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Simvastatin alters membrane cholesterol distribution and beta-amyloid levels in brains of female APP751$_{SL}$ mice

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Dedicated to Prof. Dr. Theo Dingermann, Frankfurt, on the occasion of his 65th birthday.

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Statins (HMG-CoA reductase or CSE-inhibitors) strongly reduce the cellular amyloid-beta protein production by modulating the processing of amyloid precursor protein (APP) *in vitro*. Several *in vivo* studies have addressed this important issue in transgenic mouse models with inconsistent results. Recently, we showed that simvastatin alters cholesterol distribution in synaptosomal membranes (SPM) *in vivo*. In the present study, we tested whether these changes in cholesterol membrane distribution affect APP-processing *in vivo*. Female APP751$_{SL}$ mice were force-fed with simvastatin (50 mg/kg b.wt.) by oral gavage over a time period of 3 weeks. Our data show that chronic simvastatin treatment decreased cholesterol levels in the brain and affected cholesterol distribution within SPM. Simvastatin significantly increased the levels of insoluble A$\beta_{1\text{-}40}$ and A$\beta_{1\text{-}42}$ but reduced levels of soluble A$\beta_{1\text{-}40}$ in the brain. The reduction of soluble A$\beta_{1\text{-}40}$ levels in the brain was associated with an increase of plasma-levels of A$\beta_{1\text{-}40}$ in simvastatin-treated animals that may indicate enhanced A$\beta_{1\text{-}40}$-clearance from the brain. Although the observed alteration in transbilayer cholesterol is likely to be involved in changes of APP processing by α-, β- and γ-secretase, we cannot exclude other potential mechanisms of statins such as lipid and non-lipid related, pleiotropic effects. Our data were evaluated in reference to published studies and a possible gender effect was identified.

1. Introduction

Alzheimer's disease (AD) is one the most common neurodegenerative disorders. Neuropathological hallmarks of AD are amyloid plaques (Mattson 2004), which contain beta-amyloid peptides (Aβ). Amphipathic Aβ tend toward self-aggregation and accumulation, which initiates a cascade that triggers complex pathological reactions eventually leading to neuronal dysfunction and cell death (Golde et al. 2006; Haass and Selkoe 2007; Hardy and Selkoe 2002). Aβ aggregation is a concentration-dependent phenomenon, which is initiated via a seeded polymerization reaction (Caughey and Lansbury 2003). Monomeric Aβ initially forms poorly characterized nuclei that assemble into larger aggregates. The nucleation of Aβ is followed by oligomer and protofibril formation, which ultimately leads to insoluble amyloid fibril assembly (Cernescu et al. 2012; Golde et al. 2006). Current research suggests that soluble oligomeric forms of Aβ may play a major role in AD pathophysiology (Golde et al. 2006; Haass and Selkoe 2007). Soluble amyloid oligomers bind specifically to neurons, disrupt dendritic spines (Lacor et al. 2007) and inhibit hippocampal long-term potentiation (Li et al. 2009). Most Aβ is composed of 38-43 amino acid residues, all deriving from the transmembrane amyloid precursor protein (APP) after the sequential proteolytic cleavage by different secretases. There are two concurrent cleavage pathways. In the non-amyloidogenic pathway, APP is first cleaved at the N-terminus by α-secretase within the Aβ sequence and precludes the formation of Aβ. Alternatively, Aβ is produced in the amyloidogenic pathway that involves first APP cleavage at the N-terminus by β-secretase. β-Secretase is a membrane-anchored aspartyl protease with its active site in its ectodomain and was shown to be a member of the memapsin family. Subsequent cleavage by γ-secretase, releases Aβ with varying lengths, the most common being A$\beta_{(1\text{-}40)}$ and A$\beta_{(1\text{-}42)}$. This model of Aβ formation is now widely accepted. However, the presence and exact contribution of both intracellular and extra-cellular Aβ to the pathological outcome is still an issue of controversy and interest in the field (Haass and Selkoe 2007; Savonenko et al. 2012).

APP processing by α-, β-, and γ-secretase, which are strictly associated with cellular membranes, strongly depends on membrane biophysical properties (Peters et al. 2009) and is modified by the membrane disordering effects of Aβ peptides (Müller et al. 2001). The activity of the γ-secretase is especially sensitive to alterations of hydrophobic membrane lipid domains (Gamerdinger et al. 2008). Hydrophobic membrane lipid domains include lipid rafts, caveloae, and two other domains of the membrane namely the two leaflets of the bilayer (Wood et al. 2011). There are substantial differences in the two leaflets including for example electrical charge, thickness, fluidity, and lipid distribution. Cholesterol, a major lipid in membranes accounting for over 40 mol% of synaptic plasma membrane lipids is also asymmetrically distributed (Wood et al. 2011). We have shown that the cholesterol distribution within lipid bilayers can be pharmacologically modified by administration of statins *in vivo* (Kirsch et al. 2003). Burns et al. (2006) confirmed these results and reported that statin-induced changes

in membrane cholesterol distribution led to a reduction of murine $A\beta_{1-40}$ and $A\beta_{1-42}$ brain levels in non-transgenic mice. To further explore the relation between statin, membrane cholesterol, and APP processing, we investigated the effects of subchronic simvastatin treatment on synaptosomal membrane cholesterol distribution and Aβ levels in a transgenic mouse model that harbours human APP with the Swedish and London mutation ($APP751_{SL}$).

2. Investigations and results

2.1. Cholesterol levels and distribution in synaptosomal plasma membranes

Cholesterol levels of synaptosomal plasma membranes (SPM) isolated from simvastatin treated mice decreased significantly by about 15 % (Fig. A). This reduction in total cholesterol levels is due to a depletion of the cytofacial membrane leaflet of SPM as shown in Figure B. In SPM of the control group, approximately 78% of cholesterol was located in the cytofacial leaflet, whereas after simvastatin treatment approximately 60% was found in the cytofacial membrane leaflet (Fig. B). Cholesterol levels in the exofacial membrane leaflet increased, but did not change significantly after simvastatin treatment (Fig. B).

2.2. Simvastatin affects $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in the brain of treated mice

The values for soluble and insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ are given in Table 1. In general, levels of insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ in brains of control and simvastatin treated Thy-1-APP_{751SL} mice were significantly higher than levels of soluble $A\beta_{1-40}$ and $A\beta_{1-42}$. Soluble Aβ tends to form highly toxic oligomers (Cernescu et al. 2012). Against this background the finding that simvastatin treatment led to a small, but significant reduction of soluble $A\beta_{1-40}$ in the brain of Thy-1-APP_{751SL} mice might indicate a beneficial effect (Table 1). However, levels of insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ were significantly increased in the brain of simvastatin-treated mice (Table 1).

2.3. Simvastatin affects $A\beta_{1-40}$ levels in the plasma of treated mice

In the plasma of simvastatin treated mice, levels of $A\beta_{1-40}$ were significantly elevated compared to controls (Table 1).

3. Discussion

We report that chronic simvastatin treatment affects membranous cholesterol homeostasis in the brain of transgenic Thy1-$APP751_{SL}$ mice. Cholesterol is not uniformly distributed within the plasma membrane. About 75 % of total membrane cholesterol are located within the cytofacial leaflet of the plasma membrane (Wood et al. 2011). Accordingly, we report that in SPMs of the control group, approximately 78% of cholesterol was located in the cytofacial membrane leaflet, which is in line with earlier findings (Burns et al. 2006; Igbavboa et al. 1996; Kirsch et al. 2003). Our data show that levels of free cholesterol in SPM of simvastatin-treated mice were significantly decreased and the depletion of membrane cholesterol predominantly occurred in the cytofacial membrane leaflet. These observations confirm our earlier findings in SPM isolated from non-transgenic C57BL/6J mice (Kirsch et al. 2003). In agreement with our findings Burns et al. (2006) reported that simvastatin reduced cytofacial cholesterol levels. However, their data further show that simvastatin correspondingly increased exofacial leaflet cholesterol levels. However, in the present study we also detected a slight but non-significant increase of membrane cholesterol in the exofacial leaflet. So far, it is unknown how cholesterol distribution is regulated between the two membrane leaflets and how statins specifically change the leaflet distribution of cholesterol (Wood et al. 2011). However, several candidates have been proposed as regulators of the asymmetric cholesterol membrane distribution such as ApoE, the LDL-receptor, the sterol carrier protein-2 (SCP-2) and polyunsaturated fatty acids (Wood et al. 2011).

It is well known that cholesterol modulates the membrane biophysical properties and thereby the activity of various membrane proteins including α-, β-, and γ-secretases (Peters et al. 2009). Since the cleavage site of γ-secretase for the APP transmembrane domain is located in the lipid leaflet interface of plasma membranes (Kaether et al. 2006), changes in the equilibrium of exofacial and cytofacial cholesterol may change the accessibility of the γ-secretase cleavage site within the APP domain. This γ-secretase cleavage site determines the production of $A\beta_{1-40}$ and $A\beta_{1-42}$. In the current study simvastatin treatment is associated with a significant decrease of soluble $A\beta_{1-40}$ levels in the brain and a corresponding increase of $A\beta_{1-40}$ levels in the plasma of transgenic $APP751_{SL}$ mice. These findings indicate that the reduction of soluble $A\beta_{1-40}$ levels in the brain might partly be due to an enhanced clearance of $A\beta_{1-40}$ from the brain. $A\beta_{1-40}$ is the dominant Aβ-species in the plasma of healthy humans (Hoglund et al. 2004) and the clearance of $A\beta_{1-40}$ through the blood brain barrier (BBB) is mediated via lipoprotein receptor protein (LRP) and its ligands ApoE and α_2-macroglobulin (Zlokovic 2004). Accordingly, Shinohara et al. (2010) recently reported that fluvastatin up-regulated LRP1 levels in the BBB through an isoprenoid-dependent mechanism, contributing to increased LRP1-mediated Aβ clearance at high Aβ levels.

Levels of insoluble $A\beta_{1-40}$ and $A\beta_{1-42}$ were significantly increased in brains of simvastatin treated mice. Our findings are in line with Park et al. (2003), who demonstrated that lovastatin treatment for 3 weeks enhanced insoluble Aβ levels and senile plaque deposition only in brains of female but not in male Tg2576 mice with the Swedish APP mutation (Table 2). Thus, statin treatment enhanced the processing of APP in female mice similar to our study, which indicates a potential gender effect. Interestingly, another study that also used female Tg2576 mice reported no statin effects on Aβ levels (Li et al. 2006), while most studies using male transgenic models reported diminished Aβ levels after statin treatment (Table 2). As already discussed by Schuessel et al. (2005) in several different APP transgenic mouse models, female mice have been reported to exhibit increased or accelerated plaque formation compared to male transgenic mice. Despite differences in length of APP, number of APP mutations, and promotors used to drive transgene expression as well as divergent genetic background between the different mouse models, female mice consistently show elevated formation of Aβ and plaque deposition (Schuessel et al. 2005). The authors concluded that it is likely that these observations are not merely artifacts of some special property of a single mouse model but rather that sex or unknown endocrine factors modulate APP processing toward the amyloidogenic pathway in general, especially since similar gender differences were found in humans (Schuessel et al. 2005).

Enhanced insoluble Aβ levels can be explained by moderate changes of cholesterol levels in SPM induced by simvastatin. Abad-Rodriguez et al. (2004) reported that a moderate reduction of membrane cholesterol (<25%) results in enhanced Aβ generation in hippocampal neurons whereas a strong cholesterol reduction (>35%) results in a significant decrease in Aβ generation. The authors of this study concluded that a moder-

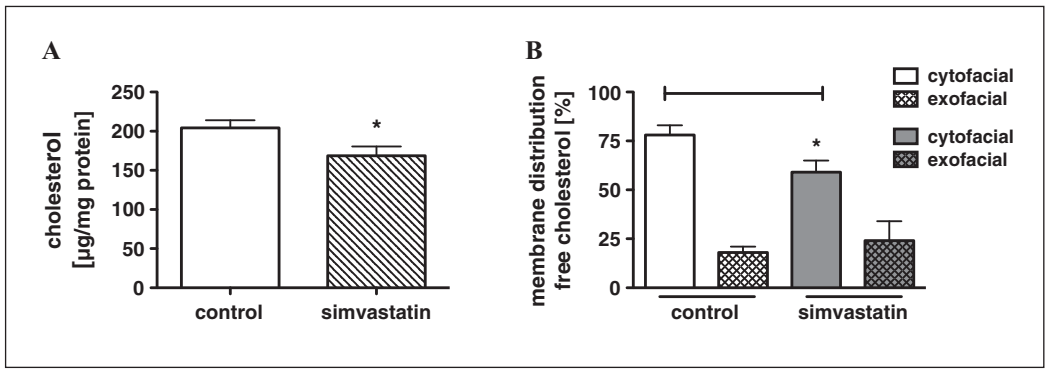

Fig. A: Levels of free, unesterfied cholesterol in synaptosomal plasma membranes isolated from simvastatin and vehicle (control) treated mice. B. Transbilayer distribution of unesterfied membrane cholesterol in SPM of vehicle (control) and simvastatin-treated mice. Data are shown as means ± SEM (n = 6): p* < 0.05 vs. control (Student's t-test).

Table 1: Concentrations of soluble and insoluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ in the brain of control and simvastatin treated $APP751_{SL}$ mice

	Control	Simvastatin
Soluble $A\beta_{1\text{-}40}$ [ng/g brain tissue]	19.69 ± 0.863	17.46 ± 0.337 *
Soluble $A\beta_{1\text{-}42}$ [ng/g brain tissue]	1.187 ± 0.221	1.587 ± 0.182 n.s.
Insoluble $A\beta_{1\text{-}40}$ [ng/g brain tissue]	1094 ± 128.5	1613 ± 147.2 *
Insoluble $A\beta_{1\text{-}42}$ [ng/g brain tissue]	621.0 ± 56.6	781.9 ± 58.1 +
Plasma $A\beta_{1\text{-}40}$ [ng/ml plasma]	0.139 ± 0.029	0.3088 ± 0.027***

Levels of Aβ were determined in brain homogenate and plasma samples as described in material & methods. Values are means ± SEM, n = 11 per group; student's t-test, *p < 0.05, +p < 0.0647, ***p < 0.001, n.s. = not significant.

ate reduction in membrane cholesterol causes a disorganisation of rafts enhancing the access of β-secretase to APP. A strong reduction of membrane cholesterol is supposed to inhibit β- and γ-secretase activity.

So far, only one *in vivo* study has investigated the impact of statin-induced membrane transbilayer changes on APP-processing. Burns et al. (2006) reported that simvastatin and lovastatin induced changes in membrane cholesterol distribution were associated with a significant reduction in total $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ levels in brains of non-transgenic mice. Differences between our data and the results of Burns et al. might be due to the different mouse strains, genetic background and different quantification methods for Aβ.

Results from other *in vivo* studies using APP transgenic mice investigating statin-effects on Aβ generation also give controversial results (Table 2). In general, mice harbouring the human Swedish APP mutation (K670N/M671L substitution) show elevated levels of both total $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ whereas the London APP mutation (V7171 substitution) or presenilin mutations (PS1) specifically increase the generation of $A\beta_{1\text{-}42}$ (Table 2). In PS/APP mice, atorvastatin treatment led to a significant decrease of brain $A\beta_{1\text{-}40}$- and $A\beta_{1\text{-}42}$-levels accompanied by a decrease of serum but not brain cholesterol levels (Petanceska et al. 2002). In TgCRND8 mice, pravastatin and lovastatin treatment for 1 month resulted in dose-dependent reductions in total Aβ levels (Chauhan et al. 2004), whereas the same treatment with simvastatin did not change Aβ levels in Tg2576 mice (Li et al. 2006). It should be noted that the latter study reported beneficial effects of simvastatin on cognition although Aβ levels were unchanged further indicating that pleiotropic effects of statins play a role for its efficacy (Li et al. 2012).

Table 2: Overview of recent *in vivo* studies investigating the effect of statins on APP processing

Model	Mutation	Gender	Brain cholesterol	Total Aβ (brain)	Statin	Reference
PSAPP	APP-KM670/671N & PS1M146V	♀, ♂	↔	↓	Atorvastatin, 30 mg/kg, p.o, 8 weeks	(Petanceska et al. 2002)
Tg2576	APP-KM670/671NL	♀, ♂	n.d.	♀: ↑ ♂: ↔	Lovastatin, 100 mg/kg, p.o. (food), 3 weeks	(Park et al. 2003)
Tg2576	APP-KM670/671NL	♀	n.d.	↔	Simvastatin, 50 mg/kg, p.o., 12 weeks	(Li et al. 2006)
TgCRND8	APP-KM670/671NL & V717F	♂	n.d.	↓	Pravastatin, Lovastatin 0.5-10 mg/kg, p.o (food), 4 weeks	(Chauhan et al. 2004)
APP23	APP-KM670/671NL	♀, ♂	n.d.	↓	Fluvastatin, 5 mg/kg, p.o., 4 weeks	(Shinohara et al. 2010)
C57BL/6	Wild type	♂	n.d.	↓	Fluvastatin, 5 mg/kg, p.o., 4 weeks	(Shinohara et al. 2010)
Swiss Webster	Wild type	♂	SPM-cholesterol ↓	↓	Atorvastatin, Lovastatin, Simvastatin, 50 mg/kg, p.o., 3 weeks	(Burns et al. 2006)
$APP751_{SL}$	APP & KM670/671NL & V717I	♀	SPM-cholesterol ↓	↑	Simvastatin, 50 mg/kg, p.o., 3 weeks	

n.d. = not determined.

Epidemiological data indicated that statins significantly decreased the incidence of AD: Long-term treatment of patients with coronary heart disease (CHD) treated with lovastatin, pravastatin or simvastatin lowered the risk of developing AD up to 70% compared to control subjects receiving other antiatherosclerotic medication (Eckert et al. 2007). Subsequently, prospective studies were initiated to evaluate the ability of statins to prevent AD. Meta-analysis systematically identified relevant studies, and data were abstracted according to predefined criteria. The authors concluded that statin use did not show a beneficial effect on the risk of AD (Rockwood 2006; Zhou et al. 2007). A more recent review concluded that human studies of statins show highly variable outcomes, making it difficult to draw firm conclusions. Several confounding factors among the human studies were identified that contribute to the substantial variability observed to date and future human studies of this still important therapeutic topic were recommended (Shepardson et al. 2011).

In conclusion, our data show that chronic simvastatin treatment affected the distribution of cholesterol within SPM, significantly increased the levels of insoluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ but reduced the ratio of soluble $A\beta_{1\text{-}40}$ levels in the brain of female $APP751_{SL}$ mice. The reduction of soluble $A\beta_{1\text{-}40}$ levels in the brain was associated with an increase of plasma-levels of $A\beta_{1\text{-}40}$ in simvastatin-treated animals which may indicate that simvastatin treatment enhanced the $A\beta_{1\text{-}40}$-clearance from the brain. Although the observed alteration in transbilayer cholesterol is likely to be involved in changes of APP processing by α-, β- and γ-secretase, we cannot exclude other potential mechanisms of statins such as lipid and non-lipid related, pleiotropic effects (Wood et al. 2010).

4. Experimental

4.1. Animal model and statin administration

$APP751_{SL}$ mice (C57B/6J background) are transgenic for the 751 amino acid form of APP with the Swedish (KM670/671NL) and London (V7171) mutations under control of a neuron-specific murine Thy-1 promoter. Young (3 months) female $APP751_{SL}$ mice (Sanofi-Aventis, Vitry-sur-Seine, France) were treated with simvastatin (50 mg/kg bw.) by oral gavage once a day for 21 days. Simvastatin was selected since it has the highest availability in the brain of mice (Johnson-Anuna et al. 2005). Free access to water and food was provided and mice were weighed daily for dose adjustment. Mice of the control group received the same volume of 0.2% (w/v) agarose gel according to their weight. All experiments on animals were performed according to the standards of the European Communities and Council Directive (86/609/EEC). At the end of the study, 24 hours after the last treatment, blood samples were collected retrobulbary and mice were decapitated. Blood was collected into heparin tubes and centrifuged at 10,000 g at 4 °C for 10 min to obtain the serum fraction. After removal of brain stem and cerebellum, brain hemispheres were stored frozen at -20 °C until tissue preparation. One brain hemisphere of each animal was used for the preparation of synaptosomal plasma membranes and for the quantification of Aβ, respectively.

4.2. Determination of cholesterol and protein levels

Cholesterol was determined enzymatically using a modified CHOD-PAP method as previously reported (Hooff et al. 2012). Protein content was measured according to the Lowry method.

4.3. Synaptosomal plasma membranes and transbilayer distribution of cholesterol

Synaptosomal plasma membranes (SPM) were prepared by density gradient centrifugation and labelling was performed as previously described (Kirsch et al. 2003). Dehydroergosterol (DHE), a fluorescent analogue of cholesterol, and trinitrobenzensulfonic acid (TNBS), a fluorescence quencher, were used for the investigation of cholesterol transbilayer distribution in SPM (Kirsch et al. 2003). Fluorescence intensities were measured in a SLM Luminescence Spectrometer Aminco-Bowman® Series 2 (excitation 324 nm, emission 375 nm).

4.4. Quantification of $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$

Brain homogenate aliquots were mixed with 2 vol of 9 M guanidin hydrochloride (GH) solution in 50 mM Tris-HCl, pH 7.4. The homogenate was mixed for 1 h with three sonification cycles followed by centrifugation at 50,000 g at 4 °C for 120 min. Aβ peptides were quantified in guanidin-HCl supernatants (soluble Aβ) or guanidin-HCl brain extracts (insoluble Aβ) by electrochemiluminescence assays using different Aβ antibodies and an Origen M8 analyzer (IGEN Europe Inc., Oxford, England) according to the procedure described by Blanchard et al. (2003). Briefly, the 4G8 antibody was ruthenylated with TAG-NHS ester and used in conjunction with 6E10 bionylated antibody to detect total Aβ. To specifically quantify $A\beta_{1\text{-}42}$, 6E10 was replaced by the $A\beta_{1\text{-}42}$-specific antibody 22F9 which binds to the C-terminus of $A\beta_{1\text{-}42}$. Aβ-levels were expressed as ng/g wet tissue or as ng/ml plasma.

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