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Characterization of A7r5 cell line transfected in a stable form by hSSAO/VAP-1 gene (A7r5 hSSAO/VAP-1 cell line)

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Summary A smooth muscle cell line (A7r5) was stably transfected with the human SSAO/VAP-1 (hSSAO/VAP-1) gene. The expressed protein was located solely in the membrane fraction of the cell. However it was also shown to be released into the cell-culture medium. Both the membrane-bound and released, soluble, forms had SSAO enzyme activity. Although MAO-A is present in wild-type A7r5 cells, it was undetectable in the transfected cells.

Keywords: Monoamine oxidase, semicarbazide-sensitive amine oxidase, smooth muscle cells, gene transfection, vascular-adhesion protein 1

Abbreviations

Clor	clorgyline
Dep	deprenyl
MAO	monoamine oxidase
Sc	semicarbazide
SSAO	semicarbazide-sensitive amine oxidase
VAP-1	vascular-adhesion protein 1

Introduction

Semicarbazide sensitive amine oxidase (SSAO, [E.C 1.4.3.6]), is a multifunctional enzyme (O'Sullivan et al., 2004) found in almost all mammalian tissues (Andres et al., 2001), specially in fat and in highly vascularized tissues (adipocytes, endothelial and smooth muscle cells). It is a glycoprotein that has different biological roles, which depend on the tissue where it is expressed. SSAO metabolizes primary amines (Lyles, 1996), generating hydrogen peroxide (H_2O_2), ammonia (NH_3) and the corresponding aldehyde as final products. In adipocytes, SSAO shows an insulinomimetic effect (Enrique-Tarancon et al., 1998) and it behaves as an inducible vascular-adhesion protein (VAP-1) under inflammatory conditions in endothelial cells (Salmi et al., 2001). However, its physiological function is still far from clear. SSAO/VAP-1 exists as a membrane-bound form and also as a soluble enzyme in plasma. It has been postulated (Abella et al., 2004) that the soluble form is derived from the membrane-bound one by a metalloprotease-dependent shedding process. Although high expression of SSAO/VAP-1 is observed *in vivo*, cultured cells lose this phenotype. In order to characterize the enzyme present in smooth muscle cells, we have stably transfected a smooth muscle cell line (A7r5) with the human SSAO/VAP-1 (hSSAO/VAP-1) gene.

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Materials and methods

Construct

Human SSAO/VAP-1 cDNA contained in pCMV-SPORT6 vector (ATCC; Image Clone ID: 6193046, GenBank ID: BC050549) was subcloned into pcDNA3.1(+) vector (Invitrogen; Barcelona, Spain). Recombinant vector was introduced in transformed *E. coli* DH5a cells. Cells were allowed to grow in LB medium plus ampicillin (50 μg/ml), and positive clones were selected and amplified to extract DNA. Plasmid DNA was confirmed by restriction enzyme digestions with *Eco*RI, *Not*I and *Spe*I and agarose gel electrophoresis.

Cell culture and transfection

The immortalized non-tumorigenic rat aortic smooth muscle cell line A7r5 (ECACC) was grown in DMEM supplemented with 2 mM glutamine,

50 U/ml penicillin, 50 µg/ml streptomycin, and 10% FBS (Gibco BRL; Grand Island, NY, USA). Cultures were maintained at 37°C in a humidified incubator, containing 95% room air and 5% CO_2 atmosphere. Transfections were performed using Fugene6 (Roche) in 50% confluent 100 mm dishes. Cells were allowed to grow for 1–2 months in medium containing the selection antibiotic geneticine (G418; 400 µg/ml). Cells were then diluted and allowed to grow in the presence of 100 µg/ml G418 in isolated colonies, which were amplified separately.

Total cell lysates, subcellular fractionation and concentrated culture media

To obtain total cell lysates, cells were washed twice with PBS, scrapped into a buffer containing 1% Triton X-100, 10 mM EDTA, 50 mM Tris-HCl, pH 7.5, supplemented with protease inhibitor cocktail (Sigma Aldrich; St. Louis, Mo, USA) and sonicated for 10 s. For subcellular fractionation, the buffer contained 10 mM Hepes, 1.5 mM $MgCl_2$ and 10 mM KCl, pH 7.9, supplemented with protease inhibitor cocktail. Cells were lysed by Dounce homogenization and centrifuged at 2,000 *g* for 15 min at 4°C to pellet unlysed cells and nuclei. The supernatant was centrifuged at 100,000 *g* (Sorvall Discovery M120 SE) for 1 h at 4°C to separate a soluble cytosolic fraction from a membranous pellet. Culture medium was collected from the cells and concentrated by means of successive centrifugations at 20,000 *g* in 10 KDa porus Centricon® centrifugal filter units.

Western blot analysis

Cell lysate protein determinations were made using the Bradford method. Samples were separated by SDS-PAGE, for culture media, the maximum volume was loaded, and transferred onto nitrocellulose membrane. Membranes were incubated with specific antibodies for VAP-1 (1:500) (C-terminal E-19 from Santa Cruz Biotech; Heidelberg, Germany), IGF-I receptor (1:1000) (Santa Cruz Biotech; Heidelberg, Germany), or GAPDH (1:20000) (Ambion; Cambridgeshire, UK) overnight at 4°C and developed using ECL® detection reagents, from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

SSAO, MAO-A and MAO-B activity determination

A modification of the radiochemical method of Otsuka and Kobayhashi (1964) was used. SSAO and MAO-B activities were determined using 100 µM (^{14}C)-benzylamine (2 mCi/mmol, Amersham, UK), and MAO-A activity using 5-(2-^{14}C)-hydroxytryptamine binoxalate (0.5 mCi/mmol, Perkin Elmer, USA) as substrates. 1 µM *l*-deprenyl was used to inhibit MAO-B, 1 µM semicarbazide to inhibit SSAO and 1 µM clorgyline to inhibit MAO-A. Cell lysate activities are expressed as pmol product/min · mg protein and culture medium activities as pmol product/min · ml medium.

Statistics

Results are given as means ± SEM. Statistical analysis were done by one-way ANOVA and further Newman-Keuls Multiple Comparison Test, using the program Graph-Pad Prism 3.0. Significance was accepted at $P < 0.05$.

Results

SSAO/VAP-1 expressed in A7r5 hSSAO/VAP-1 transfected cells is localized in the plasma membrane and released into culture medium

Although high expression of SSAO/VAP-1 is observed *in vivo*, cells lose this phenotype in culture. In order to study the membrane-bound form of this enzyme present in smooth muscle cells, it was necessary to obtain a smooth muscle cell line transfected in a stable form with the human SSAO/VAP-1 gene. To achieve this, the human SSAO gene was subcloned into pcDNA3.1 vector, which allows selection in eukaryotic cells, and the recombinant vector obtained was checked by means of restriction enzyme digestions (data not shown). The A7r5 smooth muscle cells were then transfected using the FuGene6 method (Roche) and selected with the antibiotic G418.

After isolation and amplification of cell colonies, different clones were checked and selected by their SSAO expression. The specific markers: IGF-1 receptor for membrane fraction and GAPDH for cytosolic fraction were used in order to elucidate the subcellular localization of transfected SSAO in the cytosolic and membrane fractions of A7r5 hSSAO/VAP-1 cells. Figure 1A shows the presence of SSAO only in the membrane fraction. Because it has been postulated that the soluble SSAO is derived from the membrane-bound form, the possibility that the clones released SSAO into culture medium was investigated. Western-blot analysis (Fig. 1B) showed that SSAO

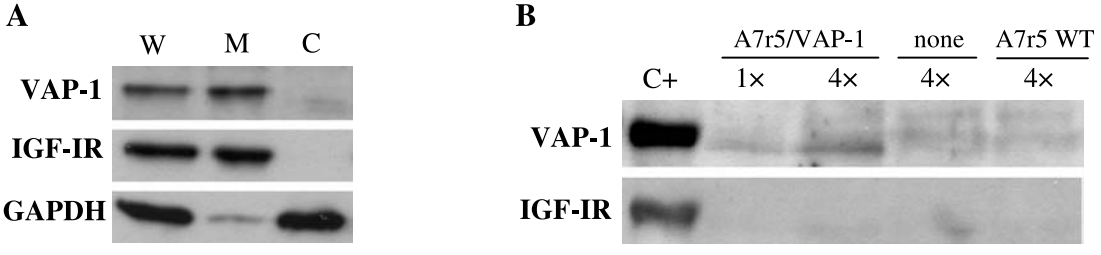

Fig. 1. Distribution of SSAO/VAP-1 in A7r5 hSSAO/VAP-1 cells. **A** SSAO/VAP-1 is present in the cell membrane but not in cytosol. Whole lysate (*W*), membrane (*M*) and cytosolic (*C*) subcellular fractions. IGF-IR and GAPDH were used as membrane and cytosolic subcellular fraction markers. **B** The enzyme is released into the culture medium from transfected cells. hSSAO/VAP-1 presence in: A7r5 hSSAO/VAP-1 cell lysate (C+) and conditioned medium from A7r5 hSSAO/VAP-1 cells [not concentrated (1×) and 4 times concentrated (4×)], control medium, and from A7r5/WT cells. IGF-IR was used as control for any lysed cells in the medium

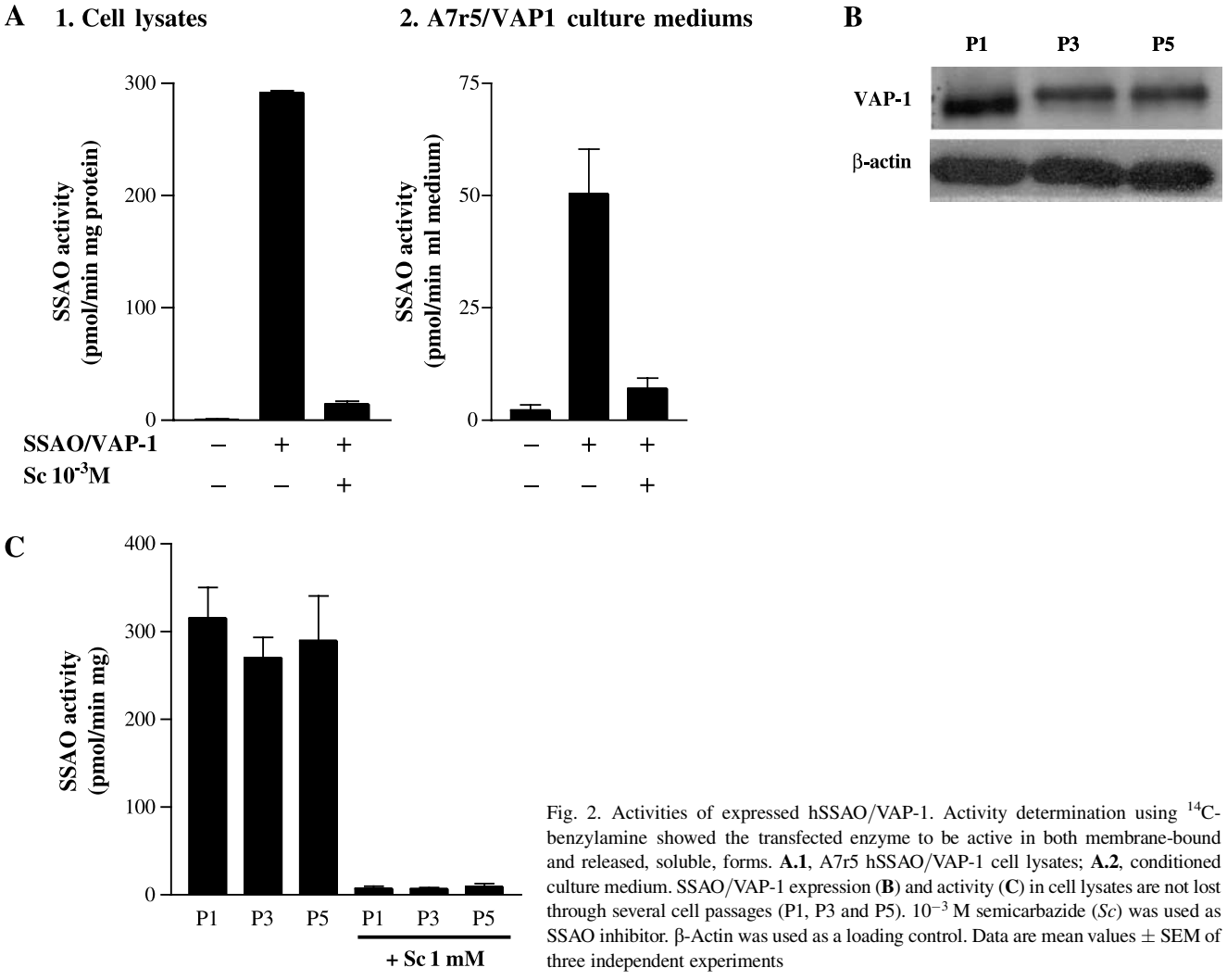

Fig. 2. Activities of expressed hSSAO/VAP-1. Activity determination using ^{14}C-benzylamine showed the transfected enzyme to be active in both membrane-bound and released, soluble, forms. **A.1**, A7r5 hSSAO/VAP-1 cell lysates; **A.2**, conditioned culture medium. SSAO/VAP-1 expression (**B**) and activity (**C**) in cell lysates are not lost through several cell passages (P1, P3 and P5). 10^{-3} M semicarbazide (*Sc*) was used as SSAO inhibitor. β-Actin was used as a loading control. Data are mean values ± SEM of three independent experiments

was present in the medium from transfected cells [not concentrated (1×) and four times concentrated (4×)], but was absent from control medium or medium from wild-type (WT) cells.

Transfected hSSAO/VAP-1 is enzymatically active in both cell lysates and culture medium

After checking the protein expression in different clones, the enzyme activity towards benzylamine as substrate was determined. Both cell lysates and culture medium from transfected cells showed SSAO activity, which was completely inhibited by the specific SSAO inhibitor semicarbazide (Fig. 2A), whereas neither cell lysates, nor culture media of WT cells showed any SSAO activity. To check the stability of SSAO expression in transfected cells, successive cell passages of different clones were analyzed for SSAO expression (Fig. 2B) and activity (Fig. 2C). There was no loss of expression or activity through several passages.

A7r5 WT and A7r5 hSSAO/VAP-1 have different amine oxidase pattern activities

MAO-A and MAO-B activities were also assayed in the transfected and WT cells. There was significant MAO-A activity in WT cells (Fig. 3A), but SSAO and MAO-B activities were absent. Surprisingly, this pattern changed in transfected cells (Fig. 3B), which showed SSAO activity, as a consequence of the transfection, but a lack of MAO-A activity. These results suggest a possible modulation between SSAO and MAO-A activities. However, treatment of WT cells with the MAO-A inhibitor, clorgyline (10^{-7} M) for ten days did not induce SSAO activity,

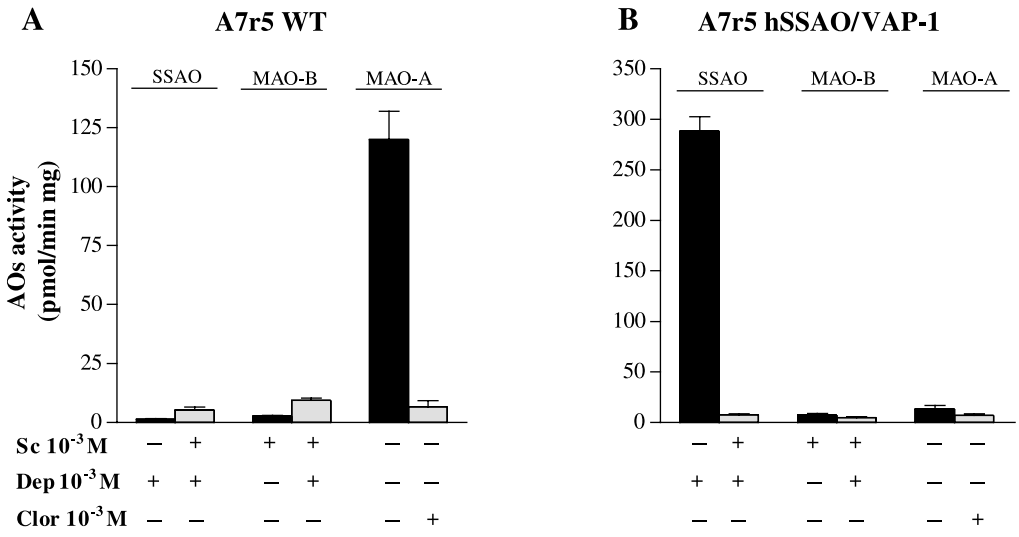

Fig. 3. Enzyme activities of SSAO, MAO-B and MAO-A in A7r5 WT and hSSAO/VAP-1 cell lysates. SSAO and MAO-B activities were measured with 100 μM ^{14}C-benzylamine, and MAO-A with 100 μM ^{14}C-5-HT. Total inhibition of SSAO, MAO-B and MAO-A activities with their specific inhibitors semicarbazide (*Sc*), deprenyl (*Dep*) and clorgyline (*Clor*), respectively, confirmed the specificity of the activities detected. **A**, MAO-A activity was detected in WT cells, but MAO-B and SSAO activities were not present. **B**, SSAO activity was detected in hSSAO/VAP-1 cells, but there was no MAO-A or -B activity. Data are mean values ± SEM of three independent experiments

and treatment of transfected cells with semicarbazide (10^{-3} M) was unable to induce MAO-A activity (data not shown).

Discussion

In order to elucidate the role of SSAO in cerebrovascular tissue in pathological conditions such as in cerebral amyloid angiopathy linked to Alzheimer's disease (CAA-AD), and its contribution to vascular damage, it was necessary to work with vascular cells that express SSAO/VAP-1. Although SSAO is constitutively expressed in vascular smooth muscle cells (Conklin et al., 1998), A7r5 cell line and HVSM (human vascular smooth muscle) cells did not exhibit any detectable SSAO activity or expression. This may result from the difficulty of maintaining a differentiated contractile phenotype in culture. It has been widely reported that vascular SMC show an *in vivo* and *in vitro* plasticity, which allow them to change its phenotype in response to environmental changes (Langford et al., 2001). Moreover, the loss of SSAO/VAP-1 expression in other smooth muscle and endothelial cell types has been previously observed by other authors (Blaschko, 1962; Owens, 1995; Yu and Zuo, 1993).

The results obtained in this study showed SSAO/VAP-1 expression in cell lysates of transfected A7r5 cells and in its culture medium, and this expression correlated with the catalytic activity towards ^{14}C-benzylamine. This activity was of the same order as that previously reported in aorta (Andres et al., 2001). Since WT cells did not show any SSAO activity in cell lysates or in culture medium, our results suggest that overexpression of membrane-bound SSAO in the transfected cells enhances the enzyme release into the culture medium. These data are in agreement with studies reported using transgenic mice overexpressing SSAO/VAP-1 in smooth muscle (Gokturk et al., 2003), endothelial cells and adipocytes (Stolen et al., 2004a, b).

Although SSAO expression and activity were not lost in transfected cells through several cell passages, they showed the same morphology than A7r5 WT cells. After subcellular fractionation, SSAO/VAP-1 expression was only observed in the membrane fraction indicating the same subcellular localization as *in vivo* conditions.

Some authors have suggested that it could exist a compensatory activity between MAO-A/MAO-B and SSAO (Fitzgerald et al., 1998; Fitzgerald and Tipton, 2002). Although the transfected cell line showed high SSAO/VAP-1 activity, MAO-A and MAO-B activities were not detected. In contrast, WT A7r5 cells showed significant levels of MAO-A, but no SSAO and MAO-B activities. When activities were determined in human aorta homogenates, we detected high SSAO/VAP-1 activity but no MAO-A activity. This amine oxidase activity pattern, also observed in our transfected cell line, could suggest a possible modulation between the enzymes. We tried to induce SSAO expression in WT cells and MAO-A expression in transfected cells by means of clorgyline and semicarbazide treatments, respectively, but this was not successful. The

possible modulation between these enzymes may be explained by another, activity independent, mechanism, probably at the gene expression level.

Taken together, these results show that the hSSAO/VAP-1-transfected A7r5 cell line is a valid model for studying the membrane-bound form of SSAO/VAP-1 enzyme *in vitro*.

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