An amidoxime reductase system in adipocyte mitochondria containing cytochrome b₅ type B (CYB5B) and molybdenum cofactor sulfurase C-terminal containing 2 (MOSC2) of importance for lipid synthesis.

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Background: High amidoxime reductase activity is found in liver and fat tissue, although its composition in cells is unknown.

Results: Amidoxime reductase is upregulated during adipogenesis and requires MOSC2 and CYB5B.

Conclusion: MOSC2 and CYB5B are essential components of the mitochondrial amidoxime reductase and MOSC2 is important for lipogenesis.

Significance: We suggest a role of MOSC2 in adipogenesis and fatty acid synthesis and as a potential novel drug target.

SUMMARY
Reduction of hydroxylamines and amidoximes is important for drug activation and detoxification of aromatic and heterocyclic amines. Such a reductase system was previously found to be of high activity in adipose tissue and liver and, furthermore, in vitro studies using recombinant truncated or purified enzymes suggested the participation of cytochrome b₅ reductase (CYB5R), cytochrome b₅ (CYB5) and molybdenum cofactor sulfurase C-terminal containing 1 and 2 (MOSC1 and 2). Here we show that purified rat liver outer mitochondrial membrane (OMM) contains high amidoxime reductase activity and that MOSC2 is exclusively localized to these membranes. Moreover using the same membrane fraction we could show direct binding of a radio-labeled benzamidoxime substrate to MOSC2. Following differentiation of murine 3T3-L1 cells into mature adipocytes, the MOSC2 levels as well as the amidoxime reductase activity were increased, indicating that the enzyme is highly regulated under lipogenic conditions. siRNA-mediated down-regulation of MOSC2 and the mitochondrial form of cytochrome b₅ type B (CYB5B) using siRNA significantly inhibited the reductase activity in the differentiated adipocytes, whereas down-regulation of MOSC1, cytochrome b₅ type A (CYB5A), CYB5R1, CYB5R2 or CYB5R3 had no effect. Down-regulation of MOSC2 caused impaired lipid synthesis. These results demonstrate for the first time the direct involvement of MOSC2 and CYB5B in the amidoxime reductase activity in an intact cell system. We postulate the presence of a novel reductive enzyme system of importance for lipid synthesis that is exclusively localized to the outer mitochondrial membrane and is composed of CYB5B, MOSC2.
and a third unknown component (a CYB5B reductase).

An increasing number of drugs and drug candidates contain basic functional groups such as amidines and guanidines that decrease their oral bioavailability by preventing efficient gastrointestinal adsorption as these groups are protonated under physiological pH. Instead prodrugs have been developed by N-hydroxylation of these amidine groups, converting them into amidoximes in order to improve their oral bioavailability as exemplified by the direct thrombin inhibitor ximelagatran (1,2), its follow-up compound AZD0837 (3) and a platelet inhibitor sibrafidan (4). Once these prodrugs are absorbed into the body they are reduced back to their bioactive amidines by a reductive enzyme system believed to be composed of cytochrome b₅ (CYB5), cytochrome b₅ reductase (CYB5R) and a third unknown component and is associated with both the microsomal fraction (5) as well as the mitochondrial fraction (6,7). The reductive metabolism of nitrogen-containing groups is not only of importance for the activation of prodrugs but also for reduction of hydroxylamines and for detoxification of carcinogenic N-hydroxylated aromatic and heterocyclic amines (8,9). The endogenous role of this activity however is still unknown.

In a previous study we found high amidoxime reductase activity preferentially in outer mitochondrial membranes (OMM) associated with adipose tissue, liver and kidney (6). Furthermore, the reductase activity was dependent on the cofactor NADH and inhibited by potassium cyanide (KCN), while typical CYP inhibitors, such as carbon monoxide, were ineffective (6) indicating that the reductase is unlikely to be a cytochrome P450 as was previously suggested (10). Several studies have attempted to identify the third component and the involvement of stearoyl-CoA desaturase (SDC) (11) and cytochrome P450 isoenzyme CYP2D (10) in the microsomal fraction as well as a novel molybdenum containing enzyme in the mitochondrial fraction (12) were suggested. In subsequent studies, two highly homologous molybdenum containing proteins, namely molybdenum cofactor (MOCO) sulfatase C-terminal containing 1 and 2 (MOSC1 and MOSC2 respectively, referred to as mARC1 and mARC2 by the authors) were shown to display amidoxime reductase activity in vitro when incubated in a reconstituted system with CYB5 and CYB5R3 (cytochrome b₅ reductase 3) (13,14). In addition, Kurian et al. showed that in a reconstituted system purified soluble CYB5 and purified soluble CYB5R were able to reduce amidoximes to amidines without the involvement of a third component (15). However all studies mentioned above were performed using reconstituted systems consisting of purified and soluble truncated components, without showing the direct involvement of these components in more integrated physiological systems.

In the current report we demonstrate using a cell model commonly used to study adipogenesis and siRNA knock-down experiments that MOSC2, but not MOSC1, is critically involved in the amidoxime reductase activity in differentiated adipocytes. In addition, the mitochondrial form of cytochrome b₅ type B (CYB5B) is also shown to be essential for the reductase activity in this intact cell system which appears to be of importance for lipid formation. In contrast the microsomal form of cytochrome b₅ type A (CYB5A) and CYB5R3 as well as its homologues cytochrome b₅ reductase 1 and 2 (CYB5R1 and CYB5R2) are not involved in the reductase activity in the adipocyte cell system.

**EXPERIMENTAL PROCEDURES**

**Reagents** - Dulbecco’s Modified Eagles Medium (DMEM), fetal bovine serum (FBS), sodium pyruvate, penicillin and streptomycin, insulin and OptiMem were obtained from Invitrogen (Life Technologies Europe BV, Stockholm, Sweden). Bovine calf serum (BCS) was from American Type Culture Collection (LGC Standards AB, Borås, Sweden). Cell culture flasks and plates were from Corning Life Science and Sarstedt (VWR International AB, Stockholm, Sweden). DharmaFECT Duo and ON-TARGETplus SMARTpool siRNA constructs (mouse MOSC1 L-044395-01, mouse MOSC2 L-044395-01, mouse CYB5R3 L-058184-01, mouse CYB5R1 L-046143-01, mouse CYB5R2 L-056941-01, mouse CYB5 L-050436-01 (CYB5A), mouse CYB5B L-059461-01 and Non-targeting Pool D-001810-10) were purchased from Thermo Scientific (VWR International AB). Dexamethasone, 3-isobutyl-methylxanthine,
benzamidoxime, 6-propyl-2-thiouracil (PTU) were from Sigma-Aldrich (Sigma-Aldrich Sweden AB, Stockholm, Sweden). Complete protease inhibitor cocktail was from Roche Applied Science (Roche Diagnostics Scandinavia, Bromma, Sweden). Rabbit anti-MOSC2 and anti-CYB5B antibodies were from Atlas Antibodies (Stockholm Sweden), mouse anti-mHSP70 and rabbit anti-VDAC from Affinity Bioreagents (Thermo Scientific), rabbit anti-CYB5 was from Santa Cruz Biotechnology (AH Diagnostics AB, Skärholmen, Sweden). Rabbit anti-CYB5R3 (16), rabbit anti-ERp27 (17) and rabbit anti-calnexin (18) were previously described. Secondary horseradish peroxidase coupled anti-rabbit and anti-mouse antibodies were from Dako (Dako Sweden AB, Stockholm, Sweden). AR-H069637, AR-H069927 and AZ13228184-14C were from AstraZeneca R&D Mölndal, Sweden.

**Purification of outer mitochondrial membranes from rat liver** - Outer mitochondrial membranes (OMM) were purified as described by Hovius et al. (19) and de Kroon et al. (20). Purified OMM vesicles were aliquoted and snap frozen in liquid nitrogen and stored at -70°C. Also collected during fractionation were the homogenate (the 600 x g supernatant), P10 (the 10,000 x g pellet, containing mitochondria), the inner mitochondrial membrane (IMM)/mitochondrial matrix fraction and the microsomes (100,000 x g supernatant).

**Protein concentration determination** - Protein concentrations were determined according to the method of Lowry (21) using bovine serum albumin as standard.

**Western blot analysis** - Subcellular fractions isolated from rat liver and cell lysates from adipocytes and pre-adipocytes were subjected to Western blot analysis as described (22).

**Amidoxime reduction assay** - Amidoxime reductase activity was determined following the benzamidoxime reduction to benzamidine as previously described (6). The amidoxime reductase activity was also monitored by the reduction of the intermediate metabolite of AZD0837, the N-hydroxylated amidine AR-H069927 to the corresponding amidine AR-H067637 (Supplemental Data, Fig S1) (23). Incubations were composed of 0.25 μg OMM and 20 μM AR-H069927 in 50 mM phosphate buffer pH 6.3 in a volume of 100 μl. After a 2 min pre-incubation at 37°C, the reaction was started by the addition of 250 μM NADH and samples were incubated for 20 min at 37°C. Reactions were terminated by the addition of 10 μl 6 M formic acid and precipitated proteins were settled by centrifugation 17,000 g at 4°C for 15 min. The supernatant was mixed with an equal volume of acetonitrile containing 1% (v/v) acetic acid. The formation of AR-H067637 was monitored by HPLC analysis using a Varian ProStar model 410 autosampler, Varian ProStar 310UV-visible detector and Varian ProStar model 240 solvent delivery module (Agilent Technologies Sweden AB, Kista, Sweden). Samples were separated on a LiChrospher® 60 RP-select B (5 μm) column (Merck Chemical International, Merck AB, Solna, Sweden) using an isocratic mobile phase composed of 0.1% (v/v) acetic acid and 3.8 mM ammonium acetate containing 18% acetonitrile. Metabolite and parent compound were detected at 229 nm and quantified using purified standards.

**Cross-linking studies** - Purified OMM (0.5 mg) was incubated with a radio-labeled (carbon-14) and cross-linkable (azide) benzamidoxime analogue (AZ13228184-14C, Supplemental data Fig S2) in order to identify putative components of the amidoxime reductase complex. OMM were diluted in phosphate buffered saline (PBS) to a concentration of 1 mg/ml and incubated with 25 μM (1.42 μCi/ml or 52.5 kBq/ml) 14C-benzamidoxime azide in the presence and absence of 250 μM NADH for 2 min at 37°C in the dark, and samples were cross-linked on ice by exposure to UV-light for 2.5 min. The cross-linked samples were subjected to sequential detergent extraction using Triton X-100 followed by Zwittergent 3-14 (Calbiochem, VWR International AB) as described in the Supplemental Data (Supplemental Experimental Procedures and Fig S3). The detergent extracted samples were diluted in Laemmli sample buffer, boiled and separated by SDS-PAGE using a 10% Tris-Tricine gel. The gel was stained with Coomassie blue R-250, dried and protein-14C-benzamidoxime complexes were visualized by autoradiography (Fuji-BAS 1800, FujiFilm, Stockholm, Sweden).
**Analysis of proteins by mass spectrometry** - Coomassie stained protein bands with an apparent molecular mass of 30-40 kDa, which were identified by cross-linking to the radio-labeled benzamidoxime analogue, were excised from the gel and processed for mass spectrometer analysis essentially as described (24). In brief, the gel pieces were destained, dried and trypsin (porcine, modified, sequence grade from Promega Biotech AB, Nacka, Sweden) was allowed to soak into the swelling gel pieces on ice. After overnight incubation at 30°C and acidification, the proteolytic peptides were subjected to mass analysis by matrix assisted laser desorption/ionization time of flight mass spectrometry on a Bruker Ultraflex III TOF/TOF instrument from Bruker (Stockholm, Sweden), applying the manufacturer’s recommendations. Alpha-cyano-4-hydroxy-cinnamic acid was used as matrix, and the spectra were externally calibrated using a 7 peptide mixture. The generated peptide mass lists were used to scan the current NCBInr sequence database for protein identities, employing the search engine ProFound (http://prowl.rockefeller.edu).

**Immunoprecipitation analysis** - 14C-benzamidoxime azide was incubated and cross-linked to OMM (700 µg) as described and cross-linked samples were solubilized in 1.2% digitonin (Sigma-Aldrich) on ice for 30 min, conditions known to solubilize the MOSC2 protein from the OMM (data not shown). Non-solubilized material was settled by centrifugation for 15 min at 17,000 x g and supernatant was transferred to fresh eppendorf tubes. The pre-cleared supernatant was subjected to immunoprecipitation using anti-MOSC2 antibodies (0.5 µg) overnight at 4°C. The next day protein complexes were captured by incubation with 25µl Protein A/G gel (Thermo Scientific) for 1.5 h at 4°C and washed 2 times in PBS containing 1.2% digitonin and 2 times in PBS. The collected protein complexes were eluted by boiling in Laemmli sample buffer, separated by SDS-PAGE and analyzed by western blotting for MOSC2 and subsequent autoradiography of the same nitrocellulose membrane to detect radio-labeled MOSC2.

**Culture and differentiation of 3T3-L1 cells** - Murine 3T3-L1 pre-adipocytes were obtained from American Type Culture Collection and cultured in DMEM with high glucose containing 10% BCS, 1% sodium pyruvate, and 100 IU/ml penicillin and 100 µg/ml streptomycin and subcultured before they reach 80% confluency. For differentiation pre-adipocytes were cultured in complete medium supplemented with 10% FCS and fully differentiated adipocytes were obtained by treating 3 days confluent cells with a standard MDI cocktail of 3-isobutyl-Methylxanthine (0.5 mM), Dexamethasone (0.5 µM) and Insulin (1.7 µM) for two days, followed by treatment with complete medium containing only insulin for another 5-7 days. Medium was changed every 2 days. Cells were cultured and differentiated in 6 well-cluster plates or 10 cm dishes for preparation of subcellular fractions.

**Subcellular fractionation of 3T3-L1 cells** - Pre-adipocytes or differentiated adipocytes were washed twice with PBS, scraped into 1 ml of PBS and centrifuged 5 min at 750 x g and 4°C. The cell pellet was resuspended in buffer A (10 mM Tris-HCl, pH 7.4 containing 20% (w/v) glycerol, 1 mM EDTA and protease inhibitor cocktail) and sonicated on ice with 20 x 1 sec bursts. Cell lysates were centrifuged at 800 x g and 4°C for 10 min, supernatants transferred and further centrifuged at 6,500 x g and 4°C for 15 minutes to obtain mitochondrial pellets, which were resuspended in mitochondrial buffer (10 mM Tris-acetate pH 7.4, 0.25 M sucrose, 0.5 mM EDTA) and the 6,500 x g supernatant containing the microsomes and cytosol.

**siRNA transfection of 3T3-L1 adipocytes** - Transfection of differentiated 3T3-L1 adipocytes with siRNA was adapted from Kilroy et al. (25). Three days confluent 3T3-L1 preadipocytes were differentiated using the standard MDI protocol for 5 days, after which they were trypsinized and resuspended in DMEM with high glucose containing 10% FCS and insulin at 6 x 10⁵ cells/ml. Transfections were performed in 6 well cluster plates and the transfection mix for one well (9.6 cm²) was composed of 200 pmol siRNA, 14 µl DharmaFect Duo diluted in 400 µl OptiMEM to which 12 x 10⁵ cells (2 ml) were added. Cells were analyzed for reductase activity and protein expression 5 days post-transfection (10 days post-differentiation).
Real time PCR - The mRNA expression levels of several genes in siRNA-treated or control adipocytes was determined by ready to use TaqMan Gene Expression Assays according to the manufacturer’s instructions (Applied Biosystems, Stockholm, Sweden) using β-actin as a housekeeping gene.

Cytochrome b5 reductase assay - The activity of NADH cytochrome b5 reductase (CYB5R) was determined by monitoring the reduction of the artificial electron acceptor 2,6-dichlorophenolindophenol at 590 nm. The incubation mixture contained 1 mM NADH, 3 mg/ml 2,6-dichlorophenolindophenol and 15 µg rat liver microsomes in a 100 mM phosphate buffer pH 7.4. The CYB5R activity was determined without and in the presence of the CYB5R inhibitor 6-propyl-2-thiouracil (PTU).

Oil Red O Staining – Intracellular lipids were measured through staining with Oil Red O (Sigma-Aldrich). Cells were fixed with 10% formalin for 10 min, and incubated in fresh formalin for at least 1 h. After washing twice with ddH2O and 60% isopropanol, cells were stained for 10 min in freshly diluted Oil Red O solution (5.2 mM). Oil Red O was eluted by adding isopropanol for 10 min and the extracted dye was measured at 510 nm on a Varian spectrophotometer (Agilent Technologies).

Fatty acid determination – Cell homogenates were prepared from mature adipocytes collected 5 days after transfection with control or MOSC2 siRNA. To homogenates corresponding to 0.36 mg cell protein, nonadecanoic acid (C19:0, 20 mg) was added as an internal standard. After Folch extraction the extracts (500 ml) were transmethylated by addition of 0.5 ml 0.5 M sodium methoxide in methanol. Following incubation at 56°C for 15 minutes 1 ml of 1M HCl/methanol was added to esterify free fatty acids. After incubation at 56°C for 15 minutes, 2 ml of water was added and the samples were extracted three times with 3 ml of hexane. The samples were dried under a gentle stream of argon and redissolved in 500 ml hexane. The relative contents of fatty acids were determined on a Hewlett Packard HP 6890 Gas Chromatograph connected to a HP 5977 Mass Selective Detector. The gas chromatograph was equipped with a HP-5MS column (30 m x 0.25 mm, 0.25 mm film thickness). Ratios of fatty acids (C14:0, C16:0, C16:1, C18:0 and C18:1) to the internal standard (C19:0) were calculated based on peak area. Authentic fatty acids were used to establish retention times and mass spectra were recorded for the different fatty acids to ensure identity.

RESULTS

Enzyme kinetics of the amidoxime reductase - The enzyme kinetics of the amidoxime reductase enzyme system was characterized using subcellular fractions isolated from rat and human liver in the absence and presence of the inhibitor potassium cyanide (KCN) (6). The reductase activity observed in the presence of KCN is considered to be the non-specific reductase activity, especially at higher benzamidoxime concentrations. The kinetics of benzamidine formation by rat liver microsomes and mitochondria as well as human liver microsomes displayed Michaelis-Menten kinetics (Supplemental data, Fig S4) and the apparent $K_m$ and $V_{max}$ values are listed in Table 1. The KCN-inhibitable $K_m$ values for the rat liver microsomes and mitochondria are very similar 24.4 and 24.6 µM respectively, while the observed $K_m$ in human microsomes is much higher namely about 430 µM. The highest $V_{max}$ value was detected in rat liver mitochondria 3.2 nmol/min/mg protein, being 2-3-fold higher than those observed in rat liver microsomes and human liver microsomes.

MOSC2 is localized in the outer mitochondrial membrane - In previous studies the highest amidoxime reductase activity was found to be associated with fractions enriched with the outer mitochondrial membrane (OMM) (6,12). We determined the amidoxime reductase activity as well as the MOSC2 protein levels in highly purified OMM isolated from female rat liver, since female rat liver displays higher activity levels than males (6). Different subcellular fractions were prepared and characterized by western blot (Fig 1A). A very pure OMM fraction, heavily enriched in the OMM marker protein VDAC, was isolated that was devoid of significant inner mitochondrial membrane (IMM)/mitochondrial matrix and
microsomal contamination (Fig 1A, left panel). Next the amidoxime reductase activity in the different fractions was determined using the N-hydroxyamine AR-H069927 as a substrate (Fig 1B). Over 20-fold enrichment in reductase activity was observed in the OMM fraction as compared to the homogenate (H) and the mitochondrial fraction (P10), whereas only low activity was observed in the microsomal fraction (MIC). Similar results were obtained using benzamidoxime as a substrate (data not shown). The protein levels for MOSC2, CYB5 and CYB5R3 were determined in these fractions (Fig 1A, right panel). MOSC2 is exclusively localized to the OMM and completely absent from the IMM and microsomal fractions, much like the OMM marker protein VDAC and correlated well with the amidoxime reductase activity. Since two forms of CYB5, the microsomal form (CYB5A) and the mitochondrial form (CYB5B) exist, the presence of both forms was determined in the different subcellular fractions. CYB5B was found to be present only in the OMM fraction (much like MOSC2), whereas CYB5A as well as CYB5R3 were found both in the microsomes and the OMM. The specificity of the two different CYB5 antibodies was confirmed using cDNA expressed CYB5A and CYB5B (Supplemental data, Fig S5). The distribution in the different subcellular fractions of both MOSC2 and CYB5B thus correlated well with the amidoxime reductase activity.

MOSC2 binds a benzamidoxime analogue - A proteomic approach using a radio-labeled (carbon-14) and cross-linkable benzamidoxime analogue (Supplemental data, Fig S2A) was set up to identify the amidoxime reductase. Incubation of the analogue with OMM in the presence of NADH resulted in the appearance of a single metabolite, much like its parent compound benzamidoxime (Supplemental data, Fig S2B) showing that it was a substrate for the enzyme, and, in addition, AR-H069927 was able to inhibit the metabolism of the radio-labeled benzamidoxime analogue (data not shown).

The radio-labeled benzamidoxime was incubated with the OMM in the presence of NADH in order to enhance binding of the substrate to the putative reductase candidate after which cross-linking was induced by exposure to UV-light, as described under experimental procedures. The cross-linked protein-substrate complexes were extracted by sequential detergent extraction using Triton X-100 followed by Zwittergent 3-14, a procedure that was able to extract and partially preserve the amidoxime reductase activity from the OMM (Supplemental data, Fig S3). Several proteins were cross-linked in the presence of NADH to the radio-labeled benzamidoxime, in particular in the 30-40 kDa region (Fig 2A, numbered 1-6). The protein bands corresponding to the radiolabeled bands were excised from the Coomassie stained part of the gel containing non-labeled OMM and were identified by mass spectrometric analysis (Fig 2B). Amongst the proteins tentatively identified were MOSC2 and CYB5R3. In addition, when MOSC2 was immunoprecipitated from OMM cross-linked with the radio-labeled benzamidoxime analogue, the immunoprecipitated MOSC2 protein band was shown to contain the radiolabeled substrate (Fig 2C). These results confirm that MOSC2 is able to bind to its substrate benzamidoxime.

Amidoxime reductase activity is regulated during adipogenesis - Previously we observed high amidoxime reductase activity not only in liver and kidney isolated from both male and female rats, but the highest specific activity was observed in the adipose tissue from both genders (6). We decided to use the 3T3-L1 cell line, an established cell model to study conversion of pre-adipocytes into mature adipocytes (26) to monitor the amidoxime reductase activity during this process. The NADH-dependent benzamidoxime reductase activity was analyzed in the mitochondrial fraction and the 6,500 x g supernatant (containing microsomes and cytosol) isolated from pre-adipocytes (day 0) and after 2 and 8 days of differentiation (Fig 3A). Very low benzamidoxime reductase activity levels were detected in undifferentiated pre-adipocytes or after 2 days of differentiation. After 8 days of differentiation however, a dramatic increase in benzamidoxime reductase activity was seen in both the mitochondrial fraction and the 6,500 x g supernatant. Moreover a 3-fold higher amidoxime reductase activity was associated with the mitochondrial fraction as compared to the 6,500 g supernatant (Fig. 3A) and was effectively inhibited by KCN. In addition, incubations with ximelagatran, a substrate with an amidoxime
mitochondria, in intact differentiated adipocytes revealed a 50-fold increased reduction rate as compared to undifferentiated pre-adipocytes (Supplemental data, Fig S6). Together these results suggest that the amidoxime reductase activity is present at high amounts in adipocyte mitochondria and regulated under adipogenic conditions.

Expression of MOSC2 and CYB5B in differentiated adipocytes - The expression of proteins suggested to be involved in the amidoxime reductase activity was also determined in the differentiated adipocyte cell model. The MOSC2 protein was undetectable in pre-adipocytes, but highly expressed in differentiated mature adipocytes (Fig 3B) and its presence correlated well with the amidoxime reductase activity. The MOSC1 levels were determined at the mRNA level since antibodies recognizing murine MOSC1 are unavailable. The MOSC1 mRNA level increased 170-fold in differentiated adipocytes compared to pre-adipocytes (data not shown), demonstrating that MOSC1 is also induced under adipogenic conditions. CYB5A was also strongly induced under adipogenic conditions, much like MOSC2 (Fig 3B). In contrast, the mitochondrial form CYB5B as well as CYB5R3 were expressed at similar levels in both pre-adipocytes and mature adipocytes and appear not to be regulated during adipogenesis (Fig 3B). Besides CYB5R3 two homologous cytochrome b₅ reductases exist in the mammalian genome namely CYB5R1 and CYB5R2. Since no commercial antibodies were available that recognize these proteins their mRNA levels were determined. Both genes were induced in mature adipocytes at the mRNA level 140-fold and 21-fold for CYB5R1 and CYB5R2 respectively (data not shown). Stearoyl-CoA desaturase (SCD1) was included as a positive control for adipocyte differentiation and as expected, was only expressed in mature adipocytes (Fig 3B).

MOSC2 and CYB5B are both required for amidoxime reductase activity - To investigate the involvement of MOSC2, CYB5A, CYB5B and CYB5R3 in the amidoxime reductase activity further, these proteins were down-regulated by siRNA in differentiated 3T3-L1 adipocytes. In brief, pre-adipocytes were differentiated for 5 days, after which they were trypsinized and transfected with the different siRNA constructs specifically targeting their respective targets (25). At first the efficiency of the siRNA-mediated down-regulation was evaluated by western blot analysis (Fig 4A). The efficiency of down-regulation was quantified by densitometry and is shown in Fig S7. Compared to the cell lysates from control siRNA transfected adipocytes, MOSC2, CYB5A and CYB5B were effectively down-regulated at the protein level by the siRNA treatment. Down-regulation of CYB5R3 also resulted in significant decrease in both CYB5R3 mRNA and protein levels with approximately 21% of the protein remaining after siRNA transfection while at the mRNA level 25% remained (Figs 4A, 5A and S7). Simultaneous down-regulation of CYB5A together with CYB5B or MOSC2 together with CYB5B also resulted in effective down-regulation of both of the targeted proteins (Fig 4A and Fig S7).

Down-regulation of MOSC2 alone resulted in a 65% decrease in the amidoxime reductase activity as compared to control transfected cells (Fig 4B), while the protein levels were down-regulated to 6% of those present in control transfected cells (Figs 4A and S7). Down-regulation of CYB5A had a slightly simulating effect on the reductase activity and although reproducible this was not statistically significant (Fig 4B), although protein levels were down-regulated to 6% of the levels present in control cells (Figs 4A and S7). In contrast down-regulation CYB5B resulted in a significant decrease in activity of about 50% (Fig 4B), with 31% of the protein remaining (Figs 4A and S7). Also when both forms of CYB5 (CYB5A + CYB5B) were simultaneously knocked-down, activity was decreased by about 50% (Fig 4B) with 10% or less of both proteins remaining (Figs 4A and S7). These results clearly indicated that MOSC2 and the mitochondrial form of cytochrome b₅ (CYB5B), but not the microsomal CYB5A are involved in the amidoxime reductase reaction.

Simultaneous down-regulation of MOSC2 and CYB5B did not further decrease the reductase activity as compared to down-regulation of the individual proteins (Fig 4B). Down-regulation of CYB5R3 did not significantly alter the reductase activity, even though the observed activity was slightly lower than control (Fig 4B). All activities were efficiently inhibited by KCN indicating that
the reductase activity was indeed specific (data not shown). Together, these data clearly demonstrate that MOSC2 as well as CYB5B, but not CYB5A and CYB5R3, are important components of the amidoxime reductase enzyme complex in differentiated adipocytes.

**MOSC1, CYB5R1 and CYB5R2 are not involved in the amidoxime reductase activity -** MOSC1 has been shown to be able to catalyze the reduction of amidoximes in reconstituted systems containing soluble and truncated forms of MOSC1, CYB5 and CYB5R3 (13,14). The involvement of MOSC1 in the amidoxime reductase activity in mature adipocytes was studied also by down-regulation of MOSC1 in these cells. As shown in Fig. 5A MOSC1 siRNA down-regulated MOSC1 mRNA levels to about 20 % of the control levels. The amidoxime reductase activity in MOSC1 down-regulated adipocytes was however not significantly affected (Fig 5B).

The involvement of CYB5R1 and CYB5R2 was also examined by siRNA-mediated down-regulation in the mature adipocyte cell model. Both CYB5R1 and CYB5R2 are efficiently down-regulated at the mRNA level with 20% or less of levels observed in control transfected cells (Fig 5A). The amidoxime reductase activity in these CYB5R1 and CYB5R2 down-regulated adipocytes was unaffected as compared to control cells (Fig 5B). In addition simultaneous down-regulation of CYB5R1, CYB5R2 and CYB5R3 did not inhibit the amidoxime reductase activity either (data not shown).

Finally the involvement of CYB5R in the amidoxime reductase activity was studied using the specific CYB5R inhibitor PTU (27). Although PTU was able to efficiently inhibit the CYB5R activity with almost 90% efficiency (Fig 6B), it was not able to significantly inhibit the benzamidoxime reductase activity in the OMM fraction (Fig 6A), again confirming that CYB5R is not an essential component of the amidoxime reductase enzyme system in these cells.

**Down-regulation of MOSC2 affects intracellular lipid levels.** Since MOSC2 is up-regulated during adipogenesis we investigated the effect of down-regulation of MOSC2 on the intracellular lipid content in mature adipocytes. Adipocytes were transfected with MOSC2 siRNA and 5 days after transfection cells were stained for intracellular lipids using Oil Red O (Fig 7A). Down-regulation of MOSC2 resulted in a significant decrease (17%, n=6; p<0.001) of the intracellular lipid levels as compared to control transfected cells. The intracellular fatty acids were also analyzed by GC/MS and the amounts for C14:0, C16:0, C16:1, C18:0 and C18:1 were determined relative to the internal standard (C19:0) (Fig 7B). Down-regulation of MOSC2 resulted in decreased levels of these fatty acids, the reduction being most pronounced for C16:0 and C16:1 with about a 28 % decrease.

**DISCUSSION**

In the present study we demonstrate for the first time in an intact cell system (mature adipocytes) that the mitochondrial amidoxime activity is dependent on the expression of MOSC2 and CYB5B. CYB5A, the microsomal form of cytochrome b5, although present in the outer mitochondrial membrane (OMM), is not critically involved in the mitochondrial amidoxime reductase activity. Furthermore, MOSC1, CYB5R1, CYB5R2 or CYB5R3 were found not to be crucial components of the mitochondrial amidoxime reductase enzyme system in mature adipocytes. In addition, our data show that MOSC2 affects the fatty acid levels in adipocytes suggesting a role of MOSC2 in lipogenesis, a finding consistent with its lipogenic regulation in the adipocytes.

Further characterization established that MOSC2 is exclusively localized to the OMM isolated from rat liver and its expression correlated well with the amidoxime reductase activity. Using a cross-linkable benzamidoxime analogue and immunoprecipitation we could show direct binding of a benzamidoxime substrate to MOSC2. Differentiation of pre-adipocytes into mature adipocytes resulted in an over 100-fold increase in mitochondrial associated amidoxime reductase activity and was paralleled by an increase in MOSC2 expression. Moreover siRNA-mediated down-regulation of MOSC2 attenuated the amidoxime reductase activity. An interesting observation was that while CYB5A was regulated under adipogenic conditions and CYB5B was not, down-regulation of CYB5B and not CYB5A inhibited the reductase activity, showing the involvement of the mitochondrial form but not the
microsomal form of cytochrome b$_5$ in the amidoxime reductase activity. In our adipocyte cell model we were unable to demonstrate the involvement of MOSC1 in the reductase activity, MOSC1 was like its homologue MOSC2 up-regulated in differentiated adipocytes but down-regulation of MOSC1 in these cells did not affect the amidoxime reductase activity. MOSC1 has previously been shown to be able to reduce amidoximes to amidines in vitro (13,14). The reason for this discrepancy is unclear but in the in vitro system the amidoxime reductase activity was reconstituted with recombinantly expressed truncated and soluble components CYB5B, CYB5R3 and MOSC1 or MOSC2.

Although soluble CYB5R3 can support the amidoxime reductase activity in vitro together with truncated soluble MOSC2 and CYB5B (13,14) we were unable to confirm its involvement in the adipocyte cell system. This despite the fact that we identified CYB5R3 as one of the proteins that was cross-linked by the radiolabeled substrate in the OMM and might possibly reflect not direct binding of the substrate to CYB5R3 but background caused by non-specific cross-linking. The closely related genes CYB5R1 and CYB5R2 were also found not to be crucially involved in the reductase activity, although they were up-regulated in mature adipocytes. Moreover the selective CYB5R inhibitor PTU (27) was unable to inhibit the amidoxime reductase activity in the OMM. Together these data suggest that another at present unknown reductase is involved in the NADH supported mitochondrial amidoxime reductase activity. Since the other two components MOSC2 and CYB5B are shown to be exclusively localized to the mitochondrial outer membrane, it is suggested that the unknown reductase is also present in this compartment.

CYB5B and CYB5A share considerable homology with each other (28) and although much is known about the function of CYB5A not much is known about the function of CYB5B. CYB5B has been suggested to be involved in the semidehydroascorbic acid reductase activity (29) and is thought to function as an activator of androgen synthesis by CYP17A1 (30). More recently increased CYB5B levels were observed in Hodgkin and aggressive non-Hodgkin lymphomas (31) and luteinizing hormone stimulated prostate cancer cell lines (32), though the exact role of elevated CYB5B levels in these cancers remains unclear. Here we show that CYB5B is exclusively associated with the OMM, although it has been suggested that CYB5B was present in both mitochondria and microsomes (30). In contrast CYB5A and CYB5R3 display a less defined localization and are found to be associated with both the microsomal membrane and the OMM, confirming previous observations (16,33). Based on the findings presented here we propose a novel function for CYB5B, namely in the reduction of N-hydroxylated amines together with MOSC2.

The amidoxime reductase enzyme kinetic parameters differed considerably between human liver microsomes and rat liver microsomes and mitochondria, with rat liver mitochondria being the most efficient. The amidoxime reductase activity in isolated rat liver OMM (data not shown) and in mitochondria isolated from differentiated adipocytes was effectively inhibited by KCN. Cyanide has been shown to be an inhibitor of the reduced form of molybdenum containing proteins such as xanthine oxidase, aldehyde oxidase and sulfite oxidase (34). The inhibitory effect of KCN on the amidoxime reductase activity in our system is in good agreement with these observations. It was recently reported that in an in vitro recombinant system using truncated MOSC1 and MOSC2 that cyanide was not able to inhibit the reductase activity and it was subsequently concluded that MOSC1 and MOSC2 do not contain a terminal sulfide ligand on the molybdenum as is the case in e.g. xanthine oxidase (14). One explanation for this inconsistency might be that we included the cyanide directly in the incubation mixture containing OMM or the mitochondrial fraction from adipocytes together with cofactor and substrate under aerobic conditions, while Wahl et al. incubated the recombinant proteins with cyanide under anaerobic conditions prior to the incubation with cofactor and substrate (14).

Molybdenum (Mo) is an essential trace element for nearly all organisms and Mo-dependent enzymes, such as sulfite oxidase and xanthine oxidase, are conserved in all three domains of life and usually catalyze redox reactions on carbon, nitrogen or sulfur centers (35,36). The MOSC (molybdenum cofactor sulforase C-terminal) family of proteins is conserved in most eukaryotes and is responsible for the sulfuration of the MOCO.
(molybdenum cofactor) present in xanthine dehydrogenase and aldehyde oxidase, a modification essential for their catalytic activity (37). In eukaryotes this modification is catalyzed by MOCOS (molybdenum cofactor sulfurrase) a protein consisting of two domains: an NH2-terminal NifS domain which possesses cysteine desulfurase activity and a C-terminal MOSC domain responsible for the transfer of the sulfur to the MOCO (35,37). Two other conserved members of the MOSC family only possess the MOSC domain and are called MOSC1 and MOSC2. The physiological role of these two proteins is at present not known, but MOSC2 or CDK7 (candidate diabetes associated kidney gene 7) was shown to be up-regulated in the kidney in a type II diabetes rat model and by glucose in human kidney cells (38). In Escherichia coli hypersensitivity towards N-hydroxylated derivatives of purines and pyrimidines was shown to be caused by inactivating mutations of two members of the MOSC superfamily namely YcbX and YiiM (39). Moreover it was suggested that both proteins were involved in the detoxification of these N-hydroxylated base analogues by reducing them to their corresponding amines. This finding together with ours presented in the present study suggests that MOSC domain containing proteins are capable of catalyzing reduction of N-hydroxylamines and that this activity could be one of the physiological functions of these proteins. In a recent integrated proteomic and transcriptomic high-throughput study, MOSC2 mRNA and protein levels were found to be down-regulated in colon cancer and it was suggested that MOSC2 could be used as a colon tumor biomarker (40).

Down-regulation of MOSC2 in mature adipocytes not only affected the amidoxime activity but also decreased the intracellular lipid levels as shown by Oil Red O staining and also the fatty acid levels were decreased in these MOSC2 down-regulated adipocytes. Together with the fact that high amidoxime reductase activity was found in the adipose tissue in rats (6), these results imply that this activity is the result of an lipogenic enzyme. In contrast the amidoxime reductase activity in liver fractions from starvation treated rats was decreased by 75% as compared to liver fractions from rats that received food ad libitum suggesting that during lipolytic conditions the activity is down-regulated (data not shown). Recently a genome-wide association study (GWAS) identified a single nucleotide polymorphism (SNP) linked to the MOSC1 locus that was associated with altered plasma concentrations of total cholesterol and low-density lipoprotein cholesterol, two important risk factors for coronary artery disease, suggesting a possible role of MOSC in lipoprotein metabolism (41). It must be noted that in the 3T3-L1 adipocyte cell model, the up-regulation of the reductase activity also coincided with that of the adipocyte differentiation marker SCD1 suggesting its regulation during adipogenesis. The exact role of the outer mitochondrial electron transport chain and MOSC2 in lipid synthesis requires further research.

In conclusion, we demonstrate using an intact cell system that MOSC2 and CYB5B are essential components of the mitochondrial amidoxime reductase enzyme system. The identification of MOSC2 in the amidoxime reductase system is in accordance with results obtained with soluble protein components in a reconstituted system (13,14). Our findings are schematically summarized in Fig. 8. Electrons from NADH are transferred via an as of yet unidentified reductase (FAD) to CYB5B, which in its turn will reduce the MOSC2-benzamidoxime complex and produce the reduced product benzamide. As both MOSC2 and CYB5B are exclusively localized in the OMM it is hypothesized that all components of the amidoxime reductase enzyme system are embedded in the outer mitochondrial membrane. Moreover the reductase activity is regulated under adipogenic conditions and down regulation of the terminal component MOSC2 resulted in decreased lipid synthesis, suggesting a possible physiological role of this enzyme system and its component MOSC2 in lipogenesis.

REFERENCES


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**FOOTNOTES**

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2 Both authors contributed equally to this work

3 The abbreviations used are: CYB5: cytochrome b5; CYB5R: cytochrome b5 reductase; OMM: outer mitochondrial membrane; PTU: 6-propyl-2-thiouracil.

**FIGURE LEGENDS**

**FIGURE 1. Amidoxime reductase activity is enriched in the outer mitochondrial membrane (OMM) isolated from female rat liver.** A. Characterization of the different sub-cellular fractions that were isolated from female rat liver as described under experimental procedures. Equal amounts of protein from the different fractions were analyzed by western blot for the presence of specific marker proteins for the different cellular compartments (left panel). Voltage-dependent anion-selective channel protein (VDAC) was used as a marker for the OMM, mitochondrial heat shock protein 70 (mHSP70) for the mitochondrial matrix/inner mitochondrial membrane (IMM) fraction and calnexin and ERp29 for the microsomal fraction (MIC). The MOSC2, CYB5A, CYB5B and CYB5R3 protein levels in the different subcellular fractions are shown in the right panel. Arrow head indicates CYB5B, star indicates non-specific band. B, The amidoxime reductase activity in the different sub-cellular fractions isolated from female rat liver was determined by monitoring the reduction of the hydroxylamine AR-H069927. The homogenate (H), mitochondrial pellet (P10), OMM, IMM/matrix (IMM) and microsomes (MIC)
were incubated with the substrate AR-H069927 and formation of the reduced metabolite AR-H067637 was determined by HPLC analysis. Results are presented as mean ± S.D. (n=3). ***, p < 0.001 compared to the homogenate.

**FIGURE 2.** MOSC2 binds to its amidoxime substrate. **A,** Rat liver OMM was incubated with carbon-14 radio-labeled benzamidoxime azide (AZ13228184-14C) in the presence (+) or absence (-) of NADH and the substrate was cross-linked by exposure to UV light. The cross-linked OMM proteins were extracted by sequential detergent extraction and cross-linked complexes were separated by SDS-PAGE. The gel was dried and 14C-labeled benzamidoxime cross-linked proteins were visualized by autoradiography. Bands that showed increased labeling in the presence of NADH are numbered 1 to 6. Molecular weight markers are indicated. **B,** Coomassie blue stain of the detergent extracted OMM fraction where the indicated bands that corresponded to the radio-labeled bands shown under A, were excised and analyzed by mass spectrometric analysis. The proteins that were identified are shown to the right. ADT2: ADP/ATP translocase 2; CYB5R3: cytochrome b5 reductase 3; VDAC: voltage-dependent anion-selective channel protein 1; NDUFA9 protein: NADH dehydrogenase [ubiquinone] 1 alpha subcomplex subunit 9; MOSC2: molybdenum cofactor (MOCO) sulfurase C-terminal containing 2; ATP6V0D1: ATPase H+, lysosomal 38 kDa, transporting V0 subunit d1. Further details for the protein identification are shown in the supplemental data Table S1. **C,** MOSC2 is cross-linked to the radio-labeled benzamidoxime analogue. 14C-labeled benzamidoxime azide was UV cross-linked to the OMM in the presence of NADH, proteins were solubilized and immunoprecipitated with a MOSC2 specific antibody. The immunoprecipitate was analyzed by western blot for MOSC2 (middle panel) and the same membrane was also analyzed by autoradiography (left panel). As a control for MOSC2, 5 µg of OMM was included (right panel). Arrow head indicates MOSC2; star indicates IgG.

**FIGURE 3.** Amidoxime reductase activity is regulated during differentiation of pre-adipocytes into mature adipocytes. **A,** Benzamidoxime reductase activity measured in the mitochondria and the 6,500 x g supernatant containing both the microsomes and cytosol isolated from pre-adipocytes (0 days) and 2 and 8 days differentiated adipocytes. Incubations were performed with 20 µM benzamidoxime in cells at 0, 2 and 8 days after differentiation start for 30, 20 and 10 min, using 30, 25 and 10 µg mitochondrial or 6,500x g supernatant proteins, respectively. Incubations were performed in the absence (grey bars) and in the presence (white bars) of KCN. Results are presented as mean ± S.D. (n = 3). ***, p < 0.001. **B,** Protein expression levels of MOSC2, CYB5A, CYB5B and CYB5R3 in the cell lysates isolated from pre-adipocytes (0 days) and 8 days differentiated adipocytes. Also shown are the levels for SCD1 (stearoyl-CoA desaturase 1), a marker for the adipocyte differentiation and calnexin as a loading control.

**FIGURE 4.** MOSC2 and CYB5B are essential for the amidoxime reductase activity. **A,** The efficiency of siRNA-mediated down-regulation of MOSC2, CYB5A, CYB5B, CYB5R3, CYB5A together with CYB5B and CYB5B with MOSC2 in differentiated 3T3-L1 adipocytes as determined by western blot analysis. Cell lysates prepared from siRNA transfected adipocytes were separated by SDS-PAGE and analyzed by western blot using specific antibodies as shown. Calnexin is included as a loading control. Quantification of the down-regulated proteins is shown in supplemental data Fig S7. **B,** The effect of siRNA-mediated down-regulation of MOSC2,
CYB5A, CYB5B, CYB5R3, CYB5A together with CYB5B and CYB5B with MOSC2 in differentiated 3T3-L1 adipocytes on the benzamidoxime reductase activity. The same cell lysates analyzed by western blot under A were used. Activity data is shown relative to the benzamidoxime reductase activity in control siRNA transfected adipocytes (0.56 nmol benzamidine formed/min/mg protein). Results are presented as mean ± S.D. (n = 3). ***, p < 0.001.

FIGURE 5. **MOSC1 is not involved in the amidoxime reductase activity.** A, The efficiency of the siRNA-mediated down-regulation of MOSC1, CYB5R1, CYB5R2 and CYB5R3 in differentiated adipocytes as determined by RT-PCR. B, The effect of the siRNA-mediated down-regulation of MOSC1, CYB5R1 and CYB5R2 on the benzamidoxime reductase activity in differentiated adipocytes. Activity data is shown relative to the benzamidoxime reductase activity in control siRNA transfected adipocytes. Results are presented as ± S.D. (n = 3). *, p < 0.05.

FIGURE 6. **Inhibition of the CYB5R activity by PTU does not affect the amidoxime reductase activity.** A, Increasing concentrations of 6-propyl-2-thiouracil (PTU) do not inhibit the benzamidoxime reductase activity. OMM was incubated with benzamidoxime in the absence (control) and presence of 0.05 mM, 0.5 mM and 5.0 mM PTU and the benzamidoxime reductase activity was determined. Activity data is shown relative to the benzamidoxime activity in control OMM. B, The efficiency of the CYB5R inhibitor PTU was evaluated in rat liver microsomes by monitoring the reduction of 2,6-dichlorophenolindophenol in the absence (control) and the presence of 0.05 mM, 0.5 mM and 5.0 mM PTU. Activity data is shown relative to control microsomes. Results are presented as mean ± S.D. (n = 3). **, p < 0.005.

FIGURE 7. **MOSC2 levels affect the intracellular lipid content.** A, Mature adipocytes were transfected with control or MOSC2 siRNA and 5 days post-transfection stained with Oil Red O. The dye was eluted in isopropanol and quantified spectrophotometrically at 510 nm. Results are presented as mean ± S.D. (n = 6). ***, p < 0.001. B, Relative amounts of fatty acids in control and MOSC2 siRNA transfected adipocytes. Transfected adipocytes were harvested 5 days post-transfection and cellular fatty acids were extracted, converted into methyl esters and analyzed by GC-MS. An internal standard (IS), nonadecanoic acid (C19:0) was added to the cells before extraction as described under Experimental Procedures.

FIGURE 8. **Proposed reaction scheme for the mitochondrial amidoxime reductase enzyme system.** For more details see discussion. FAD: flavin adenine dinucleotide (oxidized), FADH₂: flavin adenine dinucleotide (reduced).

TABLES

**TABLE 1.** Kₘ and Vₘₐₓ values for the amidoxime reductase activity in microsomes and mitochondria isolated from rat and human liver.** Shown are the values in the absence (-KCN) and the presence (+KCN) of potassium cyanide. Values in the absence of KCN are considered non-specific activity. The specific benzamidoxime activity is represented by the KCN inhibitable values. Further details can be found in supplemental data Fig S4.
### Benzamidoxime reductase activity

<table>
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<td>24.6 µM</td>
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<tr>
<td>$V_{\text{max}}$</td>
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<td>2.01</td>
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<tr>
<td>$K_m$</td>
<td>837</td>
<td>4953</td>
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Figure 1

A

VDAC
mHSP70
calnexin
ERP29

H P10 OMM IMM MIC

MOSC2
CYB5A
CYB5B
CYB5R

H P10 OMM IMM MIC

B

Formation of AR-H067637 (nmol/mg protein/min)

0 10 20 30 40 50

***

H P10 OMM IMM MIC
Figure 3

A

Formation of benzamidine (nmol/min/mg protein)

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B

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<td>Calnexin</td>
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Figure 4

A

MOSC2
CYB5A
CYB5B
CYB5R3
calnexin

control CYB5A CYB5B CYB5R3 MOSC2 CYB5A+CYB5B CYB5B+MOSC2

siRNA

B

Relative benzamidoxime reductase activity (% of control)

control CYB5A CYB5B CYB5R3 MOSC2 CYB5A+CYB5B CYB5B+MOSC2

siRNA
Figure 5

A

Fold difference ($2^{-\Delta\Delta C_T}$)

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B

Relative benzamidoxime reductase activity (% of control)

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<td>CYB5R2</td>
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Figure 6

A  Relative benzamidoxime reductase activity (% of control)

B  Relative cytochrome b: reductase activity (% of control)
Figure 7

A

Oil Red O staining absorbance at 500 nm

control MOSC2

B

Fatty acid / IS

control siRNA MOSC siRNA2

C14:0 C16:0 C16:1 C18:0 C18:1 C19:0 (IS)
Figure 8

NADH → FAD → FADH₂ → Fe(II) → Fe(III) → CYB5B → MOSC2 → benzamidine → benzamidoxime
An amidoxime reductase system in adipocyte mitochondria containing cytochrome b5 type B (CYB5B) and molybdenum cofactor sulfurase C-terminal containing 2 (MOSC2) of importance for lipid synthesis
Etienne P.A. Neve, Asa Nordling, Tommy B. Andersson, Ulf Hellman, Ulf Diczfalusy, Inger Johansson and Magnus Ingelman-Sundberg

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