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Semicarbazide-sensitive amine oxidase substrates in obesity and diabetes

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1. Content

2. Abbreviations

2-DG	**2**-**D**eoxy**g**lucose
3-MTPPA	**3**-(4-**M**ethyl**t**hio**p**henyl)**p**ropylamine
4-PBA	**4**-**P**henyl**b**uthyl**a**mine
5'-AMP	**A**denosine 5'-**M**ono**p**hosphate
AC	**A**denyl **C**yclase
ACC	**A**cetyl-**C**oA **C**arboxylase
Acetyl-CoA	**Acetyl**-**Co**enzyme **A**
ACS	**A**cetyl-**C**oA **S**ynthase
AGE	**A**dvanced **G**lycation **E**ndproduct
AMPK	**AMP**-activated Protein **K**inase
AO	**A**mine **O**xidase
AOC3	This gene codes SSAO
APJ	7 transmembrane domains receptor of apelin
AR	**A**mplex **R**ed
ATGL	**A**dipocyte **T**ri**glyceride L**ipase
ATP	**A**donosine **T**ri**p**hosphate
AUC	**A**rea **U**nder the **C**urve
B_6V_{10}	**B**enzylamine-**V**anadate complex salt by Gene Medica
BSA	**B**ovine **S**erum **A**lbumin
BzA	**B**en**z**yl**a**mine
cAMP	**C**yclic **A**denosine **M**ono**p**hosphate
CAO	**C**opper **A**mine **O**xydase
cd31	Adipocyte differentiation marker
cd45	Leucocyte marker
cGMP	**C**yclic **G**uanosine **M**ono**p**hosphate
CRP	**C**-**R**eactive **P**rotein
CTRL	**C**on**tr**o**l**
DAG	**D**i**a**cyl-**g**lycerol
DAO	**D**iamine **O**xidase
db -/-	Mice having null mutation for db gene
db -/?	Heterozygotes and mice having null mutation
db -/+	Heterozygote mice for db gene mutation
DG	**D**i**g**lyceride
DNA	**D**eoxy**r**ibonucleic **A**cid
ECD	**E**ndothelial **C**ell **D**ysfunction
ECM	**E**xtra**c**ellular **M**atrix
eNOS	**E**ndothelial **NOS**
ER	**E**ndoplasmic **R**eticulum
ERK1/2	**E**xtracellular signal-**r**egulating **K**inase
F 4/80	Adipocyte differentiation marker
FAD	**F**lavin **A**denine **D**inucleotide
FAS	**F**atty **A**cid **S**ynthase
FATP	F**FA t**rans**p**orter
FFA	**F**ree **F**atty **A**cid
GAPDH	**G**lyceryn**a**ldehyde-**p**hosphate **D**e**h**ydrogenase
Gi	**I**nhibiting **G**-protein
GLUT-1	**Glu**cose **T**ransporter-**1**
GLUT-4	**Glu**cose **T**ransporter-**4**
GPCR	**G**-**p**rotein **C**oupled **R**eceptor

Gs	**S**timulating **G**-protein
H_2O_2	Hydrogene peroxide
HbA1c	**H**emoglo**b**ine **A 1**C
HFD	**H**igh **F**at **D**iet
HOMA index	**H**omeostasis **M**odel **A**ssessment **I**ndex
HSL	**H**ormone **S**ensitive **L**ypase
IKK	**I**nhibitor **K**appaB **K**inase
IL-6	**I**nter**l**eukine-**6**
Ins	**Ins**ulin
INWAT	**In**tra-abdominal **W**hite **A**dipose **T**issue
IPGTT	**I**ntra**p**eritoneal **G**lucose **T**olerance **T**est
IRS-1	**I**nsulin **R**eceptor **S**ubstrate **1**
IRS-2	**I**nsulin **R**eceptor **S**ubstrate **2**
IRS-3	**I**nsulin **R**eceptor **S**ubstrate **3**
Iso	**Iso**prenaline
JNK	**J**anus **K**inase
Km	Michaelis-Menten konstant
KO AOC3	**K**nock **O**ut for **AOC3** gene
LO	**L**ysyl **O**xidase
LPL	**L**ipo**p**rotein **L**ipase
LTQ	**L**ysine **T**yrozyl**q**uinone
Malonyl-CoA	**Malonyl**-**Co**enzyme **A**
MAO	**M**ono**a**mine **O**xydase
MAPK	**MAP**-**K**inase
MG	**M**ono**g**lyceride
MG	**M**ethyl**g**lyoxal
MGL	**M**ono**g**lyceride **L**ipase
MRI	**M**agnetic **R**esonance **I**maging
NC-IUBMB	**N**omenclature **C**omittee of the **I**nternational **U**nion of **B**iochemistry and **M**olecular **B**iology
NO	**N**itric **O**xide, **N**itrogen Mon**o**xide
NOS	**NO S**ynthase
NS	**N**ot **S**ignificant
OH •	Hydroxyl Radical
PAGE	**P**oly**a**crlyamide **G**el **E**lectrophoresis
PAI-1	**P**lasminogen **A**ctivator **I**nhibitor **1**
PAO	**P**oly**a**mine **O**xidase (FAD-containing AOs)
PBS	**P**hosphate **B**uffer **S**aline
PDE-3B	**P**hospho**die**stherase **3B**
PGWAT	**P**eri**g**onadal **W**hite **A**dipose **T**issue
PI-3 kinase	**P**hosphatidyl **I**nositol **3 Kinase**
PKA	**P**rotein **K**inase **A**
PKB	**P**rotein **K**inase **B**
PKC	**P**rotein **K**inase **C**
PRWAT	**P**eri**r**enal **W**hite **A**dipose **T**issue
RNS	**R**eactive **N**itrogen **S**pecies
ROS	**R**eactive **O**xygen **S**pecies
SCWAT	**S**ub**c**utaneous **W**hite **A**dipose **T**issue
SDS	**S**odium **Di**s**u**lphide
SOCS	**S**upressors **O**f **C**ytokine **S**ignaling
SOD	**S**uper**o**xide **D**ismutase
SSAO	**S**emicarbazide **S**ensitive **A**mine **O**xydase

STAT	**S**ignal **T**ransducers and **A**ctivators of **T**ranscription
STZ	**St**repto**z**otocin
TBS	**T**RIS **B**uffer **S**aline
TBST	**T**RIS **B**uffer **S**aline **T**ween
TE	**T**RIS **E**DTA Buffer
TGs	**T**ri**g**lyceride**s**
Tie 2	Endothelial cell differentiation marker
TNF-α	**T**umor **N**ecrosis **F**actor **α**
TPQ	**T**o**pa**q**u**inone
UCP	**U**n**c**oupling **P**rotein
GlcNAc	**N**-**Ac**etyle **Gl**u**c**oseamine
V	**V**anadate
VAP-1	**V**ascular **A**dhesion **P**rotein **1**
VHFD	**V**ery **H**igh **F**at **D**iet
VLDL	**V**ery **L**ow **D**ensity **L**ipoprotein
Vmax	**Max**imal Reaction **V**elocity
WAT	**W**hite **A**dipose **T**issue
WT	**W**ild **T**ype
α2-AR	**α2**-**A**drenergic **R**eceptor
β-AR	**β**-**A**drenergic **R**eceptor

3. Introduction

The number of obese individuals increases worldwide, leading to consider obesity as a global epidemic. It has been estimated that, in 2005, approximately 1.6 billon adults were overweight and more than 400 million adults were obese. Nowadays, around 15 % of the world's population is overweight or obese, according to the International Association for the Study of Obesity (website: http://www.iaso.org). Obesity is recognized as a major risk factor for type 2 diabetes, cardiovascular diseases, non-alcoholic fatty liver diseases and certain types of cancers. Insulin resistance (namely the deficiency of response to insulin, leading to very blunted glucose uptake into cardiac or skeletal muscles and adipose tissues) is a metabolic disturbance that firmly links excess of white adipose tissue (WAT) with the obesity-related complications.

When considering the number of diabetic patients, type 1 diabetes represents a minor part, approximately 10% of all patients with diabetes. However, taking into account that these patients suffer from their disease from early on in their lives, they also merit serious attention because of late complications. Today's modern lifestyle with greatly reduced physical activity resulted in the increasing number of obese and thus insulin-resistant subjetcs, also among type 1 diabetic patients. Moreover, diabetic patients have very high risk for vascular complications. Therefore new therapeutic targets to mimic the effetcs of insulin, but acting independently of insulin are of great interest.

Some biogenic amines are well-established neurotransmitters, having a key role in central appetite regulation. Other amines are modulators or pertubators of neuronal functions, not only in mammals but also in insects or crustaceans where they are naturally present. They are also abundant in plants as products of secondary metabolism. Therefore, foods (meat, fish, vegetables), food additives or various medicinal plants that are world-wide ingested bring substantial amounts of alimentary amines. Once ingested, these dietary amines are mainly metabolised by amine oxidation. In spite of a great amount of amines metabolized in the gut, some of them may reach the circulation and affect different peripheral or central receptors or may also be degraded by different enzymes.

Benzylamine (BzA), a synthetic exogenous amine, has never been used in clinical studies or proposed as nutraceutic by para-medical companies, but has been recently described that it is capable to reduce hyperglycemia in diverse animal models of diabetes. The effect of this amine can be extended to diverse amines which are substrates of monoamine oxidases (MAO) and mainly of semicarbazide sensitive amine oxidases (SSAO). Since the enzymes MAO and SSAO are abundantly expressed in adipose tissue, this anatomical location is a key feature in the action of BzA and other amine oxidase (AO) substrates. Further studies are necessary before extraplolating to man the promising anti-hyperglycemic effects of this family of agents.

3.1. Amine oxidases

AOs are widely used by both autotrophic and heterotrophic life forms. They catalyse the metabolism of different mono- and polyamines. Their physiological importance is still not entirely understood, though their investigation grew more and more intensive in recent years. Their substrates are endogenous or exogenous, both decomposing by oxidative deamination that can be described by the following equation:

$$R\text{-}CH_2\text{-}NR'R'' + O_2 + H_2O \rightarrow R\text{-}CHO + H_2O_2 + R'R''NH$$

During the process aldehyde, hydrogen peroxide and an amine or ammonium (in case of a primary amine) are formed. AOs can be classified by the chemical structure of their substrates: monoamine oxidases (MAOs), diamine oxidases (DAOs), polyamine oxidases (PAOs) and lysyl oxidases (LOs).

Table 1. Classification of amine oxidases. Adapted from Jalkanen *et* al., 2001. For abbreviations see the text.

	Amine oxidases (AOs)						
	FAD-containing AOs			CAOs			
	MAO	PAO	renalase	DAO	SSAO	RAO	LO
Cofactor	FAD	FAD	FAD	TPQ	TPQ	TPQ	LTQ
Coding human gene	**MAO-A, MAO-B, chromosome X**			**AOC1, chromo-some 17**	**AOC3, chromosome 17**	**AOC2, chromosome 17**	**LOX1-4, chromosome 10**
Presence	**mitochondria**	**intracell.**	**soluble**	**intracell.**	**extracell., soluble**	**extracell.**	**extracell.**
Substrates	**noradrenaline dopamine adrenaline β-phenylethyl -amine tyramine tryptamine BzA octopamine**	**spermine spermi-dine**	**nor-adrenaline dopamine adrenaline**	**putrescine, cadeverine histamine**	**BzA, methylamine aminoacetone**	**β-phenylethyl-amine tryptamine p-tyramine**	**lysine**
Inhibitors	**pargyline clorgyline selegyline**	**carbonyl reactive agents**	**not known**	**semi-carbazide, hydroxyl-amine**	**semicarbazide, hydroxylamine**	**semi-carbazide**	**semicarbazide hydroxylamine**
Effect	**neuro-transmission**	**cell growth**	**cardiac function, blood pressure**	**Histamine-degradation cell division**	**amine oxidation, glucose uptake, leucocyte-adhesion**	**not known**	**forming of ECM**

The traditional classification of AOs divides them into two main groups, based on the chemical nature of the attached cofactor (Table 1.). The FAD- and topa-quinone containing (TPQ) AOs not only differ in their cofactors, but are also distinct in terms of their subcellular distribution, substrates, inhibitors and biological functions. A third classification based on the enzyme reaction is proposed by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB).

The flavin adenine dinucleotide (FAD)-contaning enzymes are also classified as amine:oxygen oxidoreductase (deaminating) EC 1.4.3.4 by NC-IUBMB:

MAO-A and –B are well known mitochondrial enzymes that have firmly established roles in the metabolism of neurotranssmitters and other biogenic amines (Shih *et al.*, 1999).

FAD-containing PAOs use secondary amines spermine and spermidine as their preferred substrates, and thereby possibly regulate cell growth (Seiler, 1995).

Renalase is the most recently found FAD-containing AO (Xu *et al.*, 2005). It is released by the kidney, oxidises chatecholamines and regulates cardiac function and blood pressure, however independently of its enzyme ctivity (Boomsma *et al.*, 2007).

The TPQ containing enzymes are collectively designated as SSAOs due to their characteristic sensitivity of inhibition by a carbonyl-reactive compound, semicarbazide. They were also cited as copper amine oxidases (CAOs), however NC-IUBMB has recently divided them to EC 1.4.3.21 - primary-amine: oxygen oxidoreductase (deaminating) and EC 1.4.3.22 -histamine:oxygen oxidoreductase (deaminating).

The TPQ-containing enyme DAO prefers diamines putrescine, cadaverine and histamine as its substrate. Diamine oxidase is mainly an intracellular enzyme preferentially synthetised in the placenta, kidney and intestine. The secreted form binds in a heparin-dependent manner to endothelial cells. It is important in regulating infammation and allergic reactions.

The other SSAOs are mostly soluble or expressed on the cell surface, have methylamine, aminoaceton, BzA as substrates, are insensitive or only weakly sensitive to classical MAO inhibitors, like clorgyline or deprenyl, and mediate different biological functions.

The retina specific amine oxidase (RAO) is a cell surface enzyme, present in the retina. It prefers β- phenylethylamine, tryptamine and p-tyramine as substrates. Its proper function is yet to be understood (Imamura *et al.*, 1997; Kaitaniemi *et al.*, 2009).

LO, another copper containing enzyme is also inhibited by SSAO inhibitors, but only to a limited extent, compared to other SSAOs. Its classification is still disputed, hence there are several points where it differs from other SSAOs: its structure misses some conservative motifs and its cofactor lysine tyrozylquinone (LTQ) is also slightly different. The ε-amino groups are its excellent substrates. It is able to establish cross-links between collagen and elastin during the formation of the extracellular matrix (ECM) (Smith-Mungo *et al.*, 1998). It is also classified as EC 1.4.3.13-protein-L-lysine:oxygen 6-oxidoreductase (deaminating).

3.1.1. Semicarbazide-sensitive amine oxidases

SSAOs are widely present in nature: they are expressed by plants, microorganisms, animals and men. SSAOs convert primary amines to aldehydes (the only exception is histamine). During the process the concomitant hydrogen peroxide and ammonium are formed. There are two mammalian isoforms known: the membrane-bound and soluble (plasma) isoforms. The majority of the tissular SSAOs can be found in the vascular walls, particularly in smooth muscle, but endothelial cells, adipocytes, chondrocytes, fibroblasts, the retina, the sclera, the kidney, the spleen, the placenta and the bone marrow also show membrane-bound SSAO activity.

TPQ containing SSAOs were discovered some decades ago. The enzyme was cloned and its primary structure was determined. The results were surprising: the structure was identical with that of another protein, mapped before, the vascular adhesion protein (VAP-1) (Smith *et al.*, 1998). The deaminating enzyme activity was also proven in case of the VAP-1 and it also turned out that its distribution in the body is also similar to other SSAOs.

Genes and protein structure

Most SSAOs are dimeric glycoproteins with molecular masses of 140-180 kDa, containing two atoms of copper per dimer (Klinman *et al.*, 1994; Lyles, 1996). An unknown amino acid 6-hydroxydopa (2,4,5-tridydroxyphenylalanine quinone or TPQ) was identified (Janes *et al.*, 1990; Klinman, 1996) being the cofactor. TPQ is generated

from an intrinsic tyrosine molecule by a self-processing event that only requires bound copper ion and molecular oxygen (Mu *et al.*, 1992).

SSAO enzyme has been cloned from several species: cow (Klinman *et al.*, 1994; Mu *et al.*, 1992), mouse (Bono *et al.*, 1998a; Bono *et al.*, 1998b; Moldes *et al.*, 1999), rat (Morris *et al.*, 1997; Ochiai *et al.*, 2005), human (Smith *et al.*, 1998; Zhang *et al.*, 1996). At the moment full length cDNA sequences are available from seven mammalian SSAOs. The human SSAO contains 762 amino acids.

The products of two genes AOC3 and AOC4 have been identified to be responsible for SSAO activity. In man AOC3 and AOC4 are situated in cluster with AOC2, the gene coding the RAO, all in the long arm of chromosome 17. AOC3 gene codes membrane-bound SSAO. So far it is not clear if soluble SSAO is derived from AOC3 or it is encoded by a separate gene. The large extracellular part of the SSAO protein was shown to be released by proteolytic cleavage from endothelial (Stolen *et al.*, 2004b), vascular smooth muscle (Göktürk *et al.*, 2003), liver endothelial cells or adipocytes using transgenic and cell culture models (Abella *et al.*, 2004; Stolen *et al.*, 2004b). At least it is the case in humans and rodents where plasma SSAO activity is very low compared to other mammalian species like cow, dog or pig. In pig, the product of AOC4 gene, situated in a cluster with AOC3 was found to be responsible for such high plasma SSAO activity (Schwelberger, 2006). The mature protein derives from the primary translation product by cleavage of a 19 or 22 amino acid signal peptide and addition of N-linked carbohydrate residues. 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 (Schwelberger *et al.*, 2008). It is intriguing that a functional AOC4 gene is also present in cow and dog, which, similary to the pig, have a high serum SSAO activity, whereas humans and rodents, which have a defective or absent AOC4 gene, respectively, have only a very low serum SSAO activity. Proteolytic AOC3 gene product release may also contribute to a minor part of plasma SSAO activity in cow, dog and sheep.

Conserved regions of the protein

Although we know that SSAOs are not exacly the same depending on the species, the isoform (membrane-bound or soluble) and even the age, there are regions that are the same regardless of these factors.

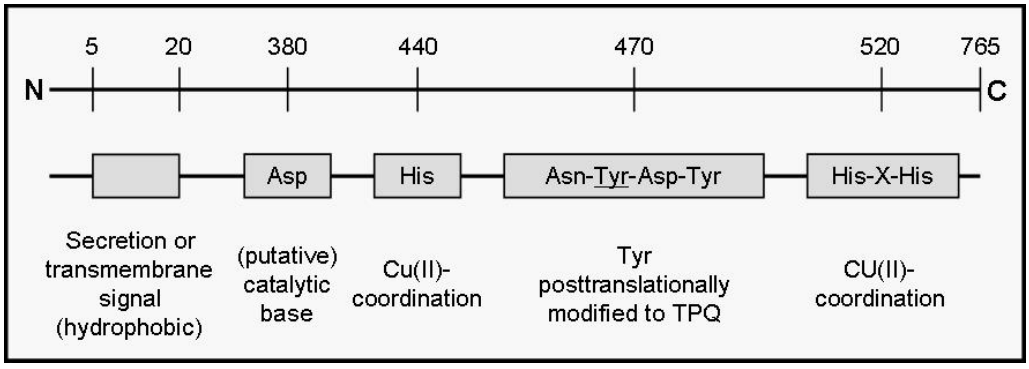

Figure 1. Conserved regions of SSAO protein. Adapted from Jalkanen 2001.

It has been proven that the active site of the enzyme remained practically unchanged during the evolution (Klinman *et al.*, 1994). Figure 1 shows the conserved main caracteritics:

- The protein consists of 763-765 amino acids, 90 kDA, foming a homodimer where the subunits are attached in a bond disulphur.
- 5 amino-acids at N terminal part are positioned intracellular and a transmembrane domain, which is a secretion signal is to 5-20 amino acids from N terminal part.
- The carbon atom has been shown to be in the second position of TPQ is situated 6 Å away from the Cu^{2+} ion.
- There is a Asn-TPQ-Asp/Glu-Tyr sequence in the active site of the enzyme.
- Tyr is situated at 470 or 471 amino acids from N terminal which is post-trancriptionally modified to TPQ.
- The protein contains three conserved histidine-binding Cu^{2+} ion (there is a His-X-His motif situated approximately 50 residues C-terminal from the cofactor and the third one 20-30 residues N-terminal from it).
- Asp is approximately 100 residues N-terminal from TPQ, the catalytic base of the deamination in its reductive half-reaction.
- 6 sites of N-glycosilation (Asn)
- Arg-Gly-Asp motive for binding with integrines.

Generally, amino acid identity among SSAOs isolated from different species but being of the same cellular origin is higher than that of different isoforms of the same species.

3D protein structure

Bovine serum amine oxidase was the first mammalian SSAO characterised 3D (Lunelli *et al.*, 2005). The 3D structure of human SSAO has recently been described (Jakobsson *et al.*, 2005). The SSAO is a homodimer. The TPQ and the Cu^{2+} ion are situated in the active site. The protein is composed of 4 domains:

- N terminal D1 domain of each monomer is a transmembrane helix.
- D2-D4 are located extracellularly.
- The monomers are bound by the D4 domain.
- Leucine residue (Leu 469) located adjacent to the active site has been revealed to function as a gate controlling its accessibility.

An Arg-Gly-Asp motif is displayed on the surface, where it could be involved in integrin binding and possibly play a role in the shedding of SSAO from the membrane. Carbohydrate moieties are observed at five of six potential N-glycosylation sites. Carbohydrates attached to Asn 232 flank the active-site entrance and might influence substrate specificity.

SSAO substrates

SSAOs accept primary amines as substrates, although there may be exceptions to this rule (like histamine). Nevertheless, different species show a wide variety of substrate preferences. Therefore, some caution must be taken when concluding results with SSAOs of different species. In 1996, Lyles summarized that BzA, phenethylamine, tyramine, dopamine, which are also MAO substrates, tryptamine and histamine are good SSAO substrates in rat or human tissue (Figure 2.) (Lyles, 1996). Generally, it would appear that SSAO plays only a small role in metabolizing substrates that are also oxidised by MAO, although it could be more important when MAO is inhibited, or in the rare genetic condition where the MAO genes are deleted (Fitzgerald *et al.*, 2002). Although serotonin is not a substrate for SSAO from most sources, it is a good substrate for the enzyme from pig and human dental pulp (O'Sullivan *et al.*, 2002). The enzyme may also play a role in the metabolism of some xenobiotics, such as mescaline and primaquine (O'Sullivan *et al.*, 2004; Tipton *et al.*, 2004). Among all, BzA has been the preferred substrate for most SSAOs for a long time.

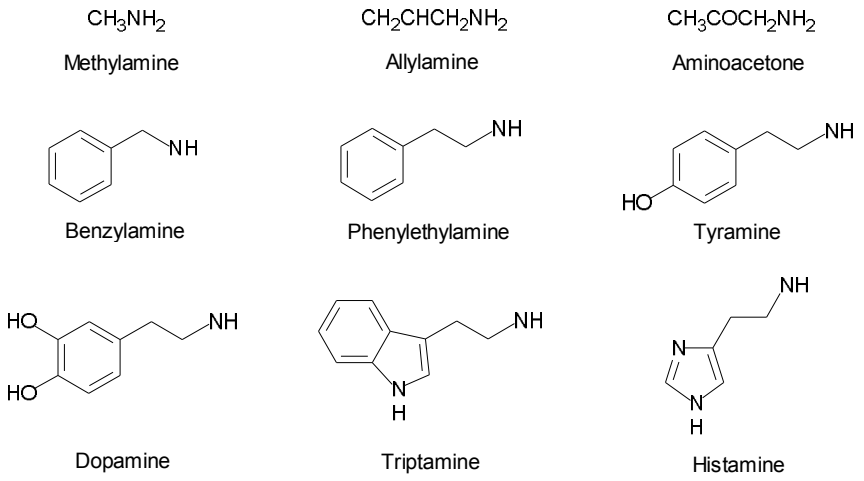

Figure 2. Well-caracterized substrates of SSAO.

SSAO also recognises aliphatic amines. Allylamine, an industrial chemical has been described to cause serious vascular and myocardial damage in several species (Boor *et al.*, 1982; Boor *et al.*, 1979). Vascular SSAO oxidises allylamine to acrolein, acting as a distal toxin, (Boor *et al.*, 1987) responsible for the lesions caused by allylamine intoxication (Boor *et al.*, 1980).

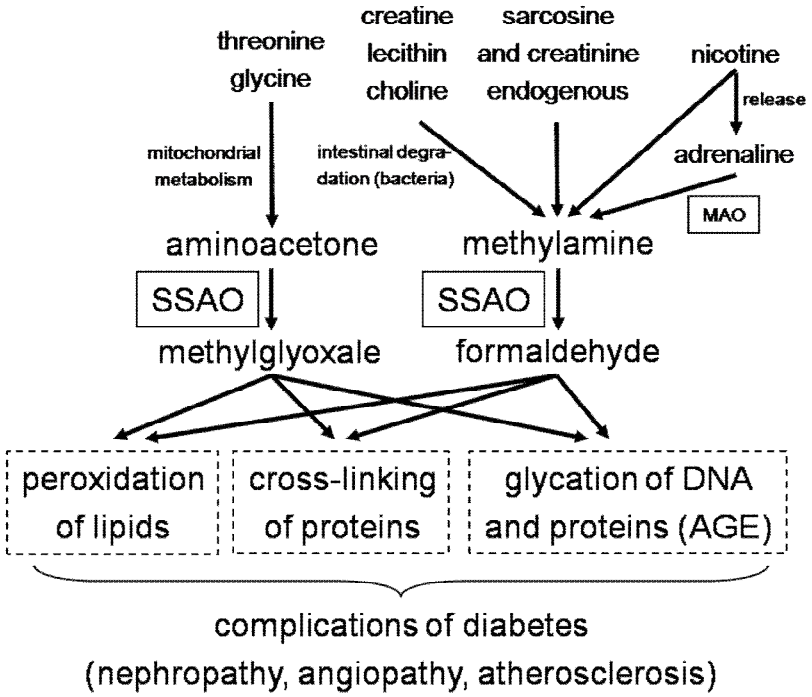

Figure 3. Role of endogenous substrates in the development of angiopathy.

Aminoacetone and methylamine do not have affinity to MAOs (Lizcano *et al.*, 1998). The major source of methylamine is endogenous with contributions from the diet. The role of gut microflora, which varies both within and between individuals, in

processing several chemicals or food supplements to methylamine or suitable precursors should also be considered. Methylamine is produced in several catabolic reactions, including the breakdown of adrenaline (catalysed by MAO) sarcosine, creatine, lecithin and choline. It is also a common component of some foods and beverages, and is an atmospheric pollutant therefore some sources suggested the high levels of SSAO in the lung as a protection against inhaled methylamine and other volatile amines (Lizcano *et al.*, 1998). Methylamine is also a constituent of cigarette smoke and is considered as the major end product of nicotine metabolism. Smoking or nicotine can induce the release of adrenaline, which is in turn deaminated by monoamine oxidase, also producing methylamine (Yu, 1998b). Plasma levels of methylamine vary between 11.5 and 123.4 ng/ml (Li *et al.*, 2004), and may be altered in some pathological conditions such as diabetes, typhoid fever, and liver or renal insufficiency or uremia (Baba *et al.*, 1984; Mitchell *et al.*, 2001).

Methylamine has in fact been shown to be converted to formaldehyde *in vivo* by SSAO (Yu *et al.*, 1997):

$$CH_3NH_2 + O_2 + H_2O \rightarrow HCHO + H_2O_2 + NH_3.$$

The deaminated product formaldehyde is extremely reactive, forming adducts with lysyne side chain of proteins and nucleic acids (Gubisne-Haberle *et al.*, 2004). It is potentially carcinogenic and is a subject of major environmental concern. Some reactive free radicals can be generated from formaldehyde in the presence of hydrogen peroxide:

$$2\ HCHO + H_2O_2 \text{ -»} 2\ H\text{-*C=O} + 2\ H_2O.$$

Formaldehyde and hydrogen peroxide simultaneously generated from deamination of methylamine may act synergistically causing cellular damage (Yu *et al.*, 2003b). Yu et al. found that methylamine by itself is relatively nontoxic towards cultured endothelial cells obtained from both human umbilical vein and calf pulmonary artery. It becomes very toxic, however, in the presence of SSAO. In some pathologies there is an increased risk for formaldehyde formation and consequent oxidative stress (Yu, 1998a; Yu *et al.*, 1998).

It has been proposed that excessive deamination of methylamine (*e.g.* due to increased occurrence or impaired disposal of the amine) may become a potential risk factor responsible for initiation of endothelial injury of diabetic complications (Yu *et al.*, 1993) (see in Figure 3.), and probably disorders associated with stress-related

behaviour (Yu *et al.*, 1997). The association of nicotine or disease-mediated increase of methylamine can contribute to the potential adverse health effects. Selective SSAO inhibitors, e.g. MDL-72974A or aminoguanidine, can effectively protect the cells from SSAO-methylamine induced damage (Yu *et al.*, 1993).

Aminoacetone, which may be formed as a result of threonine or glycine metabolism, is oxidised by SSAO to form methylglyoxal:

$$CH_3COCH_2NH_2 + O_2 + H_2O \rightarrow CH_3COCHO + H_2O_2 + NH_3.$$

Methylglyoxal (MG) is cytotoxic and mutagenic. Although there are other pathways of aminoacetone breakdown, SSAO makes a significant contribution to the process (Mathys *et al.*, 2002). MG is suggested to induce formation of oxygen free radicals and chemical modification of essential proteins by reacting with arginine, lysyne and cystein residues. MG modifies cell proteins non-enzymatically through the Maillard reaction, in which aldehydes and ketones react with ε-amino groups of lysine residues and guanidino groups of arginine residues resulting in stable chemical adducts in proteins known as advanced glycation end products, or AGEs (Monnier *et al.*, 1992). AGEs have been implicated in diverse long-term complications of diabetes, including atherosclerosis, retinopathy, nephropathy, and cataract formation. Because MG appears to be a major precursor of AGEs *in vivo* (Brownlee, 2001), any reaction that increases MG levels in tissue or plasma could ultimately lead to these complications.

There is a growing number of evidence proving the existence of *in vivo* SSAO-dependent oxidation of amonoacetone and methylamine:

- Urinary formaldehyde was in correlation with plasma SSAO activity when testing different mouse models (Yu *et al.*, 1998).
- Methylamine and aminoacetone administration resulted in higher urinary excretion of formaldehyde and methylglyoxal in diabetic rats (Deng *et al.*, 1998).
- Increased urinary levels of methylamine were found following SSAO inhibition in different rodent models (Yu *et al.*, 1998).
- Some studies report an increase of urinary excretion of methylamine following administration of nicotine or creatine and the subsequent production of formaldehyde and cross-linkage with tissue constituents (Poortmans *et al.*, 2005; Yu, 1998b).

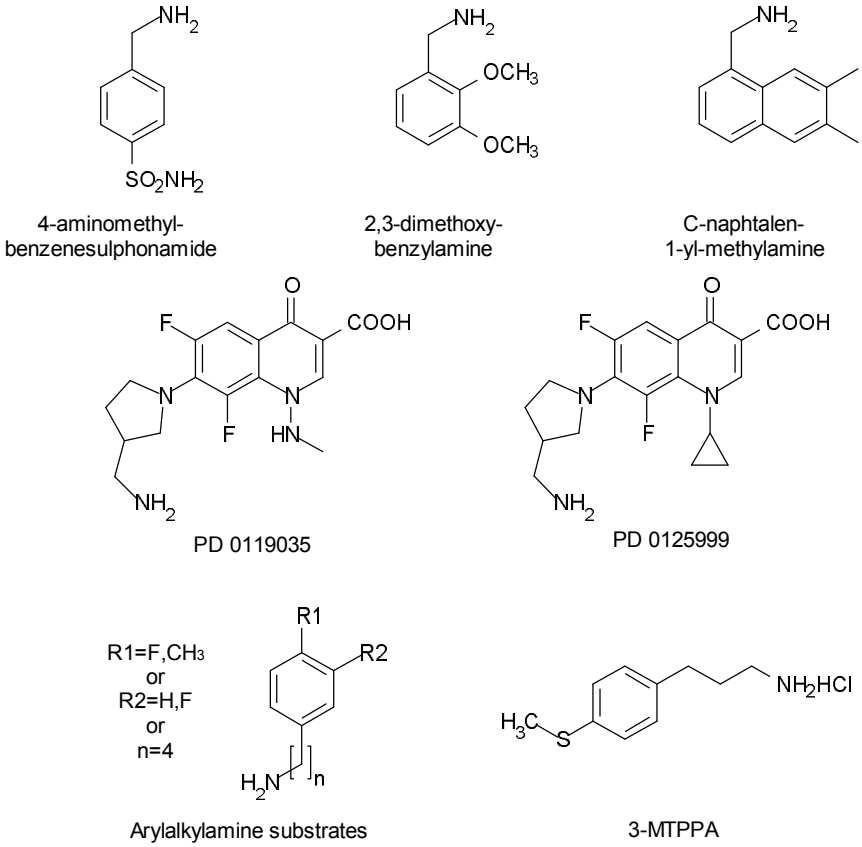

Figure 4. Novel subtrates identified after Dunkel *et al.*, 2008 and Yraola *et al.*, 2009.

The characterisation of the 3 D structure of the protein promoted the chemical design of new SSAO substrates. Homology modeling of the catalytic site of the mouse SSAO was performed by the group of Marti and pharmacophoric motif based on BzA was constructed (Marti *et al.*, 2004). Their model resulted in 5 substrates having arylalkylamine or aminomethyl-pyrrolidine structure. Yraola has then published structure-activity relationship of arylalkylamines in human and mouse enzyme preparations (Yraola *et al.*, 2006), the best substrates of the screening are illustrated in Figure 4. Unzeta and coworkers synthesised 3-(4-methyltiophenyl) propylamine (Gallardo-Godoy *et al.*, 2004).

SSAO inhibitors

The enzyme is characterised by its sensitivity towards semicarbazide, however, this hydrazine derivative has a relatively weak inhibitor capacity. Inhibitors can be conveniently subdivided into the main groups of hydrazine derivatives, arylalkylamines, propenyl- and propargylamines, oxazolidinones, and haloalkylamines.

Table 2. Some important inhibitors of SSAO.

Pharmacons having SSAO-inhibiting capacity				
chemical structure/group	Compounds	SSAO inhibition	MAO inhibition	other characteristics
Hydrazine Derivatives	semicarbazide	weak (1mM), irreversible	no	inhibits LO and DAO
	aminoguanidine	potent, irreversible	weak	weak NOS inhibitor
	isoniazid	Weak (1mM)	weak	antituberculoticum
	hydralazine (neprezole)	irreversible (μM)	reversible	antihypertensive drug
	phenelzine		irreversible MAO	antidepressant
	carbidopa			anti-Parkinson drug
	benserazide			anti-Parkinson drug
	procarbazine	selective reversible strong	no	anticancer drug
	LJP-1207	potent (nM) competitive, irreversible		
Arylalkylamine Derivatives	B24	potent (μM), reversible	no	
	mexiletine			antiarrythmic drug
Propenylamines	MDL 72974A	potent (10 nM)		
	MDL 72145	potent (10 nM)	MAO-B irreversible, competitive MAO-A	anti-Parkinson drug candidate
Propargylamine Derivatives	propargylamine	potent (μM), irreversible	weak	
	β-aminopropionitrile	weak (200 μM), competitive reversible		LO inhibitor at μM
Haloalkylamine Derivatives	2-bromoethylamine	potent, irreversible	no	
Oxazolidinone Derivatives	MDL 220662	potent (μM)		
Antidepressants with different chemical structures	imipramine			antidepressant
	maprotiline			antidepressant
	zimeldine			antidepressant
	nomi fensine			antidepressant
Hydrazinoalcohols	BTT-2052	potent		
	amikacin tobramycin puromycin gentamicin			aminoglycoside antibiotic

Of them, aryl(alkyl)hydrazines, and 3-halo-2-phenylallylamines are generally very strong SSAO inhibitors. Most of these inhibitors of SSAO have been originally

developed for other purposes, or they are simple chemical reagents with highly reactive structural element(s); these compounds have not been able to fulfil all criteria of high potency, selectivity, and acceptable toxicity. New potent compounds have recently been developed with the structures: oxazolidinone; hydrazinoalcohols; 4,5,6,7-tetrahydroimidazo[4,5-c]pyridines; thiocarbamoyl derivatives; carboxamides and sulfonamides; tetraphenylphosphonium analogues; 1,3,4-oxadiazines. There are also alternative ways to inhibit SSAO. Some biomolecules with a free NH_2 group, such as peptides with lysine side-chain, aminohexoses or aminoglycoside antibiotics can interact with the enzyme. Function-blocking antibodies to hVAP-1, as well as small interfering RNAs could be applied for modulation of the adhesion molecule. There is a demand for novel selective and non-toxic inhibitors, which may be useful tools for further understanding the roles and function of SSAO. SSAO inhibitors may even be valuable substances for the treatment of various diseases (Dunkel *et al.*, 2008; Matyus *et al.*, 2004). Table 2. presents the most important SSAO inhibitors.

The physiological and pathophysiological role of semicarbazide sensitive amine oxidases

The physiological role of SSAOs is not fully understood yet. Until recently it was assumed that their most important effect is to decrease the level of monoamines by oxidative deamination. The elevated level of the known endogenous substrates, methylamine and aminoacetone, is harmful, therefore it seems reasonable to keep them under control. Some novel functions of SSAO have been characterised: they may play a role in inflammatory processes, in the regulation of the glucose transport of adipocytes and in the maturation of collagen and elastin.

3.1.2. The oxidative deamination

SSAOs catalyse oxidative deamination of mainly primary amines. The reaction consists of two parts. In the first step the enzyme is reduced by the substrate, itself being oxidated to the corresponding aldehyde (Figure 5, 1-4). In the second half-reaction the enzyme is reoxidated by molecular oxygen with conformant release of hydrogen peroxide and ammonium (Figure 5, 5-6).

During the reductive half-reaction multiple transition stages exist among others a transient but covalent Schiff base is formed, thus substrate is *ad interim* trapped in a covalent bond. During the oxidative half-reaction, the reduced cofactor returns in its former oxidised TPQ form, and hydrogen peroxide and ammonium are released.

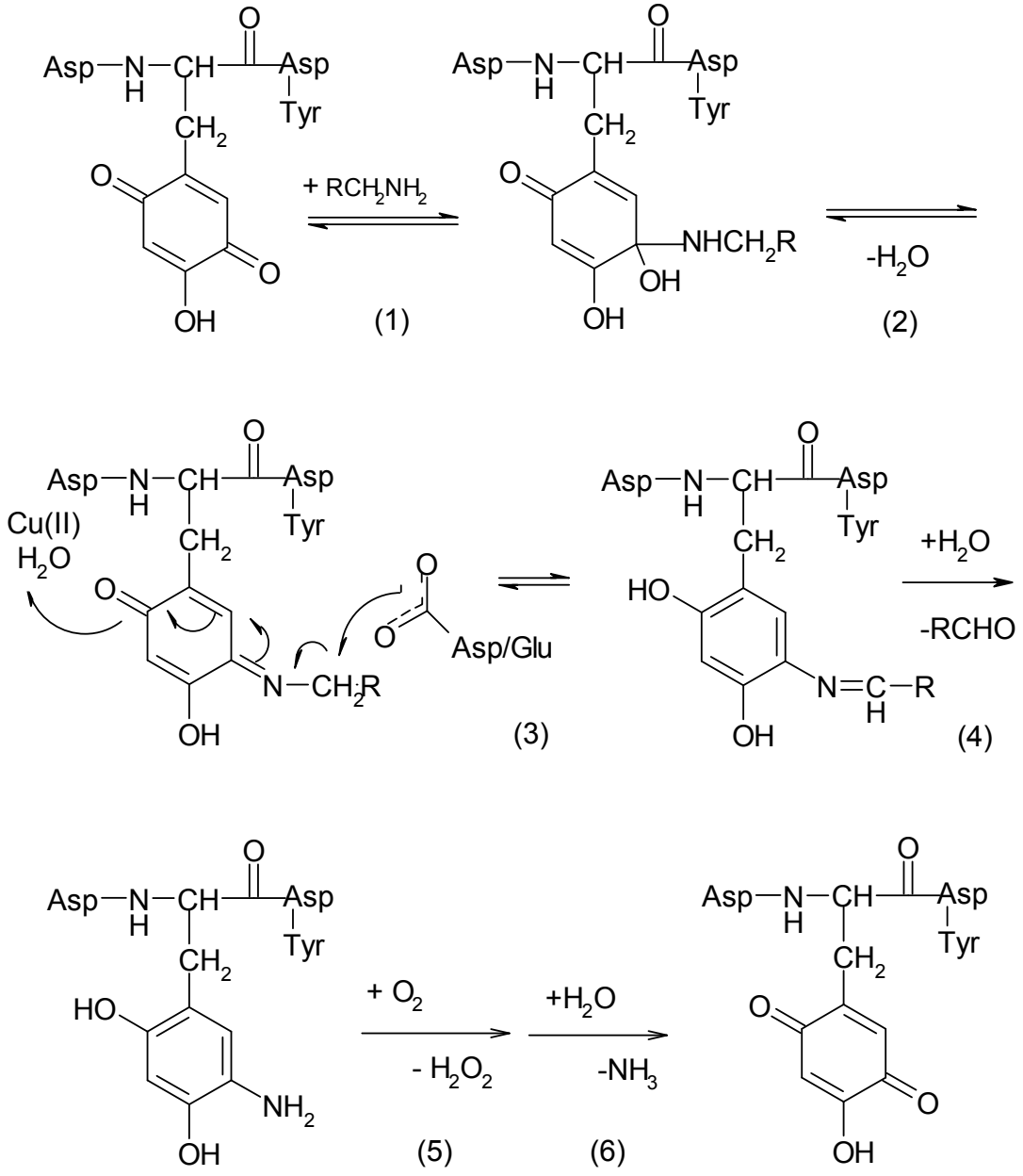

Figure 5. The oxidative deamination catalysed by SSAO. After Matyus *et al.*, 2004.

Membrane-bound SSAOs

The biological role of SSAOs, with the exception of LO, has remained enigmatic for decades in mammals. In bacteria and yeast, the SSAO reaction provides a source of nitrogen (and carbon) when growing. On the other hand the hydrogen peroxide is used

for wound healing in plants. In mammals novel functions of SSAO has been revealed besides amine oxidation. Although the significance of all SSAO mediated biological effects is far from clear.

Mammals express SSAO in a wide variety of tissues (Jaakkola *et al.*, 1999; Lewinsohn, 1984; Lyles, 1996; Salmi *et al.*, 1993). The most prominent synthesis takes place in smooth muscle (both vascular and non-vascular) and adipocytes, endothelial cells and follicular dendritic cells. Leucocytes, epithelial and fibroblastoid cells lack SSAO. Brain is also devoid of SSAO (except microvessels). Unlike in other mammals, in man, the protein is also absent from chondrocytes and odontoblasts.

Membrane-bound SSAOs may be involved in the regulation of glucose metabolism in adipose cells, in the regulation of leucocyte trafficking in endothelial cells as well as vascular elasticity.

Glucose metabolism

SSAO is involved in glucose metabolism in adipocytes and smooth muscle cells, with H_2O_2 being responsible for the effect. The mechanism involves insulin-responsive glucose transporter (GLUT-4) translocation to the plasma membrane in fat cells, while GLUT-1 accumulation on the surface of smooth muscle cells (El Hadri *et al.*, 2002). The involvment of adipocyte SSAO in the glucose metabolism is a major topic of this thesis. For more details see chapter 3.2.

The role of SSAO/VAP-1 in the regulation of leucocyte adhesion

As mentioned previously, SSAO has been found identical to VAP-1 adhesion molecule. SSAO induces cell adhesion and regulates leucocyte trafficking through the endothelial barrier. Circulating leucocytes reversibly tether to and roll on the endothelial cells under conditions of blood flow as a first step of a well-orchestrated cascade (Salmi *et al.*, 1997a). Reception of appropriate activation signals is followed by firm adhesion to the vessel wall and transmigration into the tissue by leucocytes. Multiple adhesion and activation molecules on the leucocytes and endothelial cells regulate the molecular execution of the process (Figure 6.) (Jalkanen *et al.*, 2001).

SSAO is constitutively present in intracellular granules within endothelial cells (Salmi *et al.*, 1993). However, with *in vivo* inflammation models, SSAO seems to be translocated lumenally from intracellular storage granules only upon elicitation of

inflammation (Salmi *et al.*, 1997b). SSAO mediates leucocyte subtype specific adhesion of $CD8^+$ T-cells, NK-cells and granulocytes. SSAO is also present on sinusoidal endothelial cells in liver (McNab *et al.*, 1996). The SSAO molecule seems to be important for the rolling phase and most likely later at the transmigration step of leucocyte extravasation/transmigration (Tohka *et al.*, 2001).

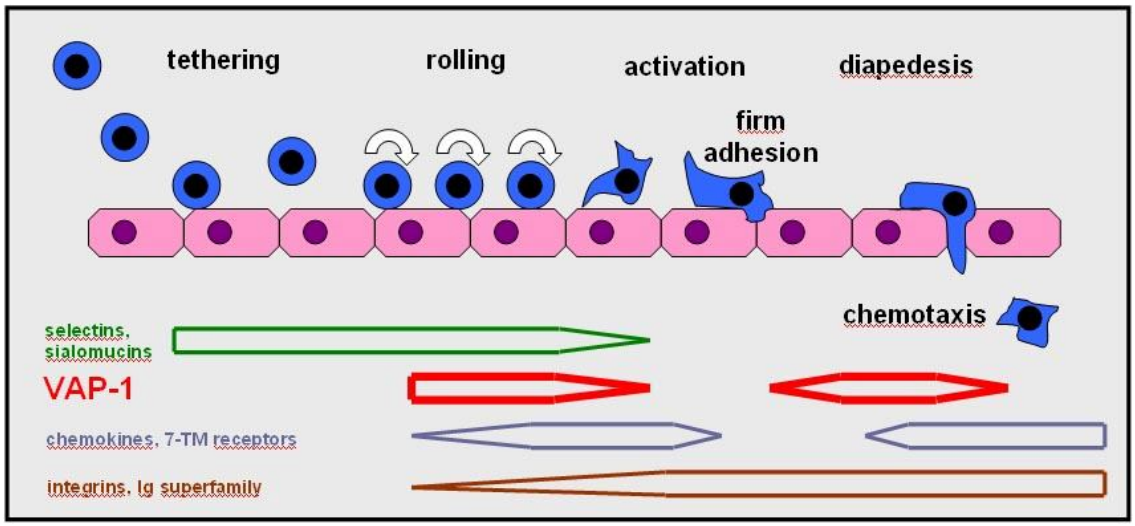

Figure 6. Role of VAP-1 and other molecules in the extravasation of leucocytes. Adapted from Jalkanen 2001.

The protein was also shown to oxidise BzA and methylamine. Moreover, the reactions catalysed by SSAO have been shown to be important in the leucocyte adhesion (Salmi *et al.*, 2001b). It is interesting that artificial SSAO inhibitors decrease rolling and adhesion of leucocytes by about 50% and anti-VAP-1 antibodies inhibit lymphocyte rolling but do not affect enzyme activity. On the other hand products of the catalytic reactions, like aldehydes or hydrogen peroxide do not alter the lymphocyte-endothelium adhesion. Leucocyte adhesion can also be decreased by SSAO substrate pretreatment suggesting that during the multistep extravasation cascade there is a transitional covalent bond between the enzyme (the endothelial VAP-1) and a membrane-bound substrate of leucocyte origin. It is therefore the membrane-bound isoform and not the soluble one that plays the important role in the adhesion to the endothelial cells. This hypothesis was substantiated by an experiment using a synthetic peptide blocking the catalytic cavity of VAP-1. It is possible that surface bound amines (like N-termini of proteins, NH_2-containing amino acid side chains, amino sugars etc.) may be SSAO substrates in addition to soluble amines (Salmi *et al.*, 2001b). Moreover, aminosugars, also promoting the adhesion, bind reversibly to SSAO in the presence of

H_2O_2. The enzyme catalysed oxidation of substrates may thus considerably contribute to leucocyte binding to the endothelial cell (O'Sullivan *et al.*, 2003; O'Sullivan *et al.*, 2004). SSAO, in this case an endothelial ectoenzyme, can directly and indirectly regulate the leucocyte extravasation. VAP-1 is a dual function molecule: it binds lymphocytes to the endothelium and utilises the catalytic reaction between the surface-bound amine and VAP-1 to transiently link the interacting cells. The VAP-1-catalysed reaction may further contribute to cross-linking if the protein-bound aldehydes formed on lymphocytes interact with molecules on the endothelial cell surface (Jalkanen *et al.*, 2001).

Organisation of the arterial wall

SSAO may be involved in vascular smooth muscle cell differentiation (El Hadri *et al.*, 2002), organization of the extracellular matrix (Vidrio, 2003) and regulation of vascular tone (Langford *et al.*, 1999; Langford *et al.*, 2002; Vidrio, 2003). Langford and his coworkers have shown that pharmacological inhibition of SSAO induced by semicarbazide in a growing rat model led to striking elastic fiber disorganisation. Semicarbazide, however, inhibits both SSAO and LO and its effect on collagen cross-linking is mainly attributed to LO inhibition (Mercier *et al.*, 2007; Mercier *et al.*, 2006). Alternatively, it has been suggested that SSAO could influence arterial vascular tone, although contradictory results have been reported: Vidrio and his group have proposed that SSAO-mediated H_2O_2 production could increase vascular tone and enhance hydralazine-induced vasodilation (Vidrio *et al.*, 2003); In contrast, Conklin *et* al. have recently suggested that SSAO activation by methylamine mediated vasorelaxation in isolated human arteries (Conklin *et al.*, 2004). At a molecular level, it is conceivable, in view of previous results using molecular modeling (Salmi *et al.*, 2001a) that in addition to soluble primary amines, such as methylamine or aminoacetone, SSAO may act on amino acids, including matrix proteins. SSAO may, thus, contribute to physiological cross-linking of elastic and collagen fibers. Formaldehyde-induced protein cross linkage (Gubisne-Haberle *et al.*, 2004) might also be involved in the process. Nevertheless, SSAO-deficient mice have normal elastic fiber network, elastin cross-linking processes, and vascular endothelial or smooth muscle function (Mercier *et al.*, 2006). These results do not support a major role of SSAO in the rodent arterial wall, even if unknown compensatory mechanisms may contribute to the phenotype in this model. However, the

only detectable alteration in KO AOC3 mice was an increase in carotid diameter, in line with a potential involvement of SSAO in arterial growth and/or the pathophysiology of aneurysms, as suggested (Langford *et al.*, 1999; Sibon *et al.*, 2004).

Soluble SSAOs

Soluble SSAOs may have an important function in several biological mechanisms. First of all, increased levels of soluble serum SSAO were found in specific diseases, for instance in diabetes and liver diseases. Soluble SSAO may also be involved in the production of non-enzymatic addition of oligosaccharides to proteins during formation of advanced AGEs, typical of diabetic lesions. Finally, it has been proven, that soluble SSAOs also modulate lymphocyte adhesion to endothelial cells, presumably by triggering positive signals on the lymphocyte (Kurkijarvi *et al.*, 1998).

Plasma SSAO activity varies considerably in mammals (Boomsma *et al.*, 2000b). It is high in species with a functional AOC4 gene and low in humans and rodents that lack a functional AOC4 gene. Despite that human plasma amine oxidase activity is lower than in tissues, the easier plasma accessibility has resulted in a substantial number of clinical reports in various pathologies (Boomsma *et al.*, 2003).

In healthy adults with BMI ≤ 25, SSAO activity varies in a narrow range. It is interesting, that the activity is much higher in children, with a sharp decrease at the age of 16 to finally settle at the adult values. There is a slight increase in the activity from the age of 50. Enzyme activity is independent from gender, posture (standing, sitting, lying) or circadian rhythm. A higher activity can be measured during pregnancy (Boomsma *et al.*, 2003).

Obesity, diabetes and atherosclerosis

Obesity is not a condition associated with clear-cut increase in plasma SSAO, despite adipose tissue has been proposed as a source of soluble SSAO, essentially in diabetes (Stolen *et al.*, 2004b). While plasma SSAO clearly increases in diabetic conditions, its association with obesity is less consistent (Carpéné, 2009). Therefore, the correlation found between plasma SSAO activity and BMI in type 2 diabetic patients (Mészáros *et al.*, 1999b) might be the consequence of a parallel increase in insulin resistance and adiposity. An elevation in SSAO activity has been found in non-diabetic, obese patients and has been proposed as a cardiovascular risk factor (Weiss *et al.*,

2003). However, such an association between plasma SSAO and BMI was not confirmed by other studies conducted with non-diabetic (Dullaart *et al.*, 2006) or young obese (Visentin *et al.*, 2004) subjects. When morbidly obese patients (BMI of 38.8) received vertical banded gastroplasty and lost weight after the surgery (BMI lowering to 30.8), there was only a weak tendency to correlate the individual changes in plasma SSAO with the decrease in different anthropometric measurements of adiposity (Li *et al.*, 2005). A more substantial increase in plasma SSAO than those reported in the above mentioned studies should be expected in obese subjects, regarding to the dramatic increase in TNF-α in obesity and owing to *in vitro* TNF-α stimulation of SSAO shedding from adipocytes (Abella *et al.*, 2004).

High plasma SSAO level was measured in type 1 (Boomsma *et al.*, 1995), type 2 (Mészáros *et al.*, 1999b), and type 1 juvenile diabetes. This phenomenon has also been observed in animal models of diabetes (Hayes *et al.*, 1990). It has been proven that oxidative stress is important in the development of diabetic complications. It is assumed that diabetic vascular complications are caused partially by elevated blood level of the toxic formaldehyde, MG and hydrogen peroxide, all deriving from the SSAO mediated oxidative deamination of methylamine and aminoacetone. SSAO activity is elevated in patients with diabetic complications such as retinopathy (Garpenstrand *et al.*, 1999; Gronvall-Nordquist *et al.*, 2001), hypertension and atherosclerosis (Mészáros *et al.*, 1999a), therefore plasma SSAO has been suggested to be a possible clinical marker of diabetic complications.

Serum SSAO levels correlate with Crouse score and intima media thickness, both reliably marking the progression of atherosclerosis (Mészáros *et al.*, 1999a). Experiments done on human umbilical vein showed that SSAO catalyses LDL oxidation by endothelial cells (Exner *et al.*, 2001). A more intensive oxidative stress can help to progress the disease. Oxidation of LDL increases the atherogenity of the lipoprotein and this can hasten the formation of atherosclerotic plaques.

Other diseases

Since there is a significant amount of SSAO in the vascular wall, great interest has been given to patients suffering from all types of vascular diseases. In patients with congestive heart failure plasma SSAO has been found considerably increased (Boomsma *et al.*, 2000a) and was in correlation with severity of the disease. According

to certain observations, SSAO activity of individuals with essential and renovascular hypertension is normal (Lyles, 1996), SSAO levels of patients with chronic renal insufficiency are lower compared to controls. There is a very high level in stroke and malignant hypertension, which can be a consequence of the cardiac insufficiency. SSAO activity is increased in pre-eclampsia (Boomsma *et al.*, 2003) or in cerebral autosomal dominant arteriopathy with subcortical infarcts as well as in leukoencephalopathy (Ferrer *et al.*, 2002), inflammatory liver diseases (Kurkijarvi *et al.*, 1998), multiple sclerosis with ongoing inflammatory activity (Airas *et al.*, 2006), psoriasis (Madej *et al.*, 2007), atopic eczema (Madej *et al.*, 2006), Alzheimer's disease (del Mar Hernandez *et al.*, 2005), myopathies (Olive *et al.*, 2004) and also bone cancer with skeletal metastases (Ekblom *et al.*, 1999).

SSAO activity is unchanged in Sjögren syndrome or colitis ulcerosa. Decreased levels are rare, *e.g.* in children born with hernia diaphragmatica, in cases of severe burns, in different malignomes and in lung cancer, and decreased serum levels have also recently been reported in highly depressed (Roessner *et al.*, 2006) and schizophrenic patients treated with second-generation "diabetogenic" antipsychotics (a possible link to the role of SSAO in glucose metabolism was suggested) (Roessner *et al.*, 2007).

In pathologies with high plasma SSAO activity some of the enzyme substrates are also elevated: methylamine and aminoacetone in diabetes, creatinine in uremia and adrenaline in stress-related diseases. It is possible that the substrates mentioned above cause the increase of SSAO activity.

It is not clear how a moderate increase of SSAO activity observed in a number of human diseases could possibly cause any damage (Boomsma *et al.*, 2003; Yu *et al.*, 2003b). However, the increase in released SSAO could just be indicative of an increase of the membrane-associated SSAO at the cell surface where the actual damage is done.

The regulation of plasma SSAO levels is yet to be defined. Although several correlations have been set up between plasma SSAO activity and other clinical markers like ACE activity (Boomsma *et al.*, 2005b), thyroid hormone (Boomsma *et al.*, 2005a), TNF-α, insulin and blood glucose levels, glycated haemoglobin (Dullaart *et al.*, 2006; Salmi *et al.*, 2002) etc, only TNF-α and insulin seems to be involved in the regulation of plasma SSAO activity. TNF-α promoting insulin resistance, causes an increase in the release of SSAO by adipocytes (Abella *et al.*, 2004; Garcia-Vicente *et al.*, 2005). On the

other hand insulin appears to hamper shedding from tissues or increase the clearance of plasma SSAO levels. Finally, some recent trials showed that acute glucose load itself may increase plasma SSAO activity in non-diabetic individuals (Li *et al.*, 2009a; Li *et al.*, 2009b).

3.2. Adipose tissue

In vertebrates white adipose tissue (WAT) is the primary site of energy storage. The lipid droplets of the adipocytes contain mostly triglycerides (TG). When energy expenditure exceeds energy intake WAT releases free fatty acids (FFA). WAT is also a secretory organ as it produces several adipocytokines permitting self-regulation (autocrine effect), a cross-talk with local (paracrine effect) and distant adipocytes and other cells in the brain, the liver, the muscles, the pancreas, etc. (endocrine effect) (Kim *et al.*, 2000).

WAT contains different cell types. Only one third of the cells are adipocytes and the rest is a mixture of stromal vascular fraction: fibroblasts, macrophages, stromal cells, monocytes and preadipocytes. Adipose tissue seems to derive from an embryonic stem cell precursor (Pittenger *et al.*, 1999). The development of white adipose tissue begins early during embryogenesis and continues throughout life resulting in an increase in fat cell size (hypertrophy) as well as in fat cell number (hyperplasia) depending on the energy status and the storage needs of the body.

Fat develops in many different sites and has different functions: subcutaneous adipose tissue (SCWAT) is an effective insulating layer located between the muscles and dermis while visceral adipose tissue fills in space gaps between organs, like gonads (PGWAT), heart, kidneys (PRWAT), or gut, protecting them against mechanical impacts and maintaining them in the adequate position.

3.2.1. Metabolic function

Lipogenesis

Figure 7 shows the lipogenic processes grouped on the left hand side. In postprandial state adipocytes produce TG. Lipid accumulation in adipose tissue depends on circulating FFA and glucose uptake. Fatty acids may be synthesised from carbohydrate precursors (*de novo* lipogenesis). Insulin stimulates glucose uptake by

stimulating the translocation of GLUT-4 to the plasma membrane. Glucose undergoes glycolysis resulting in acetyl-coenzyme A (acetyl-CoA). Acetyl-CoA is transformed to Malonyl-CoA by the Acetyl-CoA carboxylase (ACC). Further chain prolongation is catalysed by the fatty acid synthase (FAS). In human, only a fraction of the TG stock is formed by *de novo* lipogenesis. Insulin also favours fatty acid uptake from circulating lipoproteins by stimulating lipoprotein lipase (LPL) activity. FFAs are released by lipoprotein lipase hydrolysing TGs from chylomicrons or VLDLs and are finally taken up by FFA transporters (FATP). Be the source of FFAs carbohydrates or TGs, reesterification is necessary to form TGs suitable for lipid storage (Vazquez-Vela *et al.*, 2008).

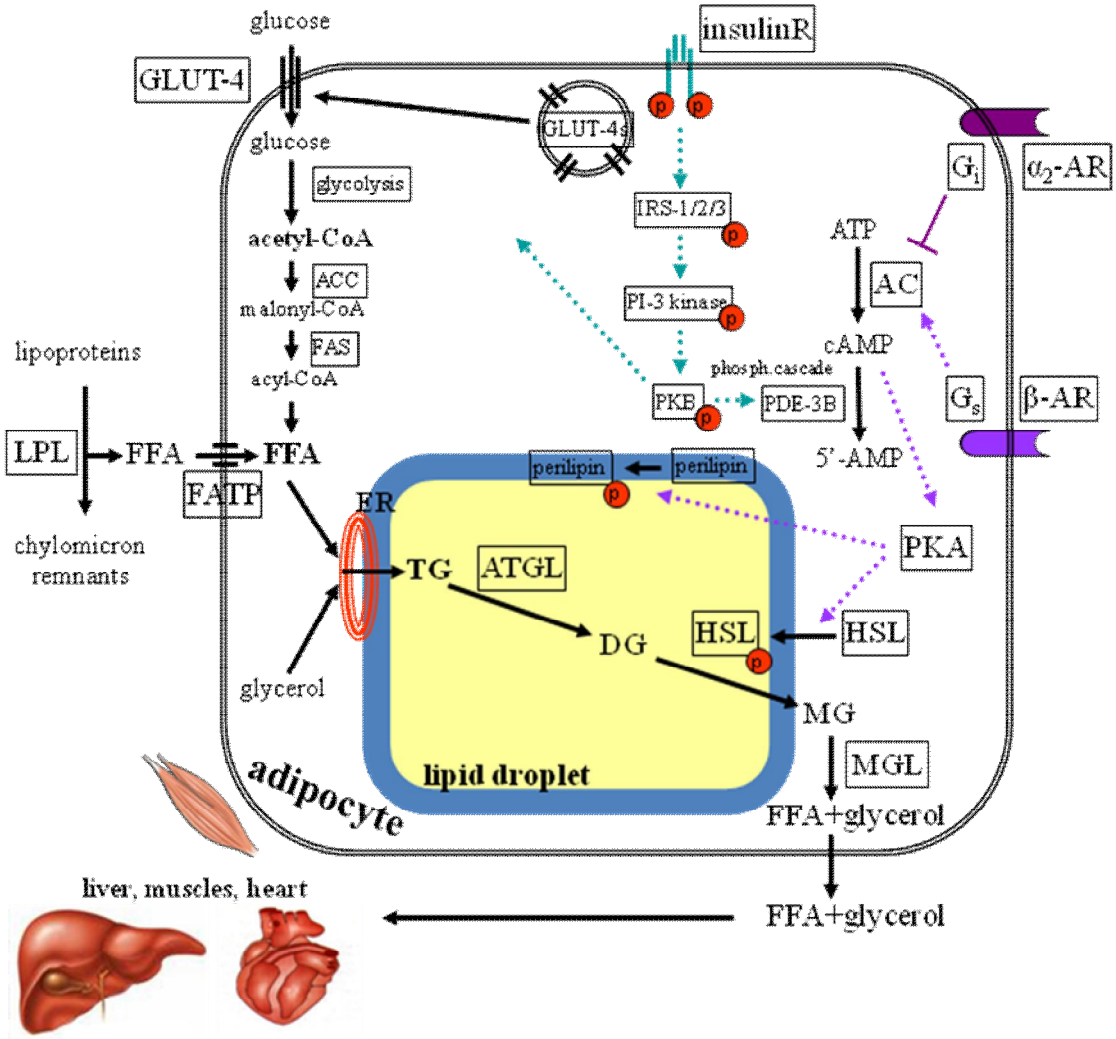

Figure 7. Major biochemical pathways involved in the regulation of energy homeostasis in the adipocyte (Vazquez-Vela *et al.*, 2008). For abbreviations see the text.

Lipolysis

During fasting WAT hydrolyses TGs to supply other organs with FFAs (Figure 7. right hand side). TGs stored in the lipid droplet are first hydrolysed by the adipose triglyceride lipase (ATGL), resulting in diacylglycerol moiety and FFA, then further hydrolysed sequentially by the hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL) producing FFAs and glycerol. Receptors enhancing cyclic adenosine monophosphate (cAMP) formation stimulate lipolysis. β-adrenergic stimulation (Gs) of adipocytes and the subsequent protein kinase A-dependent (PKA) phosphorylation of HSL and perilipin trigger the translocation of HSL from the cytoplasm to the lipid droplet and induce neutral lipid hydrolysis. Opposingly, molecules decreasing intracellular cAMP inhibit lipolysis. Insulin induces PDE-3B (phosphodiesterase 3B) which degrades cAMP. α_2-adrenergic agonists are also anti-lipolytic, as they inhibit adenyl cyclase (AC) enzyme activity.

3.2.2. Endocrine function

Beyond its essential function in lipid storage and the supply of free fatty acids, WAT produces numerous bioactive molecules (lipids or peptides). Among these molecules, we can find hormones, like leptin, adiponectin, resistin and apelin (Table 3.). Leptin is considered as a metabolic signal for energy sufficiency by directly acting on the hypothalamus. Adiponectin enhances insulin-sensitivity, decreases FFA influx, increases β-oxidation in liver and muscle. Adiponectin decreases atherogenic risk via depressing the expression of adhesion molecules within the vascular wall, as well. Resistin acts on skeletal muscle and WAT and its high plasma levels are associated with insulin resistance. Apelin increases cardiac contractility, decreases blood pressure and hyperglycemia (Dray *et al.*, 2008; Vazquez-Vela *et al.*, 2008). Obesity might reduce physiological response of some adipokines (resistance).

In addition to the above mentioned molecules, adipose tissue also produces and secretes a variety of other peptides, cytokines and complement factors, including tumour necrosis factor α (TNF-α), interleukine 6 (IL-6), plasminogen activator inhibitor 1 (PAI-1), angiotensinogen, etc. TNF-α is involved in the pathophysiology of insulin resistance via modifying insulin sensitivity which induces abnormal phosphorylation of

insulin receptor substrate (IRS)-1. IL-6 comes from the stromal vascular fraction and it controls the hepatic production of inflammatory proteins (Antuna-Puente *et al.*, 2008).

Table 3. Some biologically active molecules of WAT. See abbreviations in chapter 2.

	Source	Receptors	Pathway	Main effects	In obesity
TNF-α	adipocytes macrophages lymphocytes	membrane bound and soluble cytokine receptors	JNK, IKK	block insulin's action (IRS-1)	↑
IL-6	fibroblasts endothelial cells monocytes	cytokine receptor	JNK	induce liver inflammatory protein production (CRP) alters tyr phosphatase activity and SOCS	↑
Leptin	adipocytes	long, short, soluble cytokine receptors	JNK/ STAT	β-oxidation ↑ UCP expression↑ hypothalamus-food intake↓ insulin secretion↑	↑ RESISTANCE
Resistin	adipocytes macrophages	not known	not known	WAT, skeletal muscle-insulin resistance (IRS-2↓) liver-glycogen synthesis ↑	↑
Adiponectin	adipocytes	GPCR	AMPK/MAPK	liver and skeletal muscle-insulin sensitivity, β-oxidation ↑ hypothalamus-food intake↑	↑ RESISTANCE
Apelin	adipocytes	GPCR	AMPK/PKB	blood pressure↓ blood glucose↓	↑ RESISTANCE?

3.2.3. Obesity

The abundance of nutrients and the decreased physical activity coupled with modern western life, result in excessive accumulation of adipose mass. Obesity, the excessive storage of energy as fat caused by the imbalance between energy intake and expenditure, has become a prevalent health hazard in industrialised countries.

Disturbed energy balance alters WAT activity especially that of adipocytes or macrophages, inducing low-grade chronic inflammation which is supposed to play a central role in obesity-linked insulin resistance. Insulin resistance may be featured in many pathologies complications such as hypertension, hyperlipidemia, atherosclerosis, metabolic syndrome, polycystic ovarian disease, certain cancers, and type 2 diabetes mellitus. Especially, increase in visceral fat mass seems to contribute to such complications.

3.2.4. Hydrogen peroxide

The majority of the bioactive H_2O_2 is derived from spontaneous or superoxide dismutase-catalyzed (SOD) dismutation of superoxide, a side product of several enzyme reactions: mitochondrial electron transport chain, lipoxygenase, cycloxygenase, cytochrome P450s, xanthine oxidase, NADPH oxidases, uncoupled eNOS, etc. Some other enzymes, like xantine oxidase, glucose oxidase or amine oxidases produce H_2O_2 directly.

H_2O_2 has long been known to mimic several of insulin's actions in adipocytes (Czech *et al.*, 1974; May *et al.*, 1979). H_2O_2 acts through insulin signaling: it enhances insulin receptor phosphorylation and kinase activity (Hayes *et al.*, 1987) and subsequent tyrosine phosphorylation of intracellular proteins. Insulin can induce NADPH oxidase-dependent H_2O_2 release, H_2O_2 thus can be regarded as a second messenger of insulin (Mukherjee *et al.*, 1977). The membrane-bound NADPH oxidase is coupled to the insulin receptor via a Gi/Go heterotrimeric G protein (Krieger-Brauer *et al.*, 1997) regulating insulin action (Moxham *et al.*, 1996).

Despite its unquestionable biological function, it cannot be denied that H_2O_2, as a reactive oxygen species (ROS), may also have deleterious effects on the cells. As a matter of fact, H_2O_2 is poorly reactive in chemical terms. Its danger comes mainly from its conversion into hydroxyl radical ($OH^{\bullet}$) either in the Fenton or the Haber-Weiss reaction. Insulin resistance, as well as its complications have also been associated with high levels of H_2O_2. This might be the consequence of impaired antioxidant systems (catalase, glutathione peroxidase) and/or increased ROS production (Eriksson, 2007).

3.2.5. Expression of SSAO in adipocytes and in white adipose tissue

An increase of SSAO during adipocyte differentiation in rodents has been described as early as 1990 by Raimondi and co-workers on cells isolated from the stroma-vascular fraction of rat adipose tissue and cultured under adipogenic conditions (Raimondi *et al.*, 1990). Afterwards, an impressive increase of SSAO mRNA, protein and activity was detected during adipocyte differentiation in the murine lineages 3T3-F442A and 3T3-L1 (Moldes *et al.*, 1999). More recently, it has been evidenced that the product of the AOC3 gene dramatically increases during adipogenesis of human

preadipocytes (Bour *et al.*, 2007a) resulting in high SSAO activity in mature adipocytes (Bour *et al.*, 2007a; Morin *et al.*, 2001). The increase in SSAO activity found during adipogenesis has still a poorly defined role.

In human adipose tissue, there is no doubt that fat cells exhibit higher levels of SSAO than any other cells belonging to the stromal vascular fraction (Bour *et al.*, 2007a). This observation confirms previous findings made on rat brown adipose tissue (Barrand *et al.*, 1984). The very high expression of SSAO in adipocytes has been confirmed by independent approaches studying protein expression profile in different subcelullar compartments: SSAO has been detected in plasma membrane (Barrand *et al.*, 1984), glucose transporter-containing vesicles (Enrique-Tarancon *et al.*, 2000), caveolae (Souto *et al.*, 2003) and in diverse microvesicles of adipocytes (Morris *et al.*, 1997).

The variations of obesity-induced changes in SSAO expression of WAT are still unknown and need further investigation. In the subcutaneous adipose tissue, no change in SSAO was found when comparing young obese subjects with age-matched controls. (Visentin *et al.*, 2004). Since the visceral, intra-abdominal adipose depots are especially involved in the serious complications of massively obese patients, it would be worth to obtain information on pathology-related SSAO expression in these tissues, as well.

Our limited knowledge about the regulation of SSAO in human obesity is hardly compensated by animal studies (Carpéné, 2009). An upregulation of the protein has been detected in omental adipose tissue of dogs after 9 weeks of high-fat feeding: both SSAO mRNA and activity increased. Intriguingly simultaneous upregulation in subcutaneous WAT has not been observed (Wanecq *et al.*, 2006). On the contrary, high-fat diet did not induce changes of SSAO activity or expression in mice (Visentin *et al.*, 2005). To further complicating the puzzle, a decrease in SCWAT was showed in obese Zucker rats (activity and mRNA) (Moldes *et al.*, 1999), as well as a decreased SSAO mRNA and increased SSAO activity were found in PGWAT of db-/- animals when compared to lean littermates (Cioni *et al.*, 2006). Although, some *in vitro* experiments proposed TNF-α, forskolin and isoproterenol to decrease SSAO activity of adipocytes by releasing the protein to the culture medium (Abella *et al.*, 2004; Garcia-Vicente *et al.*, 2005; Mercier *et al.*, 2003; Moldes *et al.*, 1999). Among all, only TNF-α has been proven *in vivo* to downregulate SSAO activity in WAT (Mercier *et al.*, 2003). Despite

of the proposed regulation by TNF-α, it is still a very hard task to clarify changes in WAT SSAO activity in different animal models of obesity.

3.2.6. Insulin-like actions of SSAO substrates

The two first papers reporting the insulin-like action of tyramine (Marti *et al.*, 1998) and BzA (Enrique-Tarancon *et al.*, 1998) in rat adipocytes clearly showed that SSAO and MAO activity was responsible for promoting glucose transporter translocation and glucose uptake: the synergistic action between SSAO substrates and vanadate is due to either extracellularly or intracellularly generated active compounds, possibly peroxovanadate. Such active compounds may activate a protein-tyrosine kinase or inhibit a protein-tyrosine phosphatase, which in turn causes tyrosine phosphorylation of IRS-1 and IRS-3, rapidly interacting with the p85 subunit of PI 3-kinase, crucial in insulin-signal transduction. The p85–p110 PI 3-kinase activity is also stimulated resulting in GLUT-4 recruitment to the cell surface (Figure 8.). Such activation of a protein-tyrosine kinase or inhibition of a protein-tyrosine phosphatase causes a weak activation of insulin-receptor kinase (Zorzano *et al.*, 2003).

Diverse *in vitro* insulin-like actions were reported for the combination of 0.1-1 mM SSAO substrates and 0.1 mM vanadate while these agents were without clear effect when tested separately (Enrique-Tarancon *et al.*, 2000; Enrique-Tarancon *et al.*, 1998; Visentin *et al.*, 2003a; Yu *et al.*, 2004). Enzyme-dependent generation of H_2O_2 was proven to be responsible for such effects. Insuline-like effect of SSAO substrates and vanadate was also investigated *in vivo*: acute injection improved glucose tolerance in non-diabetic or diabetic animals, and chronic injection reduced hyperglycemia of diabetic animals (Abella *et al.*, 2003a; Marti *et al.*, 2001).

In some other experiments, effects of BzA were observed even in the absence of vanadium. First, in human adipocytes, the insulin stimulation of glucose transport was partially reproduced by 0.1-1 mM BzA and methylamine in a manner that was not potentiated by vanadate (Iglesias-Osma *et al.*, 2005; Morin *et al.*, 2001). Second, SSAO substrates alone induced adipogenesis of the murine preadipocyte 3T3-F442A or 3T3-L1 cell lines (Carpéné *et al.*, 2006; Fontana *et al.*, 2001). Lastly, lipolysis inhibition, another insulin-like action, was observed with methylamine and BzA in human, rabbit and mouse fat cells or murine preadipocyte cells without any need for vanadate

(Carpéné *et al.*, 2006; Iglesias-Osma *et al.*, 2005; Iglesias-Osma *et al.*, 2004; Morin *et al.*, 2001).

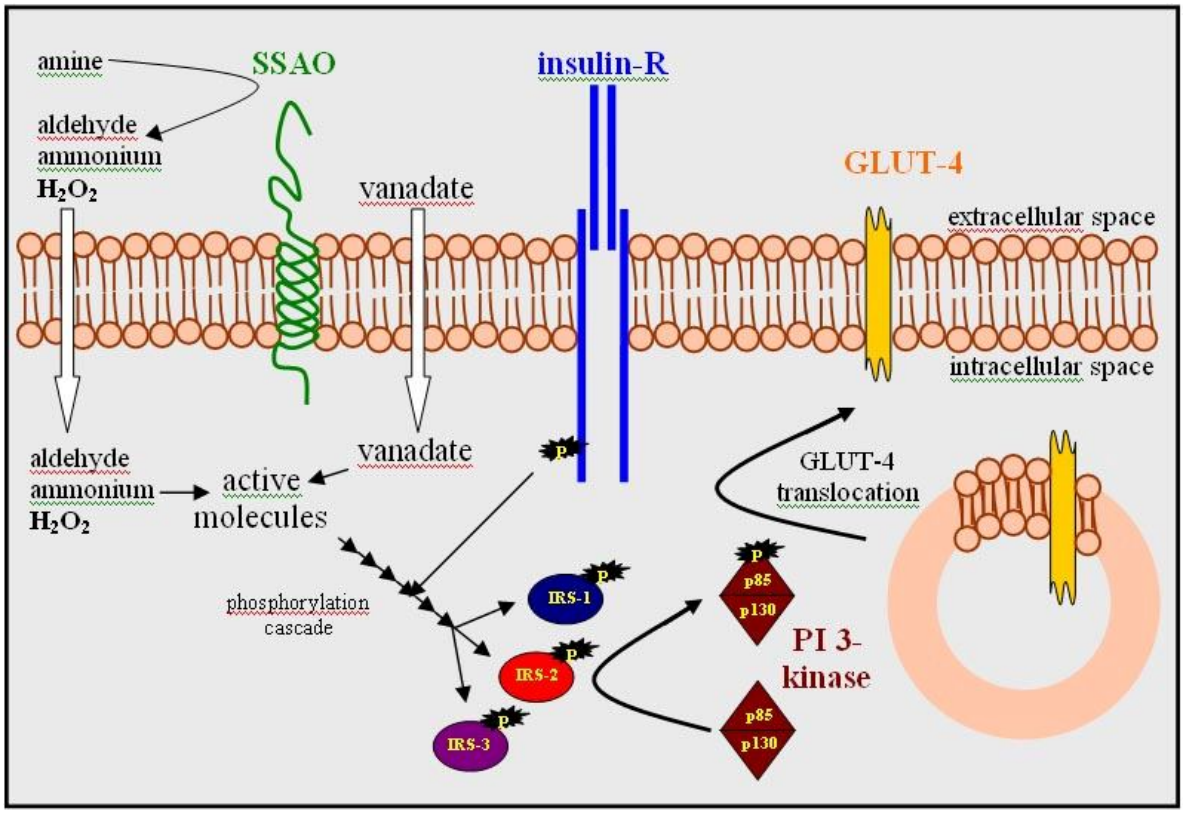

Figure 8. The role of SSAO in the regulation of glucose uptake in adipocytes. Adapted from Zorzano, 2003.

Most importantly, SSAO substrates exhibited insulin-like actions even when responses were studied *in vivo*. In the conscious rabbit and mice, the single infusion or injection of 7 µmol/kg BzA (without vanadate) before a glucose challenge is sufficient to lower the hyperglycaemic response, in an SSAO-dependent manner (Iglesias-Osma *et al.*, 2004). Furthermore, chronic oral administration of BzA improved glucose-tolerance in Wistar rats (Bour *et al.*, 2005).

3.3. Diabetes induced oxidative stress and vasculopathy

Vascular complications are the main causes of morbidity and mortality in diabetic patients (Head *et al.*, 1990; Ruderman *et al.*, 1992). The pathophysiology of diabetic angiopathy is complex. Chronic hyperglycemia, characteristic feature of the disease, initiates processes responsible for the early functional impairment of the blood vessels, the endothelial cell dysfunction (ECD). ECD is characterised by reduced

endothelium-dependent vasodilatation, as well as altered anti-coagulant, anti-inflammatory properties and disturbed barrier function of the endothelium, and is recognized as the common initial pathophysiological change in the development of diabetic microangiopathy and macroangiopathy. Decreased availability of the vasodilator nitric oxide (NO), as well as increased production and action of vasoconstrictor agents, such as ROS are suggested to be responsible for ECD (Cosentino *et al.*, 1998; Santilli *et al.*, 2004).

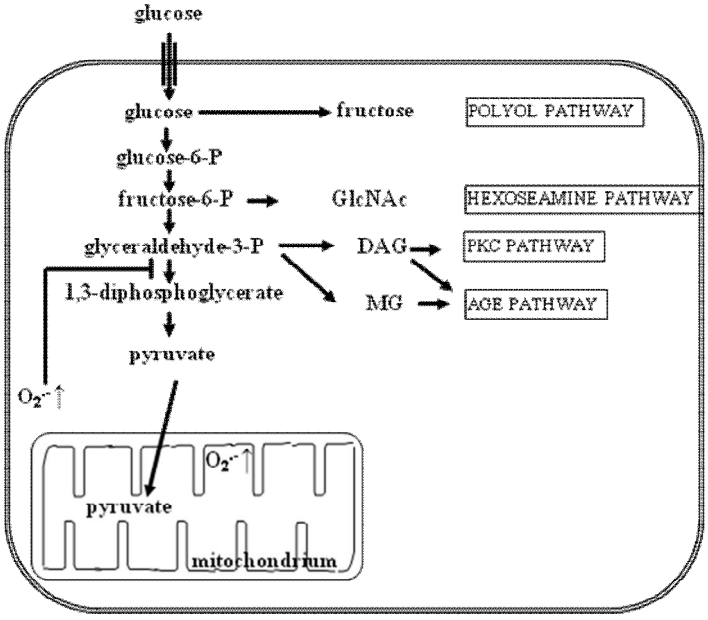

Figure 9. Mechanism by which hyperglycemia-induced mitochondrial superoxide overproduction activates four pathways of hyperglycaemic damage. After Brownlee, 2001.

The key event in hyperglycemia-induced oxidative stress is the increased oxidative metabolism of glucose, resulting in the overproduction of superoxide anions in the mitochondria (Nishikawa *et al.*, 2000), and activation of biochemical pathways responsible for the elevated formation of ROS, as well as decreased antioxidant defense mechanisms. Excess superoxide partially inhibits the glycolytic enzyme glyceraldahyde-phophate dehydrogenase (GAPDH), thereby diverting upstream metabolites from glycolysis into pathways of glucose overutilization (Figure 9.). Increased glucose flux activates the polyol pathway and excess of fructose-6-phosphate transforms to N-acetylglucosamine (hexoseamine pathway). Triose phosphates are converted to either diacyl-glycerol (DAG) an activator of protein kinase C (PKC), or to methylglyoxal (MG), the main intracellular AGE precursor (Brownlee, 2001; Ceriello, 2003; Sheetz *et al.*, 2002).

The impaired biological effects of NO can be the consequence of its *1.)* reduced production by NO synthase (NOS), *2.)* increased inactivation related mainly to oxidative stress, or *3.)* decreased responsiveness to its cellular actions. There are conflicting data in the literature about the alteration of the expression of NOS isoforms and their activity to produce NO in tissues of experimentally induced diabetic animals (Honing *et al.*, 1998; Santilli *et al.*, 2004).

H_2O_2 causes endothelium-dependent and independent vasodilation of vessels. Endothelium-dependent dilatory mechanisms include enhanced eNOS expression via PKB or ERK1/2 activation or potassium channel-induced hyperpolarization. Of note, endothelium-independent vasorelaxation may involve cGMP activation or other currently unknown pathways. H_2O_2 strongly upregulates eNOS expression *in vitro* and *in vivo*. On the other hand H_2O_2 may derive from uncoupled eNOS (Drouin *et al.*, 2007; Matoba *et al.*, 2000). The strong interplay between eNOS and H_2O_2 is involved in the physiological adaptation to exercise (Lauer *et al.*, 2005) but may have detrimental pathophysiological consequences, as well.

Some authors propose that ROS production down-regulates endothelial NOS (eNOS) expression and activity (Brownlee, 2001; Srinivasan *et al.*, 2004). However, it is well established, that metabolic transformation of NO is accelerated during oxidative stress. Oxidative stress leads to NOS uncoupling and NO-quenching by excess superoxide resulting in peroxynitrite and further reactive nitrogen species (RNS) production. These compounds do not have vasoactive properties, but are strong oxidant and nitrating agents, thus may damage endothelial cells (Beckman *et al.*, 1990; Pacher *et al.*, 2005; Soriano *et al.*, 2001).

Direct measurement of NO and peroxynitrite production in the tissues is difficult because of their reactivity and short half-life. However, the biological availability and oxidative metabolism of NO can be estimated by the measurement of its stable end-products. Under physiological conditions, nitrite is the primary decomposition product of NO, which is further oxidised only in the presence of oxidizing species such as oxyhaemoglobin and superoxide (Ignarro *et al.*, 1993). Contrary to NO, peroxynitrite and other RNS decompose directly to nitrate (van der Vliet *et al.*, 1995). Tissue level of nitrite thus indicates the effectiveness of NO, while the ratio of nitrate to nitrite can be used to assess the oxidative metabolism of NO (Kobayashi *et al.*, 2001).

The complex interplay of these mechanisms results in a perturbation of the physiological properties of NO in the maintenance of endothelial homeostasis, such as vasodilation, anticoagulation, leucocyte adhesion, smooth muscle cell proliferation, and antioxidant capacity.

4. Research Objectives

My work aimed at studying the insulin-like effect of semicarbazide-sensitive amine oxidase (SSAO) substrates on adipocyte metabolism (glucose uptake, lipolysis), on *in vivo* glucose homeostasis and on certain markers of vascular complications developing during obesity and diabetes.

Semicarbazide-sensitive amine oxidase activity is very high in white adipose tissue (WAT). It is present at the surface of fat cells and independent approaches have evidenced its impressive increase during adipogenesis. However, the factors that might regulate the expression of SSAO in adipose tissue are still poorly defined.

- The first part of my thesis thus investigates the link between adipose tissue extension (increased in obesity or reduced during fasting) and SSAO activity in the adipose depots of different anatomical location.

Insulin-like effect of amine oxidase substrates has already been reported *in vitro* as well as *in vivo*. BzA, a very good substrate of the enzyme, was used for further experimental approaches aiming at the better understanding the role of SSAO in adipose tissue physiology and in regulation of energy balance. Our questions were:

- Could the effect of BzA be abolished in adipocytes by the pharmacological inhibition or the genetic invalidation of SSAO protein?
- Is a single injection of BzA able to improve glucose tolerance in mice *in vivo*?
- How does chronic BzA injection influence markers of vascular complication in streptozotocin-induced diabetes?
- Could the chronic oral BzA administration restore the defective glucose handling and vascular complication markers in different mouse models of insulin resistance?

On the basis of previous experiments with BzA on human adipocytes, and owing to the anti-hyperglycemic properties of BzA evidenced on the above mentioned models, we propose SSAO substrates as possible drug candidates to restore glucose homeostasis in adipose tissue.

- In this context, we wanted to know whether recently synthesised amines can act more efficiently to produce insulin-like effects such as anti-lipolysis or glucose uptake stimulation on human fat cells.

5. Materials and Methods

5.1. Chemicals

Benzylamine hydrochloride (BzA), semicarbazide, pargyline, collagenase, bovine insulin, fatty-acid-free bovine serum albumin, and other reagents were obtained from Sigma-Aldrich (Saint Quentin Fallavier, France and Budapest, Hungary). Enzymes for glycerol assay and liberase blendzyme 3 were from Roche Diagnostics (Indianapolis, USA). 2-[1,2-^{3}H]-deoxyglucose (2-DG, 29.7 Ci/mmol) and [3-^{3}H]-glucose were purchased from Perkin Elmer Life Science Products (Boston, USA).

[^{14}C]-benzylamine (57 mCi/mmol) came from Amersham Biosciences (Buckinghamshire, UK). Amplex red fluorescent dye was from Flouoprobes/Interchim (Montlucon, France).

Antibodies for immunoblot analysis (TK-1079) and for immunohistochemistry (7-88) were a very kind gift of Professor Sirpa Jalkanen (Turku University, Finland).

Hexaquis (benzylamonium) decavanadate (B_6V_{10}) salt was synthesised at GenMedica Therapeutics (Barcelona, Spain) and provided by Dr. Alec Mian.

4-phenylbuthylamine hydrochloride (4-PBA), 3-(4-methylthiophenyl)-propylamine hydrochloride (3-MTPPA) and substrates A-D were synthesised at Semmelweis University, Department of Organic Chemistry. The latter are presented without complete chemical description in order to keep the confidentiality until complete data acquisition and patent application.

5.2. Animals

5.2.1. Housing

All of the mice (C57BL/6 or transgenic) or rats (Wistar) used for our studies were handled in accordance with the European Communities Council Directives for experimental animal care with the principles and guidelines established by the French National Institute of Medical Research (INSERM) or by Semmelweis University. They were housed under specific pathogen-free/conventional conditions with constant temperature (20 to 22°C) and with a 12-hour light-dark cycle. All mice and rats had free

access to food and water. Normal and special diet compositions are presented in Table 4.

Table 4. Diet compositions

	% of energy		
	Fat	Protein	Carbohydrate
Normal diet (ND): 3.85 kcal/g (Gannat, France)	11	19	70
High fat diet (HFD): 4.73 kcal/g (UAR, France)	45	20	35
Very high fat diet (VHFD): 5.24 kcal/g (Safe, France)	72 (Corn oil 14.6, lard 46.5%)	28	<1

5.2.2. Rat models

Streptozotocin-induced diabetes model

Diabetes was evoked by a single *i.p.* injection of 75 mg/kg streptozotocin (STZ) in citrate-buffered saline, pH 5.0. Diabetes induction was verified by the measurement of blood glucose; animals with blood glucose higher than 20 mmol/L were considered being diabetic. The animals were assigned in five groups and were treated for four weeks, starting four days after the induction of the disease. One group of diabetic animals was treated with *s.c.* insulin ultralente 15 IU/animal/day, one group was given BzA 10 mg/kg/day *s.c.*, one group received BzA 10 mg/kg/day *s.c.* and sodium orthovanadate (V) in the drinking water 50 mg/L, the control group received 0.9 % sodium chloride *s.c.* injection (CTRL).

Fasting conditions

Five groups of male Wistar rats were constituted according to their age (7 weeks) and similar initial body weight. They were sacrificed under fed condition or after 1 to 4 days of starvation. None of the rats died during prolonged starvation.

5.2.3. Mouse models

VHFD model

C57BL/6 male mice were purchased from Charles River (l'Arbresle, France) and were housed at 4-6 animals per cage. For one descriptive experiment, one group of 15 mice received standard rodent chow while the other group of 45 mice was fed on VHFD (see Table 4.). As previously reported, VHFD can produce in C57BL/6 mice diverse states of obesity and/or diabetes (Burcelin *et al.*, 2002). For studies on chronic effect of BzA on glycemic control and oxidative stress, mice turned on VHFD for at least three months were screened by intraperitoneal glucose tolerance test (IPGTT) and only those presenting glucose intolerance were used for further studies. These VHFD-fed mice were separated in two groups of equivalent body weight and hyperglycemic response to IPGTT. 16 mice were fed VHFD without any additional treatment (control group), and their water consumption was determined weekly. 16 other mice fed VHFD were given BzA 4000 µmoles/kg/day in the drinking water for 12 weeks (BzA-treated). BzA amount (in mg) to be dissolved in 400 ml drinking solution was weekly adjusted according to mouse body mass (in g) and daily water intake (in ml). Lastly, two other groups of VHFD were constituted in a similar manner: 12 VHFD and 12 VHFD drinking 2000 µmoles of BzA/kg/day (Figure 10.).

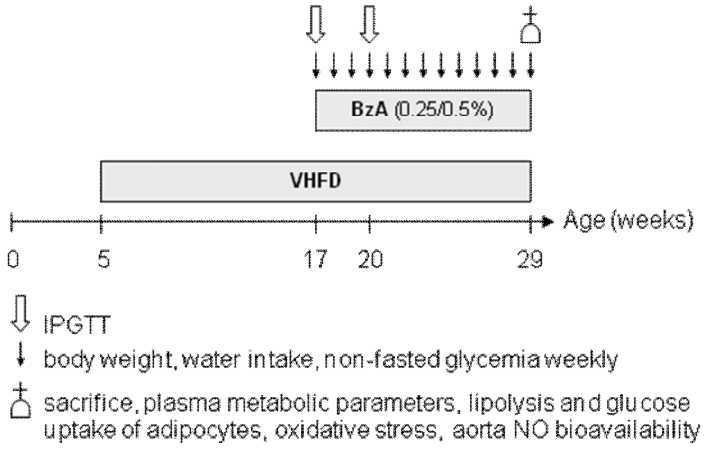

Figure 10. Protocol of VHFD model

Characteristics of the VHFD model

Mice were fed VHFD for three months before receiving BzA injection or chronic *per os* treatment. At this stage they were 17-week old, hyperglycemic, hyperlipidemic, and mildly obese when compared to standard diet-fed control mice. However, they were not hyperinsulinemic since they ingested only very limited amount of carbohydrates, practically absent in the VHFD. Nevertheless, the glucose intolerant

state of VHFD mice was readily evidenced during IPGTT: their hyperglycemic response was exaggerated when compared to controls (respective AUC was 920 ± 28 *vs* 628 ± 17 arbitrary units, $p < 0.001$). Once installed, glucose intolerance remained constant for at least 3 months (Table 5.).

Table 5. Main characteristics of the VHFD model. 15 C57BL/6 male mice received standard rodent chow (ND) and 15 mice very high fat chow (VHFD).VHFD was statistically different from ND: * $p < 0.05$; *** $p < 0.001$.

	ND	VHFD	
Body weight (g)	**24.8 ± 0.3**	**27.8 ± 0.3**	***
Glucose (mmol/l)	**6.60 ± 0.22**	**8.27 ± 0.33**	***
Insulin (µIU/ml)	**19.2 ± 2.6**	**22.7 ± 1.5**	**Ns**
Cholesterol (mmol/l)	**2.92 ± 0.20**	**4.54 ± 0.36**	*
FFA (mmol/l)	**0.23 ± 0.02**	**0.57 ± 0.09**	*

HFD model

HFD (diet composition in Table 4.) was purchased from UAR (UAR, Lyon, France) or Research Diets Inc (New Brunswick, USA) and replaced the standard rodent chow (ND) at the age of 8 weeks for a group of 36 C57BL/6 mice housed at 3 animals per cage with free access to food and water.

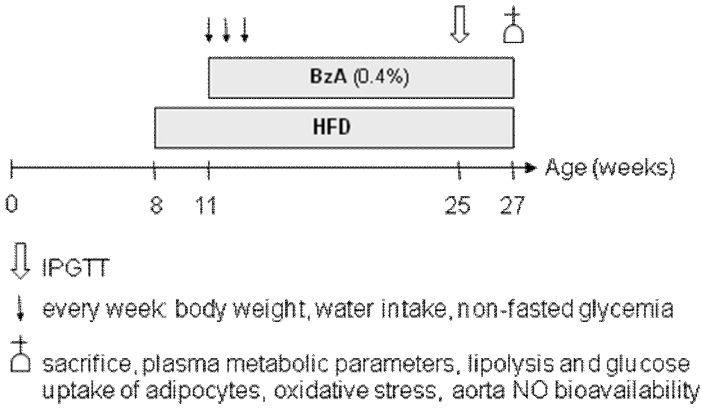

Figure 11. Protocol of HFD model

Three weeks after the nutritional switch, one group of 12 mice was kept untreated (control), while another group of 12 weight-matched mice received BzA in the drinking water for a 17-week period in order to ingest approximately 3600 µmol/kg/day (BzA-treated). For that, the amount of BzA (in mg) to be dissolved in 400 ml of the drinking solution was adjusted taking into account body mass (in g) and daily

water intake (in ml); this resulted in an approx. 0.4 % BzA solution that was changed weekly (Figure 11.).

Characteristics of the HFD model

Mice were fed HFD for three weeks before receiving BzA *per os*. At sacrifice, mice were 27-week old, hyperglycemic, hypercholesterolemic, and mildly obese when compared to control mice fed a standard diet. However, they were not hyperinsulinemic (Table 6.).

Table 6. Main characteristics of the HFD model. 12 C57BL/6 male mice received standard rodent chow (ND) and 12 mice high fat chow (HFD). HFD was stratistically different from ND: * $p < 0.05$; *** $p < 0.001$.

	ND	HFD	
Body weight (g)	**30.4 ± 1.3**	**36.0 ± 1.8**	*
Glucose (mmol/l)	**8.00 ± 1.10**	**15.90 ± 1.00**	***
Insulin (µIU/ml)	**19.0 ± 3.0**	**23.0 ± 6.0**	Ns
Cholesterol (mmol/l)	**2.92 ± 0.20**	**5.63 ± 0.26**	***
FFA (mmol/l)	**0.70 ± 0.07**	**0.61 ± 0.06**	Ns

db-/? mice

Mice that develop a severe hyperglycemia and insulin resistance have been shown to have a recessive inheritance (db-/-) of the gene encoding for the leptin receptor leading to the loss of leptin signaling and altered food consumption. Among many other disturbances, these mice have a lot of reproductive problems therefore the reproduction consisted in using heterozygotes db-/+ (Table 7.).

Table 7. Representation of the reproduction of db-/? mice.

	db-/	db /+
db-/	**db-/-**	**db-/+**
db /+	**db-/+**	~~**db+/+**~~

After weaning, the littermates (3-15) were separated by gender at four weeks of age and the litters were further divided in two groups, one receiving BzA in the drinking water (2 g BzA hydrochloride in 400 ml drinking water, changed weekly). The protocol was conducted on db-/- and their db-/+ littermates as their controls. db+/+ were discarded from the experiment based on their smaller body weight and their grey fur.

At the age of 6 weeks, the db-/- animals, phenotyped on their overweight, were separated from their lean db-/+ littermates to facilitate the follow-up of food and liquid consumption. It means that during a while parameters were collected without knowing

if they would become obese or not. We could thus reconstitute *post hoc* the body weight and non-fasting glycemia of the complete groups as soon as week 4 (Figure 12.).

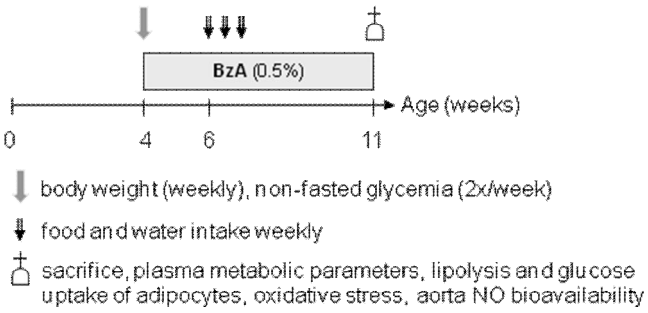

Figure 12. Protocol of db-/? model

Characteristics of the db-/? model

At sacrifice db-/- mice were 11 week old, hyperglycemic, hyperlipidemic, hyperinsulinemic and obese when compared to db-/+ ones. Since the diabetes and obesity observed in this model were of genetic origin, standard diet (ND) was served *ad libitum* to the animals (Table 8.).

Table 8. Main characteristics of the db-/? model. n = 21 (both genders) for db-/+ and n = 28 (both genders). Statistically different from heterozygotes: * $p < 0.05$; *** $p < 0.001$.

	db -/+	db -/-	
Body weight (g)	**21.8 ± 0.8**	**32.9 ± 1.0**	***
Glucose (mmol/l)	**6.09 ± 1.42**	**31.47 ± 2.70**	***
Insulin (µIU/ml)	**9.7 ± 1.2**	**69.3 ± 7.6**	***
Cholesterol (mmol/l)	**1.94 ± 0.13**	**2.29 ± 0.22**	*
FFA (mmol/l)	**0.64 ± 0.05**	**0.90 ± 0.05**	***

Mice invalidated for the AOC3 gene

Mice deficient in SSAO/VAP-1 (KO AOC3) were produced by the group of Prof. S. Jalkanen (Turku Univ., Finland) on a pure 129 background by replacing a portion of the first exon of the mouse AOC3 gene with a neomycin-resistance cassette. KO AOC3 mice, homozygous for the null mutation, were backcrossed onto C57BL/6 for eight generations and compared with wild-type mice with a C57BL/6 background. Mouse VAP-1 mutant allele (null), and endogenous mouse VAP-1 allele (WT) were all identified by polymerase chain reaction (PCR) screening of purified genomic DNA with specific primers and verified immunohistochemically with human and mouse SSAO/VAP-1 antibodies and enzymatic assays (Amplex Red-based fluorometric

detection). Comparisons between genotypes were made on mice sacrificed after overnight fasting, at the age of 6 months, unless stated otherwise.

As for db-/? mice, problems in reproduction prompted us to breed heterozygous animals and after genotyping, the homozygous offsprings bearing two copies of the invalidated gene were compared to homozygous wild type littermates, when possible or, when precised to WT of the same strain.

Characteristics of the model

At sacrifice mice were 27-week old, normoglycemic, normolipidemic, not hyperinsulinemic and not obese when compared to WT littermates. Note that, in several mature females, an overweight occurred lately as already observed during the preliminary work of Bour and colleagues (Bour *et al.*, 2009) (Table 9.).

Table 9. Main characteristics of the KO AOC3 model. Data for 10 male mice.

	WT	KO AOC3
Body weight (g)	**31.5 ± 1.8**	**29.3 ± 1.2**
Glucose (mmol/l)	**6.11 ± 0.39**	**6.83 ± 0.17**
Insulin (µIU/ml)	**13.4 ± 4.3**	**10.3 ± 1.9**
Cholesterol (mmol/l)	**3.29 ± 0.28**	**3.00 ± 0.33**
FFA (mmol/l)	**0.70 ± 0.09**	**0.61 ± 0.08**

5.3. *In vivo* non invasive studies

5.3.1. Intraperitoneal glucose tolerance tests (IPGTT)

Fasted mice (5 h) were *i.p.* injected with saline or BzA (0.7 to 700 µmol/kg), BzA (7 µmol/kg) + vanadate (22 µmol/kg), B_6V_6 (7 umol/kg) 15 min before *i.p.* glucose (1 g/kg) load. Blood was collected from the tail vein of conscious mice after 0, 15, 30, 45, 60, 90 and 120 min for determination of glucose levels with a glucosemeter (Roche Diagnostic, Mannheim, D). Blood was also collected before any *i.p.* injection to determine basal glycemia. Area under the curve (AUC) of hyperglycemic excursion was calculated and expressed in arbitrary units as previously detailed (Morin *et al.*, 2002).

5.3.2. *In vivo* metabolic follow up

In many protocols unfasted blood glucose, body weight, water consumption and food intake were determined weekly, except in HFD and VHFD conditions due to the

sticky nature of the pellets. Some db-/? mice were chosen randomly and put in individual metabolic cages for 24 hours to collect urine.

5.3.3. Determination of fat and lean mass

To measure total body fat and lean mass by nuclear magnetic resonance, living mice were placed into a Plexiglass tubular holder inserted into an Echo MRI NMR machine (100TM3; Echo Medical Systems, Houston, TX) and measured during less than 1 minute.

5.4. Blood and tissue sampling

Most comparisons between genotypes or between untreated and treated animals were made on mice/rats sacrificed after overnight fasting by cervical dislocation, at the stated age. When rodents were sacrificed at the end of treatment, tissues and plasma/serum were immediately frozen until determination of diagnostic parameters using the described kits and methods.

5.4.1. Plasma/serum markers

Ultrasensitive Insulin Elisa kit was obtained from Mercodia (Uppsala, Sweden), NEFA C Wako Free fatty acid kit was from Oxoid (Dardilly, France). Plasma glucose was measured using the glucose oxidase method (Biomériux, France). Analyses of circulating fructosamine (using a colometric assay based on nitrotetrazolium blue reduction) and of uric acid (using a uricase-peroxidase method) were adapted to 96-well microplates for use in the multi-analyser. Plasma total cholesterol, HDL, LDL and TGs were determined using a Cobas-Mira+ multi-analyser, according to the manufacturer' instructions (Roche, Neuilly, France). AGE levels in the sera/plasma were measured by their native fluorescence (370/440 nm) (Wu *et al.*, 1996). Serum samples were diluted 30 times with pH 7.4 phosphate buffered saline (PBS), mixed with chloroform to remove lipoproteins, and centrifuged (15 000 g, 4°C, 5 min). The aqueous phase was used for the fluorimetric analysis. Glycated hemoglobin levels (HbA_{1C}) were determined by a commercially available laboratory assay using immunoturbidimetry.

5.4.2. RNA Extraction, Reverse Transcription, and Real-Time PCR

400 mg of WAT/liver were homogenised in 2 ml of a highly denaturing guanidine-thiocyanate-containing buffer supplied by the RNeasy mini kit (Qiagen, Courtaboeuf, France) plus 20 µl of β-mercaptoethanol. After lipid elimination by chloroform extraction, total RNA was extracted according to the supplier's instructions. Then, 0.5 µg of total RNA was reverse-transcribed using random hexamers and Superscript II reverse transcriptase (Invitrogen, Cergy Pontoise, France). The same reaction was performed in parallel without reverse transcriptase (RT-) to estimate genomic DNA contamination. Real-time PCR was performed starting with 6.25 ng of cDNA and both sense and antisense oligonucleotides in a final volume of 20 µl using the SYBR Green TaqMan Universal PCR Mastermix (Eurogentec, Angers, France). Fluorescence was monitored in GeneAmp 7500 detection system instrument (Applied Biosystems, Foster City, CA). Oligonucleotide primers were designed using Primer Express (Perkin- Elmer Life Sciences, Courtaboeuf, France) and verified on Blast Nucleotide software. Primer specificity was checked by the occurrence of a single peak of expected size during dissociation experiments. Analysis of the 18S ribosomal RNA was performed using the Ribosomal RNA Control TaqMan assay kit (Applied Biosystems) to normalize gene expression. Results were expressed as arbitrary units relative to 18S expression, as reported (Bour *et al.*, 2007a) using the following equation:

$$2^{(Ct18S-Ctgene"RT+")} \times \left[1 - \frac{1}{2^{\{Ctgene"RT-"-Ctgene"RT+"\}}}\right],$$

where Ct corresponds to the number of cycles needed to generate a fluorescence threshold.

5.4.3. Human adipose tissue

Samples of subcutaneous adipose tissue were obtained from a total of 6 healthy nonobese women: mean body mass index (BMI) was 28.0 ± 1.2 kg/m^2, and age ranged from 30 to 48 years. Mammary or abdominal adipose tissue was obtained from patients undergoing dermolipectomy (not suction lipectomy) of localized fat depots for cosmetic

reasons in the Department of Plastic Surgery of Toulouse Rangueil Hospital. All patients had fasted overnight, and the operations were performed in the morning under general anesthesia. The study was approved by the Ethical Committee of Toulouse University Hospital. Samples of subcutaneous tissues were transported to the laboratory in less than 15 min and were promptly subjected to homogenisation or adipocyte preparation through collagenase digestion.

5.5. *Ex vivo* and *in vitro* determinations

5.5.1. Determination of DNA content from adipose tissue

30-50 mg of adipose tissue (SCWAT or PGWAT) was digested in 1 ml preheated (30 min at 55°C) TBS (50 mM TRIS-HCl + 150 mM NaCl, pH 7.5) containing 0.1 mg/ml proteinase K (Macherey-Nagel GmbH, Düren, Germany) shaked overnight at 55°C. 10 µl of DNA samples were diluted in 90 µl TE buffer (10 mM TRIS-HCl + 1 mM EDTA, pH 8). 10 µl of the diluted samples and 90 µl of TE were put in wells. 100 µl of mouse genomic DNA (Bioline, London, UK) were used for calibration (0-500 ng/ml). 100 µl of PicoGreen (Invitrogen Life Technologies, Glasgow, UK) solution was used once 200-fold diluted as reagent. After 2 minutes the fluorescence (excitation: 460 nm, emission: 530 nm) was detected in 96-well dark plates by a Fluoroscan Ascent FL plate reader (Thermo Electron Corporation, Vantaa, Finland).

5.5.2. Immunohistochemistry

For immunohistological analysis, WAT was fixed in PBS containing 4% paraformaldehyde. The sections were first incubated in PBS 2% bovine serum albumin (BSA) for 30 minutes, then with PBS 2% BSA 50 µg/ml rat anti-VAP-1 (7-88 mAb recognizing mouse VAP-1) for 90 minutes. Sustained lavages were performed in PBS 2% Tween (4x10 min) and PBS (5 min). Further incubation in PBS 2% BSA for 30 minutes then in PBS + 2% BSA containing peroxidise-coupled anti-rat IgG (Vector Laboratories, Peterborough, UK) was used as secondary antibody. Samples were exposed to another wash block of PBS 2% Tween (4x) and PBS (1x) then incubate in DAPI 1X (Invitrogen) to mark nuclei. Whole tissue parts were then analysed using fluorescence microscopy (inversed microscop Eclipse TE2000-U, Nikon).

5.5.3. Immunoblotting assays

WAT was homogenised in sample buffer (Tris-HCl 62,5 mM, pH 6.8, SDS, 10 % w/v, glycerol 0,01 % v/v, bromophenol blue), then kept at 95°C for 5 min. SDS-PAGE electrophoresis was performed using BioRad polyacrylamide gels (acrylamide gradient 4-12 %). After loading 20 µg of protein per lane, the electrophoretic separation in 25 mM TBS + 20% SDS was allowed to proceed at 100 V/gel during 1 hour then 125 V during 2 hours.

Separated proteins were transferred to a nitrocellulose membrane in TBS + 20% methanol using an electric field of 60 V during 1 h then 80 V for 30 minutes. The nitrocellulose membranes were then incubated for 60 minutes under gentle shaking in blocking solution. The blocking solutions used were TBST (25 mM Tris Base, 140 mM NaCl, 0.1% v/v Tween 20) containing 5% w/v non-fat dry milk. The saturated membranes were subsequently incubated overnight, at 4°C with the corresponding primary antibodies against (4.8 ug/ml TK 10-79: rat antibody against mouse SSAO; or β-actine rabbit) mouse in TBST containing 5% of the corresponding blocking solution. After repeated washing in TBST, membranes were incubated with the secondary antibody monoclonal anti-rabbit (for β-actin) or anti-rat IgG coupled to horseradish peroxidase (for SSAO) (2 h, at 4°C, gentle shaking), Sigma Detection was performed using enhanced chemoluminescence (ECL, Amersham Pharmacia Biotech.). Blots were subsequently scanned and quantified using Image'Quant TL (Molecular Dynamics, Sunnyvale, CA, USA).

5.5.4. Isolation of adipocytes

Samples of adipose tissue were dissected and digested by 60 µg/ml liberase (rodents) or 40 µg/ml liberase (human) (Blendzyme3, Roche Diagnostics, Mannheim, Germany) in Krebs-Ringer solution, pregassed with 95% O_2 / 5% CO_2, containing bovine serum albumin (35 mg/ml), 15 mM sodium bicarbonate, 10 mM Hepes (pH 7.4), and 2 mM pyruvate or 5 mM glucose. After digestion for 35 to 45 min at 37°C under agitation, isolated fat cells were filtered and washed three times in the same buffer without liberase. Freshly isolated adipocytes were adjusted to a suitable dilution and immediately dispensed in plastic vials for the determination of lipogenesis, glucose transport, or lipolytic activities in intact cells.

5.5.5. Lipolysis

After being washed, the floating fat cells were diluted in around 10-fold their volume of Krebs-Ringer containing 15 mM sodium bicarbonate, 10 mM HEPES, and bovine serum albumin, and 400 µl of the cell suspension was immediately distributed under shaking into plastic incubation vials containing 4 µl of drug dilutions at 100-fold the final concentration to be tested. After a 90-min incubation, the glycerol released into the medium was enzymatically assayed as described (Morin *et al.*, 2001). The results were expressed as percentage of responses to 10 nM isoprenaline, which is independent of fat cell size and basal triglyceride breakdown (Prévot *et al.*, 2007).

5.5.6. Hexose Transport

Isolated adipocytes were diluted 10-fold in Krebs-Ringer buffer and 400 µl of cell suspension were distributed in vials containing the tested agents and incubated for 45 min at 37°C. The addition of 2 mM pyruvate replaced the removal of glucose in order to avoid any competition with its non-metabolisable analogue added under the form of an isotopic dilution of [^{3}H] 2-Deoxyglucose (2-DG, 26.5 Ci/mmol, PerkinElmer Life Sci.,Boston, MA). Thus, 2-DG was added in 100 µl portions for an exact period of 10 min at a final concentration of 0.1 mM (0.6 mCi/100 ml). Assays were then stopped by 100 µl of 100 µM cytochalasin B and aliquots were centrifuged in microtubes containing dinonyl-phthalate to separate adipocytes from buffer by flotation. Intracellular radioactive 2-DG was counted as described. After centrifugation, the fat cells (upper part of the tubes) were placed in scintillation vials, and the intracellular radioactivity was counted as described. Extracellular 2-DG present in the cell fraction, which was determined using adipocytes whose transport activity had been previously blocked by cytochalasin B, did not exceed 1% of the maximum 2-DG transport in the presence of insulin.

5.5.7. Lipogenesis

Isolated adipocytes were diluted 10-fold in Krebs-Ringer buffer and 400 µl of cell suspension were distributed in vials containing the tested agents and [^{3}H]-glucose (400 000 dpm/tube), and were incubated for 120 min at 37°C. Again, to better detect the glucose incorporation into lipids, the labelled hexose was present at 0.55 mM final

instead of 5.5 mM for lipolysis measurements. Reaction was stopped by a cocktail for liquid scintillation that is non miscible in aqueous solutions, allowing extraction into an organic phase and the measurement of [^{3}H]-glucose incorporated and metabolized into lipids. Since the radiolabelling element is tritium, the radioactive glucose which was not metabolized remained in the aqueous phase and was not in contact with the scintillation organic mixture, present in the upper phase and the only detected during radioactivity counting as already described (Moody *et al.*, 1974).

5.5.8. Measurement of SSAO activity and H_2O_2 production

Radioactive method

SSAO activity was measured based on the conversion of radiolabelled substrate [^{14}C]-benzylamine to [^{14}C]-benzaldehyde according to the method of (Yu *et al.*, 1992). Tissue homogenates were used for the enzyme activity measurements. The concentration of substrate used in the assay was 0.01-1 mM. Enzyme activity was expressed as pmol [^{14}C]-benzaldehyde produced/mg protein/min, since only the benzaldehyde was extracted in the toluene/ethyl acetate-based extraction medium while the BzA that escaped from oxidation remained in the lower aqueous phase and was not counted.

Amplex Red method

Amplex Red (10-acetyl-3,7-dihydrophenoxazine) was used as a fluorescent probe for the detection of hydrogen peroxide. 50 µl tissue homogenate was preincubated in 50 µl 0.2 M pH 7.4 phosphate buffer with or without inhibitors (1 mM semicarbazide or 0.1 mM pargyline) for 10 minutes at 37°C. The reaction was started by adding 50 µl substrate/buffer and 50 µl reaction mixture containing 40 µM Amplex Red, 4 U/ml horseradish peroxidase. After 30 minutes of incubation at 37°C in 96-well dark plates the fluorescence (excitation: 530 nm, emission: 590 nm) was detected by a Fluoroscan Ascent FL plate reader (Thermo Electron Corporation, Vantaa, Finland). A standard H_2O_2 curve was built in a concentration range of 0–10 µM and used for calibration. Enzyme activity or H_2O_2 release was given as pmol H_2O_2/mg protein/min.

Approximately 20 mg of WAT pieces were put in 580 µl phosphate buffer for 10 minutes. 200 µl fluorogenic mixture (same as for honogenates) was added at time 0. Plastic tubes protected from light were incubated for 30 minutes at 37°C. 200 µl of the

solution was placed to 96-well dark plates for the detection of fluorescence signal using reactives and calibration as described above.

5.5.9. Measurement of tissue nitrite and nitrate concentration

Tissue nitrate and nitrite concentrations were measured by a capillary electrophoresis method (Szökő *et al.*, 2004). Briefly, samples were sonicated in 5 volumes of 0.1 M NaOH to immediately destroy proteins. pH was adjusted to about 7 by an equal volume of 0.09 M acetic acid. Samples were then placed in boiling water for 3 minutes, then cooled on ice and proteins were removed by centrifugation (20 000 g for 10 minutes at 4 °C). Supernatant was used for injection (100 mbar for 0.75 min). Electrophoretic separation was performed by Prince capillary electrophoresis system (PrinceTechnology, Emmen, The Netherlands). Separations were carried out in uncoated fused silica capillaries (75 μm i.d., 365 μm o.d., 70 cm total length, 55 cm to the detector, Polymicro Technology, Phoenix, AZ, USA) using 30 mM sulfate-β-alanine pH 3.8 buffer, constant current: -110 μA. Appartus was equipped with a UV detector, set at 214 nm. Calibration curve was fitted for 0-100 μM KNO_3 and $NaNO_2$, 100 μM $KBrO_3$ was used as internal standard.

5.6. Statistical analysis

Results are given as means ± SEM and were compared using Student's t test. NS means no significant difference between the compared samples. We used the Splus 6.1 software version (Insightful , WA) for statistical computations of the parameters of db-/? animals. A linear mixed statistical model was fitted to the data collected constitutively during the treatment period. For end-point analysis (mostly parameters at sacrifice) 2x2 ANOVA or student's *t* test was used.

6. Results

6.1. Investigation of SSAO activity in adipose tissue

6.1.1. Determination of SSAO activity in subcutaneous adipose tissue

We measured SSAO activity using BzA as a substrate, and basal SSAO-dependent H_2O_2 production in SCWAT of C57BL/6 mice.

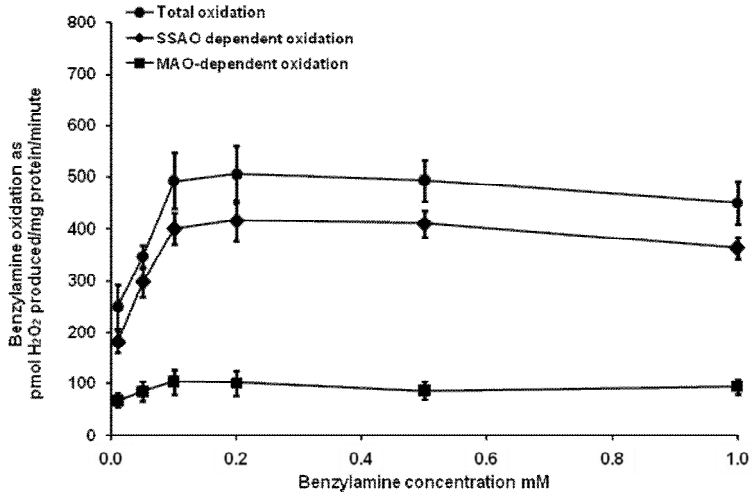

Figure 13. Benzylamine oxidation in SCWAT. Total, SSAO and MAO-dependent benzylamine oxidation was determined in SCWAT homogenate of 4 adult (15-week old) male C57BL/6 mice. H_2O_2 production was measured by Amplex Red method, data are presented as mean ± SEM.

As shown in Figure 13, BzA oxidation in SCWAT, in the concentration range of 0.02-1 mM, is very sensitive to 1 mM semicarbazide (SSAO-dependent oxidation), while a very little part of its oxidation is sensitive to 0.1 mM pargyline (MAO-dependent oxidation). Maximum amine oxidation capacities have been reached already at 0.1 mM BzA concentration. As it can be seen on the Figure 11, a detectable H_2O_2 production in the mouse SCWAT in the absence of exogenous BzA was found, which could be inhibited by semicarbazide. It was decided to verify whether this hydrogen peroxide production, detected without any addition of exogenous amine, was really related to a basal, spontaneous SSAO activity.

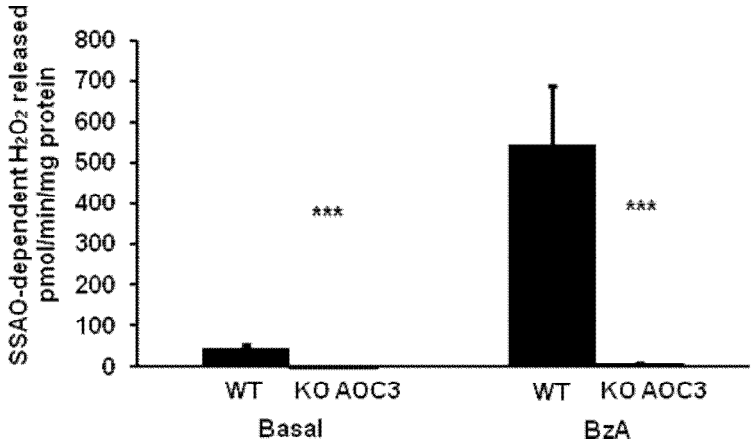

Figure 14. SSAO-dependent H_2O_2 production. Basal SSAO-dependent H_2O_2 release is measured in the absence and in the presence of 0.1 mM benzylamine (BzA) in SCWAT homogenate of 3 WT and 5 KO AOC3 27 week-old mice. Data are presented as mean ± SEM, difference between genotypes is marked: *** $p < 0.001$.

The basal SSAO-dependent hydrogen-peroxide production was totally abolished in KO AOC3 mice (Figure 14.).

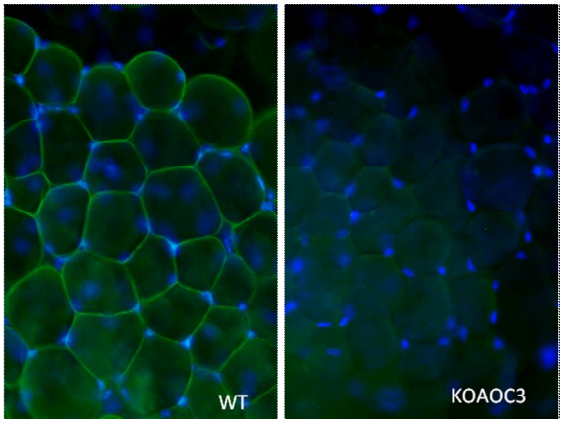

Figure 15. Effect of genetic invalidation of AOC3 on immunoreactive SSAO in white adipose tissue. AOC3 protein (SSAO) is not present at the surface of adipocytes of KO AOC3 mice while it is aboundantly expressed in wild type mice (WT). Semicarbazide-sensitive amine oxidase protein labelled in subcutaneous mouse fat cells by the anti SSAO-VAP-1 antibody 7-88 (Merinen 2005)+ DAPI (Magnification: 20x).

Since the KO AOC3 mice are totally lacking SSAO-dependent BzA oxidation (Figure 14.) and SSAO protein (Figure 15.) in their adipocytes, one could exclude the possibility that another amine oxidase, sensitive to semicarbazide (DAO or LO), would be responsible for such semicarbazide-inhibitable basal hydrogen peroxide production.

6.1.2. Influence of the adipose mass on the SSAO activity in different adipose tissues

High fat diet induced obesity

The effect of high fat diet-induced obesity (see characteristics in chapter 5.2.3) on SSAO activity and basal SSAO-dependent H_2O_2 release was investigated in PGWAT and SCWAT of C57BL/6 mice. Mice eating normal chow served as controls in the experiment.

Table 10 shows that BzA oxidation was more intensive in PGWAT than in SCWAT in control, as well as in obese mice. Obesity caused an increase in SSAO activity in SCWAT but not in PGWAT. This difference was also seen in basal SSAO-dependent H_2O_2 release (in absence of BzA).

Table 10. H_2O_2 production of SC and PGWAT homogenates. SSAO activity was assessed as 1 mM semicarbazide inhibitable hydrogen peroxide release. Results are expressed in mean ± SEM, n=5, statistically different from normal diet at: * $p < 0.05$; ** $p < 0.01$.

	HFD	ND	
Basal SSAO-dependent H_2O_2 release (pmol/mg prot/min) in PGWAT	**25 ± 2**	**29 ± 6**	**ns**
Basal SSAO-dependent H_2O_2 release (pmol/mg prot/min) in SCWAT	**48 ± 8**	**18 ± 5**	******
SSAO activity of PGWAT (0.1 mM BzA used-pmol H_2O_2/mg prot/min)	**210 ± 26**	**152 ± 35**	**ns**
SSAO activity of SCWAT (0.1 mM BzA used-pmol H_2O_2/mg prot/min)	**100 ± 17**	**43 ± 14**	*****

Genetic obesity

Effect of obesity on SSAO activity and basal SSAO-dependent H_2O_2 release was investigated in different white adipose tissue depots, namely PGWAT, PRWAT and SCWAT in genetically obese mice owing to the use of the db-/? strain (Table 11.). To assess SSAO activity BzA, was added in different concentrations between 0.02-0.5 mM. Lineweaver-Burk plot was used for Km and Vmax determination. Mice with a naturally occurring mutation of db gene with loss of function became obese and diabetic (see Materials and Methods for further information). Heterozygotes for the mutation were used as controls in the protocol.

Table 11. H_2O_2 production of SC, PG and PR WAT tissue homogenates. Results are expressed in mean±SEM, n=6 for PG and PR ,7 for SCWAT, statistically different from db-/+ * $p < 0.05$.

	db-/-	db-/+	
Basal SSAO-dependent H_2O_2 release (pmol/mg prot/min) in PGWAT	28 ± 9	29 ± 5	ns
Basal SSAO-dependent H_2O_2 release (pmol/mg prot/min) in PRWAT	31 ± 9	39 ± 9	ns
Basal SSAO-dependent H_2O_2 release (pmol/mg prot/min) in SCWAT	92 ± 18	68 ± 14	ns
SSAO activity of PGWAT (0.1 mM BzA used-pmol H_2O_2/mg prot/min)	356 ± 63	252 ± 37	ns
SSAO activity of PRWAT (0.1 mM BzA used-pmol H_2O_2/mg prot/min)	273 ± 41	294 ± 55	ns
SSAO activity of SCWAT (0.1 mM BzA used-pmol H_2O_2/mg prot/min)	299 ± 47	152 ± 10	*
Km PGWAT (µM)	23 ± 1	28 ± 4	ns
Km PRWAT (µM)	28 ± 3	24 ± 1	ns
Km SCWAT (µM)	33 ± 4	22 ± 5	ns
Vmax PGWAT (pmol H_2O_2/protein/min)	413 ± 75	306 ± 41	ns
Vmax PRWAT (pmol H_2O_2/protein/min)	339 ± 38	345 ± 61	ns
Vmax SCWAT (pmol H_2O_2/protein/min)	440 ± 72	212 ± 22	*

Based on our measurements, db-/- mice had increased SSAO activity in SCWAT (Table 11.). Both calculated Vmax and the measured H_2O_2 production during oxidation of 0.1 mM BzA were increased in the obese mice. PGWAT and PRWAT SSAO activities were not affected by the recessive mutation of db gene. Increased SSAO activity in SCWAT was in line with both the enhanced expression of AOC3 mRNA (db-/- 78.22 ± 7.58 *vs* db+/- 30.68 ± 3.37 arbitrary units, n = 9, $p < 0.001$, not shown) and enriched content in immunoreactive SSAO proteins detected by Western Blot analysis : (2.65 ± 0.06 *vs* 2.04 ± 0.11 arbitrary units normalized for β-actin, n = 6, $p < 0.001$) (Figure 16.).

Figure 16. Immunoblot analysis of SSAO protein in SCWAT of female db -/- and db -/+ mice, representative for 6 cases.

Influence of fasting on INWAT SSAO activity

Influence of 1-4 days of fasting on SSAO activity was investigated in INWAT of Wistar rats.

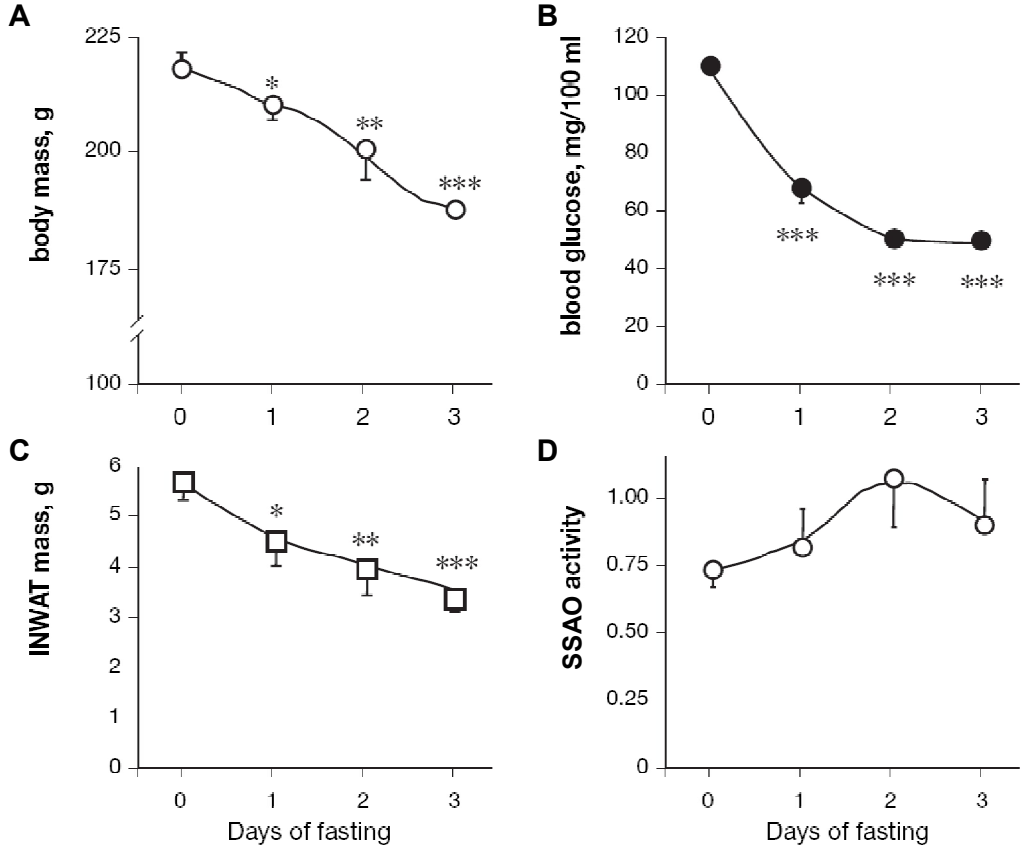

Figure 17. Influence of fasting duration on body mass (A), blood glucose (B), mass of intra-abdominal white adipose tissues (INWAT) (C), and SSAO activiy in INWAT (D). Seven-week old rats were sacrificed under fed state (day 0) or after the indicated days of fasting. Semicarbazide sensitive amine oxidase activity was determined on crude INWAT homogenates and are expressed as oxidised (^{14}C benzaldehyde) product formed in nmol/mg protein /min. Mean ± SEM of 6 animals in fed state (day 0) and of 3 for each fasting duration. Statistically different from fed control at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

Fasting-induced slimming (Figure 17. A) and glucose lowering (Figure 17. B) effects were statistically significant even after the first day of starvation. As expected, longer fasting periods provoked a continuous fall in body and dissectible INWAT mass (Figure 17. A, C) while blood glucose level remained stable (Figure 17. B). Under these conditions, no change in SSAO activity was observed in the INWAT of male rats (Figure 17. D).

6.2. *In vitro* insulin-like effect of benzylamine

In adipocytes, several amines may exert insulinomimetic effects (see 3.2.6) when oxidised. BzA-induced *in vitro* insulin-like effects were thus investigated in mouse adipocytes. To see the role of SSAO in these processes adipocytes of AOC3 null mice were also tested.

Lipolysis in rodent fat cells

Effect of insulin, BzA, vanadate and their combination on lipolytic response of isolated adipocytes was investigated. 10 nM isoprenaline was used for stimulation of lipolysis.

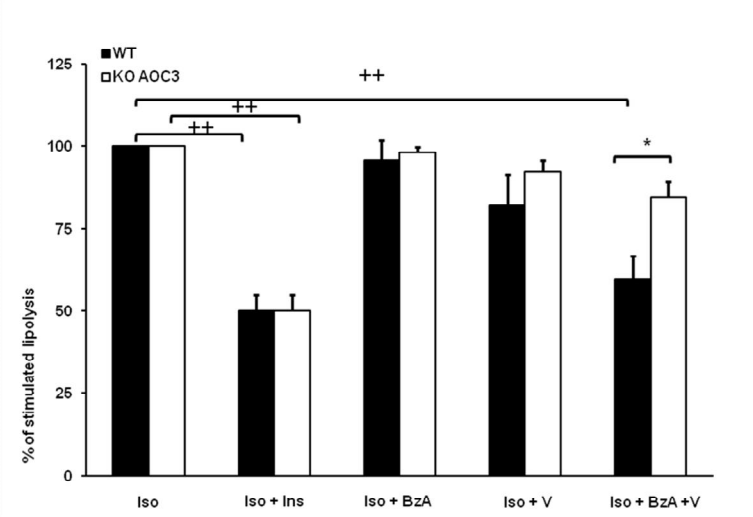

Figure 18. Inhibition of 10 nM isoprenaline-induced lipolysis (Iso) by 100 nM insulin (Ins), 0.1 mM benzylamine (BzA), 0.1 mM vanadate (V) and their combination (BzA + V) in mouse adipocytes (n = 10 WT and 7 KO AOC3, age 27 weeks). Data are expressed as mean ± SEM in % of 10 nM isoprenaline (Iso) stimulation above the basal lipolysis (which was 0.67 ± 0.07 and 0.44 ± 0.04 μmol glycerol released/100 mg lipid/90 min in WT and KO AOC3, mean ± SEM, n = 29 and 25, respectively, $P < 0.01$) , Statistically different (t test) from 10 nM isoprenaline's effect: ++ $p < 0.01$;and different from WT * $p < 0.05$.

Figure 18. shows that BzA or vanadate alone could not inhibit β-adrenergic-stimulated lipolysis, while the anti-lipolytic effect of BzA + vanadate was abolished in KO AOC3 mice. The anti-lipolytic effect of 100 nM insulin was identical in both genotypes.

Glucose uptake in rodent adipocytes

Impact of insulin, BzA, vanadate and their combination on glucose uptake of isolated adipocytes was studied.

BzA or vanadate alone exerted only 20-30 % of 100 nM insulin's effect on glucose uptake. When they are used in combination, glucose uptake stimulation reached 85% of that of 100 nM insulin (Figure 19.). Such stimulation of glucose uptake by amines was found to be abolished in KO AOC3 mice (Bour *et al.*, 2007b).

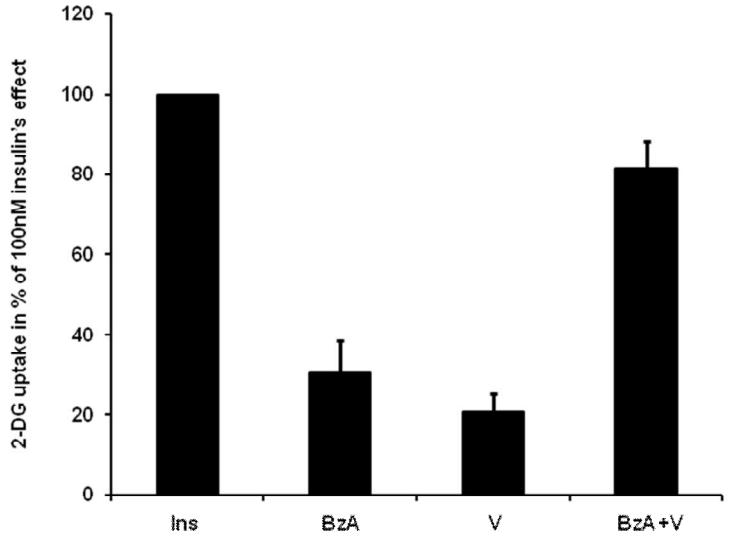

Figure 19. Effect of 0.1 mM benzylamine (BzA) and 0.1 mM vanadate (V) on glucose uptake in isolated mouse adipocytes from INWAT of HFD mice (n = 12, age 28 weeks). Data are expressed as mean ± SEM in % of 100 nM insulin's (Ins) effect.

Lipogenesis in rodent adipocytes

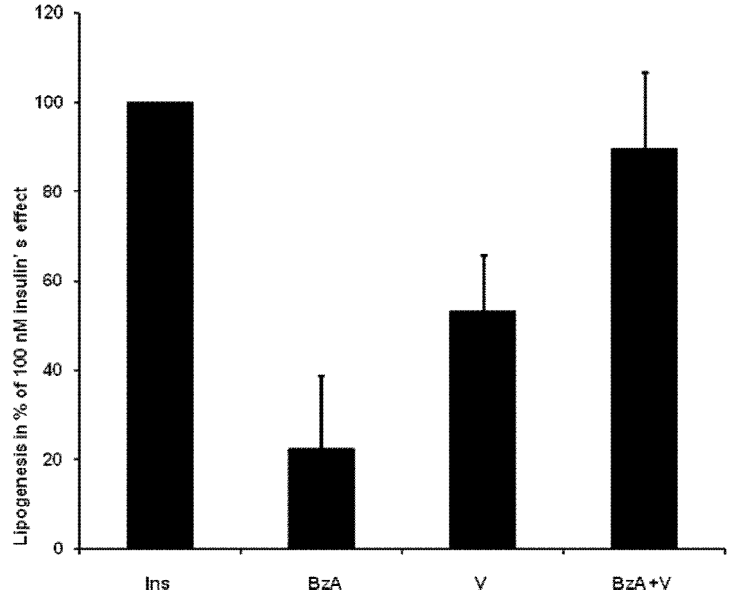

Figure 20. Lipogenic effect of benzylamine (BzA) and vanadate (V) and their combination (BzA + V) in isolated mouse adipocytes from INWAT of C57BL/6 mice (n = 10, age 27 weeks). Data are expressed as mean ± SEM in % of 100 nM insulin's effect.

Lipogenesis measurements were performed in adipocytes isolated from mouse INWAT to study the effect of BzA. BzA or vanadate alone had partial effect on lipogenesis compared to that of their combination. BzA + V, which reached 89 ± 17% of 100 nM insulin's effect (Figure 20.). 100 nM insulin stimulation was similar in KO AOC3 and WT mice adipocytes (1.9 ± 0.2 *vs* 2.0 ± 0.1 fold increase over basal ^{3}H glucose incorporation). In KO AOC3 mice, the lipogenic effect of BzA + vanadate was

compared to WT animals, and reached only the 37 ± 13% of the insulin's effect. Stimulatory effect of vanadate was 20 ± 6% of that of insulin in SSAO invalidated mice, while 53 ± 13% of that of insulin in WT animals.

Together, these results show that the absence of SSAO alters the *in vitro* insulinomimetic effects of BzA + vanadate, thus these effects are at least partly due to BzA's oxidation.

6.3. *In vivo* insulin-like effect of benzylamine

6.3.1. Effect of single injection of benzylamine on glucose handling

We have observed in adult normoglycemic mice that, when injected 15 min before a glucose load of 1 g/kg, 70 µmol/kg *i.p.* BzA was able to lower the hyperglycemic response (area under the curve of the hyperglycaemic excursion was : 2375 ± 295 arbitrary units in BzA treated *vs* 3386 ± 252 arbitrary units in controls, n = 6 and 12, $p < 0.05$).

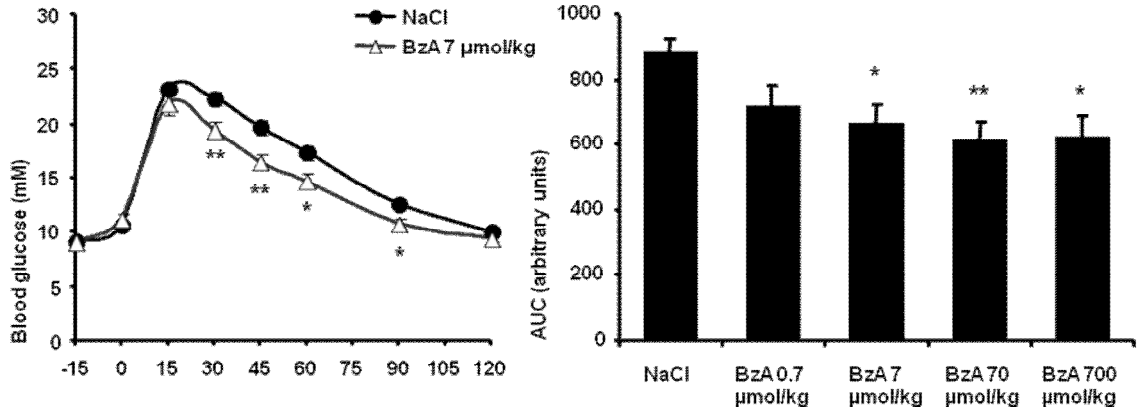

Figure 21. Dose-dependent effect of benzylamine (BzA) on provoked hyperglycemic responses in VHFD mice. After 5 hour fasting, mice *i.p.* received saline (0) or the indicated dose of BzA 15 min before IPGTT consisting of a load of glucose at time 0 (1 g/kg, *i.p.*). Blood samples were collected from the tail vein of conscious animals for immediate blood glucose assay. Areas under the curve (AUC) of the hyperglycemic responses were expressed in arbitrary units. Means ± SEM of 4 to 8 mice per group, age 17 weeks. Different from control at: * $p < 0.05$, ** $p < 0.01$.

The dose-response of the anti-hyperglycemic action of BzA was studied in mice rendered glucose intolerant by 3 months of VHFD. Bolus *i.p.* injection of BzA did not lower blood glucose in overnight fasted mice which was 9.2 ± 0.5 mM 15 min after saline bolus, and remained at 8.7 ± 0.3, 9.5 ± 0.5, 8.5 ± 0.5 and 9.1 ± 0.2 mM after receiving 0.7, 7, 70 or 700 µmol/kg BzA, respectively (ns). However, BzA improved

the somewhat altered glucose tolerance of these mice adapted to a calory intake almost devoid of carbohydrates. In fact, BzA dose-dependently reduced the hyperglycemic response following an *i.p.* glucose load (Figure 21.). The anti-hyperglycemic action of BzA occurred without a detectable change in plasma insulin level. Stimulation of insulin secretion thus was not the mechanism by which BzA exerted its anti-hyperglycemic effect.

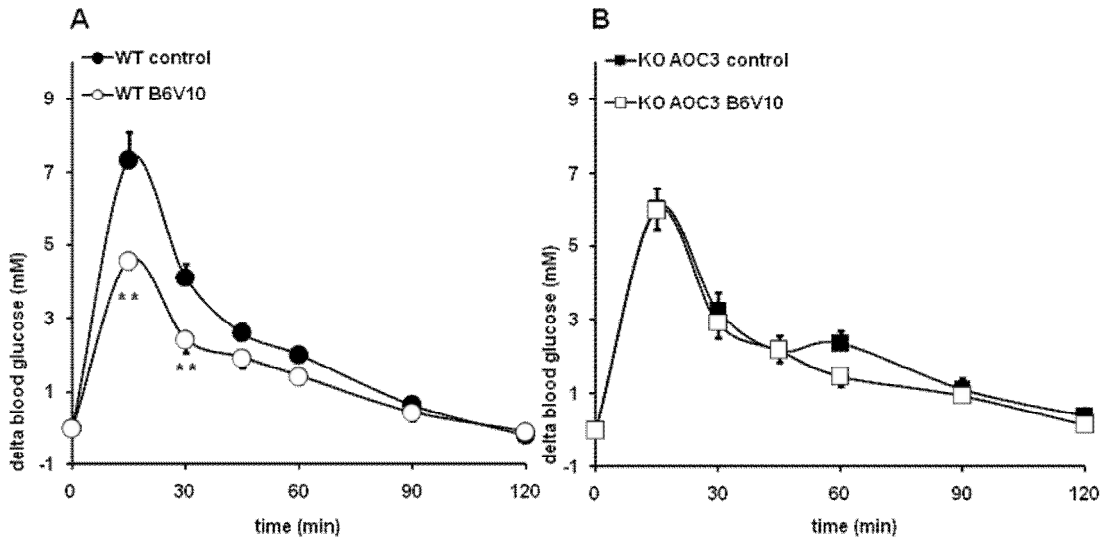

Figure 22. Glucose tolerance test after single injection of hexakis (benzylammonium) decavanadate (B_6V_{10}) or saline (control) in WT (A) and KO AOC3 (B) mice. B_6V_{10} (7 µmol/kg) or NaCl 0.9% were *i.p.* injected 15 min before glucose load (1 g/kg, *i.p.*) at time 0. Mean ± SEM of 12 mice per group. Different from corresponding control at : ** $p < 0.01$.

It was also tested whether the response to 7 µmol/kg BzA could be potentiated by simultaneous administration of vanadate. No greater anti-hyperglycemic effect was found when BzA 7 µmol/kg was combined with vanadate 22 µmol/kg (respective AUC of IPGTT were 469 ± 38 and 441 ± 35 arbitrary units, n = 10-14, ns).

Since a benzylamine-vanadate complex salt synthesised by GenMedica Therapeutics (Barcelona, Spain) named hexaquis (benzylamonium) decavanadate (B_6V_{10}) has been shown to exert similar or even improved insulinomimetic effect than the combination of benzylamine and vanadium (Garcia-Vicente *et al.*, 2007; Yraola *et al.*, 2007), we took advantage of the availability of this compound to further verify whether its mechanism of action was really dependent on amine oxidase activity. 7 µmoles of B_6V_{10} were injected to WT and KO AOC3 mice. As Figure 22 shows the compound improved glucose tolerance in WT but not in KO AOC3 mice. The presence of SSAO is thus crucial for the *in vivo* effect of the benzylamine compound. Insulin

levels measured at 15 minutes after glucose load were similar in all studied groups (WT control: 0.74 ± 0.10, WT B_6V_{10}: 0.59 ± 0.08, KO AOC3 control: 0.72 ± 0.05, KO AOC3 B_6V_{10}: 0.71 ± 0.07 μg/l, n=9-11), thus the effect is not the consequence of an increased insulin release.

Our studies have shown that BzA can directly interact with adipose tissue, by increasing the hydrogen peroxide production, and by activating glucose transport and lipogenesis. Moreover, BzA improves glucose tolerance *in vivo*. We supposed that chronic BzA treatment might affect not only hyperglycemia but also hyperglycemia related vascular complications.

6.3.2. Effect of chronic benzylamine injection in streptozotocin-induced diabetes in rats

Diabetes was evoked by streptozotocin in rats. Effect of 4 weeks' treatment with *s.c.* 15 IU/animal/day insulin (Ins), *s.c.* 10 mg/kg/day benzylamine (BzA) and *s.c.* 10 mg/kg/day benzylamine combined with 50 mg/L vanadate in the drinking water (BzA+V) was investigated in streptozotocin-induced diabetic rats (STZ). Blood glucose, glycated haemoglobin, serum AGE and NO bioavailability in the kidney were measured to clarify whether the anti-hyperglycemic or the potential cytotoxic effects of the SSAO substrate - through the production of H_2O_2 - have greater impact on diabetic vascular complications.

Blood glucose, HbA_{1C} and serum AGE levels

Four weeks treatment with Ins or BzA reduced but did not normalize the elevated blood glucose in diabetic rats. HbA_{1C} level was significantly reduced only by the insulin treatment. The amount of the serum AGEs in the diabetic animals was higher compared to the controls. Ins and BzA + V treatments were found to decrease the elevated serum AGE levels, whereas BzA alone was ineffective (Table 12).

Table 12. Diagnostic parameters of control, diabetic and treated rats. CTRL: control animals, STZ: diabetic animals, STZ + Ins: diabetic animals having insulin treatment, STZ + BzA: diabetic animals having benzylamine treatment, STZ + BzA + V: diabetic animals having benzylamine and vanadate treatment. Mean ± S.E.M of five groups. * $p < 0.05$ *vs* Control group, † $p < 0.05$ *vs* STZ group.

Group	Glucose (mM)	HbA_{1C}(%)	AGE(RFU)
CTRL	9.00 ± 0.75	3.80 ± 0.30	280.89 ± 6.94
STZ	38.10 ± 2.40 *	9.25 ± 0.53 *	408.00 ± 16.74 *
STZ + Ins	27.90 ± 4.70 †	6.86 ± 0.99 †	296.00 ± 21.93 †
STZ + BzA	27.50 ± 5.87 †	9.69 ± 1.50 *	384.63 ± 19.60 *
STZ + BzA + V	28.40 ± 1.61 †	9.12 ± 0.63 *	331.67 ± 14.53 †

NO availability in diabetic animals

Impaired NO bioavailability and increased formation of RNS were indicated by the measurements of the stable metabolic end products of NO, nitrite and nitrate in kidney tissue of the diabetic animals. The nitrite concentration was found to be reduced, while the ratio of nitrate/nitrite was elevated in diabetic rats compared to controls (Table 13.).

Table 13. Nitrite level and nitrate/nitrite ratio in kidney tissue of control, diabetic and treated rats. STZ: diabetic animals, STZ + Ins: diabetic animals having insulin treatment, STZ + BzA: diabetic animals having benzylamine treatment, STZ + BzA + V: diabetic animals having benzylamine-vanadate treatment. Mean ± S.E.M of five animals per group. * $p < 0.05$ *vs* Control group, † $p < 0.05$ *vs* STZ group.

Group	Nitrite nmol/g kidney	Nitrate/nitrite ratio
Control	10.72 ± 0.94	2.35 ± 0.28
STZ	6.51 ± 1.14 *	4.90 ± 1.00 *
STZ + Ins	5.67 ± 1.09 *	3.74 ± 0.51 †
STZ + BzA	7.48 ± 1.12 *	4.82 ± 0.92 *
STZ + BzA + V	9.80 ± 0.54 †	3.67 ± 0.50 †

Although insulin treatment failed to restore the kidney nitrite concentration, it was effective in reducing the elevated nitrate/nitrite ratio in the diabetic animals. Similarly, four week BzA+V treatment enhanced NO bioavailability in the kidney of diabetic rats. The nitrite level was markedly increased in these animals, compared to the untreated diabetic controls. BzA treatment alone caused no significant change in the nitrite and nitrate levels of the diabetic rats (Table 13.).

In conclusion, BzA + V treatment, similarly to Ins, lowered blood glucose and improved NO bioavailability, the impairement of which is contributing to endothelial dysfunction and vascular complications.

6.3.3. Effect of oral benzylamine treatment in various animal models of insulin resistance

We have shown that BzA alone has anti-hyperglycemic properties *in vivo*, although for *in vitro* insulin-like effects, the presence of vanadate is crucial in rodents. As we intended to study the effect of orally administered BzA, we have performed some initial experiments which have shown that a remarkable amount of BzA could cross the intestinal barrier without being oxidised by the amine oxidases expressed in the gut wall protecting the body against dietary amines (Bour, 2006). BzA was also detected in urine of BzA-drinking animals (measured in collaboration with Dr. Marianna Budai who performed HPLC-MS determinations; data not shown).

Oral administration of benzylamine in VHFD-fed mice

In this first model of insulin resistance mice were fed a very high fat diet for three months (see chapter 5.2.3 for model caracteristics) before they were given BzA (2000 or 4000 μmol/kg/day) in their drinking water for 12 weeks. BzA was supposed to improve glucose handling in these glucose-intolerant mice.

Glucose handling in VHFD mice

After three weeks of treatment, mice treated or not with BzA were fasted for 5 hours and submitted to IPGTT (Figure 23. A).

The overall hyperglycemic excursion was reduced after prolonged treatment with BzA at 2000 μmol/kg/day, since the area under the curve presented in Figure 23. B was reduced in BzA-treated mice. This modest improvement of glucose tolerance was apparently not the consequence of increased insulin sensitivity since injection of insulin at 0.75 IU/kg lowered blood glucose similarly in control and BzA-treated mice, reaching a minimum concentration of 6.0 ± 0.2 and 6.7 ± 0.3 mM within 30 min ($n = 9$, NS). In the VHFD animals, glycemia was lower in fed than in fasted state, and a significant glucose lowering effect of BzA-treatment could be evidenced under fed conditions at several time points of the treatment. For instance, four weeks after the start of treatment, non-fasting blood glucose was 8.27 ± 0.33 mM in untreated VHFD mice, which was lowered to 7.32 ± 0.22 mM in mice having daily 2000 μmol/kg BzA ($n = 12$, $p < 0.02$). However, no decrease in non-fasting blood glucose was detected in BzA treated animals, with the 4000 μmol/kg/day dose given during 12 weeks (not shown).

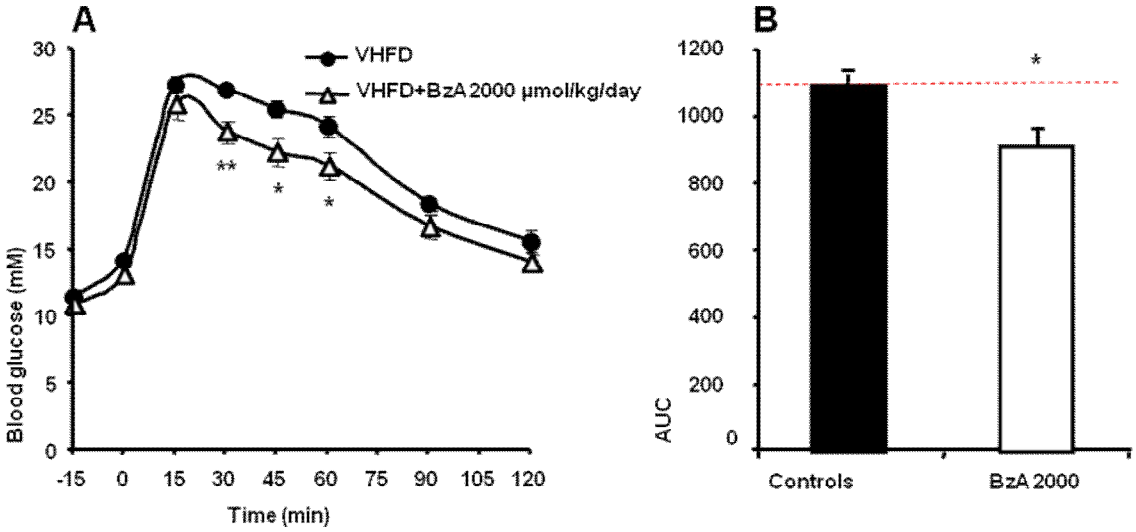

Figure 23. Influence of benzylamine (BzA) oral treatment on glucose tolerance. A panel: After three weeks of oral BzA administration, mice were fasted for 5 hours before a glucose load (1 g/kg, *i.p.* at time 0). Blood glucose values were determined on conscious animals at the indicated times. B panel: Area under the curve of the hyperglycemic excursion (AUC). Means ± SEM of 11 mice per group. Different from control at: * $p < 0.05$, ** $p < 0.01$.

Water intake and body weight gain

The average daily water intake was 4.4 ± 0.1 g in mice receiving 4000 µmol/kg/day dose of BzA (BzA-treated) in drinking water, while it was 4.8 ± 0.1 g in mice drinking only water (n = 16, $p < 0.05$). Such reduction of water intake persisted along the entire 12-week treatment. It was difficult to assess whether this reduction of water intake was concomitant with change in calorie intake, because the very high fat chow was peculiarly sticky and did not facilitate accurate determination of food consumption. Nevertheless, after 12 weeks of treatment, there was no difference in body mass between the control and the BzA-treated groups, whatever the BzA dose tested (2000 or 4000 µmol/kg/day). Similarly, there was no change in adipose tissue, liver, or heart mass.

Plasma markers of metabolic disturbances

BzA treatment did not modify plasma triglycerides (1.32 ± 0.05 *vs* 1.37 ± 0.05 mmol/l, NS), cholesterol (4.86 ± 0.18 *vs* 4.55 ± 0.36 mmol/l, NS) or non-esterified fatty acids (0.38 ± 0.04 *vs* 0.37 ± 0.01 mmol/l, NS) in VHFD mice. The treatment did not modify the amount of circulating AGEs (3.58 ± 0.55 *vs* 4.24 ± 0.25 relative units of fluorescence, NS), which were lower in both groups of VHFD mice than in animals fed a standard chow (6.05 ± 0.48, n = 5-6, $p < 0.001$), probably because of the low carbohydrate content of the diet. In adipocytes, the expression of genes encoding for

proinflammatory adipokines like TNFα, IL-6, leptin, resistin, PAI-1 was unaltered by BzA-treatment (not shown).

Lipolytic and glucose uptake activities in adipocytes

In order to test whether the sustained presence of the substrate administered to the treated mice was altering the *in vitro* insulin-like effects of BzA described above, we measured the glucose uptake and lipolysis activities in adipocytes from control and BzA-treated mice fed a VHFD.

The dose-dependent stimulation of lipolysis by isoprenaline was identical in mouse adipocytes from VHFD control and BzA-treated mice. Maximal stimulation was reached at using 100 nM beta-adrenergic agonist (not shown). The insulin-evoked inhibition of lipolysis was also unaltered by BzA-treatment (treated: 62 ± 12 *vs* untreated: $72 \pm 13\%$ inhibition of 10 nM isoprenaline's effect, $n = 5$, NS). The inhibition of lipolysis by 0.1 mM BzA plus 0.1 mM vanadate was also equivalent in the two groups, leading to 60 ± 10 and $57 \pm 15\%$ inhibition of the lipolytic response to 10 nM isoprenaline (NS).

GLUT-1 and GLUT-4 expressions, basal and insulin-stimulated glucose transport activities were also unchanged by BzA-treatment. Insulin 10 nM submaximally stimulated hexose uptake, and the combination of 0.1 mM BzA plus 0.1 mM vanadate clearly reproduced insulin activation of hexose transport in both VHFD control and BzA-treated mice. Therefore, no alteration of the adipocyte responses to insulin or insulin mimicking agents could be detected after chronic oral administration of BzA, ruling out a putative desensitisation of the pathways activated by sustained activation of SSAO.

Oxidative stress and aorta NO bioavailability

No alteration of nitrite content could be detected in the aorta even after 12-week treatment with the higher dose of BzA (55.5 ± 6.5 *vs* 52.4 ± 4.4 nmol/g tissue, $n = 6$, NS). Similarly, nitrate to nitrite ratio remained unchanged in the aortas of BzA-treated mice (2.47 ± 0.33 *vs* 2.68 ± 0.27 nmol/g tissue, $n = 6$, NS), indicating that BzA administration probably did not provoke oxidative stress in the vasculature. In liver, the gene expression of catalase or manganese superoxide dismutase was unaltered by BzA

administration. Catalase gene expression in adipocytes was also similar in BzA-treated and non-treated mice.

Taken together, VHFD mice clearly responded to BzA challenges by limiting their hyperglycemic response during IPGTT. However, the chronic oral administration did not reduce their slightly increased fasting glycemia. This was probably the consequence of the lack of carbohydrates in their special diet. As chronic BzA ingestion was devoid of adverse effects on nitric oxide bioavailability in the vasculature or on markers of inflammation in the adipose depots, our team decided to investigate this amine's effect in a mouse model of insulin resistance having more carbohydrates in the diet.

Oral administration of benzylamine in HFD-fed mice

In this second model of insulin resistance HFD mice were given 3600 µmol/kg/day BzA in their drinking water during 17 weeks (detailed in chapter 5.2.3).

Glucose handling in HFD mice

Blood glucose was followed weekly under non-fasting conditions. BzA-treated mice had significantly reduced glycemic levels on only three occasions out of 17 sample points, but the mean values of the 204 determinations made for each group during the treatment period showed significantly decreased blood glucose from 8.18 ± 0.07 mM in control to 7.71 ± 0.08 in BzA-treated mice.

Two weeks before the end of the treatment, mice were subjected to a glucose tolerance test. They were fasted for 5 hours and then received an *i.p.* glucose challenge (1 g/kg). The overall hyperglycemia was lower in BzA-treated mice as shown by Figure 24. A: the more rapid return of blood glucose to basal levels at almost all the check points tested, resulted in lower area under the curve ($n = 12$, $p < 0.05$) (Figure 24. B). Alongside the improvement in glucose tolerance it induced, BzA treatment also lowered fasting blood glucose level based on two measurements (t-15 and t-0), since the BzA-treated mice had lower values than controls before the glucose load: 8.9 ± 0.7 mM and 10.2 ± 0.7 mM *vs* 11.8 ± 0.3 mM and 12.9 ± 0.7 mM, respectively ($n = 12$, $p < 0.01$) (Figure 24. A).

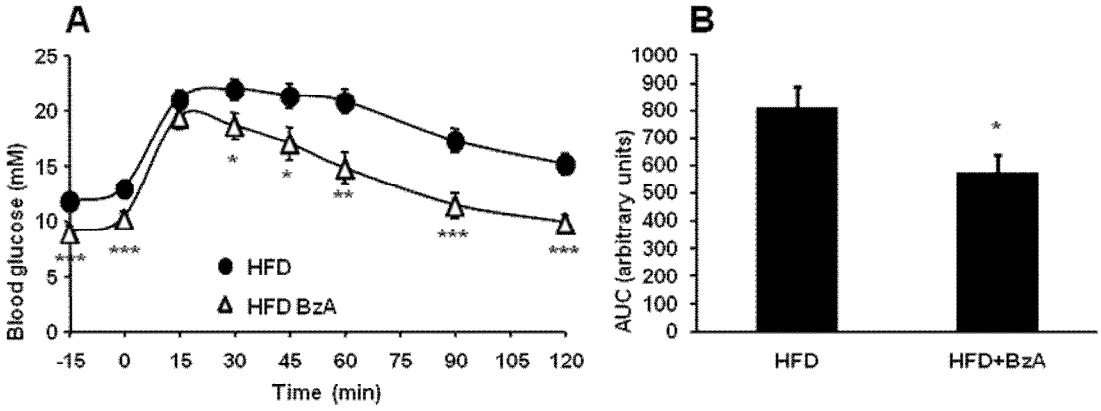

Figure 24. Influence of benzylamine (BzA) oral treatment on glucose tolerance. After 15 weeks of oral amine administration, mice were fasted for 5 hours prior to glucose load (1 g/kg, *i.p.* at time 0, arrow). A panel: glucose was determined in blood collected from the tail of conscious animals at the indicated times before and after the glucose load. B panel: Area under the curve of the hyperglycemic excursion (AUC). Mean ± SEM of 12 mice per group. Statistically different from control at: * $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$.

Further analysis of plasma samples obtained at sacrifice showed that glucose levels were clearly lower in the treated mice: they were equivalent to only 76 ± 6% of that of untreated animals ($n = 12$, $p < 0.02$). Immunoreactive insulin levels also showed a tendency to diminish but, due to high intra-group variability, there was no statistically significant effect of the treatment. Consequently, the observed decrease in HOMA, an index of insulin resistance, was at the limit of significance (7.79 ± 2.25 *vs* 18.76 ± 5.07 $n = 8$, $p < 0.07$). Therefore, the glycemic control appeared to be improved in BzA-treated animals, at least when considering the lowered non-fasting and fasting glycemia and the reduced hyperglycemic response to glucose load.

Water intake and body weight gain

The water consumption was followed weekly and exhibited small variations. There were only two instances when water intake was lower in the BzA-treated group than in the controls (6 and 10 weeks after starting treatment, $p < 0.02$). Nonetheless, the mean daily water consumption calculated over the entire 17-week treatment period was significantly lower in mice with the BzA treatment: average values were 3.9 ± 0.1 g/mouse/day for the controls and 3.5 ± 0.1 for the treated animals ($p < 0.001$, $n = 68$). BzA-treated mice thus limited their water consumption by approximately 10%.

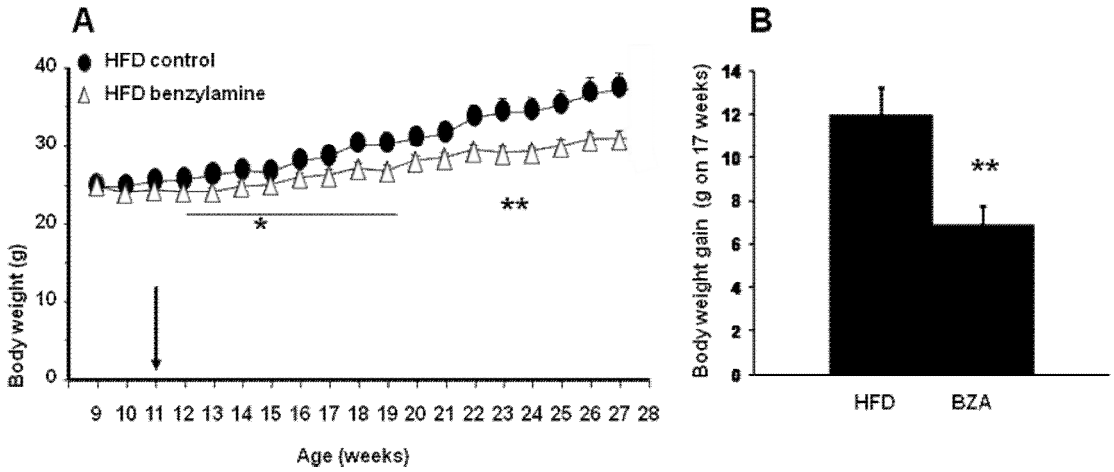

Figure 25. Body weight of mice fed a high-fat diet (HFD) and treated or not with orally administered benzylamine (BzA). Two groups of age- and weight-matched mice were constituted (4 cages of 3 animals per group) three weeks before oral administration of BzA (arrow). The amine was given as a drinking solution in order to achieve an intake of 3600 µmol/kg/day for 17 weeks. Mice had free access to water and HFD. A) Weekly body weight changes and cumulative body weight gain during the treatment (B). Each point/column is the mean ± SEM of 12 determinations. Statistically different from corresponding value in HFD control at * $p < 0.05$; ** $p < 0.01$.

Although both groups had the same initial body mass (BzA + HFD: 25.1 ± 0.6 *vs* HFD: 25.3 ± 0.6 g, n = 12, NS), BzA-treated mice weighed significantly less than the untreated age-matched controls after one week of treatment and until the end of experiment (Figure 25. A). Consequently, the overall body weight gain was clearly limited by the BzA-treatment (Figure 25. B).

Plasma markers of metabolic disturbances

At the end of the treatment, it became evident that the reduced body weight of BzA-treated mice was accompanied by a reduced lipid accumulation since the adiposomatic index, which is estimated as the percentage of dissected intra-abdominal (perirenal, retroperitoneal and epididymal) and subcutaneous fat depots relative to final body weight, was significantly lower than in the untreated animals (BzA + HFD: 6.6 ± 1.1 *vs* HFD: 11.6 ± 0.9%, n = 12, $p < 0.01$). This was due to a reduction in both intra-abdominal WAT and SCWAT. For instance, the latter weighed 1.72 ± 0.22 g in the HFD-control and 0.82 ± 0.17 g in the HFD-mice drinking BzA (n = 12, $p < 0.01$). The liver was also smaller in the BzA-treated animals than in control mice but no significant reduction could be detected when hepatic mass was expressed as a percentage of body weight (3.4 ± 0.1 *vs* 3.6 ± 0.1%, n = 12, NS).

Table 14. Effect of chronic benzylamine ingestion on plasma metabolites and cytokines. Mice fed a high-fat diet (HFD) and drinking benzylamine (HFD + BzA) were sacrificed after overnight fasting. Data are presented as Mean ± SEM of 12 mice. Significantly different from HFD at: * $p < 0.05$; *** $p < 0.001$.

Experimental group: (plasma levels)	HFD	HFD + BzA
FFA (mM)	0.61 ± 0.06	0.64 ± 0.07
TG (mg/dl)	69.2 ± 5.9	58.0 ± 3.2
Total cholesterol (mg/dl)	218.2 ± 10.2	131.0 ± 15.7 ***
Fructosamine (µmol/l)	200.4 ± 11.2	193.5 ± 6.9
Uric acid (µmol/l)	365.9 ± 32.4	234.1 ± 13.6 ***
Leptin (ng/ml)	9.78 ± 2.46	9.31 ± 2.35
Adiponectin (µg/ml)	12.92 ± 1.16	12.64 ± 1.03
Resistin (ng/ml)	5.93 ± 0.51	4.45 ± 0.29 *

The circulating levels of free fatty acids were also measured. Under fed conditions, no difference was detected between untreated and BzA-treated groups (respective values found after 15 weeks of treatment were 0.70 ± 0.07 and 0.67 ± 0.07 mM, NS). Under the fasting condition at the time of sacrifice, neither free fatty acids or triglycerides (Table 14.) were reduced in the plasma of treated mice. However, a decrease in total cholesterol was unexpectedly observed in the plasma from the BzA-treated mice. Such a clear-cut anti-hypercholesterolemic effect of BzA, leading to a reduction of 40% in the levels found in the hyperlipidemic mice was not accompanied by a significant decrease of the beneficial HDL-cholesterol: 171 ± 21 and 121 ± 18 mg/dl in HFD and HFD+BzA, respectively (n = 5, NS). The natural antioxidant compound uric acid showed increased plasma levels in HFD mice when compared with age-matched controls fed a standard chow (365.9 ± 32.4 *vs* 250.2 ± 22.7 µmol/l, n = 10-12, $p < 0.01$). BzA-treatment significantly lowered this parameter. Fructosamine levels were not modified in the BzA-treated group, suggesting that BzA treatment did not affect plasma protein glycation. Among the three adipokines measured, only resistin showed a significant decrease while adiponectin and leptin plasma levels remained unaltered in BzA-treated mice.

Lipolytic and glucose uptake activities in adipocytes

The dose-dependent stimulation of lipolysis by isoprenaline was similar in mouse adipocytes from HFD control and BzA-treated mice, in terms of increase over basal values (2.5 ± 0.2 *vs* 2 ± 0.1, n = 9 and 12, NS for 10 nM isoprenaline). Submaximal stimulation by 10 nM isoprenaline was chosen to study the anti-lipolytic actions of insulin and BzA alone or in combination with vanadium. Insulin-dependent

inhibition of lipolysis was unaltered in BzA-treated animals. BzA or vanadate alone were practically not anti-lipolytic, but the simultaneous presence of 0.1 mM BzA plus 0.1 mM vanadate mimicked insulin anti-lipolytic action and clearly reduced the effect of isoprenaline stimulation. This effect was not modified after chronic ingestion of BzA since the inhibition of lipolysis was equivalent in both groups (54 ± 4 *vs* 62 ± 7 in % inhibition of 10 nM isoprenaline action, n = 9 and 12, NS).

Dose-dependent stimulation of glucose transport by insulin was unaltered by chronic BzA-treatment (2.2 ± 0.5; 4.9 ± 0.7; 5.1 ± 0.4; *vs* 2.2 ± 0.3; 4.6 ± 0.5; 5.9 ± 0.7 fold increase over basal transport for 1; 10; 100 nM insulin, n = 9 and 12, NS). When tested separately, neither vanadium nor BzA exceeded 25% of the maximal insulin activation on hexose transport, whatever the group studied. Again, the synergism between BzA and vanadate totally reproduced insulin action in both HFD control and BzA-treated mice. Therefore, no alteration of the adipocyte responses to insulin, BzA and vanadate could be detected after prolonged oral administration of BzA.

Oxidative stress and aorta NO bioavailability

High fat diet reduced nitrite concentration in the aorta 125.08 ± 18.10 nmol/g in mice fed a normal chow *vs* 53.55 ± 2.72 nmol/g in HFD-fed mice. Table 15 shows that nitrite concentration was increased in the aortas of BzA-treated mice, indicating that BzA administration slightly improved the reduced NO bioavailability found in the vasculature of HFD-mice. Chronic BzA treatment did not modify the nitrate to nitrite ratio in aortic tissue (Table 15.). This finding argued against a putative exaggerated oxidative conversion of NO induced by hydrogen peroxide production accompanying sustained BzA ingestion and oxidation.

Table 15. Effect of chronic benzylamine ingestion on the concentration of stable end-products of NO metabolism. Mean ± SEM of 8-9 aortae per group. Statistical difference between mice fed a high-fat diet (HFD) and drinking benzylamine (HFD + BzA) at: * $p < 0.05$.

	HFD	HFD+BzA
Nitrite nmol/g aorta	**53.55 ± 2.72**	**66.23 ± 4.49 ***
Nitrate/nitrite ratio	**1.91 ± 0.22**	**1.59 ± 0.14**

Accordingly, no detectable change in the expression of SOD was found in the liver (not shown).

In HFD animals receiving BzA treatment we could observe improved glucose tolerance. Moreover, the amine also lowered fasting blood glucose, body weight and the high circulating cholesterol without causing excessive oxidative stress.

Further inverstigations aimed at studying oral BzA administration in a more drastic animal model of diabetes during the set-up of hyperglycemia.

Oral administration of benzylamine in db-/- mice

This third set of experiments on the effect of oral BzA was conducted on young littermates. After weaning mice were immediately distributed into two groups: on 0.5% BzA and tap water drinking groups and were investigated during 8 weeks.

A linear mixed statistical model was fitted to the data (blood glucose, water consumption, body weight) to describe the temporal pattern change. The initial model contained sex, treatment (BzA or not) and genotype (db-/- or db-/+) as fixed factors and it was assumed that these factors modify the individual slopes and intercepts of the changing parameter-age curve of the animals. Initial analysis revealed that sex is a clearly non-significant factor but at the same time there is a highly significant triple interaction between time (age), treatment and genotype. Independent factors were always the same, regardless what the dependent variable (water consumption, body weight or blood glucose) was. To reveal the reason of this interaction we split the data into two parts by genotype and dropped sex as a factor from the model.

Glucose handling in db-/- mice

Non fasted glycemia was monitored twice a week during the whole protocol (between week 4 and 11).

Initial fed glycemia was higher in db-/- and remained above the level of db-/+ mice throughout the entire experiment. While the glucose level of db-/+ did not change with age, non-fasted glycemia of db-/- mice continuously increased ($p < 0.0001$ for age dependence). BzA did not alter normoglycemia of db-/+, but significantly lowered the non-fasting blood glucose level of db-/- animals (Figure 26.). Applying the linear mixed statistical model, the slope for BzA db-/-: 33.81 ± 3.14 *vs* db-/-: 52.61 ± 3.90, mean ± SE, $p < 0.0005$).

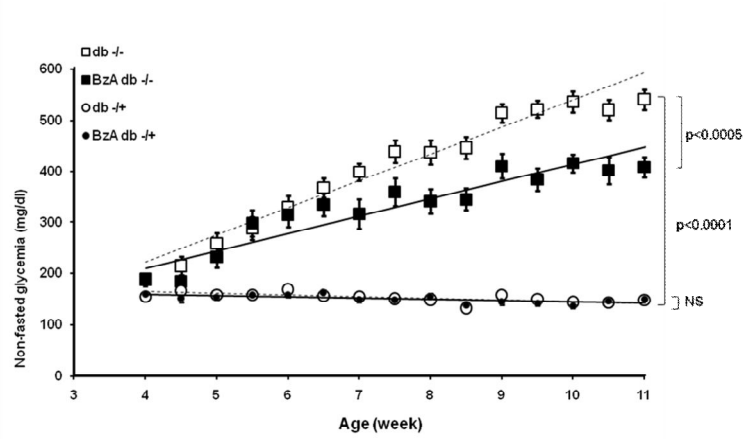

Figure 26. Non-fasted glycemia of db-/? mice at the age of 4-11 weeks. Mean ± S.E.M of experimental points. Statistical analysis: linear mixed model was fitted for 1027 observations and 89 groups, difference is indicated between slopes. Data collection db-/-: 27 (17/10); BzA db-/-: 18 (9/9); db-/+: 21 (12/9); BzA db-/+: 14 (7/7) animals (males/females) used respectively.

The daily urine excretion was increasing with age in db-/-, concomitantly with the diabetes onset, while this parameter remained at a lower level in db-/+ mice (Figure 27. A). The increase in the daily urine production was prevented by BzA treatment (Figure 27. A). Although the BzA-treated db-/- animals excreted more urine than the normoglycemic db-/+ ones, they produced significantly less than the untreated hyperglycemic mice during the last two weeks of treatment. Accordingly, the glucosuria was significantly reduced in db-/- mice drinking BzA solution, but was not normalized to a complete disappearance of glucose in urinary output (Figure 27. B).

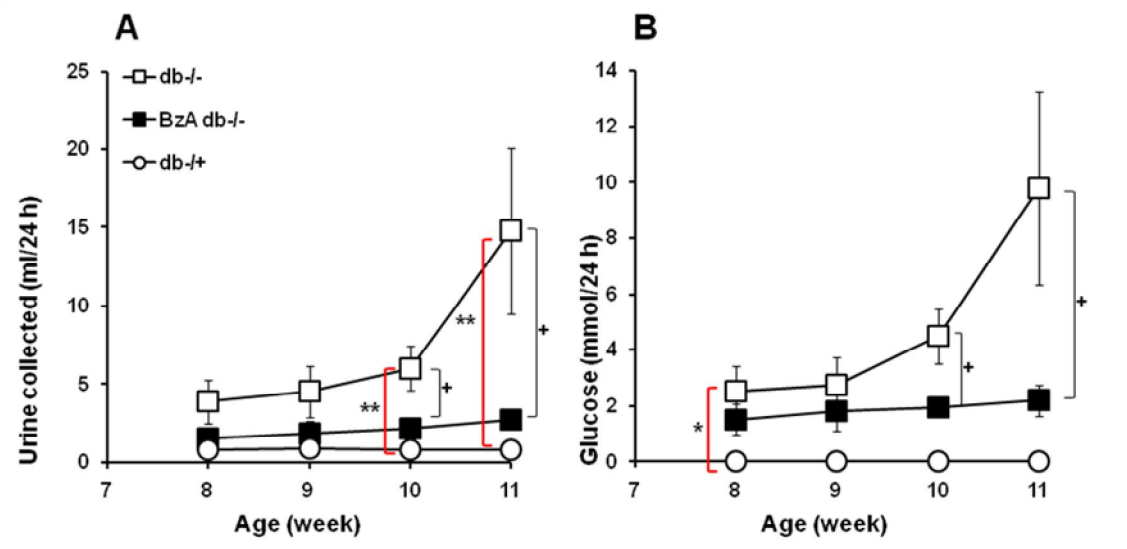

Figure 27. Urine emission (A) and daily glucose urinary output (B) of db-/- and db-/+ mice at the age of 8-11 weeks. Mean ± S.E.M for experimental points of db-/-: 11 (5/6); BzA db-/-: 9 (5/4); db-/+: 10 (5/5) animals (males/females) isolated in metabolic cages, 103 determinations altogether. * $p < 0.05$ (*vs* db-/+); ** $p < 0.01$ (*vs* db-/+); + $p < 0.05$ (*vs* db-/- mice).

Elevated fasting plasma glucose of db-/- mice at sacrifice was accompanied with increased plasma immunoreactive insulin levels (Table 17.), which is totally in agreement with the type 2 diabetes mellitus that takes place in this model. BzA did not modify fasted glycemia of heterozygotes but lowered that of db-/- animals by 42%. Circulating immunoreactive insulin was not altered by the treatment regardless of the genotype.

Water and food intake, body weight gain

Water consumption increased with age in the db-/- group ($p < 0.0001$ for age dependence) while it showed small variations but not age-dependence in the db-/+ littermates. BzA treatment did not affect water intake of db-/+ while it changed this parameter dramatically in db-/- mice (slope for BzA db-/-: 0.96 ± 0.26 *vs* db-/-: 3.72 ± 0.64, mean±SE, $p < 0.0005$ Figure 28.).

The food consumption was followed weekly (week 6-11) and exhibited small variations. The db gene invalidation resulted in two-fold increase in food intake (Figure 29.). BzA limited the average food intake by 13% in db-/+ and by 15% in db-/- mice.

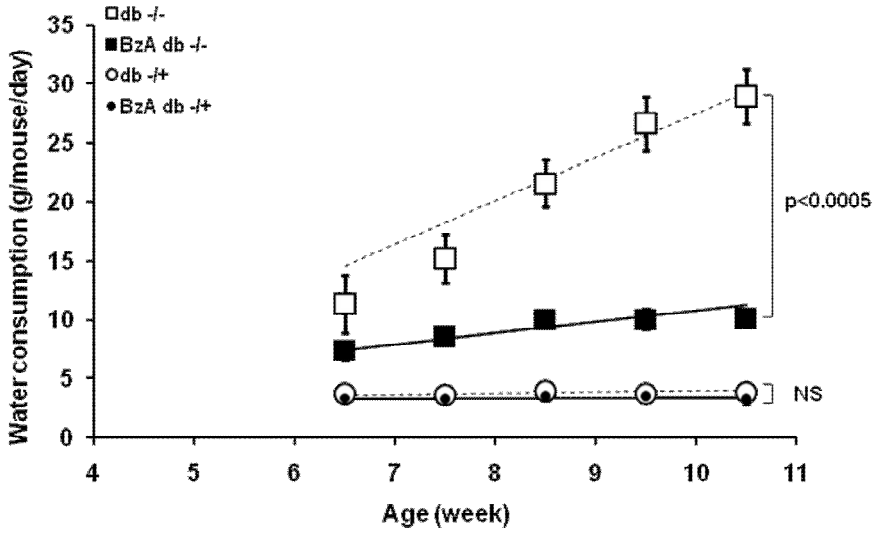

Figure 28. Influence of benzylamine supplementation (BzA) on liquid consumption of db-/? mice between the ages of 6 and 11 weeks. Mean ± S.E.M of experimental points. Data collection from db-/-: 12 (7/5); BzA db-/-: 8 (4/4); db-/+: 9 (6/3); BzA db-/+: 5 (2/3) cages (males/females). For statistical analysis raw data (141 points, 35 groups) was fitted into linear mixed model, difference is indicated between slopes of each group.

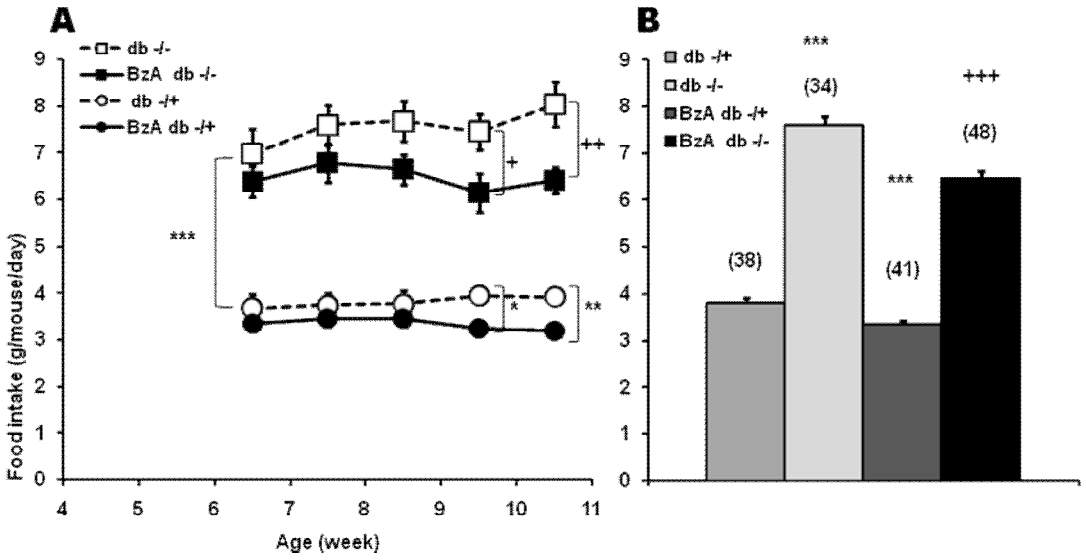

Figure 29. Daily food intake between the ages of 6 and 11 weeks in db-/- and db-/+ mice (A) and overall average of food intake (B) during the complete experimental period. Mean ± S.E.M of experimental points. Data collection from db-/-: 12 (7/5); BzA db-/-: 8 (4/4); db-/+: 9 (6/4); BzA db-/+: 5 (2/3) cages (males/females) (A). N is given between parentheses in B. Different (t test) from db-/+ at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. or from db-/- : + $p < 0.05$; ++ $p < 0.01$; +++ $p < 0.001$.

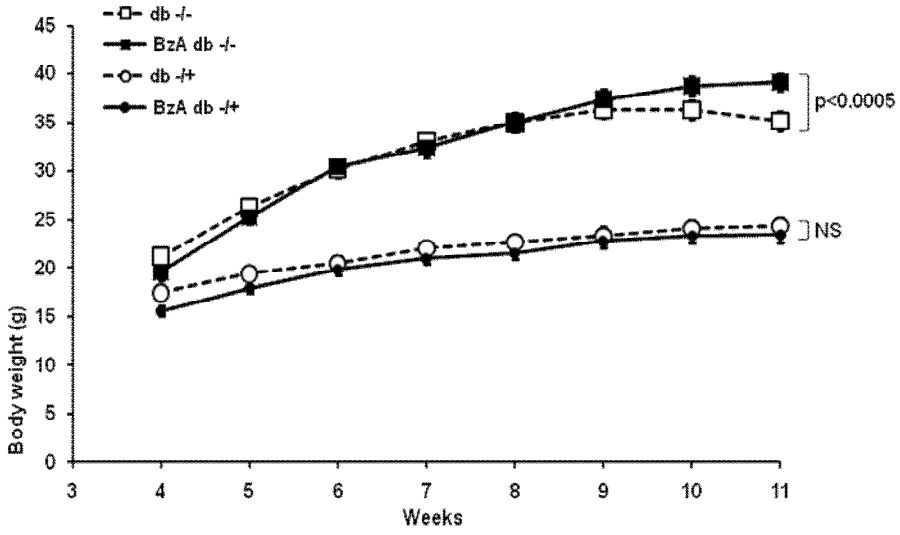

Figure 30. Body weight evolution of db-/? mice at the age of 4-11 weeks. Mean ± S.E.M of experimental pionts. For statistical analysis linear mixed model was fitted for 562 observations, 88 groups, difference is indicated between slopes. Data collection db-/-: 27 (17/10); BzA db-/-: 18 (9/9); db-/+: 21 (12/9); BzA db-/+: 14 (7/7) animals (males/females) used respectively.

Although separation of obese and lean animals according to their body weight at the age of 4 weeks was not at all evident, body mass of db-/- mice was higher than that

of the animals in the db-/+ group throughout the whole experiment/measured period. Body weight increased with age in db-/+ ($p < 0.0001$) as well as in db-/- ($p < 0.0001$), however body weight of db-/- mice reached a plateau between 9-10 weeks and even decreased slightly by the end of the experiment (Figure 30.).

Body mass at 4 weeks of age was similar in treated and untreated groups. BzA did not influence body mass of db-/+ mice (slope for BzA db-/+: 1.09 ± 0.07 *vs* db-/+ :1.09 ± 0.07, NS). db-/- BzA treated mice kept their maximum body weight between 9-11th weeks differentiating the slopes and values at the 11th week from that of BzA-treated mice (slope for BzA db-/-: 2.92 ± 0.18 *vs* db-/-: 1.84 ± 0.15, mean±SE, $p < 0.0005$). Body weight gain also confirms BzA's action (db-/- BzA: 19.7 ± 1.1 *vs* db-/-: 14.6 ± 1.1 g, n = 9+9 and 11+12 males and females, $p < 0.01$). When adiposity was assessed by Echo MRI, an increased % fat mass was found in obese mice 1 day before sacrifice (BzA db-/-: 49.6 ± 1.6 *vs* db-/-: 44.3 ± 1.4, n = 3+6 and 6+7 males and females, $p < 0.03$.)

At sacrifice, INWAT and SCWAT mass was at least 10-fold increased in db-/- *vs* db-/+ (Table 16.). As described above, BzA did not change any of these parameters in db-/+ animals. In db-/- mice, increased body weight of BzA-treated mice was accompanied by an increased lipid accumulation according to the changes in adiposomatic index, which is estimated as the percentage of dissected fat depots relative to final body weight. The adiposomatic index was significantly higher in the db-/- BzA-treated mice (BzA db-/-: 15.3 ± 0.5 *vs* db-/-: 13.7 ± 0.4%, n = 18 and 28, $p < 0.02$). This was mainly due to a 25% increase of subcutaneous white adipose tissue mass compared to the non-treated db-/- animals (Table 16).

Table 16. Effect of benzylamine supplementation (BzA) on some clinical parameters in db-/+ and db-/- at sacrifice. Statistical analysis was made by two-way ANOVA. Number of animals (males/females)/group db-/-: 28 (18/10); BzA db-/-: 18 (9/9); db-/+: 21 (12/9); BzA db-/+: 14 (7/7) used respectively. Different from db-/+ at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, or from db-/-: + $p < 0.05$; ++ $p < 0.01$, no difference found between db-/+ and BzA db-/+.

	db-/+	BzA db-/+		db-/-		BzA db-/-	
body weight (g)	**21.74 ± 0.82**	**21.52 ± 0.70**	**ns**	**32.94 ± 0.96**	*******	**36.19 ± 0.95**	**+**
Fasting blood glucose (mg/dL)	**85.00 ± 5.58**	**76.41 ± 4.20**	**ns**	**375.00 ± 22.94**	*******	**245.39 ± 23.77**	**+++**
total mass of INWAT (g)	**0.45 ± 0.02**	**0.42 ± 0.06**	**ns**	**1.93 ± 0.10**	*******	**2.24 ± 0.11**	**ns**
SCWAT mass (g)	**0.35 ± 0.03**	**0.29 ± 0.04**	**ns**	**2.66 ± 0.18**	*******	**3.31 ± 0.16**	**+**
liver total mass (g)	**0.89 ± 0.03**	**0.91 ± 0.04**	**ns**	**1.71 ± 0.07**	*******	**1.87 ± 0.07**	**ns**

Plasma markers of metabolic disturbances

Diabetes onset increased cholesterol (total and HDL), free fatty acid, uric acid and fructosamine in plasma. BzA-treatment did not affect any of the above mentioned parameters in db-/+ mice, albeit BzA reduced triglyceridemia but not FFA. The lowered TG is in accordance with increased SCWAT permitting a better storage of lipids in that tissue. BzA also lowered uric acid levels (Table 17.) as observed before in other animal model of insulin resistance (Oral administration of benzylamine in HFD-fed mice).

As BzA had no effect on glycemia, body weight, plasma metabolic parameters of db-/+ mice, but it clearly improved diabetic state of db-/- animals, further investigations aimed at studying BzA's effect in only db-/- mice.

Table 17. Plasma metabolic parameters after overnight fasting at sacrifice. Number of animals (males/females)/group db-/-: 28 (18/10); BzA db-/-: 18 (9/9); db-/+: 21 (12/9); BzA db-/+: 14 (7/7) used respectively. Statistical analysis was made by two-way ANOVA. Different from db-/+ at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, or from db-/-: + $p < 0.05$; ++ $p < 0.01$, no difference found between db-/+ and BzA db-/+.

	db-/+	BzA db-/+	db-/-	BzA db-/-
Glucose (g/l)	1.10 ± 0.08	1.07 ± 0.13 ns	5.67 ± 0.49 ***	3.30 ± 0.43 ++
Insulin (µg/l)	0.40 ± 0.05	0.38 ± 0.05 ns	2.89 ± 0.32 ***	2.62 ± 0.51 ns
Cholesterol (mmol/l)	1.94 ± 0.13	2.00 ± 0.13 ns	2.29 ± 0.22 *	2.46 ± 0.19 ns
Cholesterol LDL (mmol/l)	0.18 ± 0.04	0.20 ± 0.04 ns	0.28 ± 0.12 ns	0.23 ± 0.10 ns
Cholesterol HDL (mmol/l)	1.62 ± 0.11	1.69 ± 0.11 ns	2.01 ± 0.16 **	2.08 ± 0.15 ns
FFA (mmol/l)	0.64 ± 0.05	0.78 ± 0.07 ns	0.90 ± 0.05 ***	0.94 ± 0.06 ns
TG (g/l)	0.97 ± 0.07	1.13 ± 0.09 ns	1.27 ± 0.10 ns	0.87 ± 0.06 +
Uric acid (µmol/l)	279.31 ± 17.99	272.63 ± 17.94 ns	406.70 ± 23.54 ***	330.60 ± 24.55 +
Fructosamine (µmol/l)	204.46 ± 4.66	189.07 ± 7.69 ns	291.84 ± 11.23 ***	262.73 ± 14.28 ns

Lipolytic and glucose uptake activities in adipocytes

The influence of diabetes and oral BzA on lipolysis and glucose uptake, was studied in adipocytes freshly isolated from perigonadal WAT of db-/- mice.

Basal lipolysis was similar in db+/- and db-/- mice (not shown). Isoprenaline exerted maximal effect at 100 nM in db-/+ (not shown) while at 10 µM concentration in db-/- littermates. The basal lipolytic activity was unaltered after oral BzA treatment (0.63 ± 0.05 *vs* 0.55 ± 0.11 µmol glycerol/100 mg lipid/90 min, $n = 3$, NS). The dose-dependent stimulation of lipolysis by isoprenaline remained also unaffected (Figure 31. A).

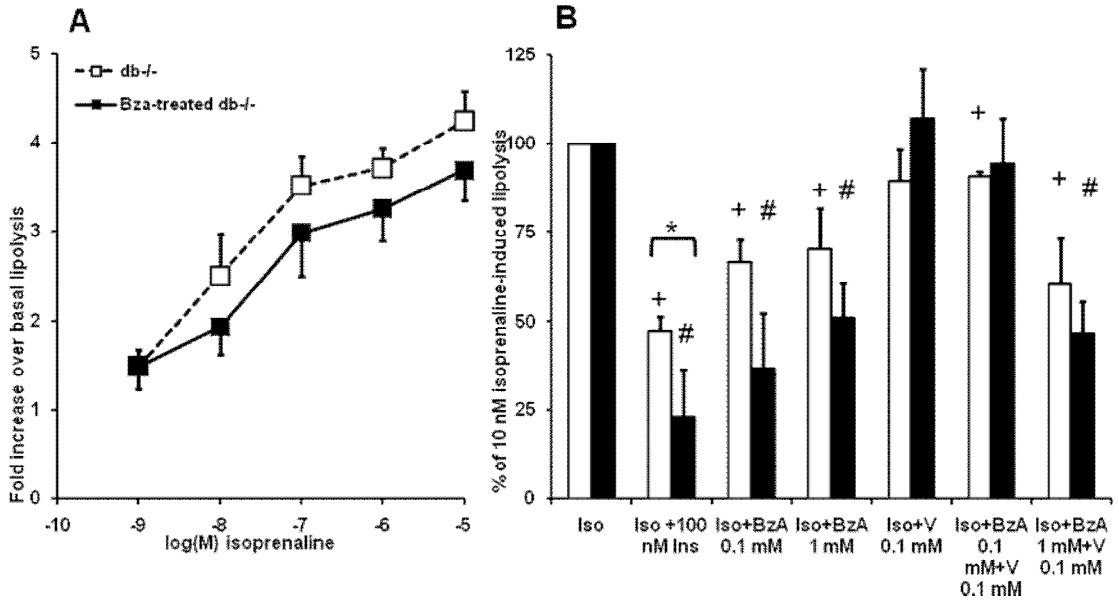

Figure 31. Isoprenaline-induced lipolysis in db-/- mice expressed as the increase over basal output (A). Anti-lipolysis of different agents on 10 nM isoprenaline's stimulated effect (B). Means ± SEM of 4 mice. Statistical analysis is made by t test. Different from 10 nM isoprenaline in db-/- at: + $p < 0.05$; different from 10 nM isoprenaline in BzA-treated db-/- at: # $p < 0.05$ and difference between db-/- *vs* BzA-treated db-/-: * $p < 0.05$.

When the anti-lipolytic effect of insulin was tested on the submaximal stimulation promoted by 10 nM isoprenaline, BzA-treated mice exhibited a greater response to 100 nM insulin than untreated db-/- mice, although insulin could only partially inhibit the β-adrenergic agonist-evoked lipolysis in both groups (Figure 31. B). BzA itself was also anti-lipolytic, since only 60 ± 12 and 46 ± 9% of the 10 nM isoprenaline-induced lipolysis remained in the presence of 1 mM BzA in db-/- and BzA-treated db-/- mice, respectively. BzA effect was not greater in the presence of vanadate.

In accordance with the well-known insulin-resistant state of db-/- mice, the 2-deoxyglucose transport was poorly activated by insulin in adipocytes of young db-/- obese mice, since 100 nM insulin induced only 2.2 ± 0.2 fold increase over basal values while in lean heterozygotes the maximal response to the hormone was 4.9 ± 0.4 fold ($n = 6$, $p < 0.001$, not shown). In fact, oral BzA treatment modified neither basal (0.47 ± 0.09 *vs* 0.35 ± 0.05 nmol 2-DG/100 mg lipid/10 min, $n = 3$, NS) nor maximal insulin-stimulated hexose uptake (2.7 ± 0.4 *vs* 2.2 ± 0.1 increase over basal 2-DG uptake, $n = 4$, NS). By contrast, BzA treatment tended to improve the adipocyte sensitivity to insulin since 10 nM insulin's effect was equivalent to 56 ± 2% of maximal insulin's effect in treated *vs* 37 ± 7% in untreated obese mice ($n = 3$, $p < 0.07$). Again,

BzA was able to partially mimic insulin action on adipocytes, since at 1 mM, it stimulated uptake up to 52 ± 3% of maximal insulin effect in db-/- mice. Taken together, these data show that BzA behaved *in vitro* as an insulin mimicking agent, while it was unable to totally prevent *in vivo* the setup of insulin resistance in adipocytes of INWAT.

As SCWAT was more affected by BzA-treatment, the gene expression in SCWAT was investigated to further explore BzA's actions. Table 18. shows that expression of HSL a protein related to adipocyte metabolism was higher in diabetic animals. FAS, Adiponectin, apelin, some adipocyte hormones, as well as inflammation (PAI-1, Il-6, F4/80) or endothelial markers (cd31, Tie2) were also overexpressed in diabetic animals.

Table 18. Gene expression in SCWAT in lean and obese mice receiving or not oral benzylamine (BzA). Mean ± SEM of 9 mice. Different from db-/+ at: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, no difference found between db-/- and BzA db-/-.

Function		db-/+	db-/-		BzA db-/-	
Adipocyte secretion	Adiponectin	207.03 ± 16.36	298.20 ± 32.30	*	302.33 ± 21.49	ns
	Apelin	0.27 ± 0.05	0.93 ± 0.15	***	0.70 ± 0.08	ns
Fatty acid metabolism	FAS	220.40 ± 43.23	77.47 ± 48.93	*	41.57 ± 4.88	ns
	HSL	55.39 ± 4.06	86.08 ± 8.57	**	88.26 ± 6.16	ns
Inflammation	PAI-1	2.04 ± 0.66	20.43 ± 4.14	***	26.59 ± 4.21	ns
	IL-6	0.01 ± 0.00	0.04 ± 0.02	ns	0.05 ± 0.01	ns
	F 4/80	0.49 ± 0.08	4.51 ± 1.60	*	2.74 ± 0.68	ns
Endothelial markers	cd31	31.52 ± 3.25	43.85 ± 4.78	*	41.22 ± 3.09	ns
	Tie 2	5.37 ± 0.62	8.06 ± 0.99	*	6.08 ± 0.85	ns
Adipocyte differentiation markers	APJ	2.06 ± 0.35	2.44 ± 0.52	ns	2.28 ± 0.46	ns
	SSAO	30.68 ± 3.37	78.22 ± 7.58	***	84.64 ± 7.10	ns

Oxidative stress and aorta NO bioavailability

Basal hydrogen peroxide production, without any added exogenous substrate was increased in db-/- *vs* db-/+ mice when expressed on a per mg protein basis (113.4 ± 19.1 *vs* 65.3 ± 13.2 pmol H_2O_2/mg protein/min, n = 7, $p < 0.06$) in SCWAT tissue homogenates. BzA treatment did not change hydrogen-peroxyde release neither in homogenates (BzA db-/-: 103.1 ± 9.2 *vs* db-/-: 113.4 ± 19.1 pmol H_2O_2/mg protein/min, n=7, NS), nor in fresh pieces of WAT: (BzA db-/- 0.45 ± 0.19 *vs* db-/- 0.46 ± 0.21 pmol H_2O_2/mg wet tissue/min, n = 4-5, NS).

Nitrite concentration was decreased, while nitrate to nitrite ratio was increased in the aortas of db-/- compared to db-/+ (Table 19.). These data are in accordance with those collected on previously reported models (HFD- and streptozotocin-induced

diabetes), indicating a decreased NO formation of the endothelial cells during the set-up of insulin resistance accompanied by an increased relative oxidative conversion of this vasoactive agent. BzA restored aortic nitrite levels (as was also seen in HFD-fed mice), and reduced nitrate/nitrite ratio, however this reduction was at the limit of significance ($p < 0.06$). BzA administration is thus suggested to improve the reduced NO bioavailability found in the vasculature of db-/- mice.

To sum up, we could show that oral BzA is able to delay the onset of hyperglycemia in db-/- mice. BzA reduced food and water intake and increased body weight gain. This latter is in accordance with the effect of the amine on insulin resistance in adipocytes. Furthermore BzA improved NO bioavailability in aorta and did not cause any excessive oxidative stress or inflammation in SCWAT.

Table 19. Nitrite concentrations and nitrate to nitrite ratio in aorta of db-/? mice. Mean ± SEM of db-/-: 14 (8/6); BzA db-/-: 11 (7/4); db-/+: 13 (7/6) mice (males/females) used respectively. Statistical analysis is made by t-test. Different from db-/+ at: * $p < 0.05$; ** $p < 0.01$ or from db-/-: ++ $p < 0.01$.

	db-/+	db-/-	BzA db-/-
Nitrite nmol/g aorta	181.21 ± 25.45	108.24 ± 11.00 *	179.36 ± 24.64 ++
Nitrate/nitrite ratio	1.97 ± 0.22	3.48 ± 0.48 **	2.28 ± 0.34 Ns

6.4. *In vitro* tests for screening SSAO substrate drug candidates

It has been described several decades earlier that inter-species variations and even inter-tissular variations exist regarding the selectivity of SSAO towards its substrates because SSAO and other copper-containig amine oxidases have been studied in numerous animal models, rat, mouse, cow, pigs, *etc* on different tissues (Boomsma *et al.*, 2000b). Curiously, less evident were the changes of inhibitor capacities between rodents and human forms of SSAO (Callingham *et al.*, 1990). In this context and with our objective to search for novel substrates of SSAO, we focused our attention in comparing biochemical and pharmacological properties of human and rodent SSAOs. We have found that human SCWAT has greater capacity to oxidise BzA even at 1 mM concentration than mouse fat depots. In both species, BzA was prominently oxidised by SSAO. Mouse SSAO Vmax is one third of human's. Km of SSAO in human SCWAT is 40 fold higher than that of mouse SCWAT (Table 20.). Another difference is that human adipocytes do not need vanadate for glucose transport stimulaton or anti-

lipolysis by SSAO substrates. Human adipose SSAO, thus can be a promising drug target.

Table 20. Comparison of enzyme kinetic parameters of murine and human SCWAT. SSAO-dependent H_2O_2 production was assessed by Amplex Red method. Mean ± SEM of n = 3-5 determinations.

	Vmax (pmol/mg protein/min)	Km µM
Human SCWAT H_2O_2 detection	**1184 ± 243**	**469 ± 44**
Mouse SCWAT H_2O_2 detection	**411 ± 11**	**14 ± 2**

Until recently, BzA was the best substrate for SSAO. Four novel (Substrate A-D) and two already known (4-phenylbuthylamine (4-PBA) (Marti *et al.*, 2004), 3-(4-methylthiophenyl)propylamine (3-MTPPA) (Gallardo-Godoy *et al.*, 2004)) SSAO substrates were designed and synthesised at the Organic Chemistry Department of Semmelweis University. Chemical structures of the new compounds are not allowed to be published because of patentship. We developed screening methods to test these 6 SSAO substrates as potential drug candidates in diabetes, in all protocols we used BzA as a reference.

First, hydrogen peroxide production in human SCWAT homogenates was tested at 0.1 and 1 mM amine concentrations.

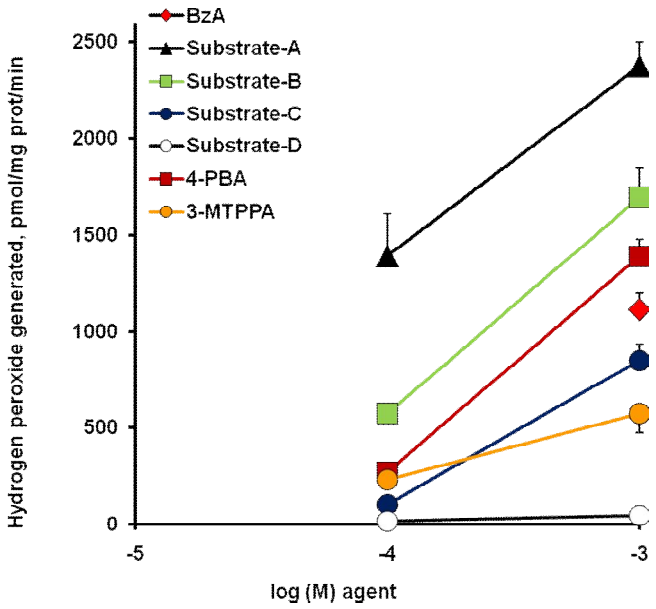

Figure 32. Effect of amines on hydrogen peroxide release of SCWAT homogenate of 6 women patiens undergoing abdominal dermolipectomy.

Most substrates were able to increase H_2O_2 production and their action depended on the used concentration (Figure 32.). Substrate-A, substrate-B and 4-PBA hydrochloride produced more hydrogen peroxyde in human fat preparations at 1 mM concentration when compared to 1 mM BzA. 4-PBA has been previously reported a better substrate of SSAO than BzA (Yraola *et al.*, 2006) .

Next, *in vitro* insulinomimetic effects of amines were measured in isolated human adipocytes.

Lipolysis in human adipocytes

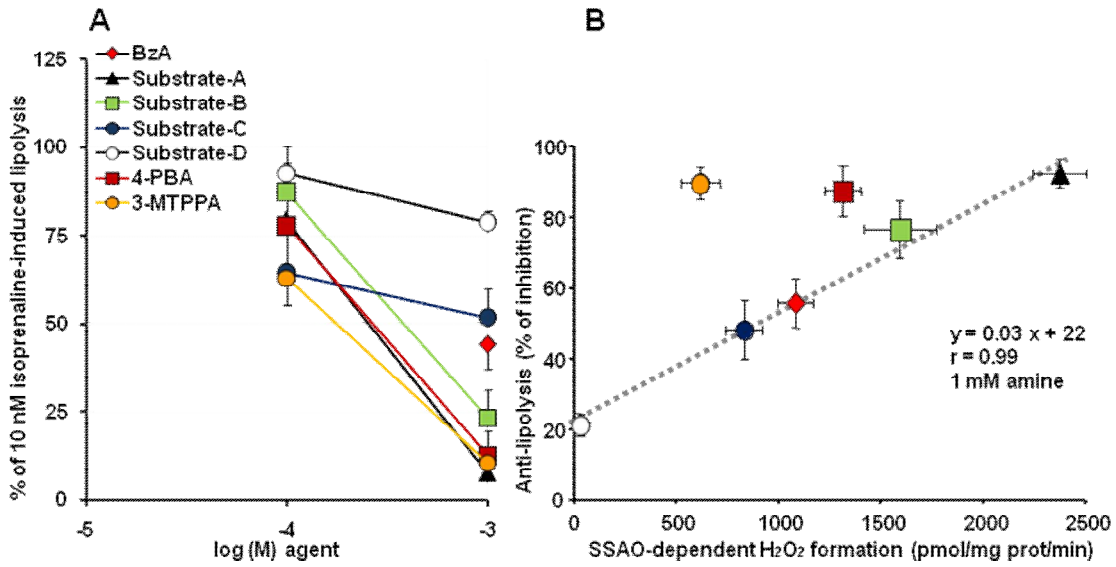

Figure 33. Anti-lipolytic effect of SSAO-interacting agents(A). Adipocytes were isolated human from SCWAT of 5 women undergoing abdominal dermolipectomy. Correlation between antilipolitic effect and SSAO-dependent H_2O_2 release in the presence of 1 mM amine concentration (B). 4-PBA and 3-MTPPA are excluded from correlation.

BzA at 1 mM inhibited the isoprenaline-stimulated lipolysis in human adipocytes by 50%. Under the same conditions, 4-PBA and 3-MTPPA totally abolished the lipolytic activation. Their anti-lipolytic effect was dose-dependent and the stimulation induced by 10 nM isoprenaline was inhibited by 100 µM of amines (Figure 33.). 4-PBA and 3-MTPPA were also anti-lipolytic in mouse adipocytes (not shown). In order to verify the involvement of SSAO in their anti-lipolytic effect, the agents were further tested in KO AOC3 mice. 3-MTPPA and 4-PBA were still anti-lipolytic at 1 mM in adipocytes fom KO AOC3 mice (the former inhibiting 22.3 ± 4.9, the latter 61.7 ± 6.2 % of 10 nM isoprenaline stimulation), while BzA (+vanadate) did not show effect. These agents may behave as MAO or other AO substrates or can hamper isoprenaline-

lipolytic response through other possible ways (β-antagonist, α_2-agonist). As both compounds inhibit the effect of the direct adenyl cyclase activator forskolin (3-MTPPA: 32.7 ± 16 and 4-PBA: 33.9 ± 15.9 in % of the effect of 10 µM forskolin, n=2) in human adipocytes, they do not interact with β-adrenoceptors. Actually, the SSAO-independent anti-lipolytic action of these two molecules is under current investigation.

In most cases hydrogen peroxyde production correlates with the anti-lipolytic effect. Substrate-A and substrate-B seems to have greater effect than BzA. 3-MTPPA and 4-PBA are excluded from the correlation as they apparently do not behave only as SSAO-interacting agents.

Glucose uptake in human adipocytes

4-PBA, but not 3-MTPPA, was as efficient as BzA regarding glucose transport activation in human adipocytes. At 100 µM, both amines promoted a glucose uptake activation that was equivalent to 27% of the maximal response to insulin.

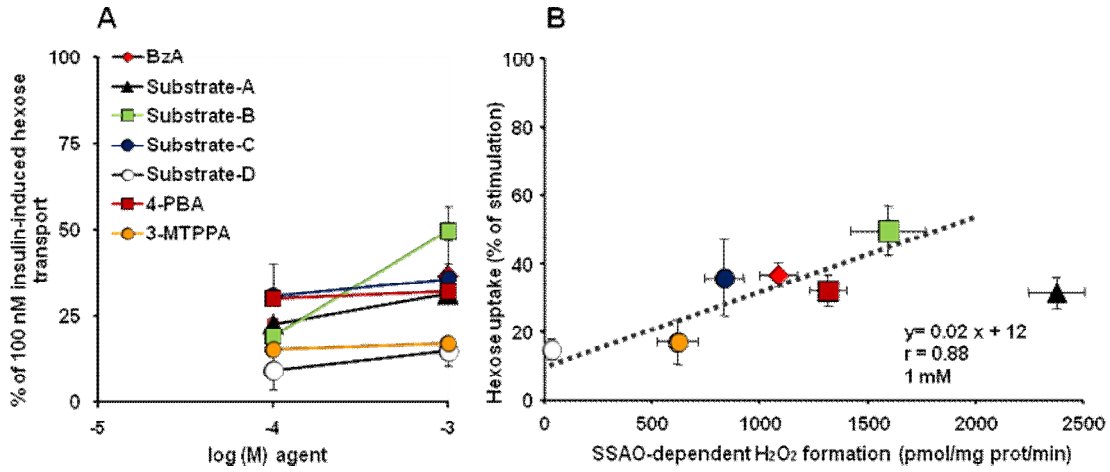

Figure 34. Effect of SSAO-interacting agents on glucose uptake(A). Adipocytes were isolaletd from SCWAT of 6 women. Correlation between glocose uptake stimulation and H_2O_2 production at 1 mM substrate concentration (B). Substrate-A does not match in correlation.

Figure 34 shows that only substrate-B has better capacity to stimulate glucose uptake than BzA.

Taking together anti-lipolysis and glucose transport, this screening method has succeeded in detecting Substrate-B as better substrate for SSAO than BzA. Ongoing research on a larger number of compounds will ascertain whether other molecules can be considered as better substrates for human SSAO than BzA.

7. Discussion

7.1. Investigation of SSAO activity in adipose tissue

High SSAO activity and subsequent H_2O_2 production has already been described in WAT (Raimondi *et al.*, 2000; Raimondi *et al.*, 1991). Our results showing that the oxidation of BzA in SCWAT is highly dependent on SSAO are in accordance with these previous reports. The maximal H_2O_2 release which is slightly diminished at higher substrate concentrations reflects the very complicated enzyme kinetics of SSAO (Holt, 2009; Holt *et al.*, 2008). Nevertheless this work reports first that the WAT SSAO produces H_2O_2 *in vitro* without any addition of exogenous amines. This finding deserves further investigations. First, the endogenous substrates present in WAT responsible for the small but detectable hydrogen peroxide production should be identified. The concentrations of two such candidates, namely aminoacetone and methylamine, have been recently determined in adipose tissue (1.9±0.9 for methylamine, 0.1±0.0 aminoacetone nmol/g tissue) (Xiao *et al.*, 2009). However because of their very small amounts, they can hardly be responsible for the basal H_2O_2 production found in our experiments. Second, it should be verified if the intact, adipose tissue shares the same spontaneous activity with the homogenate or the homogenisation process and the cell rupture are responsible for the release of a substrate (for example histamine (Fredholm *et al.*, 1971), noradrenaline from WAT nerves (Dodt *et al.*, 2003) or lysine) prone to be catabolized by the SSAO enzyme.

The soluble form of SSAO circulating in the plasma has been extensively studied in diverse pathologies, including diabetes (Obata, 2006) and obesity (Dullaart *et al.*, 2006; Li *et al.*, 2005; Mészáros *et al.*, 1999b; Visentin *et al.*, 2004) while changes in tissue-bound SSAO activity in such pathologies are less described.

The present work clearly shows that the SSAO activity is increased in SCWAT in two models of obesity and insulin resistance: the HFD-fed and the db-/- mice. The higher activity was due to an enhanced expression of the gene encoding for the copper-containing amine oxidase and to increased protein abundance as was confirmed by RT-PCR and Western Blot analysis, respectively. Enzyme activity was not changed in PGWAT and in PRWAT, indicating a complex anatomical-dependent mechanism of regulation. The fact that the basal SSAO-dependent H_2O_2 release is also increased in

SCWAT of HFD-diabetic animals but not in that of db-/- mice may be a result of the different diet composition or genetic background leading to a difference in endogenous SSAO substrate amines present in WAT.

Fasting did not alter enzyme activity in INWAT depots in rats. The lack of detectable change in SCWAT SSAO activity in adipose tissue during fasting was also observed (Iffiú-Soltész *et al.*, 2009). This preservation of SSAO capacity in fat stores during weight loss is likely the consequence of: 1) persistent SSAO expression in spite of fasting-induced metabolic disturbances, and 2) maintained total protein content in INWAT. Fasting-induced depletion of fat cell stores thus is not accompanied by SSAO/VAP-1 down-regulation, while the circulating level of insulin was found to be decreased (not shown).

These observations improve our current knowledge about amine oxidase regulation in adipocytes, which was limited so far to the repeatedly reported increase of SSAO activity and mRNA expression during adipocyte differentiation (Bour *et al.*, 2007a; Mercier *et al.*, 2001; Moldes *et al.*, 1999; Subra *et al.*, 2003).

Increased SSAO activity in SCWAT of obese mice is probably due to an enhanced expression of SSAO in the mature adipocytes, since they have higher activity and express much more mRNAs of the AOC3 gene encoding for SSAO than the other cell types found in adipose tissue (Bour *et al.*, 2007a). The SSAO enrichment that occurs in hypertrophied fat cells resembles to the SSAO emergence that takes place during adipocyte differentiation (Bour *et al.*, 2007a; Mercier *et al.*, 2001; Moldes *et al.*, 1999; Subra *et al.*, 2003). However, the overexpression of SSAO in WAT does not contribute to obese phenotype (Iffiú-Soltész et al. unpublished data).

Increased SSAO activity in SCWAT of obese mice can also be related to low grade inflammation of the tissue. This latter idea is supported by the enhanced mRNA expression of PAI-1 and F 4/80 in SCWAT of db-/- vs db-/+ mice (Table 18.) and by the fact that KO AOC3 mice possess reduced low-grade inflammation of WAT (Bour *et al.*, 2009).

Nevertheless, the relationship between fat depot extension and SSAO expression could be more complicated than it appears since PGWAT and PRWAT, which also enlarge with obesity, did not exhibit any increase in SSAO activity. However, higher SSAO activity in intra-abdominal - but not subcutaneous - fat depots has been reported

in dogs fed a high-fat diet (Wanecq *et al.*, 2006) and PGWAT of adult db-/- mice (Cioni *et al.*, 2006). Another investigation on mice rendered obese by high-fat diet feeding described no alteration in adipose SSAO activity (Visentin *et al.*, 2005) and obese Zucker rats seem to express less adipose SSAO than their lean controls (Moldes *et al.*, 1999). In SCWAT, no change in SSAO activity was found when comparing young obese subjects (Body mass index >30) with age-matched controls (Visentin *et al.*, 2004). Therefore, our observations can be regarded as one more among the numerous interspecies differences (Al-Wabel, 2008) related to SSAO.

Two distinct causes of increased SSAO may coexist: one is the upregulated expression of AOC3 gene encoding for SSAO and the other merely depends on the adipose tissue enlargement that characterises obesity. As a consequence of its hypertrophied mass, the adipose tissue of an obese individual contains much more SSAO activity than the same anatomical fat depot in a lean control, even without an increase in enzyme expression at cellular level. Obviously, this quantitative aspect must be considered for a tissue that can increase its mass 10-fold in obesity. There are no other organs capable of a change of this magnitude except for growing tumors and placenta (Carpéné, 2009).

Even if the enzyme activity does not change when expressed on a per mg protein basis, in obesity the overall capacity of WAT to oxidise BzA is higher compared to lean controls. At this point, the need for further determination of age- and adiposity-dependent changes in adipose SSAO becomes evident, especially for clinical investigations. Indeed, the numerous variations of plasma SSAO found in patients suffering from a broad variety of metabolic diseases (Boomsma *et al.*, 2003) warrant the extension of the investigations to the adipose tissue-bound form.

7.2. In vitro insulin-like effect of benzylamine

Since amine oxidation may influence adipocyte functions, there is a need to increase our knowledge on the regulation and the physiological role of amine oxidases and their substrates in adipose tissue.

In our *in vitro* rodent models, assessing anti-lipolysis, glucose transport and lipogenesis, we have evidenced that beside the SSAO-mediated oxidation of our reference amine substrate (BzA), the presence of the protein tyrosin phosphatase inhibitor vanadate was also needed to observe insulinomimetic effects. These data are in

line with previous reports about the synergism between vanadate and amines due to the formation of peroxovanadate complexes (Abella *et al.*, 2003a). We have also shown that anti-lipolysis and lipogenesis induced by BzA + V were significantly less in KO AOC3 mice. Similar difference was found on glucose uptake in adipocytes of KO AOC3 and WT mice (Bour *et al.*, 2007b), evidencing that the insulinomimetic effects were dependent on SSAO. The targeted invalidation of the AOC3 gene leads to SSAO-deficient mice that otherwise possess functional adipocytes: the β-adrenergic isoprenaline response is unaltered as well as insulin responsiveness.

Albeit several insulin-like effects of exogenous amines have been described in *in vitro* studies there is not at the present time any indication supporting the spontaneous *in vivo* occurrence or the physiological relevance of the amine-dependent H_2O_2 production. Further studies on the adipose tissue development and on the metabolic control of mice with null mutation of the AOC3 gene appear to be of first importance, especially because, under some conditions, this model can be considered as mildly obese (Bour *et al.*, 2009). Disturbances in cholesterol and methylamine metabolism, adipokine production are under current investigation (Carpéné et al., unpublished observations) and may help to unravel novel role of AOs in adipocyte physiology.

7.3. In vivo insulin-like effect of benzylamine

7.3.1. Effect of a single injection of benzylamine on glucose handling

Although the in vitro experiments have demonstrated the insulin-like actions of BzA in combination with vanadate, it seemed to be worth to clarify whether BzA alone is also effective *in vivo*, for at least two reasons: On the one hand vanadium potentiates BzA's insulin-like actions but it is potently toxic and it may alter overall phosphorus metabolism (a serious adverse effect that has limited its development as an antidiabetic drug). On the other hand the presence of vanadate does not improve the insulin-like effects of BzA in murine preadipocytes (Carpéné *et al.*, 2006) and in human adipocytes (Morin *et al.*, 2001). It might thus be suspected that the synergism observed between the amine and the transition metal in the rodent diabetic models studied so far could not be easily extrapolated to diabetic patients. In this context, it was justified to test whether a diabetic model shown *in vivo* to be responsive to arylalkylamine vanadium salts

(Garcia-Vicente *et al.*, 2007; Yraola *et al.*, 2007) could be influenced similarly by BzA alone.

Non-diabetic and VHFD mice clearly responded to BzA challenges by limiting their hyperglycemic response during IPGTT. BzA administration did not change fasting plasma glucose levels in VHFD mice (Figure 21. A). Therefore, the synthetic amine was devoid of a strong hypoglycemic activity, while it provoked a dose-dependent clear-cut anti-hyperglycemic response at 7-700 μmol/kg doses (Figure 21. B). These results are completely in line with previous findings where 7 μmol/kg BzA improved glucose tolerance in diabetic mice or non-diabetic rabbits when injected or infused (Iglesias-Osma *et al.*, 2004). In such conditions the *in vivo* insulinomimetic effect of BzA was totally abolished by pre-treatment with semicarbazide.

In our model the simultaneous presence of vanadate did not further improve the anti-hyperglycemic response to BzA. The presence of vanadate thus was not crucial for the in vivo insulin-like effect of the tested SSAO substrate. Moreover, BzA was equally efficient alone or when associated with vanadium in reducing the hyperglycemic response of mice subjected to a glucose load.

SSAO is the protein being involved in the anti-hyperglycemic effect of the benzylamine-vanadate complex B_6V_{10} (Figure 22.), as supported by previous reports (Garcia-Vicente *et al.*, 2007). The immunoreactive insulin levels clearly showed that beneficial effect of B_6V_{10} on glucose tolerance is not the consequence of a stimulated insulin secretion. This was in line with previous *in vitro* experiments where BzA was unable to stimulate insulin secretion in pancreatic islets from VHFD-fed mice (Iglesias-Osma *et al.*, 2004).

Glucose tolerance of KO AOC3 and WT mice was similar (Figure 22.), as was insulin secretion in our experiments at 0-5-15-30 minutes after glucose load. However Garcia-Vicente and coworkers experienced a delay in early insulin secretion (5 min after glucose load) in female KO AOC3 mice *vs* WT (Garcia-Vicente, 2007).

Some observations suggest that SSAO inhibitors could impair a putative action of biogenic amines and may predispose to insulin resistance, therefore explaining the hyperinsulinism observed in treated rabbits (Iglesias-Osma *et al.*, 2004), or are capable to provoke glucose intolerance, as evidenced in another model of obese diabetic mice (Yu *et al.*, 2004). It should be taken into account that SSAO substrate amines escaping

from oxidation may activate other receptors like H_2 receptors in adipocytes (Berlan *et al.*, 1981) or ion channels (Banchelli *et al.*, 2001; Pirisino *et al.*, 2001). The lack of inhibitor selectivity might be responsible for such contradictions between these models and genetic invalidation of SSAO. We should not forget that even semicarbazide inhibits DAO, LO, *etc*.

7.3.2. Effect of chronic benzylamine injection in streptozotocin-induced diabetic rats

As SSAO is present on the surface of endothelial cells, besides adipose SSAO (Abella *et al.*, 2004), endothelial SSAO can be a possible source of the excessive plasma SSAO found in diabetes (Stolen *et al.*, 2004b). While SSAO activity can have beneficial effect in adipocytes because of the insulin-mimicking properties described above, the activation of plasma or endothelial SSAO can be hazardous, because it increases the local production of ROS, aldehydes, and ammonium, which products can damage the blood vessels. These cytotoxic compounds thus may contribute to the cardiovascular and renal complications of diabetes (Yu *et al.*, 2003a). Considering the deleterious vascular effects of methylamine administration reported in mice overexpressing vascular SSAO (Stolen *et al.*, 2004a), SSAO substrates might induce oxidative stress or diabetic-like vascular injury. In diabetic conditions the study of this double action of substrates, thus have an interest. We have examined the insulinomimetic activity and effect on indicators of oxidative stress of BzA treatment in diabetic rats in the absence or presence of low concentration of vanadate.

50% increase in AGEs in the serum of diabetic rats after 4 weeks of the disease induction was found compared to the control animals. Insulin and BzA+V treatment, which was started immediately after the onset of diabetes, reduced the accumulation of the glycated proteins. The drop in the amount of AGEs induced by BzA+V treatment may be, at least partly, the consequence of the reduction in soluble SSAO activity and ROS production (Soltész *et al.*, 2007). Although, BzA alone also reduced hyperglycemia, this anti-hyperglycemic effect was not accompanied by the reduction of accumulation of AGEs in the serum.

Endothelial dysfunction, characterised by reduced NO-dependent vasodilatation, is the early sign of vascular complications. Reduced effectiveness of NO in diabetes

may be the consequence of either its decreased formation, or its increased elimination/inactivation. There are contradictory data about the altered expression or activity of NO synthase in the diabetic state (Santilli *et al.*, 2004; Tábi *et al.*, 2006). An accelerated inactivation of NO is highly probable because of the increased formation of reactive oxygen species in the disease. Increased vascular endothelial generation of superoxide anions (Hink *et al.*, 2001) has been reported and elevated amounts of peroxynitrite in the kidney of diabetic rats have also been shown (Stadler *et al.*, 2003). The measurement of the stable end products of the NO metabolism in the tissues allows the assessment of the oxidative conversion of NO. In our experiments, decreased nitrite level and increased nitrate/nitrite ratio in the diabetic rat kidney was found, indicating a reduced biological availability and accelerated oxidative inactivation of NO. Insulin and BzA+V treatments both significantly reduced this elevated nitrate/nitrite ratio. No increase of the NO production could be observed in the insulin-treated group. This contrasts with previous findings, which described increased efflux of nitrate and nitrite from aortic strips of insulin treated diabetic rats after acetylcholine stimulation, and increased expression of endothelial NO synthase (Kobayashi *et al.*, 2001). However, in contrast to insulin, BzA+V treatment increased the nitrite levels, indicating elevated production of NO. Both treatments could ameliorate the accelerated oxidative inactivation of NO, although mechanisms involved are probably different. In the case of insulin, reduction of oxidative stress and consequent decrease of the oxidative metabolic transformation of NO are probable, while BzA+V treatment may increase the production of NO.

7.3.3. Effect of oral benzylamine treatment in various animal models of insulin resistance

Though BzA is a synthetic amine it is also present in the plant Moringa oleifera, known as the “miracle tree” used in folk medicine as an antidiabetic agent. The glucose lowering property of this medicinal plant was certified in a pharmacological survey (Kar *et al.*, 2003). This latter finding together with the well-known anti-hyperglycemic effect of parenteral BzA administration led us to suppose that BzA might contribute, at least partly to the plants’s effect.

The demonstration of BzA diffusion across everted gut sacs, and the detection of BzA in the urine of BzA-drinking mice show that the intestinal barrier is not insurmountable for this amine oxidase substrate. Substantial proportion of ingested BzA is thus supposed to be absorbed in the gut, despite of the considerable amine oxidation activity in the intestine, supporting the idea that oral administration could result in plasma and tissue BzA levels sufficient to exert some of the insulin-like actions observed *in vitro* (Bour, 2006).

Chronic oral administration of BzA did not totally normalize the relative glucose intolerance of VHFD mice, since it could not reduce their slightly increased fasting glycemia. Decrease of blood glucose levels was observed only in fed state but did not consistently appeared throughout BzA-treatment. This was probably the consequence of the highly variable glycemia of fed animals, which unexpectedly remained lower than in the fasted state.

We extended our first observations made in VHFD-fed mice using another special diet composition (HFD) containing more carbohydrates to induce insulin resistance. A transgenic model of diabetes (db-/- and db-/+) was also accommodated. Orally given BzA improved the glucose tolerance in HFD-fed mice, as it did in VHFD animals. In addition, BzA slightly reduced fed and fasted glycemia in HFD-fed mice. These observations are in perfect agreement with those reported in a preliminary study, describing reduced hyperglycaemic response during glucose tolerance tests in non-obese non-diabetic rats receiving BzA *per os* (Bour *et al.*, 2005). Such pilot experiment also revealed that fasting glycemia was not influenced by BzA in non-obese, non-diabetic rats, being in complete agreement with our results found in db-/+ mice. However, hyperglycemia of db-/- animals was markedly lowered in either fed or fasting state by oral BzA supplementation. In other words, BzA supplementation delayed the onset of diabetes in this widely recognized model of severe type 2 diabetes. The anti-hyperglycemic effect of BzA in HFD-fed and db-/- insulin-resistant mice is in complete accordance with its beneficial action in streptozotocin-induced type 1 diabetic rats, the hyperglycemia of which was not normalized but significantly reduced by repeated *s.c.* administration of BzA alone (see chapter 1.3.2). The BzA-dependent activation of glucose transport and *de novo* lipogenesis in adipocytes should be responsible for such beneficial effects (Fontana *et al.*, 2001; Marti *et al.*, 2001).

The lack of the effect of BzA on fasting glucose in VHFD-fed animals is somehow in discrepancy with the above-mentioned experiments. A possible explanation for this is that VHFD mice have a peculiar glucose homeostasis, as they are starved for alimentary glucose even under fed conditions, since their diet is devoid of carbohydrates. These mice are intolerant to a glucose load although do not display all the typical features of type 2 diabetes: they do not exhibit hyperinsulinemia and show lower protein glyc(oxid)ation index (AGEs) than normoglycemic mice fed a control diet. Therefore, it might be difficult for an agent deemed to improve glucose utilization in peripheral tissues to correct the defect of VHFD mice having hepatic gluconeogenesis as practically sole source of glucose. This may explain the discrepancy between the evident beneficial action of BzA on glucose handling in the presence of a glucose load and its inconsistent action on fasting hyperglycemia in VHFD mice.

BzA treatment diminished water consumption in all studied models of insulin resistance. It can be considered as a reduced fluid intake only if spillage and dressing can be ruled out. Similar reduction has already been observed in an experiment on non-diabetic Wistar rats receiving a 0.3% BzA drinking solution for seven weeks (Bour *et al.*, 2005). In all conditions, it is difficult to assess whether BzA exerts an aversive influence on drinking behaviour or a pharmacological effect on fluid homeostasis. The former hypothesis could not be discarded due to the bad smell of BzA solutions, even smelling like putrefied fish at much higher concentrations than those given to the mice.

Unfortunately, in VHFD and HFD-fed mice, it was difficult to assess if there was a reduction in calorie intake during BzA-treatment because the very high-fat and high-fat chow given to the mice was particularly sticky and could not be accurately weighed. Nevertheless BzA was shown to decrease food intake of db-/- as well as that of db-/+ mice. This inhibitory effect of BzA on appetite is also supported by previous findings, reporting that the amine's effect is mediated by the Kv1.1 potassium channel blockade (Banchelli *et al.*, 2001; Pirisino *et al.*, 2001) and probably the release of catecholamines and serotonin in the central nervous system (Banchelli *et al.*, 2001). The hypophagic action of BzA is definitively not mediated via its oxidation by SSAO since inhibitor of the amine oxidase increased - instead of inhibiting - such effect.

Surprisingly body weight gain did not show obvious change in the three investigated mouse models as water consumption did. In fact, the limitation of fat

deposition observed in treated HFD-fed mice is in apparent contradiction with the anti-lipolytic effect of BzA detected on adipocytes in the presence of vanadate, although it could be explained by its central hypophagic action supposed that BzA passes the blood-brain barrier. On the contrary, the elevated body weight observed at the end of BzA treatment in db-/- mice is in line with the anti-lipolytic effect, although it is in contradiction with the experienced reduction in food-intake, which can be considered, as a consequence of disrupted leptin anorectic signalling, less important than that experienced by HFD mice. Unlike in other two models, BzA did not alter body weight gain in VHFD-mice. Conflicting data on body weight gain of the various obesity and diabetes models can also be the consequences of different diet composition, genetic background and physical activity.

As far as we know, the hypophagic effects of BzA have been described only in acute experiments in behavioural studies on non obese, non diabetic mice (Banchelli *et al.*, 2001; Pirisino *et al.*, 2001) and no reduction of body weight after prolonged treatment has been reported to date. In earlier studies, only a slight tendency of reduced food consumption could be detected in Wistar rats drinking BzA while their water intake was significantly reduced at the end of the seven-week treatment period (Bour *et al.*, 2005). If such a central hypophagic action of chronically ingested BzA persisted in the treated mice, it could be responsible for some of the beneficial effects observed, including reduced non-fasting glycemia or body weight gain, but it could not totally account for the improved tolerance to a glucose load. This is also supported by the results of BzA treatment in VHFD-fed and db-/- mice: the anti-hyperglycemic action of BzA was not accompanied by the reduction of body weight gain.

To better characterise oral BzA's action in insulin-resistant states several plasma markers of the metabolic disease were measured. Immunoreactive insulin was expected to be increased by BzA. Albeit, not decreased in the three models, an improvement in insulin-sensitivity was found in response to BzA supplementation in HFD-fed mice, as they showed a reduced HOMA index, and in db-/- mice, due to a better insulin-responsiveness of their adipocytes.

A decrease in plasma FFA was expected, owing to the *in vivo* anti-lipolytic effects of BzA found previously in mouse (Iglesias-Osma *et al.*, 2004) and rat (Bour *et al.*, 2005), but did not accur in any of the three models studied.

Circulating cholesterol and trigricerides were not expecteded to be altered by the treatment. Notwithstanding, oral BzA administration reduced plasma cholesterol levels in HFD but not in db-/- or VHFD mice. Although the decrease was mainly observed in the non-HDL fraction, there was no rationale of such a beneficial effect. Although it can be noted that, in a very recent study, various hydrazine derivatives used as carbonyl scavengers, but also known to inhibit SSAO and/or MAO activity, such as hydralazine, phenelzine and iproniazid, have been shown to exert anti-atherogenic effects, but without lowering circulating cholesterol (Galvani *et al.*, 2008). A putative link between amine oxidase activity and cholesterol metabolism deserves to be clarified. We can also suggest that the reduction of body weight gain and the ameliorated glucose tolerance, together might also contribute to the reduction of total cholesterol.

A decrease in leptin, adiponectin and resistin was supposed in HFD mice, because of the reduced adiposity of the treated animals. After all, only plasma resistin was found lower while leptin and adiponectine levels were unaltered in BzA-drinking HFD-fed mice. Reduced resistin and the slightly reduced insulin levels are consistent with an improvement of insulin sensitivity by BzA-treatment, leading to lowered glucose levels, enhanced glucose tolerance.

An increase in fructosamine was assumed as a consequence of the long-term SSAO substrate ingestion, generating H_2O_2. The glycated plasma protein levels were not altered by BzA-treatment in HFD-fed and db-/- mice.

Elevated plasma uric acid accompanied the onset of metabolic syndrome (db-/- *vs* db-/+, HFD-fed mice *vs* NC-fed mice) like it did in clinical studies (Yoo *et al.*, 2005). BzA decreased the high circulating level of uric acid in both models. Of note, the lowered levels of this independent marker of cardiovascular disease risk (Gagliardi *et al.*, 2009) could be regarded as protective for cardiac and renal function. Since BzA decreased the high circulating level of uric acid in treated HFD and db-/- mice, this agent can be endowed with interesting preventive properties.

In all the treated mice, BzA did not affect the β-adrenergic, dose-dependent lipolytic effect of isoprenaline in adipocytes. The effect of insulin in adipocytes of HFD and VHFD-fed mice on stimulated lipolysis was not altered by chronic BzA administration, but insulin exerted a better anti-lipolytic response in db-/-.

Moreover, the long-term oral administration, used in our protocols, may have induced desensitisation of members involved in redox regulation. Intriguingly, anti-lipolytic action of BzA (Visentin *et al.*, 2003b) persisted even after chronic administration. Adipocytes of VHFD and HFD-fed mice limited their lipolytic activity only when BzA was combined with vanadate. Neither vanadate nor BzA (0.1 mM) alone was able to hamper stimulated lipolysis. However, BzA alone was effective in db-/- mice, probably due to the increased adipose SSAO activity found in this model. This *in vitro* anti-lipolytic action clearly supports the amine's *in vivo* effect on body weight. Similarly, in db-/- BzA alone could stimulate 2-DG uptake of isolated adipocytes but simultaneous presence of vanadate was demanded in adipocytes of HFD or VHFD-fed mice. Other examples, in which vanadate is not necessary, are the cultured preadipocytes 3T3-F442A (Fontana *et al.*, 2001), 3T3-L1 adipocytes (Subra *et al.*, 2003; Visentin *et al.*, 2003a), although these lineages express much less SSAO than db-/- or human adipocytes (Carpéné *et al.*, 2001a; Carpéné *et al.*, 2001b; Morin *et al.*, 2001; Visentin *et al.*, 2001). The H_2O_2 generated during amine oxidation is required to be a sufficient trigger for the insulin-like effect of BzA, but perhaps its degradation strongly differs among the different models, and could be suspected to be very efficient in adipocytes of HFD, VHFD-fed mice or rat fat cells (Enrique-Tarancon *et al.*, 1998; Figueiredo *et al.*, 1998; Marti *et al.*, 1998). Different oxidation states of vanadate (vanadate or vanadyl) or the difference in cellular antioxidant apparatus may be hypothesised to influence the *in vitro* effect. Regarding SSAO, that one may consider as porooxidant in view of its ability to produce H_2O_2, we have never experienced this kind of adverse effect: SSAO activity and mRNA expression was unaltered in WAT of BzA-treated mice (Iffiú-Soltész *et al.*, 2007), so it was for the anti-lipolytic action of BzA+V in isolated adipocytes.

BzA treatment did not alter insulin-responsiveness of adipocytes of HFD-, and VHFD-fed mice. However, because BzA acts independently of insulin (Abella *et al.*, 2003a; Abella *et al.*, 2003b; Zorzano *et al.*, 2003), its effect on glycemia likely have delayed the set-up of insulin resistance and of glucotoxicity, at least in adipocytes of INWAT of young db-/- mice.

The decreased nitrite concentrations in the aorta of diabetic db-/- and HFD-fed mice is in line with investigations known from the literature, showing decreased

acetylcholine-induced relaxation of the aorta in diabetic animals (Miike *et al.*, 2008) and also with our results in the kidney of STZ-induced diabetes (chapter 6.3.2). The significantly elevated nitrite concentration in BzA-treated groups suggests an increased NO production. H_2O_2 has been reported to increase eNOS expression, and NO release of endothelial cells mediated by cooperative effects between PI 3-kinase/PKB-dependent eNOS serine 1179 phosphorylation and activation of MEK/ERK1/2 (Cai *et al.*, 2003; Thomas *et al.*, 2002). However, these latter observations were not confirmed in long-term studies (Boulden *et al.*, 2006). Insulin has also been described to increase nitrite concentraion in the aorta by inducing eNOS expression, as well. Alternatively, the higher aorta nitrite levels can be due to the reduction of hyperglycemia, reducing oxidative stress, thus improving local endothelial function. This latter hypothesis is in concert with the fact that in non-diabetic (db-/+) and VHFD-fed mice nitrite was unchanged by BzA such as plasma glucose at sacrifice.

The lack of excessive accumulation of nitrate in the aorta of all the three studied models of insulin resistance after chronic BzA ingestion indicated that there was no increased vascular oxidative stress caused by the treatment. The following markers of the anti-oxidant defence systems confirmed that the treatment did not promote clear-cut oxidative stress:

- unchanged SOD2 and catalase expression in the liver of HFD-fed mice, or in WAT of VHFD-fed mice receiving BzA
- unaltered H_2O_2 production in SCWAT of treated db-/-

Furthermore, no evident signs of excessive glycation or vascular damage, being the possible consequence of excessive activation of soluble or vascular SSAO and aldehyde-induced cross-linking (Gubisne-Haberle *et al.*, 2004), were detected after BzA administration. Finally, when considering the ratio of tissue nitrate to nitrite as an indicator of the conversion of NO to RNS by excessive ROS, there was no indication of such oxidative stress in the aorta of db-/- and HFD-fed mice treated with BzA.

Lowered plasma uric acid and increased nitrite concentration in the aorta of HFD-fed and db-/- mice may therefore indicate an improved endothelial function in BzA-treated animals instead of the expected damage. The lack of deleterious effects of BzA-treatment on vasculature may support the hypothesis that benzaldehyde, the product of SSAO -mediated BzA oxidation, is less cytotoxic than formaldehyde

produced by methylamine oxidation (Conklin *et al.*, 1998; Gubisne-Haberle *et al.*, 2004). Therefore, we suggest that among the different possible fates for ingested BzA, a competition with methylamine for SSAO-mediated oxidation can occur, and as a result, less cytotoxic aldehyde could be generated. In this context, it should be mentioned that BzA can also be oxidatively deaminated by reactive aldehydes themselves such as methylglyoxal (produced in part by SSAO -dependent aminoacetone oxidation which is increased in diabetic plasma) and may limit their toxicity by acting like a scavenger (Akagawa *et al.*, 2003).

We have shown that chronic BzA administration improves glucose homeostasis in three models of insulin resistance and in a model of type 1 diabetes. Moreover, these mice, prone to develop diabetes and obesity, also reduced levels of several markers of the metabolic disease such as plasma cholesterol, resistin (HFD-fed mice) and uric acid levels (HFD-fed mice, db-/-) when drinking BzA. The oral amine supplementation rather improved (HFD-fed, db-/- mice) or did not affect (VHFD-fed, db-/+ mice) nitric oxide bioavailability in the vasculature. Chronic BzA ingestion was therefore definitively devoid of adverse effects on markers of oxidative stress, at least under our experimental conditions.

The present findings show that oral amine administration merits testing, and is worthy of further investigation. As a matter of fact, the reported anti-hyperglycemic and hypocholesterolemic actions warrant future research for selective amine oxidase substrates endowed with more powerful anti-hyperglycemic properties and yet devoid of adverse effects. In this spirit, we set up an *in vitro* system to screen SSAO substrates as possible future anti-hyperglycemic drug candidates.

7.4. In vitro tests for screening SSAO substrate drug candidates

In vitro insulin-like effect of BzA has also been confirmed *in vivo*, so far. However some of our results can hardly be extrapolated to humans. Human WAT is thus a preferred model to test the SSAO-dependent insulin-like effect of several possible anti-hyperglycemic drug candidates, *in vitro*.

The very high SSAO activity we found in human SCWAT is in accordance with previous findings that AOC3 gene expression dramatically increases during

adipogenesis of human pre-adipocytes (Bour *et al.*, 2007a). As a consequence mature adipocytes express one of the highest levels of AOC3 mRNAs and SSAO activity found in the body (Bour *et al.*, 2007a; Morin *et al.*, 2001) as confirmed by various gene expression databases (e.g. SymAtlas from Genomic Institute for the Novartis Research Foundation). In our experiments, however, we used WAT homogenates for the determination of SSAO activity, since they are more readily availalable than freshly preparated adipocytes and since previous reports presented that the SSAO activity found in WAT homogenates was mainly due to SSAO activity of adipocytes, not to the stroma vascular fraction (Bour *et al.*, 2007a). In keeping with their high SSAO activity, insulin-like effects of amine oxidation have been described in freshly isolated human adipocytes (Carpéné *et al.*, 2001a; Carpéné *et al.*, 2001b; Morin *et al.*, 2001; Visentin *et al.*, 2001) but cannot be reproduced on disrupted cells, lacking intact insulin signalling pathways.

The enzyme kinetic parameters we measured and all the above mentioned preceding results validated human adipose tissue, as a relevant model for testing anti-hyperglycemic drug candidates.

Among the simultaneously tested amines, 4-PBA and 3-MTPPA were already known SSAO substrates. Originally, 4-PBA was selected as a better substrate of SSAO from an arylalkylamine database as a result of structure activity relationship kinetic studies (Yraola *et al.*, 2006). 3-MTPPA was shown to be a good substrate of bovine SSAO and MAO-B.

Most amines inhibited stimulated lipolysis and activated glucose transport. Such insulin-like effects and SSAO-dependent H_2O_2 release were in strong correlation. However, in the case of 4-PBA and 3-MTPPA the anti-lipolytic activity was not only the consequence of the amine oxidation-dependent H_2O_2 release as their strong anti-lipolytic effect was also kept in KO AOC3 mice.

We suppose that 4-PBA may involve some other possible pathways which are independent of SSAO-related H_2O_2 generation. Some possible mechanisms may be:

- MAO-dependent
- other H_2O_2-generating system
- antagonism at β-receptor
- stimulation at α_2-receptor.

β-receptor antagonism is not involved as the drug remains anti-lipolytic in the presence of forskolin which stimulates lipolysis via β-receptor-independent way. Furthermore, the drug did not activate glucose uptake better than BzA (not like anti-lipolysis) as it was reported before in mouse adipocytes (Yraola *et al.*, 2006).

Such as 4-PBA, 3-MTPPA might also act on different sites. 3-MTPPA is also a substrate of MAO-B (Gallardo-Godoy *et al.*, 2004). Nonetheless, this kind of characteristic is not likely to be responsible for its anti-lipolytic activity; first of all adipocytes do not have high MAO-B activity, second, even if it was the case, glucose uptake should be, as well, strongly stimulated. The involvement of β-receptor is also discarded, because of the pharmacon's inhibitory effect on forskolin-stimulated lipolysis.

Our results clearly show that *in vitro* functional tests are indispensable when screening for novel substrates. The measurement of SSAO activity alone does not predict all possible effects on adipocyte metabolism. As SSAO substrates are mainly monoamines, they are not only substrates for MAOs or SSAOs but they may act on biogenic amine receptors such as β or α_2 adrenoceptors, histamine, serotonine or trace amine receptors, *etc*.

During the experiments all substrates had insulin-like effect. Our screening method, however, allowed us to eliminate amines acting independently of their SSAO-dependent H_2O_2 release (4-PBA, 3-MTPPA and Substrate-A) and resulted substrate-B as a novel leader structure for the design of new future SSAO substrates.

8. Conclusions

SSAO substrates have previously been described to exert insulin-like actions *in vivo* and *in vitro*, thus SSAO has been proposed as a therapeutic target in diabetes.

Our results revealed a SSAO-dependent H_2O_2 generation in WAT homogenate of mice with addition of benzylamine at 0.01 to 1 mM. A small SSAO activity was also detectable without addition of BzA, likely due to the oxidation of endogenous amines.

SSAO activity was shown to be elevated in SCWAT but not PGWAT or PRWAT in our murine HFD-induced and genetic obesity models.

Experiments on fasting rats unravelled that SSAO activity was spared in spite of weight loss.

Investigation on isolated rodent fat cells elucidated that cells needed vanadate for the anti-lipolytic, lipogenic and glucose uptake stimulatory effect, and the genetic invalidation of SSAO hampered these BzA + V induced insulin-like properties.

The single injection of BzA was able to improve glucose tolerance in mice and simultaneous administration of vanadate did not further affect this parameter.

Chronic BzA injection lowered blood glucose in type 1 diabetic rats. Kidney endothelial function was only improved in the presence of vanadate. The latter was a result of increased NO formation rather than due to a decreased oxidative metabolism of NO.

Oral BzA improved glucose homeostasis in three models of insulin resistance: VHFD and HFD-induced obesity and in genetically obese db-/- mice. Such anti-hyperglycemic effect can be the consequence of: 1.) the oxidation of BzA in adipose tissue favouring glucose utilization and 2.) the central hypophagic action of BzA. These two, depending on the model may contribute to an *in vivo* effect in a different way. In all cases, BzA was devoid of adverse effects. Moreover, in HFD-fed and db-/- mice where BzA treatment lowered fasting glucose it also increased nitrite concentration in the aortae suggesting a strong relationship between these two parameters. Obviously, BzA can be oxidised in blood and vessels, and in WAT, which is considerably increased in overweight rodents and rich in SSAO activity. It is therefore conceivable that direct insulin-like actions of orally given BzA can occur in WAT.

Accordingly, a very high SSAO activity was measured in human SCWAT compared to murine. Our test system, based on the correlation between glucose-uptake

or anti-lipolysis and SSAO-dependent H_2O_2 production was able to select Substrate-B, as a better SSAO substrate than BzA. The system also showed that several amines can also exert SSAO-independent actions on adipocyte metabolism.

On the basis of the present findings we propose SSAO substrates as possible anti-hyperglycemic drug candidates worth for further experimental and clinical investigations.

Summary

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Obesity and diabetes are serious public health problems in developed countries and considerable efforts are put in the development of drugs improving impaired carbohydrate metabolism. Semicarbazide-sensitive amine oxidase (SSAO) may be a possible target. The enzyme catalyzes the oxidation of primary amines. SSAO is highly expressed in white adipose tissue (WAT) and in the vasculature. It has been found that its soluble form in plasma is an independent cardiovascular risk factor in diabetes. However, the administration of exogenous SSAO substrates has been shown to reduce hyperglycemia in diabetic animals. Hydrogen peroxide formed during the enzyme reaction has been proven responsible for such effect. However, this reactive oxygen species may contribute to vascular complications of obesity and diabetes, as well.

Our aim was to explore the implication of SSAO substrates on certain parameters of diabetes and obesity. We investigated the influence of WAT extension (in obesity and fasting) on its SSAO content. We have analysed the insulin-like actions of benzylamine (BzA), an exogenous substrate of SSAO, *in vitro* and *in vivo*. Furthermore, being aware of the dual action of SSAO in diabetes, we also studied the effect of long-term BzA treatment on glucose handling and on putative vascular complications. Then, we tested SSAO substrate candidates in human adipocytes.

SSAO activity was higher in subcutaneous WAT of obese than lean mice. BzA injection was effective alone to improve glucose homeostasis in type 1 and type 2 diabetic animals. Chronic BzA injections also improved endothelial function of diabetic rats when vanadate was simultaneously administered. Chronic oral administration of BzA improved glucose homeostasis in three mouse models of insulin-resistance. In such conditions, we did not observe any adverse effect of hydrogen peroxide. Moreover, increased aorta nitrite concentration, indicative of NO production, accompanied the reduction of fasting plasma glucose levels. In keeping with this, we have set up a pharmacological screening, based on human adipocytes, which allowed the detection of better SSAO substrates than BzA. The results obtained in obese and diabetic models thus confirmed the beneficial metabolic action of BzA and encouraged us to propose SSAO substrates as possible anti-hyperglycemic drug candidates.

Összefoglalás

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Az elhízás és a cukorbetegség a fejlett országokat súlyosan sújtja, ezért jelentős összegeket fordítanak olyan gyógyszerek fejlesztésére, amelyek a megváltozott szénhidrát-metabolizmust célozzák helyreállítani. Az egyik ígéretes célpont a szemikarbazid-szenzitív aminoxidáz (SSAO). Az SSAO primer aminokat oxidál és nagy mennyiségben található a zsírsejtekben és az erekben. Igazolták, hogy a plazma SSAO független kardiovaszkuláris kockázati tényező cukorbetegségben. Ugyanakkor az SSAO szubsztrátjaival sikerült csökkenteni a hiperglikémiát diabéteszes állatmodellekben. A hatásért a reakció során felszabaduló H_2O_2-t tartják felelősnek. Másfelől ez a szabadgyökök keletkezéséhez vezető anyag hozzájárulhat az elhízás és a cukorbetegség vaszkuláris komplikációinak kialakulásához is.

Célul tűztük ki az SSAO szubsztrátok elhízás és cukorbetegség egyes paramétereire gyakorolt hatásának vizsgálatát. Az elhízás és éhezés hatását vizsgáltuk a különböző lokalizációjú zsírszövetek SSAO aktivitására. Megvizsgáltuk az SSAO egyik exogén szubsztrátjának, a benzilaminnak (BzA) inzulinszerű hatásait *in vitro* és *in vivo*. Továbbá, tudtában az SSAO kettős hatásának diabéteszben, nyomon követtük a hosszútávú BzA kezelés hatásait a glükóz háztartásra, valamint a vaszkuláris komplikációkra. Végül néhány új SSAO szubsztrát-jelölt molekula hatásait vizsgálatuk emberi zsírsejteken.

Az elhízott egerek bőr alatti zsírszövete emelkedett SSAO aktivitást mutat. Diabéteszes állatok (1-es és 2-es típus) glükóz-háztartásának helyreállításához a *s.c.* BzA kezelés önállóan elegendő. A krónikus *s.c.* BzA kezelés vanadáttal kombinálva az endoteliális funkciót is javítja diabéteszes patkánymodellen. A krónikus *per os* BzA kezelés javítja a glükóz homeosztázist három inzulinrezisztens egérmodellen. Ezen körülmények között nem figyeltünk meg semmilyen H_2O_2-eredetű mellékhatást. Ráadásul az éhomi vércukorszint csökkenését az aortában mért emelkedett nitrit-koncentráció kísérte, amely fokozott nitrogén monoxid termelésre utal. A humán zsírsejteken tesztelt vegyületek között egy bizonyult a BzA-nál jobb szubsztrátnak.

A fenti eredmények megerősítették a BzA-kezelés kedvező hatását a szénhidrát anyagcserére, ami arra sarkall bennünket, hogy az SSAO szubsztrátjait lehetséges anti-hiperglikémiás gyógyszerjelölt molekuláknak tekintsük.

Résumé

Thèse pour obtenir le grade de Docteur de l'Université
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L'obésité et le diabète sont de sérieux problèmes de santé dans les pays développés. Le développement de médicaments qui pourraient normaliser un métabolisme glucidique alteré est donc une grande nécessité actuelle. L'amine oxydase sensible au semicarbazide (SSAO) semble être une cible d'intérêt pour améliorer l'homéostasie glucidique. Cette enzyme oxyde les amines primaires. Elle est fortement exprimée dans le tissu adipeux blanc (WAT) et dans les vaisseaux. Sa forme circulante est un facteur indépendant des complications cardiovasculaires du diabète et de l'obésité. Cependant, l'administration des substrats exogènes de la SSAO réduit l'hyperglycémie des animaux diabétiques. Il a été montré que le peroxyde d'hydrogène formé pendant l'oxidation des amines par la SSAO tissulaire est responsable d'un tel effet. Pourtant, cette espèce reactive de l'oxygène, à forte dose, peut aussi contribuer aux complications de l'obesité et du diabète.

Nous avons étudié l'influence des substrats de la SSAO sur certains paramètres de l'obésité et du diabète. Nous avons d'abord examiné l'influence de la masse du tissu adipeux blanc sur sa richesse en SSAO en la faisant varier expérimentalement (obésité et jeûne). Nous avons ensuite analysé les actions insulinomimétiques de la benzylamine (BzA), un substrat exogène de la SSAO, *in vitro* et *in vivo*. De plus, nous avons étudié l'effet de traitements à long terme par la BzA sur la tolerance au glucose et les éventuelles complications vasculaires. Enfin, nous avons testé des molécules nouvelles, substrats potentiels de la SSAO sur des adipocytes humains.

L'activité de la SSAO augmente dans le WAT sous-cutané de souris obèses. L'injection de BzA améliore l'homéostasie glucidique des animaux diabétiques de type 1 et 2. L'injection chronique de BzA améliore la function endothéliale de rats diabétiques si elle est effectuée simultanément avec du vanadate. L'administration chronique de BzA par voie orale améliore aussi la tolérance au glucose dans trois modèles de souris résistantes à l'insuline dans des conditions où nous n'avons pas observé d'effets délétères du peroxyde d'hydrogène. De plus, la concentration des nitrites augmente dans l'aorte, indiquant qu'une meilleure biodisponibilité du NO a

accompagné la réduction du glucose plasmatique. Ces résultats obtenus sur des rongeurs obèses et diabétiques ont confirmé l'action bénéfique de la BzA sur le métabolisme glucidique. En utilisant un système de criblage basé sur l'utilisation d'adipocytes humains, nous avons caractérisé quelques molécules plus efficaces que la BzA pour mimer les effets de l'insuline. L'ensemble de ce travail nous encourage à considérer les substrats de la SSAO comme de possibles médicaments antidiabétiques.

9. References

Abella, A, Garcia-Vicente, S, Viguerie, N, Ros-Baro, A, Camps, M, Palacin, M, Zorzano, A, Marti, L (2004). Adipocytes release a soluble form of VAP-1/SSAO by a metalloprotease-dependent process and in a regulated manner. *Diabetologia* **47:** 429-38.

Abella, A, Marti, L, Camps, M, Claret, M, Fernandez-Alvarez, J, Gomis, R, Guma, A, Viguerie, N, Carpéné, C, Palacin, M, Testar, X, Zorzano, A (2003a). Semicarbazide-sensitive amine oxidase/vascular adhesion protein-1 activity exerts an antidiabetic action in Goto-Kakizaki rats. *Diabetes* **52:** 1004-13.

Abella, A, Marti, L, Carpéné, C, Palacin, M, Testar, X, Zorzano, A (2003b). Stimulation of glucose transport by semicarbazide-sensitive amine oxidase activity in adipocytes from diabetic rats. *J Physiol Biochem* **59:** 153-60.

Airas, L, Mikkola, J, Vainio, JM, Elovaara, I, Smith, DJ (2006). Elevated serum soluble vascular adhesion protein-1 (VAP-1) in patients with active relapsing remitting multiple sclerosis. *J Neuroimmunol* **177:** 132-5.

Akagawa, M, Sasaki, T, Suyama, K (2003). Oxidative deamination of benzylamine by glycoxidation. *Bioorg Med Chem* **11:** 1411-7.

Al-Wabel, NA (2008). Comparative activities of semicarbazide-sensitive amine oxidase (SSAO) in five domestic species. *Pol J Vet Sci* **11:** 63-6.

Antuna-Puente, B, Fève, B, Fellahi, S, Bastard, JP (2008). Adipokines: the missing link between insulin resistance and obesity. *Diabetes Metab* **34:** 2-11.

Baba, S, Watanabe, Y, Gejyo, F, Arakawa, M (1984). High-performance liquid chromatographic determination of serum aliphatic amines in chronic renal failure. *Clin Chim Acta* **136:** 49-56.

Banchelli, G, Ghelardini, C, Raimondi, L, Galeotti, N, Pirisino, R (2001). Selective inhibition of amine oxidases differently potentiate the hypophagic effect of benzylamine in mice. *Eur J Pharmacol* **413:** 91-9.

Barrand, MA, Fox, SA (1984). Amine oxidase activities in brown adipose tissue of the rat: identification of semicarbazide-sensitive (clorgyline-resistant) activity at the fat cell membrane. *J Pharm Pharmacol* **36:** 652-8.

Beckman, JS, Beckman, TW, Chen, J, Marshall, PA, Freeman, BA (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc Natl Acad Sci U S A* **87:** 1620-4.

Berlan, M, Lafontan, M, Dang Tran, L, Carpéné, C (1981). Clonidine-induced lipolysis in dog adipocytes by stimulation of histamine (H2)-receptors. *Arch Int Pharmacodyn Ther* **249:** 213-20.

Bono, P, Salmi, M, Smith, DJ, Jalkanen, S (1998a). Cloning and characterization of mouse vascular adhesion protein-1 reveals a novel molecule with enzymatic activity. *J. Immunol.* **160:** 5563-5571.

Bono, P, Salmi, M, Smith, DJ, Leppanen, I, Horelli-Kuitunen, N, Plotie, A, Jalkanen, S (1998b). Isolation, structural characterization, and chromosomal mapping of the mouse vascular adhesion protein-1 gene and promoter. *J. Immunol.* **161:** 2953-2960.

Boomsma, F, Bhaggoe, UM, van der Houwen, AM, van den Meiracker, AH (2003). Plasma semicarbazide-sensitive amine oxidase in human (patho)physiology. *Biochim Biophys Acta* **1647:** 48-54.

Boomsma, F, de Kam, PJ, Tjeerdsma, G, van den Meiracker, AH, van Veldhuisen, DJ (2000a). Plasma semicarbazide-sensitive amine oxidase (SSAO) is an independent prognostic marker for mortality in chronic heart failure. *Eur Heart J* **21:** 1859-63.

Boomsma, F, Derkx, FH, van den Meiracker, AH, Man in 't Veld, AJ, Schalekamp, MA (1995). Plasma semicarbazide-sensitive amine oxidase activity is elevated in diabetes mellitus and correlates with glycosylated haemoglobin. *Clin Sci (Lond)* **88:** 675-9.

Boomsma, F, Hut, H, Bagghoe, U, van der Houwen, A, van den Meiracker, A (2005a). Semicarbazide-sensitive amine oxidase (SSAO): from cell to circulation. *Med Sci Monit* **11:** RA122-6.

Boomsma, F, Pedersen-Bjergaard, U, Agerholm-Larsen, B, Hut, H, Dhamrait, SS, Thorsteinsson, B, van den Meiracker, AH (2005b). Association between plasma activities of semicarbazide-sensitive amine oxidase and angiotensin-converting enzyme in patients with type 1 diabetes mellitus. *Diabetologia* **48:** 1002-7.

Boomsma, F, Tipton, KF (2007). Renalase, a catecholamine-metabolising enzyme? *J Neural Transm* **114:** 775-6.

Boomsma, F, van Dijk, J, Bhaggoe, UM, Bouhuizen, AM, van den Meiracker, AH (2000b). Variation in semicarbazide-sensitive amine oxidase activity in plasma and tissues of mammals. *Comp Biochem Physiol C Toxicol Pharmacol* **126:** 69-78.

Boor, PJ, Ferrans, VJ (1982). Ultrastructural alterations in allylamine- induced cardiomyopathy: early lesions. *Lab Invest* **47:** 76-86.

Boor, PJ, Moslen, MT, Reynolds, ES (1979). Allylamine cardiotoxicity: I. Sequence of pathologic events. *Toxicol Appl Pharmacol* **50:** 581-92.

Boor, PJ, Nelson, TJ (1980). Allylamine cardiotoxicity: III. Protection by semicarbazide and in vivo derangements of monoamine oxidase. *Toxicology* **18:** 87-102.

Boor, PJ, Sanduja, R, Nelson, TJ, Ansari, GA (1987). In vivo metabolism of the cardiovascular toxin, allylamine. *Biochem Pharmacol* **36:** 4347-53.

Boulden, BM, Widder, JD, Allen, JC, Smith, DA, Al-Baldawi, RN, Harrison, DG, Dikalov, SI, Jo, H, Dudley, SC, Jr. (2006). Early determinants of H2O2-induced endothelial dysfunction. *Free Radic Biol Med* **41:** 810-7.

Bour, S (2006). Rôle des amine oxydases du tissu adipeux: implications dans le diabete et l'obésité. *PhD Thesis*.

Bour, S, Caspar-Bauguil, S, Iffiú-Soltész, Z, Nibbelink, M, Cousin, B, Miiluniemi, M, Salmi, M, Stolen, C, Jalkanen, S, Casteilla, L, Penicaud, L, Valet, P, Carpéné, C (2009). Semicarbazide-sensitive amine oxidase/vascular adhesion protein-1 deficiency reduces leukocyte infiltration into adipose tissue and favors fat deposition. *Am J Pathol* **174:** 1075-83.

Bour, S, Daviaud, D, Gres, S, Lefort, C, Prévot, D, Zorzano, A, Wabitsch, M, Saulnier-Blache, JS, Valet, P, Carpéné, C (2007a). Adipogenesis-related increase of semicarbazide-sensitive amine oxidase and monoamine oxidase in human adipocytes. *Biochimie* **89:** 916-25.

Bour, S, Prévot, D, Guigne, C, Stolen, C, Jalkanen, S, Valet, P, Carpéné, C (2007b). Semicarbazide-sensitive amine oxidase substrates fail to induce insulin-like effects in fat cells from AOC3 knockout mice. *J Neural Transm* **114:** 829-33.

Bour, S, Visentin, V, Prévot, D, Daviaud, D, Saulnier-Blache, JS, Guigne, C, Valet, P, Carpéné, C (2005). Effects of oral administration of benzylamine on glucose tolerance and lipid metabolism in rats. *J Physiol Biochem* **61:** 371-9.

Brownlee, M (2001). Biochemistry and molecular cell biology of diabetic complications. *Nature* **414:** 813-20.

Burcelin, R, Crivelli, V, Dacosta, A, Roy-Tirelli, A, Thorens, B (2002). Heterogeneous metabolic adaptation of C57BL/6J mice to high-fat diet. *Am J Physiol Endocrinol Metab* **282:** E834-42.

Cai, H, Li, Z, Davis, ME, Kanner, W, Harrison, DG, Dudley, SC, Jr. (2003). Akt-dependent phosphorylation of serine 1179 and mitogen-activated protein kinase kinase/extracellular signal-regulated kinase 1/2 cooperatively mediate activation of the endothelial nitric-oxide synthase by hydrogen peroxide. *Mol Pharmacol* **63:** 325-31.

Callingham, BA, Holt, A, Elliott, J (1990). Some aspects of the pharmacology of semicarbazide-sensitive amine oxidases. *J Neural Transm Suppl* **32:** 279-90.

Carpéné, C (2009). Amine oxidases in adipose tissue-related disorders. *in: Copper Amine Oxidases: Structures, Catalytic Mechanisms and Role in Pathophysiology* **G. Floris and B. Mondovi Editors:** 177-194.

Carpéné, C, Daviaud, D, Boucher, J, Bour, S, Visentin, V, Gres, S, Duffaut, C, Fontana, E, Testar, X, Saulnier-Blache, JS, Valet, P (2006). Short- and long-term insulin-like

effects of monoamine oxidases and semicarbazide-sensitive amine oxidase substrates in cultured adipocytes. *Metabolism* **55:** 1397-405.

Carpéné, C, Fontana, E, Morin, N, Visentin, V, Prévot, D, Castan, I (2001a). Substrates of semicarbazide-sensitive amine oxidase mimic diverse insulin effects in adipocytes. *Inflammation Research* **50:** 142-143.

Carpéné, C, Morin, N, Fontana, E, Visentin, V, Prévot, D, Marti, L, Lafontan, M (2001b). Histamine weakly stimulate lipolysis and is poorly oxidized by amine oxidases in human subcutaneous fat cells. *Inflammation Research* **50:** S140-S141.

Ceriello, A (2003). The possible role of postprandial hyperglycaemia in the pathogenesis of diabetic complications. *Diabetologia* **46 Suppl 1:** M9-16.

Cioni, L, De Siena, G, Ghelardini, C, Sernissi, O, Alfarano, C, Pirisino, R, Raimondi, L (2006). Activity and expression of semicarbazide-sensitive benzylamine oxidase in a rodent model of diabetes: interactive effects with methylamine and alpha-aminoguanidine. *Eur J Pharmacol* **529:** 179-87.

Conklin, DJ, Cowley, HR, Wiechmann, RJ, Johnson, GH, Trent, MB, Boor, PJ (2004). Vasoactive effects of methylamine in isolated human blood vessels: role of semicarbazide-sensitive amine oxidase, formaldehyde, and hydrogen peroxide. *Am J Physiol Heart Circ Physiol* **286:** H667-76.

Conklin, DJ, Langford, SD, Boor, PJ (1998). Contribution of serum and cellular semicarbazide-sensitive amine oxidase to amine metabolism and cardiovascular toxicity. *Toxicol Sci* **46:** 386-92.

Cosentino, F, Luscher, TF (1998). Endothelial dysfunction in diabetes mellitus. *J Cardiovasc Pharmacol* **32 Suppl 3:** S54-61.

Czech, MP, Lawrence, JC, Jr., Lynn, WS (1974). Hexose transport in isolated brown fat cells. A model system for investigating insulin action on membrane transport. *J Biol Chem* **249:** 5421-7.

del Mar Hernandez, M, Esteban, M, Szabo, P, Boada, M, Unzeta, M (2005). Human plasma semicarbazide sensitive amine oxidase (SSAO), beta-amyloid protein and aging. *Neurosci Lett* **384:** 183-7.

Deng, Y, Boomsma, F, Yu, PH (1998). Deamination of methylamine and aminoacetone increases aldehydes and oxidative stress in rats. *Life Sci* **63:** 2049-58.

Dodt, C, Lonnroth, P, Wellhoner, JP, Fehm, HL, Elam, M (2003). Sympathetic control of white adipose tissue in lean and obese humans. *Acta Physiol Scand* **177:** 351-7.

Dray, C, Knauf, C, Daviaud, D, Waget, A, Boucher, J, Buleon, M, Cani, PD, Attane, C, Guigne, C, Carpéné, C, Burcelin, R, Castan-Laurell, I, Valet, P (2008). Apelin

stimulates glucose utilization in normal and obese insulin-resistant mice. *Cell Metab* **8:** 437-45.

Drouin, A, Thorin-Trescases, N, Hamel, E, Falck, JR, Thorin, E (2007). Endothelial nitric oxide synthase activation leads to dilatory H2O2 production in mouse cerebral arteries. *Cardiovasc Res* **73:** 73-81.

Dullaart, RP, Riemens, SC, Boomsma, F (2006). Plasma semicarbazide-sensitive amine oxidase is moderately decreased by pronounced exogenous hyperinsulinemia but is not associated with insulin sensitivity and body fat. *Scand J Clin Lab Invest* **66:** 559-65.

Dunkel, P, Gelain, A, Barlocco, D, Haider, N, Gyires, K, Sperlagh, B, Magyar, K, Maccioni, E, Fadda, A, Matyus, P (2008). Semicarbazide-sensitive amine oxidase/vascular adhesion protein 1: recent developments concerning substrates and inhibitors of a promising therapeutic target. *Curr Med Chem* **15:** 1827-39.

Ekblom, J, Gronvall, J, Lennernast, B, Nilsson, S, Garpenstrand, H, Oreland, L (1999). Elevated activity of semicarbazide-sensitive amine oxidase in blood from patients with skeletal metastases of prostate cancer. *Clin Sci (Lond)* **97:** 111-5.

El Hadri, K, Moldes, M, Mercier, N, Andreani, M, Pairault, J, Fève, B (2002). Semicarbazide-sensitive amine oxidase in vascular smooth muscle cells: differentiation-dependent expression and role in glucose uptake. *Arterioscler Thromb Vasc Biol* **22:** 89-94.

Enrique-Tarancon, G, Castan, I, Morin, N, Marti, L, Abella, A, Camps, M, Casamitjana, R, Palacin, M, Testar, X, Degerman, E, Carpéné, C, Zorzano, A (2000). Substrates of semicarbazide-sensitive amine oxidase co-operate with vanadate to stimulate tyrosine phosphorylation of insulin-receptor-substrate proteins, phosphoinositide 3-kinase activity and GLUT4 translocation in adipose cells. *Biochem J* **350 Pt 1:** 171-80.

Enrique-Tarancon, G, Marti, L, Morin, N, Lizcano, JM, Unzeta, M, Sevilla, L, Camps, M, Palacin, M, Testar, X, Carpéné, C, Zorzano, A (1998). Role of semicarbazide-sensitive amine oxidase on glucose transport and GLUT4 recruitment to the cell surface in adipose cells. *J Biol Chem* **273:** 8025-32.

Eriksson, JW (2007). Metabolic stress in insulin's target cells leads to ROS accumulation - a hypothetical common pathway causing insulin resistance. *FEBS Lett* **581:** 3734-42.

Exner, M, Hermann, M, Hofbauer, R, Kapiotis, S, Quehenberger, P, Speiser, W, Held, I, Gmeiner, BM (2001). Semicarbazide-sensitive amine oxidase catalyzes endothelial cell-mediated low density lipoprotein oxidation. *Cardiovasc Res* **50:** 583-8.

Ferrer, I, Lizcano, JM, Hernandez, M, Unzeta, M (2002). 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. *Neurosci Lett* **321:** 21-4.

Figueiredo, IV, Martinez Coscolla, A, Cotrim, MD, Caramona, MM, Callingham, BA (1998). Monoamine oxidase activities in human cystic and colonic arteries--influence of age. *J Neural Transm Suppl* **52:** 217-23.

Fitzgerald, DH, Tipton, KF (2002). Inhibition of monoamine oxidase modulates the behaviour of semicarbazide-sensitive amine oxidase (SSAO). *J Neural Transm* **109:** 251-65.

Fontana, E, Boucher, J, Marti, L, Lizcano, JM, Testar, X, Zorzano, A, Carpéné, C (2001). Amine oxidase substrates mimic several of the insulin effects on adipocyte differentiation in 3T3 F442A cells. *Biochem J* **356:** 769-77.

Fredholm, BB, Frisk-Holmberg, M (1971). Lipolysis in canine subcutaneous adipose tissue following release of endogenous histamine. *Eur J Pharmacol* **13:** 254-8.

Gagliardi, AC, Miname, MH, Santos, RD (2009). Uric acid: A marker of increased cardiovascular risk. *Atherosclerosis* **202:** 11-7.

Gallardo-Godoy, A, Hernandez, M, Sanz, E, Unzeta, M (2004). Synthesis of 4-methyl-thio-phenyl-propylamine and the evaluation of its interaction with different amine oxidases. *Bioorg Med Chem* **12:** 273-9.

Galvani, S, Coatrieux, C, Elbaz, M, Grazide, MH, Thiers, JC, Parini, A, Uchida, K, Kamar, N, Rostaing, L, Baltas, M, Salvayre, R, Negre-Salvayre, A (2008). Carbonyl scavenger and antiatherogenic effects of hydrazine derivatives. *Free Radic Biol Med* **45:** 1457-67.

Garcia-Vicente, S (2007). Fisiologia y farmacologia de las formas soluble y de membrana de la proteina SSAO/VAP-1. *PhD Thesis*.

Garcia-Vicente, S, Abella, A, Viguerie, N, Ros-Baro, A, Camps, M, Testar, X, Palacin, M, Zorzano, A, Marti, L (2005). The release of soluble VAP-1/SSAO by 3T3-L1 adipocytes is stimulated by isoproterenol and low concentrations of TNFalpha. *J Physiol Biochem* **61:** 395-401.

Garcia-Vicente, S, Yraola, F, Marti, L, Gonzalez-Munoz, E, Garcia-Barrado, MJ, Canto, C, Abella, A, Bour, S, Artuch, R, Sierra, C, Brandi, N, Carpéné, C, Moratinos, J, Camps, M, Palacin, M, Testar, X, Guma, A, Albericio, F, Royo, M, Mian, A, Zorzano, A (2007). Oral insulin-mimetic compounds that act independently of insulin. *Diabetes* **56:** 486-93.

Garpenstrand, H, Ekblom, J, Backlund, LB, Oreland, L, Rosenqvist, U (1999). Elevated plasma semicarbazide-sensitive amine oxidase (SSAO) activity in Type 2 diabetes mellitus complicated by retinopathy. *Diabet Med* **16:** 514-21.

Göktürk, C, Garpenstrand, H, Nilsson, J, Nordquist, J, Oreland, L, Forsberg-Nilsson, K (2003). Studies on semicarbazide-sensitive amine oxidase in patients with diabetes mellitus and in transgenic mice. *Biochim Biophys Acta* **1647:** 88-91.

Gronvall-Nordquist, JL, Backlund, LB, Garpenstrand, H, Ekblom, J, Landin, B, Yu, PH, Oreland, L, Rosenqvist, U (2001). Follow-up of plasma semicarbazide-sensitive amine oxidase activity and retinopathy in Type 2 diabetes mellitus. *J Diabetes Complications* **15:** 250-6.

Gubisne-Haberle, D, Hill, W, Kazachkov, M, Richardson, JS, Yu, PH (2004). Protein cross-linkage induced by formaldehyde derived from semicarbazide-sensitive amine oxidase-mediated deamination of methylamine. *J Pharmacol Exp Ther* **310:** 1125-32.

Hayes, BE, Clarke, DE (1990). Semicarbazide-sensitive amine oxidase activity in streptozotocin diabetic rats. *Res Commun Chem Pathol Pharmacol* **69:** 71-83.

Hayes, GR, Lockwood, DH (1987). Role of insulin receptor phosphorylation in the insulinomimetic effects of hydrogen peroxide. *Proc Natl Acad Sci U S A* **84:** 8115-9.

Head, J, Fuller, JH (1990). International variations in mortality among diabetic patients: the WHO Multinational Study of Vascular Disease in Diabetics. *Diabetologia* **33:** 477-81.

Hink, U, Li, H, Mollnau, H, Oelze, M, Matheis, E, Hartmann, M, Skatchkov, M, Thaiss, F, Stahl, RA, Warnholtz, A, Meinertz, T, Griendling, K, Harrison, DG, Forstermann, U, Munzel, T (2001). Mechanisms underlying endothelial dysfunction in diabetes mellitus. *Circ Res* **88:** E14-22.

Holt, A (2009). Membrane-bound copper amine-oxidases. *in: Copper Amine Oxidases: Structures, Catalytic Mechanisms and Role in Pathophysiology* **G. Floris and B. Mondovi Editors:** 69-86.

Holt, A, Smith, DJ, Cendron, L, Zanotti, G, Rigo, A, Di Paolo, ML (2008). Multiple binding sites for substrates and modulators of semicarbazide-sensitive amine oxidases: kinetic consequences. *Mol Pharmacol* **73:** 525-38.

Honing, ML, Morrison, PJ, Banga, JD, Stroes, ES, Rabelink, TJ (1998). Nitric oxide availability in diabetes mellitus. *Diabetes Metab Rev* **14:** 241-9.

Iffiú-Soltész, Z, Prévot, D, Carpéné, C (2009). Influence of prolonged fasting on monoamine oxidase and semicarbazide-sensitive amine oxidase activities in rat white adipose tissue. *J Physiol Biochem* **65:** 11-23.

Iffiú-Soltész, Z, Prévot, D, Gres, S, Bour, S, Szökő, E, Knauf, C, Burcelin, R, Fernandez-Quintela, A, Lomba, A, Milagro, FI, Carpéné, C (2007). Influence of acute and chronic administration of benzylamine on glucose tolerance in diabetic and obese mice fed on very high-fat diet. *J Physiol Biochem* **63:** 305-15.

Iglesias-Osma, MC, Bour, S, Garcia-Barrado, MJ, Visentin, V, Pastor, MF, Testar, X, Marti, L, Enrique-Tarancon, G, Valet, P, Moratinos, J, Carpéné, C (2005). Methylamine but not mafenide mimics insulin-like activity of the semicarbazide-sensitive amine oxidase-substrate benzylamine on glucose tolerance and on human adipocyte metabolism. *Pharmacol Res* **52:** 475-84.

Iglesias-Osma, MC, Garcia-Barrado, MJ, Visentin, V, Pastor-Mansilla, MF, Bour, S, Prévot, D, Valet, P, Moratinos, J, Carpéné, C (2004). Benzylamine exhibits insulin-like effects on glucose disposal, glucose transport, and fat cell lipolysis in rabbits and diabetic mice. *J Pharmacol Exp Ther* **309:** 1020-8.

Ignarro, LJ, Fukuto, JM, Griscavage, JM, Rogers, NE, Byrns, RE (1993). Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. *Proc Natl Acad Sci U S A* **90:** 8103-7.

Imamura, Y, Kubota, R, Wang, Y, S., A, Kudoh, J, Mashima, Y, Oguchi, Y, Shimizu, N (1997) Human retina-specific amine oxidase (RAO): cDNA cloning, tissue expression, and chromosomal mapping. *Genomics* **40:** 277-283.

Jaakkola, K, Kaunismaki, K, Tohka, S, Yegutkin, G, Vanttinen, E, Havia, T, Pelliniemi, LJ, Virolainen, M, Jalkanen, S, Salmi, M (1999). Human vascular adhesion protein-1 in smooth muscle cells. *Am J Pathol* **155:** 1953-65.

Jakobsson, E, Nilsson, J, Ogg, D, Kleywegt, GJ (2005). Structure of human semicarbazide-sensitive amine oxidase/vascular adhesion protein-1. *Acta Crystallogr D Biol Crystallogr* **61:** 1550-62.

Jalkanen, S, Salmi, M (2001). Cell surface monoamine oxidases: enzymes in search of a function. *EMBO J* **20:** 3893-901.

Janes, SM, Mu, D, Wemmer, D, Smith, AJ, Kaur, S, Maltby, D, Burlingame, AL, Klinman, JP (1990). A new redox cofactor in eukaryotic enzymes: 6-hydroxydopa at the active site of bovine serum amine oxidase. *Science* **248:** 981-987.

Kar, A, Choudhary, BK, Bandyopadhyay, NG (2003). Comparative evaluation of hypoglycaemic activity of some Indian medicinal plants in alloxan diabetic rats. *J Ethnopharmacol* **84:** 105-8.

Kaitaniemi, S, Elovaara, H, Gron, K, Kidron, H, Liukkonen, J, Salminen, T, Salmi, M, Jalkanen, S, Elima, K (2009) The unique substrate specificity of human AOC2, a semicarbazide-sensitive amine oxidase. *Cell Mol Life Sci* **66**(16)**:** 2743-2757.

Kim, S, Moustaid-Moussa, N (2000). Secretory, endocrine and autocrine/paracrine function of the adipocyte. *J Nutr* **130:** 3110S-3115S.

Klinman, JP (1996). New quinocofactors in eukaryotes. *J Biol Chem* **271:** 27189-92.

Klinman, JP, Mu, D (1994). Quinoenzymes in biology. *Annu Rev Biochem* **63:** 299-344.

Kobayashi, T, Kamata, K (2001). Effect of chronic insulin treatment on NO production and endothelium-dependent relaxation in aortae from established STZ-induced diabetic rats. *Atherosclerosis* **155:** 313-20.

Krieger-Brauer, HI, Medda, PK, Kather, H (1997). Insulin-induced activation of NADPH-dependant H_2O_2 generation in human adipocytes plasma membranes in mediated by Gαi2. *The Journal of Biological Chemistry* **272:** 10135-10143.

Kurkijarvi, R, Adams, DH, Leino, R, Mottonen, T, Jalkanen, S, Salmi, M (1998). Circulating form of human vascular adhesion protein-1 (VAP-1): increased serum levels in inflammatory liver diseases. *J Immunol* **161:** 1549-57.

Langford, SD, Trent, MB, Balakumaran, A, Boor, PJ (1999). Developmental vasculotoxicity associated with inhibition of semicarbazide-sensitive amine oxidase. *Toxicol Appl Pharmacol* **155:** 237-44.

Langford, SD, Trent, MB, Boor, PJ (2002). Semicarbazide-sensitive amine oxidase and extracellular matrix deposition by smooth-muscle cells. *Cardiovasc Toxicol* **2:** 141-50.

Lauer, N, Suvorava, T, Ruther, U, Jacob, R, Meyer, W, Harrison, DG, Kojda, G (2005). Critical involvement of hydrogen peroxide in exercise-induced up-regulation of endothelial NO synthase. *Cardiovasc Res* **65:** 254-62.

Lewinsohn, R (1984). Mammalian monoamine-oxidizing enzymes, with special reference to benzylamine oxidase in human tissues. *Braz J Med Biol Res* **17:** 223-56.

Li, H, Luo, W, Lin, J, Lin, Z, Zhang, Y (2004). Assay of plasma semicarbazide-sensitive amine oxidase and determination of its endogenous substrate methylamine by liquid chromatography. *J Chromatogr B Analyt Technol Biomed Life Sci* **810:** 277-82.

Li, HY, Lee, WJ, Chen, MJ, Chuang, LM (2005). Change in vascular adhesion protein-1 and metabolic phenotypes after vertical banded gastroplasty for morbid obesity. *Obes Res* **13:** 855-61.

Li, HY, Lin, MS, Wei, JN, Hung, CS, Chiang, FT, Lin, CH, Hsu, HC, Su, CY, Wu, MY, Smith, DJ, Vainio, J, Chen, MF, Chuang, LM (2009a). Change of serum vascular adhesion protein-1 after glucose loading correlates to carotid intima-medial thickness in non-diabetic subjects. *Clin Chim Acta* **403:** 97-101.

Li, HY, Wei, JN, Lin, MS, Smith, DJ, Vainio, J, Lin, CH, Chiang, FT, Shih, SR, Huang, CH, Wu, MY, Hsein, YC, Chuang, LM (2009b). Serum vascular adhesion protein-1 is increased in acute and chronic hyperglycemia. *Clin Chim Acta* **404:** 149-53.

Lizcano, JM, Tipton, KF, Unzeta, M (1998). Purification and characterization of membrane-bound semicarbazide-sensitive amine oxidase (SSAO) from bovine lung. *Biochem J* **331 (Pt 1):** 69-78.

Lunelli, M, Di Paolo, ML, Biadene, M, Calderone, V, Battistutta, R, Scarpa, M, Rigo, A, Zanotti, G (2005). Crystal structure of amine oxidase from bovine serum. *J Mol Biol* **346:** 991-1004.

Lyles, GA (1996). Mammalian plasma and tissue-bound semicarbazide-sensitive amine oxidases: biochemical, pharmacological and toxicological aspects. *Int J Biochem Cell Biol* **28:** 259-74.

Madej, A, Reich, A, Orda, A, Szepietowski, JC (2006). Expression of vascular adhesion protein-1 in atopic eczema. *Int Arch Allergy Immunol* **139:** 114-21.

Madej, A, Reich, A, Orda, A, Szepietowski, JC (2007). Vascular adhesion protein-1 (VAP-1) is overexpressed in psoriatic patients. *J Eur Acad Dermatol Venereol* **21:** 72-8.

Marti, L, Abella, A, Carpéné, C, Palacin, M, Testar, X, Zorzano, A (2001). Combined treatment with benzylamine and low dosages of vanadate enhances glucose tolerance and reduces hyperglycemia in streptozotocin-induced diabetic rats. *Diabetes* **50:** 2061-8.

Marti, L, Abella, A, De La Cruz, X, Garcia-Vicente, S, Unzeta, M, Carpéné, C, Palacin, M, Testar, X, Orozco, M, Zorzano, A (2004). Exploring the binding mode of semicarbazide-sensitive amine oxidase/VAP-1: identification of novel substrates with insulin-like activity. *J Med Chem* **47:** 4865-74.

Marti, L, Morin, N, Enrique-Tarancon, G, Prévot, D, Lafontan, M, Testar, X, Zorzano, A, Carpéné, C (1998). Tyramine and vanadate synergistically stimulate glucose transport in rat adipocytes by amine oxidase-dependent generation of hydrogen peroxide. *J Pharmacol Exp Ther* **285:** 342-9.

Mathys, KC, Ponnampalam, SN, Padival, S, Nagaraj, RH (2002). Semicarbazide-sensitive amine oxidase in aortic smooth muscle cells mediates synthesis of a methylglyoxal-AGE: implications for vascular complications in diabetes. *Biochem Biophys Res Commun* **297:** 863-9.

Matoba, T, Shimokawa, H, Nakashima, M, Hirakawa, Y, Mukai, Y, Hirano, K, Kanaide, H, Takeshita, A (2000). Hydrogen peroxide is an endothelium-derived hyperpolarizing factor in mice. *J Clin Invest* **106:** 1521-30.

Matyus, P, Dajka-Halász, B, Földi, A, Haider, N, Barlocco, D, Magyar, K (2004). Semicarbazide-sensitive amine oxidase: current status and perspectives. *Curr Med Chem* **11:** 1285-98.

May, JM, de Haen, C (1979). The insulin-like effect of hydrogen peroxide on pathways of lipid synthesis in rat adipocytes. *J Biol Chem* **254:** 9017-21.

McNab, G, Reeves, JL, Salmi, M, Hubscher, S, Jalkanen, S, Adams, DH (1996). Vascular adhesion protein 1 mediates binding of T cells to human hepatic endothelium. *Gastroenterology* **110:** 522-8.

Mercier, N, El Hadri, K, Osborne-Pellegrin, M, Nehme, J, Perret, C, Labat, C, Regnault, V, Lamaziere, JM, Challande, P, Lacolley, P, Fève, B (2007). Modifications of arterial phenotype in response to amine oxidase inhibition by semicarbazide. *Hypertension* **50:** 234-41.

Mercier, N, Moldes, M, El Hadri, K, Fève, B (2003). Regulation of semicarbazide-sensitive amine oxidase expression by tumor necrosis factor-alpha in adipocytes: functional consequences on glucose transport. *J Pharmacol Exp Ther* **304:** 1197-208.

Mercier, N, Moldes, M, El Hadri, K, Fève, B (2001). Semicarbazide-sensitive amine oxidase activation promotes adipose conversion of 3T3-L1 cells. *Biochem J* **358:** 335-42.

Mercier, N, Osborne-Pellegrin, M, El Hadri, K, Kakou, A, Labat, C, Loufrani, L, Henrion, D, Challande, P, Jalkanen, S, Fève, B, Lacolley, P (2006). Carotid arterial stiffness, elastic fibre network and vasoreactivity in semicarbazide-sensitive amine-oxidase null mouse. *Cardiovasc Res* **72:** 349-57.

Mészáros, Z, Karádi, I, Csanyi, A, Szombathy, T, Romics, L, Magyar, K (1999a). Determination of human serum semicarbazide-sensitive amine oxidase activity: a possible clinical marker of atherosclerosis. *Eur J Drug Metab Pharmacokinet* **24:** 299-302.

Mészáros, Z, Szombathy, T, Raimondi, L, Karádi, I, Romics, L, Magyar, K (1999b). Elevated serum semicarbazide-sensitive amine oxidase activity in non-insulin-dependent diabetes mellitus: correlation with body mass index and serum triglyceride. *Metabolism* **48:** 113-7.

Miike, T, Kunishiro, K, Kanda, M, Azukizawa, S, Kurahashi, K, Shirahase, H (2008). Impairment of endothelium-dependent ACh-induced relaxation in aorta of diabetic db/db mice--possible dysfunction of receptor and/or receptor-G protein coupling. *Naunyn Schmiedebergs Arch Pharmacol* **377:** 401-10.

Mitchell, SC, Zhang, AQ (2001). Methylamine in human urine. *Clin Chim Acta* **312:** 107-14.

Moldes, M, Fève, B, Pairault, J (1999). Molecular cloning of a major mRNA species in murine 3T3 adipocyte lineage. differentiation-dependent expression, regulation, and identification as semicarbazide-sensitive amine oxidase. *J Biol Chem* **274:** 9515-23.

Monnier, VM, Sell, DR, Nagaraj, RH, Miyata, S, Grandhee, S, Odetti, P, Ibrahim, SA (1992). Maillard reaction-mediated molecular damage to extracellular matrix and other tissue proteins in diabetes, aging, and uremia. *Diabetes* **41 Suppl 2:** 36-41.

Moody, AJ, Stan, MA, Stan, M, Gliemann, J (1974). A simple free fat cell bioassay for insulin. *Horm Metab Res* **6:** 12-6.

Morin, N, Lizcano, JM, Fontana, E, Marti, L, Smih, F, Rouet, P, Prévot, D, Zorzano, A, Unzeta, M, Carpéné, C (2001). Semicarbazide-sensitive amine oxidase substrates stimulate glucose transport and inhibit lipolysis in human adipocytes. *J Pharmacol Exp Ther* **297:** 563-72.

Morin, N, Visentin, V, Calise, D, Marti, L, Zorzano, A, Testar, X, Valet, P, Fischer, Y, Carpéné, C (2002). Tyramine stimulates glucose uptake in insulin-sensitive tissues in vitro and in vivo via its oxidation by amine oxidases. *J Pharmacol Exp Ther* **303:** 1238-47.

Morris, NJ, Ducret, A, Aebersold, R, Ross, SA, Keller, SR, Lienhard, GE (1997). Membrane amine oxidase cloning and identification as a major protein in the adipocyte plasma membrane. *J. Biol. Chem.* **272:** 9388-9392.

Moxham, CM, Malbon, CC (1996). Insulin action impaired by deficiency of the G-protein subunit $G_{i\alpha 2}$. *Nature* **379:** 840-844.

Mu, D, Janes, SM, Smith, AJ, Brown, DE, Dooley, DM, Klinman, JP (1992). Tyrosine codon corresponds to topa quinone at the active site of copper amine oxidases. *J. Biol. Chem.* **267:** 7979-7982.

Mukherjee, SP, Lynn, WS (1977). Reduced nicotinamide adenine dinucleotide phosphate oxidase in adipocyte plasma membrane and its activation by insulin. Possible role in the hormone's effects on adenylate cyclase and the hexose monophosphate shunt. *Arch Biochem Biophys* **184:** 69-76.

Nishikawa, T, Edelstein, D, Du, XL, Yamagishi, S, Matsumura, T, Kaneda, Y, Yorek, MA, Beebe, D, Oates, PJ, Hammes, HP, Giardino, I, Brownlee, M (2000). Normalizing mitochondrial superoxide production blocks three pathways of hyperglycaemic damage. *Nature* **404:** 787-90.

O'Sullivan, J, O'Sullivan, M, Tipton, KF, Unzeta, M, Del Mar Hernandez, M, Davey, GP (2003). The inhibition of semicarbazide-sensitive amine oxidase by aminohexoses. *Biochim Biophys Acta* **1647:** 367-71.

O'Sullivan, J, Unzeta, M, Healy, J, O'Sullivan, MI, Davey, G, Tipton, KF (2004). Semicarbazide-sensitive amine oxidases: enzymes with quite a lot to do. *Neurotoxicology* **25:** 303-15.

O'Sullivan, M, Tipton, KF, McDevitt, WE (2002). Immunolocalization of semicarbazide-sensitive amine oxidase in human dental pulp and its activity towards serotonin. *Arch Oral Biol* **47:** 399-406.

Obata, T (2006). Diabetes and semicarbazide-sensitive amine oxidase (SSAO) activity: a review. *Life Sci* **79:** 417-22.

Ochiai, Y, Itoh, K, Sakurai, E, Tanaka, Y (2005). Molecular cloning and characterization of rat semicarbazide-sensitive amine oxidase. *Biol Pharm Bull* **28:** 413-8.

Olive, M, Unzeta, M, Moreno, D, Ferrer, I (2004). Overexpression of semicarbazide-sensitive amine oxidase in human myopathies. *Muscle Nerve* **29:** 261-6.

Pacher, P, Obrosova, IG, Mabley, JG, Szabo, C (2005). Role of nitrosative stress and peroxynitrite in the pathogenesis of diabetic complications. Emerging new therapeutical strategies. *Curr Med Chem* **12:** 267-75.

Pirisino, R, Ghelardini, C, Banchelli, G, Galeotti, N, Raimondi, L (2001). Methylamine and benzylamine induced hypophagia in mice: modulation by semicarbazide-sensitive benzylamine oxidase inhibitors and aODN towards Kv1.1 channels. *Br J Pharmacol* **134:** 880-6.

Pittenger, MF, Mackay, AM, Beck, SC, Jaiswal, RK, Douglas, R, Mosca, JD, Moorman, MA, Simonetti, DW, Craig, S, Marshak, DR (1999). Multilineage potential of adult human mesenchymal stem cells. *Science* **284:** 143-7.

Poortmans, JR, Kumps, A, Duez, P, Fofonka, A, Carpentier, A, Francaux, M (2005). Effect of oral creatine supplementation on urinary methylamine, formaldehyde, and formate. *Med Sci Sports Exerc* **37:** 1717-20.

Prévot, D, Soltész, Z, Abello, V, Wanecq, E, Valet, P, Unzeta, M, Carpéné, C (2007). Prolonged treatment with aminoguanidine strongly inhibits adipocyte semicarbazide-sensitive amine oxidase and slightly reduces fat deposition in obese Zucker rats. *Pharmacol Res* **56:** 70-9.

Raimondi, L, Banchelli, G, Sgromo, L, Pirisino, R (2000). Possible different fates for the hydrogen peroxide produced by rat white adipocyte amine oxidases. *Neurobiology (Bp)* **8:** 99-107.

Raimondi, L, Pirisino, R, Banchelli, G, Ignesti, G, Conforti, L, Buffoni, F (1990). Cultured preadipocytes produce a semicarbazide-sensitive amine oxidase (SSAO) activity. *J Neural Transm Suppl* **32:** 331-6.

Raimondi, L, Pirisino, R, Ignesti, G, Capecchi, S, Banchelli, G, Buffoni, F (1991). Semicarbazide-sensitive amine oxidase activity (SSAO) of rat epididymal white adipose tissue. *Biochem Pharmacol* **41:** 467-70.

Roessner, V, Weber, A, Becker, A, Beck, G, Frieling, H, Bleich, S (2007). Decreased serum activity of semicarbazide-sensitive amine oxidase (SSAO) in patients treated with second generation antipsychotics: a link to impaired glucose metabolism? *Eur J Clin Pharmacol* **63:** 425-9.

Roessner, V, Weber, A, Becker, A, Beck, G, Kornhuber, J, Frieling, H, Bleich, S (2006). Decreased serum semicarbazide sensitive aminooxidase (SSAO) activity in

patients with major depression. *Prog Neuropsychopharmacol Biol Psychiatry* **30:** 906-9.

Ruderman, NB, Williamson, JR, Brownlee, M (1992). Glucose and diabetic vascular disease. *Faseb J* **6:** 2905-14.

Salmi, M, Jalkanen, S (1997a). How do lymphocytes know where to go: current concepts and enigmas of lymphocyte homing. *Adv Immunol* **64:** 139-218.

Salmi, M, Jalkanen, S (2001a). VAP-1: an adhesin and an enzyme. *Trends Immunol* **22:** 211-6.

Salmi, M, Kalimo, K, Jalkanen, S (1993). Induction and function of vascular adhesion protein-1 at sites of inflammation. *J. Exp. Med.* **178:** 2255-2260.

Salmi, M, Stolen, C, Jousilahti, P, Yegutkin, GG, Tapanainen, P, Janatuinen, T, Knip, M, Jalkanen, S, Salomaa, V (2002). Insulin-regulated increase of soluble vascular adhesion protein-1 in diabetes. *Am J Pathol* **161:** 2255-62.

Salmi, M, Tohka, S, Berg, EL, Butcher, EC, Jalkanen, S (1997b). Vascular adhesion protein 1 (VAP-1) mediates lymphocyte subtype-specific, selectin-independent recognition of vascular endothelium in human lymph nodes. *J Exp Med* **186:** 589-600.

Salmi, M, Yegutkin, GG, Lehvonen, R, Koskinen, K, Salminen, T, Jalkanen, S (2001b). A cell surface amine oxidase directly controls lymphocyte migration. *Immunity* **14:** 265-76.

Santilli, F, Cipollone, F, Mezzetti, A, Chiarelli, F (2004). The role of nitric oxide in the development of diabetic angiopathy. *Horm Metab Res* **36:** 319-35.

Schwelberger, HG (2006). Origins of plasma amine oxidases in different mammalian species. *Inflamm Res* **55 Suppl 1:** S57-8.

Schwelberger, HG, Feurle, J (2008). Characterization of human plasma amine oxidase. *Inflamm. Res.* **57 Suppl 1:** S51-52.

Seiler, N (1995). Polyamine oxidase, properties and functions. *Prog.Brain.Res.* **106:** 333-344.

Sheetz, MJ, King, GL (2002). Molecular understanding of hyperglycemia's adverse effects for diabetic complications. *Jama* **288:** 2579-88.

Shih, JC, Chen, K, Ridd, MJ (1999). Monoamine oxidase: from genes to behavior. *Annu. Rev. Neurosci.* **22:** 197-217.

Sibon, I, Larrieu, D, El Hadri, K, Mercier, N, Fève, B, Lacolley, P, Labat, C, Daret, D, Bonnet, J, Lamaziere, JM (2004). Semicarbazide-sensitive amine oxidase in annulo-

aortic ectasia disease: relation to elastic lamellae-associated proteins. *J Histochem Cytochem* **52:** 1459-66.

Smith, DJ, Salmi, M, Bono, P, Helmann, J, Leu, T, Jalkanen, S (1998). Cloning of vascular adhesion protein-1 reveals a novel multifonctional adhesion molecule. *Journal of Experimental Medicine* **188:** 17-27.

Smith-Mungo, LI, Kagan, HM (1998). Lysyl oxidase: Properties, regulation and multiple fonctions in biology. *Matrix Biology* **16:** 387-398.

Soltész, Z, Tábi, T, Halász, AS, Pálfi, M, Kocsis, E, Magyar, K, Toth, M, Szökő, E (2007). Studies on the insulinomimetic effects of benzylamine, exogenous substrate of semicarbazide-sensitive amine oxidase enzyme in streptozotocin induced diabetic rats. *J Neural Transm* **114:** 851-5.

Soriano, FG, Virag, L, Szabo, C (2001). Diabetic endothelial dysfunction: role of reactive oxygen and nitrogen species production and poly(ADP-ribose) polymerase activation. *J Mol Med* **79:** 437-48.

Souto, RP, Vallega, G, Wharton, J, Vinten, J, Tranum-Jensen, J, Pilch, PF (2003). Immunopurification and characterization of rat adipocyte caveolae suggest their dissociation from insulin signaling. *J Biol Chem* **278:** 18321-9.

Srinivasan, S, Hatley, ME, Bolick, DT, Palmer, LA, Edelstein, D, Brownlee, M, Hedrick, CC (2004). Hyperglycaemia-induced superoxide production decreases eNOS expression via AP-1 activation in aortic endothelial cells. *Diabetologia* **47:** 1727-34.

Stadler, K, Jenei, V, von Bolcshazy, G, Somogyi, A, Jakus, J (2003). Increased nitric oxide levels as an early sign of premature aging in diabetes. *Free Radic Biol Med* **35:** 1240-51.

Stolen, CM, Madanat, R, Marti, L, Kari, S, Yegutkin, GG, Sariola, H, Zorzano, A, Jalkanen, S (2004a). Semicarbazide sensitive amine oxidase overexpression has dual consequences: insulin mimicry and diabetes-like complications. *Faseb J* **18:** 702-4.

Stolen, CM, Yegutkin, GG, Kurkijarvi, R, Bono, P, Alitalo, K, Jalkanen, S (2004b). Origins of serum semicarbazide-sensitive amine oxidase. *Circ Res* **95:** 50-7.

Subra, C, Fontana, E, Visentin, V, Testar, X, Carpéné, C (2003). Tyramine and benzylamine partially but selectively mimic insulin action on adipose differentiation in 3T3-L1 cells. *J Physiol Biochem* **59:** 209-16.

Szökő, E, Tábi, T, Halász, AS, Pálfi, M, Magyar, K (2004). High sensitivity analysis of nitrite and nitrate in biological samples by capillary zone electrophoresis with transient isotachophoretic sample stacking. *J Chromatogr A* **1051:** 177-83.

Tábi, T, Soltész, Z, Magyar, K, Szökő, E (2006). [Study on the altered nitric oxide metabolism in experimental diabetes]. *Acta Pharm Hung* **76:** 19-23.

Thomas, SR, Chen, K, Keaney, JF, Jr. (2002). Hydrogen peroxide activates endothelial nitric-oxide synthase through coordinated phosphorylation and dephosphorylation via a phosphoinositide 3-kinase-dependent signaling pathway. *J Biol Chem* **277:** 6017-24.

Tipton, KF, Boyce, S, O'Sullivan, J, Davey, GP, Healy, J (2004). Monoamine oxidases: certainties and uncertainties. *Curr Med Chem* **11:** 1965-82.

Tohka, S, Laukkanen, M, Jalkanen, S, Salmi, M (2001). Vascular adhesion protein 1 (VAP-1) functions as a molecular brake during granulocyte rolling and mediates recruitment in vivo. *Faseb J* **15:** 373-82.

van der Vliet, A, Eiserich, JP, O'Neill, CA, Halliwell, B, Cross, CE (1995). Tyrosine modification by reactive nitrogen species: a closer look. *Arch Biochem Biophys* **319:** 341-9.

Vazquez-Vela, ME, Torres, N, Tovar, AR (2008). White adipose tissue as endocrine organ and its role in obesity. *Arch Med Res* **39:** 715-28.

Vidrio, H (2003). Semicarbazide-sensitive amine oxidase: role in the vasculature and vasodilation after in situ inhibition. *Auton Autacoid Pharmacol* **23:** 275-83.

Vidrio, H, Medina, M, Gonzalez-Romo, P, Lorenzana-Jimenez, M, Diaz-Arista, P, Baeza, A (2003). Semicarbazide-sensitive amine oxidase substrates potentiate hydralazine hypotension: possible role of hydrogen peroxide. *J Pharmacol Exp Ther* **307:** 497-504.

Visentin, V, Boucher, J, Bour, S, Prévot, D, Castan, I, Carpéné, C, Valet, P (2005). Influence of high-fat diet on amine oxidase activity in white adipose tissue of mice prone or resistant to diet-induced obesity. *J Physiol Biochem* **61:** 343-52.

Visentin, V, Marq, P, Bour, S, Subra, C, Prévot, D, Morin, N, Valet, P, Monje, MC, Nepveu, F, Carpéné, C (2003a). Effect of prolonged treatment with tyramine on glucose tolerance in streptozotocin-induced diabetic rats. *J Physiol Biochem* **59:** 225-32.

Visentin, V, Morin, N, Fontana, E, Prévot, D, Boucher, J, Castan, I, Valet, P, Grujic, D, Carpéné, C (2001). Dual action of octopamine on glucose transport into adipocytes: inhibition via beta3-adrenoceptor activation and stimulation via oxidation by amine oxidases. *J Pharmacol Exp Ther* **299:** 96-104.

Visentin, V, Prévot, D, De Saint Front, VD, Morin-Cussac, N, Thalamas, C, Galitzky, J, Valet, P, Zorzano, A, Carpéné, C (2004). Alteration of amine oxidase activity in the adipose tissue of obese subjects. *Obes Res* **12:** 547-55.

Visentin, V, Prévot, D, Marti, L, Carpéné, C (2003b). Inhibition of rat fat cell lipolysis by monoamine oxidase and semicarbazide-sensitive amine oxidase substrates. *European Journal of Pharmacology* **466:** 235-243.

Wanecq, E, Bour, S, Verwaerde, P, Smih, F, Valet, P, Carpéné, C (2006). Increased monoamine oxidase and semicarbazide-sensitive amine oxidase activities in white adipose tissue of obese dogs fed a high-fat diet. *J Physiol Biochem* **62:** 113-23.

Weiss, HG, Klocker, J, Labeck, B, Nehoda, H, Aigner, F, Klingler, A, Ebenbichler, C, Foger, B, Lechleitner, M, Patsch, JR, Schwelberger, HG (2003). Plasma amine oxidase: a postulated cardiovascular risk factor in nondiabetic obese patients. *Metabolism* **52:** 688-92.

Wu, JT, Tu, MC, Zhung, P (1996). Advanced glycation end product (AGE): characterization of the products from the reaction between D-glucose and serum albumin. *J Clin Lab Anal* **10:** 21-34.

Xiao, S, Yu, PH (2009). A fluorometric high-performance liquid chromatography procedure for simultaneous determination of methylamine and aminoacetone in blood and tissues. *Anal Biochem* **384:** 20-6.

Xu, J, Li, G, Wang, P, Velazquez, H, Yao, X, Li, Y, Wu, Y, Peixoto, A, Crowley, S, Desir, GV (2005). Renalase is a novel, soluble monoamine oxidase that may regulate cardiac function and blood pressure. *J. Clin. Invest.* **115:** 1275-1280.

Yoo, TW, Sung, KC, Shin, HS, Kim, BJ, Kim, BS, Kang, JH, Lee, MH, Park, JR, Kim, H, Rhee, EJ, Lee, WY, Kim, SW, Ryu, SH, Keum, DG (2005). Relationship between serum uric acid concentration and insulin resistance and metabolic syndrome. *Circ J* **69:** 928-33.

Yraola, F, Garcia-Vicente, S, Fernandez-Recio, J, Albericio, F, Zorzano, A, Marti, L, Royo, M (2006). New efficient substrates for semicarbazide-sensitive amine oxidase/VAP-1 enzyme: analysis by SARs and computational docking. *J Med Chem* **49:** 6197-208.

Yraola, F, Garcia-Vicente, S, Marti, L, Albericio, F, Zorzano, A, Royo, M (2007). Understanding the mechanism of action of the novel SSAO substrate (C7NH10)6(V10O28).2H2O, a prodrug of peroxovanadate insulin mimetics. *Chem Biol Drug Des* **69:** 423-8.

Yraola, F, Zorzano, A, Albericio, F, Royo, M (2009). Structure-activity relationships of SSAO/VAP-1 arylalkylamine-based substrates. *ChemMedChem* **4:** 495-503.

Yu, PH (1998a). Deamination of methylamine and angiopathy; toxicity of formaldehyde, oxidative stress and relevance to protein glycoxidation in diabetes. *J Neural Transm Suppl* **52:** 201-16.

Yu, PH (1998b). Increase of formation of methylamine and formaldehyde in vivo after administration of nicotine and the potential cytotoxicity. *Neurochem Res* **23:** 1205-10.

Yu, PH, Cauglin, C, Wempe, KL, Gubisne-Haberle, D (2003a). A novel sensitive high-performance liquid chromatography/electrochemical procedure for measuring

formaldehyde produced from oxidative deamination of methylamine and in biological samples. *Anal Biochem* **318:** 285-90.

Yu, PH, Deng, YL (1998). Endogenous formaldehyde as a potential factor of vulnerability of atherosclerosis: involvement of semicarbazide-sensitive amine oxidase-mediated methylamine turnover. *Atherosclerosis* **140:** 357-63.

Yu, PH, Fang, CY, Yang, CM (1992). Semicarbazide-sensitive amine oxidase from the smooth muscles of dog aorta and trachea: activation by the MAO-A inhibitor clorgyline. *J Pharm Pharmacol* **44:** 981-5.

Yu, PH, Lai, CT, Zuo, DM (1997). Formation of formaldehyde from adrenaline in vivo; a potential risk factor for stress-related angiopathy. *Neurochem Res* **22:** 615-20.

Yu, PH, Wang, M, Fan, H, Deng, Y, Gubisne-Haberle, D (2004). Involvement of SSAO-mediated deamination in adipose glucose transport and weight gain in obese diabetic KKAy mice. *Am J Physiol Endocrinol Metab* **286:** E634-41.

Yu, PH, Wright, S, Fan, EH, Lun, ZR, Gubisne-Harberle, D (2003b). Physiological and pathological implications of semicarbazide-sensitive amine oxidase. *Biochim Biophys Acta* **1647:** 193-9.

Yu, PH, Zuo, DM (1993). Oxidative deamination of methylamine by semicarbazide-sensitive amine oxidase leads to cytotoxic damage in endothelial cells. Possible consequences for diabetes. *Diabetes* **42:** 594-603.

Zhang, X, McIntire, WS (1996). Cloning and sequencing of a copper-containing, topa quinone-containing monoamine oxidase from human placenta. *Gene* **179:** 279-286.

Zorzano, A, Abella, A, Marti, L, Carpéné, C, Palacin, M, Testar, X (2003). Semicarbazide-sensitive amine oxidase activity exerts insulin-like effects on glucose metabolism and insulin-signaling pathways in adipose cells. *Biochim Biophys Acta* **1647:** 3-9.

10. List of publications

Publications related to Thesis work

Iffiú-Soltész, Z, Prévot, D, Carpéné, C (2009). Influence of prolonged fasting on monoamine oxidase and semicarbazide-sensitive amine oxidase activities in rat white adipose tissue. *J Physiol Biochem* **65:** 11-23.

Iffiú-Soltész, Z, Prévot, D, Grès, S, Bour, S, Szökő, E, Knauf, C, Burcelin, R, Fernandez-Quintela, A, Lomba, A, Milagro, FI, Carpéné, C (2007). Influence of acute and chronic administration of benzylamine on glucose tolerance in diabetic and obese mice fed on very high-fat diet. *J Physiol Biochem* **63:** 305-15.

Soltész, Z, Tábi, T, Halász, AS, Pálfi, M, Kocsis, E, Magyar, K, Tóth, M, Szökő, E (2007). Studies on the insulinomimetic effects of benzylamine, exogenous substrate of semicarbazide-sensitive amine oxidase enzyme in streptozotocin induced diabetic rats. *J Neural Transm* **114:** 851-5.

Publications not related to Thesis work

Bour, S, Caspar-Bauguil, S, Iffiú-Soltész, Z, Nibbelink, M, Cousin, B, Miiluniemi, M, Salmi, M, Stolen, C, Jalkanen, S, Casteilla, L, Penicaud, L, Valet, P, Carpéné, C (2009). Semicarbazide-sensitive amine oxidase/vascular adhesion protein-1 deficiency reduces leukocyte infiltration into adipose tissue and favors fat deposition. *Am J Pathol* **174:** 1075-83.

Carpéné, C, Abello, V, Iffiú-Soltész, Z, Mercier, N, Fève, B, Valet, P (2008). Limitation of adipose tissue enlargement in rats chronically treated with semicarbazide-sensitive amine oxidase and monoamine oxidase inhibitors. *Pharmacol Res* **57:** 426-34.

Carpéné, C, Iffiú-Soltész, Z, Bour, S, Prévot, D, Valet, P (2007). Reduction of fat deposition by combined inhibition of monoamine oxidases and semicarbazide-sensitive amine oxidases in obese Zucker rats. *Pharmacol Res* **56:** 522-30.

Nemcsik, J, Szökő, E, Soltész, Z, Fodor, E, Tóth, L, Egresits, J, Tábi, T, Magyar, K, Kiss, I (2007). Alteration of serum semicarbazide-sensitive amine oxidase activity in chronic renal failure. *J Neural Transm* **114:** 841-3.

Prévot, D, Soltész, Z, Abello, V, Wanecq, E, Valet, P, Unzeta, M, Carpéné, C (2007). Prolonged treatment with aminoguanidine strongly inhibits adipocyte semicarbazide-sensitive amine oxidase and slightly reduces fat deposition in obese Zucker rats. *Pharmacol Res* **56:** 70-9.

Tábi, T, Soltész, Z, Magyar, K, Szökő, E (2006). [Study on the altered nitric oxide metabolism in experimental diabetes]. *Acta Pharm Hung* **76:** 19-23.

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