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RESEARCH ARTICLE

Activation of the PI3K/Akt Pathway is Essential for the Survival of *Brucella melitensis* 16M In Vitro and In Vivo

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ABSTRACT

Brucellosis is a zoonotic pathogen that causes both animal and human diseases. *Brucella* pathogenesis depends on the bacterial ability of inhibiting apoptosis and establishes a replicative niche inside host cells. The PI3K/Akt pathway regulates various cellular activities (cell growth, survival, and apoptosis). However, the role of the PI3K/Akt pathway in the survival of 16M in RAW264.7 macrophages remains largely unknown. In our report, we demonstrated that the PI3K/Akt pathway was activated by 16M in RAW264.7 macrophages. We found that 16M infection induced the phosphorylation of both Thr-308 and Ser-473 of Akt in a time-dependent manner. This phosphorylation was inhibited by LY in a dose-dependent manner. Inhibition of PI3K/Akt with LY significantly reduced the internalisation and replication of 16M and induced 16M-dependent inhibition of apoptosis, and induced Th1 and proinflammatory responses both in vitro and in vivo and protected mice against 16M infection. These results indicated that the PI3K/Akt pathway plays an important role in 16M survival both in vitro and in vivo, which will help to unravel the pathogenic mechanisms of 16M.

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INTRODUCTION

Brucella spp are facultative intracellular bacteria that can multiply within the specialised phagosome of both humans and animals, resulting in heavy economic losses (Smith *et al*., 2013). *Brucella spp* cause acute, transmissible infections in animal reservoirs and abortion in pregnant animals (Iqbal *et al*., 2013; Zhang *et al*., 2014). The human disease is also caused by *Brucella spp*, specifically, *Brucella melitensis*, *Brucella abortus* or *Brucella suis*. Infected humans can have fever, arthritis, dementia, and meningitis or endocarditis under rare circumstances (Hamdy *et al*., 2002; Godfroid *et al*., 2005). There is no effective and safe *Brucella* vaccine for animals, and the pathophysiology of brucellosis remains poorly understood.

PI3K comprises a family of lipid kinases that play critical roles in many biological processes (Kitamura *et al*., 2014). PI3K is composed of a regulatory subunit (p85) and a catalytic subunit (p110) (Sun *et al*., 2010). The PI3K/Akt pathway plays an important role in the regulation of cellular processes (proliferation, programmed cell death, and metabolism) (Wang *et al*., 2013; Zhang *et al*., 2014). Mammalian Akt contains three isoforms, Akt1, Akt2, and Akt3. Akt1, normally called Akt, contains two phosphorylation sites: Thr-308 in the kinase domain and Ser-473 in the regulatory domain (Datta *et al*., 1999). Phosphorylation of either Thr-308 or Ser-473 can activate Akt (Bayascas and Alessi, 2005). Akt often regulates the effects of PI3K in promoting survival (Brunet *et al*., 2001). Activated Akt phosphorylates various downstream substrates such as caspase-3, BAD, and glycogen synthase kinase 3β (GSK-3β). In addition, activated Akt prevents apoptosis and leads to cell survival and growth.

In this study, we investigated whether the PI3K/Akt pathway plays a role in 16M survival in vitro and in vivo. Our results indicated that infection with 16M activates the PI3K/Akt pathway in a PI3K-dependent manner in RAW264.7 macrophages. Inhibition of PI3K activation reduced 16M survival, induced Th1 and proinflammatory cytokines, and enhanced apoptotic responses in 16M-

[§]These authors contributed equally to this work.

infected RAW264.7 macrophages. Furthermore, inhibition of PI3K activation in vivo protected mice against 16M infection.

MATERIALS AND METHODS

Mice: Eight-week-old female BALB/c mice were purchased from the Experimental Animal Centre of Academy of Military Medical Science (Beijing, China).

Cell culture: RAW264.7 macrophages were obtained from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China) and were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum. The RAW264.7 cells were cultured at 37°C under humidified 5% CO_2 conditions.

***Brucella* infection:** To analyse the effect of LY (a competitive inhibitor of the ATP binding site of PI3K; PI3K/AKT inhibitor) on 16M internalisation, RAW264.7 cells in 6-well plates were pre-treated with LY (20 μM) at 37 °C for 1 h and then infected with 16M at a 50:1 MOI (multiplicity of infection) for 30 min and 45 min. Cells were washed twice with medium and then incubated in DMEM with gentamicin (30 μg/ml) for 45 min to kill any remaining extracellular and adherent bacteria. The infected cells were washed three times with PBS and lysed, and the live 16M were enumerated by plating on TSA plates. To determine the effect of LY on 16M intracellular growth, infections of RAW264.7 cells were performed at an MOI of 50 for 4, 12, and 24 h either in the absence or presence of LY (20 μM). The cell lysis and plating procedures were the same as for the analysis of 16M internalisation. At 24 h postinfection, the levels of IFN-γ and IL-12 in the supernatants were detected using an ELISA Quantikine Mouse kit (R&D Systems).

MTT assay: RAW264.7 cells were cultured in 96-well plates (6×10^4 cells/ml) and were washed three times with PBS. RAW264.7 cells were treated with LY (10, 20, 40, 80 μM) for the experiment groups and 0.1% dimethyl sulfoxide (DMSO) (Sigma, USA) for the control group, and RAW264.7 cells were cultured at 37°C in a humidified 5% CO2 incubator prior to assessment for cell viability using an MTT assay (Sigma). MTT (50 μl/well) was added, and RAW264.7 cells were incubated at 37°C for 4 h. Then, the supernatants were removed, and 100 μl of DMSO was added to each well. Optical density was measured by eluting the dye with DMSO, and the OD570 was determined.

Assessment of apoptosis: The rates of apoptosis were evaluated using an Annexin V-EGFP Apoptosis Detection Kit (Bestbio, Shanghai). Annexin V/PI double-positive staining and fluorescence intensity measurements were performed on RAW264.7 cells. The rates of apoptosis were determined using a FASC Aria II flow cytometer (BD Biosciences).

Western blot analysis: To determine the p-Akt (Ser473/Thr308) expression in RAW264.7 macrophages, cells were lysed in ice-cold RIPA lysis buffer for 30 min and centrifuged at 12,000 rpm for 25 min at 4°C. The protein was boiled for 7 min, and 20 μl of sample was subjected to 12% SDS-PAGE. The protein was electrotransferred onto a nitrocellulose membrane (Bio-Rad) at 200 mA and 20 V for 1 h. Unbound sites on the membrane were blocked with 2% BSA in borate-buffered saline for 1 h at 37°C. After washing three times with TBST buffer for 30 min, the membrane was incubated overnight at 4°C with a 1/1000 dilution of specific primary antibodies raised against Akt, p-Akt (Ser473/Thr308), Bax, or Bcl-2 purchased from Cell Signaling Technology (Beverly, MA). The membrane was incubated with goat anti-rabbit IgG for 1 h at 37°C. Bound conjugate was visualised with ECL (Thermo Fisher Scientific, USA).

16M infection in mice: Female BALB/c mice (7-8 weeks) (n=10) were injected i.p. with 20 mg/kg/day LY dissolved in DMSO/1×PBS buffer (diluent, 1:1; v/v), and the control group (n=10) received diluent only. For the survival model, daily treatment of BALB/c mice with LY started 5 days before logarithmically dividing 16M were injected i.p. At 3 and 6 days postinfection, the mice were euthanised, and spleens were removed aseptically. The spleens were collected aseptically for quantitation of bacterial CFU, and the spleens were weighed. The suspensions were diluted in sterile saline and plated onto TSA. The plates were incubated at 37°C, and the number of CFU was counted after three days. At 6 days postinfection, the levels of IFN-γ and IL-12 in the blood of mice were detected as previously described.

Statistical analysis: Student's t-test was used for comparisons among different groups. $P<0.05$ was considered statistically significant.

RESULTS

Cell viability is inhibited by LY294002 in a concentration-dependent manner: We determined the cell viability using MTT assay with a PI3K/Akt inhibitor, LY. RAW264.7 macrophages were treated with LY for 24 h at a range of concentrations. The results demonstrated that the LY inhibitor can inhibit cell viability, and the inhibitory effect was in a concentration-dependent manner (Fig. 1). In addition, LY at a concentration of 20 μM had no apparent effect on cell viability.

16M activates the PI3K/Akt pathway: We initially tested whether 16M could regulate the PI3K/Akt pathway. We analysed the induction of Akt phosphorylation at 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, and 24 h. As shown in Fig. 2A, 16M induced the phosphorylation of Akt at threonine 308 and serine 473 between 30 min and 4 h postinfection, and the amount of phosphorylated Akt was visible at 30 min postinfection and peaked at 1 h postinfection (Fig. 2A). At 8, 12, and 24 h postinfection, the phosphorylation of Akt was similar to basal levels. The results showed that 16M infection resulted in the enhanced transient phosphorylation of Akt protein.

We further investigated whether Akt phosphorylation is regulated by PI3K inhibition in 16M-infected cells by treating cells with LY, an inhibitor of PI3K. LY inhibited the phosphorylation of the Akt protein in a dose-dependent manner (Fig. 2B), and without changing the Akt protein

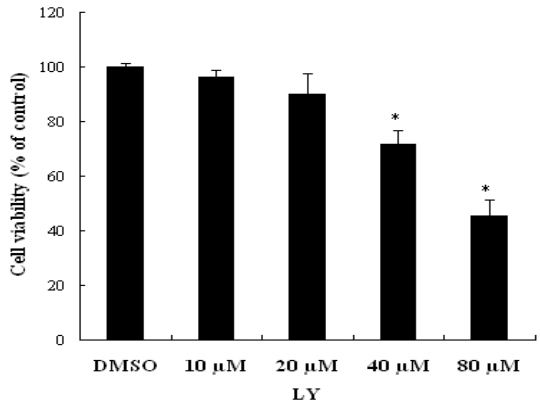

Fig. 1: The viability of RAW264.7 cells was inhibited by LY in a concentration-dependent manner. The cell viabilities of LY in 40 and 80 μM were significantly different from those of the DMSO group (*P<0.01).

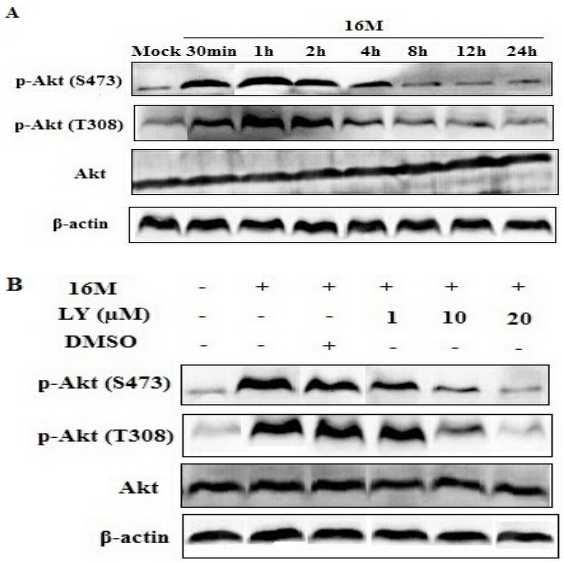

Fig. 2: Infection with 16M activates the PI3K/Akt pathway in RAW264.7 macrophages. (A) RAW264.7 cells were left uninfected or infected with 16M at an MOI 50:1 for the indicated time periods. The levels of phosphorylation of Akt (Ser473/Thr308) were analysed using Western blotting. The amounts of β-actin were assessed to monitor the equal loading of protein extracts. (B) RAW264.7 cells were pre-incubated with inhibitor LY (1, 10, and 20 μM) for 1 h and then infected with 16M at a MOI 50 for 1 h. Inhibition of 16M-induced Akt phosphorylation by treatment with LY is shown.

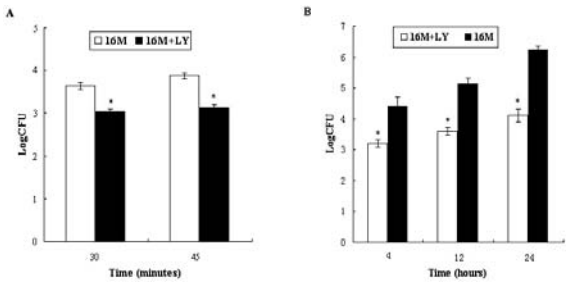

Fig. 3: Evaluation of the 16M internalisation and intracellular growth inhibited by LY. RAW264.7 macrophages were infected with 16M as a control. Bacterial internalisation (A) and bacterial intracellular growth (B) were determined after different infection times. Significant differences between the number of 16M for the LY-treated macrophages and the controls are indicated as follows: * P<0.01.

levels. After treatment with 20 μM LY, phosphorylation of Akt was blocked entirely. These results showed that 16M activated the Akt protein.

The PI3K/Akt pathway inhibits 16M infection in RAW264.7 macrophages: We next evaluated whether inhibition of the PI3K/Akt pathway affected 16M internalisation and intracellular growth. At 30 min postinfection, a significant reduction (75%) in the amount of 16M internalisation was observed inside the LY-treated cells compared to that of the control cells (P<0.01), and similar results were obtained at 45 min post-infection (Fig. 3A). For the intracellular replication assay, at 4 h post-infection, a 1.20-log decrease (P<0.01) was observed in the bacterial CFU representing 16M replication inside of the LY-treated macrophages compared with that of the control cells. At 24 h post-infection, the decrease inside of the LY-treated macrophages was even greater (2.13-log; P<0.01; Fig. 3B). These results show that inhibition of the PI3K/Akt pathway inhibits the 16M internalisation and intracellular growth in RAW264.7 macrophages.

Inhibition of PI3K/Akt induces apoptotic responses in 16M-infected cells: The PI3K/Akt pathway was inhibited by preincubating cells with LY for 1 h prior to 16M infection for 4 h, 12 h, and 24 h. The apoptotic rate of the 16M-infected LY-treated cells had a range of 8.3 to 24.3 %, the 16M-infected untreated cells had a range of 2.4 to 6.3 %, and the mock-infected cells had a range of 2.7 to 7.2% (Fig. 4A).

To investigate whether either anti-apoptotic Bcl-2 or pro-apoptotic Bax expression can be mediated by the PI3K/Akt pathway in 16M-infected cells, we next examined the protein expression levels of Bcl-2 and Bax at 24 h postinfection. When the PI3K/Akt pathway was inhibited by LY, 16M induced protein expression of Bax and inhibited expression of Bcl-2 (Fig. 4B). In addition, changes in mRNA levels and changes in protein expression of Bcl-2 and Bax were similar (data not shown). It is conceivable to confirm that the PI3K/Akt pathway indeed plays a role in apoptosis in 16M-infected cells. These results indicate that inhibition of the PI3K/Akt pathway enhances 16M-dependent apoptosis in RAW264.7 macrophages.

Inhibition of the PI3K/Akt pathway induces a cytokine response in 16M infected RAW264.7 macrophages: To determine to the role of the PI3K/Akt pathway involved in the production of IFN-γ and IL-12 in RAW264.7 macrophages, RAW264.7 cells were preincubated for 1 h with 20 μM LY and then infected with 16M for 12 and 24 h. The levels of IFN-γ and IL-12 were assessed in the supernatants of the 16M-infected LY-treated macrophages (Fig. 5). At 12 and 24 h, the 16M-infected LY-treated macrophages produced significantly higher levels of IFN-γ and IL-12 than the 16M-infected cells (P<0.01; Fig. 5). The results showed that LY could induce Th1 and proinflammatory responses in 16M-infected macrophages.

Inhibition of PI3K/Akt pathway protects mice from 16M infection: To further evaluate whether inhibition of the PI3K/Akt pathway could protect BALB/c mice from 16M infection, administration of LY for 5 days before injecting an LD100 dose (4.6×10^8 CFU) of 16M significantly

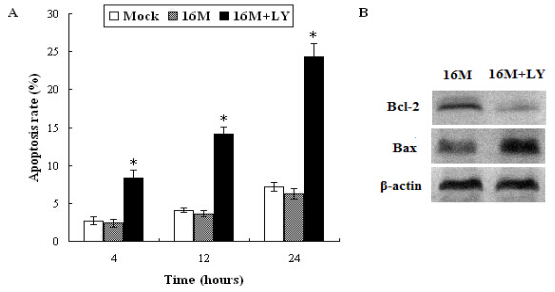

Fig. 4: Inhibition of the PI3K/Akt pathway promotes apoptosis caused by 16M infection. (A) RAW264.7 cells were infected with 16M in the absence or presence of LY for 4, 12, or 24 h, then the apoptosis rate was determined using flow cytometry after being dyed with Annexin/PI. Compared with the 16M-infected untreated cells controls, the apoptosis rates of the LY-treated 16M-infected cells were significantly different (*P<0.01) (B) Compared with the 16M-infected untreated cells, the protein expressions of Bax and Bcl-2 in the LY-treated 16M-infected cells were determined using Western blot analysis at 24 h post infection.

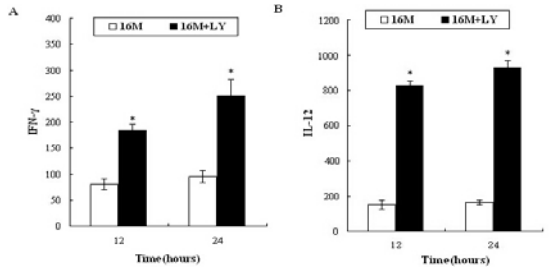

Fig. 5: The analysis of IFN-γ and IL-12 secretion was measured using ELISA from the supernatant of the 16M-infected LY-treated cells and the 16M-infected cells at 12 and 24 h postinfection. Significant differences were observed when the 16M-infected LY-treated cells were compared with the 16M-infected cells, indicated as follows: *P<0.01.

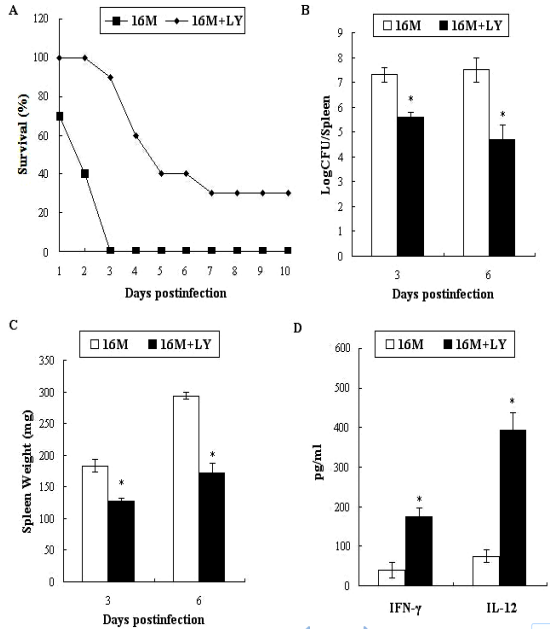

Fig. 6: LY protects BALB/c mice from 16M infection. (A) BALB/c mice were pretreated i.p. with LY for 5 days, as indicated, and then challenged with 4.6 × 10^8 16M (n = 10). Survival was observed for 10 days. Statistical significance was indicated as P<0.01. (B and C) The numbers of 16M in the spleen and spleen weight were determined (n = 10). (D) Serum concentrations of IFN-γ and IL-12 were determined from infected mice 6 day s post infection (mean ± SEM; n = 10). Statistical significance was indicated as *P<0.01.

improved the survival of the experimental mice compared to that of the control mice given only 16M (Fig. 6A). Improved survival of the LY-treated mice infected with 16M was confirmed by a significant reduction in the bacterial CFU in the spleen and the weight of the spleen 3 and 6 days after injecting 1 × 10^6 CFU of 16M (P<0.01; Fig. 6B and C).

We next examined the levels of IFN-γ and IL-12 in LY-treated mice through the administration of LY for 5 days before injecting 1 × 10^6 CFU of 16M into the animals. At 6 days postinfection, the levels of serum IFN-γ and IL-12 were significantly higher in the LY-treated mice (P<0.01; Fig. 6D). The results showed that PI3K/Akt pathway inhibition in vivo could also induce Th1 and proinflammatory responses and protect mice from 16M infection.

DISCUSSION

Brucellae do not possess classic virulence factors, and their virulence is therefore determined by their ability to invade and survive in host cells (Gao *et al*., 2013; Gul *et al*., 2013; 2014; Asif *et al*., 2014). PI3K has been reported to be involved in phagosome trafficking and maturation (Weber *et al*., 2009). Previous studies show that the uptake of *B. abortus* and *B. melitensis* into HeLa and J774.A1 cells were blocked by the PI3K inhibitor wortmannin, which indicates that PI3K plays a role in the entry of smooth *Brucella* strains into cells (Pei *et al*., 2008; Qin *et al*., 2008). However, it is still poorly understood whether the PI3K/Akt pathway regulates 16M internalisation and intracellular growth in RAW264.7 cells. This study demonstrates that 16M activates the PI3K/Akt pathway, and the activation of the PI3K/Akt pathway plays a major role in 16M survival *in vitro* and *in vivo*. In addition, the PI3K/Akt pathway is essential for apoptosis delay in 16M-infected RAW264.7 cells.

PI3K/Akt contributes to an anti-apoptosis strategy and plays a major role in growth factor-promoted cell survival by inhibiting apoptosis signalling depending on cell types (Datta *et al*., 1999; Brunet *et al*., 2001; Mannová and Beretta, 2005). Cellular adhesion to extracellular matrix components may lead to the activation of PI3K, recruitment of Akt to the plasma membrane, Akt phosphorylation, and protection against apoptosis (Datta *et al*., 1999; Cary and Guan, 1999). It has been reported that TNF-a induced phosphorylation of BAD through the PI3K/Akt pathway and that phosphorylated BAD lost its ability to bind to Bcl-XL, which is known to act on mitochondria to block the apoptotic signalling cascade (Green and Reed, 1998). Akt can regulate the activity of molecules of the BCL-2 family, which plays a major role in the release of cytochrome c from the mitochondria (Song *et al*., 2005). The PI3K/Akt pathway may also play a major role in reducing apoptosis by regulating the Bcl-2 family (Takatani *et al*., 2004). Some bacteria, such as *Salmonella enterica*, M*ycobacterium tuberculosis*, and *Escherichia coli*, have also been shown to promote cell survival through the PI3K/Akt pathway (Crane *et al*., 1999; Forsberg *et al*., 2003; Maiti *et al*., 2001). Previous studies have indicated that virulent 16M inhibits apoptosis in macrophages, which promotes *Brucella* survival after the initial killing stage (He *et al*., 2006; Pei *et al*., 2014). In this study, inhibition of the PI3K/Akt pathway induces

apoptosis of 16M-infected RAW264.7 macrophages, which is confirmed by the protein expression of Bax and BCl-2 and confers host cell resistance to survival and replication of 16M.

The Th1 immune responses characterised by IFN-γ levels are related to the host immune response to *Brucella*, which are activated by vaccines (Zhang *et al*., 2014). Previous studies showed that IFN-γ is a critical cytokine required for macrophage bactericidal activity (Yamasaki *et al*., 2013). IL-12 is a key mediator of cell-mediated immunity and can enhance the immune response against infections. IL-12 can promote Th1 cell responses and inhibit redundant Th2 cell responses (Langrish *et al*., 2004). It has been reported that PI3K signalling negatively regulate IL-12 levels (Ruhland *et al*., 2009), and so we assessed whether the inhibition of the PI3K pathway by LY promotes IL-12 levels in 16M-infected macrophages. Upon the inhibition of the PI3K/Akt pathway, we observed that IFN-γ and IL-12 were significantly unregulated, which enhances the anti-microbial activity of macrophages and is responsible for the decreased survival of 16M in vivo and in vitro.

Conclusion: In this study, we demonstrated that the PI3K/Akt signalling pathway activated by 16M infection plays a major role in 16M survival both in vivo and in vitro. The PI3K/Akt pathway also regulates anti-apoptotic responses in RAW264.7 macrophages. Inhibition of the PI3K/Akt pathway enhances Th1 and proinflammatory molecules. Our study may be useful for developing new drugs against *Brucella* infections, including prevention and therapeutic treatment.

Author's contribution: Junbo Zhang, Shuanghong Yin, Fei Guo, Zhiqing Li executed the experiment. Chuangfu Chen and Hui Zhang analyzed the data. All authors interpreted the data, critically revised the manuscript for important intellectual contents and approved the final version.

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