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Soluble Semicarbazide Sensitive Amine Oxidase (SSAO) catalysis induces apoptosis in vascular smooth muscle cells

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Abstract

Semicarbazide sensitive amine oxidase (SSAO) metabolizes oxidative deamination of primary aromatic and aliphatic amines. It is selectively expressed in vascular cells of blood vessels, but it is also circulating in blood plasma. SSAO activity in plasma is increased in some diseases associated with vascular complications and its catalytic products may cause tissue damage. We examined the effect of the oxidation of the SSAO substrate, methylamine, on cultured smooth muscle cells. Cell incubation with methylamine plus soluble SSAO, contained in bovine serum, resulted toxic to rat aorta A7r5 and human aortic smooth muscle cells, as measured by MTT reduction. This effect was completely reverted by specific SSAO inhibitors, indicating that the toxicity was mediated by the end products generated. Moreover, SSAO-mediated deamination of methylamine induced apoptosis in A7r5 cells, detected by chromatin condensation, Caspase-3 activation, PARP cleavage and cytochrome *c* release to cytosol. Formaldehyde, rather than H_2O_2, resulted to be a strong apoptotic inducer to A7r5 cells. Taken together, the results suggest that increased plasma SSAO activity in pathological conditions, could contribute to apoptosis in smooth muscle cells, leading to vascular tissue damage.

Keywords: Semicarbazide Sensitive Amine Oxidase; Methylamine; Formaldehyde; Hydrogen peroxide; Apoptosis; Aortic smooth muscle cell

1. Introduction

Semicarbazide Sensitive Amine Oxidase [E.C.1.4.3.6, oxidoreductase (deaminating) (copper-containing), SSAO] constitutes a large family of enzymes present in almost all mammalian species studied. All these enzymes are inhibited by semicarbazide [1,2]. SSAO catalyses the oxidative deamination of primary aromatic and aliphatic amines. Its catalytic action requires oxygen and generates ammonia, hydrogen peroxide (H_2O_2) and the corresponding aldehyde. Aminoacetone and methylamine (MA) are considered the physiological substrates of SSAO [3] and their oxidation generates the toxic end products, methylglioxal and formaldehyde, respectively [4]. SSAO is associated with cell membranes and it is also present in blood plasma [2,5]. Membrane-bound SSAO is predominantly expressed in adipocytes, smooth muscle and endothelial cells from blood vessels [1,2,6].

The physiological role of SSAO is still far from clear and it is considered to be a multifunctional enzyme, depending on the tissue where it is expressed [7]. In adipocytes, SSAO activity stimulates glucose transport, mimicking the insulin effect through the H_2O_2 generated during the catalytic process [8]. In addition, SSAO, also known as vascular adhesion protein-1 (VAP-1) [9], is involved in lymphocytes trafficking [10] and its expression in endothelial cells is induced during inflammation.

Some authors have proposed that soluble SSAO is derived from the membrane-bound enzyme [11,12]. Recently, it has been reported that soluble SSAO is shed from the membrane by a metalloprotease activity in adipocytes [11]. Furthermore, transgenic mouse models expressing human VAP-1 in

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endothelial cells showed that VAP-1 from vascular cells could be the major source of circulating SSAO in mice [12].

Plasma SSAO activity is increased in several pathological conditions; diabetes type I and II [13], congestive heart failure [14] and non diabetic morbid obesity [15], and it has also been implicated in atherosclerosis [16,17]. The products generated by SSAO, formaldehyde and H_2O_2, have been considered a potential risk factor for stress-related angiopathy [18,19]. H_2O_2, a major reactive oxygen species, is the principal generator of oxidative stress, which is widely implicated in several diseases. On the other hand, formaldehyde is a highly reactive aliphatic aldehyde, which is considered to be a powerful inflammatory agent [20]. The combined effect of these products could contribute to vascular degeneration associated to several disease states. Alzheimer's disease (AD) patients exhibit significant cerebrovascular pathology, such as microvascular degeneration affecting smooth muscle cells and endothelial cells, hyalinosis and fibrosis. In this context, we have previously reported that membrane-bound SSAO is overexpressed in the cerebrovascular tissue of AD and CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) patients, with the subsequent perturbation of the brain vasculature [21]. Moreover, we have recently reported that soluble SSAO activity is increased in patients suffering of severe AD [22]. There is a need to examine if the increase in SSAO activity is correlated to the vascular damage in this pathology.

Since MA and plasma SSAO levels are increased in certain diseases [23,24], we examined whether soluble SSAO, through its catalytic action, induces cell death in cultured smooth muscle (SMC). As A7r5 cells and HASMC (Human Aortic Smooth Muscle Cells) do not show SSAO activity or expression, bovine serum (BS) with high SSAO activity was used as the source of soluble enzyme. Different amines; MA, tyramine (TYR) and benzylamine (BZ), were used as SSAO substrates.

2. Methods

2.1. Materials

Dulbecco's modified Eagle's medium, fetal bovine serum (FBS), L-glutamine, penicillin, streptomycin and trypsin were obtained from Gibco BRL (Grand Island, NY, USA). Bovine Serum (BS) was from Biosystems (Barcelona, Spain). Methylamine, tyramine, benzylamine, semicarbazide, H_2O_2, formaldehyde, Hoechst 33258 and other chemicals were purchased from Sigma Aldrich (St. Louis, Mo., USA). MDL72974A ((E)-2-(4-fluorophenethyl)-3-fluoroallylamine hydrochloride) was a kind gift from Dr. P.H. Yu (University of Saskatchewan, Saskatchewan, Canada). The primary antibodies used were anti-cleaved Caspase-3 antibody from Cell Signaling (Beverly, MA, USA), anti-PARP from Upstate Biotechnology, Inc. (Lake Placid, NY, USA), anti-cytochrome *c* from BD Biosciences Pharmingen (San Diego, CA, USA) and anti-β-actin from Sigma Aldrich (St. Louis, Mo., USA). The secondary antibodies used were goat anti-rabbit IgG Alexa Fluor 594 from Molecular Probes (Eugene, OR, USA), HRP anti rabbit IgG from BD Biosciences Pharmingen and HRP anti-mouse IgG from Dako Cytomation (Glostrup, Denmark).

2.2. SSAO activity determination

SSAO activity was determined radiochemically at 37 °C as previously described [25] using 100 μM [^{14}C]-benzylamine (3 mCi/mmol, Amersham, UK) as substrate. Samples were preincubated for 30 min at 37 °C with 1 μM L-deprenyl to inhibit possible platelet MAO B contamination. The reaction was carried out at 37 °C in a final volume of 225 μl 50 mM Tris–HCl buffer, pH 9, and stopped by the addition of 100 μl 2 M citric acid. Radiolabelled products were extracted into toluene/ethyl acetate (1:1, v/v) containing 0.6% (w/v) 2,5-diphenyloxazole (PPO) before liquid scintillation counting. Different BS batches were tested and selected only when SSAO specific activity was 60 pmol/min mg protein. SSAO activity in cell treatments is expressed as U/ml (1 Unit produces 1 μmol/min of the catalytic product).

SSAO kinetic constants towards benzylamine, methylamine and tyramine (0.1–10 mM) as substrates were determined using a continuous spectrophotometric method coupled to peroxidase [26]. 4-Aminoantipyrine is oxidized by the hydrogen peroxide formed during amine oxidation and then condenses with vanillic acid to give a red quinone imine dye. The absorbance at 498 nm is proportional to the amount of hydrogen peroxide generated. Product concentrations were measured using a Cary spectrophotometer and K_m and V_{max} values were calculated using the Graph-Pad Prism 3.0 program.

2.3. Cell culture

Human Aortic Smooth Muscle Cells (HASMC) from normal adult thoracic aortas were obtained from control donor heart transplants. Samples were provided by Hospital de la Vall d'Hebron, Barcelona, according to the Rules and Procedures of its Ethics Committee. The smooth muscle cells from aorta were isolated by the explant method and cultured as described previously [27]. Cells were used at passages 3–8 and characterized as smooth muscle by morphologic criteria and expression of smooth muscle -actin. Rat aortic smooth muscle cells A7r5 were obtained from ATCC. Cells were grown in high glucose (4,500 mg/l) DMEM supplemented with 10% (v/v) FBS, 2 mM L-glutamine, 1000 U/mL penicillin, 1000 μg/mL streptomycin. Cells were seeded at 50000 cell/ml and grown at 37 °C in a humidified atmosphere containing 5% CO_2. For experiments, cells were grown for 2 days and starved with DMEM containing 0.2% (v/v) FBS for H_2O_2 or formaldehyde treatments. For MA treatments, cells were replaced with DMEM (0% FBS) containing 5, 10 or 15% (v/v) of Bovine Serum (BS), which corresponds to 3×10^{-4}, 6×10^{-4} and 9×10^{-4} U/ml of SSAO activity, respectively. SSAO inhibitors, semicarbazide or MDL72974A, were coincubated with MA in DMEM supplemented with BS.

2.4. Cell viability

Mitochondrial activity was estimated by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reduction assay. After treatments, MTT (0.5 mg/ml) was added to cells and after 90 min incubation at 37 °C the medium was replaced by dimethyl sulfoxide. The amount of formazan blue formed after MTT reduction was quantified spectrophotometrically at 560 nm [28].

2.5. Active Caspase-3 immunocytochemistry and detection of apoptotic nuclei using Hoechst 33258 staining

Analysis of active Caspase-3 was performed on treated A7r5 cells seeded on coverslips. Cells were fixed with 4% paraformaldehyde for 30 min at room temperature and washed in PBS containing 0.1% Tween 20. Coverslips were then incubated with a blocking solution containing 1% (w/v) Bovine Serum Albumin. Anti-cleaved Caspase-3 diluted 1:100 in blocking buffer was incubated overnight at 4 °C, washed in PBS/0.1% Tween 20, and thereafter incubated with the secondary antibody anti-rabbit IgG Alexa Fluor 594 diluted 1:1000 for 1 h. For nuclear staining, coverslips were washed in PBS and incubated with Hoechst 33258 (1 μg/ml) for 10 min at room temperature in the dark. Three independent experiments were performed and three fields of each treatment in duplicate were counted. At least 1000 cells were counted for each treatment.

2.6. Detection of PARP cleavage and cytochrome c release by Western Blot analysis

For cleaved PARP detection in total cell lysates, cells were washed in cold PBS and lysed in a buffer containing 50 mM Tris–HCl pH 6.8, 10%

glycerol, 2% SDS, 10 mM dithiothreitol and 0.01% bromofenol blue. To detect cytochrome *c* release, cells were harvested in 250 μl of ice-cold buffer containing 50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM dithiothreitol, complete protease inhibitor, and 250 mM sucrose, after washing once in cold PBS. Cells were incubated for 30 min on ice and then disrupted by douncing 10 times with a tight pestle in a 7 ml Weathon douncer. After centrifugation at 800×*g* for 10 min at 4 °C, supernatants were centrifuged at 20,000×*g* for 40 min at 4 °C. The resulting supernatants were saved as cytosolic extracts. Protein determinations were made using the Bradford method. Samples were size fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked for 1 h with Tris-buffered saline (TBS), 0.1% Tween 20 and 5% (w/v) defatted powdered milk, and incubated overnight with the corresponding antibody diluted 1:1000 in blocking buffer. Membranes were developed using ECL® detection reagents from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

2.7. Statistics

Results are given as means±S.E.M. Statistical analysis was done by one-way ANOVA and further Newman–Keuls Multiple Comparison Test using the program Graph-Pad Prism 3.0. A P value of less than 0.05 was considered to be statistically significant.

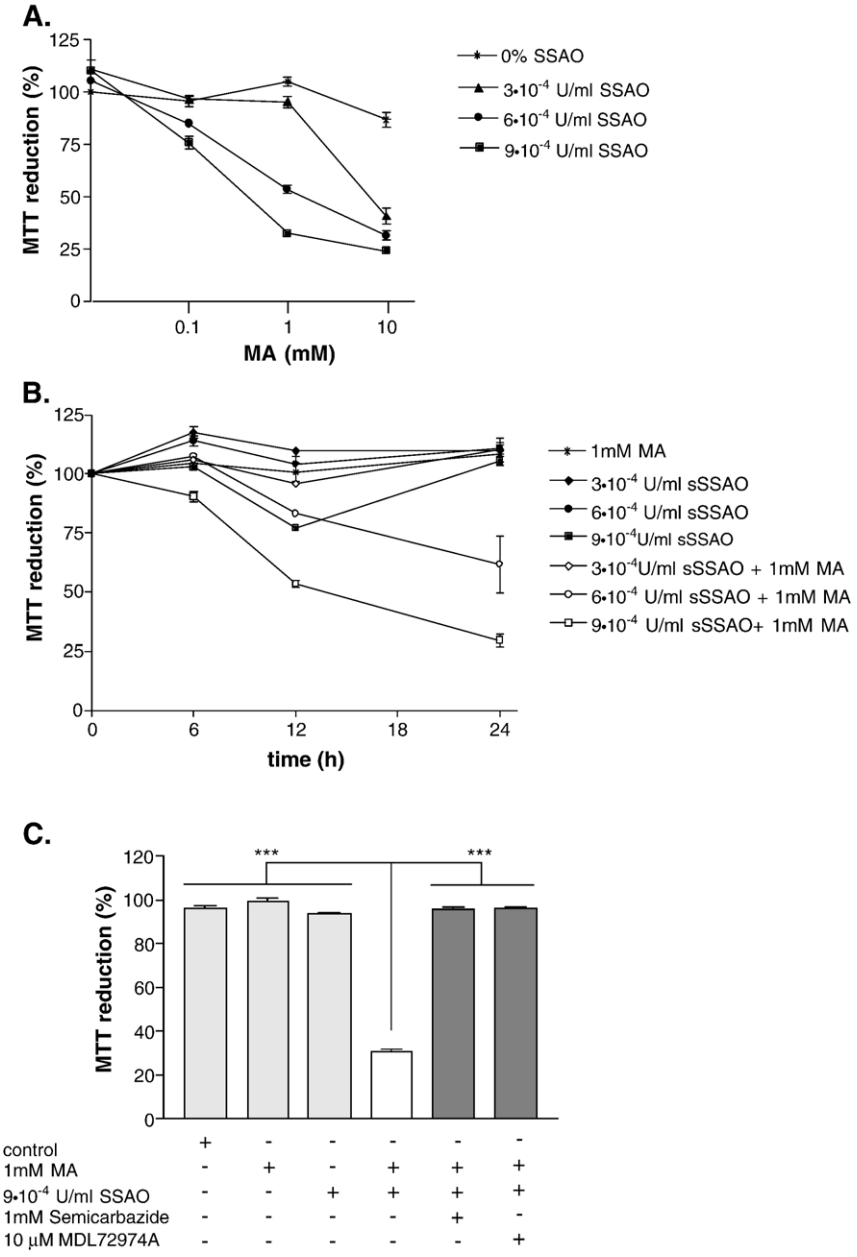


Fig. 1. MA oxidation by soluble SSAO induces cytotoxicity in A7r5 cells expressed as MTT reduction percentages. Cells were incubated with (A) different methylamine (MA) and soluble SSAO concentrations for 24 h (B) 1 mM MA and different soluble SSAO concentrations for 6, 12 and 24 h, and (C) 1 mM MA plus 9×10^{-4} U/ml of soluble SSAO and SSAO inhibitors, 1 mM semicarbazide and 10 μM MDL72974A, for 24 h. Data are mean±S.E.M. values of three separate experiments performed in triplicate. ***$P<0.001$ by a one-way ANOVA test and the addition of Newman–Keuls Multiple Comparison Test.

3. Results

3.1. MA oxidation by soluble SSAO induces cytotoxicity in A7r5 cells

Although the smooth muscle cell line A7r5 showed some MAO-A activity (100 pmol/min mg protein) determined radiochemically by serotonin oxidation, cells did not present MAO-B or SSAO activity assayed towards benzylamine as substrate. These conditions allowed us to study the soluble SSAO catalytic action on vascular cells. For this purpose, a bovine serum (BS) was used as the enzyme source, due to the large homology (81%) between soluble bovine amine oxidase and human SSAO [29]. We first studied methylamine (MA) oxidation by soluble SSAO on A7r5 cell viability, because this aliphatic amine is considered as a common substrate of bovine and human plasma SSAO [30].

A7r5 cells were treated with increasing concentrations of soluble SSAO and MA, and cell viability was measured by the MTT reduction assay. Soluble SSAO (3×10^{-4}, 6×10^{-4} and 9×10^{-4} U/ml) and 1 mM MA did not affect cell viability by themselves. However, 1 mM MA plus 9×10^{-4} U/ml of soluble SSAO induced a 70% decrease of total MTT reduction after 24 h treatment (Fig. 1A). The toxic effect was dose-dependent, obtaining a significant decrease in MTT reduction with 0.1 mM MA plus 6×10^{-4} U/ml or 9×10^{-4} U/ml of soluble SSAO (20% and 25% respectively on MTT reduction percentages). On the other hand, cell viability decrease was time dependent in the range studied (6, 12 and 24 h) (Fig. 1B). In order to confirm that cell viability loss was mediated by the SSAO activity contained in the BS, the experiment was performed using two specific SSAO inhibitors. Enzymatic activity was completely inhibited by 1 mM semicarbazide and 10 μM MDL-72974A, but cell viability was not affected at these concentrations. These SSAO inhibitors completely reverted the loss of cell viability caused by 1 mM MA plus 9×10^{-4} U/ml of soluble SSAO (Fig. 1C).

3.2. Effect of benzylamine and tyramine oxidation by soluble SSAO on A7r5 cells

Other SSAO substrates, benzylamine (BZ) and tyramine (TYR), were tested in the same experimental conditions. Incubation of A7r5 cells with 1 mM TYR and 9×10^{-4} U/ml of soluble SSAO resulted in a 90% decrease of total MTT reduction after 24 h. This effect was almost totally reverted by the presence of SSAO inhibitors (Fig. 2A). MAO inhibition by 1 μM clorgyline did not affect the cell viability, which ruled out the involvement of other amine oxidases activity in the process. In contrast, the non-physiological substrate BZ 1 mM, showed a slight toxic effect by itself, but no significant changes were observed in the presence of soluble SSAO (Fig. 2B). Inhibition of MAO or SSAO activity did not protect the cell death induced by BZ, demonstrating that they were not involved in the cytotoxicity. The enzyme kinetic parameters were determined towards the different SSAO substrates; MA, BZ and TYR. BZ was the best bovine serum SSAO substrate in terms of V_{max}/K_m ratio (Fig 2C), as previously described [30], followed by TYR and MA. In this context, the non cytotoxic effect observed after BZ metabolism suggests that the aldehyde produced by SSAO, rather than H_2O_2 or ammonia, is the main factor responsible for the reduction in cell viability.

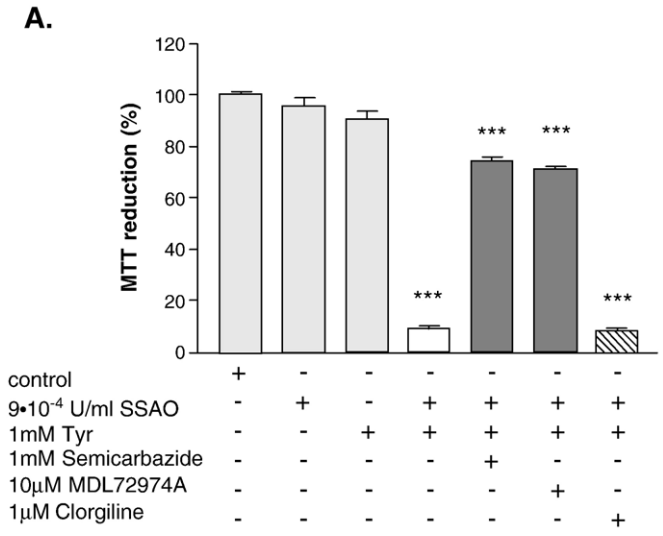
3.3. MA oxidation by SSAO induces apoptosis in A7r5 cells

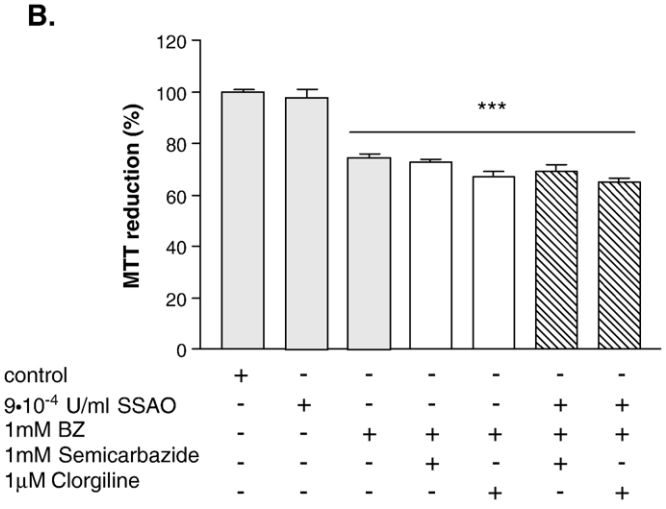
We then evaluated whether apoptosis would be involved in such toxic effect. Fig. 3 shows the double staining with Hoechst 33258 and anti-cleaved Caspase-3 antibody. Cleavage of the executer Caspase-3 into the active form is considered as a classical apoptotic feature. On the other hand, Hoechst 33258 staining display apoptotic cells with condensed, crescentic-aggregated, segmented or fragmented nuclei characteristic of apoptosis. Cell incubations with 1 mM MA or 9×10^{-4} U/ml of soluble SSAO separately for 24 h did not show stained positive cells (Fig. 3D and E). However, co-incubation with 1 mM MA and 9×10^{-4} U/ml of soluble SSAO resulted in numerous cells displaying a strong cytoplasmic red staining for cleaved Caspase-3 (Fig. 3B). The detailed micrograph shows that stained positive cells overlap with condensed nuclei stained with Hoechst 33258 (see Fig. 3B inset). Although 1 mM semicarbazide had no toxic effect by itself (Fig. 3F), it significantly diminished the number of cleaved Caspase-3-positive cells, indicating that the amine oxidase activity mediated the apoptotic process (Fig. 3C). The high percentage of active Caspase-3-positive cells (Fig. 3G) indicated that apoptosis was the main factor responsible for the cell death observed.

We also evaluated other apoptotic features; Poly (ADP-ribose) polymerase (PARP) is one of the essential substrates cleaved by executioner caspases and it is involved in maintaining DNA stability and repair. Western blot analysis revealed PARP cleavage only in cells treated with 1 mM MA plus 9×10^{-4} U/ml of soluble SSAO for 24 h (Fig. 4A), confirming the Caspase-3 activation observed. On the other hand, cytosolic fractions of cells showed a time-dependent increase in cytochrome *c* levels (Fig. 4B), as a consequence of its release from mitochondria to cytosol. Cytochrome *c* appeared after 12 h of soluble SSAO and MA co-treatment, indicating that it could be an early event in the intrinsic apoptosis pathway, before Caspase-3 activation and PARP cleavage determined at 24 h.

3.4. Effect of SSAO catalytic products on A7r5 cells

In order to study the mechanism involved in the toxicity observed, we next analyzed the direct effect of the final products generated by SSAO catalytic activity: H_2O_2, ammonia and different aldehydes (formaldehyde or benzaldehyde, which are the products of MA or BZ oxidation respectively). Considering the oxidative deamination stoichiometry (1:1) of primary amines [30] and our previous assays, which were performed at 1 mM MA, the products concentration range selected for treatments was from 0.1 to 1 mM. When cells were incubated in the presence of ammonia, no toxic effect was observed (Fig. 5A). In contrast, cells incubated with H_2O_2 for 24 h presented a





C.

SSAO substrates	Vmax (nmol/min)	Km (mM)	Vmax/ Km
Methylamine	7.32 ± 0.44	1.04 ± 0.28	7.04
Benzylamine	86.79 ± 5.22	0.85 ± 0.13	102.11
Tyramine	11.85 ± 0.73	0.42 ± 0.11	28.21

Fig. 2. Effect of different SSAO substrates oxidation in A7r5 cells. Cells were incubated for 24 h with (A) 9×10^{-4} U/ml of soluble SSAO plus 1 mM benzylamine (BZ) and SSAO inhibitors, 1 mM semicarbazide and 10 µM MDL72974A, or MAO-A inhibitor, 1 µM clorgyline, (B) 9×10^{-4} U/ml of soluble SSAO plus 1 mM tyramine (TYR) and 1 mM semicarbazide, 10 µM MDL72974A or 1 µM clorgyline. Data are mean±S.E.M. values of three separate experiments performed in triplicate. Statistically differences expressed as ***$P<0.001$ by a one-way ANOVA test and the addition of Newman–Keuls Multiple Comparison Test compared to control samples. (C) Kinetic constants of soluble SSAO towards BZ, MA, TYR as substrates (0.1, 0.5, 1, 5, 7.5, 10 mM) determined spectrophotometrically following the H_2O_2 generated.

slight decrease in cell viability, reaching a 40% decrease of total MTT reduction at 1 mM H_2O_2 (Fig. 5B). When formaldehyde was assayed at the same concentration range, the percentage of MTT reduction decreased drastically at much lower concentrations, compared to H_2O_2 treatment (Fig. 5C). Formaldehyde 0.5 mM induced almost 100% loss of cell viability. On the other hand, benzaldehyde treatment did not show changes in cell viability by itself (Fig 5D), demonstrating that BZ oxidation did not induce cytotoxicity because of the inert effect of the aldehyde generated.

We then examined whether the cytotoxic products induced apoptosis in A7r5 cells. Although it has been widely reported

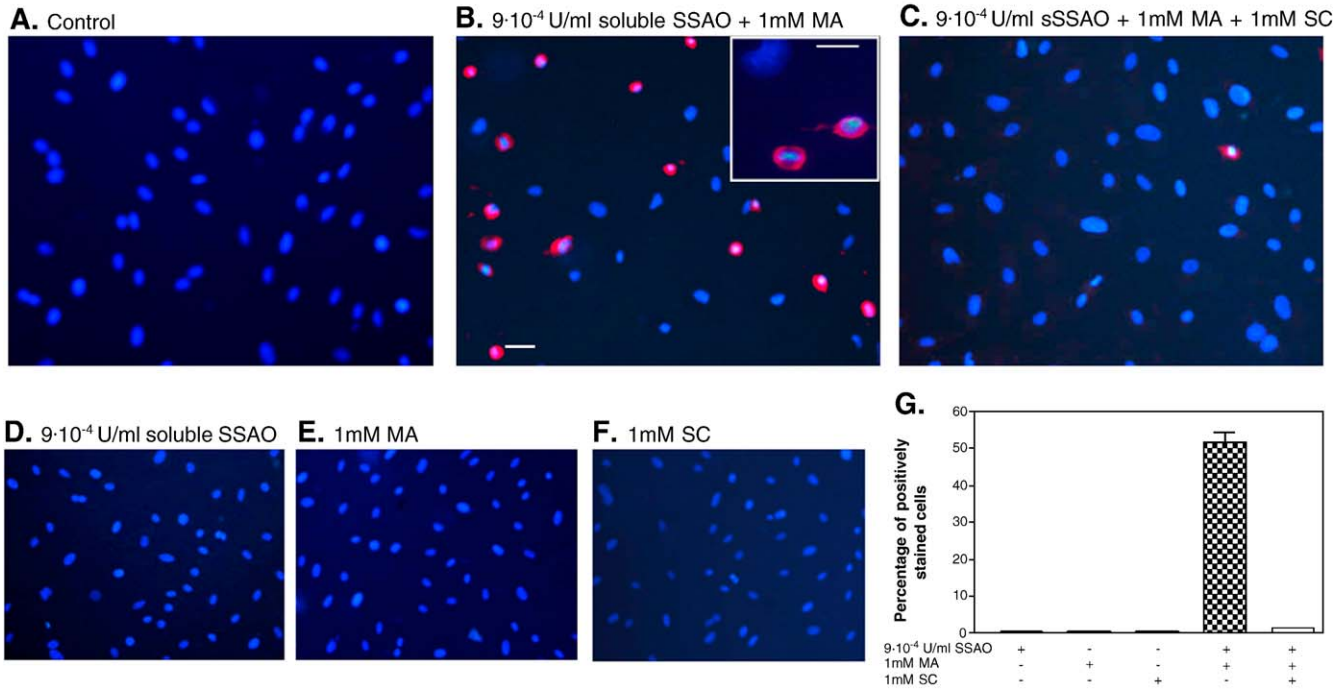


Fig. 3. MA oxidation by soluble SSAO induces Caspase-3 activation and nuclei condensation in A7r5 cells. Representative immunostaining with anti-cleaved Caspase-3 antibody and Hoechst 33258 of (A) control, (B) 9×10^{-4} U/ml of soluble SSAO+1 mM MA, (C) 9×10^{-4} U/ml of soluble SSAO+1 mM MA+1 mM semicarbazide (SC), (D) 9×10^{-4} U/ml of soluble SSAO, (E) 1 mM MA, and (F) 1 mM SC, for 24 h. Scale bar=50 μm in A–E and F and 25 μm in B inset. (G) Percentages of cleaved Caspase-3-positive cells. Data are mean±S.E.M. values of two separate experiments performed in triplicate.

that H_2O_2 induces such a cell death in many cell types, including smooth muscle cells [31], it was not known if formaldehyde was able to induce apoptosis in this cell type. 0.5 mM formaldehyde treatment for 24 h induced a 75% cleaved Caspase-3 stained cells (Fig. 6A). Hoechst 33258 staining of positive cells also revealed the characteristic condensed morphology of apoptotic nuclei. In contrast, when cells were treated with H_2O_2 in the same experimental conditions, only 10% of cells were positive for cleaved Caspase-3 cells (Fig. 6B). On the other hand, formaldehyde treatment for 24 h showed a clear PARP cleavage at all concentrations studied (Fig. 7A). Since this is one of the latest events in the apoptosis pathway, cells treated with H_2O_2 only showed PARP cleavage at longer time (42 h) (Fig. 7B).

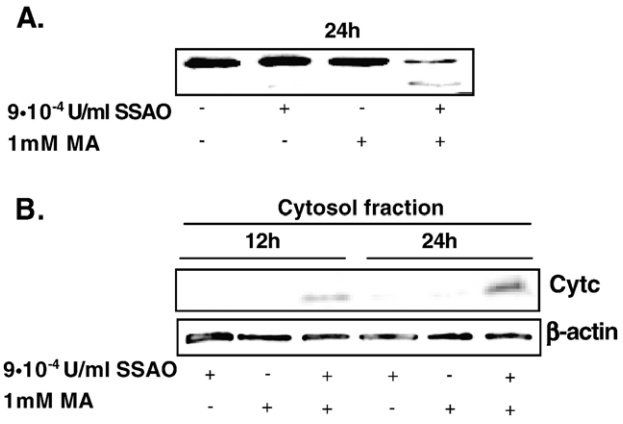


Fig. 4. MA oxidation by soluble SSAO induces apoptosis in A7r5 cells. Representative immunoblot of (A) PARP cleavage after 24 h treatment, and (B) time-dependent (12 and 24 h) cytochrome *c* release to cytosol. β-Actin is used as loading control.

3.5. MA oxidation by soluble SSAO induces cytotoxicity in HASMC

To examine whether the toxic effect induced by MA oxidation was attributable to the cell line, cell viability experiments were performed on primary cultures of smooth muscle cells from human aorta (HASMC). When cells were incubated in the presence of 1 mM MA plus 9×10^{-4} U/ml of soluble SSAO, only a 35% decrease of total MTT reduction was observed at 24 h (Fig. 8), in comparison with the 75% observed on A7r5 cells. These results indicate that cells from the primary culture are more resistant to the toxicity mediated by SSAO metabolic products. SSAO inhibitors, semicarbazide 1 mM and MDL72974A 10 μM, had no toxic effect by themselves, but recovered the loss of cell viability induced by SSAO catalysis. Since these cells do not present membrane-bound SSAO activity, MA oxidized by soluble SSAO was again the only factor responsible for the cell damage.

4. Discussion

Plasma levels of SSAO and its substrate MA are increased in certain diseases [13,22–24]. Although oxidation of the synthetic

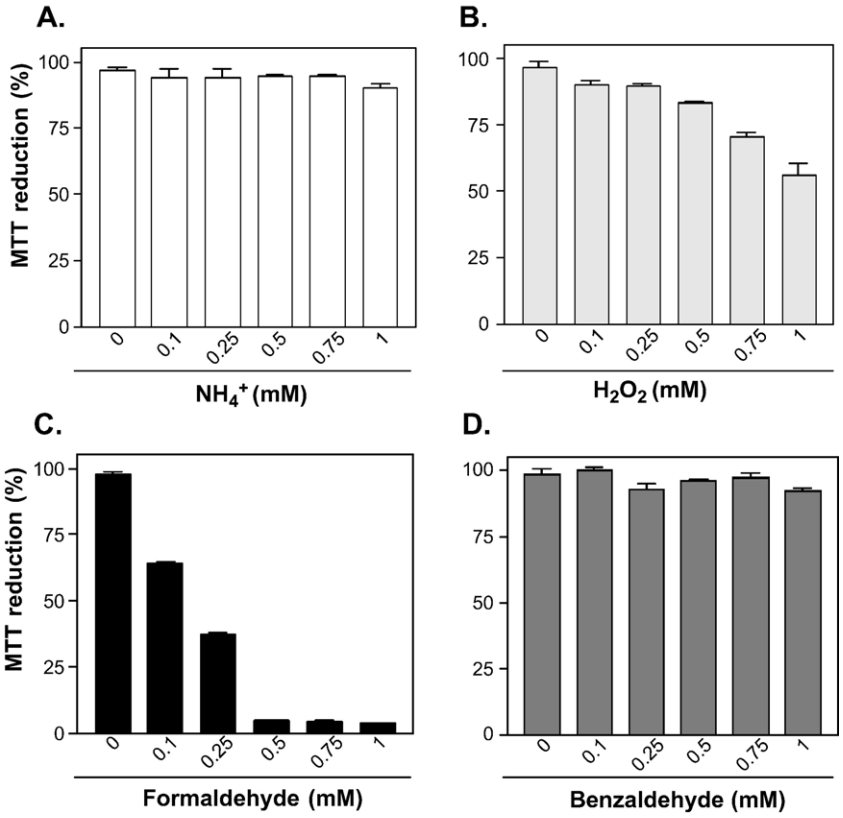


Fig. 5. Catalytic products generated by SSAO activity induce cytotoxicity in A7r5 cells expressed as MTT reduction percentages obtained from the incubation with different concentrations of (A) H_2O_2, (B) NH_4^+, (C) formaldehyde and, (D) benzaldehyde for 24 h. Data are mean±S.E.M. values of three separate experiments performed in triplicate.

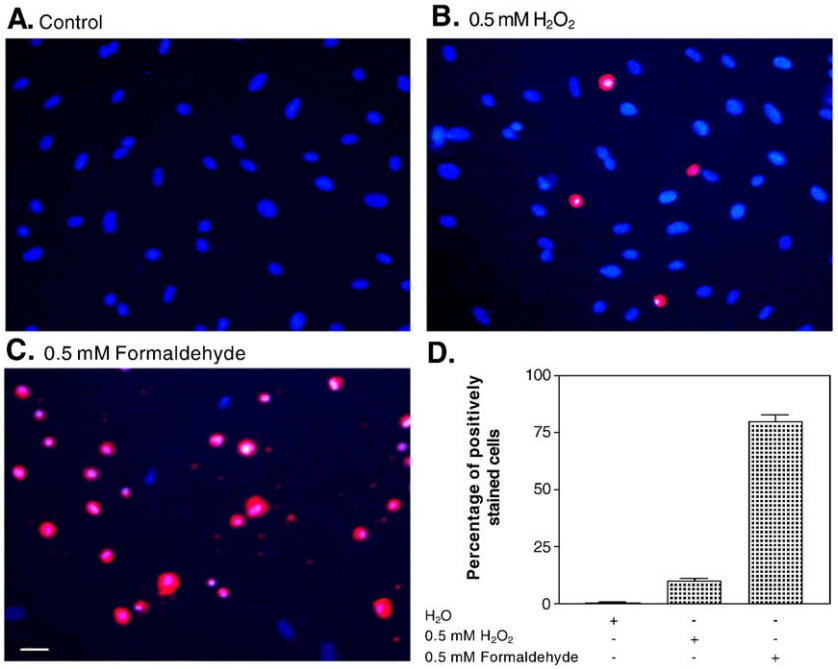


Fig. 6. SSAO cytotoxic catalytic products (H_2O_2 and formaldehyde) induce Caspase-3 activation and nuclei condensation on A7r5 cells. Representative immunostaining with anti-cleaved Caspase-3 antibody and Hoechst 33258 of (A) control, (B) 0.5 mM H_2O_2 and (C) 0.5 mM formaldehyde, after 24 h treatment. Scale bar=50 μm. (D) Percentages of cleaved Caspase-3-positive cells. Data are mean±S.E.M. values of two separate experiments performed in triplicate.

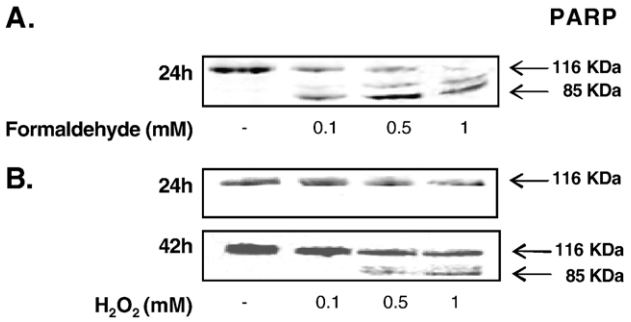


Fig. 7. H_2O_2 and formaldehyde induce apoptosis on A7r5 cells. Representative immunoblot of PARP cleavage after formaldehyde treatment for 24 h and H_2O_2 treatment for 24 and 42 h.

aliphatic amine allylamine is cytotoxic in smooth muscle cells (SMC) [32,33], these cells are resistant to MA toxicity [34], probably because of the low basal SSAO activity in cultured SMC. However, MA was toxic to endothelial cells in the presence of soluble SSAO [35]. Here we examine the effect of MA deamination by circulating SSAO on SMC as a potential risk factor of vascular damage, when the enzyme level is significantly increased in pathological conditions.

Although SSAO is constitutively expressed in vascular smooth muscle [2], A7r5 and HASMC do not show SSAO activity or expression. This could be explained by the difficulty of maintaining a differentiated contractile phenotype in culture. It has been widely reported that vascular SMC show plasticity in vivo and in vitro, which allows them to change phenotype in response to environmental changes [36]. Moreover, the loss of SSAO/VAP-1 expression in vascular cells has also been reported [35,37].

In this study, we show that soluble SSAO plus 1 mM MA was cytotoxic in A7r5 and HASMC cells. Because our experimental model is based on cultured cells, we studied an acute MA treatment for 24 h, using a higher amine concentration than those previously reported in human plasma [23]. Although this MA concentration is outside the physiological range, it has been widely used in cell culture studies [33–35,37]. The cytotoxicity observed was reverted when cells were preincubated with the specific SSAO inhibitors, semicarbazide or MDL 72974A, showing that cell death induction was due to the SSAO catalytic action, discarding other damage sources in the BS used. Furthermore, the catalytic products assayed separately, formaldehyde and H_2O_2, were cytotoxic, while ammonia was not. Formaldehyde, generated by MA oxidation, was much more toxic than H_2O_2 in the same experimental conditions, as reported in endothelial cells [35]. However, free radicals can be generated from formaldehyde and H_2O_2, which may contribute synergically to oxidative stress [38] and vascular damage. Only the oxidation of physiological substrates, MA and TYR, was toxic to A7r5 cells. In contrast, oxidation of the non-physiological substrate, BZ, by SSAO did not affect cultured cells. Because SSAO showed the highest affinity towards BZ, the differences in the cytotoxicity may be attributed to the specific reactivity and the chemical structure of the aldehyde generated. This hypothesis was confirmed by the lack of cytotoxicity obtained with benzaldehyde in comparison with formaldehyde.

Apoptosis of vascular SMC occurs during normal blood vessel development and maturation, but it has also been implicated in vascular disease [39]. Biogenic amines appear to be important for apoptosis triggering through the catalytic action of mitochondrial monoamine oxidase [40]. However, up to now, no direct evidence had shown that circulating SSAO induces apoptosis in SMC. In the present study apoptosis induced by MA oxidation was observed by chromatin condensation, Caspase-3 activation and PARP cleavage. Moreover, the release of mitochondrial cytochrome *c* to cytosol suggests that mitochondrial-mediated apoptosis is involved, probably through the oxidative stress generated by SSAO catalytic action. However, further studies are needed to elucidate whether other mechanisms or apoptotic pathways are also involved.

In pathological conditions, such as neurodegenerative diseases, stroke, traumatic brain injury, atherosclerosis and hypertension, oxidative stress contributes to apoptosis. Among the various factors that can induce oxidative damage, H_2O_2 plays a key role because it is generated in nearly all sources of oxidative stress and can diffuse freely in and out of cells and tissues [41]. In this study, we show that formaldehyde is a stronger inducer of apoptosis than H_2O_2. Formaldehyde can generate covalent interactions with macromolecular constituents in biological samples [42], altering cellular structures and inducing cell death. The ability of formaldehyde to generate cross-linking with proteins, DNA and other macromolecules, could explain the alteration of mitochondrial membrane structures, inducing the opening of the mitochondrial transition pore, promoting cytochrome *c* release to the cytosol, caspase activation and cell death. The apoptotic rate obtained after formaldehyde treatment is consistent with reports of the formation of glucose degradation products, such as methylglyoxal and

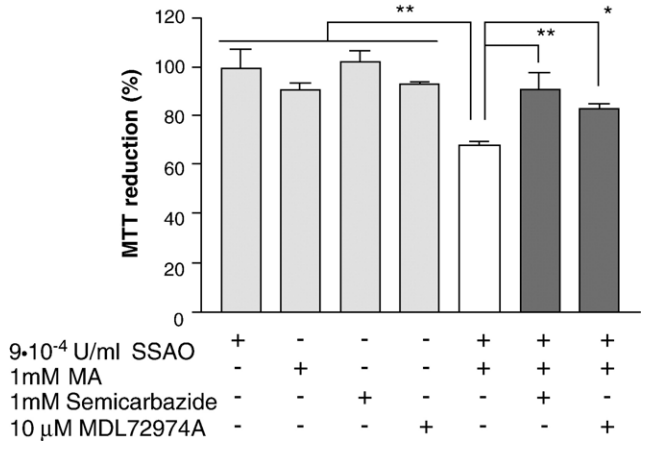


Fig. 8. MA oxidation by soluble SSAO induces cytotoxicity in HASMC expressed as MTT reduction percentages obtained with the incubation of 9×10^{-4} U/ml of soluble SSAO plus 1 mM MA and SSAO inhibitors, semicarbazide and MDL72974A, for 24 h. Data are mean±S.E.M. values of three separate experiments performed in triplicate. $^{**}P<0.01$, $^{*}P<0.05$ by a one-way ANOVA test and the addition of Newman–Keuls Multiple Comparison Test.

formaldehyde, which induced apoptosis in mesothelial cells [43].

Transgenic mice models that express full-length human VAP-1/SSAO in smooth muscle cells [44], endothelial cells or adipose tissue [12], show an increase in hVAP-1/SSAO in serum. This increase is dramatically higher when transgenic animals are treated with experimental diabetes inducers [12]. These results are in agreement with those data previously reported in human dysfunctions [13].The increase of blood MA and the resulting blood formaldehyde is potentially harmful because of the absence of formaldehyde dehydrogenase in blood plasma [45]. In vivo studies corroborate the toxic consequences of MA: chronic MA administration to SSAO/VAP-1 transgenic mice produced vascular complications related to diabetes pathology [46]. Moreover, there is evidence that increased SSAO-mediated deamination of MA contributes to protein deposition, formation of plaques and inflammation [47]. In this concern, transgenic mice overexpressing VAP-1/SSAO in SMC present pathological changes in the elastic fibers of aorta, suggesting the contribution of tissue-bound SSAO in the development of vascular damage [44].

Herein, we report for the first time, at molecular level, that plasma SSAO, through its catalytic action on the physiological substrate MA, induces apoptosis in SMC. The formaldehyde generated by SSAO seems to be the main contributor to cell death, by altering the mitochondria homeostasis and inducing apoptosis. However, a synergic effect of formaldehyde and H_2O_2 cannot be rule out.

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