J Neural Transm (2007) 114: 757–762
DOI 10.1007/s00702-007-0684-x
Printed in the Netherlands

Journal of Neural Transmission

The origin of mammalian plasma amine oxidases

H. G. Schwelberger

Labor für Theoretische Chirurgie, Universitätsklinik für Chirurgie, Medizinische Universität Innsbruck, Innsbruck, Austria

Received: September 30, 2006 / Accepted: November 20, 2006 / Published online: March 26, 2007

Summary Mammalian blood plasma contains considerable activity of soluble copper-containing amine oxidase (AOC) referred to as plasma or serum amine oxidase (SAO). The identity and origin of SAO was investigated based on the recent characterization of four porcine AOC genes with AOC1 encoding diamine oxidase (DAO), AOC2 retina-specific amine oxidase (RAO), AOC3 vascular adhesion protein-1 (VAP-1), and AOC4 a VAP-1 homologue that is expressed mainly in the liver and has a signal peptide sequence instead of a transmembrane domain at its N-terminus. Purification and characterization of the major amine oxidase activity from porcine serum showed that it is the product of the AOC4 gene. Intriguingly, all mammals possessing a functional AOC4 gene exhibit high plasma amine oxidase activity. Humans and rodents lack a functional AOC4 gene and have comparably low plasma amine oxidase activity that is probably derived from partial proteolytic release of the membrane-associated AOC3 gene product VAP-1.

Keywords: Amine oxidase, diamine oxidase, DNA, gene, retina-specific amine oxidase, vascular adhesion protein-1

Abbreviations

AOC	copper-containing amine oxidase
DAO	diamine oxidase
kDa	kilodalton
RAO	retina-specific amine oxidase
SAO	serum or plasma amine oxidase
sVAP-1	soluble or secretory VAP-1
TPQ	2,4,5-trihydroxyphenylalanine quinone
VAP-1	vascular adhesion protein-1

Introduction

The blood plasma of mammals contains variable but individually relatively constant amounts of amine oxidising activity. Values for plasma or serum amine oxidase (SAO) activity range from several thousand μU/ml in most animals to just a few hundreds in humans and only about 20–30 in rodents (Boomsma et al., 2003). SAO preferentially converts monoamines and it is a member of the class of copper-containing amine oxidases (AOC) that possess the active-site cofactor 2,4,5-trihydroxyphenylalanine quinone (TPQ), formed post-translationally from a conserved tyrosine residue (Janes et al., 1990; Klinman, 1996; Lyles, 1996). Recently new interest in SAO was triggered by the finding that its activity is increased in various human pathologies including diabetes, congestive heart failure, liver cirrhosis, and obesity and by the possibility that this increased activity may lead to cellular damage and cardiovascular complications in these patients (Boomsma et al., 2003; Weiss et al., 2003; Yu et al., 2003).

Previously three genes were characterized in mammals encoding AOC proteins: the AOC1 or ABP1 gene encodes diamine oxidase (DAO), a soluble enzyme that inactivates histamine and other diamines (Schwelberger, 2004), the AOC2 gene encodes retina-specific amine oxidase (RAO), a membrane protein of unknown function (Imamura et al., 1997), and the AOC3 gene encodes vascular adhesion protein-1 (VAP-1), a peripheral plasma membrane protein that has been implicated in leukocyte adhesion and glucose uptake (Salmi and Jalkanen, 2001; Zorzano et al., 2003). So far it was not clear if SAO is derived from one of these known AOC members or if it is encoded by a separate gene. Using transgenic and cell culture models it was shown that the large extracellular part of the VAP-1 protein can be released by proteolytic cleavage giving rise to a soluble enzyme detectable in the circulation (Abella et al., 2004; Stolen et al., 2004). Although these studies demon-

Correspondence: Dr. Hubert Schwelberger, Labor für Theoretische Chirurgie, Universitätsklinik für Chirurgie, Medizinische Universität Innsbruck, Schöpfstraße 41, 6020 Innsbruck, Austria
e-mail: Hubert.Schwelberger@i-med.ac.at

strated that VAP-1 can be released from endothelial cells and adipocytes at least in humans and rodents and that this proteolytic VAP-1 release may be the only source of SAO activity in mice it was questionable if this process also leads to the high SAO activity found in most other mammals.

Recently we cloned and characterized four genes encoding homologous AOC proteins in the pig and obtained evidence that there are no further AOC genes in this species (Schwelberger, 2006). This knowledge of the genetic repertoire and the characterization of the amine oxidase enzyme from porcine serum facilitated the investigation of the identity and source of SAO in the pig and the evaluation of the situation in other mammalian species.

Materials and methods

Four complete genes and the corresponding cDNAs encoding AOC proteins were cloned from porcine DNA libraries by screening with partial AOC cDNA probes and by PCR based techniques and their sequences were determined and assembled as will be described in detail elsewhere (manuscript in preparation). The porcine sequences were used to identify and compare orthologous genes in other mammalian species deposited in the NCBI genome database (www.ncbi.nlm.nih.gov/mapview) employing the BLAST program with customized parameters (Altschul et al., 1990).

For purification of porcine SAO, blood was collected from the jugular vein of adult female pigs, allowed to clot for 30 min at 37°C, centrifuged for 10 min at 10,000 × *g* at 4°C, and the serum was stored at −20°C until used. SAO was purified to near homogeneity from porcine serum by consecutive chromatography on Concanavalin A-Sepharose (affinity binding), Phenyl-Sepharose (hydrophobic interaction), Heparin-Sepharose (affinity exclusion), and CIM-QA (anion exchange) using an FPLC instrument (details of the procedure available upon request). All chromatographic equipment and media were obtained from Amersham Biosciences (Vienna, Austria) except for CIM-QA that was purchased from BIA Separations (Ljubljana, Slovenia). Proteins in the chromatographic fractions were analyzed by SDS polyacrylamide gel electrophoresis (Laemmli, 1970). For analysis of SAO under non-reducing conditions, 2-mercaptoethanol was omitted from the sample buffer.

The apparent molecular weight of native SAO was determined by size exclusion chromatography on a Superdex 200 HR 10/30 column (Amersham Biosciences). Protein concentration was determined according to Bradford (1976). Amine oxidase activity was determined radiometrically with [7-^{14}C]benzylamine as the substrate (Weiss et al., 2003) and specific activity was calculated in U/mg protein (1 U = 1 µmol/min). SAO was degly-

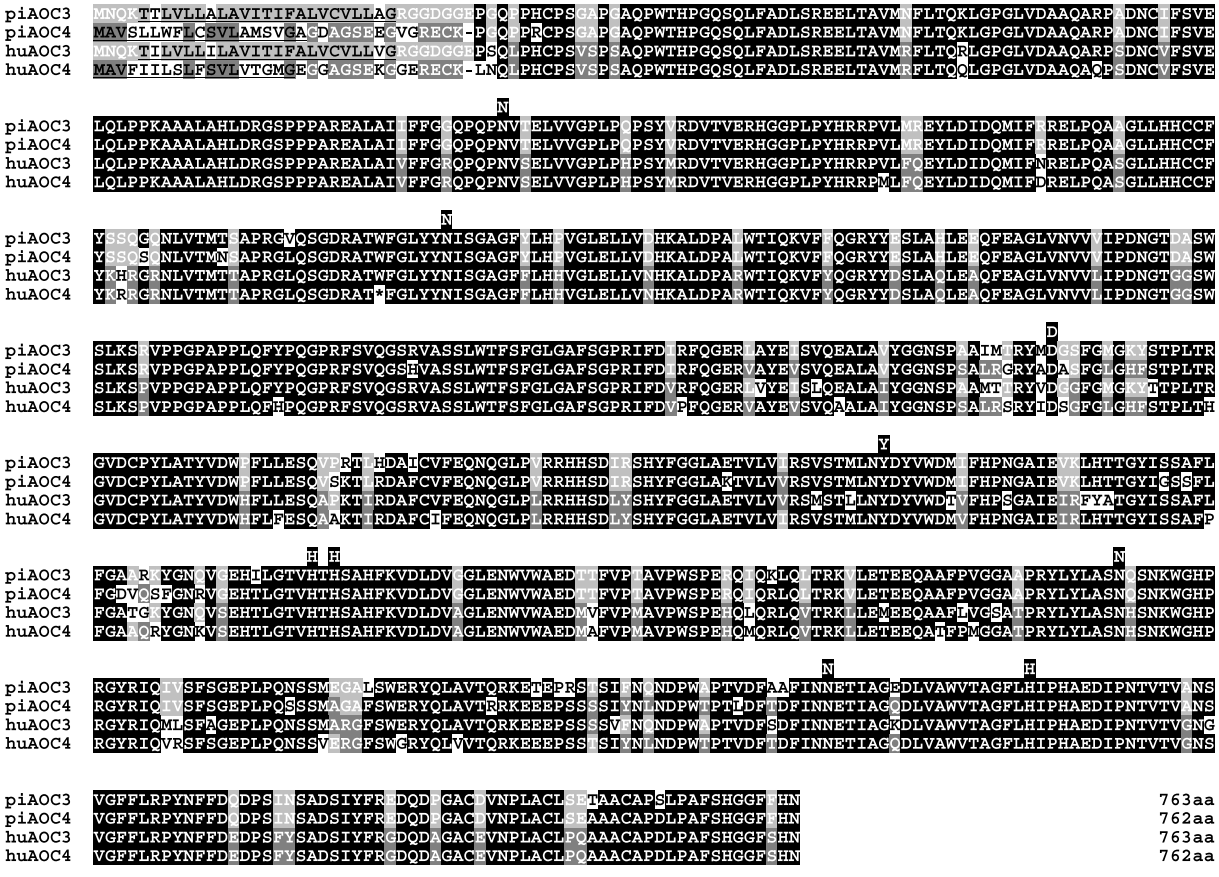


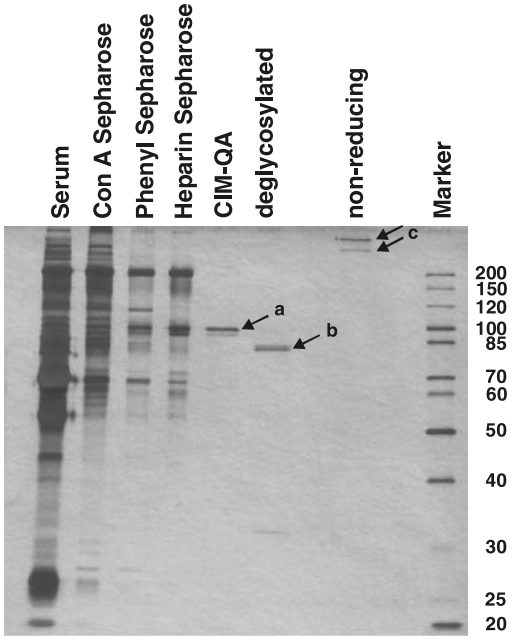
Fig. 1. Sequence alignment of porcine and human AOC3 and AOC4 proteins. Polypeptide sequences deduced from the cloned cDNAs and genomic DNAs were aligned and identical amino acid residues are indicated by black and grey shading. Amino acid residues important for catalytic function, including the TPQ precursor tyrosine (*Y*), the three histidines (*H*) binding the copper ion, and the catalytic base (*D*) as well as four conserved N-glycosylation consensus sites (*N*) are marked above the sequences. The predicted N-terminal transmembrane domains in AOC3 and the signal peptide sequences in AOC4 are underlined. The position of the stop codon in human AOC4 is marked by an asterisk (*)

cosylated after heat denaturation of 10 µg protein in 20 mM bis-Tris-hydrochloride pH 7.0 containing 1% 2-mercaptoethanol and 0.5% sodium dodecylsulfate for 5 min at 95°C by incubation with one unit N-glycosidase F (Roche, Vienna, Austria) for 60 min at 30°C. The N-terminal amino acid sequence of porcine SAO was determined from 10 µg of the purified protein by Edman degradation using an Applied Biosystems Procise 492 sequencer.

Results

Nucleic acid hybridization with cDNA probes, PCR amplification with specific primers and DNA sequencing analyses showed that the porcine genome contains four complete genes encoding copper-containing amine oxidases designated AOC1-4 (Schwelberger, 2006; Schwelberger, unpublished results). Analysis of the sequences and comparison with genomic database entries showed that the AOC1 gene has five exons and encodes diamine oxidase (Schwelberger, 2004). The AOC2, AOC3 and AOC4 genes each have four exons and are tandemly arranged on the same chromosome. AOC2 encodes retina-specific amine oxidase (Imamura et al., 1997), AOC3 encodes vascular adhesion protein-1 (Salmi and Jalkanen, 2001), and AOC4 encodes a protein of 762 amino acid residues highly homologous to VAP-1 but with a predicted signal peptide sequence instead of a transmembrane domain at its N-terminus (Bendtsen et al., 2004) (Fig. 1), hence the name soluble or secretory VAP-1 (sVAP-1). The porcine AOC4 cDNA is highly homologous also to bovine SAO cDNA that was cloned from a liver cDNA library (Mu et al., 1994) and it was found to be expressed almost exclusively in the liver.

Examination of the genome sequences of other mammals revealed the presence of complete orthologs of the porcine AOC1, AOC2, and AOC3 genes. Complete AOC4 genes in a cluster with AOC2 and AOC3 were found in the genomes ofcow, dog, macaque, and man. However, in human AOC4 a single base change converts a codon for a conserved tryptophan at position 225 to a stop codon thus leading to a truncated and non-functional protein (Fig. 1). Interestingly, expression of the human AOC4 mDNA could be detected in the liver and even splice variants of this gene

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Position   1         10        20        30        40        50
cDNA       MAVSLLWFLCSVLAMSVGAGDAGSEEGVGRECKPGQPPRCPSGAPGAQPW
SEQ 1                        GDAGSEEGVGR.....................
SEQ 2                          GSEEGVGR.....................
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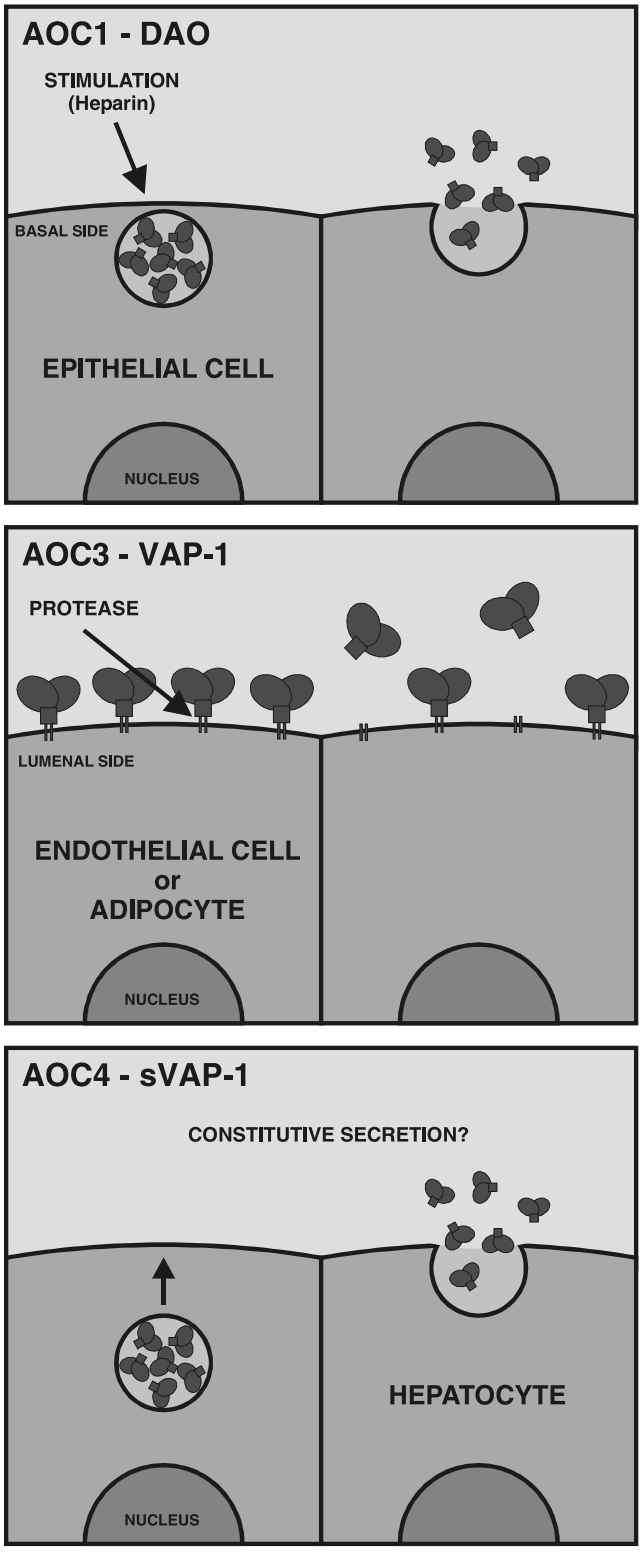
Fig. 2. Characterization of the amine oxidase enzyme from porcine serum. (Upper) Proteins from each chromatographic step of the purification of pig SAO were separated on a 10% SDS polyacrylamide gel and silver-stained. The migration positions of (*a*) monomeric SAO (97 kDa), (*b*) deglycosylated monomeric SAO (82 kDa), and (*c*) oligomeric SAO (400–800 kDa) are indicated by arrows. The sizes of marker proteins in kDa are shown on the right. (Lower) Alignment of the polypeptide sequence deduced from the porcine AOC4 cDNA (cDNA) and of the N-terminal sequences determined for the purified porcine SAO protein (SEQ1, SEQ2)

had been reported earlier (Cronin et al., 1998). The genomes of mouse and rat contain only small fragments of an AOC4 gene.



To determine which of the four porcine AOC genes encodes SAO, the amine oxidase enzyme from porcine serum was purified to near homogeneity (Fig. 2). When analyzed under reducing conditions, porcine SAO has a subunit molecular weight of 97 kDa. Removal of N-linked oligosaccharide chains by treatment of the denatured protein with N-glycosidase F resulted in a subunit molecular weight of 82 kDa, which is very close to the 81.8 kDa calculated from the AOC4 cDNA sequence. Whereas the protein migrates at 400 and 800 kDa under non-reducing conditions, an apparent molecular weight of 211 kDa was determined for native porcine SAO by size exclusion chromatography, which corresponds with a homodimeric structure as found for other copper amine oxidases.

Determination of the N-terminal amino acid sequence of the purified protein yielded two sequences of comparable intensity that turned out to be identical to the sequences deduced from the AOC4 cDNA sequence starting at positions 20 and 23, respectively, after the translation start codon (Fig. 2). The N-terminal sequences of purified porcine SAO did not match any amino acid sequences deduced from the AOC1, AOC2 or AOC3 cDNAs or any other sequences deposited in the NCBI databases. Thus the amine oxidase present in pig serum or plasma is the product of the AOC4 gene and the mature protein is derived from the primary translation product by cleavage of a 19 or 22 amino acid signal peptide and addition of N-linked carbohydrate residues.

Interestingly, a minor band of 92 kDa was consistently co-purified on all chromatographic media indicating that this protein should have very similar molecular properties

Fig. 3. Models for the cellular release of AOC proteins. (Upper) DAO, the product of the AOC1 gene, is expressed mainly in intestinal and kidney epithelial cells where the enzyme is stored in vesicles at the basolateral plasma membrane. DAO is released locally upon stimulation with heparin being the best characterized DAO release stimulator. Only a minor fraction of the released DAO will appear in the peripheral circulation where it disappears with a half-life of about an hour. (Middle) VAP-1, the product of the AOC3 gene, is anchored in the plasma membrane by its N-terminal transmembrane domain with the main portion of the protein facing the cell exterior. Recently it has been shown that the extracellular part of VAP-1 can be released by proteolytic cleavage and this can occur in both endothelial cells and adipocytes. In humans and rodents that lack a functional AOC4 gene, this proteolytic VAP-1 release likely leads to the low constitutive plasma amine oxidase activity present in these species. If the same proteolytic release process can happen with retina amine oxidase, the product of the AOC2 gene, is presently not known. (Lower) The source of the high plasma amine oxidase activity present in pigs and probably in most other mammals is the product of the AOC4 gene. This gene is expressed mainly in the liver and encodes a protein that is highly homologous to VAP-1 but has at its N-terminus a signal peptide sequence rather than a transmembrane domain. The AOC4 protein is a soluble enzyme that is probably constitutively produced and secreted by hepatocytes

to SAO (Fig. 2). It is tempting to speculate that this co-purifying protein could be the product of the partial proteolytic release of the membrane-associated VAP-1 (AOC3 gene product) that shares 93% amino acid sequence identity with the AOC4 protein (Fig. 1). Unfortunately the amounts obtained of this minor band were insufficient for amino acid sequence analysis.

Discussion

The cloning of four genes encoding porcine copper-containing amine oxidases facilitated the re-evaluation of the identity and source of plasma amine oxidase in this species. The results show that in the pig the major part of SAO is the product of the AOC4 gene designated sVAP-1. This newly discovered member of the AOC family is expressed mainly in the liver, has an N-terminal signal peptide sequence and carries asparagine-linked carbohydrate residues. Considering that the enzymatic activity of the protein in liver tissue is quite low it appears that the AOC4 gene product is a constitutively secreted enzyme produced by hepatocytes.

It is intriguing that a functional AOC4 gene is also present in cow and dog, which like the pig have a high SAO activity, whereas humans and rodents, which have a defective or absent AOC4 gene, respectively, have only a very low SAO activity that is probably derived from proteolytic release of the large extracellular part of the membrane associated AOC3 gene product VAP-1 (Abella et al., 2004; Stolen et al., 2004). Proteolytic VAP-1 release may also contribute to a minor part of SAO activity in other species and analysis of the minor 92 kDa band co-purifying with porcine SAO might confirm this. For other species with high SAO activity the AOC4 gene status is presently not known but it can be predicted that these very likely possess a functional AOC4 gene. Definite proof of the notion that the AOC4 gene product is responsible for the high SAO activity present in most mammals requires determination of both the genomic sequences and of partial polypeptide sequences of the purified plasma enzymes as done here for the pig.

The conservation of the DNA and polypeptide sequences and of the gene structures implies that the mammalian AOC genes were derived by duplication events from an ancestral gene with AOC3 and AOC4 having split off last. It is interesting that mouse and rat have either not obtained or already lost a complete copy of AOC4 whereas humans have retained a defective copy of this gene. Apparently humans and rodents do quite well without the AOC4 gene and the resulting low SAO activity. Considering the much higher SAO activity in most other mammals it is not clear how a moderate increase of SAO activity observed in a number of human diseases could possibly cause any damage (Boomsma et al., 2003; Yu et al., 2003). However, the increase in released VAP-1 could just be indicative of an increase of the membrane-associated VAP-1 at the cell surface where the actual damage is done.

Figure 3 schematically summarizes the contribution of the AOC gene products to the amine oxidase activity present in mammalian blood plasma. DAO, the product of the AOC1 gene, is usually stored inside cells, released only upon stimulation and rapidly cleared from the circulation after release (Schwelberger, 2004). So DAO is normally not present in the plasma and in contrast to SAO it prefers diamines not monoamines as substrates. Both the AOC2 and AOC3 gene products are membrane proteins that should not appear in the circulation in soluble form. However, it has been shown that the AOC3 gene product VAP-1 can be proteolytically released from endothelial cells and adipocytes resulting in low SAO activity (Abella et al., 2004; Stolen et al., 2004). Whether this can also happen with the AOC2 gene product RAO has not been studied. Finally, it was shown here that the AOC4 gene product, termed soluble or secretory VAP-1, is responsible for the high constitutive plasma amine oxidase activity in the pig and this is likely true also for most other mammals.

Acknowledgements

This work was supported by grant P14923-B05 from the Austrian Science Fund. I would like to thank A. Drasche and J. Feurle for excellent technical assistance and H. Lindner for protein sequence analysis.

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