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The role of ceramide and SEW 2871 in the transcription of enzymes involved in amyloid β precursor protein metabolism in an experimental model of Alzheimer's disease

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Abstract

Alzheimer's disease (AD) is characterized by alterations of amyloid precursor protein (APP) metabolism, accumulation of amyloid β peptides (Aβ), hyperphosphorylation of Tau proteins and also by sphingolipids disturbances. These changes lead to oxidative stress, mitochondria dysfunction, synaptic loss and neuro-inflammation. It is known that Aβ may promote ceramides formation and reversely, ceramides could stimulate Aβ peptides release. However, the effect of ceramide and sphingosine-1-phosphate (S1P) on APP metabolism has not been fully elucidated. In this study we investigated the role of ceramide and S1P on APP metabolism. Moreover, the effect of ceramide and SEW 2871 (agonist for S1P receptor-1) on Sirt1 (NAD^+-dependent nuclear enzyme responsible for stress response) gene expression under Aβ toxicity was analyzed. Experiments were carried out using pheochromocytoma cells (PC-12) transfected with: an empty vector (used as a control), human wild-type APP gene (APPwt) and Swedish mutated (K670M/N671L) APP gene (APPsw). Our results indicated that C2-ceramide significantly decreased the viability of the APPwt, APPsw as well as empty vector-transfected PC12 cells. It was observed that C2-ceramide had no significant effect on the mRNA level of α- and β-secretase in APPwt and APPsw cells. However, it significantly decreased transcription of α-secretase in control cells. Results also showed a significant increase in Psen1 (crucial subunit of γ-secretase) gene expression in APPsw cells after incubation with C2-ceramide. We observed that SEW 2871 significantly upregulated the mRNA level of α-secretase in control-empty vector-transfected cells subjected to C2-ceramide toxicity. The same tendency, though insignificant, was observed in APPwt and APPsw cells. Moreover, SEW 2871 enhanced the mRNA level of β-secretase and Psen1 in APPsw cells after C2-ceramide treatment. Additionally, SEW 2871 significantly upregulated a gene expression of Sirt1 in APPwt and also APPsw cells subjected to C2-ceramide toxicity. Furthermore, it was observed that SEW 2871 significantly enhanced the viability of all investigated cells' lines probably through its positive influence on Sirt1.

Key words: *amyloid β peptide, ceramide, sphingosine-1-phosphate, secretases, sirtuins.*

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Introduction

The metabolic turnover of sphingolipids produces several signaling molecules that profoundly affect the proliferation, differentiation and death of cells. In particular, a lot of evidence is available that defines diversified roles of ceramide and sphingosine-1-phosphate (S1P) in cell death and survival [6,7,13,33,38]. For the last two decades ceramides have been most intensively studied in relation to different types of cell death signaling. Enhanced ceramides' levels have been observed in Alzheimer's disease (AD) and in other neurodegenerative disorders [10,34,61]. Our last results demonstrated the molecular alterations evoked by ceramides in neuronal cells [11]. In physiological conditions ceramides do not accumulate, but they are used as a precursor for the synthesis of other sphingolipids such as sphingomyelin, glycosphingolipids, sphingosine and pro-survival S1P. The alterations in the ceramides' level may occur during the following processes: *de novo* synthesis, activation of enzymes that catalyze its synthesis from sphingosine and hydrolysis of sphingomyelin with participation of acid (aSMase) and neutral (nSMase) sphingomyelinases [15,23].

Ceramides may be broken down by ceramidases, which leads to the formation of sphingosine that subsequently can be phosphorylated by sphingosine kinases (Sphk1 and Sphk2) to S1P. S1P is an important bioactive phosphosphingolipid recognized as a critical regulator of a variety of signaling pathways, which play major roles in diverse physiological and pathophysiological processes. S1P is involved in many cellular processes including survival, proliferation, adhesion, migration and differentiation [13]. It is known that S1P acts in an autocrine/paracrine manner *via* a family of five S1P-specific cell-surface G-protein-coupled receptors (GPCRs, termed S1PR1-5) [30,48]. The recent studies have pointed out the importance of S1P signaling dysregulation in pathogenesis of AD [16,54]. In the last decade, the role of sphingolipids in the cellular survival was described using the sphingolipid rheostat model where pro-apoptotic ceramide and sphingosine are offset by the pro-survival signaling of their closely linked metabolites S1P and ceramide-1-phosphate (C1P). The disturbances of balance between ceramide and S1P are observed in many diseases such as AD, multiple sclerosis, ischemia [16,40,55]. Both of those bioactive sphingolipids are engaged directly or indirectly in the regulation of amyloid β peptides (Aβ) metabolism. Aβ is released from β-amyloid precursor protein (βAPP), a membrane protein involved in regulation of synapse formation, neuronal growth and repair. βAPP is metabolized by two separate pathways: nonamyloidogenic and amyloidogenic. In the nonamyloidogenic pathway, α- and γ-secretases are responsible for βAPP degradation to peptide p3 and αAPP. Several zinc metalloproteinases, including ADAM10, can mediate βAPP cleavage at α-secretase sites [1]. In the amyloidogenic pathway, βAPP is degraded by β-site AβPP-cleaving enzyme 1 (BACE1) and by γ-secretase to Aβ. The γ-secretase is a tetrameric protein complex that contains two presenilins (PSEN1 and PSEN2), the most important components of this secretase [4,26,62].

Ceramides and Aβ share many common features, especially in induction of oxidative stress and inflammatory response. Both of them are responsible for activation of inducible NO synthase (iNOS) and proinflammatory cytokines tumor necrosis factor (TNF)-α, interleukin (IL)-1β [19]. Elevated ceramides' levels are observed in the AD brain and blood samples [10,35,61]. Panchal *et al*. observed that Cer(d18:1/18:0) and Cer(d18:1/20:0) ceramides' levels are enriched within senile plaques in comparison with neutrophils [44]. The authors suggest that the increased sphingomyelin hydrolysis leads to ceramide accumulation. Moreover, the elevated aSMase and nSMase activities and gene expressions are observed in AD brains [12,18,45]. Additionally, Aβ generation may be also regulated indirectly by ceramides. Ceramides could post-translationally stabilize the β-secretase (BACE1), increase its half-life and thereby enhance Aβ biogenesis [46]. Recent data of Takasugi *et al*. study suggest that a synthetic ceramide analogue can be responsible for elevated Aβ production *via* modulation of γ-secretase production [50]. Reversely, Aβ peptides could stimulate ceramide synthesis by activation of nSMase [18,28].

It is common knowledge that oxidative stressors play a crucial role in the pathology of neurodegenerative diseases. Oxidative stress evoked by Aβ peptides may lead to disturbance of many key processes involved in regulation of the cell viability and death [3]. Among many pro- and anti-oxidative enzymes, the NAD^+-dependent sirtuins play an important role in stress response [9,39,58]. These enzymes, depending on stress conditions, may exert a neuroprotective effect or may lead to the cell degenera-

tion and death [20,21,25,37,49]. SIRTs are a family of seven enzymes that are type III histone deacetylases (HDAC). Three of them (SIRT3, SIRT4, SIRT5) primarily exist in mitochondria. SIRT1, mainly a nuclear enzyme, can be also present in mitochondria [21].

SIRT1 is one of most-studied and recognized sirtuins, which plays an important role in regulation of oxidative defense, inflammatory response and cells' fate. SIRT1 levels are downregulated in the AD brain and its expression correlates with the stage/duration of the disease. Moreover, downregulation of SIRT1 parallels with the accumulation of tau protein in AD [22,29]. SIRT1 is also partially involved in reduction of Aβ formation by activation of ADAM10 (α-secretase) and promotion of a non-amyloidogenic pathway. Moreover, SIRT1 may revert the negative consequences of Aβ-generated oxidative stress by the increase in mRNA and protein levels of antioxidative enzymes, such as manganese superoxide dismutase (SOD2), catalase (Cat), peroxiredoxin 5 (Prx5) and thioredoxin reductase 2 (TR2) [41,47,57]. SIRT1 could also be responsible for reduction of Aβ-induced inflammation *via* inhibition of the NF-κB-dependent gene expression [5,37]. Recent data of *in vitro* studies have shown that SIRT1 could also downregulate BACE1 expression levels and thus decrease Aβ levels [32]. SIRT1 is also an important regulator of Tau-protein phosphorylation – one of the pathological hallmarks of AD [36].

In this study, we investigated the effect of C2-ceramide and S1PR1 agonist (SEW 2871) on cells' viability and gene expression of enzymes, which are responsible for APP metabolism and stress response (NAD^+-dependent nuclear *Sirt1*). The studies were carried out using empty vector-transfected cells (PC12), cells transfected with the human wild-type APP gene (APPwt), and cells transfected with a Swedish mutation of the APP gene (APPsw).

Material and methods

Cell culture

Rat pheochromocytoma PC12 cells transfected with a human gene for APP wild-type (APPwt) and cells transfected with a Swedish mutation of *Homo sapiens* Beta-Amyloid Precursor Protein Gene (K670M/N671L) APP gene (APPsw) were used in this study. Cells transfected with an empty vector was used as a control. PC12 cells were a kind gift from Prof. Arkadiusz Orzechowski (Warsaw University of Life Sciences, Warsaw, Poland). The procedure of cell transfection was described previously [43]. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 5% heat-inactivated horse serum (HS), 2 mM L-glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, gentamicin sulfate 20 µg/ml and Fungizone (amphotericin B, 1 µg/ml). G-418 (400 µg/ml) and ampicillin (100 µg/ml) were additionally present in the medium for growth and selection of positively transfected PC-12 cells. Cells were cultured at 37°C in a 5% CO_2 atmosphere.

Cell treatment protocols

The cells were used for experiments between 5 and 12 passage numbers. Prior to treatment, the cells were cultivated in a low serum (2% FBS) medium containing 1% penicillin/streptomycin and 2 mM L-glutamine in order to stop proliferation of cells. Then, PC12 cells were treated with (25 µM) C2-ceramide or/and with (10 µM) SEW 2871 for 24 h. The cells in the C2-ceramide + SEW 2871 group were treated with SEW 2871 one hour prior to the C2-ceramide treatment.

Cell viability analysis using MTT test

Cellular viability was evaluated using 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). After incubation with the appropriate compounds, MTT (2.5 mg/ml) was added to all of the wells. The cells were incubated at 37°C for 2 h. Then the medium was removed, formazan crystals were dissolved in DMSO and measurement of absorbance at 595 nm was performed.

Quantitative real-time PCR assays

PC12 cells were washed twice with ice-cold PBS and suspended in 1 ml of TRI reagent (Sigma-Aldrich). Then, RNA was isolated according to the manufacturer's protocol. The concentration and purity of RNA were assessed spectrophotometrically (A260/A280 method). Digestion of DNA contamination was performed by using DNase I according to the manufacturer's protocol (Sigma-Aldrich). Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). The level of mRNA for selected genes was analyzed using TaqMan Gene Expression Assays (Applied

Biosystems, Foster City, CA, USA) on Applied Biosystems 7500 Real-Time PCR System using TaqMan Gene Expression Master Mix according to the manufacturer's instructions. The following TaqMan assays were used: *Sirt1* (Rn01428096_m1), *Adam10* (Rn01530753_m1), *Bace1* (Rn00569988_m1), *Psen1* (Rn00569763_m1). *Actb* (Rn00667869_m1) was selected and used in all of the studies as a reference gene. The relative level of mRNA was calculated using the ΔΔCt method.

Statistical analysis

The presented data are the means ± SEM. For statistical comparisons, one-way ANOVA followed by Newman-Keuls *post-hoc* test were used. *P* values < 0.05 were considered statistically significant ($^{*,\ \#}p < 0.05$; $^{**,\ \#\#}p < 0.01$; $^{***,\ \#\#\#}p < 0.001$). The statistical analyses were performed using Graph Pad Prism version 6.0 (Graph Pad Software, San Diego, CA, USA).

Results

In the first step of our research we investigated the cells' viability after C2-ceramide treatment. It was observed that C2-ceramide induced cell death in both APP-transfected cells and in PC12 control cells with an empty vector. The S1PR1 agonist (SEW 2871) enhanced cells' viability affected by C2-ceramide (Fig. 1A-C).

Then, the effect of C2-ceramide and SEW 2871 on the gene expression of APP metabolizing enzymes which regulate Aβ concentration was analyzed. It was observed that C2-ceramide alone significantly decreased the *Adam10* mRNA level in PC12 cells with an empty vector (Fig. 2A). Moreover, S1PR1 agonist significantly enhanced the mRNA level of *Adam10* after C2-ceramide treatment (Fig. 2A). A similar, though not significant, tendency was also observed in APP transfected cells (Fig. 2B,C).

In the next step of our research we investigated the influence of C2-ceramide and S1PR1 agonist

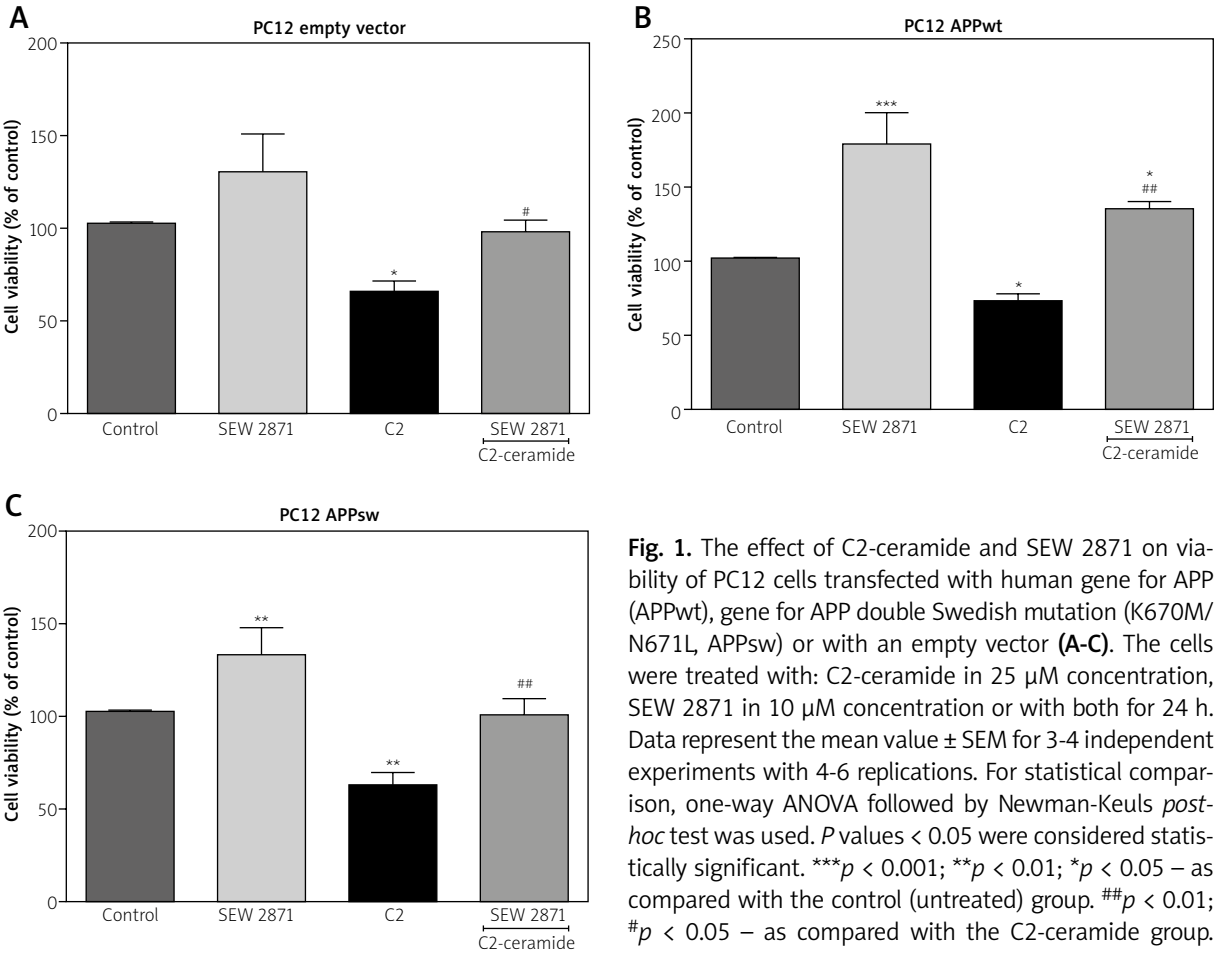
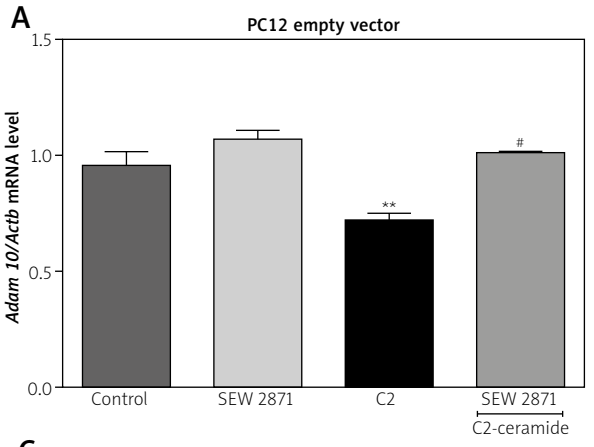
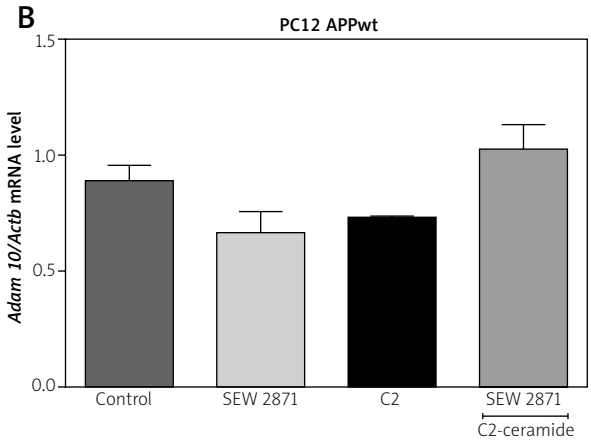


Fig. 1. The effect of C2-ceramide and SEW 2871 on viability of PC12 cells transfected with human gene for APP (APPwt), gene for APP double Swedish mutation (K670M/N671L, APPsw) or with an empty vector **(A-C)**. The cells were treated with: C2-ceramide in 25 μM concentration, SEW 2871 in 10 μM concentration or with both for 24 h. Data represent the mean value ± SEM for 3-4 independent experiments with 4-6 replications. For statistical comparison, one-way ANOVA followed by Newman-Keuls *post-hoc* test was used. *P* values < 0.05 were considered statistically significant. $^{***}p < 0.001$; $^{**}p < 0.01$; $^{*}p < 0.05$ – as compared with the control (untreated) group. $^{\#\#}p < 0.01$; $^{\#}p < 0.05$ – as compared with the C2-ceramide group.





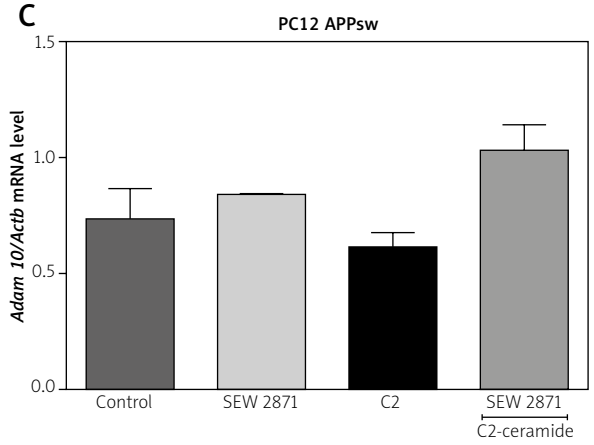
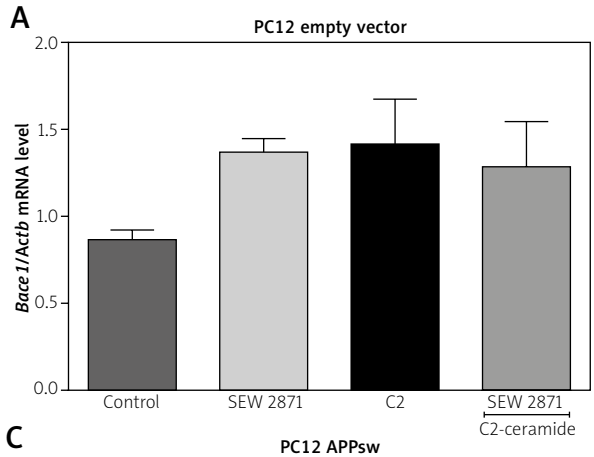
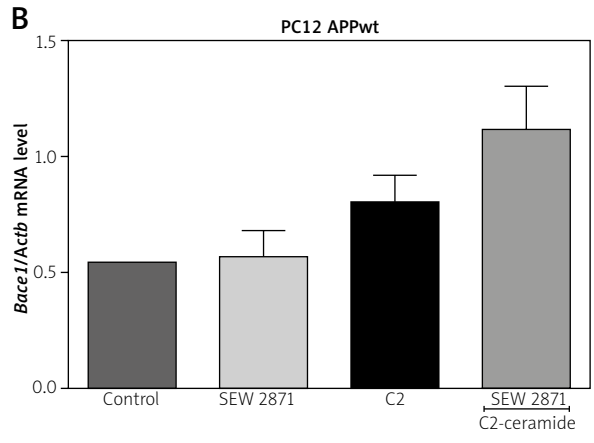


Fig. 2. The effect of C2-ceramide and SEW 2871 on *Adam10* gene expression. PC12 cells were incubated in the presence of C2-ceramide in 25 μM concentration, SEW 2871 in 10 μM concentration or with both for 24 h (**A-C**). The levels of α-secretase (*Adam10*) mRNA were analyzed *via* quantitative RT-PCR. The results of RT-PCR were normalized to *Actb* gene expression. Data represent the mean value ± SEM for 2-4 independent experiments with 3 replications. For statistical comparison, one-way ANOVA followed by Newman-Keuls *post-hoc* test was used. *P* values < 0.05 were considered statistically significant. $^{**}p < 0.01$ – as compared with the control (untreated) group. $^{\#}p < 0.05$ – as compared with the C2-ceramide group.





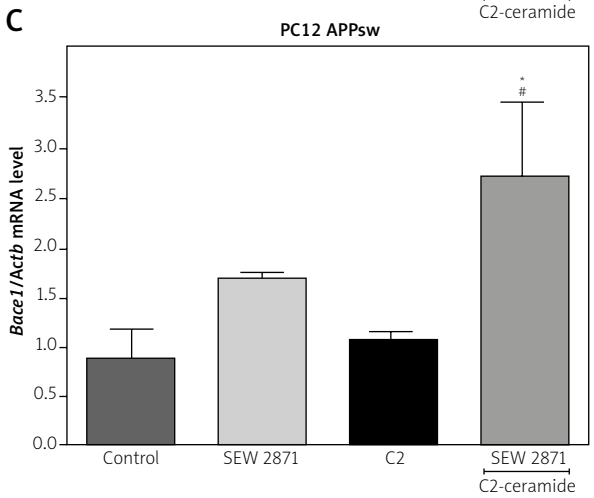


Fig. 3. The effect of C2-ceramide and SEW 2871 on *Bace1* gene expression. PC12 cells were incubated in the presence of C2-ceramide in 25 μM concentration, SEW 2871 in 10 μM concentration or with both for 24 h (**A-C**). The levels of β-secretase (*Bace1*) mRNA were analyzed *via* quantitative RT-PCR. The results of RT-PCR were normalized to *Actb* gene expression. Data represent the mean value ± SEM for 2-4 independent experiments with 3 replications. For statistical comparison, one-way ANOVA followed by Newman-Keuls *post-hoc* test was used. *P* values < 0.05 were considered statistically significant. $^{*}p < 0.05$ – as compared with the control (untreated) group. $^{\#}p < 0.05$ – as compared with the C2-ceramide group.

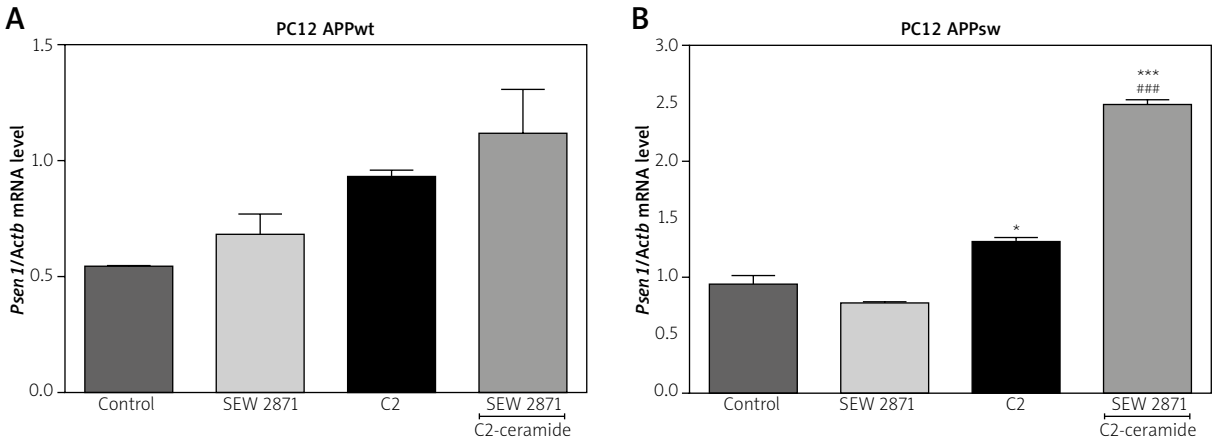


Fig. 4. The effect of C2-ceramide and SEW 2871 on *Psen1* gene expression. PC12 cells were incubated in the presence of C2-ceramide in 25 µM concentration, SEW 2871 in 10 µM concentration or with both for 24 h **(A, B)**. The levels of genes coding crucial subunits of γ-secretase (*Psen1*) mRNA were analyzed *via* quantitative RT-PCR. The results of RT-PCR were normalized to *Actb* gene expression. Data represent the mean value ± SEM for 2-4 independent experiments with 3 replications. For statistical comparison, one-way ANOVA followed by Newman-Keuls *post-hoc* test was used. *P* values < 0.05 were considered statistically significant. ***$p < 0.001$; *$p < 0.05$ – as compared with the control (untreated) group. ###$p < 0.001$ – as compared with the C2-ceramide group.

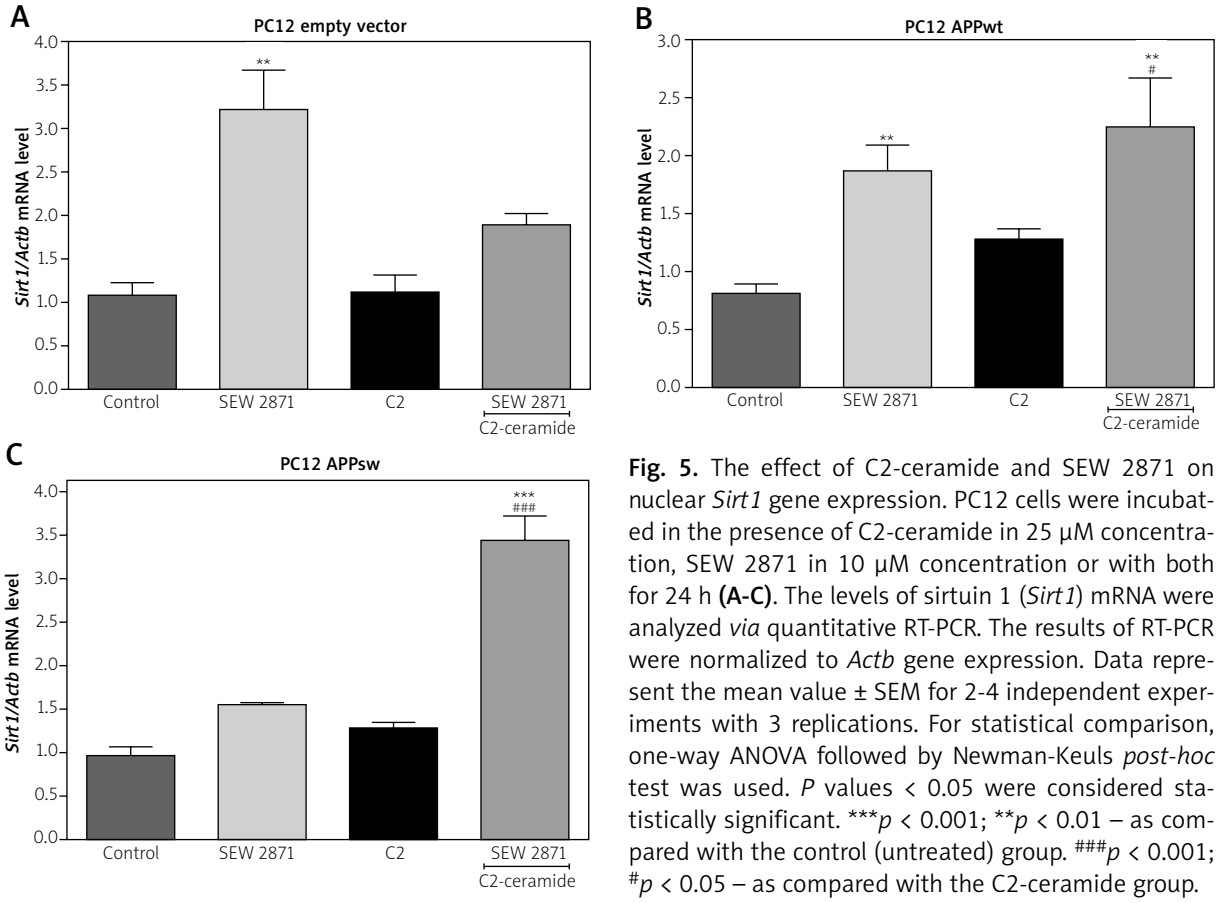


Fig. 5. The effect of C2-ceramide and SEW 2871 on nuclear *Sirt1* gene expression. PC12 cells were incubated in the presence of C2-ceramide in 25 µM concentration, SEW 2871 in 10 µM concentration or with both for 24 h **(A-C)**. The levels of sirtuin 1 (*Sirt1*) mRNA were analyzed *via* quantitative RT-PCR. The results of RT-PCR were normalized to *Actb* gene expression. Data represent the mean value ± SEM for 2-4 independent experiments with 3 replications. For statistical comparison, one-way ANOVA followed by Newman-Keuls *post-hoc* test was used. *P* values < 0.05 were considered statistically significant. ***$p < 0.001$; **$p < 0.01$ – as compared with the control (untreated) group. ###$p < 0.001$; #$p < 0.05$ – as compared with the C2-ceramide group.

on the expression of enzymes responsible for APP processing in amyloidogenic pathway. It was found that C2-ceramide exerted a stimulatory effect, though not significant, on the gene expression of β-secretase (*Bace1*) in PC12 control (with empty vector) and APPwt cells (Fig. 3A,B). This effect was not observed in APPsw cells (Fig. 3C). In APPsw cells, SEW 2871 significantly increased the mRNA level of *Bace1* only in the presence of C2-ceramide (Fig. 3C).

Additional analysis of ceramide action on APP processing indicated a stimulatory, though not significant, effect of C2-ceramide on the gene expression of *Psen1* in APPwt cells (Fig. 4A). Also, in APPsw cells, the mRNA level of *Psen1* after C2-ceramide treatment increased significantly (Fig. 4B). This effect was not observed in PC12 control cells with an empty vector (data not shown). Moreover, SEW 2871 had also a significant stimulatory effect on *Psen1* mRNA levels in the presence of C2-ceramide in APPsw cells (Fig. 4B).

Subsequently, we analyzed the role of the C2-ceramide and S1PR1 agonist in regulation/alterations of the gene expression of stress response proteins such as NAD^+ dependent *Sirt1* in PC12 cells with overexpressed human APP. We observed small insignificant changes in the mRNA level of *Sirt1* after C2-ceramide treatment. However, the S1PR1 agonist in the presence/absence of C2-ceramide exerted significant activation of *Sirt1* in APP transfected PC12 cells (Fig. 5B,C).

Discussion

In our study we have observed decreased cells' viability after ceramide treatment in all cells' lines used in the experiment. Concomitantly, the use of SEW 2871 significantly increased the viability of APP-transfected cells as well as control cells (with an empty vector). These results point out the importance of bioactive sphingolipids, especially S1P-mediated signaling as a protection against cell death. In the presented study S1PR1 agonist improved cells' viability in PC12 control cells with an empty vector and in APP-transfected cells after ceramide treatment.

It has been published that SIRT1 shifts the balance between amyloidogenic and non-amyloidogenic processing of APP *in vitro* and in transgenic mouse models [37,56]. SIRT1 up-regulates the α-secretase ADAM10, and through inhibition of NF-κB down-regulates the expression of the β-secretase (BACE1) [8,27,53]. *In vitro* studies on human cells' lines and rat primary cortical neurons have shown that γ-secretase is also regulated by SIRTs [60]. SIRT1 occurs to reduce the level of Aβ, oxidative stress and in consequence neuronal loss [14]. Our results demonstrated that S1PR1 agonist, SEW 2871, in the presence of C2-ceramide significantly increased the mRNA level of *Sirt1* in both transfected cells' lines. These data correlate with a recent study of Gao *et al*. [13] where Sphk1 inhibition results in SIRT1 downregulation. Moreover, this effect could be reversed by addition of exogenous S1P. Authors suggest that upregulation of SIRT1 may be mediated by alteration of phosphorylation levels of P38 MAPK, ERK and AKT [13]. Another *in vivo* study reveals that the activation of sirtuins expression by resveratrol may result in an enhanced level of S1P [24]. These results suggest the important link between S1P and SIRT1 expression.

The results of our study show that the mRNA level of *Adam10* is downregulated in PC12 control cells and it shows a tendency to be decreased in APPwt and APPsw cells after C2-ceramide treatment. At the same time, the mRNA expression of *Sirt1* does not change in the presence of C2-ceramide. However, the use of SEW2871 leads to an increased expression of *Adam10* during C2-ceramide stress in cells with an empty vector and also show a tendency to increase its expression levels in APP-transfected cells. This result points out the modulatory role of S1PR1 on α-secretase gene expression during stress conditions evoked by C2-ceramide. This result may also be a consequence of elevated *Sirt1* expression after modulatory action of SEW 2871 on the S1PR1.

The recent data provide a lot of evidence that exogenous addition of ceramide and/or increased level of endogenous ceramide (induced by *de novo* synthesis or sphingomyelin hydrolysis) increase the level of Aβ [31,46,50]. Processing of APP to Aβ peptide occurs predominantly in lipid rafts and BACE1 is a rate-limiting enzyme in this process. Membrane ceramides embedded in lipid rafts facilitate production of Aβ by increasing the half-life of BACE1 through posttranslational stabilization [42,46]. In our current study we indicated that C2-ceramide leads to activation of genes coding enzymes responsible for APP metabolism. C2-ceramide enhanced the mRNA level of *Psen1* crucial subunit of γ-secretase involved in degradation of APP in both APP-transfected cells'

lines. Moreover, C2-ceramide exerted a tendency to increase *Bace1* gene expression in APPwt cells. It is documented that oxidative stress can stimulate BACE1 expression in cells through the c-jun N-terminal kinase pathway in a mechanism which requires the presence of presenilin [52]. A lot of data also showed that C2-ceramide induced oxidative stress and activated the c-jun kinase pathway [2,11,59]. It is highly possible that in our research the regulation of BACE1 expression occurred through these pathways. Literature data show that another cell-permeable analog of ceramide – C6-ceramide increases the rate of Aβ biosynthesis by affecting β-cleavage of APP [46]. It was also documented that synthetic ceramide analogues may influence APP metabolism through its modulatory effect on γ-secretase activity [50]. Thus, it seems to be very important to maintain the right balance between ceramide and pro-survival molecule S1P. The use of S1P receptor modulators seems to be a promising strategy in counteracting the negative effects of elevated ceramide and amyloidogenic APP enzyme levels. In our study, S1PR1 agonist SEW2871 alters the expression of genes involved with APP processing, which may suggest that SEW2871 accelerates APP/Aβ metabolic rate.

There are a few scientific data about the S1P action on APP degrading enzymes. Takasugi *et al*. [51] revealed the correlation between Sphk2 and APP processing, where S1P pool produced by Sphk2 may activate β-secretase (*Bace1*) and lead to a higher generation of Aβ peptide [51]. The data from *in vitro* studies showed that S1P could increase the activity of ADAM17 which also possesses the properties of α-secretase [17]. Summarizing, the role of S1P in the regulation of enzymes involved in APP metabolism is complex and poorly understood. S1P may exert its effect not only by activation of its specific receptors, but also acting as a secondary messenger and regulator of the gene expression.

Disclosure

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