



Neuroprotective Effect of Duloxetine on Chronic Cerebral Hypoperfusion-Induced Hippocampal Neuronal Damage

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Abstract

Chronic cerebral hypoperfusion (CCH), which is associated with onset of vascular dementia, causes cognitive impairment and neuropathological alterations in the brain. In the present study, we examined the neuroprotective effect of duloxetine (DXT), a potent and balanced serotonin/norepinephrine reuptake inhibitor, on CCH-induced neuronal damage in the hippocampal CA1 region using a rat model of permanent bilateral common carotid arteries occlusion. We found that treatment with 20 mg/kg DXT could attenuate the neuronal damage, the reduction of phosphorylations of mTOR and p70S6K as well as the elevations of TNF- α and IL-1 β levels in the hippocampal CA1 region at 28 days following CCH. These results indicate that DXT displays the neuroprotective effect against CCH-induced hippocampal neuronal death, and that neuroprotective effect of DXT may be closely related with the attenuations of CCH-induced decrease of mTOR/p70S6K signaling pathway as well as CCH-induced neuroinflammatory process.

Key Words: Duloxetine, Chronic cerebral hypoperfusion, Hippocampus, Neuroprotection, mTOR/p70S6K signaling pathway, Pro-inflammatory cytokines

INTRODUCTION

Vascular dementia, mainly caused by alteration of blood supply to the brain, is the second most common type of dementia (Plassman *et al.*, 2007). To investigate vascular dementia, animal models of chronic cerebral hypoperfusion (CCH), induced by permanent bilateral common carotid arteries occlusion, have been widely used, because CCH causes a marked chronic reduction in cerebral blood flow (Farkas *et al.*, 2004, 2007; Cechetti *et al.*, 2012; Yang *et al.*, 2014). It has been well known that CCH can lead to cognitive impairment as well as neuropathological alterations in some brain regions including hippocampus and cerebral white matter (Farkas *et al.*, 2004, 2007; Yang *et al.*, 2014; Ueno *et al.*, 2015; Lee *et al.*, 2016a). Especially, among some brain regions, the hippocampal CA1 region is thought to be one of the most vulnerable region following CCH (Farkas *et al.*, 2004; Cechetti *et al.*, 2012; Lee *et al.*, 2016a; Park and Lee, 2016). Some of underlying mechanisms, which related to the CCH-induced hippocampal neuronal damage, have been suggested to include oxidative stress and neuroinflammation (Cechetti *et al.*, 2012; He *et al.*, 2012; Qu *et al.*, 2014; Lee *et al.*, 2016a). In addition, recent studies reported that a significant alterations of the mamma-

lian target of rapamycin (mTOR) signaling pathway, such as decrease of phosphorylated mTOR (p-mTOR), occurred in the hippocampus following CCH (Jia *et al.*, 2015; Park and Lee, 2016). However, the precise mechanisms about CCH-induced neuronal damage have not been fully elucidated yet.

Duloxetine (DXT), a potent and balanced serotonin/norepinephrine reuptake inhibitor (SNRI), has been used in the treatment of major depressive disorders, painful diabetic neuropathy and urinary incontinence (Nemeroff *et al.*, 2002; Goldstein *et al.*, 2005; Guay, 2005). Recently, we showed that DXT had a neuroprotective effect against kainic acid-induced excitotoxic neuronal death in the mouse hippocampus, and that the neuroprotective effect of DXT was related with its anti-inflammatory action (Choi *et al.*, 2015). In addition, we also reported that a neuroprotective effect of DXT was closely related with decreases of glial activation and oxidative stress in the hippocampal CA1 region following transient global cerebral ischemia (Lee *et al.*, 2016b).

Although a recent study reported that venlafaxine, another SNRI, could attenuate cognition impairment, brain oxidative stress and inflammation, as well as cerebral damage in a rat model of vascular dementia induced by renovascular hypertension (Singh and Sharma, 2016), to the best of our knowl-

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edge, there is no study on the neuroprotective effect of SNRI against CCH-induced neuronal damage as yet. Therefore, in the present study, we examined whether DXT had a neuroprotective effect on CCH-induced neuronal damage in the rat hippocampal CA1 region.

MATERIALS AND METHODS

Experimental animals

Male Sprague-Dawley rats (12 weeks old), obtained from RaonBio (Yongin, Korea), were used for this experiment. The animals were housed in a conventional state under adequate temperature ($23 \pm 3^\circ\text{C}$) and relative humidity ($55 \pm 5\%$) control with a 12 h light/12 h dark cycle, and provided with free access to food and water. All experimental procedures for animal handling and use were approved by Institutional Animal Care and Use Committee at Dankook University (Cheonan, Korea). All of the experiments were conducted to minimize the number of animals used and the suffering caused by the procedures used in the present study.

Experimental animal groups and DXT treatment

To elucidate the effect of DXT (Eli Lilly, Indianapolis, IN, USA) against CCH-induced hippocampal neuronal damage, animals were divided into 4 groups ($n=10$ in each group ($n=5$ for immunohistochemistry, $n=5$ for western blot analysis and ELISA assay in each group); 1) vehicle (sterile normal saline; 0.9% w/v NaCl)-treated sham-operated group (Sham-group), 2) vehicle-treated CCH-operated group (CCH-group), 3) 20 mg/kg DXT-treated sham-operated group (DXT-Sham-group), 4) 20 mg/kg DXT-treated CCH-operated groups (DXT-CCH-group). Vehicle and DXT were administered intraperitoneally once a day from the day of surgery (day 1) to the day of scarification (day 28). The dosage of DXT was selected based on previous studies (Choi *et al.*, 2015).

Surgery for chronic cerebral hypoperfusion

The surgical procedure for CCH was performed by 2 vessel occlusion (2VO) according to the method of our previous studies (Lee *et al.*, 2016a; Park and Lee, 2016). In brief, the animals were anesthetized with a mixture of 2.5% isoflurane (Baxter, Deerfield, IL, USA) in 33% oxygen and 67% nitrous oxide. Through a midline cervical incision, both common carotid arteries were exposed and ligated with 5/0 silk suture. The body (rectal) temperature was monitored with a rectal temperature probe (TR-100; Fine Science Tools, Foster City, CA, USA) and maintained using a thermometric blanket before, during and after the surgery until the animals completely recovered from anesthesia. The rats of the Sham- and DXT-Sham-groups were subjected to the same surgical procedures except that the common carotid arteries were not occluded. In case of death of experimental animals after the surgery, additional animals were added.

NeuN immunohistochemistry for neuronal damage

At day 28 after the surgery, the animals were anesthetized with zoletil 50 (40 mg/kg, Virbac, Carros, France) and perfused transcardially with 0.1 M phosphate-buffered saline (PBS, pH 7.4) followed by 4% paraformaldehyde in 0.1 M PBS. The brains were removed and postfixed in the same fixative for 6 h. The brain tissues were cryoprotected by infiltration with

30% sucrose overnight. Thereafter, frozen tissues were serially sectioned on a cryostat (Leica, Wetzlar, Germany) into 30 μm coronal sections, and they were then collected into six-well plates containing PBS.

To examine CCH-induced neuronal damage and neuroprotective effect of DXT in the hippocampal CA1 region, NeuN immunohistochemistry was performed according to the method of previous studies (Choi *et al.*, 2015; Lee *et al.*, 2016a, 2016b). In brief, the brain sections were incubated with diluted mouse anti-NeuN (1:1000, Chemicon, Temecula, CA, USA) overnight at 4°C and subsequently exposed to biotinylated goat anti-mouse IgG and streptavidin peroxidase complex (1:200, Vector, Burlingame, CA, USA). And they were visualized by staining with 3,3'-diaminobenzidine (Sigma-Aldrich, St. Louis, MO, USA) in 0.1 M Tris-HCl buffer (pH 7.2). And then, digital images of the hippocampal CA1 region were captured with an Axio Imager 2 microscope (Carl Zeiss, Göttingen, Germany) equipped with a digital camera (Axiocam, Carl Zeiss) connected to a PC monitor. According to the method of our previous study (Lee *et al.*, 2016a), NeuN-immunoreactive neurons were counted in a $250 \times 250 \mu\text{m}$ square applied approximately at the center of the CA1 region. Six coronal sections with 150- μm interval per animal were selected, and cell counts were obtained by averaging the counts from each animal.

Immunohistochemistry and western blot analysis for p-mTOR and p-S6K

According to the above-mentioned method, immunohistochemistry for p-mTOR and phosphorylated-p70S6 kinase (p-S6K) was performed with rabbit anti-p-mTOR (ser2448) (1:100, Santa Cruz Biotechnology, Santa Cruz, CA, USA) or rabbit anti-p-S6K (Thr389) (1:500, Abcam, Cambridge, MA, USA) as a primary antibody. In order to establish the specificity of the immunostaining, a negative control test was carried out without a primary antibody. The negative control resulted in the absence of immunoreactivity in any structures. Six sections per animal were selected with 150- μm interval to quantitatively analyze p-mTOR and p-S6K immunoreactivity, and digital images of the hippocampal CA1 region were captured with an Axio Imager 2 microscope (Carl Zeiss) equipped with a digital camera (Axiocam, Carl Zeiss) connected to a PC monitor.

To examine changes in p-mTOR and p-S6K protein levels in the hippocampal CA1 region following CCH, experimental animals were used for western blot analysis at 28 days after surgery. After sacrificing animals and removing the brains, brains were serially and transversely cut into a thickness of 400- μm on a vibratome (Leica), and the hippocampal CA1 regions were then dissected with a surgical blade. The tissues were homogenized and centrifuged, and then the supernatants were used to western blot analysis. Rabbit anti-mTOR (1:200, Santa Cruz Biotechnology), rabbit anti-p-S6K (1:500, Abcam) or mouse anti- β -actin (1:5,000, Sigma-Aldrich) was used as a primary antibody. Western blot analysis was performed with three repetitions. After scanning the result of the western blot analysis, densitometric analysis of western blot bands was performed using Image J 1.46 software (National Institutes of Health, Bethesda, MD, USA), to measure relative optical density (ROD). Ratios of the ROD of each groups were represented as %, with Sham-group designated as 100%.

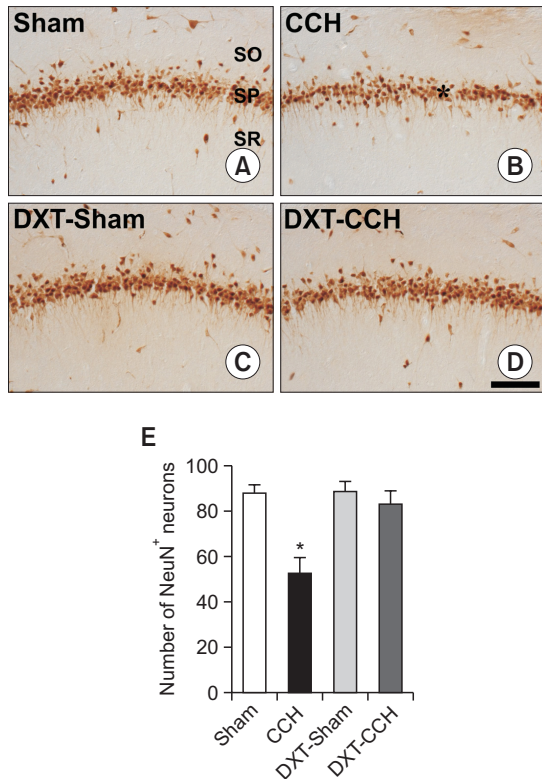


Fig. 1. NeuN immunohistochemistry in the hippocampal CA1 region of the Sham- (A), CCH- (B), DXT-Sham- (C) and DXT-CCH- (D) groups. NeuN⁺ neurons are decreased in the stratum pyramidale (SP, asterisk) of the CCH-group. However, in the DXT-CCH-group, NeuN⁺ neurons are similar to those in the Sham- and DXT-Sham-groups. SO; stratum oriens, SR; stratum radiatum. Scale bar=100 μ m. (E) Analysis in the number of NeuN⁺ neurons in the hippocampal CA1 region of the Sham-, CCH-, DXT-Sham- and DXT-CCH-groups (* p <0.05, significantly different from the Sham-group). Bars indicate the means \pm SEM.

Measurement of pro-inflammatory cytokines

To examine the effect of DXT on pro-inflammatory cytokines in the hippocampus after CCH, ELISA assay for TNF- α and IL-1 β was performed according to the previous study (Qu *et al.*, 2014; Lee *et al.*, 2016a). Briefly, the animals were sacrificed at day 28 after the surgery, and the hippocampus was removed and homogenized. After centrifugation of the homogenates at 14,000 g for 20 min at 4 $^{\circ}$ C, the supernatant was collected. The levels of TNF- α and IL-1 β were examined using commercial ELISA kit (Invitrogen, Camarillo, CA, USA). The concentration of TNF- α and IL-1 β in 100 μ L samples was determined according to the manufacturer's instruction. The levels of TNF- α and IL-1 β were expressed as pg/mg protein.

Statistical analysis

The data shown here represent the means \pm SEM. The differences among the means were statistically analyzed by a two-way analysis of variance followed by a post Dunnett test to elucidate differences among the groups. Statistical significance was considered at p <0.05.

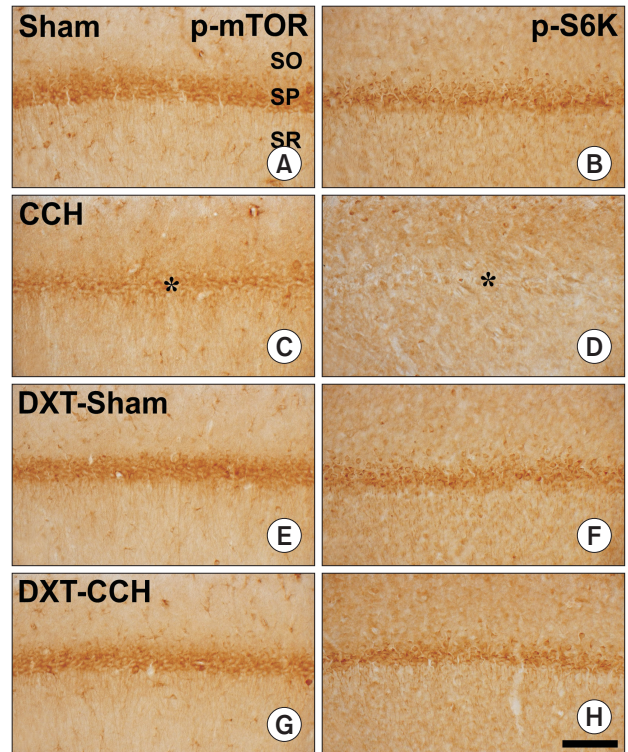


Fig. 2. p-mTOR (left column) and p-S6K (right column) immunohistochemistry in the hippocampal CA1 region of the Sham- (A, B), CCH- (C, D), DXT-Sham- (E, F) and DXT-CCH- (G, H) groups. Marked reduction of p-mTOR and p-S6K immunoreactivity are observed in the stratum pyramidale (SP, asterisk) of the CCH-group. However, p-mTOR and p-S6K immunoreactivity are maintained in the DXT-CCH-group compared with the Sham- and DXT-Sham-groups. SO; stratum oriens, SR; stratum radiatum. Scale bar=100 μ m.

RESULTS

Neuroprotective effect of DXT on CCH-induced hippocampal neuronal damage

The neuroprotective effect of DXT on CCH-induced neuronal damage was examined in the hippocampal CA1 region at 28 days after surgery using NeuN immunohistochemistry (Fig. 1). In the Sham-group, NeuN-immunoreactive (+) cells were abundantly observed in the hippocampal CA1 region (Fig. 1A, 1E). However, a significant reduction in the number of NeuN⁺ neurons was detected in the stratum pyramidale (SP) of the hippocampal CA1 region in the CCH-group (Fig. 1B, 1E). In the DXT-Sham-group, NeuN⁺ neurons was well observed in the SP of the the hippocampal CA1 region (Fig. 1C, 1E). In addition, there was no significant difference in the number of NeuN⁺ neurons in the DXT-CCH-group, compared with that in the Sham- and DXT-Sham-groups (Fig. 1D, 1E).

Changes of p-mTOR and p-S6K protein expression

Both p-mTOR and p-S6K immunoreactivity were easily observed in the SP of the hippocampal CA1 region in the Sham-group (Fig. 2A, 2B). However, in the CCH-group, p-mTOR and p-S6K immunoreactivity in the SP were markedly

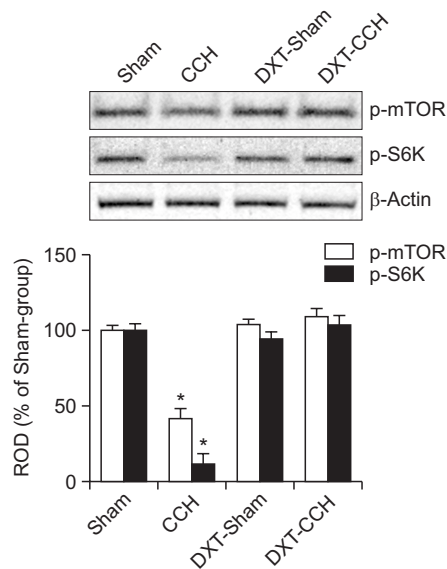


Fig. 3. Western blot analysis of p-mTOR (~289 kDa) and p-S6K (~75 kDa) in the hippocampal CA1 region derived from the Sham-, CCH-, DXT-Sham- and DXT-CCH-groups. Relative optical density (ROD) as % values of immunoblot band is also represented (* $p < 0.05$, significantly different from the Sham-group). The bars indicate the means \pm SEM.

decreased in the SP; especially, p-S6K immunoreactivity was hardly detected in the SP of the CCH group (Fig. 2C, 2D). There was no marked differences in the distribution and immunoreactivity of p-mTOR and p-S6K between Sham- and DXT-Sham-groups (Fig. 2E, 2F). In addition, both p-mTOR and p-S6K immunoreactivity in the DXT-CCH-group were similar to those in the DXT-Sham-group (Fig. 2G, 2H).

From western blot analysis, we observed that the patterns of changes in p-mTOR and p-S6K protein levels in the hippocampal CA1 region following CCH were similar to those observed in the immunohistochemical data. Both p-mTOR and p-S6K protein levels were significantly decreased in the CCH-group, compared with those in the Sham-group. However, there were no significant differences of p-mTOR and p-S6K protein levels in the DXT-CCH-group, compared with those in the Sham- and DXT-Sham-groups (Fig. 3).

Levels of TNF- α and IL-1 β

TNF- α and IL-1 β levels were 21.37 ± 1.31 pg/mg protein and 14.25 ± 1.16 pg/mg protein, respectively, in the hippocampal CA1 region of the Sham-group. In the CCH-group, both TNF- α (33.41 ± 2.96 pg/mg protein) and IL-1 β (24.34 ± 2.47 pg/mg protein) levels were significantly increased, compared with those in the Sham-group. However, TNF- α (23.61 ± 2.72 pg/mg protein) and IL-1 β (16.48 ± 2.14 pg/mg protein) levels in the DXT-CCH-group were similar with those in the Sham- and DXT-Sham-groups (Fig. 4).

DISCUSSION

It has been widely accepted that the marked neuronal damage occurs in the hippocampal CA1 region following CCH, and that CCH-induced cognitive impairment is associated

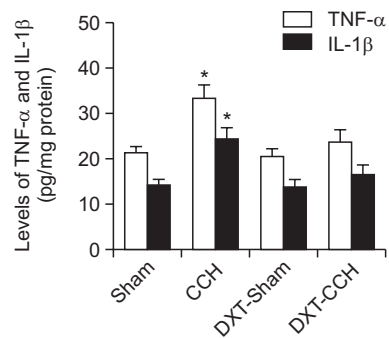


Fig. 4. TNF- α and IL-1 β levels in the hippocampal CA1 region of the Sham-, CCH-, DXT-Sham- and DXT-CCH-groups. The levels of TNF- α and IL-1 β were expressed as pg/mg protein (* $p < 0.05$, significantly different from the Sham-group). The bars indicate the means \pm SEM.

with a significant reduction of hippocampal CA1 cells (Farkas *et al.*, 2007; Cechetti *et al.*, 2012; He *et al.*, 2012; Qu *et al.*, 2014; Lee *et al.*, 2016a; Park and Lee, 2016). In this study, we observed that the number of NeuN-immunoreactive neurons was significantly decreased in the hippocampal CA1 region at 28 days after CCH. This result was consistent with that of the previous studies, which showed the marked reduction of hippocampal pyramidal neurons at 28 days after CCH (Yang *et al.*, 2014; Park and Lee, 2016). In addition, in the present study, we examined the neuroprotective effect of DXT on CCH-induced hippocampal neuronal damage, and we found that DXT could attenuate the CCH-induced neuronal damage of pyramidal neurons in the hippocampal CA1 region. This is the first report on the neuroprotective effect of DXT, one of SNRI, against CCH-induced neuronal damage, and this result is in line with our previous study, which reported that pre-treated DXT could protect against ischemia-induced delayed neuronal death of pyramidal neurons in the hippocampal CA1 region following transient global cerebral ischemia (Lee *et al.*, 2016b).

The mammalian target of rapamycin (mTOR), a downstream target of Akt, and phosphorylated mTOR by Akt has been thought to be essential for protein synthesis, cell growth, cell survival and diverse cellular functions (Gingras *et al.*, 2001; Laplante and Sabatini, 2012). In addition, mTOR phosphorylates S6K, which is also known to play important roles in protein synthesis, cell growth and cell cycle progression (Pullen and Thomas, 1997; Dufner and Thomas, 1999). It has been well known that mTOR/S6K signaling pathway is affected following cerebral ischemic insult. Some previous studies showed that phosphorylations of mTOR and p70S6K were significantly decreased in transient focal cerebral ischemia, which may be related with progression of cerebral ischemia-induced neuronal death (Janelidze *et al.*, 2001; Koh, 2008; Koh *et al.*, 2008; Koh, 2013). It has been also suggested that up-regulation or maintenance of phosphorylations of mTOR and S6K by treatment with some pharmacological agents may could reduce brain injury after cerebral ischemic insult (Koh, 2008; Koh *et al.*, 2008; Shi *et al.*, 2011; Koh, 2013). In the present study, we investigate whether DXT could affect the changes of p-mTOR and p-S6K protein expression in the hippocampal CA1 region following CCH. We found that both immunoreactivities and protein levels of p-mTOR and p-S6K

were significantly decreased in the CCH-group at 28 days following CCH. This result is in line with our previous finding, which showed that both mTOR and p-mTOR protein expressions were significantly decreased in the hippocampal CA1 region at 28 days following CCH (Park and Lee, 2016). In addition, it was also reported that p-mTOR were down-regulated in the rat hippocampus at 20 days following CCH (Jia *et al.*, 2015). On the other hand, in this study, we observed that there were no significant changes of both p-mTOR and p-p70S6K protein expressions in the DXT-CCH-group, compared with those in the Sham- and DXT-Sham-groups. Therefore, based on the results and suggestions of previous studies (Koh, 2008; Koh *et al.*, 2008; Shi *et al.*, 2011; Koh, 2013), it can be thought that the neuroprotective effect of DXT against CCH-induced hippocampal neuronal damage may be closely associated with maintenance of mTOR/S6K signaling pathway.

It has well been known that CCH causes a significant neuroinflammatory response in the rat brain, and that CCH-induced neuroinflammatory response promotes the overproduction of pro-inflammatory cytokines such as TNF- α and IL-1 β , which are thought to be closely related with neuronal damage (Farkas *et al.*, 2007; Qu *et al.*, 2014; Lee *et al.*, 2016a). In our previous and present studies, we found that both TNF- α and IL-1 β levels were significantly increased in the CCH-group (Lee *et al.*, 2016a). Whereas, in this study, we observed that both TNF- α and IL-1 β levels were measured similarly in the DXT-CCH-group, compared with those in the Sham- and DXT-Sham-group. Previously, we reported that the anti-inflammatory activity of DXT prevents kainic acid-induced excitatory neuronal damage in the mouse hippocampal CA3 region (Choi *et al.*, 2015). Therefore, it can be postulated that the anti-inflammatory activity of DXT might be also associated with the neuroprotective effect of DXT against CCH-induced neuronal damage in the rat hippocampal CA1 region.

In summary, our present study indicates that DXT could attenuate the CCH-induced hippocampal neuronal damage, and that the neuroprotective effect of DXT might be due to the maintenance of CCH-induced decrease in mTOR/S6K signaling pathway as well as the suppression of CCH-induced increases in pro-inflammatory cytokines levels.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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