Lipoplysaccharide Stimulates Surfactant Protein-A in Human Renal Epithelial HK-2 Cells through Upregulating Toll-like Receptor 4 Dependent MEK1/2-ERK1/2-NF-κB Pathway

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Background: Surfactant protein-A (SP-A) contributes to the regulation of sepsis-induced acute kidney injury. In a previous study, we demonstrated that the expression of SP-A in the human renal tubular epithelial (HK-2) cells can be stimulated by lipopolysaccharide (LPS). The present study evaluated the possible signal-transducing mechanisms of LPS-induced SP-A biosynthesis in the HK-2 cells.

Methods: Tetrazolium salt colorimetry (MTT) assay was used to detect cell viability of HK-2 cells after LPS stimulation on different time points. HK-2 cells were stimulated with 100 ng/ml of LPS for different durations to determine the effects of LPS on SP-A and toll-like receptor 4 (TLR4) messenger RNA (mRNA) expression, as well as phosphorylation of mitogen-activated/ extracellular signal-regulated kinase (MEK) 1, extracellular signal-regulated kinase 1/2 (ERK1/2), p38 mitogen-activated protein kinase (p38MAPK), and nuclear factor-kappa B (NF-κB) inhibitor-alpha (IkB-α). Then, HK-2 cells were pretreated with CLI-095, a TLR4 inhibitor, to analyze mRNA and protein levels of SP-A and TLR4 and expression of NF-κB in the cytoplasm and nucleus of HK-2 before LPS exposure.

Results: HK-2 cells exposed to 100 ng/ml of LPS for 1, 6, and 24 h did not affect cell viability which showed no toxic effect of 100 ng/ml LPS on cells (P = 0.16); however, the biosynthesis of SP-A mRNA and protein in HK-2 cells was significantly increased (P = 0.02). As to the mechanism, LPS enhanced transmembrane receptor TLR4 protein expression. Sequentially, LPS time dependently augmented phosphorylation of MEK1, ERK1/2, and p38MAPK. In addition, levels of phosphorylated IkB-α and nuclear NF-κB were augmented with LPS exposure for 2 h. LPS-induced SP-A and TLR4 mRNA as well as NF-κB expression were significantly inhibited by pretreatment with CLI-095.

Conclusions: The present study exhibited that LPS can increase SP-A synthesis in human renal epithelial cells through sequentially activating the TLR4-related MEK1-ERK1/2-NF-κB-dependent pathway.

Key words: Acute Kidney Injury; HK-2 Cells; Lipopolysaccharide; Signal Pathway; Surfactant Protein-A

INTRODUCTION

Acute kidney injury (AKI) is always caused by sepsis and septic shock, the incidence of which is closely related to the severity of infection. About 19% of sepsis patients, 23% of severe sepsis patients, and 51% of patients with septic shock would accompany with AKI.1 Clinical studies have showed that AKI may be an independent predictor of death, and the mortality of patients accompanied with AKI is as high as...
There is growing evidence showing that 62.5% of sepsis in Chinese patients is caused by Gram-negative bacteria, and the lipopolysaccharide (LPS) on the bacterial cell wall is a major pathogenic factor of these Gram-negative bacteria.

Surfactant protein-A (SP-A) was initially identified in the pulmonary epithelial cells. It is synthesized and secreted by alveolar Type II epithelial cells and encoded by two genes (SP-A1 and SP-A2). SP-A can reduce alveolar surface tension to keep the alveoli expanded. In addition, it also plays important roles in the pulmonary immunity response and inflammatory regulation. Levels of tumor necrosis factor-alpha (TNF-α), pulmonary capillary permeability, and apoptotic cells in pulmonary were significantly increased in SP-A knockout mice caused by cisplatin-induced acute lung injury. LPS-induced inflammation and apoptosis of pulmonary epithelial cells could be effectively depressed by intra-tracheal administration of exogenous SP-A. Our previous study indicated that SP-A knockout mice exhibited more severe acute kidney injury (AKI) induced by sepsis. Meanwhile, SP-A prevents renal tubular epithelial cell injury from sepsis through inhibiting nuclear factor-kappa B (NF-kB) activity to modulate TNF-α expression. However, the underlying mechanisms of LPS-induced SP-A expression in renal tubular epithelial cells are still unclear.

Toll-like receptors (TLRs) are Type I transmembrane protein and composed of extracellular domain rich in leucine repeat sequence and intracellular domain. TLR4 expression has been found in the epithelium of multiple organs (such as alveolar epithelial cells and renal tubular epithelial cells). LPS can bind to the TLR4/MD-2 complex on the membrane of renal tubular epithelial cells to activate intracellular signaling pathways. Mitogen-activated protein kinase (MAPK) belongs to serine/threonine kinase extracellular signal-regulated kinase (ERK). MAPK family consists of ERK, p38MAPK, and C-Jun N-terminal kinase (JNK). ERK, including ERK1 and ERK2, is crucial for the signal transmission from extracellular to nucleus. It has been confirmed that ERK1/2 can be activated by mitogen-activated protein/ERK kinase (MEKs), and then may cause the translocation of cytoplasmic nuclear factor-κB (NF-κB) into nucleus, leading to NF-κB activation. A variety of signaling pathways participated in TLR4 activation, our previous study showed that the secretion of SP-A could be significantly induced by LPS in HK-2 cells, but the possible mechanism is still unknown. In this study, we attempted to investigate the effects of LPS on the SP-A expression in human renal tubular epithelial (HK-2) cells and its potential mechanisms.

**Methods**

**Cell culture and drug treatment**

Renal tubular epithelial cells (HK-2 cells, a proximal tubular cell line derived from normal kidney; CRL-2190) were purchased from ATCC and cultured at 37°C in a humidified atmosphere of 5% CO₂ with Dulbecco’s modified Eagle’s medium (HyClone Pierce, USA) containing 10% fetal bovine serum (FBS; Invitrogen, USA) and 1% ampicillin. The culture medium was refreshed every 2 days until the cell confluence reached 70–80%. Cells were harvested after digestion with 0.25% trypsin/EDTA. The messenger RNA (mRNA) and protein expression were detected after LPS exposure for different durations. LPS extracted from serotype *Escherichia coli* 0111:B4 was purchased from Sigma (St. Louis, MO, USA). To prepare LPS suspension, LPS was dissolved in dimethyl sulfoxide (DMSO). Concentration of DMSO was <0.1% to reduce its toxicity to HK-2 cells. CLI-095 (Invitrogen, San Diego, CA, USA) is a cyclohexene derivative and an inhibitor of TLR4. CLI-095 was dissolved in DMOS at 10 μmol/l. Cells in control group were cultured in the medium only with DMSO at the same concentration.

**Detection of cell viability**

Cell viability was determined using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay as previously described. HK-2 cells were digested with 0.25% trypsin and then suspended in the medium containing 10% FBS at 5–8 × 10⁴ cells/ml. Cells were cultured into 96-well plates (200 μl/well), followed by incubation for 4–5 h. After cells attached, 100 ng/ml of LPS was added to each well (100 μl/well) and incubated at 37°C with 5% CO₂ for 0, 1, 6, and 12 h. Before the detection, 5 μg/ml MTT solution was added to each well (20 μl/well), and then incubated in dark for 4 h. The absorbance was measured at 490 nm by MTT enzyme-linked immunometric meter (Bio Rad, USA). The cell viability was calculated according to the standard curve.

**Real-time polymerase chain reaction**

Cultured HK-2 cells were used for real-time polymerase chain reaction (PCR) analysis of SP-A and TLR4 mRNA. Total RNA was extracted from each sample using Trizol (Invitrogen, USA) following the manufacturer’s instructions. Total RNA (1 μg) extracted from the tissue was used for the RT reaction (Takara, China), and then 2.5 μl of cDNA was used for amplification at a final volume of 25 μl according to the supplier’s protocol (Fermentas, Germany). Then, the amplified PCR product was used for melting curve analysis, especially SP-A primer: 5’-TGA AAGGGAGTCTAGCATCTCA CAGA-3’ and 5’-ACATATGCCC TATGTAGGCTGACT GAG-3’, TLR4 primer: 5’- TATCCAGAGCCGTGGTGAT CTA-3’ and 5’-ATTGAAGATG ATGCCA GAGCG ‑3’, and β-actin primer: 5’-GTCTACATGCTGCT GATCCCCACTA A -3’ and 5’-GGTCTTTTCTCTCTCTCAT CGGCTC-3’. The PCR was performed with 35 cycles of 94°C for 45 s, 60°C for 45 s (SP-A) or 55°C for 50 s (TLR4), and 72°C for 2 min. Each PCR product was subjected to electrophoresis on 2% agarose gel containing 0.1 μg/ml ethidium bromide. DNA bands in the agarose gel were photographed and quantified.
**Extraction of nuclear proteins**

Nuclear components were extracted on ice following the method of Chiu et al. with a little bit improvement.\(^{[17]}\) HK-2 cells were centrifuged, the deposit was collected and prepared in the 25 μl buffer A (10 mmol/L HEPES, pH 7.9; 10 mmol/L KC1; 1.5 mmol/L MgCl2; 1 mmol/L DTT; 5% glycerin; 0.2 mmol/L EDTA; 1 mmol/L PMSF; 3 mg/L aprotinin; 3 mg/L leupeptin; 2 mg/L pepstatinA; 1% NP-40) for 10 min, and then centrifuged on ice (14,000 r/min) for 1 min; the deposit was prepared in 15 μl buffer B on ice (20 mmol/L HEPES, pH 7.9; 420 mmol/L NaCl; 1.5 mmol/L MgCl2; 0.2 mmol/L EDTA; 0.5 mmol/L DTT; 25% glycerin; 0.5 mmol/L PMSF; 5 mg/L aprotinin; 5 mg/L leupeptin; 3 mg/L pepstatinA) for 30 min, and then centrifuged on ice for 15 min (14,000 r/min). Protein concentrations were quantified with a bicinchoninic acid protein assay kit (Logan, Utah, Hyclone Pierce).

**Western blotting**

Proteins from HK-2 cells were extracted using RIPA lysate (150 mmol/L sodium chloride, 1.0% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate [SDS], and 50 mM Tris at pH 8.0). The total protein concentrations were determined using the bicinchoninic acid protein assay (Logan, Utah, Hyclone Pierce). Total protein (50 μg) was resolved by reducing 12% SDS-polyacrylamide gel electrophoresis and then transferred electrophoretically at 60 mA onto nitrocellulose membranes at 4°C overnight (Bio-Rad, USA). After the samples were blocked in 5% nonfat milk in Tris-buffered saline, immunoblotting was conducted using a primary antibody against SP-A at a dilution of 1:150 or TLR4 at a dilution of 1:600, and then secondary antibody conjugated with horseradish peroxidase. Cellular β-actin was immunodetected using a human monoclonal antibody against human β-actin (St. Louis, MO, USA) as the internal standard. Immunoproducts were detected using enhanced chemiluminescence peroxidase detection reagents (Amersham, Sweden).

**Statistical analysis**

Data are expressed as mean ± standard error of mean. Results were analyzed by one-way ANOVA using the SPSS version 15.0 software (SPSS Inc., Chicago, Illinois, USA). \(P < 0.05\) was considered to indicate statistical significance.

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**Results**

**Effect of lipopolysaccharide on the cell viability of HK-2 cells**

To evaluate the cytotoxic effect of LPS on cells, the cell viability of HK-2 cells was detected after LPS exposure. HK-2 cells were stimulated with 100 ng/ml of LPS for 0, 1, 6, and 12 h, and absorbance was measured at 490 nm. The absorbance did not show significant difference at different durations after LPS treatment [Figure 1, \(P > 0.05\)], suggesting that 100 ng/ml of LPS is not cytotoxic to HK-2 cells.

**Effects of lipopolysaccharide on the messenger RNA and protein expression of surfactant protein-A in HK-2 cells**

Protein and mRNA expression of SP-A in HK-2 cells after LPS treatment were detected by Western blotting and real-time PCR, respectively. Cells were stimulated with LPS for 0, 1, 2, and 6 h, and then SP-A mRNA expression was detected; cells were treated with LPS for 0, 2, 6, and 12 h, and SP-A protein expression was assayed; and β-actin expression was detected as an internal standard. Expression of SP-A mRNA expression at 1, 2, and 6 h after LPS exposure was significantly increased by 1.5, 2.7, and 4.5 folds, respectively, as compared to that at 0 h [Figure 2, \(P < 0.05\)]. Moreover, the SP-A protein expression at 6 h after LPS treatment increased dramatically when compared with that at 0 h and 2 h [Figure 3, \(P < 0.05\)]. After LPS exposure for 12 h, the SP-A protein expression was markedly higher than that at 6 h. SP-A protein expression after LPS exposure for 6 h and 12 h was increased by 1.6 and 2.8 folds, respectively, as compared to that at 0 h. These findings suggested that LPS may upregulate SP-A expression at transcriptional level.

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**Figure 1:** Effects of lipopolysaccharide on the cell viability of HK-2 cells. HK-2 cells were exposed to 100 ng/ml of lipopolysaccharide for 0, 6, 12, and 24 h. Cell viability was assayed by MTT method. Optical density value of HK-2 cells for different durations was detected with colorimetric method. Each value represents the mean ± standard error of the mean (\(n = 5\), samples were collected from five different passages).
Effects of lipopolysaccharide with or without CLI-095 on the nuclear translocation and activation of nuclear factor-κB in HK-2 cells

To investigate the mechanism underlying LPS-induced SP-A expression in HK-2 cells, the expression of cytoplasmic and nuclear NF-κB as well as phosphorylated IκB-α was measured in HK-2 cells, and total NF-κB served as an internal standard. After LPS exposure for 0, 1, 2, and 6 h, the cytoplasmic NF-κB expression was decreased [Figure 4, P < 0.05], however the nuclear NF-κB expression and intracellular phosphorylated IκB-α expression were increased [Figure 5, P < 0.05]. The protein expression of nuclear factor-κB (NF-κB) at different time points was quantified, and results showed that the cytoplasmic NF-κB expression at 1, 2, and 6 h after LPS exposure was reduced by 32%, 48%, and 47%, respectively, but expressions of the nuclear NF-κB and phosphorylated IκB-α at 1, 2, and 6 h after LPS exposure were augmented by 117%, 155%, and 203% and by 167%, 320%, and 413%, respectively, as compared to those at 0 h. Moreover, the expression of the...
The present study revealed that LPS can cause inflammatory responses in renal tubular epithelial HK-2 cells by means of enhancing SP-A mRNA and protein syntheses. Moreover, the signal-transducing mechanisms of LPS-induced regulation of SP-A expression arise through TLR4-dependent cascade phosphorylations of MEK1, ERK1/2, and p38MAPK, leading to the nuclear translocation and activation of NF-κB. Moreover, pretreatment with TLR4 inhibitor, CLI-095, may significantly decrease mRNA and protein expression of nuclear NF-κB and phosphorylated IκB-α was depressed by CLI-095 when compared with by LPS stimulation alone [Figures 4 and 5, P < 0.05].

**Effects of lipopolysaccharide with or without CLI-095 on the expression of toll-like receptor 4 and surfactant protein-A messenger RNA and protein in HK-2 cells**

We further explored the role of TLR4-dependent MEK1/2-ERK1/2-NF-κB signaling pathway in LPS-induced SP-A expression. HK-2 cells were pretreated with CLI-095 before LPS exposure. Induction of TLR4 and SP-A mRNA expression by LPS were analyzed by real-time PCR. After LPS administration, the mRNA expression of TLR4 and SP-A was significantly increased [Figure 9, P < 0.05]. Pretreatment of HK-2 cells with CLI-095, an inhibitor of TLR4, before LPS administration could markedly inhibit the mRNA and protein expression of TLR4 and SP-A [Figure 9, P < 0.05]. Expression of TLR4 and SP-A mRNA in HK-2 cells after LPS exposure for 6 h was augmented by 458% and 542%, respectively, when compared with that at 0 h. With CLI-095 pretreatment, mRNA expression of TLR4 and SP-A in HK-2 cells was decreased by 66% and 60%, respectively, when compared with that by LPS administration for 6 h alone [Figure 10, P < 0.05].

**Discussion**

The present study revealed that LPS can cause inflammatory responses in renal tubular epithelial HK-2 cells by means of enhancing SP-A mRNA and protein syntheses. Moreover, the signal-transducing mechanisms of LPS-induced regulation of SP-A expression arise through TLR4-dependent cascade phosphorylations of MEK1, ERK1/2, and p38MAPK, leading to the nuclear translocation and activation of NF-κB. Moreover, pretreatment with TLR4 inhibitor, CLI-095, may significantly decrease mRNA and protein expression of toll-like receptor 4 protein in HK-2 cells induced by lipopolysaccharide for different durations. HK-2 cells were exposed to 100 ng/ml of lipopolysaccharide for 0, 2, 6, and 12 h. Total protein was prepared for Western blot analysis. β-actin protein was detected as the internal standard. Expression of toll-like receptor 4 protein of HK-2 was quantified and then compared. Each value represents the mean ± standard error of the mean (n = 5, samples were collected from five different passages). *: The value significantly (P < 0.05) differed from that of lipopolysaccharide exposure for 0 h.

**Effects of lipopolysaccharide on the expression of toll-like receptor 4, phosphorylated p38 mitogen-activated protein kinase, and mitogen-activated/extracellular signal-regulated kinase 1 in HK-2 cells**

To elucidate the signaling pathway involved in LPS-induced NF-κB activation in HK-2 cells, the mRNA and protein expression of upstream transmembrane receptor TLR4 and protein expression of phosphorylated ERK1/2, phosphorylated p38MAPK, and p-MEK1 were assayed in HK-2 cells. With β-actin expression served as an internal standard, quantitative analysis showed that the levels of TLR4 mRNA after LPS exposure for 1, 2, and 6 h were increased by 111%, 221%, and 489%, respectively, as compared to that at 0 h [Figure 2, P < 0.05]. Meanwhile, the expression of TLR4 protein after LPS treatment for 1, 2, and 6 h was also increased [Figure 6, P < 0.05]. Expression of TLR4 protein at 2, 6, and 12 h was increased by 74%, 91%, and 154%, respectively, when compared with that at 0 h (P < 0.05). Phosphorylation of p38MAPK [Figure 7, P < 0.05] and MEK1 [Figure 8, P < 0.05] in HK-2 cells at 2, 6, and 12 h was increased by 75%, 130%, 215%, and 74%, 183%, 262%, respectively, as compared to that at 0 h. Levels of phosphorylated ERK1 in HK-2 cells were obviously raised after exposure to LPS for 2, 6, and 12 h by 62%, 162%, and 254%, respectively. Exposure of HK-2 cells to LPS for 2, 6, and 12 h significantly increased ERK2 phosphorylation by 77%, 239%, and 392%, respectively [Figure 9, P < 0.05].
SP-A, depress IκB-α phosphorylation, and suppress NF-κB nuclear translocation. These findings indicate that LPS may induce SP-A synthesis through TLR4-mediated activation of MEK1-ERK1/2-NF-κB signaling pathway.

LPS is a major pathogenic factor in septic shock and multiple organ dysfunction syndrome (MODS) caused by Gram-negative bacteria. In vitro study indicated that the elevated SP-A expression in A549 cells could be induced by LPS in a time-dependent manner. Our previous study showed that SP-A protein could be expressed and secreted by renal tubular epithelial cells. SP-A may play important roles in anti-inflammatory and protective effects in both lung and kidney. In the present study, 100 ng/ml of LPS was used to stimulate HK-2 cells (a human renal tubular epithelial cell line), however cytotoxic effects of LPS on HK-2 cells were not observed. In addition, expression of SP-A protein and mRNA in HK-2 cells was found to be increased in a time-dependent manner by LPS administration, moreover elevated mRNA expression of SP-A occurred earlier than the elevated protein expression. This indicated that LPS may influence the SP-A expression at transcriptional level.

TLR4 is an transmembrane receptor involved in the innate immunity, which can recognize pathogen-associated molecular pattern (including LPS, a component of Gram-negative bacterial cell wall and part of Gram-positive bacteria) to modulate cytokine secretion in the immune response. Thus, the regulation of TLR4 expression may be involved in the innate immunity to pathogens. Studies have showed that LPS may induce the aggregation of TLR4 on the THP1 cells to promote the transmission of extracellular signals into cells, leading to release of a large amount of pro-inflammatory cytokines such as TNF-α.

Differential TLR subtypes would be activated by LPS in different types of cells. LPS can significantly stimulate the TLR2 expression on A549 cells to activate downstream signaling pathway. In the present study, HK-2 cells were stimulated by 100 ng/ml of LPS, significantly increased expression of TLR4 mRNA and protein in a time-dependent manner was observed, which indicated that TLR4 activation stimulated by LPS may be involved in the process of SP-A induction of HK-2 cells.

NF-κB is a well-known transcription factor that regulates the expression of many inflammation-related genes in response to various infections. When cells are stimulated, the NF-κB inhibitor-alpha (IκB) of NF-κB/IκB complex in the cytoplasm is phosphorylated and dissociated from this complex, and then NF-κB (p50 and p65) translocates from the cytoplasm into the nucleus, leading to NF-κB activation, which is related to MAPK pathway. Bacterial cell wall is able to activate NF-κB in the kidney in vivo, and the inhibition of NF-κB activity may ameliorate sepsis-induced AKI. It has been confirmed in the present study that the expression of phosphorylated IκB-α and the nuclear NF-κB protein was significantly increased, but the expression of cytoplasmic NF-κB was decreased dramatically in HK-2 cells by LPS exposure, suggesting the translocation of NF-κB from the cytoplasm to the nucleus, and the subsequent activation of NF-κB in HK-2 cells would be regulated by LPS. There is growing evidence showing that LPS-induced NF-κB activation is related to TLR4-mediated MyD88-dependent signaling pathway.
MAPK family consists of three protein kinases: ERK, p38MAPK, and c-JNK, which are the upstream kinases of NF-κB. ERK1/2 activation may further activate NF-κB. The ERK1/2-induced activation of IκB-α kinase through phosphorylating two serine residuals at the N-terminal of IκB-α would lead to the degradation of IκB-α. Then, NF-κB translocates into the nucleus, which contributes to its activation. NF-κB activation induced by LPS may be due to the phosphorylation of MAPKs. It has been validated in in vitro studies that the phosphorylation of MAPKs (including ERK1/2 and p38MAPK) in LPS-treated A549 cells was determined. In addition, p38MAPK also plays an important role in the NF-κB activation signaling pathway. In the present study, LPS time dependently increased the expression of phosphorylated ERK1/2 and p38MAPK in HK-2 cells. Thus, the phosphorylation of ERK1/2 and p38MAPK, two important members of MAPK family, may participate in NF-κB activation in HK-2 cells stimulated by LPS.

Increasing evidences indicate that ERK signaling pathway is related to the regulation of inflammation. ERK activation is modulated by three different signaling pathways: Raf/MEK-dependent signaling pathway, non-P13K/Raf-dependent signaling pathway, and unknown signaling pathway that can directly activate ERK protein kinase. MEK1 is an upstream protein kinase of ERK1/2, and the activation of MEK1 is associated with ERK1/2 phosphorylation. Roles of MEK1 and ERK1/2 in SP-A expression induced by LPS were also determined in the previous study using alveolar epithelial A549 cells. Our results exhibited that phosphorylation of ERK1/2 by LPS was due to the MEK1 phosphorylation. Thus, LPS-induced phosphorylation of MEK1 and ERK1/2 may be related to the subsequent NF-κB activation in HK-2 cells. Moreover, suppressing TLR4 expression with CLI-095 simultaneously inhibited LPS-induced NF-κB nuclear translocation and subsequent SP-A mRNA expression. Thus, our study indicated that the signal-transducing mechanisms of LPS-caused regulation of SP-A expression arise in renal tubular epithelial HK-2 cells through TLR4 activation and cascade phosphorylations of downstream kinase MEK1 and MAPK (ERK1/2 and p38MAPK). In succession, LPS induced NF-κB expression and translocation.

However, there are certain limitations of the present study. HK-2 cells are not primary culture cells, and results in this study might be a little bit different from those in vivo. Thus, more translational studies are needed to explore the effect of LPS on the secretion of SP-A in human renal tubular epithelial cells in case of AKI.

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**Conflicts of interest**

There are no conflicts of interest.

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