RESULTATS I DISCUSSIÓ CAPÍTOL 4:

L'ACCIÓ CATALÍTICA DE LA SSAO LLIGADA A MEMBRANA INDUEIX CITOTOXICITAT EN CÈL·LULES DE MUSCULATURA LLISA EN CULTIU

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El següent objectiu del treball va ser estudiar si l'efecte tòxic observat en el capítol anterior era exclusivament resultat de l'acció de la SSAO soluble, o contràriament, la SSAO lligada a membrana era igualment capaç d'induir dany cel·lular. Per altra banda, i donat que en certes patologies humanes s'ha descrit un augment de la SSAO, també es va voler estudiar si la SSAO tissular d'origen humà tenia el mateix comportament tòxic, mitjançant la seva acció catalítica sobre amines com a substrats, que la SSAO soluble d'origen boví. Prèviament, i donat que tant les cèl·lules A7r5, com el cultiu primari d'HASMC, no expressen la SSAO, es va sobreexpressar la proteïna d'origen humà transfectant la línia cel·lular utilitzada. D'aquesta manera, es va pretendre aproximar-se a un model relativament més fisiològic, ja que les cèl·lules de múscul llis de teixits vascularitzats expressen la SSAO de forma constitutiva *in vivo*.

4.1. Estandarització de la transfecció transitòria de la línia cel·lular A7r5

4.1.1. Transfecció transitòria de GFP

En primer lloc, va caldre posar a punt el sistema de transfecció en aquesta línia cel·lular, per tal de definir els paràmetres més adequats per a la transfecció de la línia. El reactiu de transfecció utilitzat va ser Fugene6 (Roche), el qual es basa en una mescla de lípids que s'acomplexa i transporta el DNA a l'interior de la cèl·lula. Es va escollir aquest reactiu per la seva elevada eficiència de transfecció en un gran nombre de línies cel·lulars i per ser un reactiu inert per les cèl·lules en cultiu (veure Material i Mètodes 4.3). Per establir aproximadament el rendiment i el percentatge de transfecció que s'obtenia amb aquest reactiu en la línia A7r5, es va utilitzar el vector comercial pEGFP (BD Bioscience Clontech) amb l'inserit de la proteïna GFP (G*reen Fluorescent Protein*), ja que les propietats fluorescents de la proteïna del gen transfectat permetia d'una manera senzilla observar i fer un recompte del percentatge de cèl·lules transfectades al llarg dels dies.

Es va dur a terme la transfecció de les cèl·lules A7r5 utilitzant dues relacions diferents entre el volum de reactiu i la quantitat de DNA, tal i com especifiquen els proveïdors. En fer el recompte de les cèl·lules fluorescents, és a dir que s'havien transfectat i expressaven la proteïna GFP, s'observava que la relació de 3 µl: 1 µg de DNA era la que donava lloc a un rendiment més elevat de transfecció, obtenint-se un màxim d'un 25% aproximadament de cèl·lules positives per GFP respecte el nombre total de cèl·lules als 3 dies de transfecció (Fig. 4.1). Aquests valors ens indicaven que la línia cel·lular A7r5 es transfectava satisfactòriament utilitzant el reactiu Fugene6.
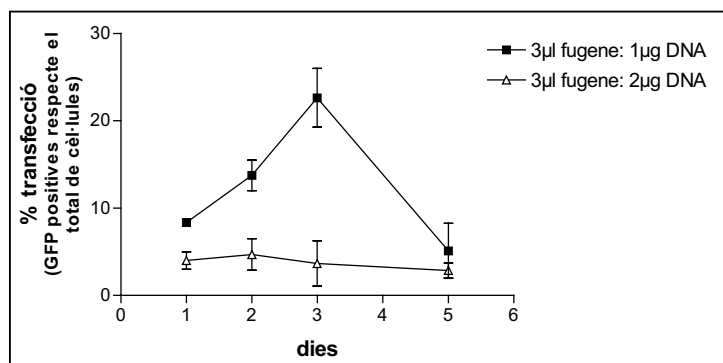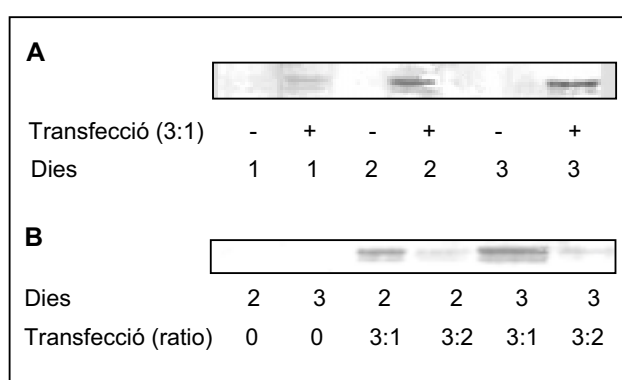

Figura 4.1. Transfecció transitòria de cèl·lules A7r5 amb Fugene6 i el plasmidi pEGFP. Percentatge de cèl·lules positives per GFP respecte el nombre total de cèl·lules utilitzant dues relacions diferents de volum de reactiu i quantitat de DNA.

4.1.2. Transfecció transitòria de VAP-1/SSAO

A continuació es va procedir a l'estandardització del mètode de transfecció utilitzant el nostre gen d'interès, VAP-1/SSAO. Es va obtenir el vector pCMV-sport6.1 amb l'inserit de la VAP-1 (*Homo sapiens amine oxidase, copper containing 3 (vascular adhesion protein 1)*), mRNA (cDNA *clone* MGC:57642 IMAGE:6193046).

Una vegada amplificat el plasmidi d'interès en cèl·lules d'*E.Coli*, es van transfectar les cèl·lules A7r5 utilitzant les mateixes relacions entre volum de reactiu de Fugene6 i quantitat de DNA utilitzades prèviament (veure Material i Mètodes 4). Es va realitzar l'anàlisi per Western-blot per tal de determinar l'expressió de la proteïna en les diferents condicions de transfecció i a diferents temps. Els resultats obtinguts (Fig. 4.2) confirmen, en primer lloc, que la línia cel·lular A7r5 no expressa la proteïna VAP-1/SSAO. En canvi,

les cèl·lules transfectades transitòriament amb aquest gen són capaces d'expressar lleugerament la proteïna. Les condicions òptimes de transfecció amb aquest plasmidi són els mateixes que les establertes amb el vector GFP, és a dir cal una relació de 3 µl de reactiu Fugene6: 1 µg de DNA. Per tant, una vegada més, cal destacar la gran importància de la quantitat de DNA a l'hora d'optimitzar el mètode de transfecció. Així mateix, en els Western-blots es va poder observar com s'obtenia una major expressió de VAP-1 en augmentar el temps de transfecció.

Figura 4.2. Transfecció transitòria del plasmidi pCMV-Sport6/VAP-1 en cèl·lules A7r5. Western-blot de la proteïna VAP-1/SSAO (anti-VAP-1) de llisats de cèl·lules transfectades amb: **(A)** una relació 3 µl de reactiu Fugene6: 1 µg de DNA en funció del temps (1, 2 i 3 dies), i **(B)** amb una relació 3:1 i 3:2 (µl de reactiu Fugene6: µg de DNA) en funció del temps (2 i 3 dies).

A continuació, es va analitzar si la proteïna expressada era funcionalment activa. Per això es va realitzar un assaig radiomètric de determinació de l'activitat SSAO en front a benzilamina com a substrat després de 48 hores de transfecció. Els resultats obtinguts confirmen que la VAP-1 humana expressada transitòriament a les cèl·lules A7r5 és capaç de metabolitzar la benzilamina com a substrat *in vitro*, obtenint una activitat específica de 10 pmol/min·mg de proteïna, contràriament al que succeeix en cèl·lules no transfectades (Fig. 4.3).
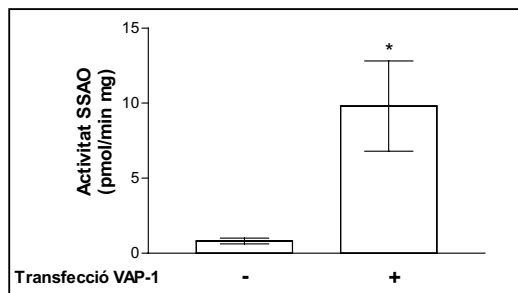
Figura 4.3. Determinació de l'activitat SSAO, mitjançant el mètode radiomètric en front a benzilamina 100 µM 2 mCi/mmol com a substrat, de cèl·lules A7r5 transfectades transitòriament amb el plasmidi pCMV-Sport6/VAP-1 durant 48 h. Les dades s'expressen com la mitja ∂ SEM de cinc experiments diferents.(*) $p<0.05$ segons el test *t*.

4.2. Efecte de l'oxidació de la MA en cèl·lules A7r5 transfectades transitòriament amb VAP-1/SSAO

Per tal de comprovar si el metabolisme de la proteïna transfectada era capaç d'induir dany cel·lular, es van tractar les cèl·lules A7r5, prèviament transfectades amb VAP-1/SSAO durant 48 hores, amb MA 1 mM durant 24 i 48 hores, i se'n va determinar la viabilitat cel·lular mitjançant el mètode de reducció de l'MTT. Els resultats mostren que el tractament no indueix cap efecte citotòxic (Fig. 4.4).
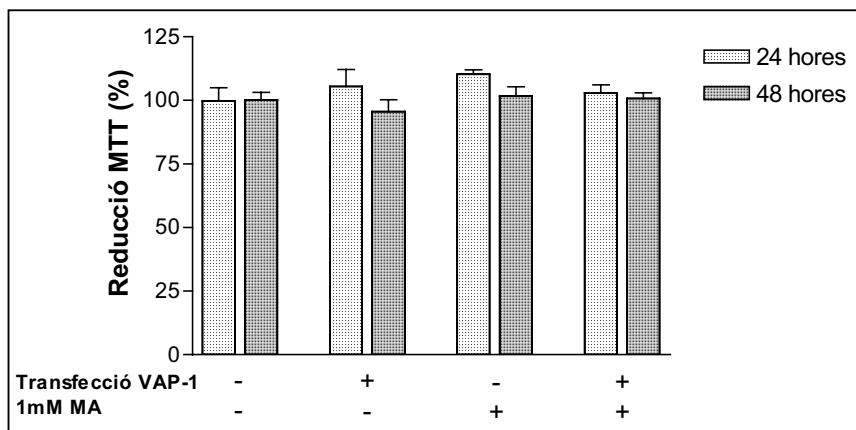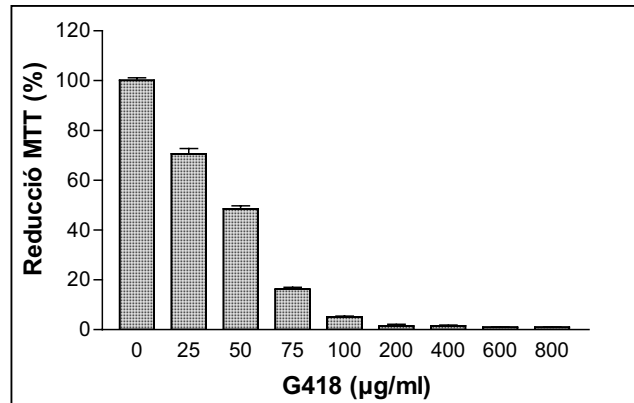

Figura 4.4. Viabilitat cel·lular expressada en percentatges de reducció d'MTT. Incubació de cèl·lules A7r5, transfectades amb el plasmidi pCMV-Sport6/ VAP-1 durant 48 h, amb 1mM MA durant 24 i 48 h. Les dades s'expressen com la mitja ∂ SEM de tres experiments diferents realitzats en triplicat.

Tenint en compte que l'activitat específica SSAO obtinguda per les cèl·lules transfectades (10 pmol/min·mg de proteïna) és molt inferior a l'activitat específica d'un homogenat de túnica media d'aorta humana (2400 pmol/min·mg de proteïna), podria ser que l'acció catalítica de la VAP-1/SSAO expressada pel mètode de transfecció transitòria no fos suficient per generar la concentració de metabòlits necessaris per induir el dany cel·lular. Donat que el percentatge de transfecció en aquesta línia es troba només al voltant del 25 %, calia augmentar el percentatge d'expressió de la proteïna, per tal de confirmar un possible efecte citotòxic de l'oxidació de la MA per part de la forma enzimàtica lligada a membrana. També cal tenir present que la determinació de la viabilitat cel·lular es va realitzar després de 48 hores del tractament amb MA, però podria ser que calgués un temps més llarg per tal que els productes de l'oxidació de la MA poguessin induir dany cel·lular.

4.3. Obtenció de línies estables VAP-1/SSAO en cèl·lules A7r5

Donat que l'expressió transitòria de la SSAO/VAP-1 no era suficient per estudiar els objectius plantejats en aquesta part del treball, es va procedir a la creació de línies cel·lulars estables VAP-1/SSAO.

Per tal d'obtenir una línia cel·lular que expressés establement la proteïna VAP-1/SSAO es va utilitzar el mateix mètode de transfecció que el descrit anteriorment. En aquest cas, les cèl·lules es transfectaven amb un vector que tingués l'insert d'interès i dos punts de selecció: resistència a ampicil·lina, per la selecció durant la transformació de bacteris amb el plasmidi, i resistència a neomicina, per la selecció de cèl·lules eucariotes que han incorporat el plasmidi (veure Material i Mètodes 5). Per tant, en primer lloc calia comprovar que les cèl·lules de treball, A7r5 sense transfectar, eren sensibles a la toxicitat per neomicina. Es va utilitzar l'antibiòtic anàleg a la gentamicina, el G418, el qual és un aminoglicòsid que bloqueja la síntesi de proteïnes en cèl·lules de mamífers. Així doncs, es va realitzar una corba de toxicitat de G418 en cèl·lules A7r5 mesurant la viabilitat cel·lular pel mètode de reducció de l'MTT després d'una setmana de tractament. Els resultats mostraven clarament que el G418 era altament citotòxic, donant lloc a una pèrdua de viabilitat cel·lular de pràcticament el 100% a una concentració de 100 µg/ml (Fig. 4.5).

Figura 4.5. Viabilitat cel·lular expressada en percentatges de reducció d'MTT. Incubació de cèl·lules A7r5 amb concentracions creixents de G418 durant 7 dies. Les dades s'expressen com la mitja ∂ SEM de dos experiments realitzats en quadruplicat.

Per obtenir un plasmidi que tingués els dos punts de selecció esmentats i poder transfectar les cèl·lules per l'obtenció de línies estables, es va utilitzar el vector comercial pcDNA3.1(+) (Invitrogen). Va caldre subclonar el gen de la VAP-1/SSAO contingut en el vector pCMV-sport6.1 al vector pcDNA3.1(+) (Fig. 4.6). Tot i que el gen d'interès havia estat inserit dins el vector pCMV-sport6.1 entre les dianes de restricció de *Not*I i *Sal*I, es van usar els enzims de restricció *Sca*I, *Not*I i *EcoR*I per la seva digestió. Es van escollir aquestes dianes perquè, en primer lloc, quedava el gen de la VAP-1 intacte, i en segon lloc, perquè en fer córrer el producte de la digestió en un gel d'agarosa 0.8% s'obtenien bandes suficientment separades entre elles per a la seva posterior purificació. Paral·lelament, es va digerir el vector pcDNA3.1(+) amb els mateixos enzims, *Not*I i *EcoR*I, amb els quals s'havia separat la banda corresponent al gen de la VAP-1 inserit a l'altre vector. En fer córrer el producte de digestió en un gel d'agarosa 0.8%, es va obtenir una única banda gran corresponent al vector obert de 5.4 Kb (Fig. 4.6). Una vegada purificades les bandes d'interès, corresponent al gen de la VAP-1 i al vector pcDNA3.1(+) obert, es va procedir al procés de lligació.

Per tal de comprovar que el producte de lligació era l'esperat, en primer lloc va caldre amplificar el material obtingut. Quan es va disposar de suficient material plasmídic, es va realitzar una digestió amb els enzims de restricció *EcoR*I, *Spe*I i *Not*IA, i es van obtenir 4 bandes del pes molecular esperat (Fig. 4.7). Es va realitzar la mateixa digestió a partir del material obtingut a partir de 6 colònies diferents resistents a ampicil·lina per

tal de comprovar que no hi haguessin mutacions en el plasmidi obtingut. Els resultats van permetre concloure que havíem obtingut el plasmidi recombinant pcDNA3.1(+)/VAP-1. A més, i com a mesura de seguretat, es va seqüenciar el nou plasmidi (Servei de Seqüenciació, SUNY at Stony Brook, NY, USA) i els resultats van confirmar els obtinguts mitjançant la digestió enzimàtica (resultats no mostrats).
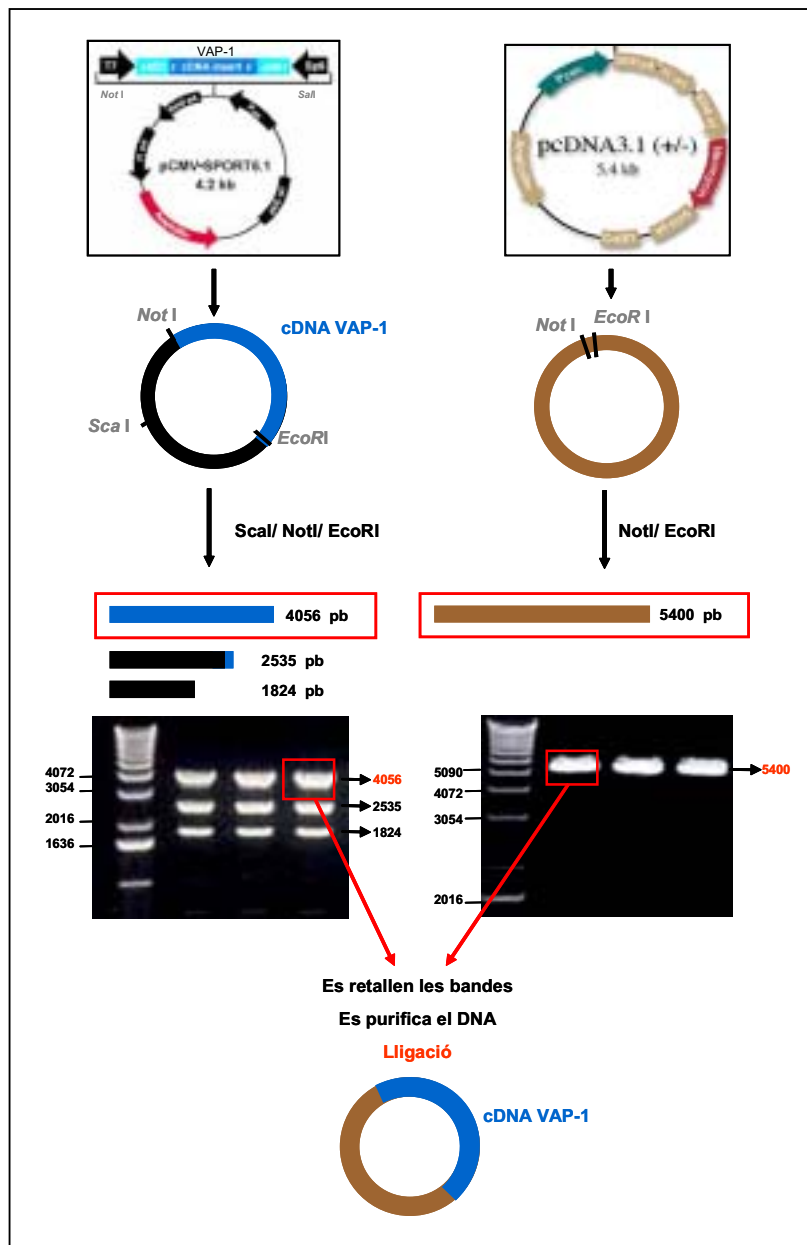
Figura 4.6. Procés de subclonatge de gen VAP-1 en el vector pCMV-sport6.1 al vector pcDNA3.1(+).
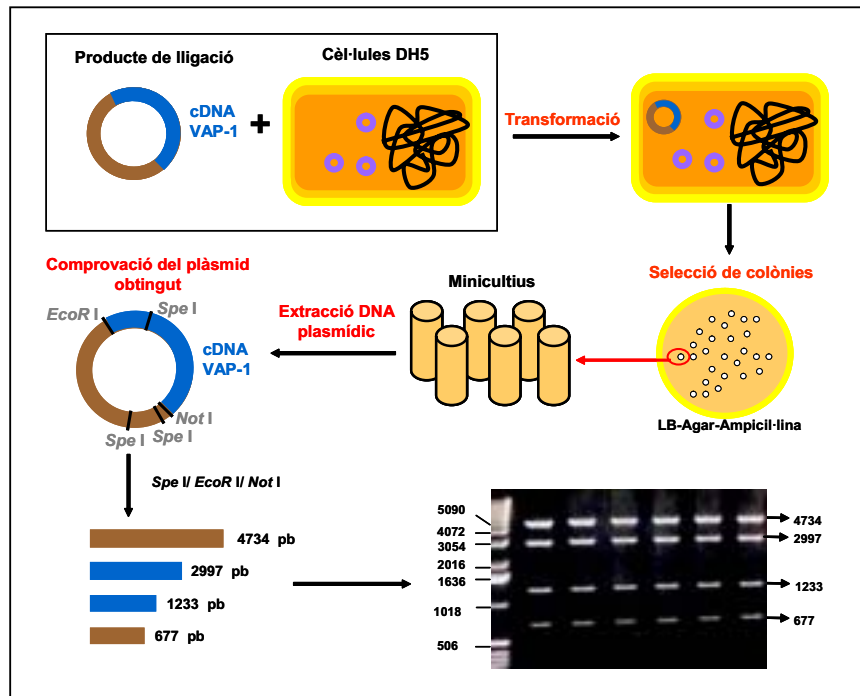
Figura 4.7. Comprovació del vector recombinant pcDNA3.1(+)/VAP-1 obtingut mitjançant digestió amb enzims de restricció

En tenir el plasmidi recombinant d'interès, es van transfectar cèl·lules A7r5 seguint el procediment descrit per la transfecció transitòria, però en aquest cas el medi de cultiu contenia G418 a una concentració de 400 μg/ml per seleccionar colònies i a una concentració de 100 μg/ml per fer créixer les cèl·lules. Es van seleccionar 15 clons diferents que es van fer créixer i analitzar per separat.

4.3.1. Caracterització de les línies cel·lulars estables VAP-1/SSAO

Tot i que els 15 clons eren capaços de subcultivar-se en presència de G418, és a dir, que havien incorporat el plasmidi en el seu genoma, i per tant tenien el gen de resistència a neomicina, es va realitzar un anàlisi per Western-blot per determinar l'expressió de la proteïna VAP-1 en els diferents clons (Fig. 4.8), després d'aproximadament 3 mesos de subcultiu.
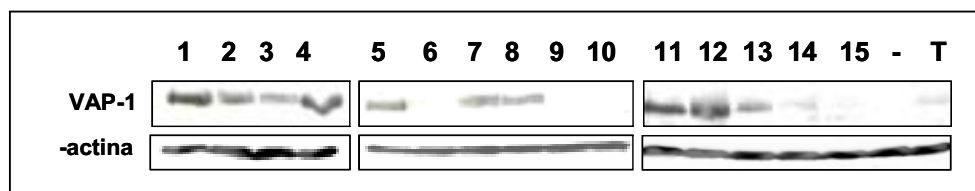
Figura 4.8. Western-blot de la proteïna VAP-1/SSAO (anti-VAP-1) de llisats de cèl·lules A7r5 transfectades establement amb el vector pcDNA3.1(+)/VAP-1 procedents de 15 clons diferents **(1-15)** resistents a G418. **(-)**; control negatiu de llisat de cèl·lules A7r5 sense transfectar. **(T)**; llisat de cèl·lules A7r5 transfectades transitòriament amb el vector pCMV-sport6.1/VAP-1 durant 48 h. L'expressió de η-actina es utilitzada com a control de càrrega.

Els resultats obtinguts van mostrar que els diferents clons expressaven de manera diferent la proteïna VAP-1; els clons 1, 2, 3, 4, 5, 7, 8, 11, 12 i 13 expressaven clarament la VAP-1, mentre que el clons 14 i 15 ho feien més lleugerament, i els clons 6, 9 i 10 semblava que no eren capaços d'expressar-la o la proteïna havia sofert un procés de degradació. En el Western-blot (Fig. 4.8) també es va carregar un control negatiu de cèl·lules A7r5 no transfectades, i una vegada més, s'observava que no hi ha expressió de la proteïna. Així mateix, també es va carregar un llisat de cèl·lules transfectades amb el vector pCMV-sport6.1/VAP-1 durant 48 hores, i es va poder observar com l'expressió era molt més suau que en el cas de les cèl·lules transfectades establement amb VAP-1.

Per altra banda, es va estudiar si la proteïna expressada en les línies estables era funcional i capaç d'oxidar un substrat *in vitro*. En aquest sentit, quan es va determinar la seva activitat amino oxidasa enfront a benzilamina com a substrat (Fig. 4.9A) s'obtenia una activitat específica de 300 pmol/min·mg proteïna dels llisats de les cèl·lules transfectades establement, en comparació a l'activitat específica de 10 pmol/min·mg obtinguda en llisats de cèl·lules transfectades transitòriament durant 48 hores. Quan els llisats cel·lulars eren preincubats durant 30 minuts amb l'inhibidor de la SSAO, semicarbazida 1 mM, s'observava com tota l'activitat amino oxidasa es veia inhibida per la semicarbazida, indicant que aquesta línia expressava clarament la forma activa de la

SSAO/VAP-1 a uns nivells 300 vegades superiors als obtinguts en llisats cel·lulars sense transfectar.

Així mateix, en determinar l'activitat SSAO dels medis de cultiu de les línies estables VAP-1, prèviament centrifugats per tal de retirar els possibles components membranosos contaminants, es va detectar una activitat SSAO de 50 pmol/min·ml de medi de cultiu, la qual s'inhibia amb la prèvia incubació de semicarbazida 1 mM (Fig. 4.9B). Contràriament, en el medi de cultiu de la línia cel·lular A7r5 sense transfectar, no es detectava activitat SSAO. Per tant, aquest resultat suggeria que la sobreexpressió de la VAP-1/SSAO en aquest tipus cel·lular induïa l'alliberació de la forma soluble al medi de cultiu.
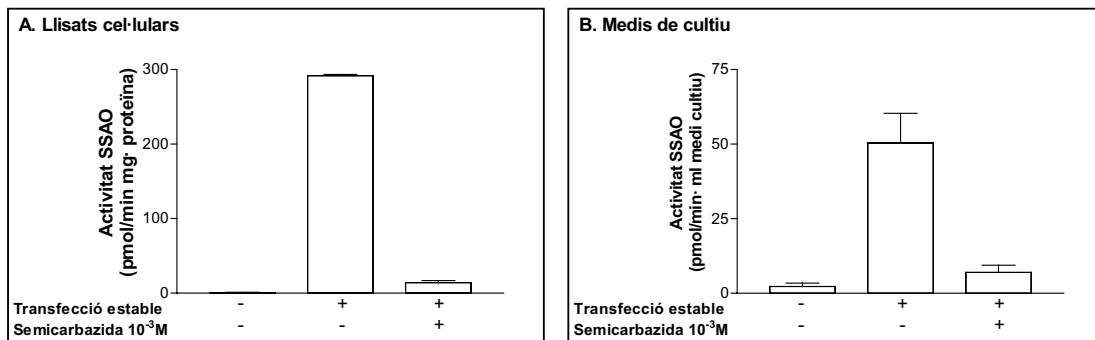
Figura 4.9. Determinació de l'activitat SSAO, mitjançant el mètode radiomètric en front a benzilamina 100 µM 2 mCi/mmol com a substrat, de **(A)** llisats cel·lulars i **(B)** medis de cultiu, de cèl·lules A7r5 transfectades establement amb el vector pcDNA3.1(+)/VAP-1 i sense transfectar. Les dades s'expressen com la mitja ∂ SEM de tres experiments diferents.

A continuació, es va analitzar si l'activitat VAP-1/SSAO era estable a mida que s'augmentaven el nombre de passatges de la línia cel·lular. A la figura 4.10 es pot observar com aquest paràmetre es manté constant al llarg del temps, indicant que realment s'ha realitzat una transfecció estable del gen de la VAP-1/SSAO al genoma de la cèl·lula.
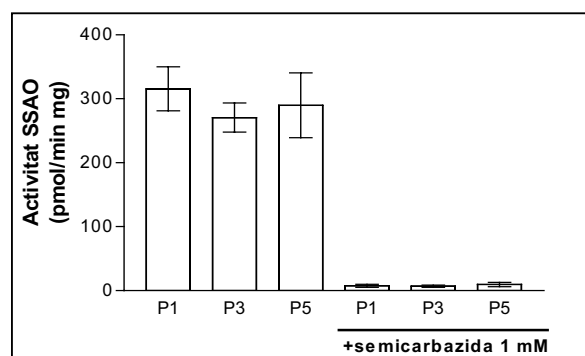
Figura 4.10. Determinació de l'activitat SSAO, mitjançant el mètode radiomètric en front a benzilamina 100 µM 2 mCi/mmol com a substrat, de llisats cel·lulars de línies estables A7r5/VAP-1 de 3 clons diferents a 3 passatges diferents (1, 3 i 5) cada un. Les dades s'expressen com la mitja ∂ SEM.

4.4. Efecte de l'oxidació de la MA en línies estables VAP-1/SSAO

Donat que la poca expressió VAP-1/SSAO obtinguda en les transfeccions transitòries, no permetia concloure si la proteïna humana recombinant lligada a membrana era capaç d'induir dany cel·lular, es va realitzar el mateix tractament en la línia cel·lular estable A7r5/VAP-1, caracteritzada per l'elevada expressió i activitat amino oxidasa de la proteïna.

Es van tractar les cèl·lules A7r5 i les corresponent línia estable VAP-1/A7r5 amb una concentració creixent de MA (1 i 3 mM) durant 3 dies i se'n va determinar la viabilitat cel·lular mitjançant el mètode de reducció del MTT. El tractament amb MA, com hem vist anteriorment, no afectava la viabilitat de les cèl·lules salvatges (Fig. 4.11A), en canvi, provocava un efecte citotòxic pronunciat en aquelles cèl·lules que expressaven la VAP-1/SSAO, depenent de les dosis de MA i del rang de temps estudiat (24, 48 i 72 hores) (Fig. 4.11B). Als 2 dies de tractament, ja s'obtenia una reducció en la viabilitat cel·lular del 25% amb MA 1 mM i del 50% amb MA 3 mM. L'inhibidor específic de la SSAO, semicarbazida 1 mM, no mostrava cap efecte tòxic en cap dels dos tipus cel·lular, però era capaç de revertir totalment la davallada en la viabilitat cel·lular observada en

presència de MA en les cèl·lules que expressaven VAP-1/SSAO a qualsevol dosi i temps estudiat. Aquest resultat demostrava que l'acció catalítica de la SSAO era la única responsable de la citotoxicitat obtinguda.
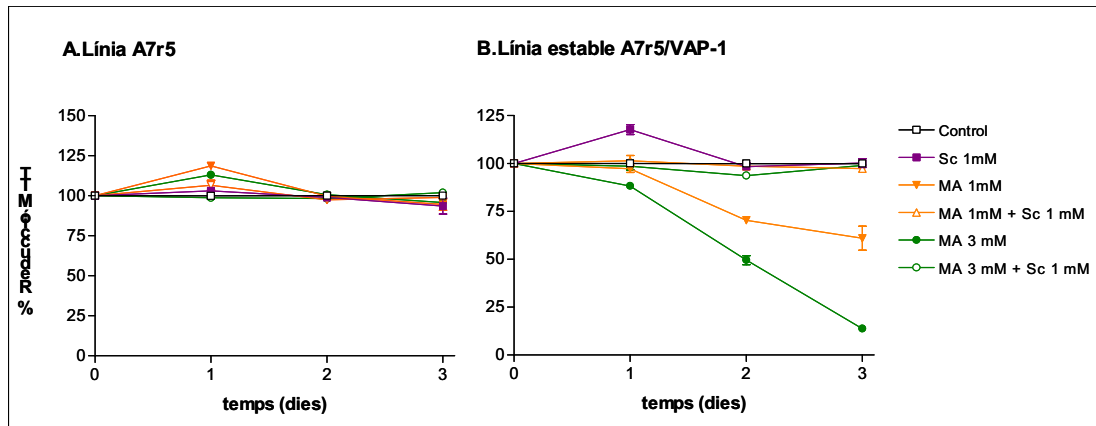
Figura 4.11. Efecte de l'oxidació de la MA sobre **(A)** la línia cel·lular A7r5 o **(B)** la línia estable A7r5/VAP-1, expressada en percentatges de reducció d'MTT després 1,2 i 3 dies de tractament amb; ( ) semicarbazida 1 mM; ( ) MA 1 mM; ( ) MA 1 mM + semicarbazida 1 mM; ( ) MA 3 mM; ( ) MA 3 mM + semicarbazida 1 mM. Les dades s'expressen com la mitja ∂ SEM de tres experiments diferents realitzats en triplicat.

DISCUSSIÓ CAPÍTOL 4

En el capítol anterior, s'havia demostrat que l'oxidació de la MA per part de la SSAO soluble d'origen boví podia induir citotoxicitat i apoptosi en cèl·lules de múscul llis en cultiu. A partir d'aquí, degut a l'augment d'expressió SSAO en vasos cerebrals de malalts d'AD, es va procedir a estudiar si la forma cel·lular de la SSAO lligada a membrana també era capaç de metabolitzar aquesta amina alifàtica i induir dany cel·lular.

Com s'ha comentat en capítols anteriors, tot i que la SSAO s'expressa constitutivament en múscul llis vascular (Clarke *et al*., 1982; Lyles i Singh, 1985; Hysmith i Boor, 1987) i presenta una activitat molt elevada, les cèl·lules A7r5 i HASMC no presenten activitat ni expressió SSAO. Per tant, per tal de dur a terme l'objectiu del treball va caldre la creació d'una línia estable de cèl·lules de múscul llis A7r5 que expressés la proteïna SSAO, ja que transfeccions transitòries no van aconseguir uns nivells d'expressió de la proteïna suficients per estudiar l'objectiu proposat.

Així doncs, es van generar línies cel·lulars A7r5 que expressaven establement la VAP-1/SSAO humana, les quals presentaven una morfologia i unes característiques de subcultiu *a priori* objectivament iguals a les cèl·lules A7r5 inicials sense transfectar. L'activitat SSAO expressada en aquestes cèl·lules era de 300 pmol/min·mg de proteïna, determinada radiomètricament en front a benzilamina com a substrat en experiments *in vitro*. Per altra banda, en cultivar les cèl·lules estables A7r5/VAP-1, es va detectar activitat SSAO en el medi de cultiu (50 pmol/min·ml medi de cultiu). Aquest resultat suggeria que la sobreexpressió de la SSAP/VAP-1 lligada a membrana podia potenciar l'alliberament de SSAO soluble al medi de cultiu, ja que les cèl·lules salvatges no presentaven activitat SSAO ni en els llisats cel·lulars ni en el medi de cultiu. Aquest resultat, que caldria confirmar determinant els nivells de proteïna en el medi de cultiu, corroborarien estudis previs en adipòcits en cultiu, on es descriu que la SSAO cel·lular pateix un procés proteolític per donar lloc a la forma soluble de l'enzim (Abella *et al*., 2003). Així mateix, en models *in vivo* utilitzant animals transgènics que sobreexpressen la SSAO, també s'ha observat que la sobreexpressió de la proteïna tant en múscul llis (Gokturk *et a*l., 2003), com en endoteli i adipòcits (Stolen *et al*., 2004a; Stolen *et al*.,

2004b), indueix un augment de la SSAO soluble en plasma. En aquest sentit, i tenint en compte els resultats anteriors d'aquest treball, l'augment de l'expressió de la SSAO observat en el teixit cerebrovascular en la malaltia d'AD (veure Resultats Capítol 1), podria induir l'augment de proteïna, i per tant, d'activitat SSAO soluble observada en plasma de pacients amb aquesta patologia (veure Resultats Capítol 2).

Per tal d'estudiar si l'acció catalítica de la SSAO expressada en aquestes línies estables era capaç d'induir dany en cèl·lules de múscul llis en cultiu, es van realitzar tractaments amb un dels seus substrats fisiològics, la MA, i els resultats obtinguts van mostrar una davallada en la viabilitat cel·lular depenent de la dosi de MA i del temps del tractament. A més, quan s'inhibia l'enzim amb l'inhibidor específic, semicarbazida, s'aconseguia revertir completament la mort cel·lular obtinguda. Aquesta dada, juntament amb el fet que el tractament amb MA no tenia cap efecte en cèl·lules que no presentaven activitat SSAO, demostrava que l'acció catalítica de la SSAO era la responsable de la mort cel·lular observada, confirmant els anteriors resultats en un model molt més heterogeni, en el qual s'incubaven les cèl·lules amb SSAO soluble d'origen boví i MA (veure Resultats Capítol 3).

En el cas del metabolisme de la SSAO cel·lular, donat que és una proteïna de membrana amb el centre actiu encarat a la part extracel·lular, els productes de la seva catàlisi s'alliberarien al medi extracel·lular, tal i com succeiria en el cas de la SSAO soluble. Per tant, la citotoxicitat esmentada, amb tractaments amb MA en cèl·lules que expressaven establement la SSAO/VAP-1, no es podria atribuir únicament a l'acció catalítica de la SSAO expressada a la cèl·lula, ja que l'activitat SSAO present al medi de cultiu també podria contribuir a la generació dels productes metabòlics inductors de la mort cel·lular. En aquest sentit, els estudis d'Abella *et al*. (2003) van demostrar que la SSAO soluble alliberada al medi era una proteïna dimèrica i altament glicosilada, fet que suggeria que era igualment activa. En el nostre treball, l'activitat SSAO present al medi de cultiu (50 pmol/min·ml, equivalent a $0.5 \cdot 10^{-4}$ U/ml) era de l'ordre de 10 vegades inferior a la utilitzada en els tractaments amb la SSAO soluble bovina o anteriors treballs amb SSAO plasmàtica humana (Yu i Zuo, 1993), però la citotoxicitat observada en aquest cas, també era inferior i més retardada en el temps. Per tant, no es pot descartar que l'oxidació de la MA en línies estables A7r5/VAP-1 en cultiu, vingui donada, almenys en part, per la proteïna alliberada al medi de cultiu. En ambdós casos, és important

assenyalar que l'efecte observat era degut a la proteïna d'origen humà, fet que corrobora la importància dels resultats descrits en relació a patologies on s'observa un augment de SSAO soluble i SSAO lligada a membrana, com seria el cas de la malaltia d'AD sever i CAA.

Cal esmentar, però, que en anteriors treballs (Conklin *et al*., 1998; Langford *et al*., 2001) s'havia descrit que les cèl·lules de múscul llis d'aorta de rata eren resistents a concentracions equivalents de MA a les utilitzades en els nostres estudis. En aquells treballs, l'activitat SSAO cel·lular era molt inferior (1.05 nmol/mg·h, equivalent a 16.7 pmol/min·mg (Conklin *et al*., 1998)), probablement degut a la pèrdua d'expressió i activitat SSAO en cèl·lules en cultiu comentada anteriorment. En aquest sentit, l'activitat específica present en les cèl·lules estables A7r5/VAP-1 (300 pmol/min·mg) seria més aproximada a l'activitat de cèl·lules de múscul llis *in vivo*, i per tant, els nivells de proteïna serien suficients per dur a terme l'oxidació de la MA.

Degut a la dificultat de mantenir l'expressió de la SSAO en cèl·lules vasculars en cultiu, el fet d'haver obtingut una línia cel·lular estable que expressi la VAP-1/SSAO, permet estudiar a nivell cel·lular el mecanisme d'acció de la forma humana de l'enzim en cèl·lules de múscul llis, i obre nous camps d'investigació per a esclarir la funció fisiològica i patològica de la SSAO en aquest tipus cel·lular. En aquests sentit, seria interessant estandarditzar aquesta nova línia cel·lular en quant a les seves propietats d'adhesió, resposta a diferents estímuls, citotoxicitat per diferents agents, etc. Així mateix, l'enzim sobreexpressat permetrà estudiar el metabolisme de la SSAO en cèl·lules en cultiu, en front a diferents substrats i inhibidors, dades que ajudaran a definir més acuradament la rellevància d'aquesta sobreexpressió en la cèl·lula de múscul llis.

Per altra banda, caldria esmentar que la citotoxicitat observada per l'oxidació de MA sembla ser específica de certs tipus cel·lulars o teixits, ja que el tractaments crònics amb MA en adipòcits en cultiu, amb una elevada expressió i activitat SSAO, no indueix citotoxicitat en aquest tipus cel·lular (Mercier *et al*., 2001). De fet, donat que es coneix poc sobre el procés d'apoptosi en adipòcits (Prins i O'Rahilly, 1997), podria ser que aquest tipus cel·lular fos més resistent a aquest tipus de senyal, i per tant, fos menys sensible a la citotoxicitat induïda pels productes catalítics de la SSAO. Aquestes dades també suggeririen que la SSAO és un enzim multifuncional, i que els productes de la

seva catàlisi poden donar lloc a estímuls o funcions diferents segons el teixit on s'expressi i les situacions fisiològiques o patològiques en els quals es trobi.

Els resultats presentats utilitzant el nostre model cel·lular, on es sobreexpressa la VAP-1/SSAO humana en cèl·lules de múscul llis, estarien d'acord amb estudis realitzats recentment en models *in vivo* utilitzant animals transgènics que sobreexpressen la VAP-1/SSAO. S'ha descrit que l'administració crònica de MA a aquests animals transgènics indueix complicacions associades a problemes vasculars, com l'augment de la pressió sanguínia, l'augment de productes AGE i la modificació de la progressió d'arteriosclerosi (Stolen *et al*., 2004a). El fet que l'activitat *in vivo* de les cèl·lules de la *tunica media* d'aorta humana sigui de l'ordre de 2400 pmol/min·mg proteïna, suggereix que l'efecte tòxic observat *in vitro*, no sigui més que una lleugera aproximació de la toxicitat que la SSAO tissular podria exercir en certes situacions patològiques, on la concentració de MA en plasma es trobi augmentada, o en situacions d'estrès, en les quals el metabolisme de l'adrenalina per acció de la MAO produeixi uns nivells anormals de MA (Yu *et al.*, 1997).

Els resultats presentats també permeten suggerir que la sobreexpressió de la SSAO en vasos cerebrals humans podria contribuir significativament al dany vascular observat en la malaltia d'AD associada a CAA.

RESULTATS I DISCUSSIÓ CAPÍTOL 5:

ESTUDI DE LA POSSIBLE IMPLICACIÓ DE LA SSAO EN MODELS *IN VITRO* I *IN VIVO* D'ANGIOPATIA CEREBRAL AMILOIDE

RESULTATS CAPÍTOL 5:

ESTUDI DE LA POSSIBLE IMPLICACIÓ DE LA SSAO EN MODELS *IN VITRO* I *IN VIVO* D'ANGIOPATIA CEREBRAL AMILOIDE

L'angiopatia amiloide cerebral (CAA) es caracteritza per dipòsits fibril·lars de -amiloide (A) en vasos cerebrals leptomeningis i corticals (Vinters *et al*., 1996; Jellinger, 2002; Rensink *et al*., 2003). Diversos estudis han implicat el dipòsits de A a la microvasculatura amb la neuroinflamació i la demència tipus AD (Jellinger, 2002; Attems i Jellinger, 2004). Diferents formes de CAA familiar resulten de mutacions puntuals en el gen del precursor del A, l'APP, com la mutació tipus *Dutch* (E22Q) o *Iowa* (D23N) (Levy *et al*., 1990; Grabowski *et al*., 2001). Concretament, la mutació *Dutch* dóna lloc a la forma familiar autosòmica dominant de CAA coneguda com Hemorràgia Cerebral Hereditària Amb Amiloidosis Holandesa (HCHWA-D) (Levy *et al*., 1990; Bornebroek *et al*., 1996). L'HCHWA-D es caracteritza per hemorràgies cerebrals recurrents, extensa d'CAA en arteries leptomeningees i arterioles corticals, i plaques difoses de A en parènquima (Maat-Schieman *et al*., 1996). En aquest context, l'HCHWA-D ha estat considerada com l'arquetipus genètic humà de l'angiopatia amiloide (Vinters, 1987; Greenberg, 1998).

Diferents evidències van fer pensar que la SSAO podia contribuir al dany vascular associat a CAA. En primer lloc, la SSAO s'expressa àmpliament en vasos sanguinis (Precious i Lyles, 1988) i concretament, en *tunica media* de múscul llis tant d'aorta (Hysmith and Boor., 1987) com de les membranes de l'espai leptomeningi cerebral humà (Castillo *et al*., 1999). Per altra banda, en resultats anteriors, havíem descrit dades que relacionaven aquest enzim amb la patologia d'AD, com l'augment d'expressió de la proteïna en vasos cerebrals de pacients d'AD, colocalitzant amb els dipòsits de A, i l'increment d'activitat de la forma soluble de la SSAO en pacients d'AD sever *in vivo*. A més, s'havia observat que la catàlisi de la SSAO era capaç d'induir citotoxicitat i que els productes metabòlics generats eren potents inductors d'apoptosi en cèl·lules de múscul llis.

Per tal d'estudiar la possible implicació de la SSAO en el dany vascular en CAA i intentar cercar l'origen molecular de la sobreexpressió de l'enzim a nivell de vasos cerebrals en l'esmentada patologia, es va utilitzar un model cel·lular *in vitro* de CAA prèviament establert i estandarditzat. El model escollit consistia en la inducció d'apoptosi en cèl·lules de musculatura llisa procedents de vasos leptomeningis humans (HLSMC) per diferent mutants del pèptid -amiloide (Davis i Van Nostrand., 1996; Davis *et al*., 1999, Van Nostrand *et al*., 2001), com el pèptid -A $_{1\ 40}$ contenint la mutació tipus *Dutch*, HCHWA-D -A $_{1\ 40}$ (D -A $_{1\ 40}$). Es va utilitzar el tractament amb aquesta darrera forma del pèptid A per la seva elevada capacitat de forma fibril·les i presentar una major patogènesi en front les cèl·lules HLSMC (Davis i Van Nostrand, 1996).

Per estudiar si la SSAO tenia alguna implicació amb el CAA *in vivo*, es va utilitzar un model d'animals transgènics (Tg-SwDI), que sobreexpressaven a nivell neuronal el gen de l'APP humà contenint les mutacions *Swidish, Dutch i Iowa*, els quals presentaven elevats dipòsits de de A a la microvasculatura (Davis *et al*., 2004).

5.1. Possible contribució de la SSAO en la citotoxicitat per -Amiloide en cèl·lules de musculatura llisa procedents de vasos leptomeningis humans

Per tal d'abordar, a nivell cel·lular, la possible implicació de la SSAO en el dany vascular associat a la malaltia d'AD, tal i com s'ha esmentat anteriorment, es va utilitzar el model cel·lular de CAA prèviament estandarditzat (Davis *et al*., 1999), en el qual s'induïa citotoxicitat en HLSMC (*Human Leptomeningeal Smooth Muscle Cells*) per la forma mutada del pèptid D -A $_{1\ 40..}$

En primer lloc, es va posar a punt el cultiu primari de cèl·lules HLSM per tal de caracteritzar la SSAO en aquest tipus de cultiu cel·lular, i posteriorment, estudiar la seva possible implicació en la mort d'aquestes cèl·lules induïda per -amiloide.

5.1.1. Caracterització del cultiu primari HLSMC

A partir dels vasos leptomeningis de cervells *post-mortem* (10 hores), (subministrats per l'Institut Anatòmic Forense, Barcelona), es va seguir el protocol de Van Nostrand *et al*.

(1994). Un cop es va tenir el cultiu i es va subcultivar (fins al passatge 3) (veure Material i Mètodes 1.1.3), va caldre identificar el tipus cel·lular i la puresa del cultiu primari.

5.1.1.1. Assaig immunocitoquímic amb l'anticòs -actina de musculatura llisa i tinció de nuclis per Hoechst 33258

Per tal de caracteritzar el cultiu primari, es va utilitzar l'anticòs monoclonal -actina de musculatura llisa, que tal i com s'ha comentat anteriorment, és específic per l'actina nativa de musculatura llisa. També es va fer una tinció del total de nuclis amb Hoetchst 33258 per tal de determinar el percentatge de cèl·lules que reconeixien l'anticòs -actina de musculatura llisa del cultiu.

Els resultats es mostren a la figura 5.1 i es pot observar clarament com el reconeixement de l'anticòs -actina era totalment específic. La diferència en la tinció d'algunes cèl·lules podria venir donada pel diferent estat proliferatiu de les cèl·lules i per la confluència entre aquestes. En quant a la tinció de nuclis per Hoechst, es podia observar que tots els nuclis corresponien a cèl·lules tenyides amb l'anti- -actina de musculatura llisa, és a dir, els resultats permetien concloure que estàvem davant un cultiu pur on totes les cèl·lules pertanyien al mateix tipus cel·lular.
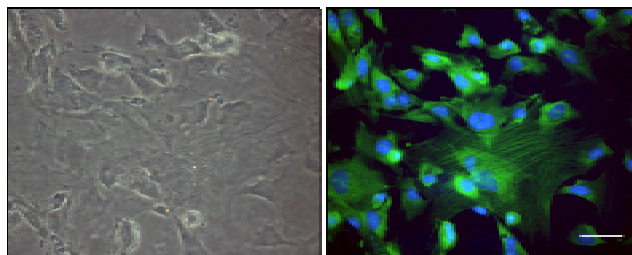

Figura 5.1. A. Contrast de fase de cèl·lules HLSMC. **B.** Immunocitoquímica d' -actina i tinció nuclear amb Hoechst 33258. Escala de barres = 50 µm en A i B.

5.1.1.2. Comprovació per mètodes immunocitoquímics de la puresa del cultiu d'HLSMC

Donat que un dels objectiu d'aquest treball era l'estudi de la SSAO en cèl·lules de musculatura llisa, era molt important confirmar, en primer lloc, l'especificitat de l'anticòs

utilitzat per caracteritzar el cultiu (anti -actina de musculatura llisa), i per tant, descartar altres possibles tipus cel·lulars que haguessin pogut contaminar el cultiu primari. Així doncs, donada la seva morfologia, el seu manteniment en cultiu, la manera de subcultivar-se i la seva presència en el teixit inicial, els dos tipus cel·lulars que es van plantejar com a possibles contaminants van ser els astròcits i els fibroblasts.

En primer lloc, es va utilitzar un cultiu pur d'astròcits de rata com a control negatiu, però tal i com es mostra en la figura 5.2, les fibres d'astròcits també reconeixien específicament l'anticòs contra ζ-actina (Fig. 5.2B), tal i com ho feien les cèl·lules del cultiu primari a estandarditzar (Fig. 5.2F). Per tant, seguidament es va haver de realitzar una immunocitoquímica utilitzant l'anticòs contra la proteïna GFAP (proteïna àcida gliofibril·lar), considerada com un marcador específic d'astròcits. En aquest cas, les cèl·lules del cultiu primari d'HLSMC presentaven una tinció negativa per GFAP (Fig. 5.2H), comparativament a la tinció específica que s'obtenia en el cultiu d'astròcits (Fig.5.2D). Per tant, tot i que l'anticòs anti- -actina de musculatura llisa no mostrava completa especificitat per múscul llis, es va poder concloure que el cultiu primari en estudi no contenia contaminació d'astròcits.
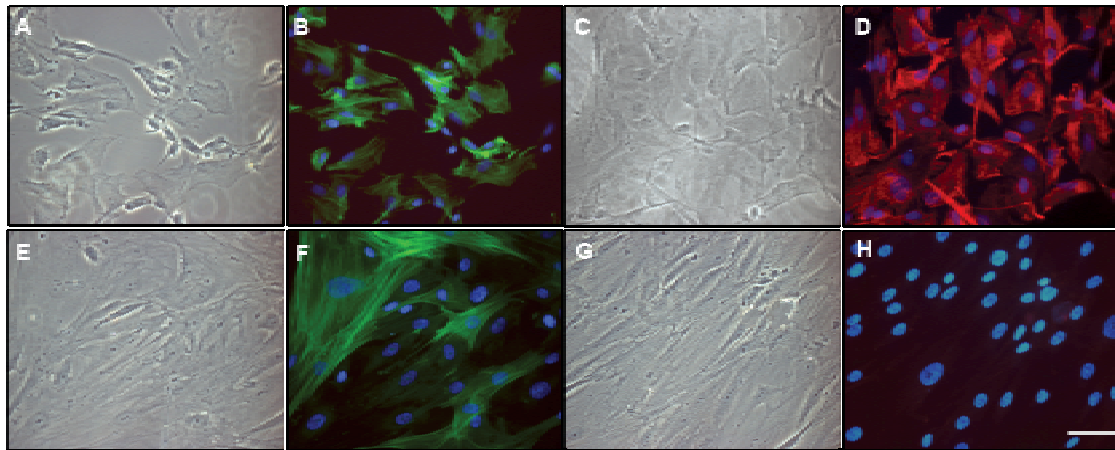
Figura 5.2. A-D: Cultiu primari d'astròcits de rata. **E-H**: Cèl·lules HLSM. Contrast de fase (A, C,E i G); immunocitoquímica d' -actina i tinció nuclear amb Hoechst 33258 (B i F); immunocitoquímica d'GFAP i tinció nuclear amb Hoechst 33258 (D i H). Escala de barres = 50 μm en A-H.

A continuació, també es va valorar la possible contaminació de fibroblasts en el cultiu, i per això, es va realitzar una tinció específica utilitzant un anticòs primari monoclonal contra una proteïna humana de superfície de fibroblasts (clon 1B10). Aquest és un anticòs que s'utilitza concretament per diferenciar els fibroblasts de les cèl·lules vasculars de múscul llis en processos de diferenciació de musculatura llisa. L'antigen que reconeix aquest anticòs s'ha detectat a la superfície cel·lular, en compartiments vesiculars de fibroblasts i en estructures endosòmiques i lisosòmiques (Ronnov-Jessen *et al*., 1992). Per altra banda, l'anticòs 1B10 reconeix un epitop tant en fibroblasts humans procedents de cultiu primari, com en línies cel·lulars de fibroblasts. És per això que també es va fer un control positiu de fibroblasts usant la línia cel·lular MRC-5 (fibroblats procedents de pulmó humà) incubats amb aquest anticòs i amb l'anticòs contra ζ-actina de musculatura llisa. En aquest cas (Fig. 5.3), es pot observar com els fibroblasts no reconeixien l'anticòs contra ζ-actina (Fig. 5.3B), tal i com calia esperar. Així mateix, les cèl·lules del cultiu primari a caracteritzar no eren capaces de reconèixer l'anticòs específic de fibroblasts (Fig. 5.3H) comparativament amb la tinció específica per 1B10 de la línia MRC-5 (Fig. 5.3D). Per tant, també es va poder concloure l'absència de fibroblasts en el cultiu primari.
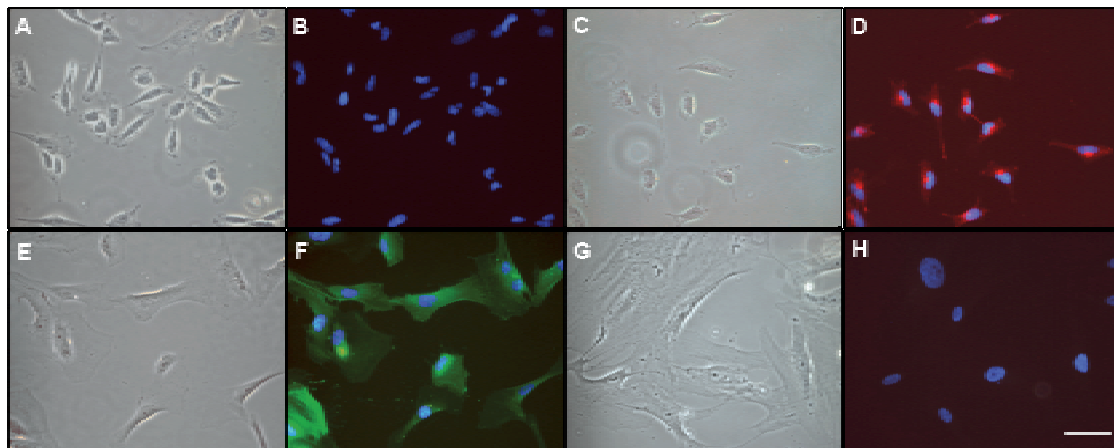
Figura 5.3. A-D: Línia cel·lular de fibroblasts MRC-5. **E-H**: Cèl·lules HLSM. Contrast de fase (A, C,E i G); immunocitoquímica d' -actina i tinció nuclear amb Hoechst 33258 (B i F); immunocitoquímica de *fibroblast surface protein* (clone 1B10) (D i H). Escala de barres = 50 μm en A-H.

Així doncs, gràcies a aquests controls i a la tinció específica utilitzant l'anticòs monoclonal anti ζ-actina de musculatura llisa, es va poder comprovar que s'estava seguint un protocol correcte (Van Nostrand *et al.*, 1994) per a la preparació d'un cultiu primari pur de cèl·lules cerebrovasculars humanes de musculatura llisa.
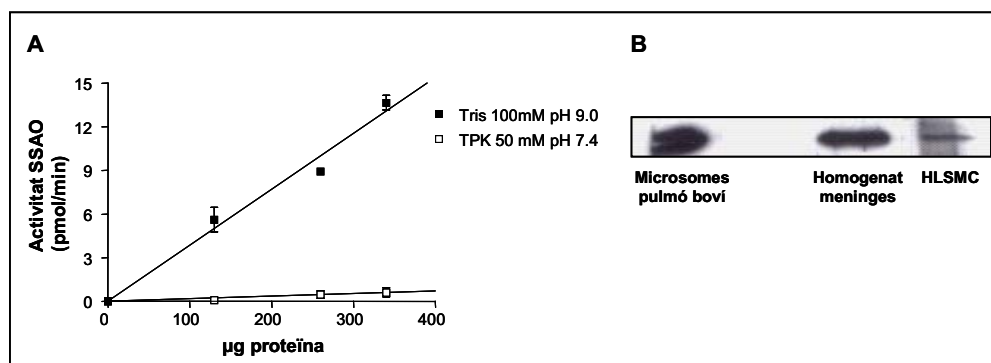
5.1.1.3. Estudi de la SSAO en el cultiu primari d'HLSMC

Es va determinar l'activitat enzimàtica SSAO de llisats cel·lulars d'HLSMC en passatges baixos (3-4) en front a benzilamina 100 μM, i es va obtenir un valor de 40 pmol/min·mg de proteïna. Aquest valor era de l'ordre de 8-10 vegades inferior a l'obtingut per l'homogenat de meninges humanes, del qual es partia per realitzar el cultiu primari. A la figura 5.4A es pot apreciar com la velocitat de la reacció presentava un comportament lineal respecte la quantitat de proteïna del llisat cel·lular. A més, s'observava que calien unes condicions de pH determinades per tal que l'enzim pogués metabolitzar el substrat d'una manera òptima. Aquestes condicions de pH s'aconseguien incubant l'enzim amb tampó Tris 100 mM, pH 9.0. Així doncs, en aquest sentit, el comportament de la SSAO de cèl·lules HLSMC aïllades era igual al de la SSAO de microvasos i meninges, on el pH òptim per la seva activitat també era de 9.0. (Castillo *et al.*, 1999).

Per altra banda, es va determinar l'expressió de la proteïna en llisats cel·lulars de passatges baixos (3-4) per tècniques immunològiques utilitzant anticossos policlonals anti-SSAO de pulmó boví (Lizcano *et al.*, 1998). A la figura 5.4B es mostra com les cèl·lules HLSM expressaven lleugerament la proteïna, en comparació a la mateixa quantitat de proteïna total d'homogenat de meninges humanes. Com a control positiu del Western-blot es va carregar 1 μg de microsomes de pulmó boví.

Cal destacar, però, que a mida que s'anaven subcultivant les cèl·lules HLSMC, es va anar perdent l'expressió de l'enzim. Aquest és un fenomen comú en cèl·lules en cultiu descrit per diversos tipus cel·lulars, i concretament per cèl·lules de musculatura llisa (Browner *et al.*, 2004). Tant processos de rediferenciació cel·lular (El Hadri *et al.*, 2002), com el subcultiu de les cèl·lules en substrats diferents, tals com col·lagen i fibronectina, no van resultar en l'augment de l'expressió de la SSAO en aquest tipus de cultiu (resultats no mostrats).

Tot i així, donat que s'havia observat un augment de l'expressió SSAO en vasos cerebrals de pacients d'AD amb CAA *in vivo*, l'objectiu de la següent part del treball va ser estudiar si un model cel·lular *in vitro* de CAA, és a dir el tractament D -A $_1$ $_{40}$ era capaç d'induir l'expressió de l'enzim en HLSMC en cultiu.


Figura 5.4. Estudi de la SSAO en el cultiu primari d'HLSMC (passatges baixos, 3-4) **A.** Determinació de l'activitat SSAO, mitjançant el mètode radiomètric en front a benzilamina 100 µM 2 mCi/mmol com a substrat, de llisats de cèl·lules HLSM (passatges inferiors a 4). Incubació amb dos tampons diferents; Tris 100 mM pH 9.0 i TPK 50 mM pH 7.4. Les dades s'expressen com la mitja ∂ SEM de tres experiments diferents. **B.** Western-Blot (anti-SSAO) de llisats d'HLMC, homogenat de meninges humanes, i microsomes de pulmó boví com a control positiu.

5.1.2. Estudi de la SSAO en el model de citotoxicitat per D -A $_1$ $_{40}$ en HLSMC

Per tal de comprovar si el tractament amb -amiloide en HLSMC induïa un augment en l'expressió de la proteïna SSAO, i aquesta a la seva vegada, contribuiria a la citotoxicitat a través dels productes de la seva pròpia catàlisi, es van tractar els cultius amb la forma mutada soluble del pèptid D -A $_1$ $_{40}$ (contenint la mutació E22Q) a una concentració de 25 µM, ja que en anteriors estudis s'havia estandarditzat els nivells i el tipus de mort amb aquesta concentració de pèptid A en aquest tipus cel·lular (Davis i van Nostrand., 1996; Davis *et al.*, 1999).

Els anàlisis per Western-blot, on s'analitzava l'expressió de la SSAO després de 6 dies de tractament amb el pèptid D -A $_1$ $_{40}$ 25 µM, no permetien detectar l'expressió de la proteïna, ni en el cas de llisats de cèl·lules control, ni en el cas de llisats amb el tractament amb A (Fig. 5.5A). Com a control del Western-blot es van utilitzar, una

vegada més, els microsomes de pulmó boví. Per tal de comprovar que les cèl·lules havien respòs al tractament amb D -A $_{1\ 40}$, es va analitzar l'expressió de la proteïna APP, ja que s'havia descrit que la degeneració cel·lular induïda per tractaments amb A anava acompanyada d'un fort augment dels nivells d'APP cel·lular i secretat al medi (Davis i Van Nostrand., 1996). Així doncs, el la figura 5.5B es pot observar com efectivament el tractament havia resultat en un augment en l'expressió d'aquesta proteïna. Per altra banda, també es va analitzar la possible presència de la SSAO en els medis de cultiu recollits i concentrats després del tractament amb D -A $_{1\ 40}$, per tal de descartar que la SSAO que no es trobava present en els llisats cel·lulars, s'hagués pogut alliberar al medis de cultiu. Els resultats obtinguts tampoc van permetre detectar la proteïna en el medi de cultiu de les cèl·lules tractades (Fig. 5.5C).
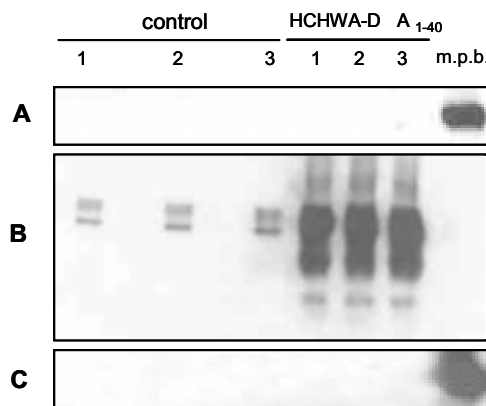
Figura 5.5. Western-Blot de llisats d'HLSMC després del tractament durant 6 dies amb D -A $_{1\ 40}$ 25 µM: **(A)** anti-SSAO (microsomes de pulmó boví (m.p.b.) com a control positiu del Western-Blot), **(B)** anti-APP. **(C)** Western-Blot (anti-SSAO) dels medis de cultiu concentrats del cultiu d'HLSMC després del tractament durant 6 dies amb D -A $_{1\ 40}$ (microsomes de pulmó boví (m.p.b.) com a control positiu del Western-Blot). Blot representatiu de dos experiments diferents realitzats en triplicat.

Paral·lelament, per tal de descartar l'efecte de la SSAO en aquest model cel·lular, es va analitzar l'efecte en la viabilitat cel·lular del pèptid D -A $_{1\ 40}$ 25 µM en presència d'inhibidors de la SSAO. Si l'activitat SSAO contribuïa parcialment al dany cel·lular, en inhibir la seva activitat catalítica, s'hauria d'observar una certa recuperació de l'estat del cultiu després del tractament amb l'inductor tòxic. Primerament, va caldre estudiar l'efecte aïllat dels inhibidors de la SSAO en cèl·lules HLSM durant 6 dies de tractament,

ja que aquest era el temps d'exposició del cultiu amb A. A la figura 5.6 es mostra la corba de toxicitat de l'inhibidor específic de la SSAO, MDL-72974A, i s'observa com a partir d'una concentració de 1 µM ja no produïa cap efecte en la viabilitat cel·lular del cultiu. Per tant, es va escollir aquesta concentració per als posteriors experiments, ja que en estudis *in vitro* s'havia observat que era suficient per inhibir completament l'activitat l'enzim. Per altra banda, l'inhibidor semicarbazida es va utilitzar a una concentració de 1 mM, ja que tampoc induïa cap efecte en la viabilitat cel·lular, i era la concentració adequada per inhibir l'acció catalítica de la SSAO (Lewinshon *et al*., 1978).
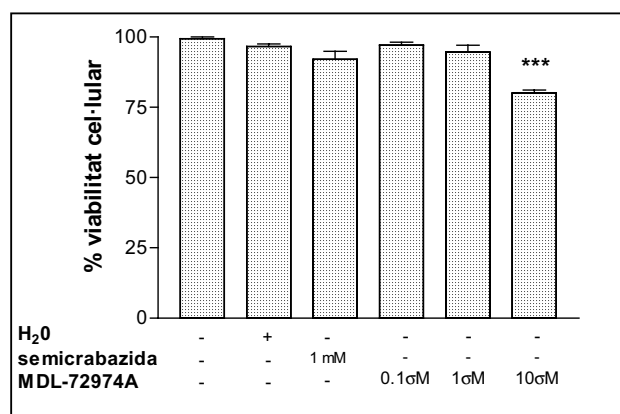
Figura 5.6. Efecte en la viabilitat cel·lular dels inhibidors de la SSAO, semicarbazida i MDL-72974A, en el cultiu HLSMC durant 6 dies de tractament, expressat com el percentatge de cèl·lules vives respecte el nombre total usant el *kit* de fluorescència LIVE/DEAD[®] Viability/Cytotoxicity . Les dades s'expressen com la mitja ∂ SEM de dos experiments diferents realitzats en triplicat. (***) *p*<0.001 segons el test One-way ANOVA amb un Newman-Keuls *post-test*.

A continuació, es va procedir a realitzar els experiments tractant les cèl·lules amb D -A $_{1\ 40}$ 25 µM durant 6 dies, en presència dels inhibidors semicarbazida 1 mM i MDL-72974A 1 µM. Els resultats mostraven clarament com el fet d'inhibir la possible activitat SSAO present en les cèl·lules HLSMC no tenia cap efecte en la toxicitat per A (Fig. 5.7). Cal assenyalar que aquests experiments també es van realitzar en presència del substrat de la SSAO, MA 1 mM, obtenint-se els mateixos resultats negatius (resultats no mostrats). De fet, aquests resultats estaven d'acord amb el fet que no s'hagués pogut detectar SSAO en el cultiu. Tal com s'havia observat anteriorment, el tractament tòxic amb D -A $_{1\ 40}$ no era capaç d'augmentar l'expressió de l'enzim.

Els resultats obtinguts en HLSMC, juntament amb l'elevada dificultat d'obtenir mostres de cervell humà per a la realització de l'esmentat cultiu primari, va suposar un important impediment per a la realització d'experiments posteriors i es va optar per un nou model cel·lular.
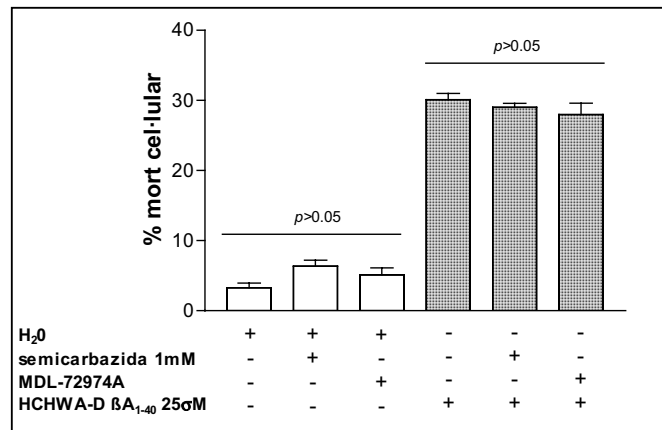
Figura 5.7. Viabilitat cel·lular d'HLSMC després del tractament durant 6 dies amb D -A $_{1-40}$ 25 µM, en presència dels inhibidors de la SSAO, semicarbazida 1 mM i MDL-72974A 10 µM, expressada com el percentatge de cèl·lules mortes respecte el nombre total usant el *kit* de fluorescència LIVE/DEAD® Viability/Cytotoxicity . Les dades s'expressen com la mitja ∂ SEM de tres experiments diferents realitzats en triplicat. *p>0.05* segons el test One-way ANOVA amb un Newman-Keuls *post-test*.

5.2. Possible contribució de la SSAO en la citotoxicitat per -Amiloide en cèl·lules A7r5

Donat que no era possible expressar la SSAO en HLSMC, es va optar per treballar amb una línia cel·lular de múscul llis on es pogués transfectar la proteïna d'interès. D'aquesta manera s'estava buscant un model, en el qual les cèl·lules musculars expressessin la SSAO, tal i com succeeix *in vivo*. Tot i que el model descrit anteriorment, tractant-se d'un cultiu primari de cèl·lules humanes, era molt més fisiològic i interessant, el fet d'utilitzar una línia cel·lular facilitava molt la manipulació i el rendiment del treball.

L'objectiu d'aquesta part de l'estudi va ser comprovar si la sobrexpressió de la SSAO era capaç d'augmentar el dany induït pel pèptid A en aquest tipus cel·lular. Per això, va caldre, en primer lloc, estandarditzar aquest tipus de mort en cèl·lules A7r5.

5.2.1. Citotoxicitat per D -A $_{1}$ $_{40}$ en cèl·lules A7r5

La mort per la forma soluble del pèptid D -A $_{1}$ $_{40}$ en HLSMC estava àmpliament caracteritzada pel laboratori del Dr. Van Nostrand, però va caldre comprovar si la línia cel·lular A7r5 responia de la mateixa manera en front aquesta forma del pèptid A. Treballs anteriors (Muñoz *et al*., 2002) ja descrivien que la forma del pèptid amb la mutació *Dutch* era la que tenia efectes més patogènics sobre la línia cel·lular A7r5, comparativament a l'efecte del A salvatge. Tot i així, aquests treballs es realitzaven amb la forma agregada del pèptid, i no amb la forma soluble, tal com s'havia treballat fins al moment.

Es va optar per seguir treballant amb la forma soluble del D -A $_{1}$ $_{40}$ a una concentració de 25 µM i no canviar les condicions del tractament emprades fins al moment. Es va realitzar una corba de toxicitat al llarg del temps utilitzant el mètode de reducció d'MTT tractant la línia cel·lular A7r5. Els resultats mostren com a partir del tercer dia de tractament s'obtenia una pèrdua de viabilitat cel·lular de l'ordre del 70%, i aquest valor no variava substancialment després de 7 dies en presència del pèptid (Fig. 5.8). Aquests resultats es van confirmar utilitzant el *kit* de viabilitat cel·lular LIVE/DEAD[®] Viability/Cytotoxicity, obtenint-te valors similars de mort cel·lular (resultats no mostrats).
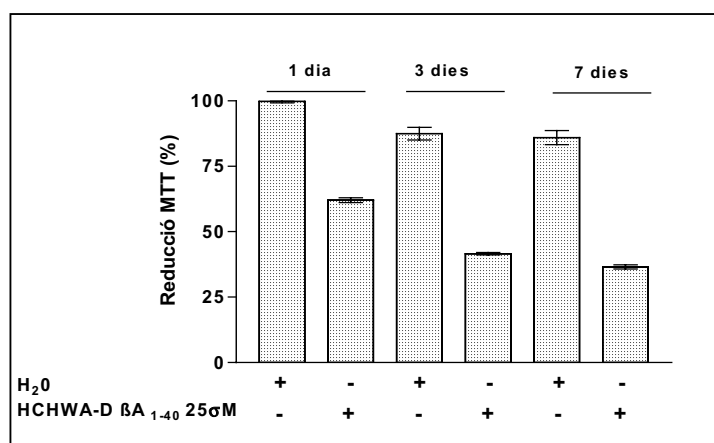
Figura 5.8. Viabilitat cel·lular d'A7r5 després del tractament durant 1, 3 i 7 dies amb D -A $_{1}$ $_{40}$ 25 µM en forma soluble, expressada com el percentatge de reducció d'MTT. Les dades s'expressen com la mitja ∂ SEM de dos experiments diferents realitzats en triplicat.

Seguidament, es va estudiar si la mort per D -A $_{1\ 40}$ en A7r5, com en el cas del cultiu d'HLSMC (Davis *et al*., 1999), era de tipus apoptòtic. A la figura 5.9 es mostra com la tinció amb Hoechst 33258 de cèl·lules tractades durant 48 hores amb D -A $_{1\ 40}$ donava lloc a nuclis condensats, refringents i fragmentats, típics de cèl·lules apoptòtiques. Així doncs, les dades obtingudes fins al moment demostraven que les cèl·lules de múscul llis, A7r5, eren susceptibles a la mort apoptòtica per D -A $_{1\ 40}$, de manera que era un model cel·lular a utilitzar per l'estudi de la possible contribució de la sobreexpressió de la proteïna SSAO en el procés de mort induït pel pèptid A.
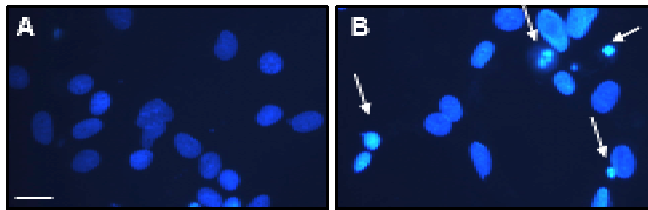

Figura 5.9. El tractament amb D -A $_{1\ 40}$ indueix mort apoptòtica en cèl·lules A7r5. Micrografies representatives del tractament amb al forma soluble del pèptid D -A $_{1\ 40}$ durant 48 h i tinció amb Hoechst 33258. Els nuclis apoptòtics, refringents i fragmentats, es mostren assenyalats amb fletxes. Escala de les barres = 25 μm.

5.2.2. Estudi de l'efecte de la sobreexpressió de la SSAO/VAP-1 en el procés de mort cel·lular per D -A $_{1\ 40}$ en A7r5

5.2.2.1. Sobreexpressió de VAP-1/SSAO per transfecció transitòria

En primer lloc, per sobreexpressar la proteïna d'interès, es va utilitzar el mètode de transfecció transitòria establert anteriorment (veure Resultats 4.1.2) per aquesta línia cel·lular. Les cèl·lules transfectades transitòriament durant 48 hores, es tractaven amb D -A $_{1\ 40}$ 25 μM durant 3 dies i se'n determinava la viabilitat cel·lular en comparació a cèl·lules sense transfectar amb el mateix tractament. Els resultats presentats a la figura 5.10 mostren com, en primer lloc, el fet que la proteïna estès sobreexpressada no afectava el grau de toxicitat induït per D -A $_{1\ 40}$ (Fig. 5.10A). Aquests experiments es van realitzar paral·lelament en presència del substrat de la SSAO, MA 1 mM, sense obtenir canvis en els resultats (resultats no mostrats). Per altra banda, l'anàlisi per

Western-blot revelava que el tractament tampoc era capaç d'induir una sobreexpressió de la proteïna endògena, ni alliberar-la al medi de cultiu durant el temps del tractament (Fig. 5.10B).
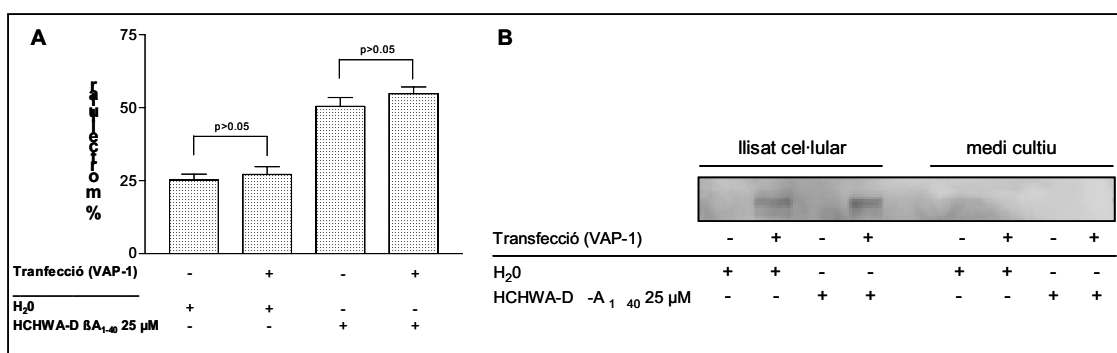
Figura 5.10. Estudi de la sobrexpressió de la SSAO/VAP-1 (transfecció amb el plasmidi pCMV-Sport6/VAP-1 durant 48h) en el tractament per D -A $_1$ $_{40}$ 25 µM de cèl·lules A7r5 **A.** Determinació de la viabilitat cel·lular expressada com el percentatge de cèl·lules mortes respecte el nombre total usant el *kit* de fluorescència LIVE/DEAD® Viability/Cytotoxicity. Les dades s'expressen com la mitja ∂ SEM de dos experiments diferents realitzats en triplicat. *p>0.*05 segons el test One-way ANOVA amb un Newman-Keuls *post-test*. **B.** Western-Blot (anti-VAP-1) representatiu de llisats de cel·lulars i medis de cultiu recollits i concentrats de dos experiments diferents.

5.2.2.2. Sobreexpressió de VAP-1/SSAO per transfecció estable

Donada la poca expressió obtinguda en transfeccions transitòries, es va optar per repetir els experiments realitzats en una línia cel·lular A7r5 que expressa la proteïna VAP-1/SSAO de manera estable (veure Resultats Capítol 4*).* Els resultats obtinguts (Fig. 5.11) demostren com una elevada expressió i activitat de la proteïna SSAO (300 pmol/min·mg proteïna) tampoc eren capaços d'augmentar la toxicitat induïda per D -A $_1$ $_{40}$, ja que tant en la línia salvatge com en la línia estable A7r5/VAP-1 s'obtenia una pèrdua de viabilitat del 35% després del tractament amb D -A $_1$ $_{40}$ 25 µM, no recuperable amb l'inhibidor de la SSAO, semicarbazida 1 mM. Una vegada més, en repetir aquests experiments en presència del substrat de la SSAO, MA 1 mM, no s'observaven canvis en els resultats ni en les conclusions de l'experiment (resultats no mostrats).
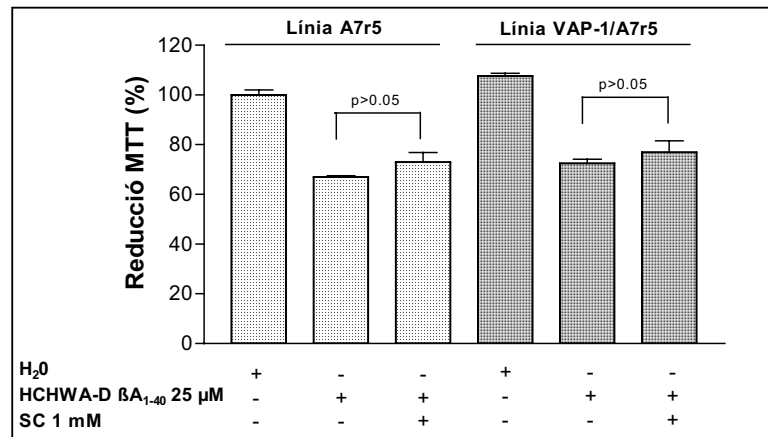
Figura 5.11. Viabilitat cel·lular de la línia A7r5 salvatge i estable per l'expressió de VAP-1/SSAO després del tractament durant 24h del pèptid D -A $_{1\ 40}$ 25 μM, expressada com el percentatge de reducció d'MTT. Les dades s'expressen com la mitja ∂ SEM de dos experiments diferents realitzats en triplicat. *p>0.05* segons el test One-way ANOVA amb un Newman-Keuls *post-test*.

5.3. Estudi de la SSAO en ratolins transgènics Tg-SwDI

A continuació, i donat que els models de CAA *in vitro* no van permetre esclarir si la SSAO podia tenir algun paper en el dany vascular associat a l'AD, es va pretendre estudiar els nivells de la proteïna en un model *in vivo* de CAA. Per aquest objectiu, es van utilitzar els ratolins Tg-SwDI, generats pel laboratori del Dr. Van Nostrand (Stony Brook University, Stony Brook, NY, USA).

5.3.1. Característiques dels ratolins Tg-SWDI

Els ratolins Tg-SWDI expressen la proteïna APP humana contenint les mutacions *Swedish* K670N/M671L i *Dutch/Iowa* E693Q/D694N en neurones, sota el control del promotor de ratolí Thy1.2, de manera que produeixen el doble mutant del pèptid A *Dutch/Iowa* E22Q/D23N en cervell (Davis *et al*., 2004). Les mutacions *Dutch/Iowa* en l'APP estan associades a una severa CAA, tendint a una acumulació preferencial de l'amiloid a nivell cerebrovascular (Levy *et al*., 1990; Grabowski *et al*., 2001). Per la seva banda, la mutació *Swedish* en aquest gen potencia la producció de pèptid A (Haas *et al*., 1995).

Els ratolins Tg-SwDI, tot i no presentar nivells d'expressió elevats del gen de l'APP humà, mostren un important augment en els nivells de A soluble, i sobretot insoluble en cervell. S'observen acusats dipòsits de A fibril·lar en microvasos cerebrals i certs dipòsits difosos de A en parènquima (Davis *et al.*, 2004). Per altra banda, estudis recents utilitzant aquests ratolins transgènics, han mostrat la presència d'astròcits i microglia reactiva associada a microvasos amb dipòsits de A fibril·lar i un augment significatiu en els nivells de IL-1 cerebral (Miao *et al.*, 2005) com a conseqüència dels dipòsits de A fibril·lar en microvasos cerebrals.

A la figura 5.12, es pot observar el forts dipòsits de A en cervell dels ratolins Tg-SwDI de dos anys d'edat, i concretament, es pot apreciar com el A s'acumula en la microvasculatura cerebral, especialment en zones com l'hipocamp, el subiculum, el tàlam, el còrtex motor i el bulb olfactori.
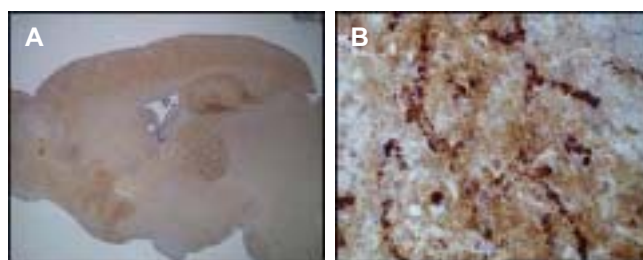
Figura 5.12. Anàlisi immunohistoquímic de l'acumulació de A en els cervells dels ratolins Tg-SwDI. Seccions d'animals de 2 anys d'edat, en les quals s'ha realitzat una doble immunohistoquímica d' A (marró) i col·lagen tipus IV (vermell). (A) 20X augments i (B) 100X augments.

5.3.2. Estudi de la SSAO en ratolins Tg-SWDI

En primer lloc, donat que havíem descrit un augment de l'expressió SSAO en vasos cerebrals de pacients d'AD, vàrem estudiar si en un model *in vivo* de CAA, com s'havia descrit pels ratolins Tg-SwDI, també hi havia canvis en l'expressió de la proteïna a nivell vascular.

Es van realitzar tincions immunohistoquímiques de seccions de ratolins de 2 anys d'edat, els quals presentaven acusats dipòsits de A microvascular, utilitzant un anticòs

contra l'extrem c-terminal de la proteïna VAP-1/SSAO. A la figura 5.13 es presenten els resultats obtinguts i es pot observar, en primer lloc, que la tinció de l'anticòs anti-VAP-1 era específica dels vasos cerebrals, ja que apareixia tinció positiva tant als microvasos (Fig. 5.13B, 5.13F) com als vasos grans leptomeningis (Fig. 5.13C, 5.13G) de ratolí, comparativament a l'absència de tinció en parènquima i en seccions control sense anticòs primari (Fig. 5.13D, 5.13H). És important remarcar que els vasos cerebrals de ratolí, mostraven una expressió de la SSAO comparable a la descrita anteriorment en vasos cerebrals bovins i humans (Castillo *et al*., 1998).

Tot i així, no es van poder observar diferencies en la tinció anti-VAP-1 en el cervell de ratolins Tg-SwDI, en comparació a la tinció en seccions de cervell de ratolins salvatges. Per tant, la VAP-1/SSAO lligada a membrana no presentava un patró d'expressió diferent en aquest model de ratolins transgènics *in vivo* a nivell de vasos cerebrals.
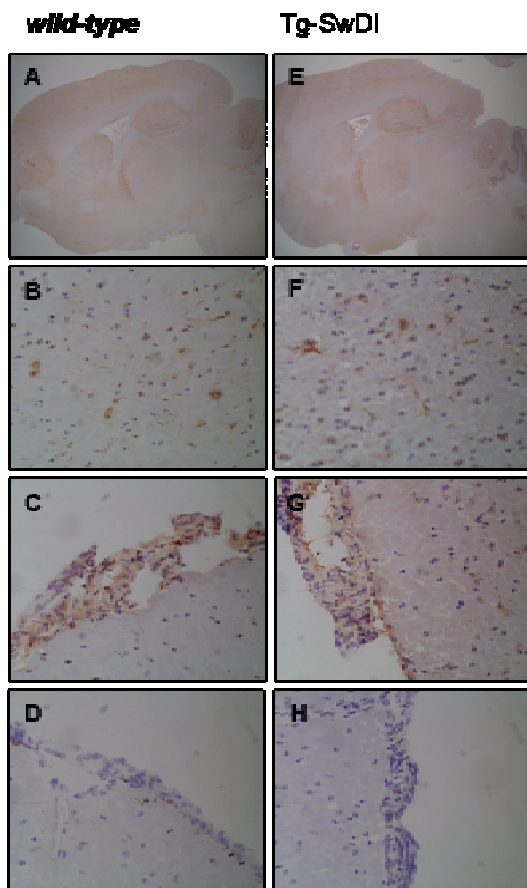
Figura 5.13. Anàlisi immunohistoquímic de la VAP-1/SSAO en seccions de cervells de ratolins, de dos anys d'edat, salvatges (A-D) i TgSwID (E-H). Tinció anti-VAP-1 en cervell (A, E), microvasos cerebrals (B, F) i vasos leptomeningis (C, G), en comparació a seccions sense anticòs anti.VAP-1 (D, H). (A i E) = 20X augments; (B-C i F-H) = 100X.

Seguidament, i en el context dels resultats presentats en el capítol 2, on s'havia descrit un augment d'activitat SSAO en plasma de pacients d'AD sever, es va valorar l'activitat SSAO plasmàtica dels ratolins transgènics Tg-SwDI (n=5) i ratolins salvatges (n=5). En ambdós casos (Fig. 5.14), es va obtenir uns nivells molts baixos d'activitat SSAO, de l'ordre 0.1 pmol/min·mg de proteïna, essent 30 vegades inferior a l'activitat específica SSAO present en plasma en humans. Per altra banda, no es van trobar poder detectar diferències significatives en el cas de l'activitat SSAO plasmàtica dels ratolins Tg-SwDI respecte els ratolins salvatges.
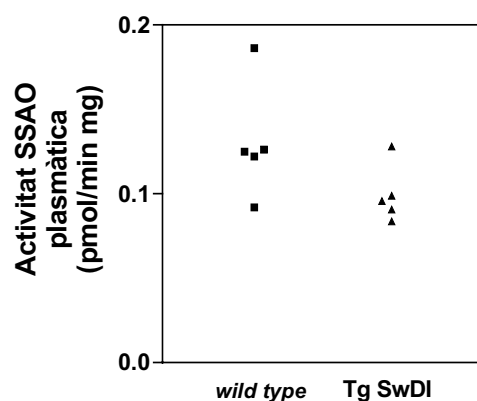
Figura 5.14. Determinació de l'activitat SSAO, mitjançant el mètode radiomètric en front a benzilamina 100 µM 2 mCi/mmol com a substrat, de plasmes de ratolins salvatges (n=5) i TgSwDI (n=5). $p>0.05$ segons el test *t.*

Així doncs, cap dels paràmetres analitzats van permetre trobar diferències en la SSAO, ni en l'expressió a nivell de vasos cerebrals, ni en l'activitat plasmàtica, en aquest model de ratolins transgènics de CAA amb el gen d'APP humà amb les mutacions vasculotròpiques de   A *Dutch/Iowa*.

DISCUSSIÓ CAPÍTOL 5

Una característica present en la majoria de casos d'AD són els dipòsits de A en vasos cerebrals, donant lloc a l'anomenada Angiopatia Amiloide Cerebral (CAA) (Vinters, 1987; Yamada *et al*., 2000). Existeixen tanmateix, casos de CAA com a conseqüència de mutacions puntuals en el gen de l'APP dins la regió del pèptid A, com la mutació *Dutch* que dóna lloc a HCHWA-D (Hemorràgia Cerebral Hereditària Amb Amiloidosis Holandesa) (Levy *et al*., 1990; Bornebroek *et al*., 1996).

Recents estudis han implicat la presència dels dipòsits de A a nivell microvascular amb la neuroinflamació i la demència en AD i CAA (Jellinger, 2002; Attems i Jellinger, 2004). De fet, diferents dades suggereixen una estreta relació entre el dany vascular i la patologia d'AD. Així, per exemple, la presència de l'al·lel 4 d'apoE-4, una proteïna de transport de colesterol, és un dels majors factors de risc, tant de patir AD i CAA (Saunders *et al*., 1993; Greenberg *et al*., 1995; Premkumar *et al*., 1996), com de patir lesions cardiovasculars i arteriosclerosi (Davignon *et al*., 1988; van Bockxmeer i Mamotte, 1992). Per altra banda, diversos estudis clínics i epidemiològics suggereixen una protecció en la incidència o en la progressió d'AD, com a conseqüència de l'administració crònica de substàncies farmacològiques que redueixen el colesterol plasmàtic (Poirier, 2005**;** Puglielli *et al*., 2003; Raffai i Weisgraber, 2003). Donat que s'ha suggerit que un dels responsables de l'acumulació de A en cervell és el seu transport, el deteriorament del teixit vascular cerebral podria ser un punt clau per transvasament anormal de pèptid A al corrent sanguini (Miao *et al*., 2005; Deane *et al*., 2004).

Així doncs, diferents estudis s'han focalitzat en la interrelació entre els riscs vasculars (ambientals i genètics) i la progressió de la malaltia d'AD. En aquest context, donat l'increment d'expressió SSAO en vasos cerebrals de pacients d'AD (veure Resultats Capítol 1) i l'augment d'activitat SSAO en plasma de pacients amb AD greu (veure Resultats Capítol 2), es va plantejar la possible implicació d'aquest enzim en aquesta patologia. Degut a que la SSAO és un enzim que es troba àmpliament expressat en múscul llis de vasos cerebrals, i s'ha demostrat que la seva catàlisi pot induir toxicitat en cèl·lules de múscul llis (veure Resultats Capítol 3) i cèl·lules endotelials (Yu i Zuo, 1993),

l'estudi es va centrar en processos de dany vascular associats a malaltia d'AD, concretament en models de CAA.

Per altra banda, diferents estudis han implicat l'estrès oxidatiu en els desordres vasculars associats a la patologia d'AD (Thomas *et al*., 1996; Suo *et al*., 1997; Muñoz *et al*., 2002). En aquest sentit, la SSAO podria considerar-se com una font d'aquest tipus d'estrès, a través de la generació d'espècies ROS, com l'H_2O_2, o la producció d'aldehids altament reactius capaços de potenciar la formació de productes AGE i la peroxidació lipídica i proteica.

Així doncs, es van escollir diferents models experimentals de CAA per tal d'investigar els processos moleculars implicats en la sobreexpressió de la SSAO *in vivo* i els possibles efectes d'aquesta en desordres tipus CAA. En primer lloc, es va utilitzar un model cel·lular *in vitro,* el qual consistia en la inducció de mort apoptòtica en cèl·lules de múscul llis procedents de vasos leptomeningis humans (HLSMC) per la forma mutada del pèptid A, contenint la mutació tipus *Dutch*, HCHWA-D -A $_{1-40}$ (D -A$_{1-40}$) (Davis i Van Nostrand, 1996; Van Nostrand *et al*, 1998). Aquesta forma peptídica és la resultant de la mutació del gen de l'APP humà en HCHWA-D, i es va utilitzar en els tractaments cel·lulars perquè resultava ser la forma més patogènica enfront les cèl·lules vasculars (Davis i Van Nostrand, 1996). A més, aquestes dades estaven d'acord amb el fet que els dipòsits de A en CAA i HCHWA-D estan formats majoritàriament per D -A$_{1-40}$ (Castaño *et al*., 1996).

En primer lloc, es va haver de posar a punt un cultiu primari pur d'HLSMC, seguint el protocol de Van Nostrand *et al.* (1994). En aquest cas, a passatges baixos, es va poder detectar certa activitat i expressió SSAO en HLSMC en cultiu. Tot i així, els valors d'activitat específica i d'expressió SSAO eren molt inferiors, de l'ordre de 10 vegades, als obtinguts amb l'homogenat inicial de meninges humanes, del qual es partia per realitzar el cultiu primari. A més, l'expressió SSAO s'anava perdent a mida que augmentaven els passatges, tal i com succeïa en el cultiu primari de cèl·lules de múscul llis d'aorta humana (HASMC), i com estava descrit per la SSAO en altres tipus cel·lulars (Buffoni *et al*., 2000), així com per d'altres proteïnes en cèl·lules de múscul llis en cultiu (Browner *et al*., 2004).

Tot i així, donat que en pacients d'AD amb CAA s'havia observat un augment d'expressió SSAO, es va pretendre comprovar si el tractament amb D -A$_{1\ 40}$ també era capaç d'induir un augment d'expressió SSAO en HLSMC en cultiu, el qual permetés investigar la seva possible implicació en aquest model de mort cel·lular. Varies dades bibliogràfiques suggerien que la SSAO/VAP-1 era una proteïna induïble sota certs estímuls, tals com citocines inflamatòries en el cas de la VAP-1 endotelial (Jaakkaola *et al*., 1999), o tractaments de diferenciació cel·lular amb insulina, en cèl·lules de múscul llis (El Hadri *et al*., 2002) o en adipòcits (Mercier *et al*., 2001). En aquest sentit, es va plantejar que la forma patogènica del A fos capaç d'induir l'expressió de la proteïna directament o a través de diferents mecanismes moleculars.

Tot i així, els resultats obtinguts al respecte, no van corroborar la hipòtesis proposada, ja que el tractament amb el pèptid D -A$_{1\ 40}$ no era capaç d'induir l'expressió de la proteïna en aquest model cel·lular. Per altra banda, també es va analitzar la possible presència de la SSAO en els medis de cultiu recollits i concentrats després del tractament amb A$_{1\ 40}$. Evidències bibliografies apuntaven que, en aquest model cel·lular, la SSAO es podia alliberar al medi de cultiu després del tractament amb D -A$_{1\ 40}$, ja que s'havia descrit que aquest tractament induïa l'activació de la proteïna MMP-2 (*matrix metalloproteinase*-2) en HLSMC, i la inhibició d'aquesta resultava en un augment en la viabilitat cel·lular (Jung *et al*, 2003). Per altra banda, en altres models cel·lulars, s'havia observat que la SSAO de membrana s'alliberava al medi en un procés depenent de l'activitat MMP (Abella *et al*., 2004). No obstant, els nostres resultats en aquest sentit tampoc van permetre corroborar aquesta teoria, ja que no es va poder detectar la proteïna en el medi de cultiu de les cèl·lules tractades. A més, la incubació amb inhibidors de la SSAO, juntament amb el tractament amb D -A$_{1\ 40}$, no presentava cap efecte en al viabilitat cel·lular, suggerint que l'acció catalítica de la SSAO no estava implicada en aquest tipus de mort, almenys en aquest model cel·lular.

El fet que les cèl·lules HLSM no expressessin la proteïna SSAO indicava que probablement no era un bon model per estudiar els possibles efectes d'aquesta proteïna en CAA. Per tant, per tal d'intentar reproduir el que succeiria fisiològicament *in vivo*, és a dir, que la SSAO s'expressi constitutivament en cèl·lules de múscul llis, es va repetir l'estudi en una línia cel·lular de múscul llis, A7r5, transfectada transitòria i establement amb la VAP-1/SSAO humana.

En primer lloc, va caldre comprovar que la línia cel·lular A7r5 responia de la mateixa manera que el cultiu primari d'HLSMC davant el tractament amb D -A$_1$ $_{40.}$ Els resultats obtinguts van mostrar que aquest tipus cel·lular era altament sensible a la toxicitat pel pèptid D -A$_1$ $_{40}$ soluble a una concentració de 25 µm, obtenint-se una reducció en la viabilitat cel·lular de l'odre del 70% després de 3 dies de tractament. En canvi, el cultiu d'HLSMC només presentava una davallada del 30-40% amb la mateixa forma peptídica i a la mateixa concentració després de 6 dies de tractament. A més, donat que la mort obtinguda en la línia a A7r5 era de tipus apoptòtic, resultava ser un bon model de mort induïda per D -A$_1$ $_{40}$ soluble, no descrit anteriorment.

El fet que les cèl·lules vasculars siguin sensibles a la toxicitat de la forma soluble del pèptid és un aspecte particularment interessant, ja que s'ha descrit àmpliament que cal una agregació prèvia del pèptid salvatge -A$_1$ $_{40}$ i -A$_1$ $_{42}$ per tal d'induir mort cel·lular en neurones (Howlett *et al*., 1995) i cèl·lules endotelials (Muñoz *et al*., 2002). Així doncs, es podria tractar d'un mecanisme diferent d'actuació del A en cèl·lules de múscul llis vasculars, tal i com ja havia estat postulat prèviament per Davis i Van Nostrand (1995).

En realitzar els experiments de toxicitat per D -A$_1$ $_{40}$ en cèl·lules transfectades amb VAP-1/SSAO humana, no es van poder apreciar diferències en els nivells de viabilitat cel·lular en cultius amb una elevada expressió i activitat SSAO, comparativament a cèl·lules A7r5 salvatges. Per tant, els resultats obtinguts també indicaven, que tot i que la SSAO estigués sobreexpressada, no presentava cap efecte en la mort cel·lular induïda per D -A$_1$ $_{40.}$

Així doncs, les dades presentades permeten concloure que l'expressió de la proteïna SSAO/VAP-1 seria un factor independent davant la mort per A en cèl·lules de múscul llis i els productes de la seva catàlisi no contribuirien al dany induït pel pèptid amiloide. A més, els resultats dels tractaments en aquest model també ens indiquen que el A *per se* no seria un estímul suficient per induir la sobreexpressió de la SSAO en un model en cultiu. Donat que no es coneix el mecanisme de regulació de l'expressió de la SSAO en cultiu, podria ser que aquest no fos el model adequat per estudiar-ne la seva implicació en la malaltia d'AD.

Cal tenir en compte però, que tal i com s'ha anat comentant al llarg de tot aquest treball, els resultats obtinguts de la VAP-1/SSAO en cèl·lules en cultiu suggereixen que estem davant d'una proteïna que requereix d'un entorn vascular complet per la seva correcta expressió i funcionalitat, per tant, el fet de no poder concloure cap implicació en la mort per A en cèl·lules de múscul llis, no descarta que els productes de la seva catàlisi no puguin tenir *in vivo* un paper rellevant en la patologia de CAA.

A més, cal tenir present que la SSAO es localitza a la membrana plasmàtica en cèl·lules de múscul llis dins d'estructures caveolars (Jaakkaola *et al.,* 1999). En aquest sentit, ha estat descrita la precisa colocalització de la SSAO en caveoles amb la proteïna (Suoto *et al.*, 2003). Per altra banda, la proteïna CD36, un receptor *scavenger* de lipoproteïnes, s'ha observat que també és capaç d'unir -amiloide fibril·lar, i s'ha suggerit que, a través de l'estimulació de la formació d'H_2O_2 en resposta a fibril·les de A en microglia i macròfags, podria intervenir en el transport de A (Coraci *et al*., 2002). En aquest context, donat que l'estructura tridimensional del CD36 és molt similar a la SSAO i les dues colocalitzen en caveoles, no es pot descartar que la sobreexpressió de l'amino oxidasa també pugui jugar un paper en el transport del A, contribuint al dany vascular a través d'un mecanisme no descrit fins ara.

Per altra banda, donat que la SSAO expressada en cèl·lules endotelials és més fàcilment induïble sota estímuls inflamatoris (Jaakkaola *et al.,* 1999), seria interessant estudiar si la proteïna SSAO expressada en aquest tipus cel·lular, podria contribuir a la mort per A descrita (Muñoz *et al*., 2002), on l'estrès oxidatiu sembla tenir un paper clau en aquest tipus de mort. Així mateix, el cocultiu de cèl·lules endotelials i de múscul llis, o el cultiu d'anells de vasos cerebrals, també serien una bona eina per estudiar i poder concloure la possible implicació d'aquest enzim en la citotoxicitat del A.

Paral·lelament als estudis en cèl·lules de múscul llis tractades amb el pèptid D -A$_{1 \ 40,}$ es va estudiar la SSAO en un model *in vivo* de CAA. Es van utilitzar uns ratolins transgènics (Tg-SwDI) que expressaven la proteïna APP humana contenint les mutacions *Swedish* K670N/M671L i *Dutch/Iowa* E693Q/D694N en neurones, els quals produïen el doble mutant del pèptid A *Dutch/Iowa* E22Q/D23N en cervell (Davis *et al*., 2004). Els ratolins Tg-SwDI, tot i mostrar importants dipòsits de A en vasos cerebrals,

no mostraven un augment en l'expressió SSAO/VAP-1 en aquest teixit, contràriament als resultats presentats en pacients d'AD.

Cal tenir present però, que aquest model de ratolí transgènic no es pot considerar com un model precís de CAA humà, ja que conté una triple mutació en el gen de l'APP i aquesta no és una condició genètica descrita en humans. El resultat de la sobreexpressió de l'APP amb la triple mutació és una extensa producció del doble mutant *Dutch/Iowa* del pèptid A, i conseqüentment, una acumulació de dipòsits de A en la microvasculatura en edat molt primerenca. Així mateix, existeixen certes diferències de fenotip pel que fa a aquests ratolins transgènics respecte els malalts de CAA. Per exemple, els Tg-SwDI presenten dipòsits de A majoritàriament en microvasos cerebrals, especialment en zones com el tàlam i el subiculum, sense mostrar una acusada acumulació de A fibril·lar en vasos leptomeningis. Per altra banda, cal remarcar la manca de microhemorràgies associades als dipòsits de A en Tg-SwDI, essent un factor present en CAA-AD.

Així doncs, les limitacions presentades per aquest model animal podrien justificar que aquest no fos el model més adequat per estudiar la possible implicació de la SSAO en el CAA. Podria ser que calgués utilitzar un model que presentés un fenotip més propi de la malaltia d'AD, com la presència de plaques senils, per tal de poder estudiar les causes moleculars de la sobreexpressió de l'enzim observades *in vivo*. Tenint en compte que la patologia d'AD és una malaltia multifactorial, probablement l'efecte del A aïllat en cèl·lules de múscul llis o en vasos cerebrals no és representatiu de les condicions patològiques *in vivo,* i per tant, no ens permet concloure si la SSAO contribueix o no a la toxicitat del A en vasos cerebrals en AD.

RESULTATS I DISCUSSIÓ CAPÍTOL 6:

ESTUDI DE LA REGULACIÓ DE L'ACTIVITAT SSAO

RESULTATS CAPÍTOL 6:

ESTUDI DE LA REGULACIÓ DE L'ACTIVITAT SSAO

6.1. Regulació de la SSAO per un component del plasma humà

Està descrit que l'activitat MAO plaquetària pot estar modulada per factors presents en plasma humà; per una banda, aquest enzim es troba inhibit per plasma de pacients esquizofrènics (Berretini *et al*., 1978). Altres autors han proposat que la seva activitat es veu activada per una molècula de tipus fosfolipídic present en plasma humà (Yu *et al*., 1979), i altres treballs (Whalund *et al*., 1984) han descrit que la MAO de plaquetes s'activa per un component de pes molecular superior a 50000 Da present en plasma.

Davant aquestes dades, i donat que l'activitat de la SSAO soluble en plasma humà es troba augmentada en certes patologies, l'objectiu d'aquesta part del treball va ser estudiar el possible efecte modulador per part d'un component present en plasma humà sobre l'enzim SSAO d'origen soluble i tissular.

Tot i que al 1983 Buffoni *et al*. van descriure la presència en plasma humà d'un inhibidor reversible de l'activitat SSAO plasmàtica, estudis anteriors del nostre grup van descriure com concentracions creixents de plasma tenien un efecte activador sobre l'activitat SSAO lligada a membrana procedent de microsomes de pulmó humà (Dalfó *et al*., 2003). De fet, gràcies al procés de concentració del plasma sota pressió de nitrogen, seguit de la utilització de centricons amb porus de 10 kDa de diàmetre i liofilització de la preparació obtinguda, es va aconseguir aïllar un component plasmàtic de baix pes molecular sense identificar capaç d'activar la SSAO.

Per altra banda, aquest component resultava ser termoestable, i després d'una sèrie d'experiments de digestió enzimàtica (amb tripsina i quimiotripsina) i tractaments amb àcid perclòric 1.2 M del plasma humà, es va poder concloure que no es tractava d'un component de naturalesa proteica, donat que seguia mantenint les propietats d'activació sobre l'enzim. En canvi, en tractar el plasma amb 35% d'àcid fòrmic, s'observava una

pèrdua en la capacitat d'activació, fet que va suggerir una possible naturalesa lipídica, glucídica, o glucolipídica de l'esmentat component present en plasma (Dalfó *et al.*, 2003).

6.1.1. Activació de la SSAO soluble i lligada a membrana per un component del plasma humà
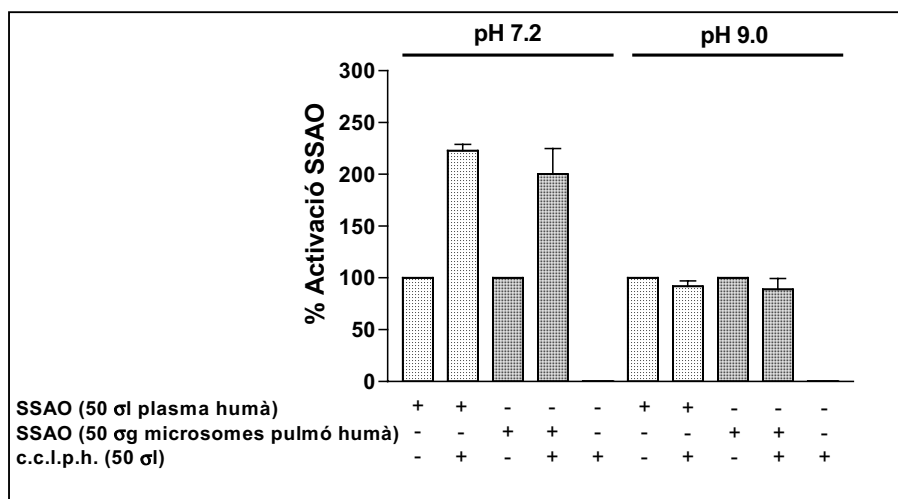
En preincubar la preparació de microsomes de pulmó i el plasma humà durant 30 minuts amb el component activador concentrat del plasma, liofilitzat i resuspès en tampó fosfat 50 mM pH 7.2 (component concentrat i liofilitzat de plasma humà: c.c.l.p.h.), i determinar-ne l'activitat SSAO radiomètricament en front a benzilamina com a substrat, es va observar que hi havia una clara activació de les dues formes enzimàtiques.

A la figura 6.1, expressada en percentatge d'activació respecte l'activitat SSAO control sense activar en cada cas, es mostra com el c.c.l.p.h. era capaç de doblar l'activitat tant la SSAO que provenia de microsomes de pulmó humà (100 ± 24 % d'activació) com la SSAO soluble present en plasma humà (122 ± 6 % d'activació), sense haver diferències estadísticament significatives en el grau d'activació en els dos casos. Cal destacar, que el c.c.l.p.h. per ell mateix no presentava activitat SSAO. Així doncs, dels resultats obtinguts s'extreia que l'efecte activador no tant sols es donava en la forma lligada a membrana de l'enzim, sinó que presentava un comportament d'activació similar sobre la forma soluble a les condicions experimentals assajades.

Aquesta activació era un procés selectiu per la SSAO, ja que la seva activitat es determinava rutinàriament amb la prèvia inhibició amb deprenil 1 mM, per tal d'inhibir la possible activitat MAO, i l'efecte es veia revertit quan es portava a terme la prèvia incubació de les preparacions amb l'inhibidor de la SSAO, semicrabazida 1 mM (Dalfó *et al.*, 2003).

Per altra banda, i donat que el màxim d'activitat de la SSAO és a pH 9.0 (Dalfó *et al.*, 2003), es va estudiar l'efecte del pH sobre la capacitat d'activació d'aquest component. La mateixa figura (Fig. 6.1) mostra com el pH era un paràmetre determinant per obtenir el fenomen d'activació, ja que a pH 9.0 l'efecte es veia completament anul·lat. Per tant, calia treballar a un pH fisiològic per tal que el component del plasma pugués modular

l'activitat de la SSAO. Aquests resultats indicaven que aquest agent activador requeria un entorn de pH neutre per tal de modular l'enzim, és a dir, l'absència de càrregues en el medi era necessària per a dur a terme la seva acció.

Figura 6.1. Activació de la SSAO de microsomes de pulmó humà i SSAO de plasma humà per part d'un component concentrat i liofilitzat de plasma humà (c.c.l.p.h). L'activitat SSAO es determina després de la prèvia preincubació durant 30 min amb el c.c.l.p.h. en tampó fosfat 50 mM pH 7.2 o en Tris 20 mM pH 9.0, en front a benzilamina 100 µM com a substrat. Les dades s'expressen en percentatge d'activació respecte les activitats SSAO control sense activar en cada cas ± SD de dos experiments separats realitzats en triplicat. *p*>0.05, segons el test One-way ANOVA amb un Newman-Keuls *post-test*.

6.1.2. Activació de la SSAO lligada a membrana del sistema cerebrovascular humà per un component del plasma humà

A continuació, i donat que un dels objectius d'aquest treball és l'estudi de la possible modulació de la SSAO present en el teixit cerebrovasculatur humà, es va estudiar l'efecte del plasma humà sobre la SSAO de microvasos i de meninges. La preparació de microvasos en tot aquest estudi es va realitzar segons el protocol modificat de Dellaire *et al*. (1991) i Grammas *et al*. (1991) (veure Material i Mètodes 2.2.2.2).

Els resultats obtinguts (Fig. 6.2), realitzant la determinació de l'activitat SSAO en tampó fosfat pH 7.2, mostraven com la preincubació durant 30 minuts amb el c.c.l.p.h. resultava en una activació de l'enzim de les diferents preparacions assajades. En tots

els casos resultaven ser un efecte pH dependent, ja que a pH 9.0 no s'aconseguia l'efecte obtingut a pH 7.2 (resultats no mostrats).

En el cas de la preparació de meninges humanes i dels microsomes de pulmó humà no s'observaven canvis significatius entre els graus d'activació (152 ± 22 % i 139 ± 9 %, respectivament), en comparació als percentatge d'activitat SSAO control sense activar en cada cas. En canvi, en el cas de la preparació de microvasos s'observava una activació inferior (95 ± 5 %), indicant que l'origen de la font enzimàtica podia ser un factor important a l'hora d'obtenir aquest efecte. Cal tenir en compte que aquesta preparació està altament enriquida en cèl·lules endotelials, i per tant la SSAO provindria bàsicament d'aquest tipus cel·lular. Contràriament, a la preparació de meninges majoritàriament trobem SSAO procedent de musculatura llisa, tal i com succeeix en el cas dels microsomes de pulmó. Per tant, el diferent origen de la forma enzimàtica podria donar lloc a diferents mecanismes de regulació o diferent accessibilitat del modulador a l'enzim. De fet, anteriors estudis ja descriuen una diferent estimulació de la SSAO/ VAP-1 en cèl·lules endotelials respecte cèl·lules de musculatura llisa (Jaakkola *et al*, 1999).

Figura 6.2. Activació de la SSAO de microvasos, meninges i microsomes de pulmó humà per part d'un component concentrat i liofilitzat de plasma humà (c.c.l.p.h). L'activitat SSAO es determina després de la prèvia preincubació durant 30 min amb el c.c.l.p.h. en tampó fosfat 50 mM pH 7.2, en front a benzilamina 100 µM com a substrat. Les dades s'expressen en percentatge d'activació respecte les activitats SSAO control sense activar en cada cas ± SD de tres experiments separats realitzats en triplicat. (**) *p*<0.001, segons el test One-way ANOVA amb un Newman-Keuls *post-test*.
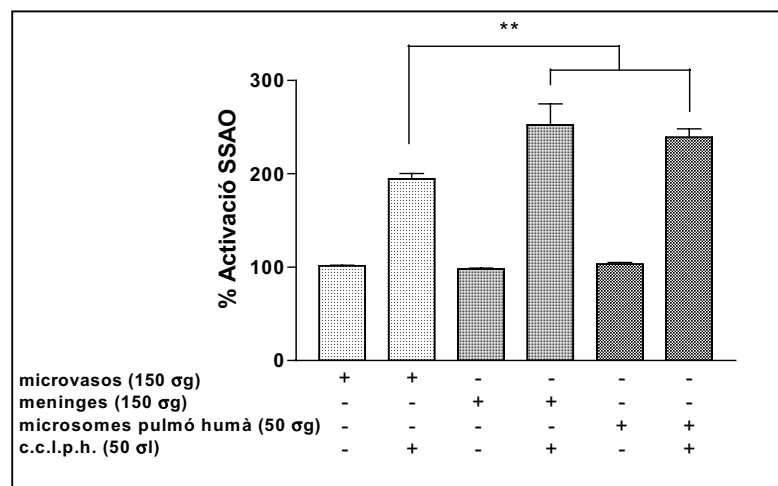
6.2. Regulació de la SSAO per la lisofosfatidilcolina

Diferents evidències van portar a pensar que la lisofosfatidilcolina (LPC) podia ser un bon candidat a ser la molècula activadora de la SSAO. En primer lloc, la LPC era una de les molècules orgàniques que es va identificar en analitzar el plasma filtrat per cromatografia en capa fina (Dalfó *et al.*, 2003). A més, la LPC és un fosfolípid que es troba en grans quantitats en plasma (Nelson, 1967) i s'ha descrit que es pot trobar acumulat en certes situacions patològiques, com arteriosclerosi i isquèmia (Katz i Messineo, 1981). Estudis anteriors del nostre grup, van descriure una certa activació de la SSAO lligada a membrana de microsomes de pulmó humà per part de LPC. El màxim efecte obtingut en aquets experiments va ser una activació del 30% amb una concentració de LPC de 40 µM, però es perdia l'efecte d'activació observat en augmentar la concentració de LPC (Dalfó *et al.*, 2003).

Donat que un dels objectius finals del treball es l'estudi de la SSAO en el sistema cerebrovascular humà, es va procedir a provar directament l'efecte d'aquest compost en l'activitat de la SSAO provinent de preparacions de meninges i microvasos cerebrals humans.
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6.2.1. Activació de la SSAO lligada a membrana de l'espai cerebrovascular humà per LPC

Les condicions experimentals dels assaigs van ser les mateixes que pels experiments d'activació per plasma. Es va provar un rang de concentracions de 0 a 250 µg/ml de LPC (Tipus V de cervell boví), i es va observar (Fig. 6.3) una activació de l'enzim únicament en el cas de al preparació de meninges a una concentració de LPC de 25 µg/ml. Aquest valor de concentració equivaldria al coeficient miscel·lar crític de la LPC de 50 µM, i és del mateix ordre que la concentració a la qual s'observava activació de la SSAO de microsomes de pulmó humà (Dalfó *et al.*, 2003).

Tot i que la LPC podria ser un bon candidat per a optar a ser l'activador de la SSAO de membrana, el percentatge d'activació resultava del 63 ± 20 % (Fig. 6.3), en comparació al valor de 130-140 % obtingut en el cas de l'activació pel c.c.l.p.h (Fig. 6.2). Tot i així,

cal esmentar que el component activador s'obtenia a partir d'un procés de concentració i liofilització del plasma, i per tant, no es podia precisar la concentració exacte de LPC que contenia. Per altra banda, l'activació per LPC només es donava en el cas de la preparació de meninges, en canvi, el c.c.l.p.h. era capaç d'activar la SSAO provinent tant de meninges com de microvasos. Aquests resultats suggerien que la LPC podria tenir un efecte diferent sobre l'enzim respecte el c.c.l.h.p. Existeix la possibilitat que la capacitat activadora del plasma provingui, no d'una única molècula amb capacitat activant, sinó de la interacció additiva de diverses molècules presents en plasma produint un canvi conformacional global de l'enzim que induiria un increment en la seva afinitat pel substrat. Tot i així, l'efecte de la LPC en meninges i microvasos revelava, un cop més, la diferent modulació de l'enzim SSAO en cèl·lules endotelials respecte cèl·lules de musculatura llisa, tal i com s'havia observat anteriorment.
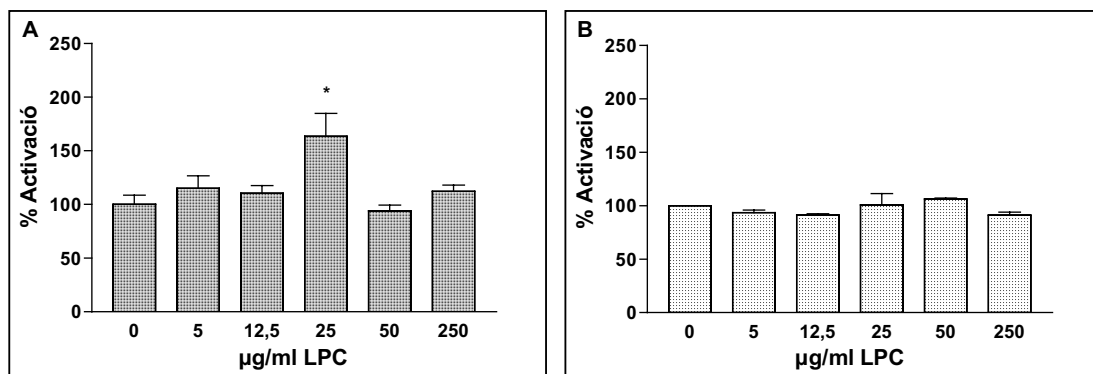
Figura 6.3. Efecte de la LPC en l'activació de la SSAO de preparacions de (A) meninges i (B) microvasos humans. L'activitat SSAO es determina després de la prèvia preincubació durant 30 min amb LPC en tampó fosfat 50 mM pH 7.2, en front a benzilamina 100 µM com a substrat. Les dades s'expressen en percentatge d'activació respecte les activitats SSAO control sense activar en cada cas ± SD de tres experiments separats realitzats en triplicat. (*) *p*<0.01 segons el test one-way ANOVA amb un Dunnett *post-test*.

## 6.3. Regulació de la SSAO soluble per un component del medi de cultiu (DMEM)

Davant les infinites possibilitats que existien de trobar un compost amb les característiques descrites en aquest treball en plasma, es feia molt difícil la identificació total d'aquest component activador. Es va optar per provar l'efecte del medi de cèl·lules en cultiu, *Dulbecco Modified Eagle Medium* (DMEM), ja que experiments publicats per

Trent *et al*. (2002) descrivien un efecte activador per part del DMEM sobre la SSAO present en sèrum fetal boví (FCS).

Així doncs, es van realitzar experiments d'activació realitzats amb el c.c.l.p.h., i en paral·lel, amb el DMEM com a possible nou agent activador. A efectes comparatius, en aquest cas, es va utilitzar la SSAO plàsmatica humana, degut a l'interès en estudiar l'efecte de l'enzim d'origen humà. A més, tal com s'havia observat prèviament, el grau d'activació per part del c.c.l.p.h. resultava ser el mateix per la SSAO lligada a membrana com per la SSAO plasmàtica (Fig. 6.1). A la figura 6.4 es pot observar com la SSAO de plasma humà es veia activada tant per la preincubació durant 30 minuts amb DMEM (137 ± 7 % d'activació) com amb el c.c.l.p.h (105 ± 4 % d'activació), en comparació al percentatge d'activitat SSAO control sense activar.
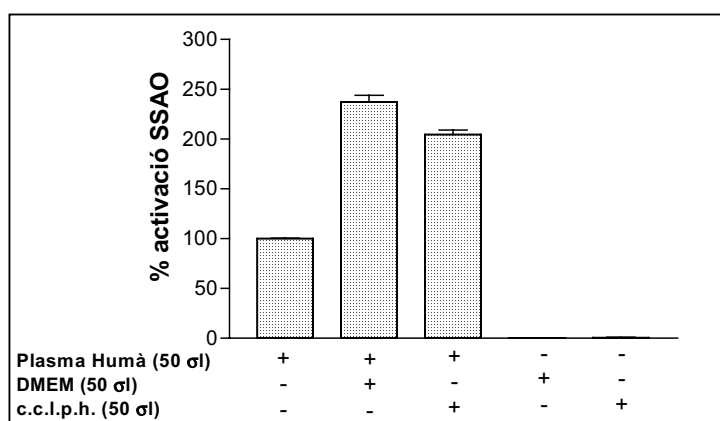
Figura 6.4. Efecte del DMEM i el component concentrat i liofilitzat del plasma humà (c.c.l.p.h.) sobre l'activitat SSAO present en plasma humà. L'activitat SSAO es determina després de la prèvia preincubació durant 30 min amb DMEM o c.c.l.p.h. en tampó fosfat 50 mM pH 7.2, en front a benzilamina 100 µM com a substrat. Les dades s'expressen en percentatge d'activació respecte les activitats SSAO control sense activar en cada cas ± SD de tres experiments separats realitzats en triplicat.

Cal remarcar que tant el DMEM com el component aïllat del plasma tenen un pH de 7, i per tant, l'efecte observat no es podia acusar a la variació de pH del medi on es duia a terme la determinació de l'activitat enzimàtica (tampó fosfat 50 mM pH 7.2).

El fet que el DMEM i el c.c.l.p.h., objecte del nostre estudi, es comportessin d'una manera similar, va suggerir la possibilitat que el component activador del plasma fos el mateix que produïa l'efecte activador del DMEM. Aquesta possibilitat simplificava molt les molècules candidates a estudiar, ja que el DMEM consta d'una composició coneguda i relativament senzilla, en comparació a la composició del plasma humà.

Així doncs, es van assajar les diferents molècules aïllades que composaven el medi DMEM que s'havia utilitzat, per tal d'estudiar l'efecte activador de la SSAO plasmàtica humana. Per cada component es va provar un rang de concentracions finals superior i inferior a la concentració present en el DMEM. A la taula 6.1 es troben resumits els resultats d'activació obtinguts per cada cas.

Composició del DMEM (D546 Sigma-Aldrich)	Concentració DMEM (1X) g/L	Solucions, compostos o molècules assajades	Efecte activador sobre la SSAO plasmàtica humana
Aminoàcids: **L-arginina·HCl** **L-cisteina· 2HCl** **Glicina** **L-Histidina·HCl·H$_2$O** **L-isoleucina** **L-leucina** **L-lisina·HCl** **L-metionina** **L-fenilalanina** **L-serina** **L-treonina** **L-tritòfan** **L-tirosina·2Na·2H$_2$O** **L-valina**	 0.084 0.0626 0.03 0.042 0.105 0.105 0.146 0.030 0.066 0.042 0.095 0.016 0.10379 0.094	**Solució d'aminoàcids:** **M5550 MEM (1X)** **L-arginina·HCl** **L-cisteina· 2HCl** **L-Histidina·HCl·H$_2$O** **L-isoleucina** **L-leucina** **L-lisina·HCl** **L-metionina** **L-fenilalanina** **L-treonina** **L-tritòfan** **L-tirosina** **L-valina**	0%
		Solució d'aminoàcids: **M7145 MEM (1X)** L-alanina L-asparagina·H$_2$O L-àcid aspàrtic **Glicina** **L-fenilalanina** **L-serina**	0%
Vitamines: **Colina** **Àcid fòlic** **Mio-inositol** **Niacinamida** **D-àcid pantotènic·1/2Ca** **Piridoxina·HCl** **Riboflavina** **Tiamina·HCl**	 0.004 0.004 0.0072 0.004 0.004 0.004 0.0004 0.004	**Solució de vitamines:** **M6895 MEM (1X)** **Colina** **Àcid fòlic** **Mio-inositol** **Niacinamida** **D-àcid pantotènic·1/2Ca** **Piridoxina·HCl** **Riboflavina** **Tiamina·HCl**	0%
Salts inorgàniques: **CaCl$_2$·H$_2$O** **Fe(NO$_3$)$_3$·9H$_2$O** **MgSO$_4$** **KCl** **NaHCO$_3$** **NaCl** **NaH$_2$PO$_4$**	 0.265 0.0001 0.09767 0.4 3.7 6.4 0.109	**Ions en solució:** **Ca^{2+}** **Mg^{2+}** **Fe^{2+}/ Fe^{3+}** **Na$^+$/ K$^+$** **Cl$^-$** **NO$_3^-$** **SO$_4^{2-}$** **HCO$_3^-$**	 0% 0% 0% 0% 0% 0% 0% 130%
Altres: **D-glucosa** **Vermell de fenol** **Àcid pirúvic**	 4.5 0.015 0.11	**D-glucosa** **Vermell de fenol** **Àcid pirúvic**	18% 0% 41%

Taula 6.1. Efecte d'activació de la SSAO plasmàtica humana (50 µl) per part dels diferents components del DMEM. L'activitat SSAO es determina després de la prèvia preincubació durant 30 min amb cada un dels components en tampó fosfat 50 mM pH 7.2, en front a benzilamina 100 µM com a substrat. Les dades s'expressen en percentatge d'activació respecte les activitats SSAO control sense activar en cada cas ± SD de tres experiments separats realitzats en triplicat.

6.3.1. Modulació de la SSAO plasmàtica humana per glucosa i àcid pirúvic

En primer lloc, es va estudiar més acuradament la lleugera activació obtinguda amb glucosa i àcid pirúvic separadament. Donat que l'objectiu final d'aquesta part del treball era trobar el component del plasma humà que activava la SSAO soluble humana, no es podia descartar que metabòlits implicats en la via glucolítica tinguessin un paper de regulació de l'enzim. A la figura 6.5 es mostren els resultats obtinguts i es pot apreciar com la glucosa 4.5 g/L era capaç d'activar la SSAO plasmàtica humana en un percentatge de 18 ± 6 %, i l'àcid pirúvic 0.1 g/L en un 41 ± 6 %. Com s'ha comentat anteriorment, l'activitat SSAO soluble es troba augmentada en malalties relacionades amb el metabolisme de la glucosa (Boomsa *et al*., 1995, 1997, 1999, 2000). El fet que l'activitat de l'enzim estigués modulada per metabòlits glucocídics, obria la possibilitat que aquests compostos, entre d'altres, fossin els responsables de l'augment d'activitat SSAO observada en certes patologies.
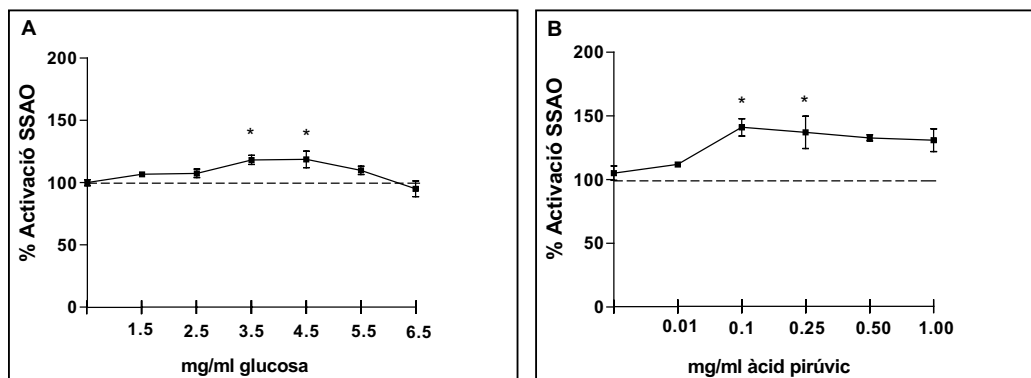
Figura 6.5. Efecte de (A) la glucosa i (B) l'àcid pirúvic sobre l'activitat SSAO plasmàtica humana (50 µl). L'activitat SSAO es determina després de la prèvia preincubació durant 30 min amb cada un dels components en tampó fosfat 50 mM pH 7.2, en front a benzilamina 100 µM com a substrat. Les dades s'expressen en percentatge d'activació respecte les activitats SSAO control sense activar en cada cas ± SD de tres experiments separats realitzats en triplicat. (*) $p<0.05$ segons el test one-way ANOVA amb un Newman-Keuls *post-test*.

Tot i així, els valors d'activació amb aquests components aïllats eren molt inferiors als obtinguts per part del DMEM (de l'ordre del 130%), fet que suggeria que hi havia d'haver algun altre component que fos el responsable de l'activació observada per part del

DMEM. En estudiar si la glucosa i l'àcid pirúvic podien donar lloc a un efecte sinèrgic en el procés d'activació, es van observar els mateixos resultats que assajant els compostos separadament (resultat no mostrat). Per tal de descartar que l'efecte observat provingués exclusivament de la glucosa i de l'àcid pirúvic, es van realitzar el mateix tipus d'experiment utilitzant medis amb diferents concentracions d'aquets components. A la taula 6.2 es resumeixen els resultats obtinguts, els quals no presentaven canvis estadísticament significatius en quant a l'activació de la SSAO plasmàtica humana depenent de la concentració de glucosa i àcid pirúvic.

Medis de cultiu	g/L Glucosa	g/L Àcid pirúvic	Activació SSAO plasmàtica humana
DMEM D6546 (Sigma-Aldrich)	4.5	0.11	130-140%
DMEM D6046 (Sigma-Aldrich)	1.0	0.11	130-140%
DMEM D5671 (Sigma-Aldrich)	4.5	0	120%
DMEM F-12 21331020 (Gibco BRL)	3.151	0.55	130%
BME (PAN Biotech)	1.0	0	120%

Taula 6.2. Efecte d'activació de la SSAO plasmàtica humana (50 µl) per part dels diferents medis de cultius cel·lulars. L'activitat SSAO es determina després de la prèvia preincubació durant 30 min amb cada un dels medis (50 µl) en tampó fosfat 50 mM pH 7.2, en front a benzilamina 100 µM com a substrat. Les dades s'expressen en percentatge d'activació respecte les activitats SSAO control sense activar en cada cas ± SD de tres experiments separats realitzats en triplicat. $p>0.05$ segons el test one-way ANOVA amb un Newman-Keuls *post-test*.

6.3.2. Modulació de la SSAO plasmàtica humana per bicarbonat

Quan es van analitzar separadament els components del DMEM per tal d'identificar el component activador, es va observar que una solució de bicarbonat 3.7 g/L era capaç d'activar la SSAO plasmàtica en el mateix ordre que ho feia el DMEM. Per corroborar aquest efecte es va provar un medi de cultiu (M199) que podia contenir o no $NaHCO_3$. Els resultats presentats a la figura 6.6 mostren com efectivament la presència de bicarbonat al medi de cultiu era determinant per obtenir l'efecte activador, ja que quan

es preincubava el plasma amb 50 µl de medi M199 s'obtenia una activació de l'ordre del 139 ± 6 %, com s'havia observat amb els altres medis analitzats, i l'efecte es veia pràcticament anul·lat quan el mateix medi de cultiu no contenia bicarbonat. Tot i així, cal observar que l'M199 sense bicarbonat mantenia un percentatge del 29 ± 3 % d'activació remanent. La concentració de glucosa en aquest medi era relativament baixa (1 g/L) i no contenia àcid pirúvic, fets que suggerien que aquests dos compostos eren independents a l'efecte activador observat. Malgrat això, no es podia descartar que l'efecte remanent observat amb el M199 sense bicarbonat vingués donat per la baixa presència de glucosa del medi. Per altra banda, tots els medis de cultiu es troben a un pH 7.2, per tant, es descartava la possibilitat que el pH intervingués en el resultat observat. Així mateix, es confirmava que l'efecte activador era selectiu per la SSAO, ja que quan es preincubava el plasma amb l'inhibidor altament específic de la SSAO, l'MDL-72974A 10 µM, s'obtenia una activitat SSAO pràcticament nul·la en tots els casos (Fig. 6.6).
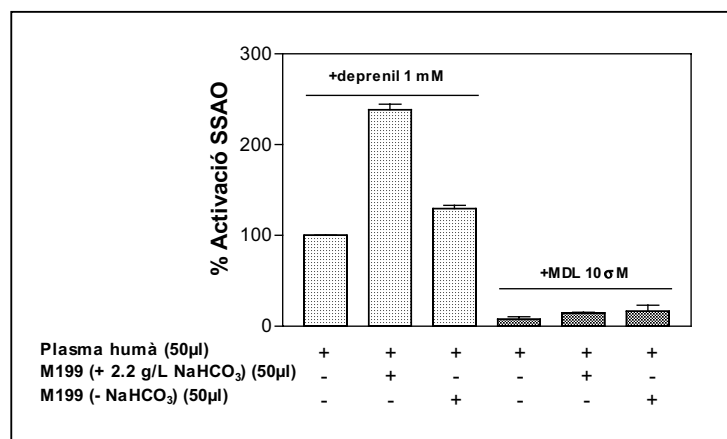
Figura 6.6. Efecte d'activació de la SSAO plasmàtica humana per part del medi de cultiu M199 ± $NaHCO_3$. El plasma utilitzat en cada cas ha estat prèviament incubat durant 30 min amb deprenil 1 mM o MDL-72974A 10 µM. L'activitat SSAO es determina després de la prèvia preincubació del plasma durant 30 min amb cada un dels medis en tampó fosfat 50 mM pH 7.2, en front a benzilamina 100 µM com a substrat. Les dades s'expressen en percentatge d'activació respecte les activitats SSAO control sense activar en cada cas ± SD de tres experiments separats realitzats en triplicat.

També cal tenir compte que tots els medis analitzats prèviament contenien una concentració elevada de $NaHCO_3$; 3.7 g/L en els medis DMEM D6546, D6046 i D5671, i 2.2 g/L en els medis DMEM F-12 21331020 i BME. Aquesta dada també suggeria que l'activació observada en tots els casos venia donada per la presència de $NaHCO_3$.

Per tal de confirmar que el bicarbonat era el responsable de l'activació observada amb els diferents medis de cultiu, es va realitzar el mateix experiment fent servir un rang de concentració creixent d'una solució de bicarbonat. Davant els resultats presentats a la figura 6.7, es podia concloure que l'efecte del bicarbonat era dosi depenent i saturant. A més, era selectiu per la SSAO, ja que es revertia l'efecte en presència de MDL-72974A 10 µM. En preincubar el plasma amb el medi de cultiu per determinar l'activitat SSAO, aquest es diluïa 1:4, de manera que les concentracions reals de $NaHCO_3$ eren de 0.6 g/L en el cas dels medis que contenien una concentració de 2.2 g/L, i 0.9 g/L en el cas dels medis que contenien una concentració de 3.7 g/L. Els percentatges d'activació obtinguts en el cas d'utilitzar una solució de bicarbonat directament eren del mateix ordre, però lleugerament superiors, als obtinguts amb els medis de cultiu, suggerint que l'efecte podria estar parcialment emmascarat per algun dels altres components que conté el medi.
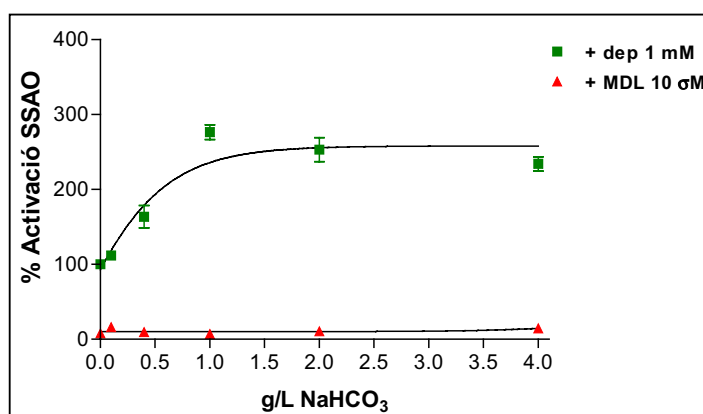
Figura 6.7. Efecte d'activació de la SSAO plasmàtica humana (50 µl) per part del $NaHCO_3$. El plasma utilitzat en cada cas ha estat prèviament incubat durant 30 min amb deprenil 1 mM o MDL-72974A 10 µM. L'activitat SSAO es determina després de la prèvia preincubació del plasma durant 30 min amb cada una de les solucions en tampó fosfat 50 mM pH 7.2, en front a benzilamina 100 µM com a substrat. Les dades s'expressen en percentatge d'activació respecte les activitats SSAO control sense activar en cada cas ± SD de tres experiments separats realitzats en triplicat.

Aquests resultats, tot i que no permetien concloure definitivament si l'efecte del $NaHCO_3$ sobre la SSAO era el mateix que l'observat en el cas del plasma i del c.c.l.p.h., suggerien que el $NaHCO_3$ podria ser un bon candidat a ser el component activador de la SSAO present en plasma.

DISCUSSIÓ CAPÍTOL 6

L'augment d'activitat SSAO plasmàtica en certes patologies presenta la possibilitat que hi hagi un augment de l'alliberament de la proteïna lligada a membrana sota certs estímuls. Per altra banda, almenys en certs casos, no es pot descartar que la regulació de l'enzim plasmàtic sigui la responsable d'aquests augments d'activitat. D'aquí, l'interès del nostre grup en trobar molècules moduladores de l'enzim, tant de la forma soluble com de la lligada a membrana.

Estudis anteriors del grup (Dalfó et al., 2003) van descriure com el plasma humà contenia un o més compostos capaços d'activar la SSAO *in vitro*. La concentració i la liofilització del plasma van permetre aïllar un preparat de molècules de baix pes molecular (component concentrat i liofilitzat de plasma humà: c.c.l.p.h.) que aconseguia activar la SSAO. En els resultats presentats en aquest camp, en primer lloc, es va poder observar que aquest c.c.l.p.h. era capaç d'activar tant la SSAO lligada a membrana de microsomes de pulmó humà, com la SSAO plasmàtica humana. Aquesta dada era particularment interessant pels nostres estudis, ja que suggeria que aquesta(es) molècula(es), a través de l'activació de la SSAO, podia augmentar la producció de metabòlits potencialment tòxics pel teixit vascular. Com s'ha vist en capítols anteriors d'aquest treball (veure Resultats Capítol 3 i 4), l'oxidació de substrats endògens de la SSAO, com la MA, per part de la forma soluble i lligada a membrana de l'enzim, resulta tòxic a cèl·lules de múscul llis en cultiu.

Donat que un dels objectius finals d'aquest treball és l'estudi de la SSAO en la patologia d'Alzheimer, es va abordar el possible efecte activador sobre la SSAO de preparacions de meninges i microvasos cerebrals humans, ja que és un dels teixits afectats en aquesta patologia. En primer lloc, es va observar que el plasma humà i el c.c.l.p.h també eren capaços d'activar la SSAO d'aquestes preparacions. Tot i així, en el cas de la SSAO provinent de microvasos s'observava una activació inferior a l'obtinguda en el cas de la SSAO de meninges cerebrals humanes. Tenint en compte que la preparació de microvasos està altament enriquida en cèl·lules endotelials, i en canvi, la preparació de meninges presenta un alt contingut en cèl·lules de múscul llis, els resultats obtinguts suggereixen que podria existir una diferent modulació de l'enzim depenent de l'origen cel·lular del qual provingués. Jaakkola *et al.* (1999) ja descriuen clares diferències

estructurals i funcionals entre la SSAO/VAP-1 en cèl·lules endotelials i en cèl·lules de musculatura llisa. La SSAO/VAP-1 de múscul llis no presenta la funció d'adhesió a limfòcits i no sembla induïble per diferents estímuls inflamatoris, trets característics descrits de la proteïna endotelial. A més, la SSAO/VAP-1 es troba localitzada únicament a la membrana plasmàtica, mentre que en cèl·lules endotelials també es troba expressada en vesícules intracel·lulars. Aquestes dades suggereixen una diferent funció i modulació de l'enzim depenent de la seva localització cel·lular i subcel·lular, que podrien ser el resultat de diferents modificacions *posttraduccionals* imprescindibles per la funció requerida en cada teixit. En aquest sentit, l'efecte activador de la(es) molècula(es) reguladores del plasma també podria tenir un comportament diferent depenent del teixit on s'expressa l'enzim.

Un dels primers candidats a ser una de les molècules amb capacitat activadora sobre la SSAO va ser la lisofosfatidilcolina (LPC), ja que mostrava un cert efecte activador sobre la forma lligada a membrana de l'enzim de microsomes de pulmó humà (Dalfó *et al.*, 2003). La LPC és un fosfolípid que deriva de la fosfatidilcolina, format durant l'oxidació de lipoproteïnes de baixa densitat (LDL) (Quinn *et al.*, 1988, Liu *et al.*, 1994). La LPC és secretada directament pel fetge (Sekas *et al.*, 1985) i es troba en grans quantitats en plasma, concretament pot representar del 5-20% del total de fosfolípids depenent de l'espècie de mamífer (Nelson, 1967).

En el context dels resultats presentats en els primers capítols d'aquest treball, es va plantejar la possible regulació de la SSAO per part de la LPC en el teixit cerebrovascular humà, ja que l'expressió de la SSAO es veia alterada en aquest teixit en pacients amb AD. La LPC va mostrar un efecte similar sobre la SSAO de meninges a l'obtingut en el cas de microsomes de pulmó humà. En canvi, la LPC no tenia cap efecte sobre l'activitat de la SSAO procedent de la preparació de microvasos humans. Aquest resultat, un cop més, suggeria la diferent modulació de l'enzim depenent de la localització cel·lular. En el cas de l'activació per LPC, l'activitat SSAO, provinent bàsicament de múscul llis, era més sensible a l'activació per estímuls externs. Tot i així, cal remarcar que en cap cas la LPC era capaç d'activar la SSAO lligada a membrana en la mesura que ho feia el plasma humà i el c.c.l.p.h. Aquest fet obre la possibilitat que l'efecte d'activació observat vingui donat per l'efecte sinèrgic de diferents molècules contingudes en el plasma. Tot i així, s'ha de tenir en compte que el lleuger efecte

d'activació per part de la LPC pot tenir una certa importància en referència a la toxicitat cel·lular. S'ha descrit que la LPC pot induir dany cel·lular a través d'un procés que implica l'estrès oxidatiu (Colles i Chisolm, 2000). En aquest sentit, una activació de la SSAO per la LPC augmentaria la producció d'H_2O_2, contribuint i accentuant el procés tòxic induït per aquest fosfolípid.

En la recerca d'altres molècules aïllades o la cooperació entre molècules que poguessin tenir un efecte equivalent a l'efecte activador del plasma sobre l'activitat SSAO, es va analitzar l'efecte del medi de cultiu DMEM (*Dulbecco Modified Eagle Medium*). Estava descrit que el DMEM presentava un efecte activador sobre la SSAO soluble continguda en FCS, i en canvi, presentava un comportament inhibitori sobre la SSAO plasmàtica de rata (Trent *et al.*, 2002). En aquest sentit, es va provar l'efecte del DMEM sobre SSAO plasmàtica humana, ja que com s'ha comentat anteriorment, l'efecte observat del plasma i el c.c.l.p.h era equivalent, tant en el cas de la SSAO lligada a membrana, com en la SSAO plasmàtica humana. Els resultats obtinguts van mostrar un efecte activador del DMEM sobre l'activitat SSAO dosi-depenent, selectiu per aquesta forma enzimàtica i comparable a l'obtingut pel c.c.l.p.h. Aquest resultat va suggerir que la(es) molècula(es) contingudes en el c.c.l.p.h, fossin les mateixes que donaven les propietats activadores al DMEM. Cal destacar que el DMEM presenta un pH de 7, i per tant, no s'estava modificant l'entorn de pH en el qual es determinava l'activitat SSAO (pH 7.2).

Així doncs, donat que el DMEM consta d'una composició coneguda i relativament senzilla, es van assajar separadament els diferents compostos com a possibles candidats a activar la SSAO. Els resultats obtinguts van mostrar que les concentracions de glucosa i d'àcid pirúvic presents en el DMEM eren capaces separadament d'incrementar molt lleugerament l'activitat SSAO plasmàtica humana *in vitro*. De fet, existeixen evidències bibliogràfiques que correlacionen els nivells de glucosa en sang i l'increment d'activitat SSAO circulant, tant en pacients diabètics (Salmi *et al.*, 2002), com en models d'animals transgènics que sobreexpressen la SSAO, als quals se'ls hi ha administrat diferents inductors de diabetis (Gokturk *et al.*, 2004). En aquest sentit, es podria considerar que metabòlits glucosídics poguessin estar modulant l'activitat de l'enzim, fet que podria explicar els lleus augments d'activitat SSAO observats *in vitro*. Un altra possibilitat que explicaria aquesta correlació entre els nivells de glucosa i l'activitat SSAO podria ser el fet que en condicions patològiques, com la diabetis, i sota estímuls

encara desconeguts, els nivells de proteïna SSAO soluble augmentessin com a conseqüència d'un major procés de *shedding* a partir de la forma enzimàtica lligada a membrana. De fet, en estudis recents s'ha demostrat que en pacients amb diabetis, no només hi ha un increment de l'activitat SSAO soluble, sinó també un augment de quantitat de proteïna (Salmi *et al.*, 2002; Boomsa *et al.*, 2003).

Tot i el moderat efecte de la glucosa i l'àcid pirúvic en l'activació de la SSAO plasmàtica *in vitro*, es va descartar que aquestes fossin les molècules responsables de l'efecte activador observat per part del DMEM, ja que medis sense aquests components presentaven el mateix efecte sobre l'activitat SSAO. En canvi, una molècula que presentava una activació similar a la del DMEM, era el bicarbonat sòdic a la concentració de 3.7 g/L. Una prova clau per atribuir l'activació de la SSAO a l'ió bicarbonat, va ser que un altre medi de cultiu amb una composició semblant al DMEM, presentava el mateix grau d'activació que aquest, però en canvi, quan es preparava el M199 sense bicarbonat, el medi perdia pràcticament les propietats activadores de la SSAO. Quan es van preparar diferents solucions de bicarbonat ajustades a un pH neutre, els resultats van mostrar un efecte dosi depenent i saturant a partir d'una concentració d'1 g/L, fet que suggeria una unió específica de l'anió a l'enzim.

Caldria realitzar estudis cinètics per tal d'estudiar el mecanisme d'unió del bicarbonat; podria ser que es tractés d'un activador competitiu que s'uniria al centre actiu, o per altra banda, es podria unir d'un segon centre d'unió, ja proposat en anteriors estudis per la SSAO lligada a membrana de pulmó boví (Lizcano *et al.*, 1994). Cal remarcar, que l'efecte obtingut amb el bicarbonat és una activació selectiva per la SSAO, ja que la prèvia incubació amb l'inhibidor MDL-72974A 10 µM reverteix completament els efectes observats. No obstant, cal tenir present que des del punt de vista estrictament cinètic, el concepte d'activació d'un enzim implica que l'enzim lliure, sense estar unit a l'activador, no ha de presentar activitat, i per tant, no s'uneix al substrat (Cornish-Bowden, 1995). En el nostre cas, l'enzim mostra una activitat basal per si mateix, la qual es veu incrementada al unir-se al bicarbonat o a un altre modulador. Per tant, el més correcte seria parlar d'un efecte modulador més que d'un efecte activador.

A la vista dels resultats presentats, seria interessant comprovar si el bicarbonat presenta les mateixes propietats sobre l'enzim lligat a membrana. De fet, estudis anteriors de la

SSAO en adipòcits de rata ja es demostra que el bicarbonat és capaç d'incrementar l'activitat SSAO en front a histamina com a substrat (Raimondi *et al.*, 1997). En el nostre estudi, també seria interessant comprovar que aquest efecte es pot donar en major o menor mesura quan la SSAO catalitza la desaminació oxidativa d'altres substrats, diferents a la benzilamina. El fet que el bicarbonat sigui el responsable de l'activació observada per part del DMEM pot ser de gran importància a l'hora de realitzar estudis de la SSAO en cèl·lules en cultiu, ja que la concentració de bicarbonat podria afectar els nivells d'activitat SSAO i per tant regular l'efecte metabòlic a estudiar.

Per altra banda, no es pot descartar que el bicarbonat mateix sigui la molècula responsable de l'efecte activador de la SSAO observat en plasma humà. Com ja s'ha comentat anteriorment, quan es concentrava i liofilitzava el plasma, s'obtenia un preparat de baix pes molecular que era capaç d'activar la SSAO en la mateixa mesura que ho fa el bicarbonat. A més, tractaments de termoestabilitat i de digestió amb proteases no alteraven la capacitat activadora del plasma. Aquesta propietat només s'anul·lava quan el plasma es liofilitzava o es tractava amb àcid fòrmic. En aquest sentit, el bicarbonat compliria tots els requisits de ser la molècula activadora.

A més, el bicarbonat és un component esmorteïdor molt important a la sang, que es troba en una concentració relativament elevada (23 mEq/L, els quals equivalen a 1.4 g/L). El bicarbonat, degut a la seva susceptibilitat a patir canvis en els seus nivells segons les necessitats fisiològiques i patològiques de l'organisme, gràcies a l'equilibri bicarbonat-àcid carbònic, podria modular l'activitat SSAO circulant en sang en aquestes determinades condicions. Però, per altra banda, donat que la concentració de bicarbonat, a la qual s'observa l'efecte màxim d'activació, coincideix amb la seva concentració en condicions fisiològiques, cabria la possibilitat que la unió del bicarbonat a l'enzim soluble sigui la condició fisiològica de la SSAO. Una altra possibilitat, i seguint la hipòtesi d'altres autors (Buffoni *et al.*, 1983), que suggerien la presència d'un inhibidor unit permanentment a la SSAO en plasma, el bicarbonat podria ser capaç de desplaçar aquest inhibidor endogen de la SSAO, obtenint-se com a conseqüència uns valors més elevats d'activitat enzimàtica. No obstant, no cal oblidar la possibilitat que en la modulació de l'activitat SSAO hi intervinguin diferents entitats moleculars presents en el plasma, contribuint sinèrgicament a la regulació de l'esmentat enzim, tant en condicions fisiològiques com patològiques.

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ANNEX I: PURIFICACIÓ D'ANTICOSSOS POLICLONALS ANTI-SSAO

ANNEX I. PURIFICACIÓ D'ANTICOSSOS POLICLONALS ANTI-SSAO

Degut a l'elevada eficiència dels anticossos prèviament generats pel nostre grup (Lizcano *et al*., 1998) i utilitzats en aquest treball, es va procedir a la nova purificació de l'anticòs a partir del mateix sèrum de conill immunitzat anteriorment. El fet de disposar novament d'aquest anticòs altament específic per la SSAO serà una eina de gran utilitat que permetrà, en un futur, caracteritzar l'enzim en condicions *in vitro*, així com estudiar la seva implicació en diferents situacions patològiques.

1. Protocol de purificació d'anticossos anti-SSAO

El sèrum immunitzat contra la proteïna SSAO purificada a partir de pulmó boví es va obtenir d'anteriors treballs del nostre laboratori (Lizcano *et al*., 1998). Totes aquelles aliquotes d'antisèrum, conservades a -20 ºC, en les quals s'observava reconeixement de la proteïna SSAO per Western-blot utilitzant mostres de microsomes de pulmó boví, es van ajuntar i diluir, per tal de procedir a la purificació de l'anticòs. Es va realitzar la purificació mitjançant una columna de proteïna A *(HiTrap rProteinA FF* d'1ml, Amersham), degut a la seva elevada especificitat en la unió a IgG de diferents espècies (Veure Materials i Mètodes 7).

Es van realitzar dues purificacions consecutives de la mateixa mostra inicial per tal d'augmentar el rendiment del procés (Fig. 1). En la primera purificació, es va recircular la mostra 3 vegades abans del tractament àcid per tal d'eluir la mostra retinguda a la columna. En aquest cas, es van recollir tres fraccions d'eluït que presentaven un màxim de concentració de proteïna, obtenint-se un total de 3 ml a una concentració de 1.72 mg/ml de proteïna total. Es va procedir a un segon procés de purificació per tal d'augmentar el rendiment de la capacitat de la columna per la unió de IgG de la mostra inicial. En aquest segon cas, es va recircular la mostra 2 vegades i es van recollir 2 ml d'eluït a una concentració de proteïna final de 1.95 mg/ml.
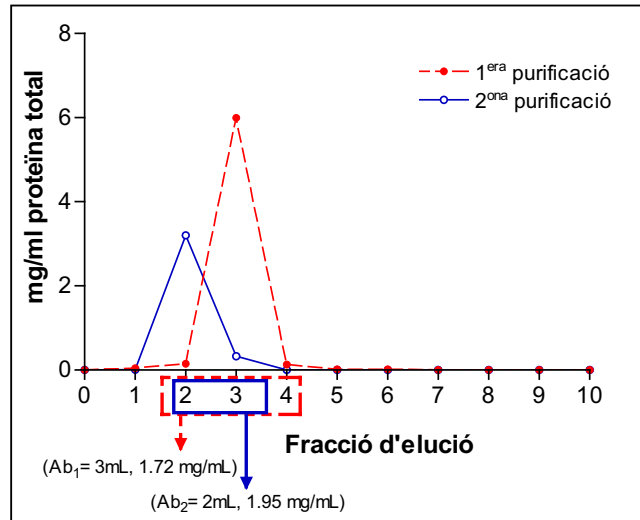
Figura 1. Procés de purificació d'anticòs anti-SSAO a partir d'antisèrum de conill immunitzant contra la proteïna SSAO purificada (Lizcano *et al.*, 1998), mitjançant una columna de proteïna A *(HiTrap rProteinA FF* d'1ml, Amersham).

2. Comprovació de l'eficiència i puresa dels anticossos obtinguts

En primer lloc, per tal de determinar el grau de purificació i de puresa de les preparacions, es va realitzar una electroforesi en un gel (10% acrilamida/bis-acrilamida) en condicions reductores de les fraccions retingudes a la columna en els dos processos de purificació, comparativament a la mostra inicial carregada a la columna i el rentat final. La tinció del gel amb el colorant Comassie (Fig. 2) permetia visualitzar les bandes obtingudes i es va observar com en els dos processos de purificació s'obtenien pràcticament dues úniques bandes de pesos moleculars d'uns 25 i 50 KDa, corresponents a la cadena lleugera i pesada de la immonoglubulina IgG. Per tant, es va poder concloure que els anticossos obtinguts dels dos processos de purificació resultaven en una gran puresa en quant al contingut en IgG.
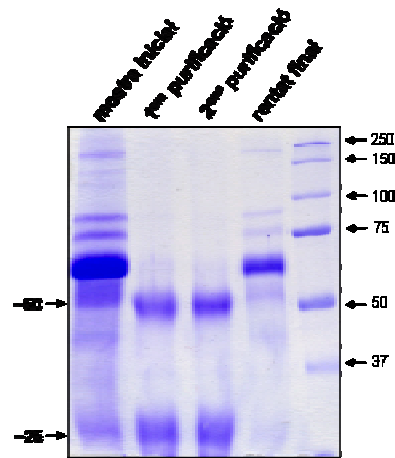
Figura 2. Tinció de Comassie del gel (10% acrilamida/bis-acrilamida) d'elecroforesi de les mostres obtingudes dels dos processos de purificació d'anticòs anti-SSAO a partir d'antisèrum de conill immunitzat amb la proteïna SSAO purificada (Lizcano *et al.*, 1998), mitjançant una columna de proteïna A *(HiTrap rProteinA FF* d'1ml, Amersham).

Seguidament, i per tal de comprovar que l'eficiència dels anticossos en el reconeixement de la proteïna SSAO, es va realitzar Western-blot utilitzant els anticossos purificats de microsomes de pulmó boví, ja que era el teixit del qual provenia la proteïna purificada amb la que s'havia realitzat la immunització del conill, i per altra banda, de preparacions de meninges cerebrals humanes, ja que pel posterior treball era particularment interessant comprovar que els anticossos purificats fossin capaços de reconèixer la proteïna expressada en el sistema cerebrovascular humà. A la figura 3, es pot apreciar com els anticossos de les dues purificacions reconeixien específicament la proteïna SSAO de 97 KDa de les dues preparacions. En canvi, aquests anticossos no eren capaços de reconèixer la proteïna quan es carregaven 100 µg d'homogenat cerebral humà total. Aquest resultat demostrava que l'anticòs era elevadament específic per la proteïna SSAO, ja que en cervell només es troba expressada en vasos cerebrals (Castillo *et al.*, 1998) i aquesta fracció no és suficientment representativa dins l'homogenat cerebral total.
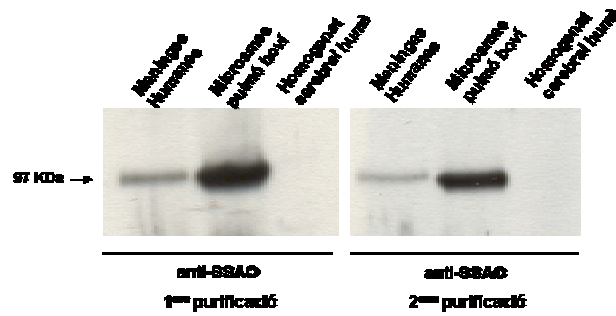
Figura 3. Western-blot anti-SSAO (1.5 µg/ml) utilitzant els anticossos obtinguts dels dos processos de purificació a partir d'antisèrum de conill immunitzat amb la proteïna SSAO purificada (Lizcano *et al.*, 1998), mitjançant una columna de proteïna A *(HiTrap rProteinA FF* d'1ml, Amersham) de les mostres; microsomes de pulmó boví (1.5 µg), preparació de meninges humanes (30 µg) i homogenat cerebral humà (100 µg).

Per altra banda, va caldre comprovar l'eficiència dels anticossos purificats per a la realització de tincions immunohistoquímiques de mostres cerebrals humanes parafinades. A la figura 4 es mostra com l'anticòs reconeixia específicament els vasos cerebrals (Fig. 4B), comparativament a un control sense la incubació amb l'anticòs primari (Fig 4A), i concretament, s'aprecia com es tenyien les cèl·lules de múscul llis dels esmentat vasos.

Figura 4. Inmunocitoquímica utilitzant l'anticòs anti-SSAO obtingut del procés de purificació a partir d'antisèrum de conill immunitzat amb la proteïna SSAO purificada (Lizcano *et al.*, 1998), mitjançant una columna de proteïna A *(HiTrap rProteinA FF* d'1ml, Amersham), de mostres de cervell humà parafinades; **(A)** control sense anticòs primari; **(B-C)** anti-SSAO (3 µg/ml). Escala de barres = 20 µm.

Les dades presentades permeten concloure que l'anticòs anti-SSAO purificat és altament pur i específic per estudiar l'expressió de la proteïna en diferents mostres d'origen humà.

ANNEX II.1. TREBALL PUBLICAT:

"Overexpression of semicarbazide sensitive amine oxidase in the cerebral blood vessels in patients with Alzheimer's disease and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy "

Ferrer I, Lizcano JM, **Hernandez M**, Unzeta M.

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Overexpression of semicarbazide sensitive amine oxidase in the cerebral blood vessels in patients with Alzheimer's disease and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy

I. Ferrer[a,c], J.M. Lizcano[b], M. Hernández[b], M. Unzeta[b],*

[a]*Institut de Neuropatologia, Hospital Princeps d'Espanya, L'Hospitalet de Llobregat (08907), Barcelona, Spain*
[b]*Unitat de Bioquímica de Medicina, Departament de Bioquímica i de Biologia Molecular, Edifici M, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*
[c]*Banc de Teixits Neurològics Universitat de Barcelona, Hospital Clinic Villarroel 170, (08036) Barcelona, Spain*

Abstract

Semicarbazide sensitive amine oxidase (SSAO) metabolizes oxidative deamination of primary aromatic and aliphatic amines, and, in the brain, it is selectively expressed in blood vessels. SSAO expression is examined, by immunohistochemistry with a purified polyclonal antibody to SSAO from bovine lung, in the brains of subjects with Alzheimer disease (AD; $n = 10$), cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL; $n = 2$), and age-matched controls ($n = 8$). SSAO immunoreactivity is restricted to meningeal and parenchymal blood vessels in control and diseased brains. Yet, a marked and selective increase in SSAO immunoreactivity occurs in association with βA4 vascular amyloid deposits in patients with AD, and in the vicinity of the typical granular deposits in the blood vessels of gray and white matter in patients with CADASIL. Oxidative deamination of primary aromatic and aliphatic amines by SSAO produces ammonia, hydrogen peroxide and the corresponding aldehyde. Moreover, increased SSAO immunoreactivity is associated with increased Cu/Zn superoxide dismutase 1 expression restricted to abnormal blood vessels in diseased brains. Therefore, it is suggested that increased SSAO expression is a source of oxidative stress in the blood vessel wall in AD and CADASIL. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Semicarbazide sensitive amine oxidase; Alzheimer disease; Cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy; βA4 Amyloid; Oxidative stress; Cerebral blood vessels; Cu/Zn superoxide dismutase 1

Semicarbazide sensitive amine oxidase (SSAO) constitutes a wide family of enzymes covering beef and sheep plasma spermine oxidase, plasma amine oxidase, lysil oxidase, diamine oxidase and tissue-bound SSAO enzyme, and which is characterized by its relatively high activity towards the non-physiological benzylamine. All of these enzymes are inhibited by semicarbazide, and this property is used to distinguish SSAO from monoamine oxidase (MAO), which is selectively inhibited by acetylenic inhibitors, such as clorgyline and L-deprenyl. Both enzymes show some overlap regarding substrate specificity; SSAO metabolizes oxidative deamination of primary aromatic and aliphatic amines, whereas MAO catalyzes primary, secondary and tertiary amines of endogenous and exogenous origin. Methylamine, resulting from the metabolism of adrenaline by MAO, is metabolized by SSAO [10]. In all of these catalytic reactions, oxygen is consumed, and ammonia, hydrogen peroxide and the corresponding aldehyde are generated as final products. SSAO is found free in blood plasma, and is associated with membranes in several tissues [1,12]. The membrane-bound SSAO shows high activity in smooth muscle cells of blood vessels [16], and a secreted soluble form of SSAO has been recovered in cultures of vascular smooth muscle cells [7]. We have recently observed that SSAO is selectively expressed in human brain blood vessels, but not in other cellular components (i.e. neurons and glial cells) of the brain [4]. It has been proposed that SSAO may play a role as a scavenger of

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* Corresponding author. Tel.: +34-93-581-1523; fax: +34-93-581-1573.

E-mail address: mercedes.unzeta@uab.es (M. Unzeta).

circulating toxic amines and as a mediator in reactions involving H_2O_2 production [14].

Alzheimer disease (AD) shows cerebrovascular amyloid deposition and microvascular degeneration [19,20]. AD is associated with increased oxidative stress in neurons, glial cells and blood vessels [2,13]. Another disease, cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) is characterized by the accumulation of basophilic and osmiophilic material between smooth muscle cells, together with thickening and fibrosis of the walls of the small and medium-sized cerebrovascular arteries [9,18]. Yet, it is not known whether the accumulation of abnormal material is associated with oxidative stress in the blood vessels in CADASIL. The present study was undertaken to gain understanding about the possible modifications of SSAO expression in the cerebral blood vessels in AD and CADASIL, and their possible implications in oxidative stress.

Patients with AD were four men and six women. Their ages ranged from 68 to 82 years, and the mean age was 72.1 years. All of them had suffered from progressive dementia consistent with AD. Age-matched control cases ($n = 8$) had not suffered from neurological disease and did not have chronic metabolic disease or congestive heart failure. The delay between death and tissue processing was between 2 and 7 h in both control and diseased brains. A complete neuropathological examination was carried out in every case (diseased cases and controls) in formalin-fixed tissue for no less than 3 weeks. AD cases were categorized as stage III (three cases) and stage VI (seven cases) of Braak and Braak. Control brains showed no abnormalities, excepting a few βA4 amyloid plaques in the hippocampus in two cases.

Two patients, one woman and one man, were members of different families with several members affected by early-onset strokes and dementia. They had complained of several episodes of cerebrovascular insufficiency that led to progressive neurological impairment with ataxia, memory loss, involuntary jerks in the head and right arm, generalized hypertonia, bilateral Babinski sign, and urinary and anal incontinence. The patients died at the age of 65 and 68 years. The neuropathological examination disclosed multiple cerebral infarcts in the cerebral cortex, subcortical white matter and centrum semi-ovale. Multiple lacunar infarcts were seen in the thalamus and striatum; status cribosus was marked in the caudate and putamen. Severe demyelination, reminiscent of Binswanger disease, was observed in the frontal, parietal, temporal and occipital lobes. No AD changes were present.

Samples of the hippocampus and neighboring entorhinal and temporal isocortex, and frontal, parietal and occipital cortices, were obtained from the ten cases with AD, the two cases with CADASIL, and the eight age-matched controls. In every case, fresh samples obtained at autopsy were fixed with 4% paraformaldehyde in phosphate-buffered saline for 48 h and embedded in paraffin. Dewaxed sections were processed following the avidin–biotin-peroxidase method (ABC; Vector, Vectastain). After blocking endogenous peroxidase with hydrogen peroxide and ethanol, the sections were incubated with normal serum and then incubated at 4°C overnight with the purified polyclonal antibody to SSAO from bovine lung [11] at a dilution of 1:100. Then, the sections were incubated for 1 h with biotinylated anti-rabbit diluted at 1:100, followed by ABC at a dilution of 1:100 for 1 h at room temperature. The peroxidase reaction was visualized with 0.05% diaminobenzidine and 0.01% hydrogen peroxide. Tissues from control and diseased brains were processed in parallel to avoid day-to-day variations in the staining procedure. Some sections were counterstained with hematoxylin.

Double-labeling immunohistochemistry was conducted by incubating the sections following a two-step protocol. The sections were first incubated with antibodies to SSAO, and the immunoreaction was visualized with diaminobenzidine and hydrogen peroxide as before. Subsequently, the sections were incubated with anti-βA4 amyloid (Boehringer-Mannheim) at a dilution of 1:10. The immunoreaction was visualized with 0.01% benzidine hydrochloride, 0.025% sodium nitroferricyanide in 0.01 M sodium phosphate buffer (pH 6.0), and 0.005% hydrogen peroxide. The first primary antibody was recognized as a brown homogeneous precipitate, whereas the second primary antibody was recognized as a dark blue granular precipitate.

Weak SSAO immunoreactivity was seen in the wall of the meningeal and parenchymal blood vessels in control brains, as described in detail elsewhere [4]. Neurons and glial cells were not stained with anti-SSAO antibodies (Figs. 1A,B and 2D). A moderate to strong selective increase in SSAO immunoreactivity was seen in the blood vessels containing amyloid deposits in AD (Fig. 1C). SSAO immunoreactivity was localized between the intima and the muscular layer of arteries with amyloid angiopathy (Fig. 1D,E), and the immunoreaction decorated the indentations of muscular cells of the vessel wall (Fig. 1F). Double-labeling immunohistochemistry to SSAO and βA4 amyloid disclosed co-localization of increased SSAO immunoreactivity and abnormal amyloid deposition, with βA4 amyloid being distributed at the periphery of SSAO deposits (Fig. 1G,H). Weak immunoreactivity (similar to control cases) occurred in blood vessels lacking amyloid deposits.

Blood vessels in gray and white matter in patients with CADASIL were thickened and fibrotic, and displayed granular deposits between the intima and the internal part of the fibrotic tunica media. These granular deposits were seen equally in hematoxylin and eosin-stained, and in trichrome-stained sections (Fig. 2A–C). Strong SSAO immunoreactivity was found in cerebral blood vessels in CADASIL (Fig. 2E,F) when compared with controls processed in parallel (Fig. 2D). In addition, increased SSAO expression in CADASIL was distributed irregularly in the blood vessels, often forming localized deposits (Fig. 2E–G) or irregular accumulations (Fig. 2H).
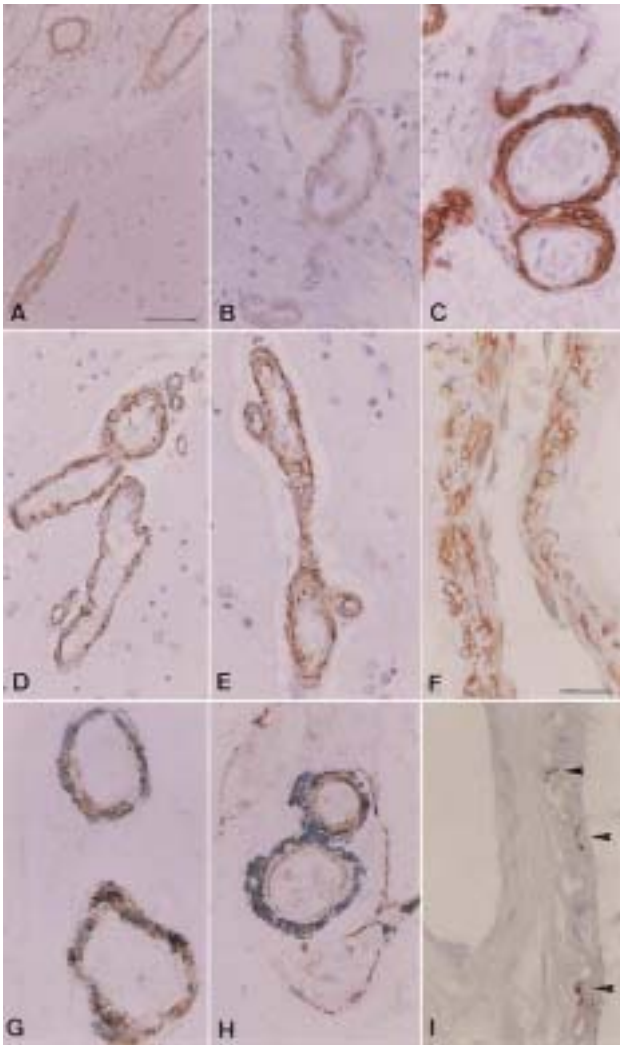
Fig. 1. SSAO immunoreactivity in the parenchymal and meningeal blood vessels in controls (A,B), and in cases with AD (D–F). Diseased vessels show βA4 amyloid deposition (C). SSAO immunoreactivity is found in the internal part of the muscular layer in control (A,B), and diseased blood vessels (D,E), and delineates the indentations of muscular cells (F). SSAO expression is markedly increased in cases with AD. Double-labeling immunohistochemistry reveals co-localization of SSAO expression (brown precipitate) and βA4 amyloid deposition (dark blue precipitate) in AD amyloid angiopathy (G,H). Yet, βA4 amyloid is usually bordering SSAO immunoreactivity in abnormal vessels. Small deposits of Cu/Zn SOD1 immunoreactivity (arrowheads) are seen in blood vessels with amyloid angiopathy in AD. Paraffin sections, slightly counterstained with hematoxylin. Bars: (A), 100 μm; (B–E,G–I), 50 μm; (F), 25 μm.

Oxidative stress is a prominent abnormality in several neurodegenerative disorders, and is a well-documented complication in AD [2,13,15]. A link between βA4 amyloid, oxidative stress and neurodegeneration has been proposed in AD [5]. Full-length βA4 peptides posses a Cu^{2+}-binding domain, and Cu^{2+} has the capacity to potentiate βA4 amyloid neurotoxicity in AD [6]. Furthermore, βA4 amyloid induces H_2O_2 formation in clonal cell lines, and catalase protects cells from βA4 amyloid neurotoxicity

and free radical formation [3]. This scenario applies for abnormal βA4 amyloid accumulation, oxidative stress responses and damaged blood vessels in AD. In line with these data, increased Cu/Zn superoxide dismutase 1 (SOD1) immunoreactivity (Novocastra NCL-SOD1; dilution, 1:50) has been found in association with amyloid deposits in senile plaques and blood vessels in AD (Fig. 1I), in agreement with previous observations (see [13], for review). Catalytic effects of SSAO on primary aromatic and aliphatic amines in the blood vessel wall may trigger the production of toxic aldehydes and H_2O_2, thus contributing to βA4 amyloid toxicity.

CADASIL is linked to chromosome 19 [18] and associated with mutations in the *Notch3* gene [8]. Interestingly, the product of this gene is related with presenilin 1, whose defect predisposes to early-onset familial AD. Electron microscopy discloses the accumulation of granular osmiophilic material
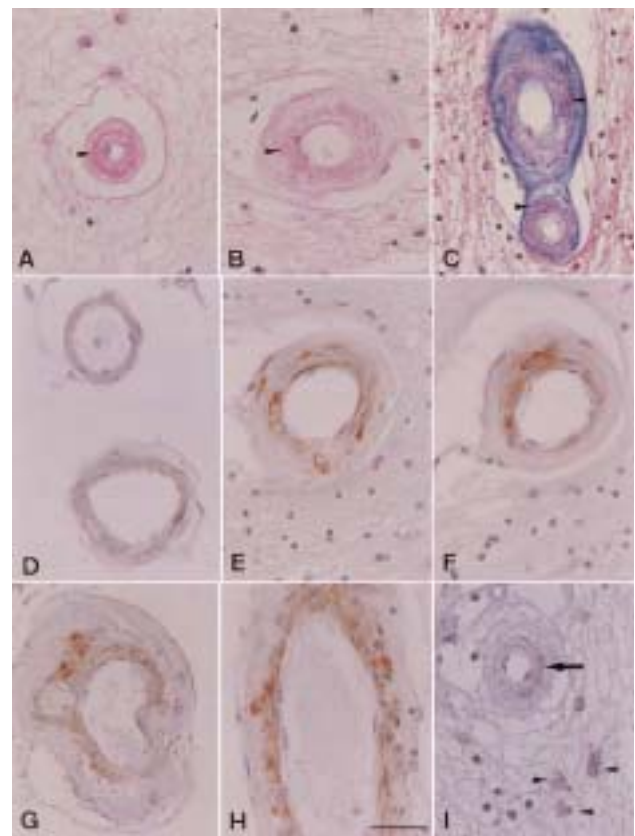
Fig. 2. SSAO immunoreactivity in CADASIL. Small and medium-sized arteries in CADASIL show a thickened and fibrotic vessel wall, as well as accumulation of granular material (arrowheads) between the intima and the inner part of the thickened tunica media (A–C). SSAO immunoreactivity is markedly increased in CADASIL (E–I) when compared with a control (D) processed in parallel. SSAO immunoreactivity is found between the intima and the muscular layer, often forming asymmetrical aggregates (F,G) or irregular deposits (H). Cu/Zn SOD1 immunoreactivity is observed in blood vessels (arrow) and in the cytoplasm of reactive astrocytes (arrowheads). Paraffin sections: (A,B), hematoxylin and eosin; (C), trichrome; (D–I), SSAO immunohistochemistry slightly counterstained with hematoxylin. Bar, 50 μm.

between degenerating smooth muscle cells or in indentations of these cells, often within the thickened basal lamina [9,17]. The composition of the accumulated product is not known, but it clearly differs from amyloid. The present results have shown increased SSAO expression in the proximity of granular deposits, but not in the fibrotic zones of afflicted brain arteries in CADASIL, suggesting that increased SSAO activity may lead to oxidative stress. Increased Cu/Zn SOD1 immunoreactivity has been observed in blood vessels bearing abnormal granular deposits and in reactive astrocytes in CADASIL (Fig. 2I), thus showing activation of mechanisms against oxidative damage probably caused by superoxide radicals in CADASIL.

In summary, we have seen a link between SSAO over-expression and vascular pathology in AD and CADASIL, and its association with oxidative stress. Yet, further studies are needed to determine the exact role of SSAO in the pathogenesis of the degenerative changes in meningeal and parenchymal blood vessels in these diseases.

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ANNEX II.2. TREBALL PUBLICAT:

"Activation of human lung semicarbazide sensitive amine oxidase by a low molecular weight component present in human plasma "

Dalfo E, **Hernandez M**, Lizcano JM, Tipton KF, Unzeta M.

Biochim Biophys Acta. 2003 Jul 30;1638(3):278-86.

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Activation of human lung semicarbazide sensitive amine oxidase by a low molecular weight component present in human plasma

E. Dalfó[a], M. Hernandez[a], J.M. Lizcano[a], K.F. Tipton[b], M. Unzeta[a],*

[a] Departament de Bioquímica i Biología Molecular, Institut de Neurociencies, Facultat de Medicina, Universitat Autónoma de Barcelona,
08193 Bellaterra, Barcelona, Spain
[b] Biochemistry Department, Trinity College, Dublin 2, Ireland

Abstract

Semicarbazide-sensitive amine oxidase (SSAO) encodes a wide family of enzymes named E.C.1.4.3.6 [amine:oxygen oxidoreductase (deaminating) (copper containing)] that metabolises primary aliphatic and aromatic amines. It is present in almost all vascularised and nonvascularised mammalian tissues, and it is also present in soluble form in plasma. SSAO appears to show different functions depending on the tissue where it is expressed. Here we describe, for the first time, the activation of the SSAO from human lung by human plasma. The extent of activation was greater when the human plasma came from diabetic and heart infarcted patients. A kinetic mechanism of such effect is proposed. The activation was lost after the plasma was dialysed, indicating a low molecular weight component (MW < 3800 Da) to be responsible. The activator component is heat stable and resistant to proteolysis by chymotrypsin and trypsin and also resistant to perchloric acid treatment. However, treatment with 35% formic acid, completely abolished activation, suggesting involvement of lipid material. The possibility of that lysophosphatidylcholine (LPC), an amphiphilic phospholipid derived from the phosphatidylcholine, the major component in plasma accumulated in pathological conditions, was studied. LPC was shown to behave as a "competitive activator" of human lung SSAO at concentrations below its critical micellar concentration (CMC value = 50 μM). Thus LPC may be a component of the SSAO activatory material present in human plasma.
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Keywords: Semicarbazide-sensitive amine oxidase; Activation; Plasma component; Lysophosphatidylcholine

1. Introduction

Several amine oxidising enzymes are inhibited by semicarbazide. These include beef and sheep plasma spermine oxidase, plasma amine oxidase, lysyl oxidase, diamine oxidase and a tissue-bound amine oxidase. All are inhibited by semicarbazide as a result of the presence of a carbonyl-group-containing cofactor. However, the term semicarbazide-sensitive amine oxidase (SSAO) is generally used to describe those enzymes that catalyse the oxidative deamination of aliphatic and aromatic primary amines. They are included in the classification as E.C.1.4.3.6 [amine:oxygen oxidoreductase (deaminating) (copper-containing)]. Semicarbazide is frequently used to distinguish the SSAOs from the monoamine oxidase (MAO) [amine:oxygen oxidoreduc-

tase (deaminating)(flavin-containing)], E.C.1.4.3.4, that is sensitive to acetylenic inhibitors such as clorgyline and *l*-deprenyl, but it is not affected by semicarbazide. The substrate specificity of MAO and SSAO overlap to some extent but, whereas MAO catalyse the oxidative deamination of primary, secondary and tertiary amines, the activity of SSAO appears to be restricted to primary amines. Methylamine, which arises from the metabolism of adrenaline, lecithin, sarcosine, and creatinine, is metabolised by SSAO from many sources [1,2] and it has been proposed to be a physiological SSAO substrate, since it is not oxidised by MAO. Both enzymes catalyse the oxidative of deamination primary amines to the corresponding aldehyde, ammonia, and hydrogen peroxide, according to the overall reaction:
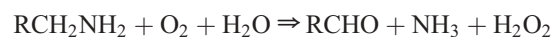
$$RCH_2NH_2 + O_2 + H_2O \Rightarrow RCHO + NH_3 + H_2O_2$$

SSAO is tightly associated with membranes in several mammalian tissues and also occurs as a soluble form in

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* Corresponding author. Tel.: +34-93-5811523; fax: +34-93-5811573.
E-mail address: Mercedes.Unzeta@uab.es (M. Unzeta).

blood plasma [3,4]. The membrane-bound SSAO shows high activity in blood vessels and appears associated with smooth muscle cells [5,6]. Vascular smooth muscle cells in culture have been reported to secrete a soluble form of SSAO [7] that may represent the origin of the plasma enzyme. SSAO activity has been also found in nonvascular cell types [8], dental pulp [9], chondrocytes [10] and in adipocytes, from rat white and brown fat [11]. Despite its wide tissue distribution in human tissue [12], the physiological role of SSAO is still far from clear. SSAO present in the microsomal fraction of human and bovine lung [13,14] may be important in the metabolism of inhaled volatile amines [15]. The SSAO located in the plasma membrane may act as a scavenger of circulating toxic amines. It appears that this enzyme is also involved in the control of cellular activities mediated by H_2O_2 [16]. It has been recently reported that SSAO colocalises with the GLUT4 glucose transporter in the endosomal compartment in rat adipocytes and that SSAO-catalysed substrate oxidation results in a marked stimulation of glucose transport, mimicking the insulin effect [17].

A vascular-adhesion protein VAP-1, present in endothelial cells, has been shown to have an identical amino acid sequence to SSAO [18]. Because VAP-1 is induced in inflammation and is involved in lymphocyte migration to lymphoid organs, a new physiological role in cellular trafficking has been proposed for SSAO [19]. However, the VAP-1 expressed in smooth muscle cells has been reported to be structurally and functionally distinct from VAP-1 present in endothelial cells and not support binding to lymphocytes [20].

SSAO activity appears to be altered in several pathological conditions. Changes in plasma SSAO activity have been reported in fibrotic liver and in patients suffering from burns [5]. SSAO from human plasma is elevated in congestive heart failure [21]. A decreased membrane-bound SSAO activity has been reported in chemically induced rat breast tumours [22]. Plasma SSAO is also increased in streptozotocin-induced diabetic rats [23] and also in patients suffering from diabetes [24]. Cardiovascular complications and retinopathy are serious consequences of diabetes [25,26], and it has been suggested that toxic metabolites arising from the SSAO-catalysed oxidative deamination of aminoacetone and methylamine may be a contributing factor. The formaldehyde generated by SSAO in vascular tissues could be a potential risk factor for stress-related angiopathy [27].

In the present study, we describe for the first time the activation of the membrane-bound SSAO from human lung, by a low molecular weight phospholipid-like component present in human plasma. The levels of this activator appeared to vary in different pathological conditions, increasing during acute phase reactions. Lysophosphatidylcholine (LPC) has been shown to activate the SSAO, suggesting that it may be a component of this plasma activator.

2. Materials and methods

2.1. Materials

Superdex 200 HR was purchased, as a pre-packed column, from Pharmacia. Centricon-10 miniconcentrators and XM-50 ultrafiltration membranes were from Amicon. The radioactive substrates [7-^{14}C]benzylamine hydrochloride (specific activity 57 mCi/mmol) and [^{14}C]methylamine (779 µCi/mg) were obtained from Amersham International. All other reagents were from Sigma-Aldrich.

2.2. Human plasma

The Ethical Committee of the Autonomous University of Barcelona has approved the experimental protocol used for human samples. Human plasma samples were obtained from the Hospital General de la Vall d'Hebron, Servei d'Hematologia, Barcelona, Spain, and stored at − 20 °C until use. Samples of pathological human plasma were obtained from the Biochemical Analysis Unit the Hospital General de la Vall d'Hebron, Barcelona, Spain.

2.3. Preparation of human lung microsomes

Human lung was obtained from the Servicio Anatómico Forense del Hospital Clínico Provincial de Barcelona, Spain, transported immediately to the laboratory and stored at − 20 °C until use. After removing the connective tissue, the lung was weighed, chopped into small pieces with scissors and washed extensively with saline (0.9%, w/v, NaCl) to eliminate blood as a potential source of contaminating plasma amine oxidase. The tissue was then homogenised in 10 volumes (w/v) of 10 mM Tris/HCl buffer, pH 7.2, containing 0.25 M sucrose, in a Waring blender, and filtered through two layers of cheesecloth. The homogenate was subjected to differential centrifugation and the microsomal fraction was obtained by adding 10 mM $CaCl_2$ to the post-mitochondrial supernatant and centrifuging as previously described [14]. The final microsomal pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.2. The protein concentration was adjusted to 10 mg/ml and samples were stored in aliquots at − 20 °C until required. The resulting preparation was referred as crude microsomes.

2.4. Concentration of plasma and activation experiments

Plasma was thawed at 37 °C and 200 ml aliquots were filtered through an Amicon ultrafiltration cell equipped with XM-50 membranes (50 kDa cutoff). The filtrate (40 ml) was recovered and filtered again, through a Centricon-10 ultrafiltration unit. The final filtrate was concentrated four to five times by lyophilisation and resuspension in 50 mM potassium phosphate buffer, pH 7.2. The filtrate containing the activator was then used for the proteolytic, formic acid and perchloric acid treatments, as described below.

2.5. Enzyme assays

SSAO activity in human lung microsomes was determined radiochemically at 37 °C, by the method of Fowler and Tipton [28], using [^{14}C]benzylamine (3 mCi/mmol) 20 μM as substrate. The reaction mixture contained SSAO enzyme and substrate in a total volume of 225 μl of 50 mM potassium phosphate buffer pH 7.2. The reaction was stopped by the addition of 100 μl of 2 M citric acid. Radioactive-labelled products were extracted into toluene/ethyl acetate (1:1, v/v) containing 0.6% (w/v) 2,5-diphenyloxazole (PPO), before liquid scintillation counting. Because of the low SSAO activity present in human plasma, activities were determined in this case by the same method described above but using 100 μM benzylamine as substrate and pH 9 in 20 mM Tris–HCl buffer.

SSAO activity in human lung microsomes was determined towards 100 μM [^{14}C]methylamine (1 mCi/mmol) as described by Precious et al. [29]. The reaction was carried at 37 °C in a final volume of 100 μl of 50 mM potassium phosphate buffer pH 7.2, and was stopped by cooling the tubes in an ice bath. The reaction product, [^{14}C]formaldehyde, was separated from any unchanged amine by passage through a 1 ml Amberlite C6–50 (carboxylic form) column. Six milliliters of Bray solution was added to the column eluate before liquid scintillation counting.

When required, SSAO activity was inhibited by preincubating samples for 30 min at 37 °C with 1 mM semicarbazide, a procedure that did not affect MAO activity.

MAO-B and MAO-A activities were selectively inhibited by preincubation, under the same conditions with 1 μM *l*-deprenyl and 1 μM clorgyline, respectively. Preincubation with 1 mM clorgyline was used to inhibit the total MAO activity, without affecting that of SSAO. Time course assays were used to ensure that initial rates of the reaction were determined, and the proportionality of the initial rates to enzyme concentration was also established in each case. Kinetic parameters were calculated by nonlinear regression analysis, using the computer program Graph-Pad Prism.

2.6. Treatment of plasma with formic acid, perchloric acid or Triton X-100

One hundred microliters of 0.4 M perchloric acid was added to 200 μl of lyophilised human plasma. The mixture was stirred for 5 min and then centrifuged at $13,000 \times g$ for 10 min. The supernatant was neutralised with 1 M NaOH, and stored at 4 °C until assay for its ability to activate human lung microsomal SSAO. The same experimental procedure was used for formic acid treatment, except that the acid concentration was 1.2 M. Samples of crude microsomes were mixed with Triton X-100 at a final concentration of 0.6% (w/v). After stirring at 4 °C for 30 min, samples were centrifuged at $105,000 \times g$ for 1 h. The resulting pellet and supernatant were collected and the protein content and SSAO activity were determined in each fraction.

2.7. Proteolytic enzyme treatment and heat treatment

For peptidase treatment, 300 μl of filtered and concentrated human plasma was incubated overnight at 37 °C with 100 μl of either trypsin or chymotrypsin, each at 1 mg/ml, in 0.1 M ammonium bicarbonate, pH 9. In controls, the peptidases were replaced by 100 μl of 0.1 M ammonium bicarbonate. After this proteolytic treatment, its activating effect was assayed on human lung SSAO.

Additional experiments were performed to confirm the lack of effect of the peptidase treatment on the SSAO activating component from plasma. Filtered and concentrated human plasma was chromatographed on Superdex-200HR-FPLC, previously equilibrated with 0.1 M ammonium bicarbonate buffer, pH 9. Fractions (0.5 ml) containing the activator were incubated 20 h at 37 °C with 100 μg of either trypsin or chymotrypsin and then assayed for their abilities to activate human microsomal SSAO, as described above. In the heat-treatment studies, samples of plasma were incubated at 100 °C for 30 min, before cooling on ice and assessing its activation effect towards human lung SSAO, as described above.

2.8. Estimation of the M_r of the activator

Filtered and concentrated human plasma was chromatographed on Superdex-200HR-FPLC, equilibrated with 50 mM potassium phosphate buffer, pH 7.2. Fractions (0.5 ml) were collected and assayed for their ability to activate human microsomal SSAO, as described above. The approximate molecular weights of the eluted material were determined by reference to a calibration curve constructed from different molecular weight (kDa) markers.

2.9. Thin layer chromatography

Concentrated human plasma samples were extracted for chromatography of lipids by the following procedure. Chloroform/methanol, 1.2 ml, was added to 0.25 ml of concentrated human plasma. The sample was allowed to stand for 10 min at room temperature and 0.5 ml chloroform and 0.5 ml of 0.25 M HCl were added. The mixture was stirred vigorously and centrifuged 2500 rpm for 5 min. The aqueous and organic phase were separated and evaporated under an atmosphere of N_2. The aqueous phase was neutralised with 1 M NaOH and then assayed for its activation effect towards human lung microsomes. The organic phase pellet was resuspended in 20 mM potassium phosphate buffer, pH 7.2, and applied to a silica-gel TLC plate. The TLC was developed with chloroform/methanol/acetone/acetic acid/water (50:10:20:10:5), and stained with ninhydrin.

2.10. Protein quantification

Protein was measured by the method of Bradford [30], using bovine serum albumin (BSA) as standard.

2.11. Statistical analysis

Data were expressed as mean ± S.D. of the values from the number of experiments as indicated in the corresponding figures. For multiple comparisons, one-way ANOVA test by the addition of Dunnett's test, was used. Significance was accepted when $P \leq 0.01$.

3. Results

3.1. Activation of SSAO from human lung by human plasma

Human lung microsomes were preincubated with different amounts of human plasma, for 30 min at 37 °C and the reaction started by adding either methylamine (100 μM) or benzylamine (20 μM), as SSAO substrates. Fig. 1 shows the percentage increase in SSAO activity with respect to controls (taken as 100%) measured in absence of human plasma. The activation was dose-dependent in both cases, but the effect depended upon the substrate used in the assay; 30 μl of human plasma caused a 5-fold activation of the SSAO activity towards the physiological substrate methylamine, whereas the activation effect on benzylamine was only 3-fold.

This activation was not simply an additive effect of the soluble SSAO activity present in human plasma, since this activity was too low at pH 7.2 (1.253 ± 0.076 pmol/min/mg protein, $n = 6$) to account for the observed activation of the human lung SSAO, which had a specific activity of 1893.8 ± 150 pmol/min/mg protein ($n = 6$) at this pH, towards benzylamine as substrate. The activities of both the plasma and lung enzymes were higher at pH 9.0, 2.598 ± 0.14 pmol/min/mg protein ($n = 6$) and 3146.8 ± 97.5 pmol/min/mg protein ($n = 6$), respectively, but no activation was observed at that pH value.
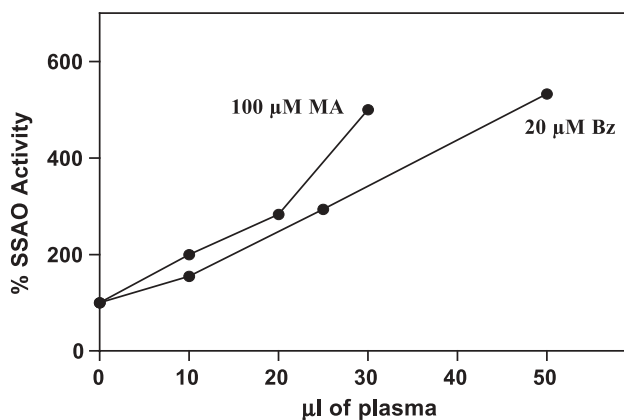
Fig. 1. Activation of human lung microsomal SSAO by human plasma. Human lung microsomes were preincubated with different amounts of filtered and lyophilised human plasma (FLHP) for 30 min, before starting the reaction by adding 100 μM methylamine or 20 μM benzylamine as substrates.
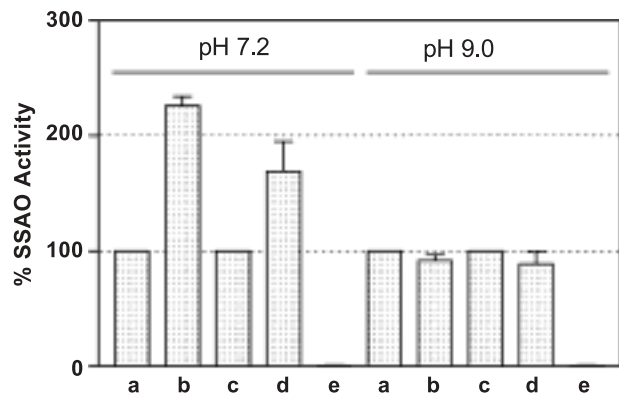
Fig. 2. Activation of human lung SSAO and human plasma SSAO by human plasma at different pH. SSAO activities were performed in 50 mM phosphate buffer, pH 7.2, or in 20 mM Tris–HCl pH 9, towards 100 μM benzylamine as substrate. In both cases, a preincubation for 30 min with 20 μl of FLHP was done to study the activation effect. The data are expressed in percentage of activation versus control SSAO activities ± S.D. of two independent experiments performed in triplicate. (a) Human plasma; (b) human plasma + FLHP; (c) human lung microsomes; (d) human lung microsomes + FLHP; (e) SSAO activity present in FLHP.

MAO-A and MAO-B are present in the human lung microsomal fraction, and because benzylamine is also a MAO-B substrate, the activation was determined after preincubation of human lung microsomes with 1 mM clorgyline, in order to fully inhibit MAO. Activation was still observed under these conditions, indicating that MAO is not involved in this effect. When the activity was determined towards 5-hydroxytryptamine, a specific MAO-A substrate, no activation by plasma was observed, confirming that this MAO isoform is not affected by this plasma component. However, when lung microsomal SSAO was inhibited with 1 mM semicarbazide, no activation effect was observed by human plasma, confirming that SSAO was the only amine oxidase present in human lung that is activated by the plasma component.

3.2. Activation of membrane-bound SSAO and plasma SSAO is pH dependent

The activating effect of the concentrated low molecular weight plasma component was determined towards human lung SSAO, and human plasma, at two different pH values (see Fig. 2). Activation was observed only at pH 7.2, but no effect was observed at pH 9 in any case. Whereas plasma SSAO was activated 2.3-fold under these conditions, the maximum activation in SSAO from human lung was only 1.7 times. The lack of agreement between these data and those reported in Fig. 1 might be explained in terms that different benzylamine concentrations have been used in each experiment. In that case, SSAO activity was assayed towards 20 μM benzylamine whereas in this experiment, 100 μM benzylamine has been used to detect SSAO in human plasma, which is very low comparing with human lung.

3.3. Activation of human lung microsomal SSAO from pathological subjects

Activation was studied in the presence of human plasma from subjects suffering different pathologies. The microsomal preparation was preincubated with 50 μl plasma sample in 50 mM potassium phosphate buffer pH 7.2, before the SSAO activity was measured towards benzylamine (20 μM) as substrate. Fig. 3 shows the increase of human lung SSAO activity after incubation with plasma from subjects with different pathologies. Plasma from patients recovering from burns gave an activating effect that was similar to that observed with healthy human plasma (about 3-fold activation), whereas the activation was about 4-fold when plasma from diabetes, heart infarcted and burns patients also suffering heart infarction were assayed.

3.4. Some characteristics of the human plasma activatory component

Samples of human plasma were dialysed overnight against 50 mM potassium phosphate buffer, pH 7.2, at 4 °C. A 50 μl sample of the dialysed plasma were assayed for their ability to activate human lung SSAO, using benzylamine (20 μM) as substrate. No activation effect was observed, suggesting that the factor responsible for the activation could be a low molecular weight component that was lost after dialysis.

The plasma component responsible of the activation was heat stable, since its ability to activate was not affected after heating the plasma at 100 °C for 30 min.
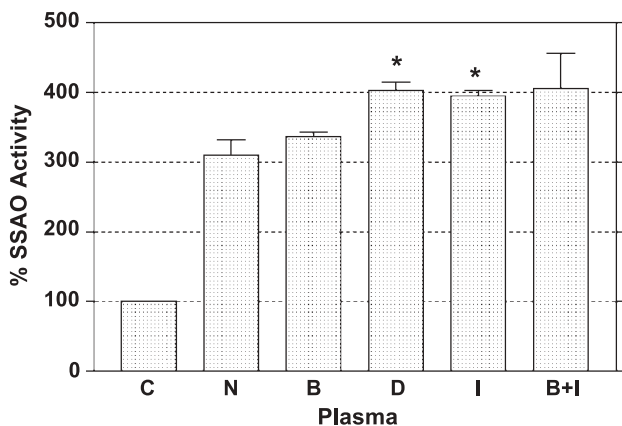
Fig. 3. Activation of human lung microsomal SSAO by healthy and pathological human plasmas. Human lung microsomes were preincubated for 30 min with 50 μl of human plasma from healthy subjects or with plasma from different pathologies, and the activation effect was determined towards benzylamine (20 μM) as substrate at pH 7.2. C = human lung SSAO activity; control (n = 3); N = C + healthy plasma (n = 5); B = C + burn plasma (n = 4); D = C + diabetic plasma (n = 6); I = C + heart infarcted plasma (n = 5); B + I = C + burns and heart infarcted plasma (n = 4). Data are mean ± S.D. (*) P ≤ 0.01 One-way ANOVA test by the addition of the Dunnett's test versus N.
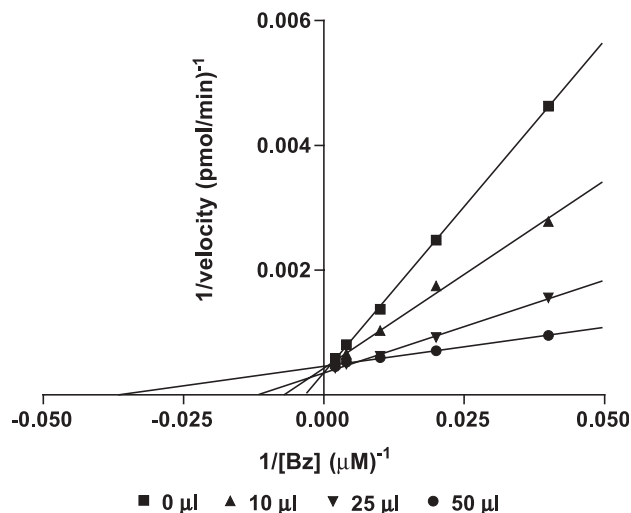
Fig. 4. Kinetic behaviour of the activating effect by human plasma on the human lung SSAO. Double-reciprocal plot of SSAO activation towards benzylamine as substrate (0.025–0.5 mM). Human lung microsomes were preincubated with different amounts of FLHP (10–50 μl) for 30 min, before starting the reaction by adding benzylamine.

In order to investigate whether the specific lipid environment of the membrane-bound enzyme SSAO from human lung microsomes might mediate the activating effect, it was solubilised with 1.2% Triton X-100 as previously described. This treatment did not affect the activation of the enzyme by the human plasma component, suggesting that interactions with the enzyme itself are involved, rather than with the lipid surrounding. Treatment of human plasma with the proteolytic enzymes, trypsin and chymotrypsin, did not reduce its activating effect. Furthermore, the activatory component was not precipitated by 1.2 M perchloric acid. Taken together, all these results suggest that the activatory component is unlikely to be a protein, although the possibility that a small peptide might be responsible for the activation cannot be excluded.
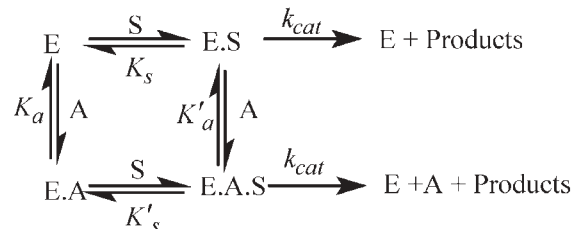
Treatment of human plasma with 35% formic acid completely abolished the activating effect, suggesting that the component(s) responsible for activation might be either a lipid or a sugar component present in human plasma. However, our latest results on the inhibitory effect on tissue-bound SSAO, by amino sugars, prompted us to discard the latest possibility (manuscript accepted for publication in BBA).

3.5. Kinetic studies

Initial-rate kinetic studies of the effects of the quantity of human plasma on the extent of activation of human lung SSAO showed the activation to be "competitive" (Fig. 4) with respect to benzylamine. Thus, the K_m values decreased as the amount of human plasma was increased, without any significant effect on the limiting velocity (V_{max}). These results indicate that the activating factor interacts with the

membrane-bound SSAO in such a way as to increase the apparent first-order rate of combination of benzylamine with the enzyme (indicated by V_{max}/K_m) without affecting the limiting rate of catalysis (indicated by V_{max}).

This nonessential, or partially competitive, activation can be represented by the scheme

$$E \underset{K_s}{\overset{S}{\rightleftarrows}} E.S \xrightarrow{k_{cat}} E + Products$$

$$K_a \Updownarrow A \qquad K'_a \Updownarrow A$$

$$E.A \underset{K'_s}{\overset{S}{\rightleftarrows}} E.A.S \xrightarrow{k_{cat}} E + A + Products$$

where the binding of the activator, A, to the enzyme results in the generation of a new species EA with a higher affinity for substrate binding ($K'_S < K_S$ and hence $K'_a < K_a$).

If one assumes that all dissociation steps are at thermodynamic equilibrium, the rate equation is quite complex [31].

$$v = \cfrac{V}{1 + \cfrac{K_S}{[S]} \cdot \left(\cfrac{1 + \cfrac{[A]}{K_a}}{1 + \cfrac{[A]}{K_a} \cdot \cfrac{K_S}{K'_S}} \right)} = \cfrac{V}{1 + \cfrac{K_S}{[S]} \cdot \left(\cfrac{1 + [A]/K_a}{1 + [A]/K'_a} \right)}$$

This predicts that the variation of the apparent slopes of the lines in Fig. 4 upon the activator concentration will be nonlinear, decreasing from an initial value of K_S/V when the activator concentration is zero to a finite value limited by K'_S/V. The apparent K_S value ($K_S^{app.}$) in the presence of activator will be:

$$K_S^{app.} = \frac{K_S(1 + [A]K_a)}{1 + [A]K_S/K_aK'_S} = \frac{K_S(1 + [A]/K_i)}{1 + [A]/K'_a}$$

Hence, if the apparent values of K_S in the presence of the activator are subtracted from the value in its absence, the values ($\Delta K_S^{app.}$) will be a rectangular hyperbolic function of the activator concentration, according to the relationship

$$^2K_S^{app.} = K_S - \frac{K_S(1 + i/K_i)}{(1 + i/K'_i)} = \frac{K_S \cdot i/K'_i - K_S \cdot i/K_i}{1 + i/K'_a}$$

Fitting the data by nonlinear regression gave a value for K'_a of 20.1 ± 4.4 µl plasma and a maximum degree of activation, with benzylamine as substrate, of 2.74 ± 0.24-fold.

3.6. Determination of the apparent molecular weight of the plasma activatory component

The activating factor was concentrated by filtering the human plasma thorough an Amicon ultrafiltration cell equipped with XM-10 membranes (110 kDa cutoff). The filtered activator was then lyophilised and taken up

in 0.1 M ammonium bicarbonate buffer pH 9, to give a further concentration of 4–5-fold. This was then gel-filtered by FPLC, through a column of Superdex-200HR equilibrated with the same buffer. Fractions of 0.4 ml were collected and each was assayed to determine the activatory effect towards the microsomal SSAO at pH 7.2. As shown in Fig. 5, the activating material was concentrated in the major, symmetrical peak eluted from the column. This corresponded to a molecular weight of about 3800 Da.

3.7. Activating effect by LPC

These results indicated that the component(s) responsible for the activation of human lung membrane-bound SSAO was of low molecular weight. Furthermore, the results obtained by TLC suggest that the activating plasma component migrated with the same R_f value as lysophoshati-
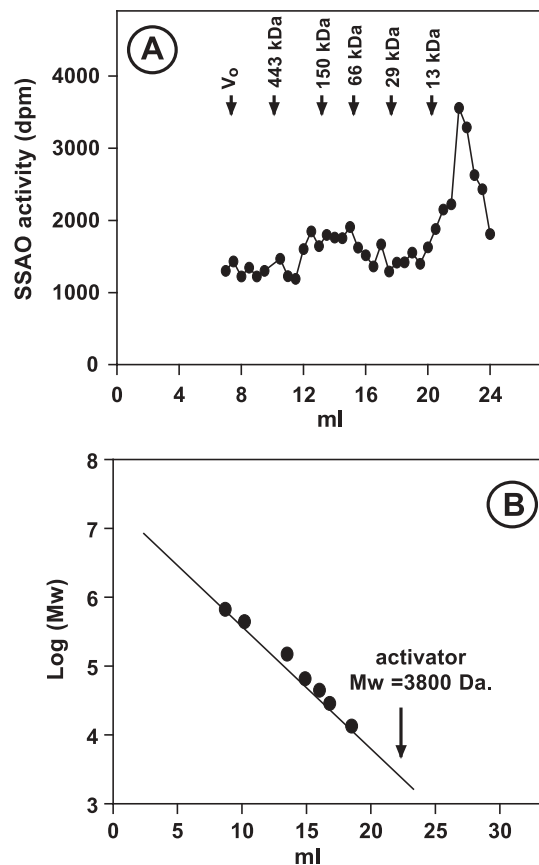

Fig. 5. Estimation of the molecular weight of the human plasma activator. (A) FLHP was chromatographed through a Superdex-200HR column connected to an FPLC system. Fifty-microliter aliquots of eluted fractions were preincubated with 25 µl human lung microsomes for 30 min before the reaction was started by adding 20 µM benzylamine. (B) Calibration of the Superdex-200HR column using the following molecular weight (kDa) markers: horse spleen apoferritin (443); yeast alcohol dehydrogenase (150); bovine serum albumin (66); erythrocyte carbonic anhydrase (29); and horse cytochrome c (13).

dylcholine (data not shown). In this context, we studied the possible activation of membrane-bound SSAO by LPC. Fig. 6 shows the effects of LPC at two different concentrations, 40 μM, below its critical micellar concentration (CMC value: 50 μM) and at 100 μM LPC, above its CMC value. Activation by LPC was higher at the lower concentration used (40 μM), resulting in a 140% increasing of the basal activity level, whereas the corresponding activation observed with 100 μM LPC was only 120%. Micelles are not formed at the lower concentration of LPC used since it is below its CMC value. Thus, the observed activation at 40 μM LPC is not a result of membrane disruption by micelles. These results are in agreement with those observed with microsomes solubilised with Triton-X-100, in which the activation effect by human plasma was maintained, indicating that this effect was not mediated by membrane components, but rather owing to a direct effect on the SSAO enzyme itself.

The total LPC concentration in human plasma from healthy subjects has been determined to be about 0.171 mmol/l and this value is increased to 0.289 mmol/l in some pathologies, such as acute asthma [32]. Thus, the LPC concentrations used in the present work is below those that may be encountered in some disease states. Thus, a greater activating effect on the tissue-bound SSAO may occur in some diseases, although the effects of LPC binding to plasma components have yet to be evaluated.

3.8. Kinetic studies on the activation by LPC

The kinetic parameters of the human lung microsomal SSAO were determined in the absence and in presence of different concentrations of LPC. The double-reciprocal plots (Fig. 7) showed the activation to be "competitive", since the K_m value was decreased without significant alteration of V_{max}. This behaviour is similar to the kinetic behaviour
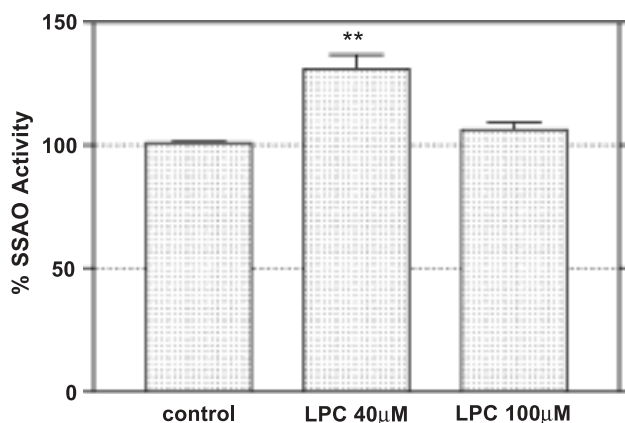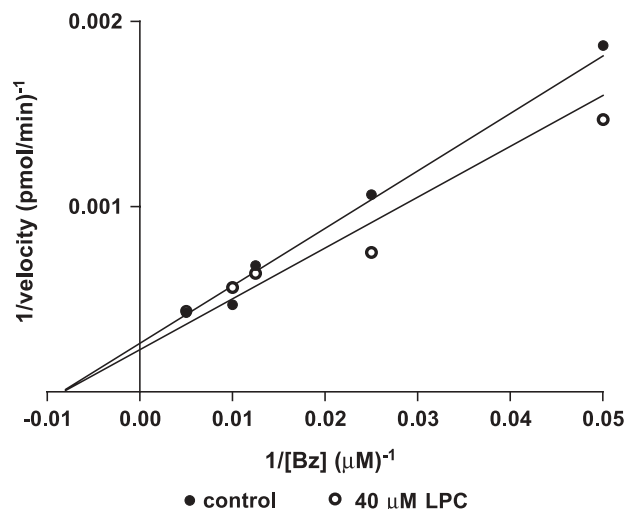
Fig. 7. Kinetic behaviour of the activation of human lung SSAO by LPC. Double-reciprocal plot of SSAO activation by LPC towards benzylamine as substrate. Human lung microsomes were preincubated in the absence and presence of 40 μM LPC for 30 min, before the assay of the activity towards benzylamine (0.025–0.5 mM) as substrate.

observed in the activation of human lung SSAO by human plasma.

4. Discussion

Semicarbazide-sensitive amine oxidase is present in almost all mammalian tissues; however, its physiological role is still far from clear. It has been reported that factors present in human plasma can modulate MAO activity in platelets. This enzyme has been shown to be inhibited by plasma from schizophrenic patients [33], whereas others have reported MAO to be activated by human plasma in a substrate-selective way [34] and have suggested that the activator is a phospholipid-like molecule. Recently, Whalund et al. [35] reported that human platelet MAO is activated by a high molecular weight fraction (M_r > 50.000) present in human plasma. Buffoni et al. [36] described the presence of a high M_r (200.000) inhibitor of SSAO in human plasma and suggested that it might account for the low activity of this enzyme under nonpathological conditions.

Some authors have described that inhibition of MAO can modulate the behaviour of SSAO, by an as yet unknown mechanism [37]. However, this is the first report on the activation of human tissue-bound SSAO by a human plasma component, without effect on either MAO-A or MAO-B. Concentrated samples of this component, which appears to have a low molecular weight (M_r 3800), also activated human plasma SSAO. The extent of activation appeared to be different when plasma samples from subjects with different disease states were assayed, which may reflect variations in the concentration of the activatory material under such conditions.

Fig. 6. Activator effect of LPC on microsomal human lung SSAO. Human lung microsomes were preincubated with 40 or 100 μM LPC before adding 20 μM benzylamine as substrate. Data are mean ± S.E. (**) $P < 0.01$ One-way ANOVA with Dunnett's post test versus Control.

Since the properties of this activatory material were consistent with the involvement of a phospholipid, we investigated the effects of LPC on the activity of the enzyme. LPC is an amphiphilic phospholipid that is derived from phosphatidylcholine, the major component in plasma, that is formed during the oxidation of low-density lipoprotein (LDL) [38,39]. LPC is secreted directly by the liver [40] and is present in large amounts in plasma, about (5–20%) of total phospholipids, depending on the mammalian species [41]. Furthermore, it can be accumulated in pathological conditions such as atherosclerosis and ischemia [42]. It has been reported that LPC regulates a variety of cellular functions, such as the activation of protein kinase C (PKC), the increase of cyclic AMP through G-protein pathways [43] and the increase of intracellular Ca^{2+} levels. It has been also reported that LPC induces NFkB-binding activity [44] and stimulates growth factor release, from vascular smooth muscle cells [45]. However, LPC can induce injury in a variety of cell types by a process that appears to involve oxidative stress [46]. In this context, the hydrogen peroxide formed in the SSAO-catalysed reaction has been suggested to contribute to oxidative tissue damage under pathological conditions (see Refs. [3,24]) and thus the activation of membrane-bound SSAO by LPC might exacerbate LPC-induced tissue damage.

LPC does resemble the endogenous plasma SSAO activator, in behaving as a partial "competitive activator" of the enzyme. However, the extent of activation by this phospholipid at concentrations below its critical-micellar concentration was lower than that observed with the plasma samples. One possible explanation could be that the LPC concentration used in these experiments is below that present in healthy controls (0.171 mmol/l) and in some disease states, such as acute asthma (0.289 mmol/l) [47]. However, other components present in plasma may also contribute to the activatory effect. In this context, it has been recently described that SSAO appears to be localised with the scavenger lipoprotein receptor, CD36, in the caveolae of adipocytes and perhaps some other cell types [48]. Further experimental work must be done in order to identify the SSAO activatory molecules present in human plasma, and how their levels are altered in different disease states, that are known to result in changes of SSAO activity. Further studies of this activating effect should be relevant to our understanding of the significance of changes in SSAO activity, in response to different pathological conditions.

Acknowledgements

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ANNEX II.3. TREBALL PUBLICAT:

"Human plasma semicarbazide sensitive amine oxidase (SSAO),
beta-amyloid protein and aging "

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Human plasma semicarbazide sensitive amine oxidase (SSAO), β-amyloid protein and aging

Maria del Mar Hernandez [a], Miriam Esteban [b], Paul Szabo [b],
Mercè Boada [c,d], Mercedes Unzeta [a,*]

[a] *Departament de Bioquímica i Biología Molecular, Institut de Neurociencies, Facultat de Medicina,
Universitat Autónoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*
[b] *Weill Medical College of Cornell University, NY, USA*
[c] *Servei de Neurologia, Hospital G.U. Vall d'Hebron, Barcelona, Spain*
[d] *Fundació ACE, Institut Català de Neurociències Aplicades, Barcelona, Spain*

Abstract

Semicarbazide sensitive amine oxidase (SSAO) metabolizes oxidative deamination of primary aromatic and aliphatic amines. The final products of its catalysis, ammonia, hydrogen peroxide (H_2O_2) and the corresponding aldehyde, may contribute to diseases involving vascular degeneration. SSAO is selectively expressed in blood vessels in the brain, but is also present in blood plasma. We have previously reported that membrane-bound SSAO is overexpressed in the cerebrovascular tissue of Alzheimer's disease (AD) patients. The aim of the present work is to study whether the circulating SSAO is also altered in this neurodegenerative disease. SSAO activity was determined in plasma of control cases ($n = 23$) and patients suffering sporadic Alzheimer dementia, distributed according to the Global Deterioration Scale (GDS): mild ($n = 33$), moderate ($n = 14$), moderate–severe ($n = 15$) and severe dementia ($n = 19$). Results show a clear increase of plasma SSAO activity ($p < 0.001$) in moderate–severe and severe AD patients, with patient age being an independent correlative factor. However, plasma SSAO activity was not altered in AD patients with mild or moderate dementia compared to controls. β-Amyloid (Aβ) (40–42) immunoreactivity in plasma samples was also determined, and no correlation was observed between Aβ 40–42 levels and the severity of the dementia or the plasma SSAO activity. Our results suggest that an increase in circulating SSAO activity could contribute to oxidative stress and vascular damage in advanced Alzheimer's disease.
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Keywords: Semicarbazide sensitive amine oxidase; Alzheimer disease; β-Amyloid; Global deterioration scale; Aging; Oxidative stress; Hydrogen peroxide

Semicarbazide sensitive amine oxidase (SSAO) [E.C. 1.4.3.6.] constitutes a large family of enzymes including beef and sheep plasma spermine oxidase, plasma amine oxidase, lysil oxidase, diamine oxidase and tissue-bound SSAO enzyme. All of these enzymes are inhibited by semicarbazide, a property that distinguishes SSAO from monoamine oxidase (MAO), which is selectively inhibited by acetylenic inhibitors such as L-deprenyl. Although SSAO and MAO show some overlap with respect to substrate specificity, SSAO metabolizes oxidative deamination of primary aromatic and aliphatic amines. Methylamine, resulting from the

metabolism of adrenaline by MAO [26], is metabolized exclusively by SSAO. The catalytic action of SSAO uses oxygen and generates ammonia, hydrogen peroxide (H_2O_2) and the corresponding aldehyde. Aminoacetone and methylamine are considered the physiological SSAO substrates. Their catalysis produces the toxic end products formaldehyde and methylglioxal, respectively [18].

SSAO is associated with cell membranes in all mammalian tissues studied and it is also present in blood plasma [14]. The membrane-bound SSAO shows high activity in endothelial and smooth muscle cells of blood vessels [7,13]. Some authors have proposed that soluble SSAO is derived from the membrane-bound enzyme. Recently, it has been reported that in adipocytes soluble SSAO is shed from the membrane by a

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Table 1
Demographic characteristics, SSAO activity (pmol/min mg) and β-amyloid concentration (pg/ml) of controls and AD patients studied

	n^a	Sex (m/f)[a]	Age ± S.E.M.[a]	SSAO activity ± S.E.M.[a]	N^b	β-Amyloid ± S.E.M.[b]
Control	23	13/10	73.5 ± 1.3	3.368 ± 0.217	8	478 ± 63.08
Mild-D	33	11/22	75.2 ± 0.9	3.269 ± 0.141	7	642.79 ± 100.24
Moderate	14	3/11	773 ± 1.5	3.755 ± 0.234	4	809.28 ± 209.23
Moderate–severe	15	6/9	79.5 ± 1.6	4.717 ± 0.235	11	621.28 ± 123.10
Severe	19	2/17	80.4 ± 1.5	4.553 ± 0.296	7	884.61 ± 241.57

[a] $N_t = 104$.
[b] $N_t = 37$.

metalloprotease activity [1]. The physiological role of SSAO is still far from clear, and it has been described as an enzyme with multifunctional behavior depending on the tissue where it is expressed. In adipocytes, SSAO activity stimulates glucose transport, mimicking the insulin effect through the H_2O_2 generated during the catalytic process [9]. On the other hand, SSAO, also known as vascular adhesion protein-1 (VAP-1), is involved in lymphocytes trafficking [22] and its expression in endothelial cells is induced during an inflammatory response.

SSAO activity is altered in several pathological conditions. Plasma SSAO is increased in patients suffering from diabetes type I and II [3] and is correlated with the severity of diabetic retinopathy [27]. Elevated plasma SSAO activity in plasma is found in patients afflicted by congestive heart failure [4], in non diabetic morbidity obese patients [24] and has been implicated in atherosclerosis [25]. Serum SSAO activity is also altered in inflammatory liver diseases [12], probably enhancing the binding of lymphocytes to the endothelial cells.

The final products of the SSAO catalysis, H_2O_2 and formaldehyde, are toxic and may contribute to diseases involving vascular degeneration. The pathophysiolgic features of Alzheimer's disease (AD) include more than neurofibrillary tangles, amyloid plaques and cerebrovascular amyloid deposition. Very often these patients exhibit significant microvascular degeneration affecting smooth muscle cells and endothelial cells [23]. Ageing is the primary risk factor for AD [6]. In addition, both β-amyloid (Aβ) 40 and 42 peptides that comprise amyloid plaques have been detected in human blood [21].

We have previously reported that membrane-bound SSAO is overexpressed in the cerebrovascular tissue of Alzheimer's disease and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) patients, with the subsequent perturbation of the brain vasculature [10]. However, it was not known whether the circulating SSAO is altered in such neurodegenerative diseases.

The present study was undertaken to determine whether the increase on SSAO activity associated with leptomeningeal vessels from AD patients correlates with an increase of circulating SSAO activity. The relationship between plasma SSAO activity, plasma Aβ (40–42 peptides) level and aging is assessed.

Controls and AD patients ($N = 104$) included 35 men and 69 women, who did not have any chronic metabolic disease or congestive heart failure. Their ages ranged from 65 to 94 years, and the mean age corresponding to each severity condition is shown in Table 1. All patients were suffering sporadic Alzheimer dementia, according to NINCDS-ADRA criteria [16]. They were distributed in five groups based on the Global Deterioration Scale (GDS) [19]: age-matched control cases ($n = 23$), who were free of neurological disease, mild ($n = 33$, GDS = 3–4), moderate ($n = 14$, GDS = 5), moderate–severe ($n = 15$, GDS = 6) and severe dementia ($n = 19$, GDS = 7). Blood samples were collected by venipuncture, by a trained phlebotomist, in sterile plunger tubes containing sodium citrate solution (0.129 mol/l) and centrifuged ($2500 \times g$, 20 min) to yield plasma samples that were aliquoted and frozen at $-80\,^{\circ}C$ until analysis.

SSAO activity was determined radiochemically at $37\,^{\circ}C$ as previously described [11] using 100 μM [14C]-benzylamine (3 mCi/mmol, Amersham, U.K.) as substrate. Plasma samples were preincubated 30 min at $37\,^{\circ}C$ with 1 μM L-deprenyl to inhibit possible platelet MAO B contamination. The reaction was carried out at $37\,^{\circ}C$ 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. Protein concentration was determined by the Bradford method, and the resulting SSAO specific activity is expressed as pmol/min mg protein.

Aβ (40–42) immunoreactivity in plasma samples was determined using a modification of a published procedure [8]. Monoclonal anti-Aβ antibody specific for the central region of the β-amyloid peptide was coated onto 96 well plates by the addition of 100 μl of the anti-Aβ antibody (15 μg/ml) in 1.7 mM NaH_2PO_4, 8 mM Na_2HPO_4 (pH 7.4) and was incubated overnight at $4\,^{\circ}C$. The wells were then blocked using 0.25% casein in PBS for 80 min at $25\,^{\circ}C$. After washing, 100 μl of undiluted plasma samples or Aβ 42 standards (0.04–1.25 ng/ml) was added. The plates were sealed and incubated overnight at $4\,^{\circ}C$. After washing, plates were first incubated with 1:1000 dilution of biotinylated N-terminal-specific antibody 6E10 (Signet) for 1 h 45 min at $25\,^{\circ}C$. This antibody recognizes the amino terminus of both Aβ 40 and Aβ 42. The plates are then incubated with Avidin-HRP (Bio-Rad) (1:6000) for 1 h 45 min at $25\,^{\circ}C$. Substrate TMB, 100 μl (Sigma), was added and the plates incubated for 8–15 min at $25\,^{\circ}C$. The reaction was stopped with 100 μl of 3N H_2SO_4 and the absorbance was read at 450 nm on an automated plate

reader. The level of Aβ was determined by comparison to the standard curve, which was linear over the indicated range.

Subject details, plasma SSAO activity and Aβ (40–42) levels are shown in Table 1. Data are expressed as mean ± S.E.M. and were analyzed using One-Way ANOVA and Newman–Keuls multiple comparison post test. Plasma SSAO specific activity from control samples showed no significant differences compared to mild or moderate patients. We observed a clear increase of SSAO activity in plasma from moderate–severe and severe AD patients ($p < 0.001$) compared to the control or mild cases. Plasma SSAO activity of the moderate group was also statistically different compared to moderate–severe and severe AD patients ($p < 0.05$) (see Fig. 1). When patient's age was compared to the severity of the dementia, the results showed that the average ages of the controls, mild and moderate groups were identical. However, as expected, AD patients with moderate–severe ($p < 0.05$) and severe ($p < 0.01$) were older than controls (Fig. 2A). Taken together, these results show that AD severity correlates with an increase in SSAO activity and with age as well. We then determined whether age was associated directly with the increase on SSAO activity observed. No correlation was found when the age of control group was compared with the SSAO activity (Fig. 2B), in agreement with previous reports [20]. These results allow us to conclude that human plasma SSAO increases in the severest cases of sporadic AD dementia, with patient age being an independent correlative factor. Gender distribution was also an independent factor in this study. Plasma SSAO activity of men and women did not change in any separate group analyzed by t-test ($p < 0.05$) (data not shown).

No correlation was observed between Aβ 40–42 plasma levels and plasma SSAO activity (Fig. 3A). When Aβ 40–42 content of plasma samples was compared with respect to the severity dementia scale, there was a tendency to increase with severity. However, this correlation did not reach statistical significance (see Fig. 3B). In our study, the method used to measure plasma Aβ levels detects both Aβ peptides. It has
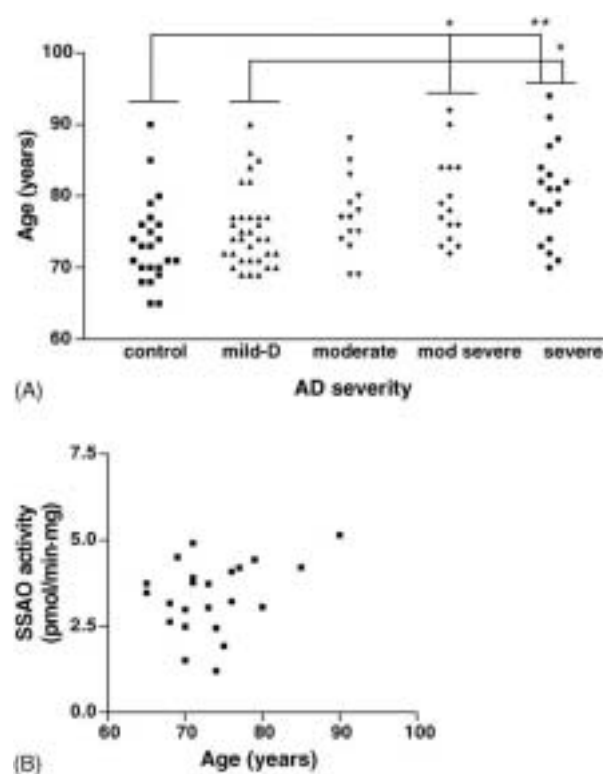

Fig. 2. (A) Scatter plot of age distribution in controls ($n = 23$) and groups with different level of AD severity classified by GDS criteria; mild-D ($n = 33$), moderate ($n = 14$), moderate–severe ($n = 15$) and severe ($n = 19$). Statistically significant differences are shown as [**]$p < 0.01$, [*]$p < 0.05$ by a one-way ANOVA test and the addition of Newman–Keuls multiple comparison test. (B) No significant correlation was observed between age of controls and SSAO activity ($n = 23$) (Pearson $r = 0.326$, p-value $= 0.130$).

been reported that Aβ 40 is the predominant peptide in plasma and its concentration does not change in plasma from patients with sporadic AD [15].

Oxidative stress contributes to AD pathophysiology [5] and has been reported to be involved in β-amyloid deposition in neuronal [2] and vascular cells [17] in vitro. In this context, the increase in circulating SSAO could contribute to oxidative stress underlying the neurodegenerative diseases via its intrinsic enzymatic activity. We had previously reported that overexpression of membrane-bound SSAO occurs in cerebrovascular tissue from AD patients [10]. Thus, the increase in circulating SSAO activity could result from shedding of membrane-bound SSAO, especially when the enzyme is overexpressed in AD. Since SSAO is an adhesion protein whose expression is induced as part of an inflammatory response, the increased plasma SSAO activity observed in the most advanced cases could be the consequence of the vascular degeneration and the inflammation process present in severe AD. Overexpression of membrane-bound SSAO by vascular cells, and its release into plasma, could amplify oxidative stress and contribute to vascular damage in AD. However, further studies are required to elucidate the molecular mechanism that controls the shedding of the membrane-bound enzyme and the possible pathological agents involved.
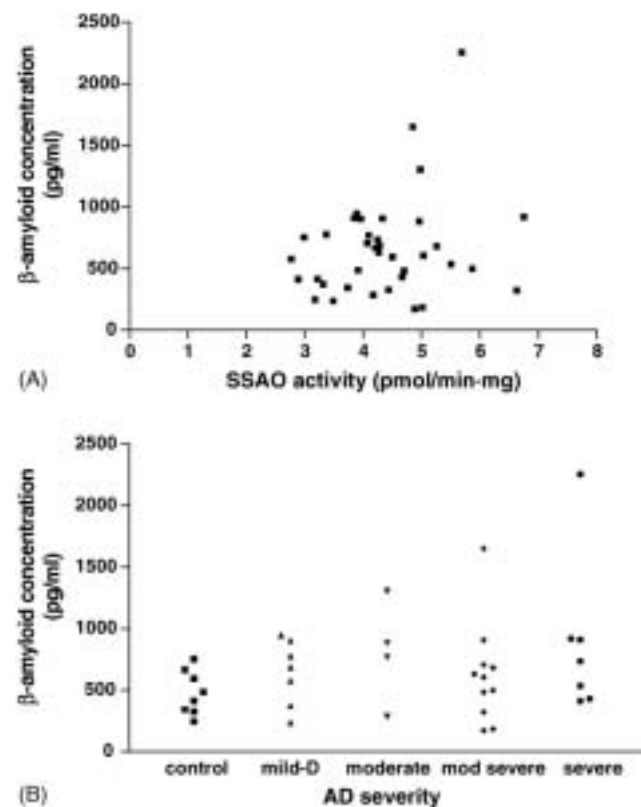

Fig. 1. Scatter plot of SSAO activity in controls ($n = 23$) and groups with different level of AD severity classified by GDS criteria; mild-D ($n = 33$), moderate ($n = 14$), moderate–severe ($n = 15$) and severe ($n = 19$). Statistically significant differences are shown as [***]$p < 0.001$, [*]$p < 0.05$ by a one-way ANOVA test and the addition of Newman–Keuls multiple comparison test.

Fig. 3. (A) No significant correlation is observed between β-amyloid concentration and SSAO activity ($n = 37$) (Pearson $r = 0.246$, p-value $= 0.428$). (B) Scatter plot of β-amyloid concentration in controls ($n = 23$) and groups with different level of AD severity classified by GDS criteria; mild-D ($n = 33$), moderate ($n = 14$), moderate–severe ($n = 15$) and severe ($n = 19$). No statistically significant differences are observed between groups analyzed by one-way ANOVA test and the addition of Newman–Keuls multiple comparison test.

Herein, we report for the first time that plasma SSAO activity increases in advanced AD-dementia. Since plasma SSAO activity is not altered in AD patients with mild or moderate dementia compared to controls, SSAO activity does not seem to be a good marker for early diagnosis of this neurodegenerative disease. However, our results suggest that an increase of circulating SSAO activity could contribute, at molecular level, to vascular damage in Alzheimer's disease through its catalytic action.

Acknowledgements

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ANNEX II. 4. TREBALL SOTMÈS A REVISIÓ EDITORIAL:

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

Hernandez M, Sole M, Boada M, Unzeta M

Manuscrit sotmès a *Biochimica et Biophysica Acta*, Juliol 2005.

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

Mar Hernandez [*], Montse Solé [*], Mercè Boada M.D [†‡] and Mercedes Unzeta [*]

[*] Departament de Bioquímica i Biología Molecular, Facultat de Medicina,

Universitat Autònoma de Barcelona, Bellaterra, Barcelona, Spain.

[†] Servei de Neurologia, Hospital G.U. Vall d'Hebron. Barcelona, Spain.

[‡] Fundació ACE. Institut Català de Neurociències Aplicades. Barcelona, Spain.

Corresponding author:

Name: Mercedes Unzeta

Mail address: Departament de Bioquímica i Biología Molecular, Institut de Neurociencies, Facultat de Medicina, Universitat Autónoma de Barcelona, (08193) Bellaterra, Barcelona, Spain.

Phone N: 34 93 5811523

Fax N: 34 93 5811573

Email address: mercedes.unzeta@uab.es

Summary

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 present in blood plasma. SSAO activity in plasma is increased in some diseases associated to vascular complications and it has been speculated that could be involved in tissue damage through its own catalytic products. The aim of the present work was to elucidate whether the soluble form of SSAO could have toxic effects on cultured smooth muscle cells by the oxidation of its substrate, methylamine. Cell incubation with soluble SSAO, contained in bovine serum, plus methylamine resulted to be dramatically toxic to rat aorta A7r5 and human aortic smooth muscle cells measured by MTT reduction. This effect was completely reverted by specific SSAO inhibitors, indicating that the toxicity was mediated by the catalytic end products generated. Moreover, SSAO-mediated deamination of methylamine induced apoptosis on 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 presented suggest that in pathological conditions, when plasma SSAO activity is increased, its catalytic activity could contribute to smooth muscle apoptotic cell death, playing an important role in vascular tissue damage.

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 of 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 SSAO substrates [3], and their oxidation generates the toxic end products, formaldehyde and methylglioxal, respectively [4]. SSAO is associated with cell membranes and it is also present in blood plasma [2, 5]. The 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 as 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]. On the other hand, 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 under inflammation.

Some authors have proposed that soluble SSAO is derived from the membrane-bound enzyme [11, 12]. Recently, it has been reported that in adipocytes, soluble SSAO is shed from the membrane by a metalloprotease activity [11]. Furthermore, transgenic mouse models expressing human VAP-1 in endothelial cells showed that VAP-1 from vascular cells can 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], patients afflicted by congestive heart failure [14], non diabetic morbidity obese patients [15], and it has also been implicated in atherosclerosis [16, 17]. The metabolism end 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 very reactive aliphatic aldehyde and it is considered a powerful inflammatory agent [20]. The combined effect of these products as vascular risk factors could be relevant in diseases related to vascular degeneration. 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]. It will be worth to elucidate whether this SSAO activity increase could have a feed-back contribution to the vascular damage in this pathology.

Since MA and plasma SSAO levels are increased in some pathologies [23, 25], the aim of this work was to elucidate whether this soluble SSAO, through its catalytic action, is able to induce cell death in cultured smooth muscle cells (SMC). Because of A7r5 cells and HASMC (Human Aortic Smooth Muscle Cells) did not present SSAO activity or expression, a bovine serum (BS) with high SSAO activity was used as the soluble enzyme source. Different amines, MA, tyramine and benzylamine, were used as SSAO substrates.

Methods

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, 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- -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).

SSAO activity. SSAO activity was determined radiochemically at 37°C as previously described [26] using 100 μM [^{14}C]-benzylamine (3 mCi/mmol, Amersham, U.K.) as substrate. Samples were preincubated 30 min at 37ºC with 1 μM L-deprenyl to inhibit possible platelet MAO B contamination. The reaction was carried out at 37ºC 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).

Cell culture. Human Aortic Smooth Muscle Cells (HASMC) from normal adult thoracic aortas were obtained from control donor heart transplants (Hospital de la Vall d'Hebron, Barcelona). 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 α-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 two 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 containing 5, 10 or 15 % (v/v) of Bovine Serum (BS), which corresponds to $3 \cdot 10^{-4}$, $6 \cdot 10^{-4}$ and $9 \cdot 10^{-4}$ U/ml of SSAO activity respectively. SSAO inhibitors, semicarbazide or MDL72974A, were coincubated with MA in DMEM supplemented with BS.

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 37ºC the medium was replaced by dimethyl sulfoxide. The amount of formazan blue formed after MTT reduction was quantified spectroscopically at 560 nm [28].

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 with 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 with 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 with PBS and incubated with Hoechst 33258 (1µg/ml) for 10 min at room temperature in the dark.

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 lysis buffer (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), 1mM EDTA, 1mM dithiothreitol, complete protease inhibitor, and 250 mM sucrose, after washing once with 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 4ºC, supernatants were centrifuged at 20000 g for 40 min at 4ºC. The resulting supernatants were saved as cytosolic extracts. Protein determinations were made using the Bradford method. Samples were size fractionated by SDS-PAGE electrophoresis 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).

Statistics. Results are given as means ± SEM. 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.

Results

Cytotoxicity of MA oxidation by SSAO on A7r5 cells

Although the smooth muscle cell line A7r5 did not present MAO-B or SSAO activity assayed towards benzylamine as substrate, it showed MAO-A activity (100 pmol/min·mg protein) determined radiochemically by serotonin oxidation. These conditions were interesting in order to study the effect of the soluble SSAO catalytic action on vascular cells. For this purpose, a bovine serum (BS), containing a high specific SSAO activity (60 pmol/min·mg protein) was used as the enzyme source. We first studied the methylamine (MA) oxidation by soluble SSAO on A7r5 cell viability, due to it is a common substrate of bovine and human plasma SSAO [29].

A7r5 cells were treated with increasing soluble SSAO and MA concentration, and cell viability was measured by MTT reduction. Soluble SSAO ($3 \cdot 10^{-4}$, $6 \cdot 10^{-4}$ and $9 \cdot 10^{-4}$ U/ml) and 1 mM MA did not show any effect on cell viability by themselves. However, 1 mM MA plus $9 \cdot 10^{-4}$ U/ml of soluble SSAO induced a 70% decrease of total MTT reduction after 24 hours treatment (Fig. 1A). This toxic effect was dose-response, obtaining a significant decrease in MTT reduction after 0.1 mM MA plus $6 \cdot 10^{-4}$ U/ml or $9 \cdot 10^{-4}$ U/ml of soluble SSAO (20% and 25% respectively on MTT reduction percentages). On the other hand, the cell viability decrease was time dependent at the range studied (6, 12 and 24 hours) (data not shown). In order to confirm that the cell viability loss observed 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, concentrations that did not show any effect on cell viability by themselves. The results obtained showed how SSAO inhibitors completely reverted the cell viability loss obtained with 1 mM MA plus $9 \cdot 10^{-4}$ U/ml of soluble SSAO (Fig. 1B).

Effect of Benzylamine and Tyramine oxidation by soluble SSAO on A7r5 cells

Next, we tested other SSAO substrates using the same experimental conditions (Fig. 1C). Tyramine, considered as another physiologic SSAO substrate, rendered a similar behavior than MA. Incubation of A7r5 cells with 1 mM tyramine and $9 \cdot 10^{-4}$ U/ml of soluble SSAO resulted in a 90% decrease of total MTT reduction after 24 hours, and this effect was almost totally reverted by the action of SSAO inhibitors. Because of tyramine is an aromatic amine which is also oxidized by both MAO isoforms, it cannot be excluded that tyramine oxidation by MAO could be contributing to the cell death observed. In contrast, the non-physiological substrate benzylamine (1 mM), showed a slight toxic effect by itself, but no significant changes were observed in the presence of SSAO. Considering that benzylamine is a better substrate of bovine serum SSAO *in vitro* [31], we assume that benzylamine oxidation produce higher H_2O_2 levels than MA deamination. In this context, the non cytotoxic effect observed after benzylamine metabolism suggests that the aldehydes produced by SSAO, rather than H_2O_2 or ammonia, are the main responsible of the cell viability reduction.

MA oxidation by SSAO induces apoptosis on A7r5 cells

We then evaluated whether apoptosis would be involved in such toxic effect. Figure 2 shows the double staining with Hoechst 33258 and anti-cleaved Caspase-3 antibody performed in untreated cells and cells treated with the selected conditions, 1 mM MA and $9 \cdot 10^{-4}$ U/ml of soluble SSAO, for 24 hours. 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 $9 \cdot 10^{-4}$ U/ml of soluble SSAO separately did not show stained positive cells (Fig. 2D & E). However, the co-incubation with 1 mM MA and $9 \cdot 10^{-4}$ U/ml of soluble SSAO resulted in numerous cells displaying a strong cytoplasmic red staining for

cleaved Caspase-3 (Fig. 2B). The detailed micrograph shows that positive cleaved Caspase-3 stained cells overlap with condensed nuclei stained with Hoechst 33258 (see Fig. 2B inset). Although the SSAO inhibitor, 1 mM semicarbazide, did not show any toxic effect by itself (Fig. 2F), its presence diminished significantly the number of Caspase-3 positive cells observed by MA oxidation, indicating that the amine oxidase activity mediated the apoptotic process (Fig. 2C). The elevate percentage of active Caspase-3 positive cells (Fig. 2G) indicated that apoptosis was the main responsible of 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, as Caspase-3, involved in maintaining DNA stability and repair. Western blot analysis revealed PARP cleavage only in cells treated with 1 mM MA plus $9 \cdot 10^{-4}$ U/ml of soluble SSAO for 24 hours (Fig. 3A), confirming the Caspase-3 activation observed. On the other hand, cytosolic fractions of cells treated with those selected conditions presented a time dependent increase in Cytochrome c release from mitochondria to cytosol (Fig. 3B). The Cytochrome c release appeared after 12 hours 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 hours.

Effect of SSAO catalytic products, H_2O_2, formaldehyde and ammonia, on A7r5 cells

In order to study the mechanism involved in the toxicity observed, we next analyzed the effect on cell viability when cells were treated with the final products generated by the MA oxidized by SSAO; H_2O_2, formaldehyde or ammonia by themselves. Considering the oxidative deamination stoichiometry (1:1) of primary amines [29] 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. Cells incubated with H_2O_2 for 24 hours presented a slight decrease in cell viability, reaching a 40% decrease of total

MTT reduction at 1 mM H_2O_2 (Fig. 4A). When formaldehyde was assayed at the same concentration range, the percentage of MTT reduction showed a drastically decrease at much lower concentrations, compared to H_2O_2 treatment (Fig. 4B). Formaldehyde 0.5 mM was enough to induce almost 100% of cell viability loss. When cells were incubated in the presence of ammonia, surprisingly no toxic effect was observed at the concentrations assayed (Fig. 4C), suggesting that this metabolic product does not contribute to vascular damage.

Although it has been widely reported that H_2O_2 induces apoptosis in many cell types, including smooth muscle cells [30], it was not known if formaldehyde was an apoptotic inducer in this cell type. Results showed that 0.5 mM formaldehyde treatment for 24 hours induced a 75% cleaved Caspase-3 positive cells (Fig. 5C). This percentage was comparable to the result obtained from cell viability assay, showing how formaldehyde was a powerful apoptosis inducer in these cells. Moreover, Hoechst 33258 staining of positive cells also presented the characteristic condensed morphology of apoptotic nuclei. In contrast, when cells were treated with H_2O_2 using the same experimental conditions, only a 10% of positive cleaved Caspase-3 cells was observed (Fig. 5B). On the other hand, formaldehyde treatment for 24 hours showed a clear PARP cleavage at all concentrations studied (Fig. 6A). 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 hours) (Fig. 6B).

Cytotoxicity of MA oxidation by SSAO on HASMC

In order to exclude that the toxic effect induced by MA oxidation was a consequence of the immortalized cell line used, cell viability experiments were performed on primary cultures of smooth muscle cells from human aorta. When cells were incubated in the

presence of 1 mM MA plus $9 \cdot 10^{-4}$ U/ml of soluble SSAO, only a 35% decrease of total MTT reduction was observed at 24 hours (Fig. 7), in comparison with the 75% observed on A7r5 cells. These results pointed out that primary culture of human cells is more resistant to the toxicity mediated by SSAO metabolic products. SSAO inhibitors, semicarbazide 1 mM and MDL72974A 10 µM, did not show any toxic effect by themselves but were enough to recover the cell viability loss induced by SSAO catalysis. Since these cells do not present membrane-bound SSAO activity, MA oxidized by soluble SSAO was again the only responsible of the cell damage.

Discussion

Human plasma SSAO levels are increased in inflammation, diabetes [13] and AD [22]. The increase of its physiologic substrate, circulating MA, it has also been reported in some pathological conditions [23, 25]. Although the oxidation of the synthetic aliphatic amine allylamine is widely described as cytotoxic agent on smooth muscle cells [31, 32], these cells seem to be resistant to MA toxicity [33]. However, MA in the presence of soluble SSAO resulted toxic to endothelial cells [34], suggesting that SMC are more resistant to this amine than endothelial cells or circulating SSAO had more toxic effect than the SSAO expressed in the SMC in culture. In this context, the aim of the present work has been to study the role of MA deamination by circulating SSAO, as a potential risk factor in vascular damage when its levels are increased in pathological conditions. We were interested to elucidate the molecular mechanism involved in such a cell death observed by MA oxidation and their catalytic products on SMC mediated by soluble SSAO.

Although SSAO is constitutively expressed in vascular smooth muscle [2], A7r5 and HASMC do not exhibit any detectable 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 an in vivo and in vitro plasticity, which allows them to change its phenotype in response to environmental changes [35]. Moreover, the loss of SSAO/VAP-1 expression in other smooth muscle and endothelial cell types has been previously observed by other authors [34, 36].

In this study, we show that incubation of soluble SSAO plus 1 mM MA induced a cytotoxic effect on A7r5 and HASMC cells. Because of our experimental model is based on cultured cells, we study an acute MA treatment during 24 hours, using an amine concentration higher than those previously reported in human plasma [23, 24]. Although this MA concentration is not at the physiologic range, it has been widely used in many cell culture studies [32, 33, 34, 36]. 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, resulted toxic to the cells, while ammonia did not show any effect in cell viability. Formaldehyde showed to be much more toxic than H_2O_2 assayed at the same experimental conditions, as other authors had previously observed on endothelial cells [18]. However, it must be taken into account that free radicals can be generated from formaldehyde and H_2O_2, contributing synergically to oxidative stress [37] and vascular damage. Only the oxidation of physiological substrates, as MA and tyramine, resulted toxic to A7r5 cells. In contrast, the oxidation of the non-physiological substrate, benzylamine, by SSAO did not show any effect. Because of all these amines tested are good SSAO substrates, the differences in the cytotoxicity observed could be determined by the specific reactivity and the chemical structure of the aldehyde generated.

Apoptosis of vascular SMC occurs during normal blood vessel development and maturation, but it has also been reported to be involved in many vascular diseases [38].

Biogenic amines appear to be important for apoptosis triggering through the catalytic action of the mitochondrial monoamine oxidase [39]. However, up to now, no direct evidence had shown that circulating SSAO is able to induce 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 must be carried out to elucidate whether different mechanisms or other apoptotic pathways could be also involved.

In pathological conditions, such as neurodegenerative diseases, stroke, traumatic brain injury, atherosclerosis and hypertension, oxidative stress is an underlying factor that contributes to the apoptotic process. Among the diverse factors capable to induce oxidative stress, 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 [40]. In this study, we show that formaldehyde induces higher apoptotic effect than H_2O_2. Formaldehyde is an extremely reactive aldehyde capable to generate covalent interactions with macromolecular constituents in biological samples [41], 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 the Cytochrome c release to the cytosol, caspase activation and cell death. The apoptotic rate obtained with formaldehyde is in agreement with some reported results where the formation of glucose degradation products, as methylglyoxal and formaldehyde, induced apoptosis in mesothelial cells [42].

Transgenic mice models, that express full-length human VAP-1/SSAO in smooth muscle cells [43], endothelial cells or adipose tissue [12], show an increase in hVAP-

1/SSAO in serum. This increase is dramatically higher when those transgenic animals are treated with different 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 [44]. Other studies in vivo corroborate the toxic consequences of MA, because chronic MA administration to SSAO/VAP-1 transgenic mice produced vascular complications related to diabetes pathology [45]. Moreover, there are evidences reporting that increased SSAO-mediated deamination of MA may contribute to protein deposition, formation of plaques and inflammation [46]. In this concern, transgenic mice overexpressing VAP-1/SSAO in SMC present pathological changes in the elastic fibers of aorta, suggesting also the contribution of tissue-bound SSAO in the development of vascular damage [43].

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

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Figure Legends

Figure 1. Cytotoxicity of SSAO catalytic action on A7r5 cells expressed as MTT reduction percentages. Cells were incubated 24h with **(A)** different methylamine (MA) and soluble SSAO concentrations, **(B)** $9 \cdot 10^{-4}$ U/ml of soluble SSAO, 1 mM MA and SSAO inhibitors, 1 mM semicarbazide and 10 µM MDL72974A, and **(C)** $9 \cdot 10^{-4}$ U/ml of soluble SSAO plus, 1 mM benzylamine (Bz), 1 mM tyramine (Tyr), and SSAO inhibitors. Data are mean ± SEM 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.

Figure 2. SSAO catalytic action ($9 \cdot 10^{-4}$ U/ml of soluble SSAO plus and 1 mM MA) induces Caspase-3 activation and nuclei condensation on A7r5 cells. Representative immunostaining with anti-cleaved Caspase-3 antibody and Hoechst 33258 of **(A)** control, **(B)** $9 \cdot 10^{-4}$ U/ml of soluble SSAO + 1mM MA, **(C)** $9 \cdot 10^{-4}$ U/ml of soluble SSAO + 1mM MA + 1mM semicarbazide (SC), **(D)** $9 \cdot 10^{-4}$ U/ml of soluble SSAO, **(E)** 1 mM MA, anf **(F)** 1 mM SC, for 24h. Scale bar = 50 µm in A, B, C, D, E & F and 25 µm in B inset. **(G)** Percentages of cleaved Caspase-3 positive cells to total cells. Data are mean ± SEM values of three separate experiments performed in triplicate.

Figure 3. SSAO catalytic action ($9 \cdot 10^{-4}$ U/ml of soluble SSAO and 1 mM MA) induces apoptosis on A7r5 cells. Representative immunoblot of **(A)** PARP cleavage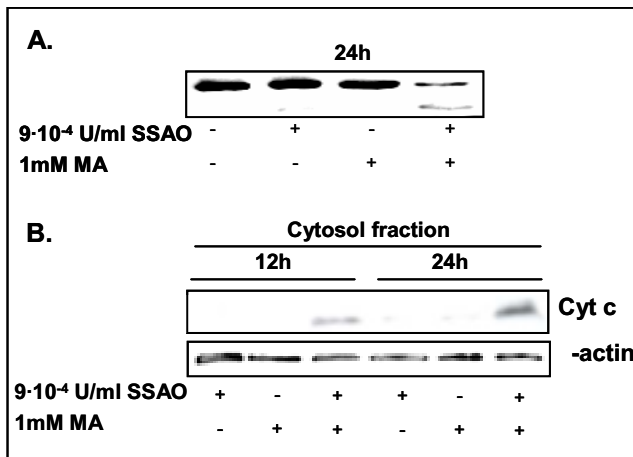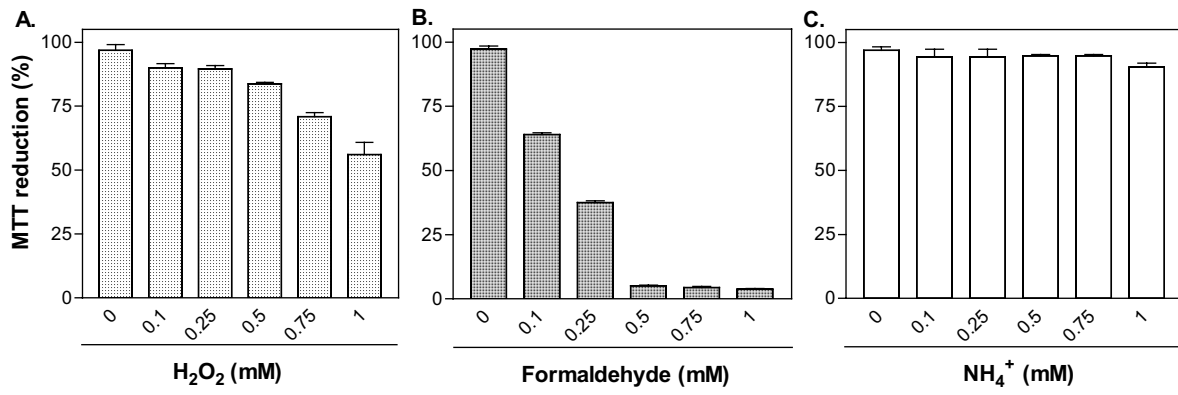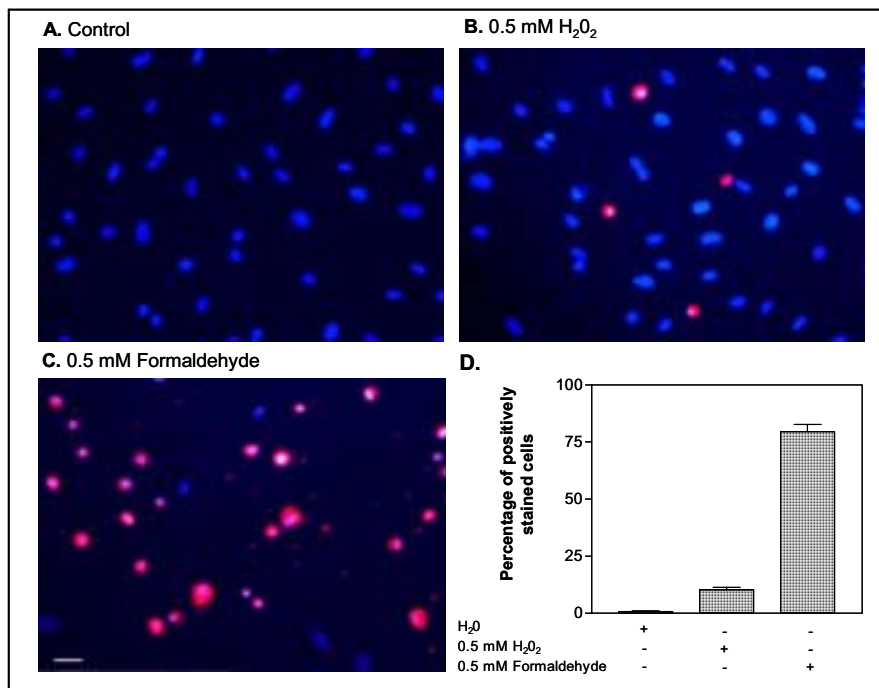 after 24h treatment, and **(B)** time-dependent (12 and 24h) Cytochrome c release to cytosol. -Actin is used as loading control.

Figure 4. Cytotoxicity of MA oxidation by SSAO catalytic products in A7r5 cells expressed as MTT reduction percentages obtained from the incubation with different concentrations of **(A)** H_2O_2, **(B)** formaldehyde, and **(C)** NH_4^+, for 24h. Data are mean ± SEM values of three separate experiments performed in triplicate.

Figure 5. 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 24h treatment. Scale bar = 50 µm. **(D)** Percentages of cleaved Caspase-3 positive cells to total cells. Data are mean ± SEM values of three separate experiments performed in triplicate.

Figure 6. H_2O_2 and formaldehyde induce apoptosis on A7r5 cells. Representative immunoblot of PARP cleavage after formaldehyde treatment for 24h and H_2O_2 treatment for 24 and 42h.

Figure 7. Cytotoxicity of catalytic SSAO action on HASMC expressed as MTT reduction percentages obtained from the incubation with $9·10^{-4}$ U/ml of soluble SSAO plus, 1 mM MA and SSAO inhibitors, semicarbazide and MDL72974A, for 24h. Data are mean ± SEM 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.

Figure 1

A.

B.

control	+	-	-	-	-	-
1mM MA	-	+	-	+	+	+
9·10⁻⁴ U/ml SSAO	-	-	+	+	+	+
1mM Semicarbazide	-	-	-	-	+	-
10 σM MDL72974A	-	-	-	-	-	+

C.

control	+	-	-	-	-	-	-	-
9·10⁻⁴ U/ml SSAO	-	+	-	+	-	+	+	+
1mM BZ	-	-	+	+	-	-	-	-
1mM Tyr	-	-	-	-	+	+	+	+
1mM Semicarbazide	-	-	-	-	-	-	+	-
10σM MDL72974A	-	-	-	-	-	-	-	+

Figure 2

A. Control
B. 9·10⁻⁴ U/ml soluble SSAO + 1mM MA
C. 9·10⁻⁴ U/ml sSSAO + 1mM MA + 1mM SC
D. 9·10⁻⁴ U/ml soluble SSAO
E. 1mM MA
F. 1mM SC

G.

Figure 3

A.

24h

| 9·10⁻⁴ U/ml SSAO | - | + | - | + |
| 1mM MA | - | - | + | + |

B.

Cytosol fraction

| | 12h | | | 24h | | |

Cyt c

-actin

| 9·10⁻⁴ U/ml SSAO | + | - | + | + | - | + |
| 1mM MA | - | + | + | - | + | + |

Figure 4

A.

MTT reduction (%)

H$_2$O$_2$ (mM)

B.

Formaldehyde (mM)

C.

NH$_4^+$ (mM)

Figure 5

A. Control

B. 0.5 mM H$_2$0$_2$

C. 0.5 mM Formaldehyde

D.

Percentage of positively stained cells

H$_2$0

0.5 mM H$_2$0$_2$

0.5 mM Formaldehyde

Figure 6

Figure 7

ANNEX III.1. ALTRES PUBLICACIONS:

"The inhibition of semicarbazide-sensitive amine oxidase by aminohexoses"

O'Sullivan J, O'Sullivan M, Tipton KF, Unzeta M, **Del Mar Hernandez M**, Davey GP.

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The inhibition of semicarbazide-sensitive amine oxidase by aminohexoses

Jeffrey O'Sullivan[a,*], Michael O'Sullivan[a], Keith F. Tipton[a], Mercedes Unzeta[b],
Maria Del Mar Hernandez[b], Gavin P. Davey[a]

[a] *Department of Biochemistry, Trinity College, Dublin 2, Ireland*
[b] *Department of Biochemistry and Molecular Biology, Faculty of Medicine, Autonomous University of Barcelona, Bellaterra, Spain*

Abstract

Semicarbazide-sensitive amine oxidase (EC 1.4.3.6; amine:oxygen oxidoreductase (deaminating) (copper-containing); SSAO) is a multifunctional protein. It acts under inflammatory conditions as a vascular-adhesion protein (VAP-1), mediating the adhesion of lymphocytes to vascular endothelial cells. The relationships, if any, between this adhesion function and the enzymatic functions (amine-substrate specificity and catalysis) of SSAO have not yet been defined. Since cell surface amino sugars and their derivatives are known to be involved in cell-to-cell recognition, we have investigated their possible effects on the enzyme activity of SSAO. The aminohexoses galactosamine, glucosamine and mannosamine were not oxidatively deaminated by SSAO. However, their presence during the assay of benzylamine oxidation resulted in a time-dependent inhibition. This inhibition was shown to follow saturation kinetics with respect to hexosamine concentration. Although time-dependent, the inhibition of SSAO activity was found to be reversible by dilution. In contrast, there is no such inhibition when the *N*-acetylamino sugar derivatives or the parent sugars (galactose, glucose and mannose) replaced the amino sugars in the reaction mixture. These results suggest that the interactions between SSAO and aminohexoses are specific and, therefore, that the cell-adhesion functions and amine-recognition functions of VAP-1/SSAO may be interlinked.

1. Introduction

The semicarbazide-sensitive amine oxidases (SSAO) are an ubiquitous group of enzymes found in plants, animals and microbes (see Refs. [1–4]). They belong to the class EC 1.4.3.6 (amine:oxygen oxidoreductase (deaminating) (copper-containing)) and contain TOPA (6-hydroxydopa; 2,4,5-trihydroxyphenylalanine) quinone as the redox cofactor (see Ref. [5]). They catalyse the overall reaction:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$
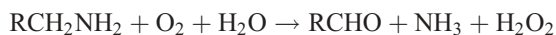
In most mammals, the enzyme exists in tissue-bound and soluble (plasma) forms, but there are wide species and tissue differences in the activities [6]. The tissue-bound form contains a short intracellular domain, a single trans-membrane domain and a long extracellular domain that includes the catalytic site [7], which might suggest a scavenging or protective role towards circulating amines. However, a proportion of SSAO in adipocytes has been shown to be associated with the intracellular vesicles containing the GLUT4 glucose transporter [8].

There are large species differences in the specificities of SSAO, but the non-physiological amine benzylamine is a good substrate for the mammalian enzymes. The physiological substrates are believed to include aminoacetone [9] and methylamine [10], 2-phenylethylamine, tyramine [11] and dopamine [12]. Many of the substrates for SSAO are also oxidatively deaminated by the monoamine oxidases (EC 1.4.3.4, amine:oxygen oxidoreductase (deaminating) (flavine-containing); MAO), but aminoacetone and methylamine are not MAO substrates. SSAO also catalyses the oxidative deamination of a number of xenobiotics (see Ref. [13]). Although SSAO has been often regarded as being involved in the detoxification of amines, the products of the reaction are potentially more toxic than the amine substrates themselves (see Refs. [2,3]).

Several alternative functions for SSAO have been proposed. It has been suggested that one of the roles of SSAO is the formation of H_2O_2, which has an insulin-like effect in stimulating the transport of the GLUT4 glucose transporter to the cell-surface and inhibiting lipolysis in adipocytes [8,14]. Amine oxidation by SSAO has also been recently shown to stimulate glucose uptake by vascular smooth muscle cells [15] and to promote adipocyte differentiation [16]. It has also been reported that H_2O_2 may modulate the activity of SSAO [17,18]. Other, less clearly defined, functions that have been suggested include involvement in the post-translational modification of some proteins [19], cardioprotection [20] and modification of neuronal K^+-channel function [21]. SSAO is also an amiloride-binding protein (see Refs. [22,23]), although the significance, if any, of this is unclear.

Recently an adhesion protein, vascular-adhesion protein 1 (VAP-1) was found to have sequence homology with SSAO at the cDNA level [24]. This protein was subsequently found to possess SSAO activity. VAP-1 is an endothelial glycoprotein which supports the adhesion of lymphocytes to endothelial cells and mediate lymphocyte re-circulation in a L-selectin-dependent manner [24,25]. VAP-1 has been shown to support sialic acid-dependent adhesion under shear stress and to mediate tethering to the tumour endothelium in human heptacellular carcinoma of T cells [26]. Its cell surface expression is induced under inflammatory conditions, and this may provide an explanation for the observations that SSAO activity is altered under such conditions (see Refs. [1,2, 27]). In order to investigate the possible sites of interaction between SSAO and the cell surface, we have investigated the effects of amino sugars, which are known to be involved in cell to cell recognition, on the enzymatic activity of SSAO.

2. Materials and methods

Amine oxidase from bovine plasma was obtained from Sigma Chemical Company along with the other chemicals used. Amino sugars were prepared in distilled water.
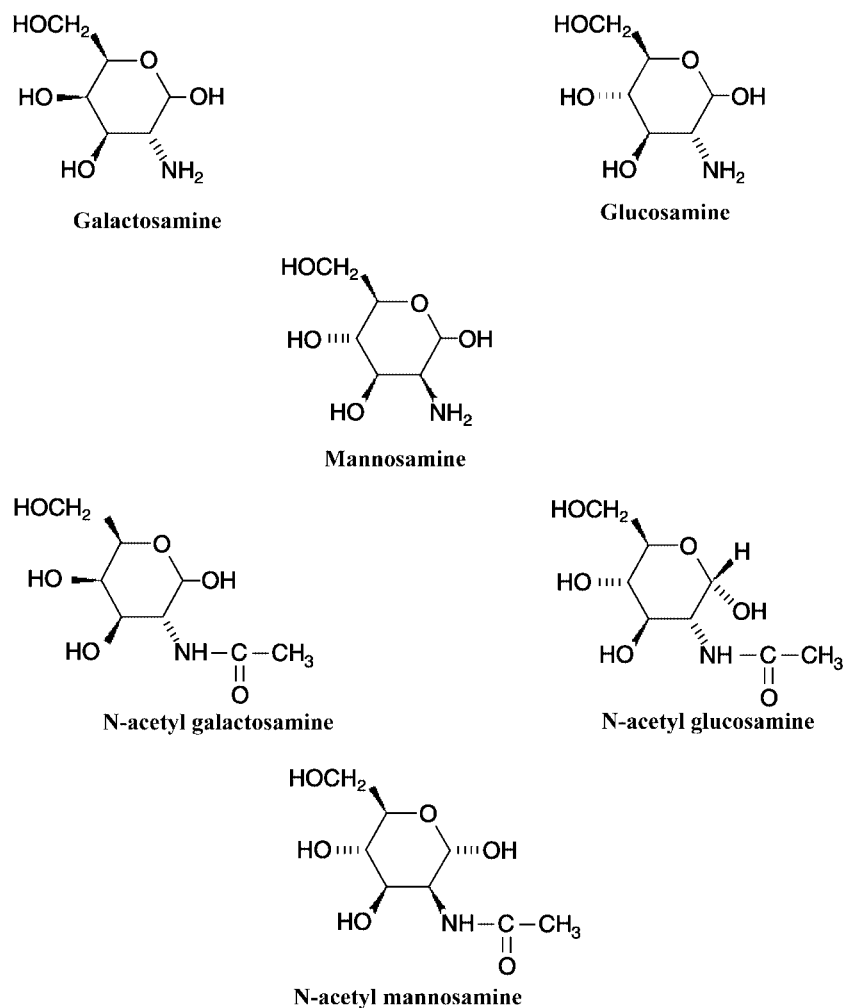

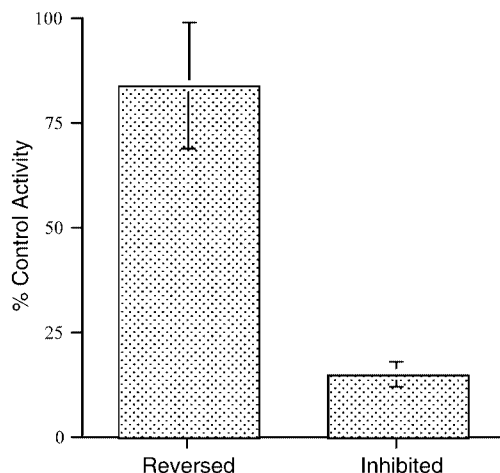Fig. 1. Chemical structures of the amino sugars used in this study.

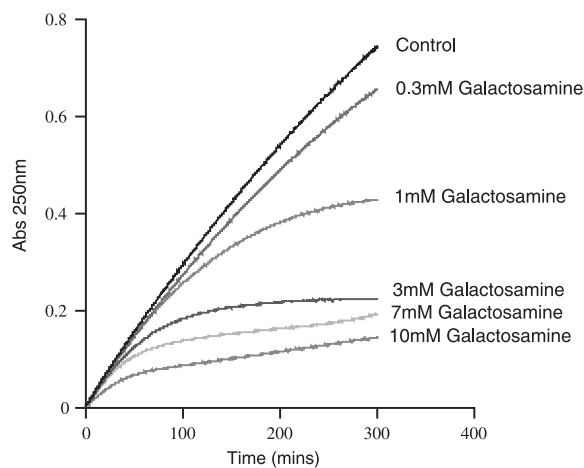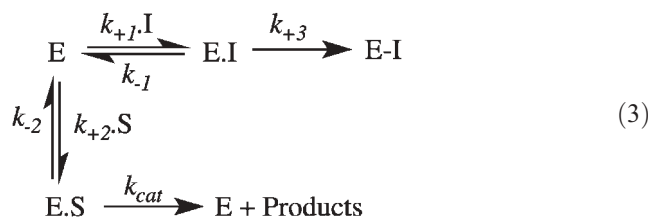Fig. 2. Reversibility of the inhibition of plasma SSAO by galactosamine.

Fig. 3. Time-courses of the oxidation of benzylamine by plasma SSAO in the presence of different concentrations of the galactosamine. Experimental details are given in the text. The results shown are direct output from a single set of directly captured progress curves and are representative of at least six replicates.

All enzyme assays were performed at 37 °C. SSAO activity towards benzylamine was assayed directly by a modification [28] of the method of Tabor et al. [29]. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.2, and SSAO (concentrations ranging from 12 to 24 μg·ml^{-1}) in a final volume of 1 ml. The reaction was initiated by the addition of the substrate benzylamine (5 mM) and the rate of change of absorbance was monitored at 250 nm in a Cary 300-Bio spectrophotometer (Varian). The molar extinction coefficient of benzaldehyde at 250 nm was taken to be 13,800 M^{-1} cm^{-1} (see Ref. [28]).

Reversibility of inhibition was assessed by dilution experiments. The enzyme (bovine plasma amine oxidase) from Sigma, at a concentration of 1.2 mg protein·ml^{-1}, was incubated with 10 mM galactosamine in the presence of 5 mM benzylamine until inhibition was essentially complete, at approximately 2 h. Aliquots of 10 μl were then removed and diluted into the standard benzylamine assay mixture, to give a final protein concentration of 12 μg·ml^{-1}, for activity determinations.

Kinetic analysis of the time-dependent inhibition of SSAO by galactosamine in the reaction was achieved by fitting the reaction–progress curves data in the presence of the amine substrate according to the equation (see Ref. [30]):

$$[P_t] = [P_\infty](1 - e^{-k't}) \tag{1}$$

where $[P_t]$ and $[P_\infty]$ are the product concentrations at any time, t, and at complete cessation of the reaction, respectively, and k' is the apparent first-order rate constant for the decline in enzyme activity.

The substrate-concentration dependence of the apparent first-order rate constants at each amino sugar concentration was fitted to the relationship,

$$k' = \frac{k_{+3}}{1 + \dfrac{K_i}{[\mathrm{I}]}\left(1 + \dfrac{[\mathrm{S}]}{K_{\mathrm{m}}}\right)} \tag{2}$$

which describes the situation where substrate and inhibitor both compete for non-covalent binding to the enzyme, according to the system:

$$
\begin{array}{c}
\mathrm{E} \xrightleftharpoons[k_{-1}]{k_{+1}.\mathrm{I}} \mathrm{E.I} \xrightarrow{k_{+3}} \mathrm{E\text{-}I} \\[2mm]
k_{-2} \big\updownarrow\, k_{+2}.\mathrm{S} \\[2mm]
\mathrm{E.S} \xrightarrow{k_{cat}} \mathrm{E} + \text{Products}
\end{array}
\tag{3}
$$

in which K_{m} is the Michaelis constant in the absence of inhibitor ($\{k_{-2}+k_{\mathrm{cat}}\}/k_{+2}$) and, assuming $k_{+3} \ll k_{-1}$ [30], $K_{\mathrm{I}} = k_{-1}/k_{+1}$. Square brackets are used to denote concentrations.
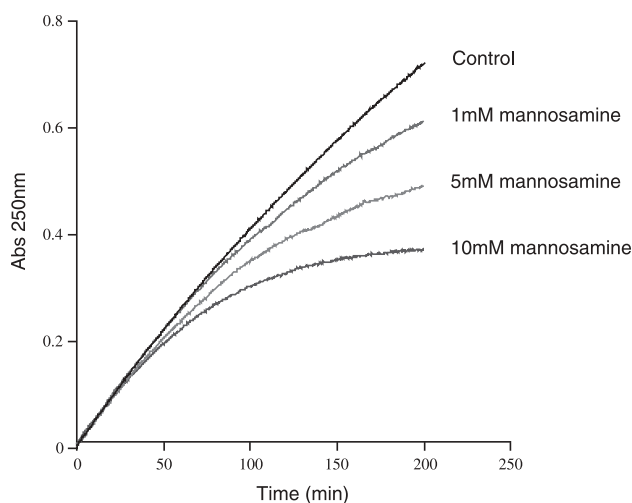

Fig. 4. Time-courses of the oxidation of benzylamine by plasma SSAO in the presence of different concentrations of the mannosamine. The results shown are direct outputs from a single set of directly captured progress curves and are representative of at least six replicates.
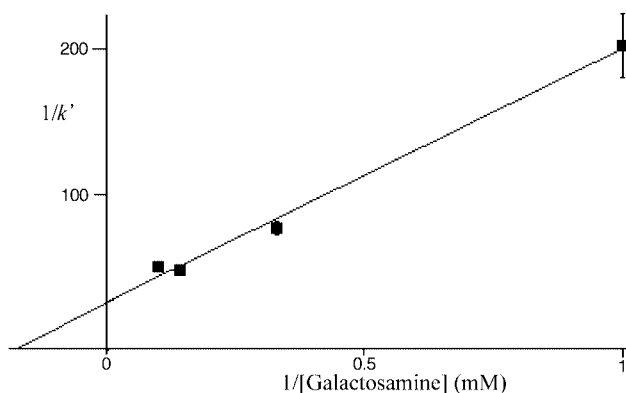
Fig. 5. The variation of the apparent first-order rate constant for time-dependent inhibition of plasma SSAO with the galactosamine concentration, presented for illustrative purposes, as a double-reciprocal plot.

K_I, k_{app} and k_{+3} values were determined using the commercial software programme MacCurveFit 1.5. (Kevin Raner Software, Australia). Data are displayed as a double-reciprocal representation of Eq. (2) for illustrative purposes only.

3. Results

Time-dependent inhibition was observed when SSAO deamination of benzylamine was performed in the presence of amino sugars. Galactosamine, mannosamine and glucosamine (Fig. 1), each at a concentration of 1 mM, all showed the ability to inhibit the plasma SSAO, as well as the activity of tissue-bound SSAO from bovine lung, both in crude microsomal fractions and purified form, prepared as previously described [23]. The parent sugars and their N-acetyl derivatives had no effect on the activity of SSAO under these conditions. Reversibility was determined by dilution experiments where the initial rate activity of SSAO returned to control levels after substantial inhibition had occurred by incubation with 10 mM galactosamine for 2 h in the presence of 5 mM benzylamine (Fig. 2).

A range of galactosamine and mannosamine concentrations (up to 25 mM) inhibited SSAO in a concentration- and time-dependent manner (Figs. 3 and 4). The K_i for the galactosamine inhibition was calculated by fitting the reaction progress curves to Eqs. (1) and (2) was found to be 3.9 ± 1.6 mM. The apparent first-order rate (k_{+3}) constant for the time-dependent inhibition was 0.029 ± 0.004 min^{-1} (see Fig. 5). Determination of hydrogen peroxide formation [28] when SSAO was incubated with the amino sugars for periods of up to 2 h indicated that they were not substrates for the enzyme.
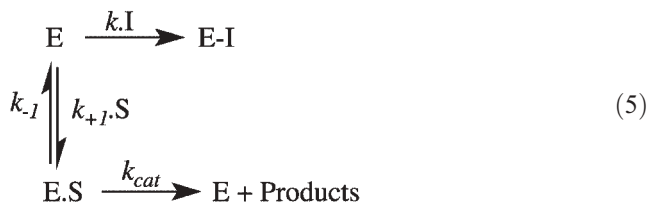
4. Discussion

The amino sugars were found to be effective time dependent inhibitors of SSAO, unlike the N-acetyl derivative and parent hexoses. This ability and the different potencies of inhibition by the amino sugars suggest that the inhibition shows specificity for the structure of the amino sugar.

The data were also fitted to the equation

$$k' = \frac{k_{app}}{1 + \dfrac{[S]}{K_m}} \qquad (4)$$

which describes the situation where the inhibitor does not form an initial non-covalent complex with the enzyme

$$\begin{array}{ccc}
E & \xrightarrow{k.I} & E\text{-}I \\
k_{-1} \Big\Updownarrow k_{+1}.S & & \\
E.S & \xrightarrow{k_{cat}} & E + Products
\end{array} \qquad (5)$$

where the *pseudo* first-order $k_{app} = k$ [I], was also attempted for comparative purposes. Such a mechanism would result in a linear dependence of the apparent first-order rate constant on the concentration of amino sugar, or a double-reciprocal plot that passes through the origin (1/rate constant) = 0 when 1/[amino sugar] = 0, which was clearly not the case (see Fig. 5).

Since the kinetics of the time-dependent inhibition follow Eqs. (2) and (3), rather than Eqs. (4) and (5), this would suggest that the amino sugar forms a weak non-covalent complex with SSAO that is then, more slowly transformed to a more tightly bound, but still reversible complex. The possibility that this interaction between SSAO/VAP-1 and amino sugars may serve as a model system for interactions with cell-surface glyco-conjugates that are involved in the adhesion process will be a subject for further studies.

Acknowledgements

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ANNEX III.2. ALTRES PUBLICACIONS:

"Synthesis of 4-methyl-thio-phenyl-propylamine and the evaluation of its interaction with different amine oxidases"

Gallardo-Godoy A, **Hernandez M**, Sanz E, Unzeta M.

Bioorg Med Chem. 2004 Jan 2;12(1):273-9.

Synthesis of 4-methyl-thio-phenyl-propylamine and the evaluation of its interaction with different amine oxidases

Alejandra Gallardo-Godoy, Mar Hernandez, Elisenda Sanz and Mercedes Unzeta*

Institut de Neurociències-Departament de Bioquímica i Biologia Molecular, Facultat de Medicina, Universitat Autónoma de Barcelona, Campus Universitari de Bellaterra, E-08193 Bellaterra, Barcelona, Spain

Abstract—A new molecule, the 4-methyl-thio-phenyl-propylamine ($PrNH_2$) was synthesized and its biological interaction with different amine oxidases such as semicarbazide sensitive amine oxidase (SSAO) [E.C.1.4.3.6], and monoamine oxidase [E.C.1.4.3.4] under its two isoforms, MAO A and MAO B, has been assessed. The substrate specifities of MAO and SSAO overlap to some extent. In this context, the search of new molecules, able to discriminate between these different amine oxidases is very important as it will allow greater elucidation of the SSAO's role in physiological and pathological conditions. We report for the first time, the synthesis and evaluation of a new molecule which has a high affinity towards the SSAO family of enzymes, more so than previously described and furthermore an ability to discriminate between the different amine oxidases.
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1. Introduction

The term semicarbazide sensitive amine oxidase (SSAO) is used to describe a group of enzymes that catalyse the oxidative deamination of aliphatic and aromatic primary amines. They are classified as E.C.1.4.3.6 (amine: oxygen oxidoreductase (deaminating) (copper-containing), and all of them have 2,4,5-trihydroxyphenylalanine (TPQ) as cofactor. Semicarbazide is frequently used to distinguish the SSAOs from the monoamine oxidase, [amine: oxygen oxidoreductase (deaminating) (flavin-containing), E.C.1.4.3.4; MAO], that contains flavin adenine dinucleotide (FAD) as cofactor and are sensitive to acetylenic inhibitors such as clorgyline and l-deprenyl, but are not affected by semicarbazide. The substrate specificities of MAO-A and SSAO overlap to some extent but, whereas MAO catalyses the oxidative deamination of primary, secondary and tertiary amines, SSAO activity appears to be restricted to primary amines. At present methylamine, which arises from the metabolism of adrenaline, lecithin, sarcosine, and creatinine, is the only physiological substrate known that can discriminate between both amine oxidases. Methylamine is exclusively metabolised by SSAO from many sources[1,2]

and does not interact with MAO. Both enzymes catalyse the oxidative deamination of amines to ammonia, hydrogen peroxide and the corresponding aldehyde, according to the overall reaction:
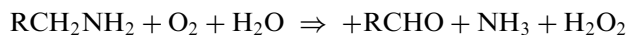
$$RCH_2NH_2 + O_2 + H_2O \Rightarrow +RCHO + NH_3 + H_2O_2$$

SSAO is tightly associated with membranes in several mammalian tissues and also occurs as a soluble form in blood plasma.[3,4] The membrane-bound SSAO shows high activity in vascular tissue and it appears to be associated with smooth muscle cells.[5,6] SSAO activity has been also found in other non-vascular cell types such as chondrocytes,[7] bovine eye,[8] in dental pulp,[9] and in adipocytes from rat white and brown fat.[10] The physiological role of SSAO is still far from clear, despite its wide distribution in mammalian tissues.[11] SSAO located in the plasma membrane may act as a scavenger of circulating toxic amines and in this context, the enzyme present in the microsomal fraction of human and bovine lung[12,13] may be important in inhaled volatile amines metabolism.[14] This enzyme could also be involved in the control of cellular activities through the generation of H_2O_2.[15] It has been recently reported that SSAO co-localizes with GLUT4 glucose transporter in endosomal compartment in rat adipocytes and that SSAO substrates cause a marked stimulation of glucose transport, mimicking the effects of insulin.[16]

The vascular-adhesion protein VAP-1, present in endothelial cells, has been shown to have an identical amino-acid sequence to SSAO.[17] Because VAP-1 is induced in inflammation and it is involved in lymphocyte migration to lymphoid organs, a new physiological role in cellular trafficking has been proposed for SSAO.[18] In this context, design and synthesis of new compounds that behave as specific SSAO substrates or inhibitors could be useful for discriminating between SSAO and MAO. Furthermore the search of new substrates with high affinity towards SSAO may have therapeutic value in diabetes, due to its importance in glucose transport mimicking insulin effects.

Starting from the 4-methyl-thio-amphetamine (4-MTA), a high selective and reversible MAO-A inhibitor and poor MAO-B inhibitor,[19] we have studied the effect of side-chain enlargement on its amine oxidase inhibitory potency. Here, we report by the first time, the design and synthesis of a new molecule, the 4-methyl-thio-phenyl-propylamine (PrNH$_2$), and its biological evaluation as inhibitor or substrate of different amine oxidases.

2. Results

2.1. Synthesis

Scheme 1 shows the route used to prepare the novel compound (3), starting from 4-methylthiobenzaldehyde, following the method published for the synthesis of *p*-methoxy-cinnamonitrile.[20] The condensation of acetonitrile with the aldehyde, catalyzed by powdered KOH, afforded a 4:1 mixture of (*E*)- and (*Z*)-4-methyl-thio-cinnamonitrile, which was used without the separation of isomers. Although there are published routes[21,22] for the synthesis of intermediate 1, this method was chosen for the simplicity and low cost of the route. Direct reduction of compound 1 by LiAlH$_4$ gave none of the desired product, and therefore a two-step sequence using NaBH$_4$ and LiAlH$_4$ successively was used, giving 3 (via 2) in very good overall yield.

2.2. Chemistry, general procedures

^{1}H NMR spectra were recorded on a Bruker ARX 300 spectrometer. Chemical shifts are expressed in parts per million downfield from internal Me$_4$Si as a reference. ^{1}H NMR data are reported in the order: multiplicity (s, singlet; d, doublet; t, triplet; m, multiplet; number of protons, and approximate coupling constant in Hz).
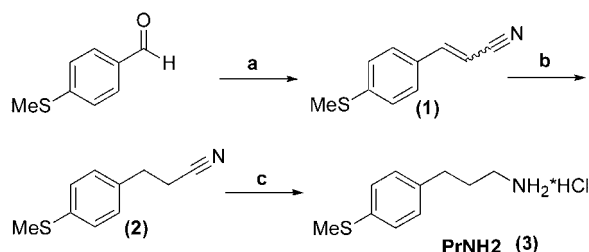
Scheme 1. (a) KOH, MeCN, reflux; (b) NaBH$_4$, MeOH, py, reflux; (c) (i) LiAlH$_4$, THF, reflux; (ii) HCl, IPA, Et$_2$O.

Elemental analyses (C, H, N) were carried out using Perkin-Elmer 240 B or 240 C instruments and the results were within ±0.4% of the calculated values. Thin-layer chromatography (TLC) was performed under standard conditions using silica gel 60-F$_{254}$ plates, 0.2 mm thickness (Merck). Abbreviations for the following solvents were used: MeCN, acetonitrile; CH$_2$Cl$_2$, dichloromethane; Et$_2$O, diethyl ether; EtOH, ethanol; IPA, propan-2-ol; MeOH, methanol; py, pyridine; THF, tetrahydrofuran.

2.2.1. 3-(4-Methylthiophenyl)-acrylonitrile (1). To a suspension of KOH (1.5 g, 26.7 mmol; fine powder) in 20 mL of acetonitrile was added 4-methylthiobenzaldehyde (4.0 g, 26.0 mmol). The reaction mixture was heated at reflux for 15 min with magnetic stirring. The mixture was poured onto 50 g of crushed ice and the organic layer separated. After washing with water, the organic layer was removed under reduced pressure resulting in a thick yellow oil, which was purified using bulb to bulb distillation (145–150 °C/0.02 Torr) to give 3.4 g (75% yield) of an *E/Z* mixture in a 4:1 ratio (by ^{1}H NMR analysis). ^{1}H NMR (CDCl$_3$): δ 7.40 (d, *J* = 8.4 Hz, 2H, Ph-3-H, Ph-5-H), 7.30 (d, *J* = 12.6 Hz, 1H, PhC<u>H</u>CHCN), 7.20 (d, *J* = 8.4 Hz, 2H, Ph-2-H, Ph-6-H), 5.80 (d, *J* = 12.6 Hz, 1H, PhCHC<u>H</u>CN), 2.50 (s, 3H, CH$_3$S).

2.2.2. 3-(4-Methylthiophenyl)-propionitrile (2). To a stirred solution of 1 (1.0 g, 5.7 mmol) in 10 mL of pyridine and 3 mL of MeOH was added in portions, NaBH$_4$ (0.22 g, 5.7 mmol). The reaction mixture was heated at reflux for 3 h. After cooling it was poured into 100 mL of 10% v/v HCl in ice water. The solution was extracted with 2 × 50 mL CH$_2$Cl$_2$, the organic layer separated and the solvent removed under reduced pressure affording a colorless oil which was purified using bulb to bulb distillation (123–125 °C/0.02 Torr) to give 700 mg (70% yield) of the title compound. ^{1}H NMR (CDCl$_3$): δ 7.24 (d, *J* = 8.4 Hz, 2H, Ph-3-H, Ph-5-H), 7.15 (d, *J* = 8.4 Hz, 2H, Ph-2-H, Ph-6-H), 2.91 (t, *J* = 7.6 Hz, 2H, PhCH$_2$C<u>H</u>$_2$CN), 2.60 (t, *J* = 7.6 Hz, 2H, PhC<u>H</u>$_2$CH$_2$CN), 2.47 (s, 3H, CH$_3$S). Anal. calcd for C$_{10}$H$_{11}$NS: C, 67.76; H, 6.25; N, 7.90; S, 18.09. Found: C 68.05; H 6.11; N, 7.84; S, 18.16.

2.2.3. 3-(4-Methylthiophenyl)-propylamine (PrNH$_2$). A solution of 2 (1.0 g, 5.6 mmol) in 5 mL of THF was added dropwise to a suspension of LiAlH$_4$ (0.5 g, 13.0 mmol) in 10 mL of freshly distilled THF. The reaction mixture was heated at reflux for 6 h. The excess LiAlH$_4$ was decomposed by successive addition of 0.5 mL of distilled water, 0.5 mL 15% w/v NaOH and 1.5 mL of distilled water. The cake was filtered and washed with 3 × 10 mL of THF. The solvent was removed under reduced pressure and the residue was purified by bulb to bulb distillation (110–115 °C/0.02 Torr). The resulting oil was crystallized as the hydrochloride in IPA/Et$_2$O to give 300 mg of white microcrystals (61% yield). ^{1}H NMR (D$_2$O): δ 7.29 (d, *J* = 8.4 Hz, 2H, Ph-3-H, Ph-5-H), 7.21 (d, *J* = 8.4 Hz, 2H, Ph-2-H, Ph-6-H), 2.95 (t, *J* = 7.6 Hz, 2H, PhCH$_2$CH$_2$C<u>H</u>$_2$NH$_2$), 2.65 (t, *J* = 7.6 Hz, 2H, PhC<u>H</u>$_2$CH$_2$CH$_2$NH$_2$), 2.45 (s, 3H, CH$_3$S), 1.88–1.90 (m, 4H, PhCH$_2$C<u>H</u>$_2$CH$_2$NH$_2$). Anal. calcd for C$_{10}$H$_{15}$NS.HCl: C, 55.16; H, 7.41; N, 6.43; S, 14.73. Found: C 55.15; H 7.39; N, 6.48; S, 14.81.

2.3. Biology

2.3.1. Inhibitory behavior of PrNH2 versus different amine oxidases.
MAO-A and MAO-B activities from rat liver mitochondria were assayed at the 0-min pre-incubationin at 37 °C, in the presence of different concentration of 4-methyl-thio-phenyl propylamine ($PrNH_2$) [10^{-8}–10^{-3} M], and the activity remaining was measured against [^{14}C]5-HT, (100 μM), serotonin, a specific MAO-A substrate, and against [^{14}C] PEA (22 μM), a specific MAO-B substrate, respectively (see Table 1). In the case of SSAO, bovine lung microsomes were used, and the activity remaining was determined towards (50 μM) [^{14}C] benzylamine as substrate. Figure 1 shows the inhibition curve of MAO A by $PrNH_2$ as representative of the inhibition curves of the rest of the oxidases. The IC_{50} values were determined from the corresponding inhibition curves and this new molecule showed the highest inhibitory potency towards MAO-A ($IC_{50} = 5.8$ μM), followed by SSAO ($IC_{50} = 38.7$ μM) and the lowest inhibition was observed with MAO-B ($IC_{50} = 175$ μM). $PrNH_2$, showed a high inhibitory selectivity towards MAO-A, expressed in terms of IC_{50} MAO-A/IC_{50} MAO-B (0.033) and a high selectivity towards SSAO compared with MAO-B (IC_{50} SSAO/IC_{50} MAO-B = 0.22). However, these enzymes were more potently inhibited by clorgyline (specific MAO A inhibitor, $IC_{50} = 10^{-8}$ M) and l-deprenyl, (specific MAO B inhibitor, $IC_{50} = 3 \times 10^{-8}$ M) than by $PrNH_2$, whereas in case of SSAO, it showed similar behaviour towards semicarbazide (specific SSAO inhibitor, $IC_{50} = 10^{-4}$ M) and $PrNH_2$. When the assays were repeated with a previous incubation of 30 min, MAO A and MAO B resulted to be no time-dependent inhibited, whereas in case of SSAO a time-dependent inhibition was observed.

2.3.2. Kinetic behavior of PrNH2 as substrate of MAO-A, MAO-B and SSAO.
Since $PrNH_2$ was an amine, it might potentially behave as a substrate of any amine oxidases. Mitochondria from rat liver that contains both MAO-A and MAO-B, were pretreated by deprenyl 10^{-6} M or clorgyline 10^{-6} M in order to inhibit MAO B and MAO A, respectively, and avoid interferences between them. When $PrNH_2$ (15–500 μM) was assayed as MAO A substrate, identical progress curves were obtained at any $PrNH_2$ concentrations used (data not shown), suggesting that this compound was not a MAO-A substrate. When $PrNH_2$ was assayed as possible MAO-B substrate, the results showed that $PrNH_2$ was oxidized by the enzyme. The kinetic parameters were calculated by non-lineal regression analysis, and they resulted to be $K_m = 91.5$ μM and $V_{max} = 1742$ pmol/min (see Fig. 2). The catalytic efficacy of MAO-B towards $PrNH_2$, expressed as V_{max}/K_m was 19.04. When SSAO bovine lung microsomes[12,13] were assayed, a concentration range of $PrNH_2$ between 1 and 30 μM was used. The kinetic parameters were $K_m = 8.35$ μM $V_{max} = 2669$ pmol/min (see Fig. 3) and the catalytic efficacy was (V_{max}/K_m) 319.6 pmol/min μM. Table 2 shows all the kinetic parameters for $PrNH_2$ in comparison with the common MAO A and MAO B substrates (5HT for MAO A and PEA for MAO B, respectively[23]). Thus, SSAO had the highest activity towards $PrNH_2$ as a substrate, which was oxidised more slowly by MAO B and not at all by MAO A. In this regard, it is worth to remark that $PrNH_2$ resulted to be the best exogenous substrate for SSAO yet discovered, with a $K_m = 8.35$ μM versus benzylamine, the commonly used SSAO substrate ($K_m = 50$ μM). Furthermore, the catalytic efficacy V_{max}/K_m of SSAO versus $PrNH_2$ as substrate, was also higher than for benzylamine as substrate.

Table 1. Chemical structures of β-phenylethylamine (PEA), 5-OH tryptamine (5HT), benzylamine (Bz), and $PrNH_2$
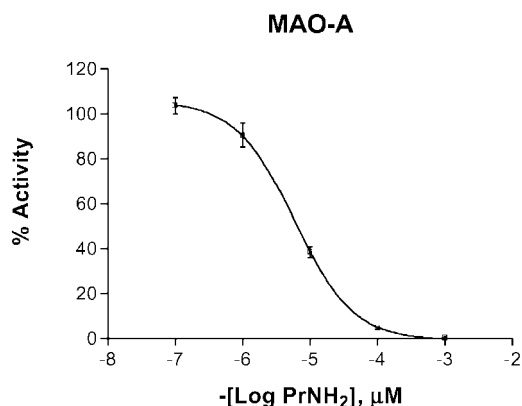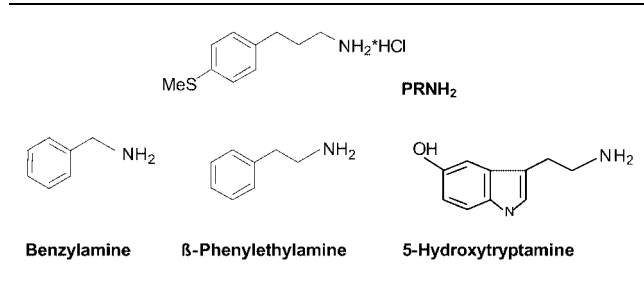
Figure 1. Amine oxidases inhibition by different concentration of $PrNH_2$ by measuring the remaining activity towards 100 μM ^{14}C-5HT (MAO A), 20 μM ^{14}C PEA (MAO B) and 50 μM ^{14}C Benzylamine (SSAO) as substrates. The data are expressed in percentage of control activities determined in absence of $PrNH_2$ as mean ±SD of three independent experiments performed in duplicate with error bar representing the standard deviation.
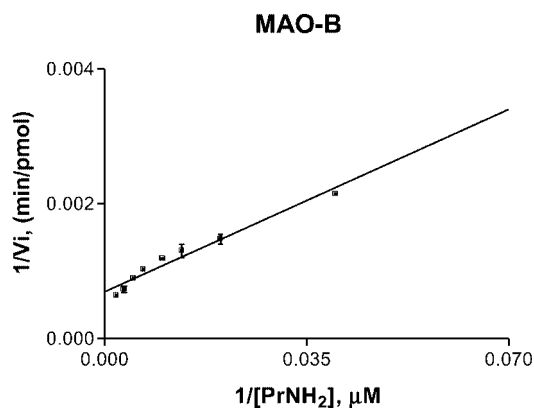
Figure 2. Double reciprocal plot of MAO B activity towards $PrNH_2$ as substrate. The concentration range used was (0–500 μM). MAO B activity was measured as described in the Experimental. Each value is the mean ±SD of two independent experiments performed in triplicate, with error bars representing the standard deviation.
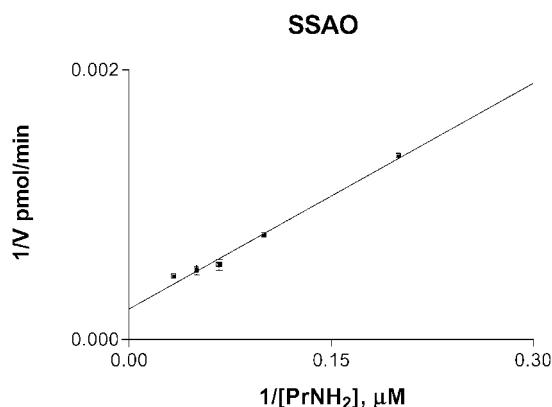
SSAO

Figure 3. Double reciprocal plot of SSAO activity towards $PrNH_2$ as substrate. The concentration range used was (0–40 µM). SSAO activity was measured as described in the Experimental. Each value is the mean ±SD of two independent experiments performed in triplicate, with error bars representing the standard deviation.

2.3.3. Kinetic behavior of $PrNH_2$ as inhibitor of MAO-A.

$PrNH_2$ was a mixed-type inhibitor of rat liver mitochondrial MAO-A, as shown in Figure 4. Plots of $1/v$ versus $PrNH_2$ concentration (Dixon, 1953)[24] (see Fig. 4 insert), confirmed a mixed-type inhibition. The K_{ic} and the K_{iu} values were calculated from the equation $V_{app} = V/1 + i/K_{iu}$ and $K_{mapp} = K_m(1 + i/K_{ic})/1 + i/K_{iu}$. The K_{iu} corresponds to the inhibition constant when the $PrNH_2$ binds to the complex ES, and its value resulted to be 13.35 µM, whereas K_{ic} expresses the inhibition constant of $PrNH_2$ when binds to the free enzyme and showed to be 2.76 µM.

3. Discussion

Several phenylisopropylamines, including amphetamine, have been evaluated as monoamine oxidase inhibitors.[25] Substituents on the aromatic ring of the phenylisopropylamine molecule (in particular at the *para* position), such as amino,[26] halogens[27] and alkylthio groups,[19] lead to an increase in the potency and selectivity towards MAO-A compared with the parent compound, amphetamine.

In the present work, we have studied the effect of the side-chain rearrangement and elongation of 4-methylthioamphetamine (4-MTA),[19] a high potent MAO-A inhibitor, and highly selective non-neurotoxic serotonin releasing and uptake blocking agent.[28] The IC_{50} values showed that this new molecule, had high potency as an inhibitor of MAO-A. It was less potent as an inhibitor

of SSAO and a relatively weak MAO-B inhibitor. The IC_{50} determined at 0 and 30 min pre-incubation time, suggested a reversible inhibitory behaviour in case of both MAO isoforms and a time-dependent inhibition in case of SSAO. $PrNH_2$ had 4 times higher selectivity towards SSAO compared to MAO-B (IC_{50} MAO-B/ IC_{50} SSAO). $PrNH_2$ had a 30 times lower inhibitory potency towards MAO-A than 4 MTA ($IC_{50} = 0.2$ µM), whereas the inhibitory potency towards MAO-B[19] was higher. These results suggest that the rearrangement and elongation of the side chain, results in a loss of affinity towards MAO-A, and an increasing affinity towards MAO-B. When $PrNH_2$ was assayed as a possible substrate, it proved to be a very good SSAO substrate followed by MAO-B, however this molecule was not metabolised by MAO-A at all. Considering the kinetic behaviour of $PrNH_2$ as a substrate, SSAO showed 11 times higher activity than MAO-B in terms of K_m values, and 1.5 times higher V_{max} values. Consequently the catalytic efficacy of SSAO towards $PrNH_2$, expressed in terms of V_{max}/K_m was 319.6 versus 19 in the case of MAO-B. When the kinetic behaviour of $PrNH_2$ was assayed as a MAO-A inhibitor, it behaved as a potent and reversible mixed-type inhibitor, indicating that $PrNH_2$ is able to bind whether to the enzyme–substrate complex or to the free enzyme at a different binding site of the active site.

The active sites of different known Cu-amine oxidases are structurally very similar. The TPQ cofactor exhibits different degrees of mobility and it is clearly flexible in all cases. Asp 383 in the active site performs multiple roles in stabilizing the TPQ in an off-copper conformation and assisting substrate binding to TPQ and abstracting the C-H proton from the substrate.[29] Recently, some authors[30] have reported the structure of human MAO-B. The structure of the enzyme shows a flat cavity lined with aromatic and aliphatic aminoacids that constitutes the substrate binding site providing a highly hydrophobic environment. Located between the active site and the protein surface, there is another smaller, hydrophobic cavity. Some residues constitute a loop that separates both cavities and this loop allows the substrate to access to the active site through the small entrance cavity.

Despite these structural differences in the active site of both types of amine oxidases, the two MAO isoforms and SSAO, all of them have in common an ability to recognize hydrophobic structures in their substrates.[31] The present data reported herein on the synthesis and biological evaluation of $PrNH_2$, shows that this mole-

Table 2. Kinetic parameters of MAO A, MAO B and SSAO towards Pr NH_2, PEA, Bz and 5Htas substrates[a]

	Pr NH_2			PEA			5-HT			Bz		
	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m	K_m	V_{max}	V_{max}/K_m
MAO A							84.2 ± 5.9	5467 ± 249	164			
MAO B	91.5 ± 91	1742 ± 180	19	9.7 ± 0.5	5057 ± 12	522						
SSAO	8.35 ± 76	2669 ± 38	320	312 ± 13	980 ± 99	3.14				40 ± 3	2010 ± 165	50.25

K_m: µM; V_{max}: pmols/min/mg prot.
[a] Each value is the mean ±SD of three independent experiments performed in duplicate.
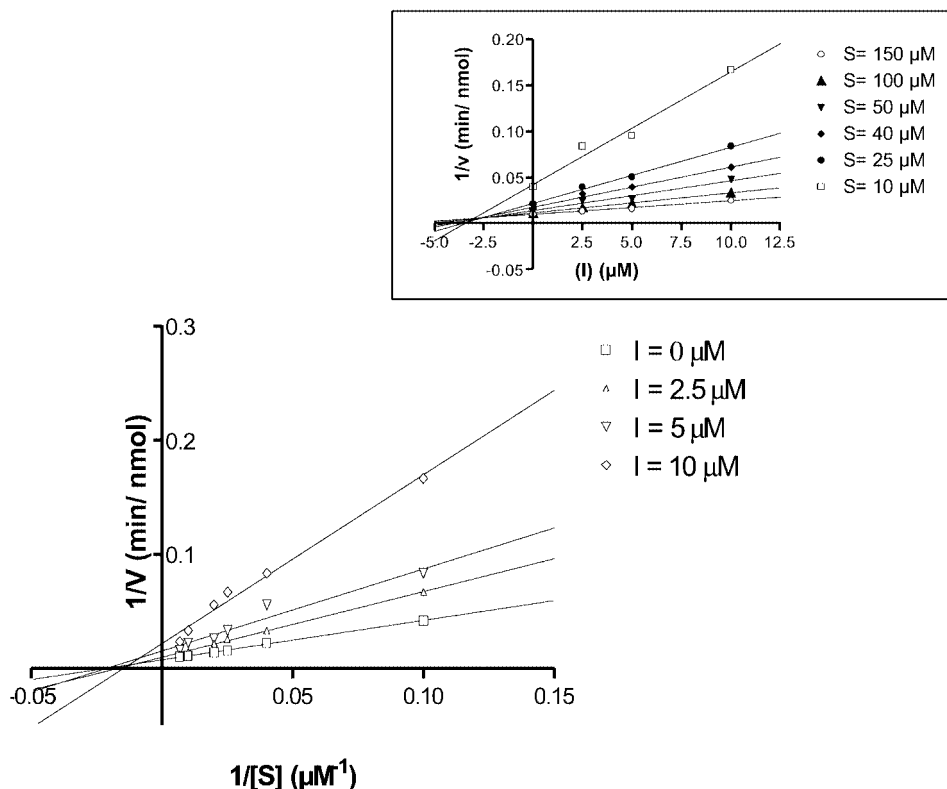
Figure 4. Double reciprocal plot of MAO A inhibition by $PrNH_2$. The $PrNH_2$ concentration range used was $(1–10\,\mu M)$. MAO A activity was measured towards Kynuramine as substrate as stated in the Experimental. Each value is the mean $\pm SD$ of two independent experiments performed in triplicate, with error bars representing the standard deviation. Insert: the Dixon plot ($1/v$ versus inhibitor concentration) of MAO A by $PrNH_2$ as inhibitor.

cule is able to interact with different types of amine oxidases. The presence of a *para* methyl-thio group, is responsible for its MAO-A inhibitory behaviour, however the rearrangement of the alpha-methyl group in the linear side chain, results in some loss of its inhibitory potency towards this MAO isoform. On the other hand, its hydrophobicity and the presence of the primary amine group on the side chain, confirm that $PrNH_2$ is a good MAO-B and SSAO substrate. However, this molecule shows better affinity towards SSAO than MAO-B, so it allows discriminating both enzymatic activities, although the amino propynyl side chain probably fits much better in the active site of SSAO than in the MAO-B's. The existence in the human MAO B structure[30] of an entrance cavity, which connects the surface of the protein to the hydrophobic substrate cavity, suggests that $PrNH_2$, could find some structural restrictions in the entry to the first small entrance cavity that could not allow the long side chains to pass to the real active site.

The design and synthesis of new substrates or inhibitors capable to discriminate between different amine oxidases, is very important to elucidate the SSAO role in physiological conditions and to develop new therapies for SSAO related disorders.

4. Experimental

4.1. Materials

The radioactive substrates [7-^{14}C]-benzylamine hydrochloride (specific activity 57 mCi/mmol), (0.2 mCi/mL),

the side-chain 2-[^{14}C]-5-hydroxy tryptamine sulphate (serotonin) (55 mCi/mmol) (0.2 mCi/mL) and ethyl-2-[^{14}C phenylethylamine HCl (57 mCi/mmol) (0.2 mCi/mL) were supplied by Amersham International. l-Deprenyl was from Research Biochemical International (R.B.I., USA) and kynuramine dihydrobromide and other common reagents, were from Sigma-Aldrich.

4.2. Preparation of bovine lung microsomes

Bovine lung was obtained from the abattoir after slaughter, packed in ice and transported immediately to the laboratory. After removal of the connective tissue, the lung was weighed, chopped into small pieces with scissors and washed extensively with saline (0.9%, w/v, NaCl) to eliminate blood as a potential source of contaminating plasma amine oxidase. The tissue was then homogenised in 1:10 (w/v) of 20 mM Tris/HCl buffer, pH 7.2, containing 0.25 M sucrose, in a Waring blender, and filtered through two layers of cheesecloth. The homogenate was subjected to differential centrifugation and the microsomal fraction was obtained by adding 10 mM $CaCl_2$ to the post-mitochondrial supernatant and centrifugation as previously described.[32] The final microsomal pellet was resuspended in 50 mM potassium phosphate buffer, pH 7.2, the protein concentration was adjusted to 10 mg/mL and samples were stored in aliquots at $-20\,°C$ until required. The resulting preparation, referred to as crude microsomes, contained the SSAO enzyme.

4.3. Preparation of rat liver mitochondria

Rat livers were recovered from male Sprague–Dawley rats (weighing 200–250 g) which had been fasted for 12 h. The liver homogenates were prepared in 10 vol (w/v) of a 50 mM potassium phosphate buffer (pH 7.2) by use of a Dounce homogenizer. The mitochondrial fraction was prepared by a standard differential centrifugation method.[33] The pellets were resuspended in the same buffer and frozen as small aliquots at −20 °C until required.

4.4. Radiochemical assays

SSAO activity in bovine lung microsomes, was determined radiochemically, by the method of Fowler and Tipton,[34] using 25 μL of [^{14}C]-benzylamine (3 mCi/mmol) (50 μM) in the assay mixture. The reaction was carried out at 37 °C in a final volume of 200 μL of 50 mM potassium phosphate buffer, pH 7.2, and the incubation was stopped by the addition of 100 μL of 2 M citric acid. Radioactive-labeled products were extracted into toluene/ethyl acetate (1:1, v/v) containing 0.6% (w/v) 2,5-diphenyloxazole (PPO) before liquid scintillation counting. In the case of MAO-A and MAO-B, [^{14}C]-serotonin (100 μM) and [^{14}C phenylethylamine (22 μM) were used as specific substrates, respectively.

Selective inhibition of SSAO without affecting MAO-B activity was achieved pre-incubating the enzyme with 1 mM semicarbazide for 30 min at 37 °C before the addition of the substrate. MAO-B activity was selectively inhibited, by preincubating the bovine lung microsomes with 10^{-6} M of l-deprenyl for 30 min at 37 °C, before adding the substrate. Time course assays were used to ensure that initial rates of the reaction were determined and proportionality to enzyme concentration was also established in each case. The IC$_{50}$ values of PrNH$_2$ towards different amine oxidases, were determined after incubating the compound with the enzyme preparation for either 0 or 30 min at 37 °C in the concentration range of 10^{-10}–10^{-3} M. The activities remaining, were then measured against the specific substrates and expressed as percentages of the controls, which have been incubated in the same way but in the absence of PrNH$_2$.

4.5. Coupled-assay method for the assay of PrNH$_2$ as substrate

PrNH$_2$ was studied as MAO-A, MAO-B and SSAO substrates, by the coupled assay method.[36] In the assay, to 808 μL of 0.2 M potassium phosphate buffer, pH 7.6, containing different concentrations of PrNH$_2$, was added 25 μL of the enzyme (final protein concentration 200 μg/mL). After pre-incubation for 5 min at 37 °C, 167 μL of cromogenic mixture containing [1 mL of vanillic acid (10 mM), 0.5 mL of 4-aminoantipyrine (10 mM) and 1 mL of Peroxidase (40 U/mL) in 2.5 mL of potassium phosphate buffer 0.2 M pH 7.6], was added. In this assay, 4-aminoantipyrine is oxidized by the H$_2$O$_2$ produced and condensed with vanillic acid to give a red quinoneimine dye. The appearance of product was measured spectrophotometrically at 498 nm and

was proportional to the amount of hydrogen peroxide released during the amine oxidase reaction. The extinction coefficient is 4654 M^{-1} cm^{-1}. Kinetic parameters were calculated by linear-regression analysis using the Graph-Pad Prism-3 program. When PrNH$_2$ was assayed as a MAO-B substrate, rat liver mitochondria was previously inhibited by clorgyline (10^{-6} M) in order to leave only MAO-B activity in the assay mixture. In order to assay PrNH$_2$ as a SSAO substrate, MAOB was previously inhibited by preincubation with deprenyl (10^{-6} M).

4.6. Kinetic behavior of PrNH$_2$ as a MAO-A inhibitor

PrNH$_2$ was assayed as a MAO-A inhibitor and the kinetic parameters determined using the spectrophotometric method with kynuramine as substrate in the presence of different PrNH$_2$ concentration (1–10 μM). Spectrophotometric assays for MAO-A activities were performed at 37 °C using kynuramine (40 μM) as substrate, in 50 mM potassium phosphate buffer (pH 7.2) containing 500 μM of mitochondrial preparation in a total volume of 3 mL. The appearance of the product was measured at 324 nm[35] and the absorbance coefficient at this wavelength was 20,000 M^{-1} cm^{-1}. Since kynuramine is a common substrate of both MAO forms, it was necessary to inhibit MAO-B with l-deprenyl (10^{-6} M) to ensure that only MAO-A activity was present.

4.7. Protein quantification

Protein was measured by the method of Bradford,[37] using bovine-serum albumin (BSA) as standard.

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