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Regulation of α- and β-secretase activity by oxysterols: Cerebrosterol stimulates processing of APP via the α-secretase pathway

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Abstract

The cholesterol 24-hydroxylase encoded by the gene *CYP46* is expressed almost exclusively in central nervous system (CNS) neurons and catalyzes the formation of 24S-hydroxycholesterol (24S-OHC) from cholesterol. This conversion corresponds to a major pathway for excretion of excess cholesterol from the brain. There is a significant flux of another oxysterol, 27-hydroxycholesterol (27-OHC) from the circulation into the brain. Polymorphisms within the CYP46A1 gene have been associated with Alzheimer's disease (AD) incidence.

In this study, we examined the effects of 24S-OHC and 27-OHC on the α- and β-secretase activity in the human neuroblastoma cell line SH-SY5Y. Furthermore, we examined the effects of the two oxysterols on the levels of extra- and intracellular proteins of secreted APPα (sAPPα). Our findings suggest that 24S-OHC may exert a unique modulatory effect on APP processing and that this oxysterol increases the α-secretase activity as well as the α/β-secretase activity ratio. The possibility is discussed that the ratio between 24S-OHC and 27-OHC is of importance for the generation of amyloid in the brain.

Keywords: 24S-Hydroxycholesterol; 27-Hydroxycholesterol; Amyloid precursor protein; APP processing; α/β-Secretase activity ratio; Alzheimer's disease

Accumulating evidence indicates the amyloid cascade as an essential event in the pathogenesis of Alzheimer's disease (AD) [1]. The proteolytic cleavage of the amyloid precursor protein (APP) is conducted by three related proteases, i.e., α-, β-, and γ-secretase. Cleavage by α-secretase (a disintegrin and metalloproteinase 10) [2,3] which occurs within the sequence of Aβ, prevents the formation of Aβ and results in the production of a secreted ectodomain (αAPP) and a shorter COOH-terminal fragment of APP (αCTF) that is further proteolysed by the γ-secretase complex. The production of amyloid β (Aβ) is mediated by the concerted action of two different secretases, namely β-secretase (BACE) [4] and γ-secretase [5], showing a proteolytic action on the amyloid precursor protein (APP).

Recent years have seen heightened interest in the molecular connection between brain cholesterol homeostasis and AD [6]. The present consensus is that local elevations in free cholesterol levels favour the cleavage of APP at the proamyloidogenic β- and γ-sites [7]. A recent report indicates that cholesterol distribution rather than total levels correlate with altered APP processing by statins [8]. In addition to high levels of cholesterol, the brain also contains remarkably high amounts of 24S-hydroxycholesterol (24S-OHC), a cholesterol oxidation product also known as cerebrosterol [9,10]. The formation of 24S-OHC is catalyzed by the cholesterol 24-hydroxylase which is expressed almost exclusively in CNS (central nervous system) neurons by the gene *CYP46* [11]. Hydroxylation of cholesterol

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in the steroid side-chain dramatically increases its capacity to pass lyophilised membranes [12,13] and conversion into 24S-OHC is considered to be a major pathway for excretion of excess cholesterol across the blood–brain barrier [14,15]. In addition to this important role in brain cholesterol balance, 24S-OHC is one of the most potent endogenous ligands for the liver X receptor (LXR), activation of which has been reported to be both anti-amyloidogenic and anti-inflammatory [16,17].

It was recently shown that there is a significant net flux of another side-chain oxidized oxysterol, 27-hydroxycholesterol (27-OHC) in the opposite direction, from the circulation into the brain [18]. Due to a very active metabolism [19] the levels of 27-OHC in the brain are relatively low.

In AD, the levels of 24S-OHC in the brain are decreased whereas the levels of 27-OHC are increased [20]. The decreased levels of 24S-OHC may be due to the decreased number of metabolic active neuronal cells, whereas the increased levels of 27-OHC may be due to decreased metabolism.

The possibility has been discussed that the changed levels of 24S-OHC and 27-OHC in the brain of AD patients may be of some pathogenetic importance [21]. In one study 24S-OHC was shown to increase the production of amyloid by cultured cells [22]. In a more recent study 24S-OHC was reported to inhibit the formation and secretion of both Aβ40 and Aβ42 by murine primary neurons [23]. The enzyme system affected was however not identified.

In the present work, we sought to address the effects of both 24S-OHC and 27-OHC on the activity of α- and β-secretase, the two key enzymes involved in APP processing in cultured SH-SY5Y neuroblastoma cells.

Material and methods

Cell culture reagents. Purified 24S-hydroxycholesterol (24S-OHC) was obtained from Bio-Nuclear Scandinavia AB (Stockholm, Sweden) and 27-hydroxycholesterol (27-OHC) from Steraloids (Newport, Rhode Island, USA). A stock of each oxysterol was prepared in ethanol and stored in the dark at −70 °C. All cell culture reagents were obtained from Invitrogen.

Cell culture treatment with 24S-OHC and 27-OHC. Human neuroblastoma SH-SY5Y cells were cultured in 60-mm cell culture dishes using Dulbecco's modified Eagle's medium:Ham's F12 with Glutamax (DMEM:F12; 1:1; v/v) and 10% FBS. No antibiotics were included in the medium. When the cells had reached 80% confluence, the cells were incubated with hydroxycholesterol by incubation for 24-h at 37 °C in DMEM:F12 with 24S-OHC, 27-OHC or a mixture of 24S- and 27-OHC (1:1) to a final concentration of 5 μM/mL. Cells incubated without hydroxycholesterol were used as control.

Cell culture transfection with CYP46. SH-SY5Y cells were transfected with the complete open-reading frame of cholesterol 24-hydroxylase of either human (CYP46A1) or murine (CYP46a1) origin under the control of a viral promoter, using lipofectamine 2000. A1:1 rations of DNA:lipofectamine was used in all transfections. The cells were transfected and allowed to recover for 72 h at 37 °C in DMEM:F12 with Glutamax and 10% FBS. To ensure that the effect was specific for the introduced plasmid, and not merely the addition of DNA or cationic lipids (i.e., the transfection reagent) we performed control experiments using an unrelated plasmid (the commonly used reporter plasmid pGL3-basic = mock) or crude lipofectamine.

Determination of α- and β-secretase activity in SH-SY5Y cells. The α- and β-secretase activities were determined using a Fluorometric α- and β-Secretase Activity Kits obtained from R&D Systems (UK). The cells were lysated using 2 mL of cell extraction buffer, centrifuged at 10,000*g* for 1 min at 4 °C and 500 μL of the supernatants was removed. Protein quantifications were made using the BCA protein assay (Pierce Biochemicals, USA). The supernatants were transferred (50 μL/well; 50 μg total protein) to a 96-well microplate (Nunc F16 Black MaxiSorpTM, Nunc, Denmark), with 50 μL 2× reaction buffer and 5 μL substrate added to a volume of 105 μL. The substrate consisted of the APP peptides YEVHHQKLV (α-secretase) and REEVNLDAEFKR (β-secretase) respectively, using the reporter system EDANS/DABCYL. The plate was sealed with a plastic film, gently mixed by tapping and incubated in the dark for 2-h at 37 °C. The fluorescent emission from EDANS was analyzed and measured at $\lambda = 345$ nm (excitation) and $\lambda = 500$ nm (emission). High fluorescence was correlated with high enzymatic activity. All measurements were performed using a Safire II microplate reader with Magellan PC software (v6.2; Tecan Austria Gmbh, Austria) at 37 °C.

Sample preparation for ELISA analysis of secreted APP. SH-SY5Y cells at 80% confluence (equivalent to approximately 1×10^7 cells per plate) were treated with the oxysterols as described above, and medium was removed and saved for ELISA analysis. The cells were rinsed with 5 mL PBS pH 7.4 and solubilized in 500 μL lysis buffer (1× protease inhibitor complete (Roche) and 1% Triton (Sigma) diluted in PBS pH 7.4). Following incubation at 4 °C for 20 min the cell lysates were collected, mixed and centrifuged at 13,000 rpm for 10 min at 4 °C. The supernatants, containing the released proteins, were saved at −20 °C until required for ELISA analyses. The protein contents of the cell lysates were measured by DC protein assay according the instructions of the manufactures (Bio-Rad).

Double-antibody sandwich ELISA analysis of secreted APP. One hundred and fifty microliters the rabbit polyclonal antibody, AB5076 (Chemicon) 1:40,000, diluted in a coating buffer (0.05 M carbonate–bicarbonate buffer, pH 9.6), were used to coat the individual wells of the 96-well microtiter plates (Immuno Modulies, Maxisorp, Cert Nunc 469949, VWR). Incubation was performed over night (ON) at 4 °C by shaking. Subsequently, the coating buffer was discarded and the wells were blocked with 250 μL/well blocking buffer (5% bovine serum albumin (BSA) in coating buffer) and incubating for 2-h at RT by shaking horizontally. The blocking buffer was discarded and the wells were washed 3× with 300 μL TBS–T (0.05% Tween 20, 0.01% NaN_3 [26628-22-8] (both from Sigma), in TBS pH 7.4) buffer. As antigen, either 150 μL cell medium or 75 μL cell lysate was added to the wells and incubated ON at 4 °C by shaking. Antigens were then discarded and the wells were washed two times with 300 μL/well PBS, pH 7.4. soluble APP-alpha (sAPPα) was detected by applying of 100 μL/well of the mouse monoclonal antibody against human soluble APP (Chemicon) 1:5000, diluted with TBT–T–BSA (0.1% BSA, 0.05% Tween 20, 0.02% NaN_3, in TBS). The plate was then incubated ON at 4 °C. The detection antibodies were discarded and the wells were washed 3× with 300 μL/well PBS–T. One hundred and fifty microliters/well of the alkaline phosphates labelled bovine anti-mouse IgG antibodies, 1:2500 diluted in TBT–T–BSA (sc-2377, Santa Cruz Biotechnology) was applied and incubated 2.5-h at RT. The wells were washed 3× with 300 μL with TBS–T. 200 μL/well substrate (4-nitrophenyl phosphate–$Na_2{\cdot}6H_2O$, Fluka, Biochemica, [71770]) diluted in DEA buffer (1 M diethanolamine, 0.5 M $MgCl_2{\cdot}6H_2O$, 0.01 % NaN_3 in H_2O pH 9.8) was applied and incubate at RT for 2.5-h in darkness. The optical density was read at 405 nm.

Direct ELISA analysis of secreted APP. In contrast to the Double-antibody sandwich ELISA, plate wells were coated directly with either 150 μL cell medium or 75 μL cell lysate and incubated ON at 4 °C. Using the above mouse monoclonal antibody against human soluble APP we were able to detect total sAPP in these wells.

Statistical analysis. The OD-values of all samples, both from cell medium and cell lysates were normalized by dividing OD-values with the respective protein content in the cell lysate from each cell-plate. Data were checked for normality and analyzed for statistical significance using Student's *t*-test and analysis of variance (ANOVA). Statistical significance was determined at $p < 0.05$. Statistical analysis was performed using

Statistica PC software (v.6.0; StatSoft Inc., USA). All values are expressed as percentage of the mean value of the control group ± SEM.

Results

α- and β-Secretase activity in SH-SY5Y cells exposed to 24S-OHC and 27-OHC

SH-SY5Y cells exposed to 24S-OHC exhibited a significant increase in the α-secretase activity ($p < 0.01$). 27-OHC and the combination of 24S-OHC and 27-OHC did not have any significant effect on the α-secretase activity. The β-secretase activity was found to be significantly decreased after incubation with 24S-OHC ($p < 0.05$). 27-OHC and the combination treatment with 24S-OHC and 27-OHC did not cause any significant changes in the activity of β-secretase. The α/β-secretase activity ratio was significantly increased in *SH-SY5Y* cells exposed to 24S-OHC (Fig. 1).

α- and β-Secretase activity after transfection with CYP46

The α-secretase activity was found to be significantly increased in *SH-SY5Y cells* transfected with CYP46A1 ($p < 0.0005$). Cells transfected with Cyp46a1 also showed a significant increase in the α-secretase activity ($p < 0.0001$), while mock transfected cells had decreased activity (Fig. 2). In contrast Cyp46a1 had no effect on β-secretase activity and CYP46A1 transfection showed an increase in the β-secretase activity which was not statistically significant. The net effect of these alterations was an increase in the α/β-secretase activity ratio in cells transfected with Cyp46a1 or CYP46A1 (Fig. 2).

sAPP ELISA analysis

The intracellular (cell lysate) and extracellular (cell medium) levels of the total secreted APP (sAPP) and the alpha form of the sAPP (sAPPα) were measured in the SH-SY5Y cells, treated with the side-chain oxidized cholesterol metabolite 24S-OHC, 27-OHC, or the combination 24S-OHC and 27-OHC. As Fig. 3 shows, the relative extra- and intracellular level of the total sAPP, in the cells treated with 24S-OH was significantly up regulated compared to that of the untreated cells ($p < 0.01$ and $p < 0.001$, respectively). In contrast, treatment with the 27-OH alone or the combination of 27-OH and 24S-OH has not effect on either intracellular of extracellular levels of total sAPP. The intra- and extracellular levels of the sAPPα were also significantly increased after 24S-OHC treatment compared to the control ($p < 0.001$). Again, 27-OHC either alone or in combination with 24S-OHC had no effect on the levels of sAPP (Fig. 3).

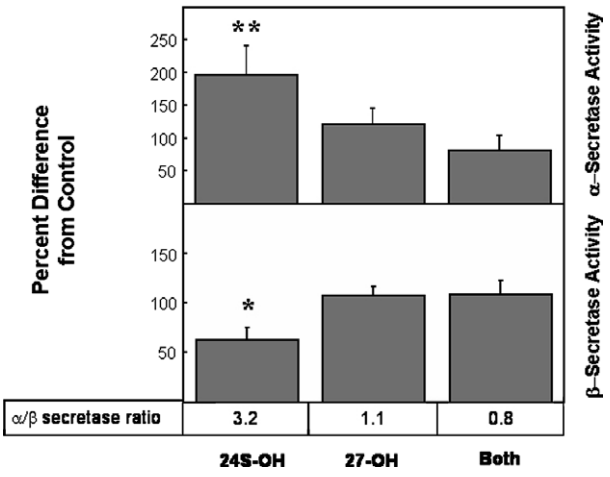

Fig. 1. α- and β-Secretase activity in cells exposed to 24S-OH and 27OH. $^{*}p < 0.05$, $^{**}p < 0.01$.

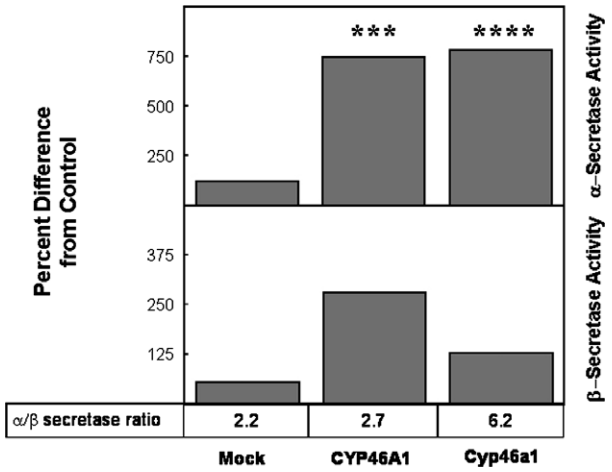

Fig. 2. α- and β-Secretase activity after transfection with human CYP46A1 and murine CYP46a1. Mock designates the unrelated plasmid (the commonly used reporter plasmid pGL3-basic). $^{***}p < 0.0005$, $^{****}p < 0.0001$.

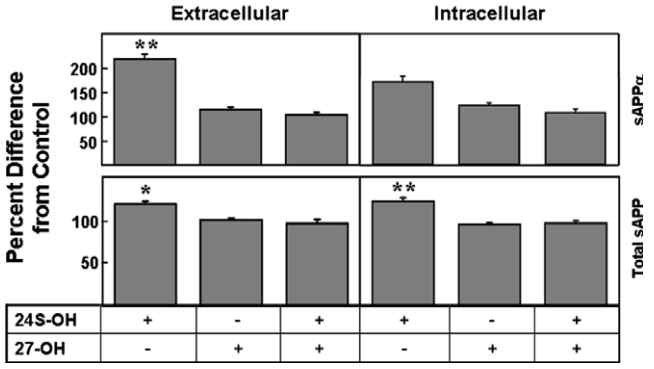

Fig. 3. Intra- and extracellular levels of sAPPα and total sAPP measured by ELISA assay in cells exposed to 24S-OH and 27OH. $^{*}p < 0.01$, $^{**}p < 0.001$.

Discussion

Under the *in vitro* conditions used here, the α-secretase activity and the relative protein values of extracellular sAPPα

and total sAPP were significantly increased, whereas the β-secretase activity was reduced by 24S-OHC, demonstrating that 24S-OHC favours APP α-cleavage. In order to compare the differential effects of cerebrosterols on the secretase activities we also calculated the α/β-secretase activity ratio. This ratio was increased after exposure of cells to 24S-OHC, and this was the case also in cells transfected with human and mouse CYP46. Although, the effects of the oxysterols were less marked than in the study by Brown et al., our study confirms and expands the latter investigation [23].

Stimulation of α-secretase activity with subsequent increased levels of soluble APP (APPsα) is a beneficial approach for the treatment of AD. In principle, proteolytic cleavage of APP within the Aβ sequence precludes formation of the amyloid peptides derived from alternative proteolysis of APP with the β-secretase cleaving at the N terminus and the γ-secretase(s) at the C terminus of Aβ peptides. On the other hand, APPsα has trophic effects on cerebral neurons in culture, stimulates neurite outgrowth, and regulates synaptogenesis [1,24]. Increased α-secretase activity has been reported previously during *in vitro* studies using cholesterol-lowering drugs [25,26].

Our results suggest that 24S-OHC modifies APP trafficking by a mechanism different from that of 27-OHC, and that this relates to its distinct regulation of cellular cholesterol metabolism. Recent data suggest that astrocytes are uniquely sensitive to 24S-OHC-mediated up regulation of LXR-responsive genes involved in cholesterol efflux such as ABCA1, ABCG1, and apoE [27].

The lower effect of 27-OHC on APP cleavage is interesting in relation to the fact that this oxysterol is fluxing from the circulation into the brain. The possibility must be considered that 27-OHC may replace the more active 24S-OHC and thus reduce the inhibitory effect on generation of amyloid. Since there is a close relation between levels of cholesterol and 27-OHC in the circulation, the possibility must be considered that the flux of 27-OHC into the brain may be part of the yet unexplained link between hypercholesterolemia and AD [21].

In summary, the stimulation of α-secretase activity and an increase in α-secretase cleaved soluble APP (APPsα) is a beneficial approach for the treatment of AD [24]. Changes in the α/β-secretase activity ratio could be a practical method for monitoring end stage alterations in the APP processing pathway in response to different pharmacological treatments. In view of the stimulatory effect of 24S-OHC on the non-amyloidogenic pathway, upregulation of CYP46 may be a possible strategy for developing future pharmacological treatment for AD.

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