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Endoplasmic reticulum (ER) stress inhibitor salubrinal protects against ceramide-induced SH-SY5Y cell death

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ABSTRACT

In the present study, we examined the mechanisms of ceramide-induced cell death in SH-SY5Y human neuroblastoma cells. Our results demonstrate a significant endoplasmic reticulum (ER) stress response in SH-SY5Y cells after short-chain ceramide (C6) treatment. Administration of ceramide (C6) to SH-SY5Y human neuroblastoma cells caused apoptotic cell death, which was inhibited by ER stress inhibitor salubrinal. Further, ceramide-induced cell death reduced significantly in stable SH-SY5Y cells expressing C/EBP homologous protein (CHOP) shRNA. Salubrinal inhibited ceramide-induced inositol-requiring enzyme 1α (IRE1α)/apoptosis signal regulating kinase 1 (ASK1)/c-Jun N-terminal kinase (JNK) phosphorylation. Taken together, these data suggest that ceramide-induced SH-SY5Y cell death may be linked to the ER stress-regulated intrinsic pathway, and proposed the potential protective effects of salubrinal.

1. Introduction

Ceramide regulates cell cycle arrest, cell differentiation and apoptosis [1–4]. Ceramide pathways are involved in a variety of neurological disorders including epilepsy, cerebral ischemia, Alzheimer's and Parkinson's diseases [5,6]. Administration of cell-permeable ceramide analogs or sphingomyelinase causes neuronal cell death in SH-SY5Y human neuroblastoma cells and various neuronal culture cells [7–14]. Despite growing evidence suggesting a role for ceramide in neuronal apoptosis, specific intracellular pathways of ceramide-induced neuronal death remain to be identified.

Normally, endoplasmic reticulum (ER) regulates the synthesis, post-translational modification and maturation of newly-synthesized proteins. It is also important for maintaining intracellular calcium homeostasis. Various stress conditions disrupt the proper functions of ER and cause ER stress. Receptors at ER membrane including double-stranded RNA-activated protein kinase (PKR)-like ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1α (IRE1α) act as the sensors of ER stresses. These receptors initiate unfolded protein responses (UPR) [15] to cause: (1) transcriptional up-regulation of ER chaperones (such as C/EBP homologous protein (CHOP); (2) translational attenuation to limit further accumulation of misfolded proteins; and (3) ER-associated degradation to eliminate misfolded proteins [15]. Although the UPR is primarily a pro-survival response, in the event of prolonged or severe ER stress, the UPR switches to cause cell apoptosis [15].

In the present study, we examined the mechanisms of ceramide-induced SH-SY5Y cell death by focusing ER stress pathway. We also tested the potential effects of ER stress inhibitor salubrinal in this condition. Our study provides further evidence in support of a role for ceramide in neuronal apoptosis.

2. Materials and methods

2.1. Chemicals and reagents

ER stress inhibitor salubrinal was purchased from Calbiochem (La Jolla, CA). Ceramide (C6) was obtained from Avanti (Alabaster, Alabama). Anti-phospho-IRE1α (Ser 724) was purchased from Novus Biologicals (Littleton, Colorado). Anti-apoptosis signal regulating kinase 1 (ASK1), tubulin, IRE1α antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). All other phospho-antibodies and their nonphospho-control antibodies were obtained from Cell Signaling Technology (Beverly, MA). GADD153 (CHOP) shRNA containing lentiviral particles (sc-35437-V) were obtained from Santa Cruz Biotech (Santa Cruz, CA).

Abbreviations: ASK1, apoptosis signal regulating kinase 1; ATF6, activating transcription factor 6; CHOP, C/EBP homologous protein; eIF2α, the eukaryotic initiation factor 2α; RE1, inositol-requiring enzyme 1; JNK, c-Jun N-terminal kinase; MTT, 3-[4,5-dimethylthylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PERK, double-stranded RNA-activated protein kinase (PKR)-like ER kinase; RNAi, RNA interference; shRNA, short hairpin RNA; TRAF2, TNF receptor-associated factor 2; UPR, unfolded protein response.

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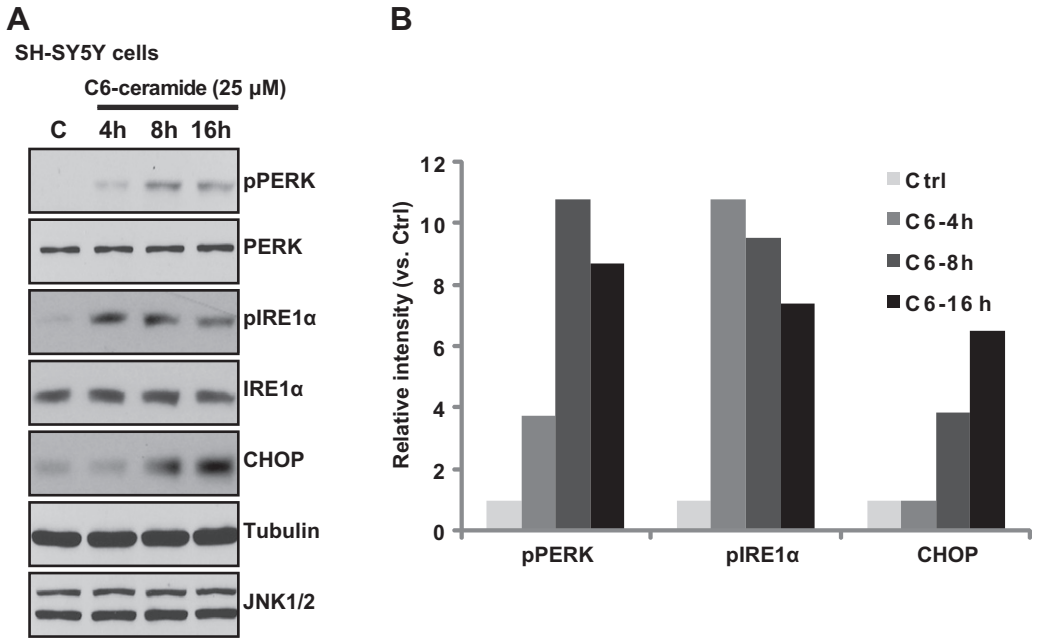

Fig. 1. Short-chain ceramide (C6) induces ER stress response in SH-SY5Y cells. SH-SY5Y human neuroblastoma cells were treated with short-chain ceramide (C6, 25 μM) for 4, 8 and 16 h, phospho- and total-levels of PERK, IRE1α, as well as expression of CHOP, tubulin and JNK1/2 were examined by Western-blots (A). Blots intensity of phospho-PERK, phospho-IRE1α and CHOP were quantified by ImageJ software after normalized their corresponding loading controls (CHOP was normalized to tubulin), the number was fold changes vs. untreated control (lane 1) (B). Experiments in this figure were repeated three times to insure consistency of results.

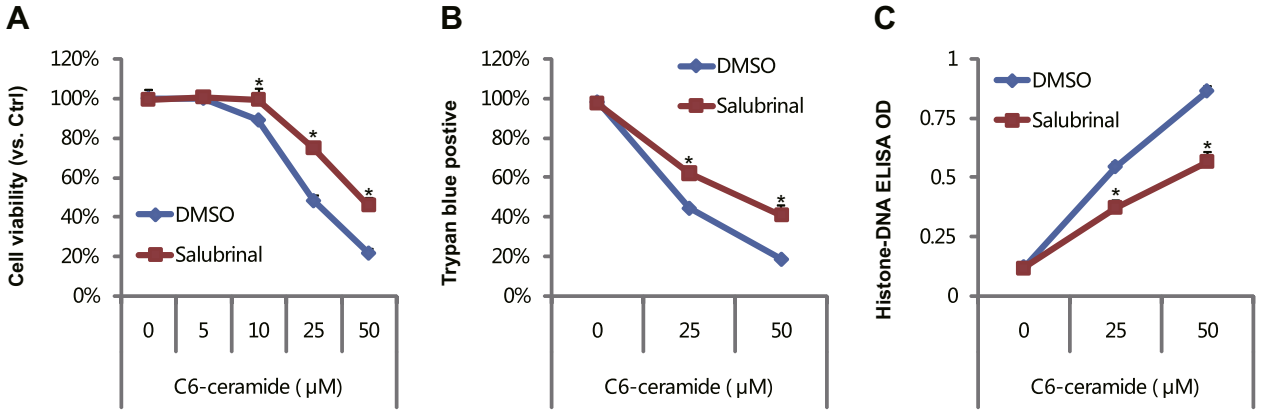

Fig. 2. ER stress inhibitor salubrinal protects against ceramide (C6)-induced SH-SY5Y cell death/apoptosis. SH-SY5Y cells were pre-treated with salubrinal (20 μM, 1 h pretreatment), followed by indicated ceramide (C6) for 48 h, cell viability was measured by MTT assay (A), the percentage of trypan blue positive cells were recorded (B), Histone–DNA ELISA assay was utilized to quantify cell apoptosis (C). Experiments in this figure were repeated three times to insure consistency of results. $^{*}p < 0.05$ vs. groups without salubrinal treatment (ANOVA).

2.2. Cell culture

SH-SY5Y human neuroblastoma cells were maintained in a DMEM medium (Sigma, St. Louis, MO), supplemented with a 10% FBS (Sigma, St. Louis, MO), penicillin/streptomycin (1:100, Sigma, St. Louis, MO) and 4 mM L-glutamine (Sigma, St. Louis, MO), in a CO_2 incubator at 37 °C.

2.3. Western-blots

Cells with indicated treatments were harvested in a lysis buffer reported early [16,17]. Protein concentration was determined by Bio-Rad protein assay (Bio-Rad, Beijing, China). Aliquots of 30–40 μg of lysates were electrophoresed on 10% SDS–PAGE and transferred to PVDF membranes. The blots were then incubated with primary antibodies at 4 °C overnight. Appropriate secondary antibodies conjugated to horseradish peroxidase (HRP) were then added. Antigen–antibody complex was detected by using enhanced chemiluminescence (ECL) reagent (Amersham Biosciences, Piscataway, NJ). All Western-blots in this study were subjected to different exposures: from 10 s to 10 min, the best exposures were selected for data presentation. The density of Western-blot bands was quantified by ImageJ software as previous reported [16,17].

2.4. Cell viability assay ("MTT" assay)

Cell viability was measured by the 3-[4,5-dimethylthylthiazol-2-yl]-2,5-diphenyltetrazolium bromide ("MTT") assay as reported [16,17]. Briefly, SH-SY5Y cells were collected and seeded in 96-well plates at a density of $2–3 \times 10^5$ cells/cm^2. After indicated treatment/s, 20 μl of MTT tetrazolium salt (Sigma, St. Louis, MO) was added to each well for 3 h at 37 °C. Afterwards, DMSO (200 μl)

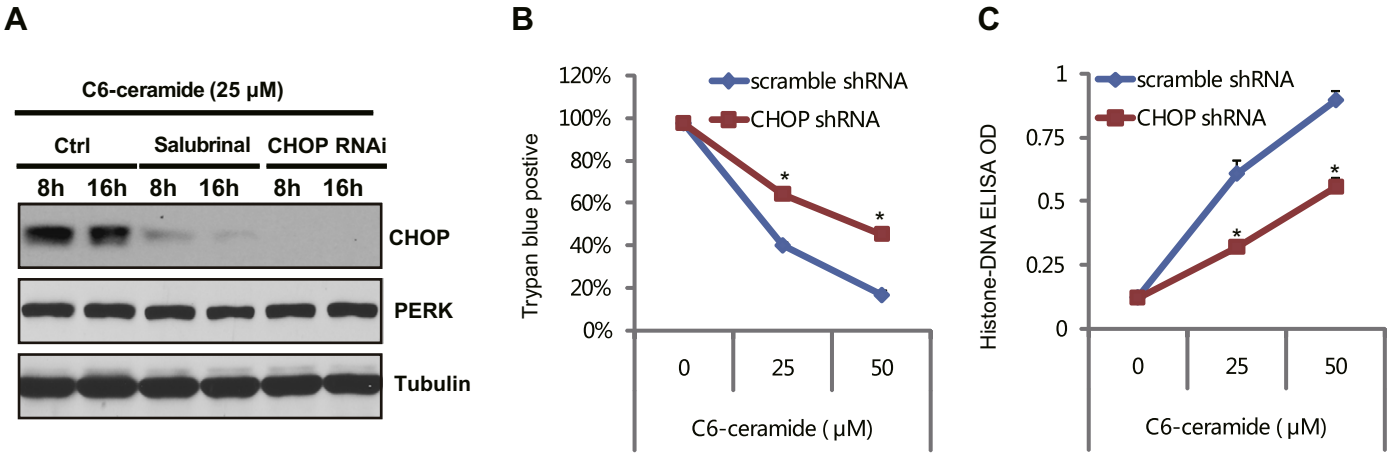

Fig. 3. CHOP RNAi knockdown cells are resistant to ceramide (C6)-induced cell death. The effect of salubrinal (20 μM, 1 h pretreatment) or CHOP shRNA (lentiviral-packed, 20 μl/ml) on ceramide (C6, 25 μM)-induced CHOP expression was detected by Western-blots, expression level of PERK and tubulin was also tested (A). Puromycin (1 μg/ml) selected stable SH-SY5Y cells expressing scramble- or CHOP-shRNA were treated with indicated ceramide (C6) for 48 h, the percentage of trypan blue positive cells were recorded (B), Histone–DNA ELISA assay was utilized to detect cell apoptosis (C). Experiments in this figure were repeated three times to insure consistency of results. $^{*}p < 0.05$ vs. groups without salubrinal treatment (ANOVA).

was added to dissolve formazan crystals, the absorbance of each well was observed by a plate reader at a test wavelength of 490 nm with a reference wavelength of 630 nm.

2.5. Live cell counting by trypan blue staining

The number of viable SH-SY5Y cells (trypan blue positive) after indicated treatment/s was counted, and the percentage (%) of viable cells was calculated by the number of the trypan blue stained cells divided by the total number of cells.

2.6. Quantification of apoptosis by Histone–DNA ELISA

The Cell Apoptosis ELISA Detection Kit (Roche, Palo Alto, CA) was used to detect apoptosis in SH-SY5Y cells according to the manufacturer's protocol. Briefly, the cytoplasmic Histone/DNA fragments from cells with/without treatments were extracted and bound to immobilized anti-Histone antibody. Subsequently, the peroxidase-conjugated anti-DNA antibody was used for the detection of immobilized Histone/DNA fragments. After addition of substrate for peroxidase, the spectrophotometric absorbance of the each sample was determined by using a plate reader at 405 nM.

2.7. Generation of CHOP knockdown stable SH-SY5Y cells by lentiviral shRNA infection

CHOP shRNA containing lentiviral particles (20 μl/ml) were added to the SH-SY5Y cells for 48 h. Afterwards, cell culture medium was replaced with puromycin (1 μg/ml)-containing fresh medium every 2–3 days until resistant stable cells were formed. The expression level of CHOP was detected by Western-blots in the resistant colonies. Same amount of scramble shRNA (sc-108080, Santa Cruz Biotech, Santa Cruz, CA) was always added in control cells.

2.8. Statistical analysis

Each experiment was repeated a minimum of three times. In each experiment, the mean value of the repetitions was calculated and this value was used in the statistical analysis. All data were normalized to control values of each assay and were presented as mean ± standard deviation (SD). Data were analyzed by one-way ANOVA followed by a Scheffe's *f*-test by using SPSS software (SPSS Inc., Chicago, IL, USA). Significance was chosen as $p < 0.05$.

3. Results

3.1. Short-chain ceramide (C6) induces ER stress response in SH-SY5Y cells

We first examined ER stress response in ceramide-treated SH-SY5Y cells by testing phosphorylation of PERK (Thr 980), IRE1α (Ser 724) and the expression of CHOP. Western-blots results in Fig. 1A demonstrated a significant ER stress response after ceramide (short-chain C6) administration in SH-SY5Y cells. Ceramide (C6) induced significant PERK/IRE1α phosphorylation and CHOP expression (Fig. 1A and B). Compared to untreated control level, after 4, 8 and 16 h of ceramide (C6, 25 μM) treatment, PERK phosphorylation increased to 3.74-, 10.80- and 8.73-fold, and CHOP expression increased to 1.00-, 3.85- and 6.52-fold, respectively (Fig. 1B).

3.2. ER stress inhibitor salubrinal protects against ceramide (C6)-induced SH-SY5Y cell death/apoptosis

To test whether ER stress is important for ceramide-induced SH-SY5Y cell death, we applied ER stress inhibitor salubrinal. Results in Fig. 2 showed the protective effects of salubrinal against ceramide (C6). Salubrinal co-administration inhibited ceramide (C6)-induced SH-SY5Y cell viability loss (Fig. 2A), the PI positive cells percentage (Fig. 2B) and Histone–DNA ELISA OD (cell apoptosis indicator) (Fig. 2C) were also decreased. For instance, the percentage of trypan blue positive cell was 18.40 ± 0.86% after ceramide (C6, 50 μM, 48 h) treatment, co-administration with salubrinal (20 μM) increased it to 40.97 ± 5.04% ($p < 0.05$) (Fig. 2B). The Histone–DNA ELISA OD decreased from 0.86 ± 0.02 (C6, 50 μM) to 0.56 ± 0.05 after salubrinal (20 μM) co-administration ($p < 0.05$) (Fig. 2C).

3.3. CHOP RNAi knockdown cells are resistant to ceramide (C6)-induced cell death

One of the major factors that contribute to ER stress-mediated cell death is CHOP. Fig. 1A demonstrated that ceramide (C6) induced a significant CHOP expression in SH-SY5Y cells. Salubrinal,

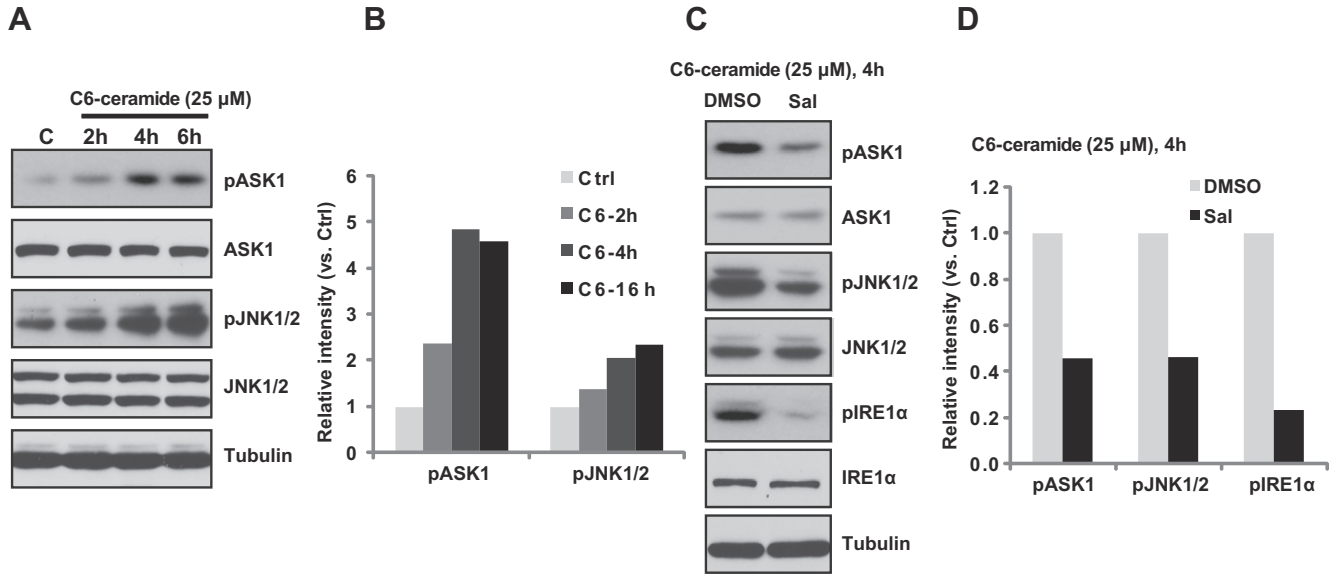

Fig. 4. Salubrinal inhibits ceramide-induced IRE1α-ASK1-JNK phosphorylation. SH-SY5Y cells were treated with short-chain ceramide (C6, 25 μM) for 2, 4 and 6 h, phospho- and total-levels of ASK1 and JNK1/2 were examined by Western-blots (A). Blots intensity of JNK1/2 (phospho-) and ASK1(phospho-) were quantified by ImageJ software after normalized their corresponding loading controls (B). The effect of salubrinal (20 μM, 1 h pretreatment) on ceramide (C6, 25 μM, 4 h)-induced IRE1α-ASK1-JNK phosphorylation was detected by Western-blots, expression level of PERK and tubulin were also tested (C). Blots intensity of IRE1α (phospho-), JNK1/2 (phospho-) and ASK1(phospho-) were quantified by ImageJ software after normalized their corresponding loading controls (B). Experiments in this figure were repeated three times to insure consistency of results.

which was protective against ceramide (C6) (Fig. 2), largely inhibited CHOP expression (Fig. 3A). We then created a stable SH-SY5Y cells expressing CHOP shRNA through lentiviral infection (Fig. 3A, CHOP RNAi) (see Section 2). Stable CHOP RNAi-knockdown cells were resistant to ceramide (C6)-induced cell death (Fig. 3B and C). Ceramide (C6)-induced loss of trypan blue positive cell was attenuated in stable SH-SY5Y cells with CHOP RNAi (Fig. 3B), and the Histone–DNA ELISA OD was also decreased (Fig. 3C). These results suggested that CHOP expression is involved in ceramide-induced SH-SY5Y cell death/apoptosis.

3.4. Salubrinal inhibits ceramide-induced IRE1α-ASK1-JNK phosphorylation

Activation of ASK1-JNK pathway is critical for ceramide-induced cell death/apoptosis [3]. In SH-SY5Y cells treated with ceramide (C6), a significant ASK1-JNK activation (phosphorylation) was also observed. After 2 h, 4 h and 6 h of ceramide (C6, 25 μM) treatment, JNK phosphorylation level increased to 1.39-, 2.07- and 2.35-fold of untreated control level, respectively (Fig. 4B). Significantly, salubrinal inhibited ceramide (C6, 25 μM, 4 h)-induced IRE1α/ASK1/JNK phosphorylation (Fig. 4C, also see quantification data in Fig. 4D). These data suggest that salubrinal inhibits ceramide-induced IRE1α-ASK1-JNK phosphorylation, which might contribute to its protective effects against ceramide.

4. Discussion

The precise role for ceramide in neuronal death and survival remains uncertain. Studies have reported the protective effects of intraventricular injections of C_2-ceramide in an immature rat brain hypoxia–ischemia model [18,19]. However, Yu et al. demonstrated beneficial effects of ceramide downregulation in mice subjected to cerebral ischemia [20]. In cultured rat cerebellar granule cells (CGC) treated with trophic support withdraw or etoposide, ceramide level was increased and was associated with apoptosis [8]. Further, ceramide increase led to apoptosis in an HN9.10e neuronal cell line [7]. To support these studies, our results confirmed that exogenously-added ceramide (C6) is pro-apoptotic in SH-SY5Y cells.

In the ER stress process, transcription factors including XBP1, ATF4 and ATF6 regulate CHOP expression to promote cell apoptosis [21]. CHOP activates mitochondrial apoptosis pathways by regulating the balance between pro-survival and pro-apoptotic Bcl-2 family members [22,23]. For instance, CHOP induces upregulation of Bim, a pro-apoptotic BH3-only member of the Bcl-2 family [23]. It is also reported that CHOP increases the expression of TRAIL receptors to promote the extrinsic apoptotic pathway [24]. In the current study, we found that ceramide (C6) induces CHOP expression in SH-SY5Y cells, and ceramide-induced apoptosis was significantly reduced in SH-SY5Y cells with CHOP deficiency. Aside from CHOP, other ER stress-mediated apoptosis mechanisms have also been proposed [15,25]. For example, under ER stress conditions, IRE1 is activated and recruits the adaptor molecule such as TRAF2 and ASK1 to activate pro-apoptotic JNK signaling [25,26]. In SH-SY5Y cells treated with ceramide, we here found a significant IRE1/ASK1/JNK activation, and it was inhibited by salubrinal. These results further confirmed the involvement of ER stress response in ceramide-induced cell apoptosis at least in SH-SY5Y cells.

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