Abstract—Cultured vascular smooth muscle cells (VSMCs) derived from rat aortic media were used to examine semicarbazide-sensitive amine oxidase (SSAO) expression during their differentiation process. In a defined serum-free medium permissive for in vitro VSMC differentiation, there was a large increase in SSAO mRNA and protein levels and in the related enzyme activity during the course of cell culture. This pattern of expression was concomitant with that of some smooth muscle–specific mRNA markers of differentiation. mRNAs in differentiated cultured VSMCs were comparable to those detected in total aorta and media. Pharmacological properties of SSAO present in VSMCs were similar to enzyme activities previously described in the aortic wall. In this model, we also demonstrated that methylamine, a physiological substrate of SSAO, activated 2-deoxyglucose transport in a time- and dose-dependent manner. This methylamine effect was reproduced by other SSAO substrates and was prevented by the SSAO inhibitor semicarbazide. It was antagonized in the presence of catalase, suggesting that SSAO-activated glucose transport was mediated through H2O2 production. In addition, methylamine promoted glucose transporter 1 accumulation at the cell surface. Thus, we demonstrate for the first time the differentiation-dependent expression of SSAO in VSMCs and its role in the regulation of VSMC glucose uptake. (Arterioscler Thromb Vasc Biol. 2002;22:89-94.)

Key Words: semicarbazide-sensitive amine oxidase ■ smooth muscle cells ■ differentiation ■ hydrogen peroxide ■ glucose transport

Semicarbazide-sensitive amine oxidases (SSAOs, EC 1.4.3.6) are copper-containing enzymes that deaminate some aromatic and aliphatic amines and differ from monoamine oxidases A and B with respect to substrate and inhibitor specificities, cofactors, and cellular distribution. At a molecular level, SSAO, which has recently been cloned in different species, is identical to vascular adhesion protein-1 (VAP-1), which displays adhesive and enzymatic properties. SSAO activities are found in blood plasma and are associated with membranes in several mammalian tissues. Despite its apparently wide tissue distribution, SSAO expression appears prominent in a limited number of cell types, including endothelial cells, smooth muscle cells, and adipocytes. In endothelial cells, SSAO/VAP-1 is essentially present in high endothelial venules of peripheral lymph nodes and in cerebral microvessels. SSAO is also highly expressed in white and brown adipose cells from different species, an observation that is in agreement with the large induction of SSAO mRNA and activity levels during the course of adipose conversion. SSAO is also abundantly found in vascular smooth muscle cells (VSMCs) and non-VSMCs. The high activity of SSAO in the aorta has been ascribed to its strong expression in the tunica media, which mainly contains smooth muscle cells. The knowledge of SSAO physiology continues to progress, and putative functions of the enzyme appear pleiotropic according to the nature of the cell type. Thus, in high endothelial venules of peripheral lymphatic tissues, SSAO/VAP-1 mediates lymphocyte binding to endothelial cells. In rat adipocytes, it has been recently shown that SSAO activation by benzylamine or tyramine can stimulate glucose transport through a hydrogen peroxide (H2O2)-dependent mechanism. In VSMCs, unlike the enzyme expressed by endothelial cells, SSAO seems unable to bind lymphocytes. It has been suggested that vascular SSAO could mediate toxicological effects. Taken together, these numerous studies illustrate that the functional diversity of SSAO partly depends on the nature of the cell type in which the enzyme is expressed.

Homogeneous populations of cultured cells represent essential tools to investigate the pharmacology, biochemistry, and function of SSAO. Cultures of VSMCs derived from the media of the aorta have been used by a limited number of laboratories to characterize SSAO expression and to study the possible toxicological effects mediated by the enzyme. However, the SSAO activities mentioned in these studies were often much lower than those reported in aortic homogenates or freshly isolated VSMCs.
Chomczynski and Sacchi. Northern blot analysis was performed to determine SSAO protein expression and function during the course of rat VSMC differentiation in vitro. We demonstrate in the present study that the emergence of SSAO in VSMCs is differentiation dependent. Furthermore, the level of SSAO expression in the present VSMC model is quite consistent with that detected in rat aortic media and provides the opportunity to study in vitro the potential functions of the enzyme in this cell type. We show that SSAO activation induces glucose transport. This effect involves SSAO-generated H$_2$O$_2$ and is accompanied by an increase in glucose transporter 1 (GLUT1) expression at the cell surface.

**Methods**

**Cell Culture**

VSMCs were isolated by enzymatic digestion of aortic media from 9-week-old male Wistar rats (300 g, Janvier Laboratories, le Genest-Saint-Isle, France) as previously described. After the initial plating at 6 × 10$^5$ cells per 100-mm-diameter dish, isolated cells were cultured in DMEM supplemented with 10% FCS. VSMCs were subcultured every 7 days before confluence, and experiments were performed between the 4th and 10th passages. At confluence, VSMCs were stimulated to differentiate in defined serum-free medium containing 1:1 (vol/vol) mixture of DMEM/F-12 Ham plus insulin (1 μmol/L), transferrin (50 μg/mL), and ascorbate (0.2 mmol/L).

Murine 3T3-L1 preadipocytes were cultured as previously mentioned. Experiments were performed on confluent or mature adipocytes.

**RNA Analysis**

Total RNA was extracted from differentiated VSMCs by the method of Cathala et al and from tissues by the procedure of Chomczynski and Sacchi. Northern blot analysis was performed as described. For extended online discussion of reverse transcription (RT)–polymerase chain reaction (PCR) analysis, please see http://atvb.ahajournals.org.

SSAO Enzyme Activity

VSMCs were washed twice in PBS and then harvested and homogenized with a glass Dounce homogenizer (pestle B) in a 4 mmol/L potassium phosphate buffer (pH 7.2) containing 250 mmol/L sucrose and 1 mmol/L EDTA. Homogenates were stored at −80°C until use. SSAO activity was assayed as described. Kinetic parameters were determined with ENZFITTER software (Biosoft-Elsevier).

Membrane Isolation and Western Blot Analysis

The cell monolayer was removed by scraping, harvested, homogenized (glass Dounce homogenizer, pestle B) in HES buffer (containing 20 mmol/L HEPES, 1 mmol/L EDTA, and 250 mmol/L sucrose, pH 7.4) plus 5 mmol/L phenylmethylsulfonyl fluoride and 5 μg/mL aprotinin, and centrifuged at 300g for 10 minutes at 4°C. The supernatant was collected and centrifuged at 200 000 g for 90 minutes at 4°C. The resulting pellet was resuspended in 30 mmol/L HEPES, pH 7.4. SDS-PAGE was performed as described (please see http://atvb.ahajournals.org).

Determination of 2-Deoxyglucose Uptake

Cells were plated at 3 × 10$^5$ to 5 × 10$^5$ cells per well in 24-well plates in DMEM containing 10% FCS until confluence. Cells were then maintained in serum-free medium for 7 to 10 days. The uptake of glucose was determined by using [1,2-3H]deoxyglucose (ICN-Biomedicals), a nonmetabolizable analogue of glucose. Differentiated VSMCs were tested for [1,2-3H]deoxyglucose uptake as described online (please see http://atvb.ahajournals.org).

**Statistical Analyses**

Results are presented as mean±SE. The statistical comparison of data between groups was assessed by ANOVA or the Kruskal-Wallis test (when mentioned) with the use of STATVIEW software.

**Results**

Expression of SSAO During Differentiation of VSMCs

In preliminary experiments, we investigated whether a serum-free differentiation medium was required to obtain an optimal expression of SSAO. At confluence, cells were either maintained in the medium containing FCS or shifted into a serum-free medium to stimulate differentiation for 10 days. Results indicated that confluent rat VSMCs maintained in a serum medium retained the properties of confluent cells (online Figure I; please see http://atvb.ahajournals.org). By contrast, shifting the cells in a defined medium promoted differentiation and SSAO gene and protein expression. Subsequent experiments were then performed on VSMCs cultured in a serum-free medium after confluence.

A more detailed study of the pattern of SSAO expression was performed during the course of the cell culture by testing the profile of SSAO mRNA expression during VSMC differentiation. Total RNA was obtained from VSMCs at intervals after confluence, and SSAO transcript levels were measured by Northern blotting and semiquantitative RT-PCR analyses (Figure 1). Both analyses indicated that terminal VSMC maturation was accompanied by an increase in SSAO mRNA levels. Thus, SSAO mRNA abundance rose by 5- to 10-fold in day-10 postconfluent VSMCs compared with confluent VSMCs. We next compared the pattern of SSAO mRNA expression with that of other well-known transcripts of the VSMC phenotype (Figure 1B). α-Actin mRNA, an early differentiation marker, was already detectable in confluent cells and remained at stable levels during the culture. The induction of calponin transcript occurred in day-2 postconfluent VSMCs. Interestingly, in parallel with the progressive induction of smooth muscle myosin heavy chain (SM-MHC) mRNA, SSAO transcript levels increased later at day 4 after confluence. Finally, the levels of GAPDH mRNA remained constant during the course of the culture.

Protein extracts were also prepared from confluent (day-0) and day-9 postconfluent VSMCs. As a control, we used the 3T3-L1 preadipose cell line that differentially expresses SSAO between an undifferentiated state and the mature adipocyte phenotype. Immunoblot analysis of SSAO protein content (Figure 2A) revealed the presence of a single band of ~95 kDa, corresponding to the molecular weight of the monomer of membrane-bound SSAO from bovine lung. Although virtually absent in confluent cells, SSAO protein was strongly expressed in day-9 postconfluent differentiated VSMCs, with a similar profile of enzyme expression being detected during adipose conversion of the 3T3-L1 preadipose cell line. SSAO activity was then determined in VSMC homogenates prepared at different intervals during the culture in serum-free medium (Figure 2B). Benzylamine (500 μmol/L) was used as a substrate in the presence of a
monoamine oxidase–selective inhibitor, pargyline. Under these conditions, benzylamine oxidase activity corresponded to SSAO activity, inasmuch as 95% of this activity was inhibited by semicarbazide (1 mmol/L). At confluence, SSAO activity was low and then progressively increased by a factor of 7 until day 10 after confluence.

Comparative Analysis of SSAO Expression Between Cultured VSMCs and Aortic Tissues

We evaluated whether the SSAO present in differentiated cultured VSMCs was expressed at levels comparable to those detectable in total aorta media. RNA was prepared from day-10 postconfluent VSMCs, from whole aorta, or from dissected media or adventitia, and a semi-quantitative RT-PCR analysis was performed on SSAO, sm-MHC, and GAPDH mRNAs (Figure 3). The levels of SSAO mRNAs detected in VSMCs differentiated in culture were quite similar to those observed in total aorta or isolated media and were consistent with the pattern of expression of sm-MHC transcripts. As a negative control, neither SSAO nor sm-MHC mRNAs were detectable in the adventitia, in agreement with a previous report on SSAO activity distribution.

Pharmacological Properties of SSAO in Cultured VSMCs

To further characterize SSAO activity in cultured VSMCs, we analyzed a variety of substrates and inhibitors. Our pharmacological studies showed that SSAOs from differentiated VSMCs had properties similar to SSAOs from freshly isolated VSMCs (please see the table in http://atvb.ahajournals.org).

Finally, in cultured VSMCs, the high levels of SSAO gene expression and the close pharmacological relationship with the SSAO activity previously described in the aorta support the idea that this in vitro culture system represents an appropriate model to study SSAO biochemistry, regulation, and function.

SSAO Activation Can Regulate Glucose Uptake in Differentiated VSMCs

It has been recently shown that in isolated rat adipocytes, SSAO activation is able to stimulate glucose transport through an H₂O₂-dependent mechanism. On the basis of

Figure 1. Comparative time course in the expression of SSAO mRNA and other smooth muscle–specific transcripts during VSMC differentiation. Cells were grown in a medium supplemented with FCS until confluence and then were stimulated to differentiate in a defined serum-free medium. VSMCs were harvested at confluence (D0, where D indicates day) and at the indicated intervals after confluence. Total RNA was extracted and analyzed by Northern blotting or semiquantitative RT-PCR. Panel A shows a representative autoradiogram of a Northern hybridization. Equivalence in RNA loading in each lane was assessed by methylene blue staining of ribosomal RNA. Panel B corresponds to a typical RT-PCR experiment (n=6), performed as described in Methods. PCR products were resolved on a 2% agarose gel and stained with ethidium bromide. Sizes of SSAO mRNA (in kilobases, panel A) and of PCR products (in base pairs, panel B) are indicated in the right margin.

Figure 2. Induction of SSAO protein expression and enzyme activity during differentiation of VSMCs. A, Immunoblot analysis of vascular and adipose SSAO expression is shown. Crude membrane proteins (25 μg) derived from confluent 3T3-L1 preadipocytes and VSMCs (D0) or from differentiated 3T3-L1 adipocytes and VSMCs (D9) were resolved on a 7.5% SDS-polyacrylamide gel. Immunoblot analysis was performed with a specific anti-SSAO antibody.22 Prestained molecular mass standards used were myosin (203 kDa), β-galactosidase (126 kDa), and BSA (71 kDa). This panel is representative of 4 separate experiments. B, VSMCs were cultured as mentioned in the legend to Figure 1. Cells were harvested at intervals, and homogenates were prepared to determine SSAO activity (n=4). *P<0.05 and **P<0.01 for postconfluent vs confluent (D0) VSMCs.

Figure 3. SSAO mRNA expression in cultured VSMCs and rat aortic tissues. Total RNA was prepared from cultured differentiated VSMCs (day 10 after confluence) and from total rat aorta and aortic medial and adventitial layers. Total RNA was tested by a semi-quantitative RT-PCR analysis for SSAO, sm-MHC, and GAPDH mRNA levels as described in the legend to Figure 1. PCR products were resolved on a 2% agarose gel stained with ethidium bromide. Experiments were repeated 3 times with similar results.
these observations, we studied the effect of methylamine, considered a physiological substrate of SSAO, on 2-deoxyglucose uptake by differentiated day-10 postconfluent VSMCs. We first investigated the time-dependent effect of exposure to 1 mmol/L methylamine (Figure 4A). The rate of glucose uptake was significantly induced after a 30-minute exposure to methylamine compared with no methylamine exposure. Methylamine-induced glucose transport was maximal between 1 and 3 hours. The effect of methylamine on glucose transport was concentration dependent: differentiated VSMCs exposed to various concentrations of the amine for 1 hour (Figure 4B) responded to methylamine at a concentration as low as 0.1 mmol/L. The maximal response was observed at 1 mmol/L methylamine, with a half-maximal action between 0.3 and 0.5 mmol/L, a concentration that correlated well with the \( K_i \) of SSAO for methylamine.

To highlight whether methylamine-induced glucose transport was due to an SSAO activation, VSMCs were exposed to 1 mmol/L methylamine for 1 hour in the absence or in the presence of the SSAO inhibitor semicarbazide (Figure 4C). We used angiotensin II, a peptide well known to stimulate glucose uptake in VSMCs, as a positive control. Angiotensin II induced a 2.2-fold increase in 2-deoxyglucose transport. Likewise, preincubation of VSMCs with 1 mmol/L methylamine for 1 hour caused a 1.6-fold stimulation of 2-deoxyglucose transport. This represented approximately half the effect of angiotensin II. The addition of semicarbazide (1 mmol/L) 30 minutes before methylamine completely prevented the stimulation of 2-deoxyglucose transport by the amine. Thus, the methylamine-induced 2-deoxyglucose uptake was mediated by SSAO activation. Semicarbazide alone was without effect on 2-deoxyglucose transport. In agreement with the pattern of SSAO expression during the course of differentiation, methylamine had no effect on 2-deoxyglucose transport in confluent (day-0) VSMCs (Figure 4C, inset).

Other SSAO substrates were also tested for their ability to modulate glucose transport in the absence or in the presence of semicarbazide. Benzylamine, \( \beta \)-phenylethylamine, and tyramine were able to stimulate 2-deoxyglucose uptake (Table). Their effect was antagonized in the presence of semicarbazide, thus indicating an SSAO-mediated effect.

To elucidate whether the methylamine-induced 2-deoxyglucose transport could involve an \( \text{H}_2\text{O}_2 \)-dependent mechanism (Figure 4D), catalase was used alone or in combination with methylamine or \( \text{H}_2\text{O}_2 \). Although catalase alone was without effect on basal 2-deoxyglucose transport, it completely abolished the glucose uptake stimulated by exogenously provided \( \text{H}_2\text{O}_2 \). Similarly, methylamine-induced 2-deoxyglucose uptake was completely prevented by catalase. Thus, 2-deoxyglucose uptake experiments indicate that SSAO activation, through \( \text{H}_2\text{O}_2 \) production, can stimulate glucose uptake in differentiated VSMCs.

**Effect of Various SSAO Substrates on 2-Deoxyglucose Transport in VSMCs**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>2-Deoxyglucose Uptake, % of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>No Semicarbazide 100, 105±10.3</td>
</tr>
<tr>
<td>Benzylamine (100 ( \mu \text{mol/L} ))</td>
<td>147±18.6*, 107±2.8†</td>
</tr>
<tr>
<td>( \beta )-Phenylethylamine (100 ( \mu \text{mol/L} ))</td>
<td>148.0±13.3*, 103±2.5§</td>
</tr>
<tr>
<td>Tyramine (250 ( \mu \text{mol/L} ))</td>
<td>154.0±11.8†, 95±4.2§</td>
</tr>
</tbody>
</table>

Values are mean±SE and are a percentage of 2-deoxyglucose transport measured in control cells. Differentiated VSMCs (day 10 after confluence) were incubated for 1 hour in KRH medium with benzylamine, \( \beta \)-phenylethylamine, or tyramine in the absence or presence of 1 mmol/L semicarbazide. Glucose uptake was then measured as described in Methods. Basal glucose transport was 706±171 pmol/\( \text{mg protein} \) (n=3–6).

*\( P<0.05 \) and †\( P<0.02 \) for amine-treated vs control cells; ††\( P<0.001 \) and ‡\( P<0.001 \) for amine plus inhibitor–treated cells vs cells treated with amine alone.
We next evaluated whether methylamine modified glucose transporter abundance at the cell surface. The effect of 1 mmol/L methylamine on the levels of GLUT1, which is highly expressed in VSMCs, was assessed by Western blot analysis. There was a rapid and clear increase in the level of GLUT1 after a 1-hour exposure to methylamine (Figure 5). This effect persisted throughout the 6-hour exposure to the amine.

Discussion
It has been established for many years that the vasculature contains a particularly high SSAO activity associated predominantly with smooth muscle cell–containing layers of the tunica media. However, only a limited number of studies have mentioned the utilization of cultured VSMCs to examine SSAO expression and function. This situation likely reflects the difficulty of maintaining a differentiated contractile phenotype of VSMCs in culture systems. VSMCs display an extreme plasticity in vivo or in vitro, which enables this cell type to undergo rapid and reversible changes in its phenotype in response to environmental influences. Thus, primary cultured VSMCs prepared by either enzymatic dispersion or explants rapidly undergo dedifferentiation under standard culture conditions, with a consistent decrease in a number of smooth muscle contractile proteins. However, in the present study, the use of a defined serum-free medium allowed the cells to differentiate, as assessed by the clear induction in the mRNA levels of sm-MHC, generally considered as the most rigorous and specific marker for identification of differentiated VSMCs. Overall, VSMC terminal differentiation was accompanied by a parallel and dramatic increase in SSAO gene and protein expression, as well as in enzyme activity. Thus, it is likely that VSMCs display a gradual loss of SSAO expression when grown in culture, whereas the shift of the committed VSMCs in a defined medium reorients the cells toward maturation.

The main advantages of using cultured cells are to allow a better control of experimental conditions with a higher reproducibility and to provide more reliable and available biological materials. In this respect, they can represent a key tool to study SSAO physiology in VSMCs, with the proviso that the enzyme is expressed at levels comparable to those present in medial layers of blood vessels. So far, the understanding of the exact functions of SSAO in VSMCs remains a major challenge. However, it has been documented that SSAO, through aliphatic amine metabolism, could exert cytotoxic effects on smooth muscle cells in vitro and cause vascular damages, which sometimes resemble the pathological changes observed in atherosclerosis. Under physiological conditions, SSAO is highly expressed in medial layers from blood vessels, suggesting that the enzyme could mediate still unknown physiological functions. Thus, SSAO-generated H₂O₂ may represent a signaling molecule controlling several biological processes in VSMCs. H₂O₂ has been shown to exert pleiotropic functions in VSMCs, including the regulation of cell growth and apoptosis.

The present work strongly suggests that SSAO activation by methylamine could stimulate glucose uptake by VSMCs through H₂O₂ production. These results extend the observation made on isolated rat adipocytes showing that SSAO activation stimulates glucose transport and glucose transporter 4 (GLUT4) recruitment to the plasma membrane by an SSAO- and H₂O₂-dependent mechanism. Activation of insulin response substrate-1 and substrate-3 and of phosphatidylinositol-3 kinase appears to be involved in this action. On the basis of these observations, it has been proposed that SSAO participates under in vivo conditions in the regulation of glucose disposal in insulin-sensitive tissues. In the present study, several experimental results support the view that SSAO could mediate an activation of glucose transport: (1) Methylamine, a physiological substrate of tissue-bound SSAO, caused a dose-dependent increase in 2-deoxyglucose cellular accumulation, with a concentration for half-maximal action that is in agreement with the Kₘ of the enzyme for this substrate. (2) Methylamine-induced 2-deoxyglucose transport is completely prevented in the presence of the specific SSAO inhibitor semicarbazide. (3) Other SSAO substrates also stimulate glucose transport, and their effect is completely reversed by semicarbazide. Moreover, evidence for the involvement of H₂O₂ in this methylamine-induced glucose transport is supplied by the inhibitory action of catalase, which is inefficient alone. The cellular mechanisms at the basis of this methylamine-H₂O₂-mediated glucose transport remain unclear. In VSMCs, H₂O₂ has also been shown to activate phosphatidylinositol-3-kine and protein kinase B. This pathway plays a key role in insulin-induced glucose transport of adipocytes through GLUT4 translocation to the plasma membrane. However, GLUT1 but no GLUT4 glucose transporters have been found in VSMCs, and it is generally considered that GLUT1 is constitutively present in the plasma membrane and does not translocate to this cell compartment in response to external stimuli. However, in some cell types, it has been reported that GLUT1 can also be translocated to the plasma membrane.

In the present study, we show that GLUT1 accumulation at the plasma membrane can occur within 1 hour after methylamine exposure. Whether this phenomenon corresponds to a GLUT1 translocation process or to a de novo protein synthesis remains to be investigated. Whatever the molecular mechanisms underlying this methylamine-induced glucose transport, it could be emphasized that in VSMCs, glucose exerts pleiotropic effects. For example, growth factor synthesis and lipid peroxidation are regulated by glucose in...
VSMCs. It would an interesting challenge to evaluate whether vascular SSAO, through this potential implication in glucose transport, could modulate some of these cellular processes.

Acknowledgments

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References

Semicarbazide-Sensitive Amine Oxidase in Vascular Smooth Muscle Cells: Differentiation-Dependent Expression and Role in Glucose Uptake
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