Semicarbazide-sensitive amine oxidase activation promotes adipose conversion of 3T3-L1 cells

Nathalie MERCIER, Marthe MOLDES, Khadija EL HADRI and Bruno FÈVE[1]

Centre de Recherches Biomédicales des Cordeliers, Université Pierre et Marie Curie, UMR 7079 CNRS, 15 rue de l'Ecole de Médecine, 75270 Paris, Cedex 06, France

Semicarbazide-sensitive amine oxidase (SSAO) is an amine oxidase related to the copper-containing amine oxidase family. The tissular form of SSAO is located at the plasma membrane, and is mainly expressed in vascular smooth muscle cells and adipocytes. Recent studies have suggested that SSAO could activate glucose transport in fat cells. In the present work, we investigated the potential role of a chronic SSAO activation on adipocyte maturation of the 3T3-L1 pre-adipose cell line. Exposure of post-confluent 3T3-L1 pre-adipocytes to methylamine, a physiological substrate of SSAO, promoted adipocyte differentiation in a time- and dose-dependent manner. This effect could be related to SSAO activation, since it was antagonized in the presence of the SSAO inhibitor semicarbazide, but not in the presence of the monoamine oxidase inhibitor pargyline. In addition, methylamine-induced adipocyte maturation was mimicked by 3T3-L1 cell treatment with other SSAO substrates. Finally, the large reversion of methylamine action by catalase indicated that hydrogen peroxide generated by SSAO was involved, at least in part, in the modulation of adipocyte maturation. Taken together, our results suggest that SSAO may contribute to the control of adipose tissue development.

Key words: amine oxidase, adipocyte, differentiation, hydrogen peroxide.

INTRODUCTION

Adipose tissue development essentially occurs after birth, but persists all along the life to contribute to energetic homoeostasis. This process involves a permanent control of pre-adipocyte growth and differentiation. Several transcription factors have been shown to act in concert to regulate adipogenesis [1]. Proteins belonging to the CCAAT-enhancer-binding protein (C/EBP), peroxisome-proliferator-activated receptor (PPAR) and helix–loop–helix families exert a sequential and co-ordinated action to regulate adipogenesis. However, environmental factors also play a key role in the initiation and the control of adipogenesis and maintenance of the adipocyte phenotype [2]. The adipose conversion process is under the tight control of numerous hormones, nutrients, cytokines and growth factors that positively or negatively regulate adipogenesis. For example, insulin is a hormonal effector that potently accelerates adipocyte differentiation [3]. Insulin regulates the expression and/or function of several major adipogenic transcription factors present during the course of adipose conversion, such as C/EBP-α, -β and -δ [4], PPARγ2 [5], and adipocyte determination and differentiation factor-1/sterol-regulatory-element-binding protein-1 [6]. Otherwise, when acting on mature adipocytes, insulin promotes intracellular lipid accumulation through the activation of glucose transport and lipogenesis, as well as through the inhibition of lipolysis. Interestingly, it has been suggested that in adipocytes, insulin effects could be partly mediated by activation of a plasma membrane hydrogen peroxide-generating NADPH oxidase [7,8]. Furthermore, it is well known that hydrogen peroxide can mimic some insulin effects. In adipose cells, hydrogen peroxide not only activates glucose transport [9], but also stimulates glucose C1-oxidation [10], glucose incorporation into glycogen [11], lipogenesis [12] and inhibits lipolysis [13]. Moreover, by using a system that chronically produces hydrogen peroxide in the culture medium, it has been documented that this reactive oxygen species directly promotes 3T3-L1 pre-adipose cell differentiation [14]. Thus in adipose tissue, hydrogen peroxide exhibits insulin-like properties and represents a messenger that could mediate pleiotropic functions.

Recently we have identified by a differential display approach a new mRNA species that is dramatically upregulated during adipocyte differentiation [15]. The corresponding full-length cDNA encodes for the murine semicarbazide-sensitive amine oxidase (SSAO), an enzyme composed of 765 amino acids, the mature form of which is glycosylated. This enzyme activity exists as a soluble and tissular form. The tissular form of SSAO contains a short intracellular domain, a single transmembrane domain and a long extracellular domain which includes the catalytic site that oxidizes some primary amines to generate the corresponding aldehyde, ammonia and hydrogen peroxide. The SSAO activity requires a cofactor with a reactive carbonyl group (topaquinone as a modified tyrosine residue) capable of reacting with semicarbazide and other carbonyl reagents acting as inhibitors. The presence of SSAO is detectable in several tissues, but this amine oxidase is preferentially expressed in the media of aorta and in adipose tissue [15]. Moreover, SSAO mRNA levels and enzyme activity are absent in pre-adipocytes, but are dramatically induced during adipose conversion [15,16]. Recent works have reported that besides its localization at the adipocyte plasma membrane, SSAO also co-localizes with GLUT4 transporter-containing vesicles [17–20]. At the functional level, it is noteworthy that SSAO, through hydrogen peroxide production, can activate glucose transport in isolated rat adipocytes [18,20]. It has also been suggested that SSAO could reduce the lipolytic properties of histamine by degradation of this potential substrate of the enzyme [21].

With regard to the sharp emergence of SSAO during the differentiation process and the capacity of this amine oxidase to produce hydrogen peroxide, we investigated the potential involvement of SSAO in the modulation of adipocyte maturation.

Abbreviations used: aP2, adipocyte lipid-binding protein; C/EBP, CCAAT-enhancer-binding protein; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; G3PDH, glycerol-3-phosphate dehydrogenase; LPL, lipoprotein lipase; MAO, monoamine oxidase; PPAR, peroxisome-proliferator-activated receptor; SSAO, semicarbazide-sensitive amine oxidase.

[1] To whom correspondence should be addressed (e-mail feve@bhdc.jussieu.fr).

Using a pharmacological approach on the murine 3T3-L1 preadipose cell line model [22], we show here that cell exposure to a physiological substrate of SSAO, methylamine, accelerates pre-adipocyte differentiation in a time- and dose-dependent manner. This effect of methylamine is mediated by SSAO activation, since it is specifically inhibited by an SSAO antagonist, and it is reproduced by several other SSAO substrates. Moreover, SSAO-generated hydrogen peroxide appears to play an important role in the modulation of adipocyte maturation. Finally, our observations support the view that in adipose tissue, SSAO could be of physiological importance not only for the control of several metabolic pathways in differentiated adipocytes, but also during the course of fat cell development.

MATERIALS AND METHODS

Cell lines and cell culture

3T3-L1 pre-adipocytes [22] were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/l glucose and supplemented with 10 % donor calf serum. For experiments, cells were grown in DMEM containing 10 % fetal calf serum (FCS). At confluence (day 0), differentiation was induced with DMEM supplemented with 10 % FCS, 170 nM insulin, 100 μM 1-methyl-3-isobutyl-xanthine and 0.25 μM dexamethasone for 48 h. Thereafter, the medium was changed every other day with DMEM supplemented with only 2.5 % FCS. At day 4 following confluence, cells were treated daily with substrates and/or inhibitors of SSAO or of monoamine oxidase (MAO) at concentrations and for periods of time described in the Results section.

[^{3}H]Thymidine incorporation

3T3-L1 cells were cultured in 6-well plates under conditions indicated above. From day 4 after confluence, cells were exposed or not exposed for 1 or 5 day(s) to 3 mM methylamine, a physiological substrate of SSAO. [*methyl*-^{3}H]Thymidine (specific activity 60 Ci/mmol, ICN Pharmaceuticals; 5 μCi/well) was then added for 1 h at 37 °C. Cells were washed twice with ice-cold PBS and solubilized in 1 % SDS (500 μl/well). After precipitation in 10 % trichloracetic acid for 45 min at 4 °C, samples were collected under vacuum on glass microfibre filters type C (Whatman, Villeneuve d'Asq, France). After 3 washes with 5 % ice-cold trichloracetic acid, filters were dried with absolute ethanol, transferred into vials and solubilized in liquid scintillation cocktail [Ecolite(+), ICN Pharmaceuticals].

Cell extracts and enzyme assays

3T3-L1 cells were washed twice in PBS, harvested and homogenized in 25 mM Tris, pH 7.5/1 mM EDTA [20 strokes in a Dounce homogenizer (Merck Eurolab, Strasbourg, France), pestle B]. A fraction of the homogenate was stored at −80 °C. The remaining fraction was centrifuged at 10000 ***g*** for 10 min at 4 °C, and the supernatant was kept at −80 °C until use. Aliquots of homogenates and supernatants were used to determine protein content by the method of Lowry, using BSA as a standard. Triacylglycerol concentration was determined with the GPO Trinder kit (Sigma Diagnostics).

SSAO activity was tested by measurement of hydrogen peroxide production by the fluorimetric method of Matsumoto et al. [23], using benzylamine as a substrate. Briefly, 50 μg of cell homogenates were pre-incubated for 30 min at 37 °C, in a final volume of 100 μl containing 40 mM sodium phosphate, pH 7.4, 1 mM homovanillic acid, 1 mM sodium azide and 1 mM pargyline to inhibit MAO-A and -B, in the absence or in the presence of the SSAO inhibitor semicarbazide (1 mM). Then, benzylamine (500 μM) was added in each assay. Incubation was carried out for 1 h at 37 °C. Reaction was stopped with 1 mM semicarbazide, and 1.2 ml of 0.1 M NaOH was added. Fluorescence intensity was measured with excitation at 323 nm and emission at 426 nm. As blank tests, assays were incubated in parallel without substrate addition. Preliminary experiments were performed to ensure that SSAO activity was tested at the initial rate of reaction.

Glycerol-3-phosphate dehydrogenase (G3PDH) activity was assayed by recording the initial rate of oxidation of NADH at 340 nm at 25 °C [24]. The standard mixture contained 50 mM triethanolamine/25 mM HCl buffer, pH 7.5, 1 mM EDTA, 0.13 mM β-NADH, 1 mM dihydroxyacetone phosphate, 1 mM 2-mercaptoethanol and variable amounts of 10000 ***g*** cell supernatants.

Western-blot analysis

Cells were washed three times in cold PBS, harvested and homogenized in 1 ml of HES buffer [20 mM Hepes (pH 7.4), 1 mM EDTA, 250 mM sucrose] supplemented with a cocktail of anti-proteases (Complete™; Roche). Homogenates were centrifuged at 200000 ***g*** for 90 min at 4 °C. The resulting pellets were resuspended in 30 mM Hepes buffer, pH 7.4, and stored at −80 °C.

SDS/PAGE was performed with a Bio-Rad mini protean III apparatus. Proteins (30 μg/lane) were boiled for 5 min at 95 °C, separated by SDS/PAGE (7.5 % gel) and electroblotted onto 0.45 μm PVDF membrane (Immobilon-P, Millipore) in 0.1 % SDS, 192 mM glycine and 25 mM Tris, pH 8.3. The membrane was kept in a methanol bath and then blocked with 5 % fish gelatine, 0.1 % (v/v) Tween 20 in TBS buffer (20 mM Tris and 137 mM NaCl, pH 7.6) for 45 min at room temperature. After an initial washing in TBS buffer containing 0.1 % Tween 20, the membrane was incubated overnight at 4 °C with primary antibodies (rabbit polyclonal antibody against bovine lung SSAO, dilution 1:2000, [25]) in TBS buffer containing 2 % fish gelatine and 0.05 % Tween 20. After four washes with 0.1 % Tween 20/TBS, the membrane was incubated with horseradish peroxidase conjugated to anti-(rabbit IgG) (dilution 1:20000 in TBS buffer containing 0.05 % Tween 20 and 2 % fish gelatine), for 1 h at room temperature. The membrane was then washed in 0.1 % Tween 20/TBS. Detection of immune complex was performed using an enhanced chemiluminescence detection kit for Western blot (Amersham International) on an X-OMAT-AR Kodak film.

RNA analysis

Total RNA was extracted according to Cathala et al. [26]. RNA samples (10 μg/lane) were electrophoresed through a 1.2 % agarose/2.2 M formaldehyde gel and transferred to a nylon membrane (Nylon+; Scheicher and Shuell). After fixation, 18 S and 28 S ribosomal RNAs were stained by Methylene Blue to ensure the similarity in RNA loading. Hybridization to DNA probes labelled by random priming was carried out as described by Church and Gilbert [27]. Final washing was performed in 0.2 × 150 mM NaCl, 15 mM sodium citrate and 0.1 % SDS for 15 min at 60 °C. Blots were then exposed at −80 °C to an X-OMAT-AR Kodak film. The SSAO cDNA probe has been described previously [15]. Adipocyte lipid-binding protein (aP2) and G3PDH cDNAs were generously given by Dr H. Green (Department of Cell Biology, Harvard Medical School, Boston, U.S.A.) [28]. The human lipoprotein lipase (LPL) cDNA probe [29] was a kind gift of Dr Arjun Singh (Department of Pediatrics,

University of Tennessee, Memphis, TN, U.S.A.). Autoradiograms and Methylene-Blue stained membranes were analysed with a videodensitometer (Vilber-Lourmat Imaging, Marne-La Vallée, France).

Statistical analysis

Results are presented as mean ± S.E.M. of at least three independent experiments. The level of significance between groups was assessed using analysis of variance.

RESULTS

It has been documented for a long time that hydrogen peroxide is able to mimic some insulin effects. Thus in adipocytes, hydrogen peroxide exerts lipogenic [12] or anti-lipolytic effects [13], and activates glucose transport [9]. Moreover, hydrogen peroxide can contribute to the maintenance of the adipocyte phenotype [14]. With regard to the insulin-like properties of hydrogen peroxide and to the strong induction of SSAO expression during adipogenesis, we hypothesized that a chronic activation of SSAO could promote or accelerate the adipocyte differentiation process. This issue was addressed on the murine 3T3-L1 cell line model. Since the present study dealt with the activation of adipogenesis, 3T3-L1 cells were cultured under conditions which are suboptimal for differentiation. Thus, immediately after removal of the induction mixture, 3T3-L1 cells were shifted to a medium containing only 2.5% FCS and insulin was omitted.

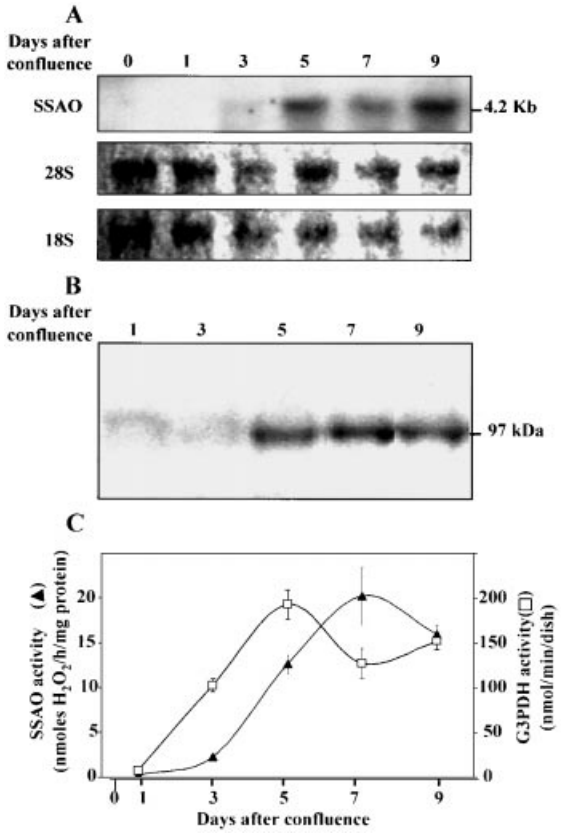

Figure 1 Characterization of SSAO expression during 3T3-L1 adipose conversion

At confluence, 3T3-L1 cells were induced to differentiate in 10% FCS supplemented with a mixture of methyl-isobutyl-xanthine (100 μM), insulin (170 nM) and dexamethasone (0.25 μM). Thereafter, cells were changed to a medium containing 2.5% FCS, and insulin was omitted. Cells were harvested at different times relative to confluence arbitrarily considered as day 0, and various cell extracts were prepared. (**A**) Northern analysis of SSAO mRNA levels. Samples of total RNA (10 μg/lane) were subjected to electrophoresis, transferred on to a nylon membrane and hybridized with a radiolabelled SSAO cDNA probe. Ribosomal RNAs were stained with Methylene Blue to assess the similarity in RNA loading. (**B**) Western-blot analysis of SSAO protein. Samples (50 μg protein/lane) were subjected to electrophoresis, blotted onto a PVDF membrane, hybridized with an anti-SSAO primary antibody (dilution 1:2000) [25], and then with a horseradish peroxidase conjugated to anti-(rabbit-IgG) (dilution 1:20000). (**C**) SSAO and G3PDH enzyme activity. Cell homogenates (50 μg of protein/assay) were tested for SSAO activity (nmol/h per mg of protein) (▲) by the fluorimetric detection of hydrogen peroxide production [23]. G3PDH activity (nmol/min per dish) (□) was measured for the supernatants obtained from centrifugation at 10000 ***g***. Results represent the mean ± S.E.M. of four independent experiments.

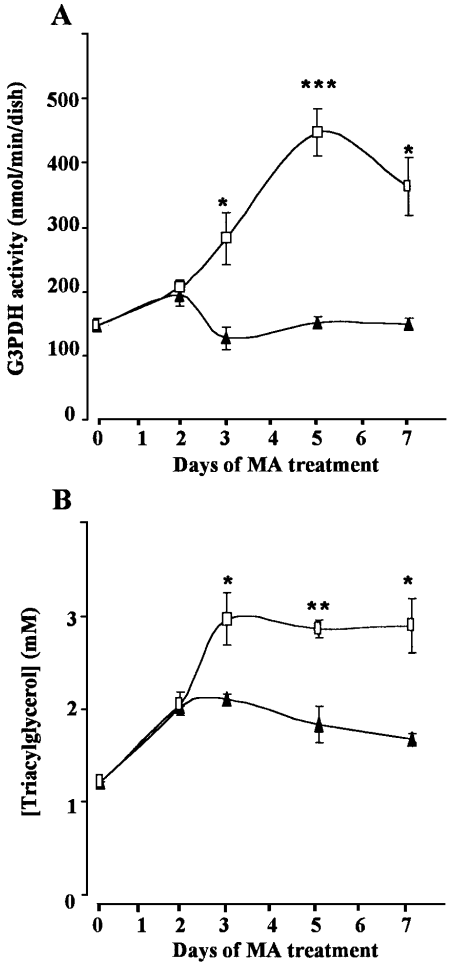

Figure 2 Time-dependent effect of methylamine on adipocyte differentiation

From day 4 following confluence, cells were treated (□) or not (▲) with 3 mM methylamine (MA), and harvested at intervals. (**A**) G3PDH activity (in nmol/min per mg of protein) was measured for the 10000 ***g*** supernatants. (**B**) Triacylglycerol concentration. Results represent the mean ± S.E.M. of 3–4 independent experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; for methylamine-treated compared with control cells.

SSAO expression during 3T3-L1 adipose conversion

Cell extracts were prepared at various intervals relative to confluence (day 0). Figure 1(A) shows that SSAO mRNA was poorly detected at day 3 and was dramatically increased during the terminal adipocyte differentiation of 3T3-L1 cells, reaching a maximal level at day 7 following confluence. In agreement with the pattern of SSAO mRNA expression, the levels of SSAO

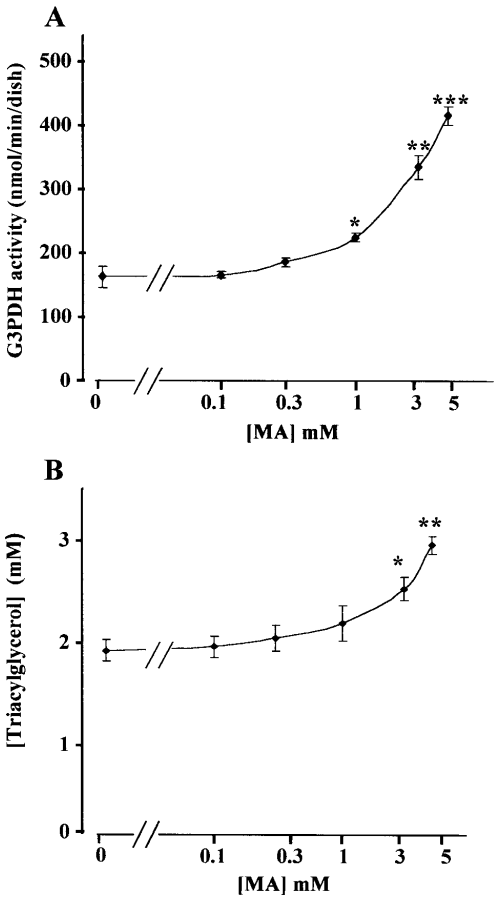

Figure 3 Dose-dependent effect of methylamine on G3PDH activity and triacylglycerol content

3T3-L1 cells were treated from day 4 after reaching confluence with various concentrations of methylamine (MA) for 5 days. Cell extracts were then prepared and tested for G3PDH activity (**A**) and triacylglycerol content (**B**). Results correspond to the mean ± S.E.M. of five separate experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ for methylamine-treated compared with control cells.

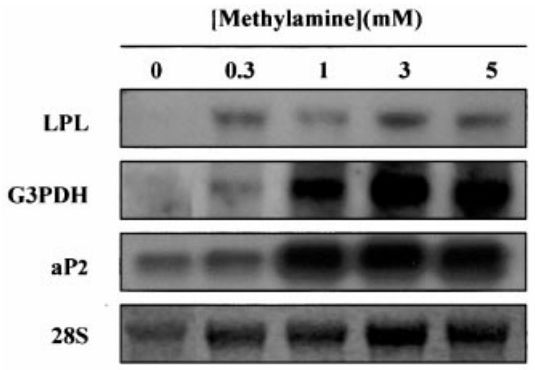

Figure 4 Dose-dependent effect of methylamine on LPL, G3PDH and aP2 mRNA levels

Day-4 post-confluent cells were exposed for 5 days to various concentrations (0–5 mM) of methylamine. Total RNA was extracted and tested by Northern analysis for LPL, G3PDH and aP2 mRNA levels. Methylene Blue staining of ribosomal RNAs was performed to ensure the similarity in RNA loading and to normalize the amounts of the specific mRNA species. Autoradiograms are representative of three separate experiments.

protein (Figure 1B) and enzyme activity (Figure 1C) were strongly induced between day 3 and day 5 and then reached a plateau. These preliminary experiments indicated that even under limiting culture conditions, there was a clear and late expression of SSAO during adipocyte differentiation, the activity of which was similar to that observed previously in 3T3-L1 cells differentiated with a classical culture protocol [15]. The setting up of the conversion process was further explicated by the emergence of G3PDH (Figure 1C), a marker that closely reflects the level of fat cell development [30].

SSAO substrate methylamine promotes the adipocyte differentiation process

Methylamine is generally considered as a physiological substrate for SSAO [31]. Since the pattern of SSAO expression indicated a late emergence of the enzyme (Figure 1 and [15]), the 3T3-L1 cells were exposed or not exposed to methylamine only from day 4 following confluence.

We first investigated the time-dependence effect of an exposure to methylamine at 3 mM, a concentration that is 3–5-fold higher than the K_m of SSAO for methylamine [15,31]. 3T3-L1 cells were treated with methylamine for 2–7 days, and cell extracts were prepared to test cell triacylglycerol content and G3PDH activity. As a preliminary control, we verified that methylamine effect on adipocyte differentiation was not related to a post-confluent increase in cell growth. Cell counts and [^{3}H]thymidine incorporation experiments were performed after various days of methylamine exposure. Methylamine did not modify cell density or [^{3}H]thymidine incorporation, even when the cells were exposed for long periods of time (results not shown). This ruled out the possibility that methylamine effect was linked to an increase in cell proliferation.

As illustrated in Figure 2(A), there was a slight, but non-significant increase in G3PDH activity after a 2-day exposure to methylamine. After a 3-day treatment with the amine, we observed a dramatic 2.2-fold increase in the enzyme activity as compared with control cells. The maximal effect of methylamine was detected following a 5-day exposure to the amine (2.85-fold increase). The pattern of methylamine effect on triacylglycerol content was very close to that observed for G3PDH activity, with a sharp induction in triacylglycerol concentration after a 3-day treatment and a maximal effect after a 5-day treatment with the amine (Figure 2B).

We next tested the dose-dependent induction of adipocyte differentiation by methylamine. From day 4 after the cells had reached confluence, 3T3-L1 cells were exposed or not exposed to various concentrations of methylamine for 5 days. As shown in Figure 3, methylamine provoked a dose-dependent increase in G3PDH activity and triacylglycerol content. The effect was detectable between 0.3 and 1 mM, and continued to increase at 5 mM. Likewise, there was a dose-dependent induction of LPL, G3PDH and aP2 mRNA levels, three typical molecular markers of adipocyte differentiation (Figure 4) [3]. The weak signals observed in control cells with G3PDH and LPL mRNAs could probably be explained by the absence of insulin during the differentiation process. Under these conditions, the effect of methylamine on these transcripts was already detectable at 0.3 mM, and was maximal at 3 mM.

Taken together, our results show that methylamine, a physiological SSAO substrate, promotes 3T3-L1 cell differentiation.

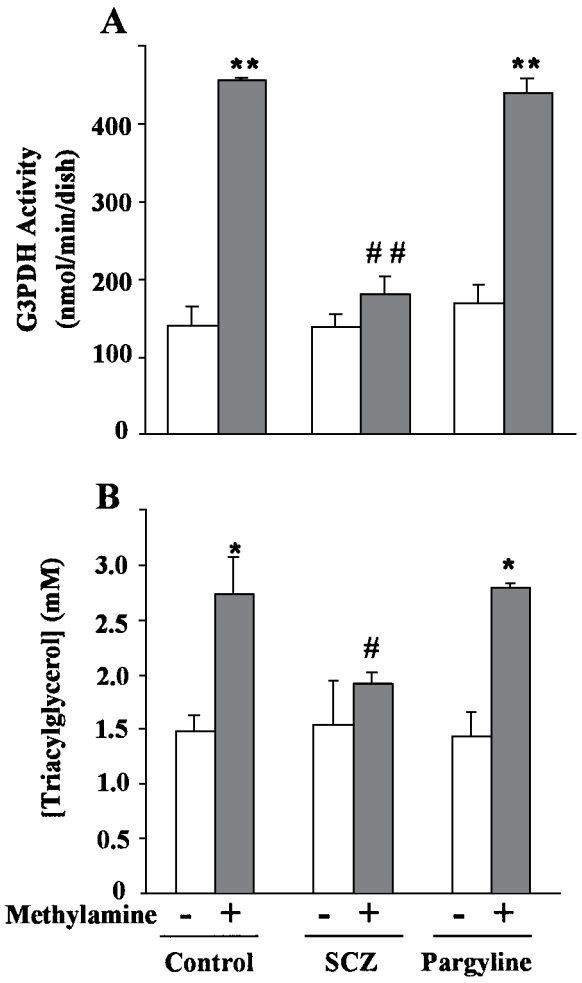

Figure 5 Methylamine effect on adipocyte differentiation depends on SSAO activation

From day 4 following confluence, 3T3-L1 cells were treated (+) or not (−) for 5 days with 3 mM methylamine, in the absence, or in the presence of 1 mM semicarbazide (SCZ) or 1 mM pargyline. Cell extracts were prepared and tested for G3PDH activity (**A**) and triacylglycerol content (**B**). Results represent the mean ± S.E.M. for three independent experiments. $^{*}P < 0.05$; $^{**}P < 0.01$; methylamine-treated compared with control cells. $\#P < 0.05$; $\#\#P < 0.01$; methylamine- and semicarbazide-treated cells compared with cells treated with methylamine alone.

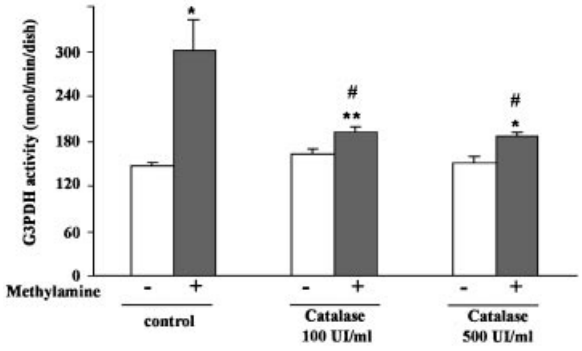

Figure 6 Methylamine effect on adipocyte maturation depends on hydrogen peroxide production

From day 4 following confluence, 3T3-L1 cells were exposed (+) or not exposed (−) to 3 mM methylamine for 5 days, in the absence or in the presence of catalase (100 or 500 units/ml). Cell extracts were then tested for G3PDH activity. Results represent the mean ± S.E.M. for four independent experiments. $^{*}P < 0.01$; $^{**}P < 0.001$; methylamine-treated compared with control cells. $\#P < 0.05$, methylamine and catalase-treated cells compared with cells treated with methylamine alone. UI, catalase units.

Methylamine effect on adipocyte differentiation is mediated by SSAO

Since methylamine is a potential substrate for both MAO-A and -B and SSAO, and as regards to the strong expression of MAOs in adipocytes [32], it was of importance to assess whether potentiation of adipose maturation caused by methylamine was mediated through SSAO or MAO activation. Thus, we compared the effects of the selective SSAO inhibitor semicarbazide to those of pargyline, a selective MAO-A and -B inhibitor, on methylamine-induced adipocyte differentiation. Day-4 post-confluent 3T3-L1 cells were exposed or not for 5 days to 3 mM methylamine, in the absence or in the presence of 1 mM semicarbazide or 1 mM pargyline (Figure 5). Neither semicarbazide nor pargyline had any effect on G3PDH activity or triacylglycerol content when used alone. As expected, methylamine provoked a sharp increase in the levels of these markers of adipocyte differentiation (3.2-fold and 1.75-fold increases in G3PDH activity and triacylglycerol content respectively). Overall, semicarbazide almost completely prevented the promoting effect of methylamine on adipogenesis. The SSAO inhibitor reduced by about 90%

Table 1 Effects of various SSAO and/or MAO substrates on adipocyte maturation

Day-4 post-confluent 3T3-L1 cells were exposed for 5 days to methylamine, benzylamine, tyramine, β-phenylethylamine or 5-hydroxytryptamine in the absence (None) or in the presence of 1 mM semicarbazide (SCZ), or 1 mM pargyline (PARG) or the combination of both (SCZ + PARG, 1 mM each). Cells extracts were prepared and assayed for G3PDH activity (in nmol/min per dish). Results represent the mean ± S.E.M. of 6–15 independent experiments.

		G3PDH activity (nmol/min per dish)			
Substrate	Inhibitors ...	None	SCZ	PARG	SCZ + PARG
Control		94 ± 6	83 ± 4	100 ± 4	101 ± 4
Methylamine (3 mM)		162 ± 5‡	96 ± 6¶	174 ± 9‡	102 ± 5¶
Benzylamine (0.5 mM)		197 ± 10‡	115 ± 9¶	162 ± 6‡§	97 ± 4¶
Tyramine (1 mM)		180 ± 7‡	116 ± 7¶	164 ± 13‡	113 ± 5¶
β-Phenylethylamine (1 mM)		163 ± 5‡	123 ± 5*‖	136 ± 8†‖	99 ± 5¶
5-Hydroxytryptamine (0.5 mM)		108 ± 3†	107 ± 2†	86 ± 7§	85 ± 7§

*, $P < 0.05$; †, $P < 0.01$; ‡, $P < 0.001$; for amine-exposed compared to control cells.
§, $P < 0.05$; ‖, $P < 0.01$; ¶, $P < 0.001$; for amine- and inhibitor(s)-treated cells compared to cells treated with the amine alone.

methylamine action on G3PDH activity, and by 70 % the amine effect on cell triacylglycerol content. By contrast, the MAO-selective inhibitor pargyline did not impair methylamine-induced adipose conversion.

Another pharmacological approach was used to establish that SSAO activation could mediate an accelerated fat cell maturation. Several SSAO and/or MAO substrates were tested for their ability to modulate the adipocyte differentiation process, in the absence or in the presence of semicarbazide or pargyline, or a combination of both. As shown in Table 1, benzylamine, tyramine and β-phenylethylamine provoked an enhancement in G3PDH activity similar to that induced by methylamine. The major part of this effect was antagonized by semicarbazide. By contrast, pargyline only weakly or partially reversed the effects of these three substrates. The MAO substrate 5-hydroxytryptamine exerted only a poor action on G3PDH activity, and its effect was antagonized by pargyline, but not by semicarbazide. In the absence of amine oxidase substrates, the antagonists, alone or in combination, had no significant effect on adipocyte maturation.

Finally, experiments performed with SSAO- or MAO-selective antagonists or with different SSAO or MAO substrates strongly support the view that a chronic SSAO activation promotes adipocyte differentiation.

Methylamine effect on adipocyte maturation depends on hydrogen peroxide production

SSAO metabolizes methylamine into formaldehyde, ammonia and hydrogen peroxide. With regards to the known lipogenic and adipogenic actions of hydrogen peroxide [9,12,14], we hypothesized that SSAO-generated hydrogen peroxide could mediate methylamine action on adipocyte differentiation. Day-4 post-confluent 3T3-L1 cells were exposed or not exposed to 3 mM methylamine for 5 days, in the absence or in the presence of catalase, an enzyme that specifically degrades hydrogen peroxide. Figure 6 indicates that catalase had no effect alone, whereas most of the methylamine-induced increase in G3PDH activity was inhibited in the presence of the hydrogen peroxide-degrading enzyme [inhibition of 70 % and 74 % of methylamine effect with 100 and 500 units/ml catalase respectively (catalase activity is expressed as 1 unit decomposes 1 μmol of hydrogen peroxide/min at pH 7.0 and 35 °C)]. By contrast, neither formaldehyde nor ammonia modulated the level of adipose maturation (results not shown). These results suggest that the effect of methylamine on 3T3-L1 adipocyte differentiation is mediated, at least in part, by the SSAO-generated hydrogen peroxide production.

DISCUSSION

The membrane-bound form of SSAO has been found in several tissues, and its activity is particularly high in vascular smooth muscle cells [31] and adipocytes from rat white and brown fat [15,33,34]. SSAO activity is also present in other cell types or tissues, such as bovine lung [25] and in high endothelial venules from peripheral lymphatic tissues [35]. In addition to this wide tissue distribution of the enzyme, it also appears that plasma membrane SSAO could exert pleiotropic actions. Besides a possible scavenging role with respect to circulating amines [31], SSAO also displays vascular adhesion [35,36] and metabolic properties [18–21]. In adipocytes, it has been reported previously that SSAO exerts a stimulatory role on glucose transport [18,20], and can even reduce plasma glucose levels in rat [19]. In this study, we demonstrate that this amine oxidase not only regulates a key metabolic pathway of adipose cells, but could also promote adipocyte development.

Several lines of evidence support the idea that SSAO could mediate an acceleration of adipocyte differentiation in response to several primary amines. (1) Methylamine, a physiological substrate of tissue-bound SSAO [31], provoked a dose-dependent increase in several biochemical or molecular markers of adipose conversion, with a range of potency that is close to the K_m of the enzyme for this substrate [15,31]. (2) The methylamine-induced adipocyte differentiation is antagonized by the carbonyl reagent semicarbazide, but is resistant to pargyline, an inhibitor of mitochondrial MAOs. (3) Different SSAO substrates such as methylamine, benzylamine, tyramine or β-phenylethylamine stimulate adipocyte differentiation. In agreement with the substrate selectivity for SSAO and/or MAO, this effect was completely or partially reversed by semicarbazide. (4) Finally, the promoting effects of methylamine on adipocyte differentiation are not related to an increased population of differentiating adipocytes. As a consequence, this really reflects a general increase in adipocyte maturation level.

Otherwise, experimental results presented here strengthen the view that hydrogen peroxide production mediates at least a major part of SSAO-activated adipocyte maturation. The main experimental argument is that methylamine-induced adipocyte maturation is inhibited by the concomitant addition of catalase in the culture medium. Moreover, neither formaldehyde, the aldehyde generated by SSAO from methylamine [31,37], nor ammonia activate adipose cell development. At a high concentration (1 mM), formaldehyde is even cytotoxic when applied to 3T3-L1 differentiating cells (results not shown). Taken together, these results strongly suggest that SSAO-generated hydrogen peroxide can promote fat cell maturation.

A critical question concerning induction of 3T3-L1 adipocyte differentiation by SSAO is the cellular and/or molecular mechanisms underlying hydrogen peroxide effects on adipocyte differentiation. In other terms, does SSAO-generated hydrogen peroxide potentiate fat cell development through its insulin-like properties on terminal adipocyte maturation, or through an initiating effect on the early events of adipogenesis? Three classes of transcription factors have been identified that directly influence fat cell development. These include C/EBPs, PPARs and the basic helix–loop–helix family [1]. It is generally considered that these factors act in a sequential and co-ordinated fashion to initiate adipogenesis and regulate the promoters of many fat cell-specific genes. Otherwise, it is now well established that reactive oxygen species, especially hydrogen peroxide, can directly regulate the expression and/or activity of many transcription factors [38,39]. Modifications of transcription factors may provide a mechanism by which cells sense these redox changes. For example, the conformation of the DNA-binding domains of nuclear factor κB, activator protein-1 and p53 can be changed by reactive oxygen species, resulting in an alteration of their affinity for their promoter targets [39]. It is also documented that hydrogen peroxide, through regulation of ubiquitin activity, could control the degradation of the hypoxia inducible factor Hif-1 [40,41]. Whether reactive oxygen species could alter the functions of PPARγ, C/EBP-α, -β, -δ, and adipocyte determination and differentiation factor-1/sterol-regulatory-element-binding protein-1, five major adipogenic transcription factors, remains an open question.

Though we cannot exclude that the SSAO-generated hydrogen peroxide could alter expression or activity of key adipogenic transcription factors, it is also possible that hydrogen peroxide acts on several signalling or metabolic pathways at later stages of adipogenesis to promote terminal differentiation. In adipocytes, the insulin-like properties of hydrogen peroxide, including activation of glucose transport [9] and lipogenesis [12], and in-

hibition of lipolysis [13], have been described for a long period of time. Interestingly, Enrique-Tarancon et al. [18,19] and Marti et al. [20] have shown previously that in adipocytes, SSAO substrates, in combination with low doses of vanadate, rapidly stimulate insulin responsive subtrates-1 and -3, phosphatidylinositol 3-kinase activities, GLUT4 recruitment to the cell surface and glucose transport through a hydrogen peroxide-dependent mechanism. Moreover, other signalling pathways, also involved in the regulation of adipocyte differentiation such as p42, p44 and p38 mitogen-activated protein kinases [42–44] or tyrosine phosphatases [45], are also modulated by hydrogen peroxide [46–48]. It is also conceivable that the expression of several genes coding for proteins essential for adipocyte metabolism could be regulated by SSAO-generated hydrogen peroxide. Thus, exogenously and chronically supplied hydrogen peroxide not only enhances 3T3-L1 adipocyte differentiation [14], but also positively and negatively regulates GLUT1 and GLUT4 expression respectively [49]. Finally, it must be emphasized that SSAO expression is a late marker of the adipocyte differentiation process, and that in our experimental protocol, exposure to SSAO substrates was concomitant with the emergence of the enzyme and delayed as compared with the early induction of C/EBPs and PPARγ [1,2]. Thus it is likely that the promoting effect of SSAO activation on fat cell development is linked to a pleiotropic effect of hydrogen peroxide on key metabolic or signalling pathways of the cell, rather than to an initiating action on the early transcriptional events of adipogenesis.

The augmentation of 3T3-L1 adipocyte differentiation induced by SSAO activation raises the question of the physiological and pathophysiological relevance of this observation. In a general context, our results point to the fact that hydrogen peroxide not only generates an oxidative stress, but could also represent a second messenger mediating the developmental or metabolic effects of extracellular factors on differentiating or mature adipocytes. Whether an alteration in the expression and/or function of adipocyte SSAO could initiate or contribute to a disorder of adipose tissue development or metabolism is still unknown. Abnormalities in SSAO expression or activity have been observed in genetic models of obesity [15,33]. Furthermore, it has recently been reported that in humans, there is a significant positive correlation between serum SSAO activity and the body mass index [50]. However, the tissular origin of the serum SSAO is still unidentified, and this correlation does not establish a causal relationship between the excess of adipocyte tissue development and high levels of SSAO activity in fat cells and serum. Further investigations in different species will be required to determine the exact physiological and pathological implications of SSAO in obesity or non-insulin-dependent diabetes mellitus. Generation of mice deficient in the SSAO gene will provide a particularly attractive model to resolve this issue.

This work was supported by the Centre National de la Recherche Scientifique and the University of Paris VI. N.M. and M.M. are recipients of research fellowships from the Ministère de l'Education Nationale et de la Recherche and from the Fondation pour la Recherche Médicale respectively. We thank Dr M. Unzeta (Universitat Autonoma de Barcelona, Spain) for the generous gift of the polyclonal antibody against SSAO, and Dr J. Pairault (Department of Physiology, University of Paris VI, France) for the critical review of the manuscript.

REFERENCES

1 Rosen, E. D., Walkey, C. J., Puigserver, P. and Spiegelman, B. M. (2000) Transcriptional regulation of adipogenesis. Genes Dev. **14**, 1293–1307
2 Gregoire, F. M., Smas, C. M. and Sul, H. S. (1998) Understanding adipocyte differentiation. Physiol. Rev. **78**, 783–809
3 Ailhaud, G., Grimaldi, P. and Négrel, R. (1992) Cellular and molecular aspects of adipose tissue development. Annu. Rev. Nutr. **12**, 207–233
4 Zhang, B., Berger, J., Zhou, G., Elbrecht, A., Biswas, S., White-Carrington, S., Szalkowski, D. and Moller, D. E. (1996) Insulin- and mitogen-activated protein kinase-mediated phosphorylation and activation of peroxisome proliferator-activated receptor γ. J. Biol. Chem. **271**, 31771–31774
5 MacDougald, O. A., Cornelius, P., Liu, R. and Lane, M. D. (1995) Insulin regulates the transcription of the CCAAT/enhancer binding protein (C/EBP) α, β, and δ genes in fully-differentiated 3T3-L1 adipocytes. J. Biol. Chem. **270**, 647–654
6 Kim, J. B., Sarraf, P., Wright, M., Yao, K. M., Mueller, E., Solanes, G., Lowell, B. B. and Spiegelman, B. M. (1998) Nutritional and insulin regulation of fatty acid synthetase and leptin gene expression through ADD1/SREBP1. J. Clin. Invest. **101**, 1–9
7 Krieger-Bauer, H. I. and Kather, H. (1992) Human fat cells possess a plasma membrane-bound H_2O_2-generating system activated by insulin via a mechanism bypassing the receptor kinase. J. Clin. Invest. **89**, 1006–1013
8 Krieger-Bauer, H. I. and Kather, H. (1997) Insulin-induced activation of NADPH-dependent H_2O_2 generation in human adipocyte plasma membrane is mediated by $G_{\alpha i}$. J. Biol. Chem. **272**, 10135–10143
9 Czech, M. P., Lawrence, Jr, J. C. and Lynn, W. S. (1974) Evidence for the involvement of sulfhydryl oxidation in the regulation of fat cell hexose transport by insulin. Proc. Natl. Acad. Sci. U.S.A. **71**, 4173–4177
10 Czech, M. P., Lawrence, Jr, J. C. and Lynn, W. S. (1974) Evidence for electron transfer reactions involved in the Cu^{2+}-dependent thiol activation of fat cell glucose utilization. J. Biol. Chem. **249**, 1001–1006
11 Lawrence, J. C. and Larner, J. (1978) Activation of glycogen synthase in rat adipocytes by insulin and glucose involves increased glucose transport and phosphorylation. J. Biol. Chem. **253**, 2104–2113
12 May, J. M. and de Haën, C. (1979) The insulin-like effect of hydrogen peroxide on pathways of lipid synthesis in rat adipocytes. J. Biol. Chem. **254**, 9017–9021
13 Little, S. A. and de Haën, C. (1980) Effects of hydrogen peroxide on basal and hormone-stimulated lipolysis in perifused rat fat cells in relation to the mechanism of action of insulin. J. Biol. Chem. **255**, 10888–10895
14 Krieger-Brauer, H. I. and Kather, H. (1995) Antagonistic effects of different members of fibroblast and platelet-derived growth factor families on adipose conversion and NADPH-dependent H_2O_2 generation in 3T3 L1-cells. Biochem. J. **307**, 549–556
15 Moldes, M., Fève, B. and 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**, 9515–9523
16 Raimondi, L., Pirisino, R., Banchelli, G., Ignesti, G., Conforti, L., Romanelli, E. and Buffoni, F. (1990) Cultured preadipocytes produce a semicarbazide-sensitive amine oxidase (SSAO) activity. J Neural Transm. Suppl. **32**, 331–336
17 Morris, N. J., Ducret, A., Aebersold, R., Ross, S. A., Keller, S. R. and Lienhard, G. E. (1997) Membrane amine oxidase cloning and identification as a major protein in the adipocyte plasma membrane. J. Biol. Chem. **272**, 9388–9392
18 Enrique-Tarancon, G., Marti, L., Morin, N., Lizcano, J. M., Unzeta, M., Sevilla, L., Camps, M., Palacin, M., Testar, X., Carpene, C. and 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**, 8025–8032
19 Enrique-Tarancon, G., Castan, I., Morin, N., Marti, L., Abella, A., Camps, M., Casamitjana, R., Palacin, M., Testar, X., Degerman, E., Carpene, C. and Zorzano, A. (2000) Substrates of semicarbazide-sensitive amine oxidase cooperate with vanadate to stimulate tyrosine phosphorylation of insulin-receptor-substrate proteins, phosphoinositide 3-kinase activity and GLUT4 translocation in adipose cells. Biochem. J. **350**, 171–180
20 Marti, L., Morin, N., Enrique-Tarancon, G., Prevot, D., Lafontan, M., Testar, X., Zorzano, A. and Carpene, C. (1998) Tyramine and vanadate synergistically stimulate glucose transport in rat adipocytes by amine-oxidase-dependent generation of hydrogen peroxide. J. Pharmacol. Exp. Therap. **285**, 342–349
21 Raimondi, L., Conforti, G., Banchelli, G., Ignesti, G., Pirisino, R. and Buffoni, F. (1993) Histamine lipolytic activity and semicarbazide-sensitive amine oxidase activities (SSAO) of white adipose tissue (WAT). Biochem. Pharmacol. **46**, 1369–1376
22 Green, H. and Kehinde, O. (1974) Sublines of mouse 3T3 cells that accumulate lipids. Cell (Cambridge, Mass.) **1**, 113–116
23 Matsumoto, T., Furuta, T., Nimura, Y. and Suzuki, O. (1982) Increased sensitivity of the fluorometric method of Snyder and Hendley for oxidase assays. Biochem. Pharmacol. **31**, 2207–2209
24 Kozak, L. P. and Jensen, J. T. (1974) Genetic and developmental control of multiforms of L-glycerol 3-phosphate dehydrogenase. J. Biol. Chem. **249**, 7775–7781
25 Lizcano, J. M., Tipton, K. F. and Unzeta, M. (1998) Purification and characterization of membrane-bound semicarbazide-sensitive amine oxidase (SSAO) from bovine lung. Biochem. J. **331**, 69–78
26 Cathala, C., Savouret, J. F., Mendez, B., Karin, M., Martial, J. A. and Baxter, J. D. (1983) A method for isolation of intact, translationally active ribonucleic acid. DNA. **2**, 329–335

27 Church, G. M. and Gilbert, W. (1984) Genomic sequencing. Proc. Natl. Acad. Sci. U.S.A. **81**, 1991–1995
28 Spiegelman, B. M., Frank, M. and Green, H. (1983) Molecular cloning of mRNA from 3T3 adipocytes. J. Biol. Chem. **258**, 10083–10089
29 Wion, K. L., Kirchgessner, A. J., Lusis, A. J., Schotz, M. C. and Lawn, R. M. (1987) Human lipoprotein lipase complementary DNA sequence. Science (Washington, D.C.) **235**, 1638–1641
30 Pairault, J. and Green, H. (1979) A study of the adipose conversion of suspended 3T3 cells by using glycerophosphate dehydrogenase as differentiation marker. Proc. Natl. Acad. Sci. U.S.A. **76**, 5138–5142
31 Lyles, G. A. (1996) Mammalian plasma and tissue-bound semicarbazide-sensitive amine oxidases: biochemical, pharmacological and toxicological aspects. Int. J. Biochem. Cell Biol. **28**, 259–274
32 Pizzinat, N., Marti, L., Remaury, A., Leger, F., Langin, D., Lafontan, M., Carpene, C. and Parini, A. (1999) High expression of monoamine oxidases in human white adipose tissue: evidence for their involvement in noradrenaline clearance. Biochem. Pharmacol. **58**, 1735–1742
33 Barrand, M. A. and Callingham, B. A. (1984) Solubilization and some properties of a semicarbazide-sensitive amine oxidase in brown adipose tissue of the rat. Biochem. J. **222**, 467–475
34 Raimondi, L., Pirisino, R., Ignesti, G., Capecchi, S., Banchelli, G. and Buffoni, F. (1991) Semicarbazide-sensitive amine oxidase activity (SSAO) of rat epididymal white adipose tissue. Biochem. Pharmacol. **41**, 467–470
35 Salmi, M. and Jalkanen, S. (1992) A 90-kilodalton endothelial cell molecule mediating lymphocyte binding in humans. Science (Washington, D.C.) **257**, 1407–1409
36 Smith, D. J., Salmi, M., Bono, P., Hellman, J., Leu, T. and Jalkanen, S. (1998) Cloning of vascular adhesion protein 1 reveals a novel multifunctional adhesion molecule. J. Exp. Med. **188**, 17–27
37 Yu, P. H. (1990) Oxidative stress deamination of aliphatic amines by rat aorta semicarbazide-sensitive amine oxidase. J. Pharm. Pharmacol. **42**, 882–884
38 Finkel, T. (1998) Oxygen radicals and signaling. Curr. Opin. Cell. Biol. **10**, 248–253
39 Marshall, H. E., Merchant, K. and Stamler, J. S. (2000) Nitrosation and oxidation in the regulation of gene expression. FASEB J. **14**, 1889–1900
40 Huang, L. E., Arany, Z., Livingston, D. M. and Bunn, H. F. (1996) Activation of hypoxia-inducible transcription factor depends primarily upon redox-sensitive stabilization of its alpha subunit. J. Biol. Chem. **271**, 32253–32259
41 Salceda, S. and Caro, J. (1997) Hypoxia-inducible factor 1α (HIF-1α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J. Biol. Chem. **272**, 22642–22647
42 Sale, E. M., Atkinson, P. G. and Sale, G. J. (1995) Requirement of MAP kinase for differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis. EMBO J. **14**, 674–684
43 Font de Mora, J., Porras, A., Ahn, N. and Santos, E. (1997) Mitogen-activated protein kinase activation is not necessary for, but antagonizes, 3T3-L1 adipocytic differentiation. Mol. Cell. Biol. **17**, 6068–6075
44 Engelman, J. A., Lisanti, M. P. and Scherer, P. E. (1998) Specific inhibitors of p38 mitogen-activated protein kinase block 3T3-L1 adipogenesis. J. Biol. Chem. **273**, 32111–32120
45 Liao, K. and Lane, M. D. (1995) The blockade of preadipocyte differentiation by protein-tyrosine phosphatase HA2 is reversed by vanadate. J. Biol. Chem. **270**, 12123–12132
46 Hecht, D. and Zick, Y. (1992) Selective inhibition of protein tyrosine phosphatase activities as target of regulation by H_2O_2 and vanadate *in vitro*. Biochem. Biophys. Res. Commun. **188**, 773–779
47 Guyton, K. Z., Liu, Y., Gorospe, M., Xu, Q. and Holbrook, N. J. (1996) Activation of mitogen-activated protein kinase by H_2O_2. J. Biol. Chem. **271**, 4138–4142
48 Wilhelm, D., Bender, K., Knebel, A. and Angel, P. (1997) The level of intracellular glutathione is a key regulator for the induction of stress-activated signal transduction pathways including Jun N-terminal protein kinases and p38 kinase by alkylating agents. Mol. Cell. Biol. **17**, 4792–4800
49 Rudish, A., Kozlovsky, N., Potashnik, R. and Bashan, N. (1997) Oxidative stress reduces insulin responsivess in 3T3-L1 adipocytes. Am. J. Physiol. **272**, E935–E940
50 Meszaros, Z., Szombathy, T., Raimondi, L., Karadi, I., Romics, L. and Magyar, K. (1999) Elevated serum semicarbazide-sensitive amine oxidase activity in non-insulin-dependent diabetes mellitus: correlation with body mass index and serum triacylglycerol. Metabolism **48**, 113–117

Received 9 April 2001/4 June 2001; accepted 27 June 2001