PI3K-Akt-mTOR signal inhibition affects expression of genes related to endoplasmic reticulum stress

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ABSTRACT. PI3K-Akt-mTOR signaling pathway is associated with endoplasmic reticulum (ER) stress. However, it is not clear how this signaling pathway affects the ER stress. The present study aimed to determine whether the PI3K-Akt-mTOR signaling pathway regulates tunicamycin (TM)-induced increases in mRNA levels of genes involved in the ER stress, to help elucidate the mechanism by which this pathway affects the ER stress in primary goose hepatocytes. Primary hepatocytes were isolated from geese and cultured in vitro. After 12 h in a serum-free medium, the hepatocytes were incubated for 24 h in a medium with either no addition (control) or with supplementation of TM or TM together with PI3K-Akt-mTOR signaling pathway inhibitors (LY294002, rapamycin, NVP-BEZ235). Thereafter, the expression levels of genes involved in the ER stress (BIP, EIF2α, ATF6, and XBP1) were assessed. The results indicated that the mRNA level of BIP
was up-regulated in 0.2, 2, and 20 µM TM treatment group (P < 0.05), whereas the mRNA levels of EIF2a, ATF6, and XBP1 were up-regulated in the 2 µM TM treatment group (P < 0.05). However, the TM mediated induction of mRNA levels of genes involved in the ER stress (BIP, EIF2a, ATF6, and XBP1) was down-regulated after the treatment with PI3K-Akt-mTOR pathway inhibitors (LY294002, NVP-BEZ235, and rapamycin). Therefore, our results strongly suggest that the PI3K-Akt-mTOR signaling pathway might be involved in the down-regulation of the TM-induced ER stress in primary goose hepatocytes.

Key words: Endoplasmic reticulum (ER) stress; Tunicamycin (TM); Goose primary hepatocytes; Unfolded protein response (UPR); PI3k-Akt-mTOR signaling pathways

INTRODUCTION

The endoplasmic reticulum (ER) plays an important role in various cellular processes including cellular homeostasis, development, synthesis and processing of proteins and their glycosylation, and stress responsiveness. It provides a site for protein modification and folding as well as functions as an intracellular Ca\textsuperscript{2+} store that plays a central role in signal transduction (Galligan et al., 2012; Pagliassotti, 2012). However, when oxidative stress, chemical toxicity, hepatic viral infections, metabolic disorders, and abuse of alcohol or drugs cause accumulation of unfolded proteins in the ER lumen and disrupt the intracellular Ca\textsuperscript{2+} homeostasis (Lai et al., 2003; Rao et al., 2004; Schröder and Kaufman, 2005b), ER stress occurs. In order to restore ER homeostasis, liver cells down-regulate the overall protein synthesis through an adaptive protective response termed the unfolded protein response (UPR) signaling system, which involves enhancement of protein folding and degradation in the ER (Shen et al., 2004). Perturbations in the environment within the ER lumen, for example a reduction in the luminal Ca\textsuperscript{2+} concentration or altered redox status, can affect protein folding and processing (Darling and Cook, 2014).

The UPR in mammalian cells is composed of three distinct signaling pathways, which are initiated by three ER transmembrane sensors, inositol-requiring enzyme 1 (IRE1), double-stranded RNA-dependent protein kinase-like ER kinase (PERK), and activating transcription factor 6 (ATF6; Schröder and Kaufman, 2005a). Activation of these sensors is dependent on the dissociation of the major ER chaperone BiP/GRP78 (binding immunoglobulin protein/glucose regulated protein 78; Hendershot, 2004). When ER stress homeostasis is perturbed, the chaperone BiP/GRPT78 interacts with unfolded proteins leading to the activation of the three implicated ER transmembrane proteins, thereby activating the UPR.

PI3K/Akt/mTOR pathway plays an important role in signal transduction pathways including those for cell growth, proliferation, survival, and metabolism. Akt (Protein Kinase B) counteracts ER stress-induced cell death (Hu et al., 2004). The cerebral ischemic post conditioning suppression of ER stress is mediated by the PI3K/Akt pathway (Yuan et al., 2011). The mammalian target of rapamycin (mTOR), a coordinator between nutritional stress and cellular growth machinery, is associated with ER stress. This suggests that the activation of the UPR pathway is an important consequence of mTORC1 activation (Li et al., 2014a). Conversely, mTORC1 can also be activated by ER stress (Flamment et al., 2012). However, how the PI3K-Akt-mTOR signaling pathway affects the ER stress, remains unclear.
Therefore, in the present study, we investigated whether the inhibition of PI3K-Akt-mTOR signaling would affect the TM-induced alterations in the mRNA levels of genes involved in ER stress in primary cultures of goose hepatocytes. We hope that the results will help in elucidating the mechanism of the effect of PI3K-Akt-mTOR signaling pathway on ER stress.

**MATERIAL AND METHODS**

**Ethics statement**

All the animal studies were approved by the Animal Care and Use Committee of Sichuan Agricultural University in China.

**Isolation and culture of goose primary hepatocytes**

Hepatocytes were isolated from three 30-day-old Sichuan White geese, procured from the Experimental Farm for Waterfowl Breeding at Sichuan Agricultural University, using a modification of the “two-step procedure” described by Seglen (1976). The modification involved the removal of the goose liver before the preperfusion step. The geese were cleaned with a disinfectant and were administered 100 IU/kg body weight heparin sodium by intravenous injection. Thereafter, anesthesia was induced by intraperitoneal injection with 3% isoflurane (35 mg/kg body weight). After the geese fell into coma, their abdominal cavity was slit along the median line of abdomen and liver was taken out rapidly and cleaned with physiological salt solution at 37°C. The remaining steps were same as in the two-step procedure. Cell viability was greater than 90%, as assessed by trypan blue dye exclusion test. Freshly isolated hepatocytes were diluted to 1 x 10^6 cells/mL. The culture medium used was dulbecco’s modified eagle’s medium (DMEM) (containing 4.5 g/L glucose; GIBCO, USA), which was supplemented with 100 IU/L insulin (Sigma, 86 USA), 100 IU/mL penicillin (Sigma, USA), 100 g/mL streptomycin (Sigma, USA), 2 mM glutamine (Sigma, USA), and 100 mL/L fetal bovine serum (Clark, Australia). The goose hepatocytes were plated in 60-mm culture dishes at a density of 3 x 10^6 cells per dish for total RNA isolation. The cultures were incubated at 4°C in a humidified atmosphere containing 5% CO_2 and the media was renewed after 3 h followed by the addition of serum-free media after 24 h. After an additional 12 h, the cells were separately treated with serum-free media supplemented with 0, 0.2, 2, and 20 µM TM (Sigma, USA) for 12 h, whereas the control cells were cultured with un-supplemented serum-free media for 12 h or media supplemented with 0, 0.2, 2, and 20 µM TM and incubated for 24 h (the serum-free medium supplemented with TM was not renewed), while the control cells were cultured with serum-free media for 24 h (serum-free media was renewed every 24 h), or supplemented with 0, 0.2, 2, and 20 µM of TM and incubated for 36 h (the serum-free medium supplemented with TM was not renewed), while the control cells were cultured with serum-free media for 24 h (serum-free media was renewed every 24 h), or supplemented with 0, 2, 20 µM of TM and incubated for 24 h (the serum-free medium supplemented with TM was not renewed), while the control cells were cultured with serum-free media for 24 h (serum-free media was renewed every 24 h), or supplemented with 0, 0.2, 2, and 20 µM of TM and incubated for 36 h (the serum-free medium supplemented with TM was not renewed), while the control cells were cultured with serum-free media for 36 h. In a similar way, the cells were treated with inhibitors (LY294002; NVP-BEZ235; rapamycin) at different concentrations (LY294002: 0, 10, 20, 30, 40, 50 µM; NVP-BEZ235: 0, 0.1, 1, 10, 500, 1000, 2000 nM; rapamycin: 0, 1, 10, 30, 50, 90 nM) for 6, 12, 24, and 36 h. Additionally, some cells were treated with serum-free media supplemented with PI3K-Akt-mTOR pathway inhibitors (LY294002: 20 µM; NVP-BEZ235: 1000 nM; rapamycin: 30 nM, respectively), followed by the addition of 2 µM TM and incubation for 24 h. At the end of the incubation period, the culture media and cells were cooled on ice and collected. All the experiments were repeated three times.
Cell viability assay

To determine the effect of TM on the viability of goose primary hepatocytes, the cells were treated with TM at different concentrations (0, 0.2, 2, and 20 µM) for 12, 24, and 36 h. To determine the effect of inhibitors on goose primary hepatocyte viability, the cells were treated with inhibitors at different concentrations (LY294002: 0, 10, 20, 30, 40, 50 µM; NVP-BEZ235: 0, 0.1, 1, 10, 500, 1000, 2000 nM; rapamycin: 0, 1, 10, 30, 50, 90 nM) for 6, 12, 24, and 36 h. Cell viability was determined using CCK-8 dye (Beyotime Institute of Biotechnology, China) according to manufacturer’s instructions. In brief, 0.5 x 10^4 cells/well were seeded in a 96-well plate, and the cultures were incubated at 4°C in a humidified atmosphere containing 5% CO₂. The medium was renewed after 3 h followed by the addition of serum-free medium after 24 h. Subsequently, cells were treated with TM for 12, 24, and 36 h. Thereafter, 10 µL CCK-8 dye was added to each well, cells were incubated at 37°C for 2 h, and the absorbance was determined at 450 nm using a microplate reader.

Isolation of total RNA and real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted using Trizol (Invitrogen, USA) and was reverse-transcribed using the Primer Script™ RT system kit for real-time polymerase chain reaction (RT-PCR; TaKaRa, Japan) as per the manufacturer instructions. The RT-PCR mixture contained SYBR Premix Ex Taq™ (TaKaRa, Japan), the newly generated cDNA template, primers for the target genes, and sterile water. The RT-PCR was performed on the on a thermocycler (Bio-Rad, USA) using the following cycling parameters: one cycle at 95°C for 10 s, followed by 40 cycles at 95°C for 5 s and 60°C for 40 s. For each experimental sample, a normalized target gene level, corresponding to the relative expression of the target gene with respect to the housekeeping genes, β-actin and 18S rRNA, was determined by the 2^-∆∆Ct method, as described previously (Livak and Schmittgen, 2001). Specific primers used for the amplification are listed in Table 1; they were designed according to the sequences determined in our lab for the genes ATF6 (GenBank accession No. KT900002), EIF2a (GenBank accession No. KT900003), XBP1 (GenBank accession No. KT900004); BIP (GenBank accession No. KT900005); 18S (GenBank accession No. L21170), and β-actin (GenBank accession No: M26111.1).

<table>
<thead>
<tr>
<th>Table 1. Primer sequences for real-time PCR.</th>
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<tr>
<td>Gene name</td>
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<td>BIP upstream</td>
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<td>EIF2a upstream</td>
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Statistical analyses

The data were subjected to ANOVA testing and the means were assessed for
significance by the Tukey test. Analysis of variance and t-tests were performed using the SAS 9.13 package (SAS Institute Inc, Cary, NC, USA). The results are reported as means ± SD. P < 0.05 was accepted as the level of significance. Every experiment was repeated with 3 biological samples, and each sample was run in triplicate.

RESULTS

TM induced ER stress

Cell viability was evaluated through the CCK-8 assay. As shown in Figure 1, there was no serious damage to cell viability after TM treatment for 12 h (P < 0.05). Compared to the untreated cells, the cell viability was decreased in a dose- and time-dependent manner after TM treatment for 24 (P < 0.01) or 48 h (P < 0.05). As shown in Figure 2, after a 24-h treatment, 0.2, 2, and 20 µM TM up-regulated the mRNA level of BIP (P < 0.05), and the mRNA level of BIP reached the highest after culture with 2 µM TM. Thus, 2 µM TM was chosen as the suitable concentration to induce ER stress. As shown in Figure 3, after the cells were treated with 2 µM TM for 24 h, the mRNAs of genes (BIP, EIF2a, ATF6, and XBP1) involved in ER stress were up-regulated in the goose primary hepatocytes (P < 0.05).

Figure 1. Effect of tunicamycin (TM) on cell viability. Cell viability was determined after incubation with different concentrations of TM for 12, 24, and 36 h by the CCK-8 assay. Data are reported as means ± SD (N = 3). Different lowercase letters in the same set indicate the differences among the treatments at P < 0.05, compared to the 12-h group.

Figure 2. Effect of different concentrations of tunicamycin (TM) on the mRNA expression of BIP. The mRNA levels were tested by real-time polymerase chain reaction after treatment with 0.2, 2, or 20 µM TM for 24 h. The expression of genes without the treatment was normalized to a value of 1. Different lowercase letters in the same set indicate the differences among the treatments at P < 0.05.
Effect of LY294002, NVP-BEZ235, or Rapamycin on cell viability

Cell viability after treatment with inhibitors of PI3K-AKT-mTOR signaling pathways was evaluated through the CCK-8 assay. As shown in Figure 4, LY294002 at concentrations of 1, 10, 20, 30, 40, or 50 µM decreased the cell viability compared to the control group. The cell viability showed a downward trend with an increasing LY294002 concentration. The cell viability showed a decreasing trend in hepatocytes treated with rapamycin at concentrations of 1, 10, 30, 50, and 90 nM compared to those in the control group. NVP-BEZ235 at concentrations of 0.1, 1, 10, 500, 1000, and 2000 nM decreased the cell viability compared to the control group in a concentration dependent manner. For use in our experiments, we determined the suitable concentration of inhibitors to be 20 µM, 1000 nM, and 30 nM for LY294002, NVP-BEZ235, and rapamycin, respectively; these inhibitors play an important restraining role in the PI3K-AKT-mTOR signaling pathways.

LY294002 abrogated TM-induced ER stress

To verify that the regulation of ER stress was linked to the PI3K signaling pathway, the mRNA level of key genes involved in ER stress was evaluated in cells co-treated with TM and a PI3K signaling inhibitor, LY294002. As shown in Figure 5, after the combined treatment with 2 µM TM and LY294002, the mRNA levels of genes were significantly lower than in the treatment with 2 µM TM alone. These results indicated that LY294002 abrogated the TM-induced ER stress and that the PI3K signaling pathway might down-regulate ER stress in the goose primary hepatocytes.
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Figure 4. Cell viability after incubation with different concentrations of LY294002, NVP-BEZ235, or rapamycin for 6, 12, 24, and 36 h as determined by the CCK-8 assay. A. Dose and time response of cell viability to LY294002. B. Dose and time response of cell viability to rapamycin. C. Dose and time response of cell viability to NVP-BEZ235. Data are reported as means ± SD (N = 3). Different lowercase letters in the same set indicate the differences among the treatments at P < 0.05.

Figure 5. Treatment with LY294002 (LY) abrogated the tunicamycin (TM)-induced ER stress. The mRNA levels were tested by real-time polymerase chain reaction after treatment with 2 μM TM, 20 μM LY294002, or 2 μM TM in combination with 20 μM LY294002 for 24 h. The expression of genes without any treatment was normalized to a value of 1. Different lowercase letters in the same set indicate the differences among the treatments at P < 0.05.
Rapamycin abolished TM-induced ER stress

To test the hypothesis that the regulation of ER stress was connected to the mTOR signaling pathway, the key genes of ER stress were evaluated in cells treated with TM and the mTOR signaling inhibitor, rapamycin. As shown in Figure 6, after the combined treatment with 2 \( \mu \)M TM and 30 nM rapamycin, the mRNA levels of genes were significantly lower than in the treatment with 2 \( \mu \)M TM alone. These results indicated that rapamycin abolished the TM-induced ER stress and that the mTOR signaling pathway might down-regulate ER stress in the goose primary hepatocytes.

![Figure 6](image)

**Figure 6.** Treatment with rapamycin (Rap) abolished the tunicamycin (TM)-induced ER stress. The mRNA levels were tested by real-time polymerase chain reaction after treatment with 2 \( \mu \)M TM, 30 nM rapamycin, or 2 \( \mu \)M TM and 30 nM rapamycin together for 24 h. The expression of genes without the addition of any treatment was normalized to a value of 1. Different lowercase letters in the same set indicate the differences among the treatments at \( P < 0.05 \).

NVP-BEZ235 prevented TM-induced ER stress

To determine whether the inhibition of both PI3K and mTOR signaling affected the regulation of the TM-induced ER stress, the key genes of ER stress were evaluated in cells treated with TM as well as with a dual PI3K and mTOR signaling inhibitor, NVP-BEZ235. As shown in Figure 7, after the combined treatment with 2 \( \mu \)M TM and 1000 nM NVP-BEZ235, the mRNA levels of genes were significantly lower than in the treatment with 2 \( \mu \)M TM alone. NVP-
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BEZ235 prevented the effect of TM-induced ER stress. Thus, we confirmed that both the PI3K and mTOR signaling pathways might down-regulate ER stress in the goose primary hepatocytes.

Figure 7. Treatment with NVP-BEZ235 (NVP) prevented the tunicamycin (TM)-induced ER stress. The mRNA levels were tested by real-time polymerase chain reaction after treatment with 2 µM TM, 1000 nM NVP-BEZ235, or 2 µM TM and 1000 nM NVP-BEZ235 together for 24 h. The expression of genes without any treatment was normalized to a value of 1. Different lowercase letters in the same set indicate the differences among the treatments at P < 0.05.

Comparison of relative mRNA levels of genes involved in ER stress after combined treatment with TM and rapamycin, NVP-BEZ235, or LY294002

To compare the effects of inhibitors of PI3K-AKT-mTOR signaling pathways on ER stress, the mRNA levels of genes involved in ER stress were compared among the combined treatment of TM and PI3K-Akt-mTOR pathway inhibitors (LY294002, NVP-BEZ235, rapamycin, respectively). As shown in Figure 8, after the combined treatment with TM and rapamycin, NVP-BEZ235, or LY294002, there was a similar result with the mRNA expression of BIP, EIF2a, ATF6, and XBP1 being decreased in the goose primary hepatocytes (P < 0.05). However, upon combined treatment with TM and LY294002, the mRNA levels of genes were significantly higher than in the combined treatment with TM and NVP-BEZ235 (P < 0.05), but it was not significantly different between the combined treatment with TM and NVP-BEZ235 and combined treatment with TM and rapamycin (P > 0.05).
DISCUSSION

ER stress has received growing attention with research suggesting the existence of a close connection between the PI3K-Akt-mTOR signaling pathways and the ER stress (Hosoi et al., 2007). TM inhibits protein glycosylation and is a commonly used ER stress inducer. Our results strongly indicate that TM induced a significant increase in the relative mRNA expression of genes involved in the ER stress in goose primary hepatocytes. This result is consistent with the findings in mouse (Wang et al., 2015) and immortalized human hepatocyte cell model (IHH; Lauressergues et al., 2012).

PI3K/Akt signaling has been reported to be involved in the ER stress (Liu et al., 2013). Previous studies have demonstrated that the activation of Akt signaling pathway might inhibit ER stress (Hu et al., 2004; Mao et al., 2008). However, the detailed mechanism of the PI3K/Akt signaling pathway inhibiting ER stress needs further study. Glucosamine-induced an increase in Akt phosphorylation, which correlated with a large increase in the expression of the endoplasmic reticulum stress marker BIP in astroglial cells (Matthews et al., 2007). On the contrary, Akt activation was unable to alleviate TM-induced ER stress (Liu et al., 2013). In another study, PI3K/Akt inhibition by LY294002 did not suppress ER stress-induced BIP mRNA expression in HEK293 cells. These data indicate that PI3K/Akt does not participate...
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in BIP mRNA induction following ER stress (Dai et al., 2010). Compared to cells exposed to thapsigargin-induced ER stress alone, SKBr3 cells treated with thapsigargin following the treatment with LY294002 and after co-treatment with NVP-BEZ235, did not show significant activation of UPR (Kumandan et al., 2013). To verify whether the PI3K/Akt signaling pathway down-regulated the ER stress in the goose primary hepatocytes, these hepatocytes were co-treated with TM and the PI3K/Akt inhibitor LY294002, or with TM and the PI3K/mTOR dual inhibitor NVP-BEZ235. The results obtained indicated that inhibiting PI3K/Akt signal pathway could decrease the TM-induced increase in the mRNA expression of genes involved in the ER stress (Figure 5). Co-treatment with TM and NVP-BEZ235 had a similar result as that obtained with the co-treatment of TM and LY294002 (Figure 7); both these treatments decreased the TM-induced increase in the mRNA expression of genes involved in the ER stress. However, the degree of decrease with TM and NVP-BEZ235 co-treatment was significantly higher than the in the TM and LY294002 co-treatment (Figure 8). Overall, the results of the present study indicated that LY294002 abrogated the effect of TM-induced ER stress and suggested that the PI3K signaling pathway might down-regulate the ER stress in goose primary hepatocytes.

Moreover, activation of mTORC1 by deletion of TSC1 or TSC2 has been shown to activate the ER stress response (Ozcan et al., 2008) and mTORC1 signaling is primarily mediated via the regulation of various factors involved in translation (Zoncu et al., 2011; Pópulo et al., 2012). The mRNA levels of BIP and XBP1 were increased, which was observed in vehicle-fed rats and the presence of rapamycin prevented this increase. These data provide evidence that feeding can activate the IRE1a-XBP1 branch of the UPR in the liver via a rapamycin-dependent mechanism (Pfaffenbach et al., 2010). Another important set of findings was that the activation of AMPK down-regulated the mTORC1 activity and attenuated the UPR in vivo, and administration of the mTORC1-specific inhibitor rapamycin suppressed the high glucose-induced ER stress response (Li et al., 2014b). On the contrary, Hwang et al. (2012) found that the inhibition of mTOR by rapamycin had no effect on the ER-stress markers in L6 myotubes. These observations suggest that the mTOR signal pathway does not always have relationships with the ER stress. To detect whether the ER stress was connected to the mTOR signaling pathway in goose hepatocytes, the key genes of ER stress were evaluated in cells co-treated with TM and the mTOR signaling inhibitor, rapamycin and also in cells co-treated with TM and the PI3K/mTOR dual inhibitor, NVP-BEZ235. Our results indicated that the mTOR signal pathway could decrease the TM-induced increase in mRNA expression of genes involved in the ER stress (Figure 6). This result is consistent with the findings of Li et al. (2014a), who showed that rapamycin inhibits light-induced ER stress and dithiothreitol-induced ER stress in 661W cells. The result of co-treatment with TM and NVP-BEZ235 was similar with the co-treatment with TM and rapamycin (Figure 7), and both the co-treatments decreased the TM-induced increase in mRNA expression of genes involved in the ER stress. However, there was no significant difference between the two co-treatments (Figure 8). Overall, the results of the present study indicated that rapamycin abolished the effect of TM-induced ER stress and suggested that the mTOR signaling pathway might down-regulate the ER stress in goose primary hepatocytes.

Besides, our results indicated that LY294002, rapamycin, and NVP-BEZ235 down-regulated the TM-induced increase in mRNA levels of genes involved in the ER stress (BIP, EIF2a, ATF6, and XBP1) in goose primary hepatocytes. In summary, inhibiting the PI3K-Akt-mTOR pathway can prevent TM-induced ER stress. There was no significant difference in
inhibition caused by rapamycin and NVP-BEZ235 treatment, This result might have been due to the competitive inhibition effect of NVP-BEZ235, an observation consistent with that of Kong and Yamori (2010) who reported that NVP-BEZ235 and ATP were competitive inhibitors of mTOR and PI3K, but caused more reactive inhibition of mTOR. but the inhibition resulting from the treatment with LY294002 was significantly different.

In summary, the mRNA levels of genes involved in ER stress (BIP, EIF2a, ATF6, and XBP1) were increased after the TM treatment in primary goose hepatocytes. However, this increase was down-regulated by PI3K-Akt-mTOR pathway inhibitors (LY294002, NVP-BEZ235, and rapamycin). Therefore, our results strongly indicate that the PI3K-Akt-mTOR signaling pathway might down-regulate the TM-induced ER stress in primary goose hepatocytes.

Conflicts of interest
The authors declare no conflict of interest.

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