAO-B and ¹⁴C-benzylamine (¹⁴C-BZ), in a concentration range 50–1,600 μM for SSAO (Figueiredo *et al.* 1998). 5-HT, β-PEA and BZ were purchased from Sigma-Aldrich (Madrid, Spain), ³H-5-HT (15.1 Ci/mmol) and ¹⁴C-

BZ (54 mCi/mmol) from Amersham International (London, United Kingdom) and ^{14}C - β -PEA (43.8 mCi/mmol) from Perkin Elmer LAS (Boston, MA). The MAO inhibitors and all other reagents used were of analytical grade.

The tissue homogenates (25 μL) were pre-incubated for 20 min, at 37° C, with selegiline (10^{-4} M) as MAO-B inhibitor or clorgyline (10^{-6} M) as MAO-A inhibitor. Increasing concentrations of the corresponding radioactive substrates (50 μL) were added and the solution was oxygenated. After incubation for 20 min at 37° C, the reaction was stopped by the addition of 10 μL HCl (3 mol/L). The deaminated products were then extracted with ethyl acetate (400 μL) followed by centrifugation at 3,000g. The resulting supernatant (200 μL aliquot) was transferred to counting vials with scintillation fluid (6 mL) for radiochemical measurements.

The SSAO activity was determined as described for MAO using clorgyline (10^{-6} M) and selegiline (10^{-4} M) as inhibitors with 20 min pre-incubation times. The final incubation with ^{14}C -benzylamine hydrochloride was 5 min. The radioactivity released from the mixtures was measured in a Packard 2000 Tri-Carb liquid scintillation counter (Canberra, Australia) and calculations were performed to express MAO (A and B) and SSAO activities in nanomoles of substrate metabolised per mg of protein per hour ($\text{nmol}\cdot\text{mg protein}^{-1}\cdot\text{h}^{-1}$). Protein content in the homogenates was determined by Lowry method (Lowry *et al.* 1951).

NA assay

The NA content in the arteries was determined by high-performance liquid chromatography (HPLC) with electrochemical detection. The small mesenteric artery segments were placed in perchloric acid (0.1 M, Merck) at 4° C for 24h. The NA content was measured using known NA standard (Sigma Chemical Co.) concentrations and chromatograms were obtained using the appropriate HPLC software (Reis *et al.* 2005). NA concentrations were expressed in $\mu\text{g/g}$ wet tissue.

Statistical analysis

The results were expressed as mean values \pm standard deviation (SD). The results from kinetic studies were plotted by a computer-generated Hanes–Wolf plots using linear regression to obtain apparent K_m and V_{max} values. Statistical evaluation between the groups was performed by the non-parametric Mann–Whitney test. Pearson correlation and multiple regression analysis were used to test the correlations. The statistics were performed using commercial Graph Pad Prism software version 4.0 (San

Diego, CA, USA) and Analysis ToolPak added to Microsoft *Office Excel 2007*[®]. Statistical significance was considered for $P < 0.05$.

Results

MAO (type A and B) and SSAO activities

Table 1 summarises the results obtained from experiments to MAO (A and B) and SSAO kinetics parameters in homogenates of inferior mesenteric arteries. The K_m values were unchanged in MAO-A ($178.30 \pm 99.17 \mu\text{M}$ vs. $200.90 \pm 122.60 \mu\text{M}$, $P > 0.05$) and MAO-B ($100.50 \pm 38.87 \mu\text{M}$ vs. $100.40 \pm 59.68 \mu\text{M}$, $P > 0.05$) for both, non-diabetic and type 2 diabetic tissues, respectively. The MAO-A (V_{max}) results in the mesenteric arteries from patients with type 2 diabetes ($n = 8$) showed significant lower values compared to the non-diabetic tissues ($n = 12$) (32.13 ± 18.24 vs. $60.06 \pm 21.43 \text{ nmol.mg protein}^{-1}.\text{h}^{-1}$, $P < 0.05$, respectively). No differences were found for V_{max} values of MAO-B between the non-diabetic ($15.20 \pm 3.03 \text{ nmol.mg protein}^{-1}.\text{h}^{-1}$) and the diabetic ($12.31 \pm 6.87 \text{ nmol.mg protein}^{-1}.\text{h}^{-1}$) tissues.

The SSAO K_m values did not show statistical significance between the groups. The SSAO V_{max} values from the diabetic tissues were significantly lower than V_{max} values of the non-diabetic tissues (148.30 ± 39.11 vs. $229.10 \pm 46.28 \text{ nmol.mg protein}^{-1}.\text{h}^{-1}$, $P < 0.05$, respectively). No sex differences in the kinetic parameters of the three enzymes were observed.

NA content

The non-diabetic tissues had higher NA content ($0.220 \pm 0.117 \mu\text{g/g tissue}$, $n = 12$) than tissue from patients with type 2 diabetes ($0.065 \pm 0.059 \mu\text{g/g tissue}$, $n = 8$, $P < 0.05$).

There was no correlation between the NA content and the donor age in the non-diabetic group (Pearson $r = 0.205$, $P = 0.522$) or the type 2 diabetic group (Pearson $r = 0.525$, $P = 0.181$).

A negative correlation was found between NA content and MAO-A (V_{max}) in the non-diabetic tissue (Pearson $r = -0.621$, $P < 0.05$), while MAO-A (K_m) was positively correlated with NA content for both non-diabetic tissue (Pearson $r = 0.593$, $P < 0.05$) and type 2 diabetic tissue (Pearson $r = 0.927$, $P < 0.01$), (Figure 1). There was no correlation between MAO-A (V_{max} and K_m) and patient age in either group.

No statistical correlation between NA content and MAO-B was found for either group. The MAO-B (V_{max}) was negatively correlated with patient age in the non-diabetic group (Pearson $r = -0.607$,

$P < 0.05$) and type 2 diabetic group (Pearson $r = -0.710$, $P < 0.05$), (Figure 2). There was no correlation between SSAO (K_m and V_{max}) and the patient age in either group.

Discussion

Oxidative stress may contribute to the development of microvascular and cardiovascular diseases in patients with diabetes (Kuroki *et al.* 2003). Moreover, increased levels of oxidative stress markers have always been related to cardiovascular problems, atherosclerosis or hypertension, although there are some controversies about the pathophysiological function. Recent research has been focused in those processes in order to clarify unsolved questions concerning the vascular biology of arteries and veins (Szasz *et al.* 2007).

The present study showed that: 1) MAO-A and SSAO activities and NA content of type 2 diabetic tissues are lower than in non-diabetic tissues, while MAO-B remained unchanged; 2) The K_m values did not show statistical significance between the groups for both MAO (type A and B) and SSAO; 3) MAO-A (K_m) showed a positive correlation with NA content in diabetic and non-diabetic tissues. There was a negative correlation between MAO-A (V_{max}) and NA content in the non-diabetic tissues, which disappears under diabetic conditions; 4) MAO-B (V_{max}) showed a negative correlation with age for tissues from both groups.

Immunohistochemical studies revealed the presence of sympathetic and sensory-motor nerves in arteries and veins obtained from human colonic specimens. The human mesenteric vessel innervation system plays a particular and important role in controlling mesenteric vein capacitance, which is reflected by their dense innervations (Birch *et al.* 2008).

MAO plays a role in the metabolism of biogenic amines, regulation of neurotransmitter levels and control of intracellular amine stores; however, in the gastrointestinal tract and circulatory system, MAO may serve a protective function by regulating dietary amines levels, which exert potent vasopressor effects (Herraiz and Chaparro 2006). Although the catalytic products from oxidative deamination of amines (hydrogen peroxide and ammonia) could exert a cytotoxic effect, the biogenic amines could be viewed as cytoprotectors and/or as toxicants. The balance between the oxidant and antioxidant enzymes appears to be very important in carcinogenesis as well as in regulation of cell growth (Pietrangeli and Mondovi 2004). Also, in an induced solid tumor breast cancer rat model, MAO-A activity increased whereas MAO-B and SSAO activities diminished with malignancy (Lizcano *et al.* 1991).

In rabbit femoral smooth muscle cells, tyramine degradation promoted by MAO-A activates stress-induced mitogenic signalling which could participate in excessive remodelling and alteration of the vascular wall (Coatrieux *et al.* 2007). MAO-A, as an intraneuronal enzyme, could participate in extraneuronal metabolism, as was suggested by the positive correlation between enzyme activity and NA content in some vascular tissues, such as human umbilical arteries, dog saphenous veins, mesenteric arteries and rabbit ear arteries (Caramona 1986). Therefore, our data concerning the MAO-A (K_m) in mesenteric arteries of diabetic and non-diabetic tissues showed a positive correlation with NA content. Furthermore, MAO-A (V_{max}) in the non-diabetic tissues was negatively correlated with NA content, the increase in MAO-A led to a decrease in NA content while in the diabetes tissues none of the correlations were found, suggesting that the terminal nerve endings are inoperative. So, in the pathogenesis of diabetes, there is a loss of NA content, a decrease of enzymatic processes and a degradation of terminal nerve endings. These data suggest that the decreases in MAO-A activity in the mesenteric arterial tissues of patients with type 2 diabetes is associated with the diabetic neurological degeneration. This possibility must be further explored, namely through immunohistochemical analysis of arteries from diabetic patients.

No statistical differences were found for MAO-B K_m and V_{max} in the arterial homogenates studied, indicating that hyperglycaemia does not interfere with the mechanism of MAO-B oxidative deamination. However, the negative correlation between MAO-B (V_{max}) and the patient age in both type 2 diabetic tissues and non-diabetic tissues has demonstrated a decrease in the MAO-B activity in those peripheral tissues which is dependent on aging. Taken together, these results demonstrate that MAO-A and MAO-B have different roles in monoamine metabolism in inferior mesenteric arteries.

The degradation of adrenaline by MAO-A produces methylamine and SSAO catalyzes the metabolism of methylamine to formaldehyde. Endothelial cells are quite sensitive to formaldehyde and may be involved in the endothelial injury (Yu *et al.* 1997). The formaldehyde may also be involved in protein structure alteration, which causes protein deposition associated with chronic pathological disorders such as diabetic complications, atherosclerosis and Alzheimer's disease (Gubisne-Haberle *et al.* 2004).

We and other have previously reported serum or plasma SSAO activity increases in type 2 diabetic patients ((Nunes *et al.* 2010, Mészáros *et al.* 1999, Gokturk *et al.* 2003, Obata 2006), which might be caused by translocation of SSAO from the tissue-bound to plasma due to changes in arterial

permeability. These results are in agreement with another study which proposed that soluble SSAO is derived from the membrane-bound enzyme (Yu *et al.* 1994). Our findings in homogenates of inferior mesenteric arteries from patients with type 2 diabetes showed a low SSAO activity when compared with the non-diabetic arteries and no correlation with the patient age.

In conclusion, we found a decrease in NA content in diabetic arteries, possibly due to damage in the arterial walls. The reported data show that the low activity of MAO-A and SSAO in inferior mesenteric arteries of patients with type 2 diabetes is associated with several vascular changes, suggesting that MAO-A and SSAO play a role in blood vessels and hence in the pathogenesis of diabetes. These studies could open new perspectives to MAO-A and SSAO as pharmacological tools for diabetes management in order to improve the life quality of patients with type 2 diabetes.

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Table 1. MAO-A, MAO-B and SSAO kinetic parameters in mesenteric arteries homogenates of non-diabetic group and patients with type 2 diabetes.

| | | Non-diabetic group (n=12) | Type 2 diabetic group (n=8) |
|--------------|--|------------------------------|--------------------------------|
| MAO-A | V_{\max} (nmol.mg protein ⁻¹ .h ⁻¹) | 60.06 ± 21.43 | 32.13 ± 18.24* |
| | K_m (μM) | 178.30 ± 99.17 | 200.90 ± 122.60 |
| MAO-B | V_{\max} (nmol.mg protein ⁻¹ .h ⁻¹) | 15.20 ± 3.03 | 12.31 ± 6.87 |
| | K_m (μM) | 100.50 ± 38.87 | 100.40 ± 59.68 |
| SSAO | V_{\max} (nmol.mg protein ⁻¹ .h ⁻¹) | 229.10 ± 46.28 | 148.30 ± 39.11* |
| | K_m (μM) | 301.00 ± 127.30 | 228.10 ± 134.30 |

Values are means ± SD. *P<0.05 (Mann–Whitney test).

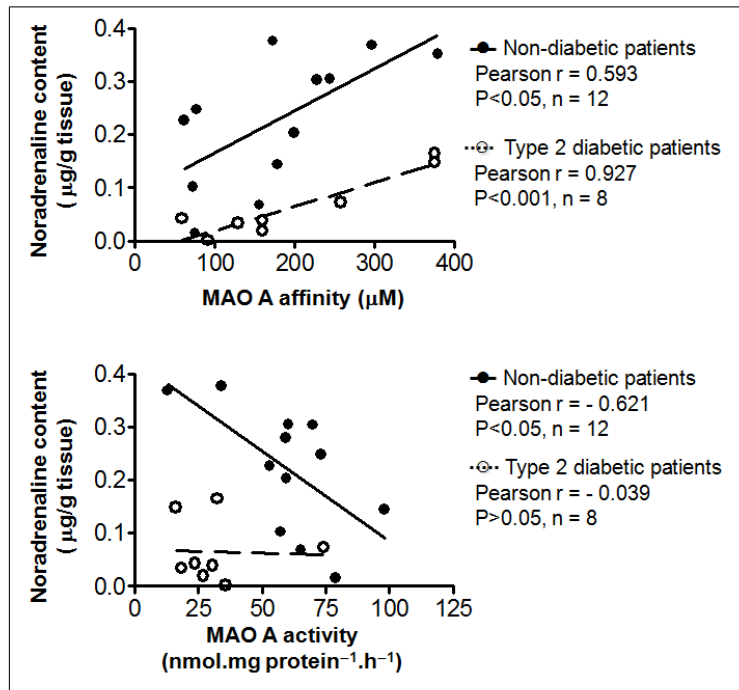


Fig. 1. Scatter plot correlations between noradrenaline content and MAO-A (K_m or V_{max}) for non-diabetic and type 2 diabetic groups.

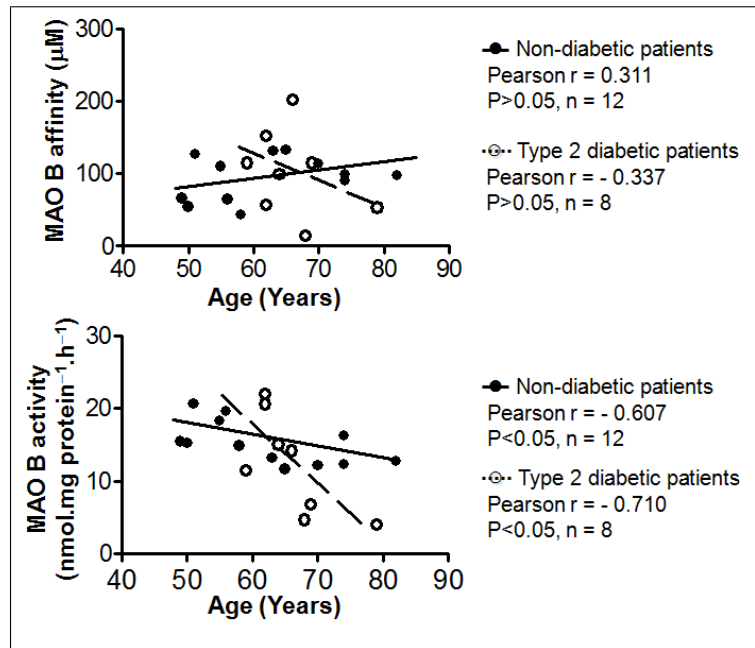


Fig. 2. Scatter plot correlations between age and MAO-B (K_m or V_{max}) for non-diabetic and type 2 diabetic groups.