Deficiency of Sphingosine-1-phosphate Lyase Impairs Lysosomal Metabolism of the Amyloid Precursor Protein*

Ilker Karaca†, Irfan Y. Tamboli†1, Konstantin Glebov‡, Josefine Richter‡, Lisa H. Fell*, Marcus O. Grimm‡, Viola J. Haupenthal‡, Tobias Hartmann‡, Markus H. Gräler‡, Gerhild van Echten-Deckert‡,* and Jochen Walter†2

From the †Department of Neurology, University of Bonn, 53127 Bonn, Germany, and the ‡German Center for Neurodegenerative Diseases (DZNE), 53175 Bonn, Germany, the §Department of Experimental Neurology, University of the Saarland, 66421 Homburg/Saar, Germany, the ¶Department of Anaesthesiology and Intensive Care Medicine, Center for Sepsis Control and Care, and Center for Molecular Biomedicine, University Hospital Jena, 07740 Jena, Germany, and the **Life and Medical Sciences, Membrane Biology and Lipid Biochemistry Unit at the Kekulé-Institute, University of Bonn, 53121 Bonn, Germany

Background: Sphingolipid metabolism is functionally linked to the proteolytic processing of APP.

Results: Inhibition of S1P-lyase decreases APP degradation in lysosomes, and mobilization of Ca²⁺ can partially rescue the accumulation of APP.

Conclusion: S1P-lyase is critically involved in the regulation of lysosomal activity and degradation of APP.

Significance: Alterations in S1P metabolism could play important roles in the pathogenesis of Alzheimer disease.

Progressive accumulation of the amyloid β protein in extracellular plaques is a neuropathological hallmark of Alzheimer disease. Amyloid β is generated during sequential cleavage of the amyloid precursor protein (APP) by β- and γ-secretases. In addition to the proteolytic processing by secretases, APP is also metabolized by lysosomal proteases. Here, we show that accumulation of intracellular sphingosine-1-phosphate (S1P) impairs the metabolism of APP. Cells lacking functional S1P-lyase, which degrades intracellular S1P, strongly accumulate full-length APP and its potentially amyloidogenic C-terminal fragments (CTFs) as compared with cells expressing the functional enzyme. By cell biological and biochemical methods, we demonstrate that intracellular inhibition of S1P-lyase impairs the degradation of APP and CTFs in lysosomal compartments and also decreases the activity of γ-secretase. Interestingly, the strong accumulation of APP and CTFs in S1P-lyase-deficient cells was reversed by selective mobilization of Ca²⁺ from the endoplasmic reticulum or lysosomes. Intracellular accumulation of S1P also impairs maturation of cathepsin D and degradation of Lamp-2, indicating a general impairment of lysosomal activity. Together, these data demonstrate that S1P-lyase plays a critical role in the regulation of lysosomal activity and the metabolism of APP.

Alzheimer disease is the most common form of dementia and is characterized by the progressive accumulation of extracellular plaques containing the amyloid-β peptide (Aβ)³ (1). Aβ derives from proteolytic processing of the amyloid precursor protein (APP) by β- and γ-secretases (2). Alternatively, APP can also be cleaved by α-secretase within the Aβ domain preventing the generation of Aβ generation (2). APP and the secretases are integral membrane proteins, and the processing occurs throughout secretory or endocytic vesicular transport routes (1, 2). APP is transported from the endoplasmic reticulum (ER) to the plasma membrane, where it is predominantly cleaved by α-secretase resulting in the generation of soluble APPα and corresponding C-terminal fragments (CTF-α) (2, 3). APP not cleaved by α-secretase can be internalized from the cell surface into endosomes where β-secretory cleavage can occur (2, 4). β-Secretase has been identified as the membrane-bound aspartic protease BACE1 (β-site APP cleaving enzyme-1) (5–8). The cleavage of APP by BACE1 generates soluble APPβ and APP-βCTF that could then be processed by γ-secretase, resulting in the release of Aβ peptides (2, 3). Importantly, significant fractions of APP and its CTFs are also targeted to lysosomes where they are degraded by lysosomal hydrolases (9–11).

Recently, sphingosine-1-phosphate (S1P) has been shown to increase the generation of Aβ by directly activating BACE1 (12). S1P is a bioactive signaling molecule regulating cell proliferation and survival as well as differentiation and motility (13, 14). S1P derives during degradation of sphingolipids from the cleavage of ceramide into fatty acid and sphingosine (15). Sphingosine is then phosphorylated by sphingosine kinases 1 and 2 (Sphk1 and Sphk2) to produce S1P (16). S1P can then be irreversibly cleaved by the S1P-lyase to phosphoethanolamine and hexadecenal (17). Alternatively, S1P can be dephosphorylated by sphingosine phosphatases back to sphingosine and

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† Present address: Dept. of Neuroscience, Georgetown University, Washington, D. C. 20057.

‡ To whom correspondence should be addressed: Dept. of Neurology, Molecular Cell Biology Unit, University of Bonn, Sigmund-Freud-Str. 25, 53127 Bonn, Germany. Tel.: 49-228-2871-9782; Fax: 49-228-2871-4387; E-mail: Jochen.Walter@ukb.uni-bonn.de.

³ The abbreviations used are: Aβ, amyloid β; APP, amyloid precursor protein; S1P, sphingosine-1-phosphate; CTF, C-terminal fragment; ER, endoplasmic reticulum; SKI II, 4-[4-(4-chloro-phenyl)-thiazol-2-ylamino]-phenol; GPN, glycyolphospholylamine 2-naphthylamide; AICD, APP intracellular domain; Lamp-2, lysosome-associated membrane protein-2; NPC, Niemann-Pick type C; MEF, mouse embryonic fibroblast; rcf, relative centrifugal force.
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described above). After 24 h cells were treated with compounds
and reagents for respective times in 100 μl of culturing
medium. Later the cells were first incubated with 550 ng/μl
MTT for 4 h in the conditioned medium and subsequently sol-
ubilized overnight by adding 100 μl of 10% SDS in 0.001 M HCl
to the medium. The metabolization of MTT was then measured
at 570 nm and statistically analyzed.

Viral Transduction of Cells—Human APPswe cDNA with the
Swedish mutation (APPsw) was cloned into a lentiviral rrl-
CMV-vector. The construct also drives the separate expression
of GFP by an internal ribosomal entry site. Cells were seeded in
6-well plates 1 day before the transduction to a 70% confluence
in DMEM medium supplemented with 10% FCS, 1% penicillin/
streptomycin. Next day, the cells were transduced with lentivi-
ral particles at 1 × 10^6 IP/100,000 cells for 15 h. Later cells were
washed four times with DMEM and cultured for an additional
48 h.

Reverse siRNA Transfection—25 μl of Sgpl1 targeting or control
siRNA (10 μM) was pipetted into a individual wells of a 24-
well plate, followed by addition of 100 μl of diluted HiPer-
fect transfection reagent (95:5% H2O:HiPerfect), and incubated
for 15 min. Then murine N9 cells (150,000 cells/well) were
seeded into the wells. After 6 h of transfection, medium was
replaced by fresh DMEM. Cells were lysed after 30 h, and pro-
teins were detected by Western immunoblotting.

Protein Extraction and Western Immunoblotting—For ex-
traction of proteins, cell were washed three times in PBS and
lysed in STEN lysis buffer (50 mM Tris-HCl, pH 7.6, 250 mM
NaCl, 20 mM EDTA, 1.2% Nonidet P-40, and 1% Triton X-100)
containing Complete® protease inhibitor (Hoffmann-La
Roche, Basel, Switzerland). For isolation of cellular membranes,
the cells were briefly washed with PBS and collected by centri-
fugation. The cells were then incubated for 10 min in hypotonic
buffer (10 mM Tris, 1 mM EDTA, 1 mM EGTA). After repeated
resuspension through a 0.6-mm cannula, the mixture was cen-
trifuged at 1300 rcf for 5 min to remove cellular debris and
nuclei. The remaining supernatant was centrifuged for 60 min
at 16,100 rcf, and the resulting membrane pellet was solved in
STEN lysis buffer containing Complete® protease inhibitor.
Proteins were separated by SDS-PAGE and detected by Western
immunoblotting using ECL imaging (Bio-Rad).

Subcellular Fractionation—Isolated membranes were resus-
pended in hypotonic buffer containing protease inhibitor mix-
ture and incubated overnight at 4 °C with constant stirring.
Vesicles were separated on a stepwise iodixanol (OptiPrep,
Sigma) gradient (50–2.5%), diluted with a sucrose buffer (0.25
M sucrose, 6 mM EDTA, 60 mM HEPES-NaOH, pH 7.4).

Measurement of Aβ Variants—Cells were grown on 24-well
culture plates until 70% confluency in DMEM as described
above. For collection of Aβ, 500 μl of fresh medium was added
overnight. Conditioned media were cleared by centrifugation
and then analyzed by electrochemiluminescence technology
(MesoScale Discovery) for Aβ40 and Aβ42 according to the
manufacturer’s protocol.

Measurement of Secretase Activity—Detection of secretase
activities in living cells was performed as described previously
with slight modifications (29, 30). Shortly, after incubation,
cells were washed two times with prewarmed life cell imaging
solution (HEPES buffer, pH 7.4). Buffer was removed, and 50 µl of cell imaging solution containing 30 µM β- and 12 µM γ-secretase fluorogenic substrate (Calbiochem, Darmstadt, Germany) was added. Incubation was performed under light exclusion using a Safire Infinity Fluorometer (Tecan, Crailsheim, Germany).

In Vitro γ-Secretase Assay—Assay was performed similar to published protocol (31). Purified cellular membranes were reconstituted in citrate buffer (150 mM sodium citrate in H₂O, pH 6.4) and incubated for the indicated time periods at 37 °C. Incubation at 4 °C for 3 h served as control. Proteins were detected by Western immunoblotting.

Extraction and Quantification of S1P—S1P measurements were performed according to an established protocol using liquid chromatography coupled to triple-quadrupole mass spectrometry (LC/MS/MS) (32). Cell suspensions in 1 ml of PBS and 1 ml of supernatants were transferred into glass centrifuge tubes. After addition of C17-base internal standards of the analytes (300 pmol/sample; Avanti Polar Lipids) samples were mixed with 200 µl of 6 N hydrochloric acid and 1 ml of methanol and vigorously vortexed for 5 min in the presence of 2 ml of chloroform. Aqueous and chloroform phases were separated by centrifugation for 3 min at 1900 rcf, and the lower chloroform phase was transferred into a new glass centrifuge tube. After a second round of lipid extraction with additional 2 ml of chloroform, the two chloroform phases were combined and vacuum-dried at 50 °C for 50 min using a vacuum concentrator. The extracted lipids were dissolved in 100 µl of methanol/chloroform (4:1, v/v) and stored at −20 °C. Detection was performed with the QTrap triple-quadrupole mass spectrometer (AB Sciex, Framingham, MA) interfaced with the Merck-Hitachi Elite LaChrom series 3.1.3 chromatograph and autosampler (VWR International). Positive electrospray ionization LC/MS/MS analysis was used for detection of all analytes. The ion source conditions and gas settings for positive electrospray ionization LC/MS/MS analysis were as follows: ion spray voltage, 5500; ion source heater temperature, 450 °C; collision gas setting, medium; ion source gases 1 and 2, settings 30 and 60, respectively; curtain gas setting, 45. Multiple reaction monitoring transitions were as follows: S1P m/z, 380/264; C17-S1P m/z, 366/250; and C17-sphingosine m/z, 286/268. Liquid chromatographic resolution of all analytes was achieved using a 2 × 60-mm MutoHigh C18 reversed phase column with 3-µm particle size (CS-Chromatography Service). The column was equilibrated with 10% methanol and 90% of 1% formic acid in H₂O for 10 min, followed by sample injection and 26-min elution with 100% methanol with a flow rate of 300 µl/min. Standard curves were generated by adding increasing concentrations of the analytes to 300 pmol of the internal standard. Linearity of the standard curves and correlation coefficients were obtained by linear regression analyses. Data analyses were performed using Analyst 1.4 (AB Sciex).

Immunocytochemistry—Cells were cultured on glass coverslips until 70% confluency and fixed in 4% paraformaldehyde. After washing with PBS, cells were first permeabilized in 0.25% Triton X-100 for 10 min and blocked with 10% BSA containing 0.125% Triton X-100 in PBS for 1 h. Primary and secondary antibodies were incubated for 1 h in 5% BSA with 0.125% Triton X-100 in PBS with three repetitive washing steps for 10 min in between (0.125% Triton X-100 in PBS). The nuclei were stained with DAPI for 10 min in PBS. Cells were embedded on a microscope slide with ImmuMount (Thermo Scientific) and analyzed by fluorescence microscopy (AxioVert 200; Zeiss; equipped with a plan-Apochromat 63×/0.75 objective and an Axiocam MRm camera). Images were acquired and processed using AxioVision 4.8 software (Zeiss).

RESULTS

Modulation of Intracellular S1P Levels Affects APP Metabolism—To assess the role of S1P in the metabolism of APP, we first used the sphingosine kinase inhibitor SKI II to reduce intracellular levels of S1P in native HEK293 or HEK293 cells overexpressing human APP<sub>695</sub> (Fig. 1A). The inhibition of SpHK induced a decrease of APP-CTFs in a dose-dependent manner, whereas APP-FL showed few, if any, changes (Fig. 1B). Cell viability was analyzed by MTT reduction assay and was not impaired by treatment with SKI II (data not shown). Next, we analyzed the expression of APP and CTFs in embryonic fibroblasts from wild-type mice and from mice with a genetic deletion of the S1P-lyase (Fig. 1C). Mass spectrometry showed a selective increase of intracellular S1P in S1P-lyase KO cells as compared with WT cells (Fig. 1D), whereas concentrations of extracellular S1P were not increased in conditioned media of S1P-lyase KO cells (Fig. 1E), suggesting that deletion of the S1P degrading enzyme predominantly affects the intracellular pool of S1P. Interestingly, levels of full-length and particularly that of APP-CTFs were below the detection limit, although levels of APP CTFs were below the detection limit, whereas concentrations of extracellular S1P were not increased in conditioned media of S1P-lyase KO cells (Fig. 1E), suggesting that deletion of the S1P degrading enzyme predominantly affects the intracellular pool of S1P. Interestingly, levels of full-length and particularly that of APP-CTFs were strongly increased in S1P-lyase KO cells (Fig. 1F). The use of specific antibodies against APP-CTFs showed that all three variants of APP-CTFs, αCTF, βCTF, and βCTF<sup>α</sup>, are increased in S1P-lyase-deficient cells. APP-βCTF<sup>α</sup> is an additional cleavage product of APP generated by alternative BACE1 processing at position Glu-11 within the Aβ domain (33, 34). Consistent with a previously described conversion of βCTF and βCTF<sup>α</sup> into αCTF by α-secretase (35), the highest accumulation was observed for αCTF.

In addition to the use of MEFs from WT and S1P-lyase KO mice, we also targeted S1P-lyase by siRNA in mouse N9 cells. Although levels of APP CTFs were below the detection limit, levels of full-length APP significantly increased upon knockdown of S1P-lyase (Fig. 1, G and H), thereby confirming the effects observed in S1P-lyase KO cells. We also determined the levels of sphingosine that could derive from dephosphorylation of S1P in WT and S1P-lyase KO cells. Consistent with previous results (23), sphingosine levels were significantly increased in S1P-lyase KO cells (Fig. 2A). Thus, we tested the effect of sphingosine on APP and its CTFs. Incubation of both WT and S1P-lyase KO cells with spingo-
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Sine led to an increase in full-length APP and CTFs (Fig. 2B). In contrast, cell incubation with extracellular S1P exerted little, if any, effect on APP or APP CTFs (Fig. 2C). Because sphingosine efficiently penetrates the plasma membrane and could be phosphorylated by intracellular SphK to S1P, these data suggest that the accumulation of APP in S1P-lyase KO cells might be caused by intracellular rather than extracellular S1P or sphingosine.

We next tested the effect of the SphK inhibitor SKI II in both WT and S1P-lyase KO cells. Mass spectrometry revealed a significant increase of endogenous sphingosine in both WT (con-
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FIGURE 1. Modulation of S1P levels affects the metabolism of APP. A, schematic showing the metabolism of S1P and enzymes involved. B, native HEK293 expressing endogenous APP751/770 isoforms (left panels) or HEK293 cells overexpressing the human neuron-specific APP695 isoform (right panels) were treated with the indicated concentrations of SKI II for 24 h. APP and its CTFs were detected by Western immunoblotting. APP695 total intracellular APP, a significant decrease in secreted APP-CTFs was observed (Fig. 3A). These data thereby confirm the observations for the endogenous APP (Fig. 1, D and E) and further indicate an impaired metabolism of APP in S1P-lyase-deficient cells.

We then measured levels of different Aβ species secreted by the transduced cells. Levels of Aβ40 were ~20-fold higher than those of Aβ42. Interestingly, the levels of Aβ40 were slightly but significantly increased in S1P-lyase KO as compared with S1P-lyase WT cells (Fig. 3A). These data thereby confirm the observations for the endogenous APP (Fig. 1, D and E) and further indicate an impaired metabolism of APP in S1P-lyase-deficient cells.

control, 3.63 ± 1.3 pmol/sample; SKI II, 17.83 ± 4.55 pmol/sample; p < 0.01) and S1P-lyase KO cells (control, 16.18 ± 1.99 pmol/sample; SKI II, 42.92 ± 11.20 pmol/sample; p < 0.01), respectively, indicating inhibition of sphingosine phosphorylation. Interestingly, SKI II strongly reduced levels of APP-FL and CTFs in S1P-lyase knock-out as well as in WT cells (Fig. 2D). Notably, the secretion of soluble APPα was also reduced upon treatment with SKI II in both WT and S1P-lyase KO cells, indicating that the decrease in cellular APP and APP-CTF levels was not caused by increased secretion of this protein (Fig. 2D).

FIGURE 2. Involvement of intracellular S1P in the accumulation of APP. A, determination of intracellular sphingosine concentration in WT and S1P-lyase KO cells by LC/MS-MS (*, p < 0.01; n = 3). B and C, WT and S1P-lyase KO cells were treated with 10 μM sphingosine or 10 μM S1P for the indicated time periods, and APP-FL and its CTF were detected by Western immunoblotting. D, WT and S1P-lyase KO MEFs were treated with 5 μM SKI II for 24 h followed by detection of APP-FL and CTFs in isolated membranes and of secreted APPα in conditioned media.

S1P-Lyase Deficiency Modulates the Generation of Aβ and Secretase Activities—To determine whether S1P-lyase deficiency also affects the generation of Aβ, we stably transduced WT and S1P-lyase KO MEFs with lentivirus containing the human APPswe cDNA with the Swedish mutation (APPswe). The construct also drives the separate expression of GFP by an internal ribosomal entry site. WT and S1P-lyase KO cells showed similar expression of GFP, indicating similar transduction and expression of the construct (Fig. 3A). The specific detection of transgenic human APPswe with the human specific antibody 6E10 revealed strongly increased levels of APPswe in S1P-lyase KO as compared with S1P-lyase WT cells (Fig. 3A). These data thereby confirm the observations for the endogenous APP (Fig. 1, D and E) and further indicate an impaired metabolism of APP in S1P-lyase-deficient cells.

We then measured levels of different Aβ species secreted by the transduced cells. Levels of Aβ40 were ~20-fold higher than those of Aβ42. Interestingly, the levels of Aβ40 were only slightly but significantly increased in S1P-lyase KO as compared with WT cells (Fig. 3B). Aβ42 levels were not significantly different (Fig. 3C). Notably, when Aβ levels were normalized to the total cellular APP, a significant decrease in secreted Aβ40 and Aβ42 was evident in S1P-lyase-deficient cells (Fig. 3, D and E), suggesting that the accumulated APP-CTFs were not efficiently processed by γ-secretase. Direct measurements of γ-secretase with a fluorogenic substrate indeed revealed slightly but significantly reduced activity of this enzyme in S1P-lyase KO cells as compared to cells with functional S1P-lyase.
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FIGURE 3. Altered Aβ generation in S1P-lyase-deficient cells. A, WT and S1P-lyase KO MEFs were stably transduced with a human APP695swedish-internal ribosomal entry site-GFP construct. Expression of APP and GFP was analyzed by Western immunoblotting. B and C, Aβ levels in conditioned media were determined by electrochemiluminescence and normalized to GFP expression (see “Experimental Procedures”). D and E, Aβ secretion was normalized to cellular APP levels. (***, p < 0.001; n = 3).

FIGURE 4. Decreased γ-secretase activity in S1P-lyase-deficient cells. A, γ-secretase activity in living cells was determined by a fluorometric assay (see “Experimental Procedures”). γ-Secretase is decreased by ~20% in S1P-lyase KO as compared with WT cells (***, p < 0.001; n = 9). B and C, in vitro γ-secretase assay with purified membranes of WT and S1P-lyase KO cells (see “Experimental Procedures”). APP, CTFs, and AICD were detected by Western immunoblotting after the indicated incubation times (B). The ratios of AICD and CTFs after 1 h of incubation at 37 °C were determined by densitometry (*, p < 0.05; n = 3). Incubation of 3 h at 4 °C efficiently blocked the cleavage of CTFs to AICD (C).

To further prove decreased γ-secretase activity in S1P-lyase KO cells, we performed an in vitro γ-secretase assay as described previously (31). Incubation of purified membranes for 3 h at 37 °C showed efficient production of APP intracellular domain (AICD) in both WT and S1P-lyase KO samples. However, at 1 h of incubation, CTFs were still detectable in samples from S1P-lyase KO samples, whereas very few, if any, CTFs were detectable in WT samples (Fig. 4B). Quantification of CTFs and AICD showed statistically significant differences (Fig. 4C). These data confirm the measurements with the fluorometric assay on reduced γ-secretase activity in S1P-lyase KO. However, the decrease in γ-secretase activity in S1P-lyase-deficient cells was low compared with the strong accumulation of APP CTFs.
Accumulation of APP-CTFs in Lysosomal Compartments of S1P-lyase KO Cells—In addition to the processing by γ-secretase, APP-CTFs can also be degraded by lysosomal proteases, including cathepsins (9, 10, 36, 37). Thus, we specifically tested whether S1P-lyase deficiency affects the lysosomal degradation of APP-CTFs. First, the association of APP CTFs with lysosomes was investigated by double staining of cells with antibodies against the lysosome-associated membrane protein-2 (Lamp-2) and the C-terminal domain of APP, respectively. As compared with WT cells, the intensity and size of Lamp-2-positive structures appeared increased in S1P-lyase-deficient cells, indicative for impaired lysosomal activity. Importantly, S1P-lyase KO cells also showed increased reactivity for the APP C terminus in vesicular structures that partly co-localized with Lamp-2. In contrast, WT cells revealed only little co-localization of APP and Lamp-2 (Fig. 5A, red frames), likely because of efficient degradation of APP and its CTFs in lysosomes.

To further prove accumulation of APP-CTFs in lysosomal compartments, we next performed subcellular fractionations. Only very low amounts of APP-CTFs were detected in cathepsin D-positive fractions in WT cells (Fig. 5B). In contrast, APP-CTFs were strongly increased in cathepsin D-positive fractions of S1P-lyase-deficient cells (Fig. 5B), indicating their selective accumulation in lysosomes.

To test whether S1P-lyase deficiency affects other lysosomal proteins, we analyzed the expression of cathepsin D and Lamp-2 in more detail. Western immunoblotting revealed decreased levels of the mature active form (25 kDa) of cathepsin D, whereas those of the immature pro-/intermediate-forms (55–45 kDa) were slightly elevated in S1P-lyase KO cells. Accordingly, the ratio of mature to immature forms of cathepsin D in S1P-lyase KO cells was significantly reduced by ~30% as compared with WT cells (Fig. 6, A and B). Reduced ratios of mature/immature forms of cathepsin D were also demonstrated upon RNAi-mediated knockdown of S1P-lyase (Fig. 6, C and D).

To further demonstrate a lysosomal impairment in S1P-lyase-deficient cells, we also detected Lamp-2 and the GM2 activator protein, two proteins also degraded in the lysosome (38). Both proteins were slightly but significantly increased by ~20–30% in S1P-lyase KO cells (Fig. 6, E–H). Together, these data strongly indicate that S1P-lyase deficiency and intracellular S1P accumulation impair lysosomal turnover of proteins.

We showed previously that induction of autophagy by cell starvation promotes the degradation of APP-CTFs (9). Thus, we tested the effects of starvation on the clearance of APP-CTFs in S1P-lyase and WT cells. Starvation strongly decreased
the levels of both APP-FL and APP-CTFs in WT, as well as S1P-lyase KO cells (Fig. 7). However, APP-CTF levels remained higher in S1P-lyase KO cells as compared with that of WT cells at each time point. To check the induction of autophagy, we monitored conversion of LC3-I to LC3-II. After 2 h of starvation, we detected almost exclusively LC3-II in both cell types,
levels of APP-CTFs in the first 1–2 h. As observed with WT cells, levels of APP-CTFs steadily increased after the initial decline upon longer incubation times (Fig. 8B). The combined data indicate that mobilization of Ca^{2+} from ER or lysosomal stores strongly promotes the degradation of APP-CTFs. However, the magnitude and time course of effects strongly differ between WT and S1P-lyase-deficient cells, indicating that the altered metabolism of APP-CTFs involves aberrant regulation of intracellular Ca^{2+} concentrations.

**DISCUSSION**

Our data demonstrate involvement of the S1P-lyase in lysosomal APP metabolism. Genetic inhibition of the enzyme results in increased levels of intracellular S1P and impaired degradation of APP and its CTFs in lysosomal compartments. Although S1P-lyase deficiency led to a slight increase in the total secretion of Aβ, we found a strongly decreased product-precursor relationship when Aβ levels were normalized to that of APP CTFs. In line with this observation, the absolute activity of γ-secretase was lower in S1P-lyase KO cells as compared with WT cells. S1P has been shown to increase the secretion of Aβ from neuronal cells, and this effect was attributed to the direct stimulation of the β-secretase BACE1 (12, 41). Indeed, we also observed increased β-secretase activity when S1P was added to isolated membranes of SH-SY5Y cells. Because the stimulatory effect of S1P has also been observed for an isolated BACE1 variant without the transmembrane domain, S1P likely exerts allosteric effects upon interaction with the catalytic ectodomain of the enzyme (12, 41).

The molecular mechanisms underlying the effects of S1P on γ-secretase remain unclear. However, cleavage of APP-CTFs to the intracellular AICD was slightly reduced in isolated membranes from S1P-lyase KO cells. Thus, it would be interesting to further assess the role of S1P or sphingosine in the regulation of γ-secretase activity. It has been shown that γ-secretase activity could be modulated by membrane lipids (42–46). However, whether these effects are caused by direct interaction of the respective lipids with γ-secretase components and its substrates or via more global changes in membrane fluidity is unknown.

In this study, we identified a more general effect of S1P-lyase deficiency on lysosomal activity, resulting in the strong accumulation of APP-CTFs in lysosomal compartments of S1P-lyase-deficient cells. The impairment of lysosomal activity was also evident from the lower maturation of cathepsin D and the accumulation of Lamp-2. Both proteins are well accepted markers to evaluate lysosomal function (38, 47, 48). In addition, we also observed accumulation of the GM2 activator protein in lysosomal compartments of S1P-lyase-deficient cells. The impairment of lysosomal activity was also evident from the lower maturation of cathepsin D and the accumulation of Lamp-2. Both proteins are well accepted markers to evaluate lysosomal function (38, 47, 48).
different as compared with WT cells. Second, addition of extracellular S1P did not affect APP levels, whereas incubation with sphingosine induced a strong accumulation of APP and CTFs. Sphingosine could have increased membrane permeability than S1P and can be subsequently phosphorylated by intracellular sphingosine kinases to increase the intracellular S1P concentration. Third, the inhibition of sphingosine kinase by SKI II decreased the levels of APP and its CTFs in both WT and S1P-lyase KO cells. Because SKI II further increased cellular sphingosine levels, it is unlikely that the accumulation of APP is directly caused by intracellular sphingosine. Thus, intracellular S1P concentrations appear to be critical for lysosomal function. However, the data do not rule out a contribution of other sphingolipid metabolites, including the cleavage products of the S1P-lyase reaction, phosphoethanolamine and hexadecenal. It would be interesting to further investigate the role of intracellular S1P and its derivatives in more detail.

Our experiments on the effects of starvation showed that both S1P-lyase-deficient cells and WT cells respond with similar induction of autophagy as indicated by efficient conversion of LC3-I to LC3-II and initial degradation of APP and its CTFs. However, whereas LC3-II and APP CTFs were almost completely cleared during starvation in WT cells, the same proteins were much more stable in the S1P-lyase KO cells. These data also strongly support inefficient protein degradation in lysosomes, despite induction of autophagy in S1P-lyase KO cells.

Delivery of molecules to lysosomes depends on transport and fusion of endocytic and secretory vesicles with the lysosomes and involves mobilization of Ca\(^{2+}\) from intracellular stores (50–54). Interestingly, NPC cells have defective Ca\(^{2+}\) mobilization from lysosomes (39) associated with impaired trafficking and metabolism of cholesterol in endolysosomal compartments. This deficit could be partially restored by elevating the cytosolic Ca\(^{2+}\) concentration (55). Of interest, NPC cells, as well as other models of lysosomal storage disorders, also show accumulation of APP and CTFs (9, 36, 56–59) similar to the S1P-lyase KO. We thus assessed the effect of Ca\(^{2+}\) mobilization in the S1P-lyase KO model. Indeed, the elevation of cytosolic Ca\(^{2+}\) concentration by thapsigargin efficiently decreased APP-CTF levels in S1P-lyase-deficient cells. More interestingly, the selective release of Ca\(^{2+}\) from lysosomal stores by GPN acutely promoted the degradation of APP-CTFs within 1 h of treatment. These data thus indicate impaired mobilization of Ca\(^{2+}\) and lysosomal function in S1P-lyase-deficient cells. In line with this findings, it has been demonstrated previously by Ca\(^{2+}\) imaging that S1P-lyase KO cells have aberrantly high levels of Ca\(^{2+}\) in the cytosol but also in intracellular stores (26).

The present study revealed an important role for the S1P-lyase in lysosomal function. It will be interesting to further dissect the molecular mechanisms underlying these effects and also explore the potential of S1P-lyase modulation and lysosomal Ca\(^{2+}\) mobilization to promote APP degradation and thereby decreased A\(\beta\) generation.

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