Original Article

Sepsis Strengthens Antagonistic Actions of Neostigmine on Rocuronium in a Rat Model of Cecal Ligation and Puncture

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Abstract

Background: The antagonistic actions of anticholinesterase drugs on non-depolarizing muscle relaxants are theoretically related to the activity of acetylcholinesterase (AChE) in the neuromuscular junction (NMJ). However, till date the changes of AChE activity in the NMJ during sepsis have not been directly investigated. We aimed to investigate the effects of sepsis on the antagonistic actions of neostigmine on rocuronium (Roc) and the underlying changes of AChE activity in the NMJ in a rat model of cecal ligation and puncture (CLP).

Methods: A total of 28 male adult Sprague-Dawley rats were randomized to undergo a sham surgery (the sham group, n = 12) or CLP (the septic group, n = 16). After 24 h, the time-response curves of the antagonistic actions of 0.1 or 0.5 μmol/L of neostigmine on Roc (10 μmol/L)-depressed diaphragm twitch tension were measured. Meanwhile, the activity of AChE in the NMJ was detected using a modified Karnovsky and Roots method. The mRNA levels of the primary transcript and the type T transcript of AChE ($AChE_T$) in the diaphragm were determined by real-time reverse transcription-polymerase chain reaction.

Results: Four of 16 rats in the septic group died within 24 h. The time-response curves of both two concentrations of neostigmine in the septic group showed significant upward shifts from those in the sham group ($P < 0.001$ for 0.1 μmol/L; $P = 0.009$ for 0.5 μmol/L). Meanwhile, the average optical density of AChE in the NMJ in the septic group was significantly lower than that in the sham group (0.517 ± 0.045 vs. 1.047 ± 0.087, $P < 0.001$). The AChE and $AChE_T$ mRNA expression levels in the septic group were significantly lower than those in the sham group ($P = 0.002$ for AChE; $P = 0.001$ for $AChE_T$).

Conclusions: Sepsis strengthened the antagonistic actions of neostigmine on Roc-depressed twitch tension of the diaphragm by inhibiting the activity of AChE in the NMJ. The reduced content of AChE might be one of the possible causes of the decreased AChE activity in the NMJ.

Key words: Acetylcholinesterase; Diaphragm; Neostigmine; Non-depolarizing Muscle Relaxants; Sepsis

Introduction

Non-depolarizing muscle relaxants (NDMRs) are commonly used in general anesthesia to improve anesthetic and surgical conditions. At the end of anesthesia, anticholinesterase drugs such as neostigmine are recommended to reverse the residual neuromuscular block, which has a high incidence rate and can lead to critical respiratory events.[1,2] Cholinesterase can be divided into two categories: acetylcholinesterase (AChE) and pseudocholinesterase. In the neuromuscular junction (NMJ), all of the cholinesterase present is AChE.[3] Anticholinesterase drugs decrease acetylcholine hydrolysis by inhibiting the activity of AChE and thus, improve the concentration of acetylcholine in the NMJ to overcome the effects of muscle relaxants; therefore, their mechanism of reversal is indirect[4] and their antagonistic actions are theoretically related to the activity of AChE in the NMJ.

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As the function of the diaphragm during sepsis is often severely impaired,[5] it is of great importance to fully reverse the residual neuromuscular block in septic patients. Sepsis decreased the activity of serum pseudocholinesterase;[6,7] however, to the best of our knowledge, the changes of AChE activity in the NMJ during sepsis have few directly investigated to date. One pharmacological study investigated the dose-response curves of anticholinesterase drugs and

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showed that the antagonistic actions of anticholinesterase drugs were weakened during sepsis.[8] However, that study did not mention the time points when these measurements were taken. As sepsis could possibly affect the time-response relationship of anticholinesterase drugs, further studies are needed to explore the possible changes of their time-response curves. In the current study, we measured the time-response curves of two clinically relevant concentrations of neostigmine to more intensively investigate the effects of sepsis on the potency of anticholinesterase drugs. Meanwhile, we detected the changes of AChE activity during sepsis, which might be the reason for these effects.

Methods

Animals and experimental design

The current study was approved by the Animal Care and Use Committee of Shanghai General Hospital Affiliated to Shanghai Jiao Tong University. A total of 28 male adult Sprague-Dawley rats, weighing 220–260 g, were used for this study. All animals were pathogen-free and acclimatized for at least 1 week before the experiments were conducted. The animals were allowed free access to food and water and housed at an ambient temperature of 23–25°C with 12 h light-dark cycles. The rats were randomly divided into two groups: (1) the sham group (n = 12) and (2) the septic group (n = 16 due to an expected mortality rate of approximately 25% within the first 24 h in the preliminary study). In the septic group, sepsis was surgically induced using the cecal ligation and puncture method as previously described.[9] Briefly, a midline abdominal incision was made, the middle portion of the cecum was ligated, and a single through and through puncture on the cecum was made with an 18-gauge needle. Droplets of feces were squeezed out of the cecum through the two penetration holes to ensure patency. In the sham group, a midline abdominal incision was made, and the cecum was manipulated but not ligated or punctured. At the end of the surgery, all rats were resuscitated with a subcutaneous injection of 10 ml prewarmed (37°C) normal saline to the back. The rats had free access to water and food after surgery and were closely observed for 24 h after the operation.

At 24 h after surgery, all of the rats that survived were euthanized by intraperitoneal injection of pentobarbital (100 mg/kg). The midcostal region of the left hemidiaphragm with the phrenic nerve attached was removed and prepared for *in vitro* measurement of the antagonistic actions of neostigmine. Meanwhile, the ventral costal region was immediately removed for histological detection of AChE activity in the NMJ. The remaining dorsal costal part was rapidly frozen in liquid nitrogen and stored at −80°C for real-time reverse transcription-polymerase chain reaction (RT-PCR).

Measurement of antagonistic actions of neostigmine on rocuronium-depressed twitch tension of diaphragm

The methods used here were modified from our previous studies.[10,11] The left hemidiaphragm with attached phrenic nerve, central tendon, and rib cage intact was rapidly moved and immediately immersed in Krebs solution that was maintained at 37°C and continuously bubbled with 95% O_2/5% CO_2. The composition of the Krebs solution was as follows (mmol/L): 137 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 1 KH_2PO_4, 12 $NaHCO_3$, and 6.5 glucose, with a pH 7.40 ± 0.5. A muscle strip of the diaphragm (approximately 8 mm wide) with the phrenic nerve attached was obtained from the midcostal region of the diaphragm by careful dissection parallel to the long axis of the fibers. The strip had intact fibers inserted at the ribs and central tendon. Then, the muscle strip was vertically suspended in a tissue chamber (40 ml in volume) containing Krebs solution as mentioned above. The rib end of the muscle was tied to a rigid support, and the central muscle tendon was connected to a force displacement transducer (ALC-AF; Alcott Biotech, Shanghai, China). The phrenic nerve was positioned on two silver stimulating electrodes that were connected to an electrical stimulator (ALC-MPA2000-S; Alcott Biotech). Isometric twitch tension was elicited by supramaximal constant-voltage stimulation at 0.1 Hz for 0.05 ms, and the signal was amplified and recorded using a data acquisition system (ALC-MPA2000 m; Alcott Biotech). Twitch stimuli were used to determine the optimal muscle length at which skeletal muscle could generate the greatest force. After 15 min of equilibration, the baseline twitch tension amplitude was recorded as the control value. Rocuronium (Roc) bromide (N.V. Organon, Oss, The Netherlands) was then added to Krebs solution in the tissue chamber with calibrated micropipettes and its ultimate concentration was 10 μmol/L. After 20 min, the twitch tension was again determined. Neostigmine bromide (Sigma-Aldrich, St. Louis, MO, USA) was then applied to the preparation at a concentration of 0.1 or 0.5 μmol/L. The twitch tension was again determined every 1 min for 30 min. All measurements were repeated in duplicate. For comparison of the effects of Roc and neostigmine, the twitch tension data were expressed as a percent of the control value. The area under the time curve (AUC) of the twitch tension for 30 min after application of neostigmine was calculated as its antagonistic potency.

Histological detection of acetylcholinesterase activity in neuromuscular junction

A modified Karnovsky and Roots method was used to detect the activity of AChE in the NMJ.[12] A muscle strip containing motor endplates was removed from the ventral costal region of the left hemidiaphragm and blotted dry. Two 25-μm-thick serial longitudinal sections were cut using a cryostat (Microtome 5030; Bright Instruments, Huntingdon, UK) at the temperature of −20°C and then fixed in 10% formalin for 5 min. After being washed 3 times in distilled water for 1 min each, the muscle sections were incubated for 1 h at 37°C in the reaction mixture containing (mmol/L) 1.73 acetylthiocholine iodide, 58.8 sodium acetate, 6.2 acetic acid, 5 sodium citrate, 3 copper sulfate, 0.5 potassium ferricyanide, and 10^{-5} Iso-OMPA (Sigma-Aldrich), which is a selective inhibitor of pseudocholinesterase. The preparations were then rinsed in distilled water and sequentially dehydrated by ethanol (75%, 95%, and

100%). After that, they were permanently mounted using neutral balsam. Zeiss upright microscopy (Axiouret S100, Carl Zeiss, Oberkochen, Germany) was used for slide examination, and images of five randomly selected visual fields containing motor endplates under ×100 magnification were collected in each muscle section. The average optical density of AChE in the NMJ was calculated using Image-Pro Plus software (version 6.0, Media Cybernetics, Rockville, Maryland, USA).

Real-time reverse transcription-polymerase chain reaction

The dorsal costal region of the left hemidiaphragm was homogenized, and total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, California, USA). Complementary DNA (cDNA) synthesis was performed using a reverse transcription reagent kit (Bio TNT, Shanghai, China). The cDNA sample was then subjected to PCR analysis using a Transitor® qPCR SybrGreen Detection Kit (Bio TNT) on an Applied Biosystems™ ViiA™ 7 Real-time PCR System (Life Technologies) according to the following program: 95°C for 5 min followed by 40 cycles at 95°C for 5 s and 60°C for 30 s. A dissociation procedure was performed to generate a melting curve that allowed for the confirmation of amplification specificity. The primers were designed and produced by Bio TNT, and the sequences were as follows: AChE (forward primer: 5'-CTG AGT GAA GAC TGC CTT TA-3', reverse primer: 5'-GTA GAA ACC ACC CCC ATA GA-3'); $AChE_T$ (type T splice subunit of AChE,[13] forward primer: 5'-GGA ATC GTT TTC TCC CCA AA-3', reverse primer: 5'-CAC AGG TCT GAG CAG CGT-3'); and β-actin (forward primer: 5'-CCT CTA TGC CAA CAC AGT-3', reverse primer: 5'-AGC CAC CAA TCC ACA CAG-3'). All reactions were repeated in triplicate. The relative mRNA level of the target gene was normalized to the endogenous control (β-actin), and the results were expressed as relative fold changes using the $2^{-\Delta\Delta CT}$ method.

Statistical analysis

Values are expressed as the mean ± standard deviation (SD), unless otherwise specified. The data were tested for normality and equality of variance. The differences in the time-response curves of neostigmine between the sham and septic groups were compared using two-way repeated measures analysis of variance (ANOVA). Other statistical comparisons between each value were performed by paired or unpaired two-tailed Student's *t*-test. All statistical analyses were performed using statistical software (SPSS, version 17.0; SPSS Inc., Chicago, Illinois, USA). A $P < 0.05$ was considered statistically significant.

Results

Mortality within 24 h

Four of 16 rats in the septic group died within 24 h. The remaining animals that survived in the septic group developed loss of movement, shortness of breath, hair erection, subconjunctival hemorrhage, and diarrhea. All rats in the sham group survived during the first 24 h.

Twitch tension of septic diaphragm and effect of rocuronium on twitch tension

Twitch tension elicited by indirect stimulation was determined in the sham and septic groups ($n = 12$, each). Although all of the muscle strips used were of roughly equal sizes (8 mm in width), the twitch tension in the septic group was less intense than that in the sham group (3.39 ± 1.08 g vs. 9.27 ± 1.13 g, $t = -13.076$, $P < 0.001$). Application of 10 μmol/L of Roc for 20 min significantly decreased the twitch tension in both the septic (15.25 ± 2.17% of the control, $t = -135.191$, $P < 0.001$) and the sham groups (16.03 ± 1.70% of the control, $t = -171.021$, $P < 0.001$), but with no significant difference between the two groups ($t = -0.978$, $P = 0.339$).

Antagonistic actions of neostigmine on rocuronium-depressed twitch tension of septic diaphragm

The antagonistic actions of two different concentrations of neostigmine on Roc-depressed twitch tension elicited by indirect stimulation were evaluated for 30 min in the sham and the septic groups [$n = 6$ in each determination, Figure 1a and 1b]. The time-response curves of both concentrations of neostigmine in the septic group showed significant upward

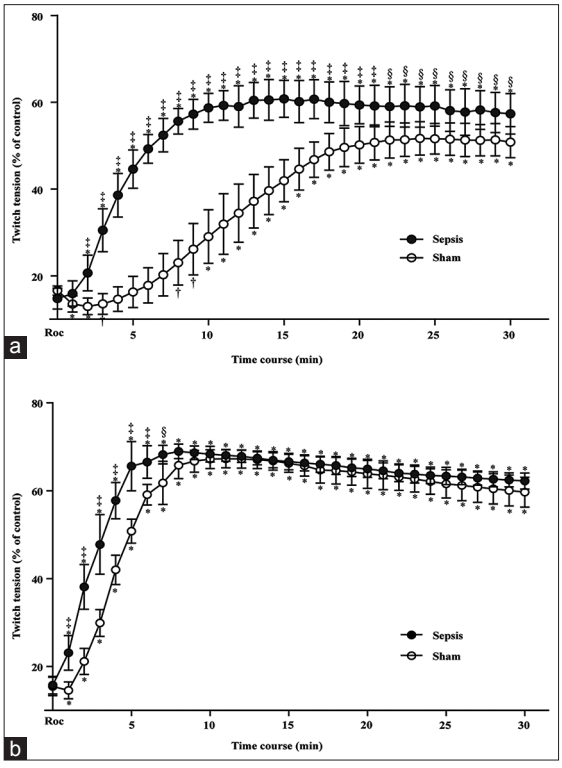

Figure 1: Time course of the antagonistic actions of 0.1 (a) or 0.5 (b) μmol/L of neostigmine on Roc (10 μmol/L)-depressed twitch tension of the sham and the septic diaphragm elicited by indirect stimulation. *$P < 0.01$ and †$P < 0.05$ versus Roc (10 μmol/L) by paired *t*-test. ‡$P < 0.01$ and §$P < 0.05$ versus the sham group by unpaired *t*-test. The data are expressed as the mean ± SD, $n = 6$ in each plot. Roc: Rocuronium; SD: Standard deviation.

shifts from those in the sham group ($F = 73.712$, $P < 0.001$ for 0.1 μmol/L; $F = 10.294$, $P = 0.009$ for 0.5 μmol/L, detected by two-way repeated measures ANOVA). At 0.1 μmol/L of neostigmine, the twitch tension in the septic group exhibited a straight and quick rise to the peak, while it underwent a fall for several min followed by a slow increase to the peak in the sham group. At 0.5 μmol/L of neostigmine, the twitch tension in both the septic and the sham groups increased more rapidly and intensely to the peak than their counterparts at 0.1 μmol/L. However, in the sham group, it still underwent a transient fall initially. Furthermore, the twitch tension AUC until 30 min was significantly higher in the septic group than that in the sham group for both 0.1 μmol/L (1586.7 ± 103.4 vs. 1107.7 ± 86.3, $t = 8.711$, $P < 0.001$) and 0.5 μmol/L (1847.3 ± 53.6 vs. 1729.6 ± 70.9, $t = 3.244$, $P = 0.009$) of neostigmine.

Acetylcholinesterase staining at neuromuscular junction of septic diaphragm

As shown in Figure 2, AChE staining was detected at motor endplate regions in rat diaphragms from both the sham and the septic groups. The average optical density of AChE in the NMJ in the septic group (0.517 ± 0.045, $n = 6$) was significantly lower than that in the sham group (1.047 ± 0.087, $n = 6$; $t = -13.298$, $P < 0.001$).

Acetylcholinesterase and $AChE_T$ mRNA expression in septic diaphragm

The mRNA expression levels of both the AChE and $AChE_T$ in the diaphragm were detected in rats from the sham and the septic groups [Figure 3]. The AChE and $AChE_T$ mRNA expression levels in the septic group were significantly lower than those in the sham group ($t = -5.828$, $P = 0.002$ for AChE; $t = -7.494$, $P = 0.001$ for $AChE_T$).

Discussion

Using a rat model of polymicrobial sepsis, this study found that sepsis could attenuate diaphragm isometric twitch tension elicited by phrenic nerve stimulation. Moreover, and somewhat surprisingly, sepsis strengthened the antagonistic actions of neostigmine on Roc-depressed twitch tension of the diaphragm. This enhancement was accompanied by decreased activity of AChE in the NMJ and decreased expression of AChE and $AChE_T$ mRNA in the diaphragm.

To date, there is no consistent conclusion as to whether sepsis within 24 h could affect the IC_{50} value of NDMRs in depressing muscle twitch tension.[14,15] We chose Roc in our study because it is widely used in clinics as an intermediate-term acting NDMR. The dosage of Roc used in the current study was based on our previous research.[10,11] In the preliminary experiment, we found that 10 μmol/L of Roc could time-dependently depress the diaphragm twitch tension within 30 min. However, the mid-grade sepsis model[9] utilized in the current study could not shift the time-response curve. In the current study, 10 μmol/L of Roc reduced the diaphragm twitch tension to a similar extent in both groups at 20 min, further illustrating that mid-grade sepsis within 24 h could not affect the potency of Roc.

In clinical practice, the dosage of neostigmine for the reversal of residual neuromuscular block is 20–70 μg/kg.[2,16] Neostigmine has a ceiling effect, and the use of doses higher than 70 μg/kg not only could not strengthen the reversal effects but also would increase the incidence of side effects.[2,16] As the molecular weight of neostigmine bromide is 303 g/mol and the steady-state volume of distribution is approximately 0.5 L/kg,[17] its plasma concentration was calculated to be 0.13–0.46 μmol/L. Therefore, the two concentrations of neostigmine (0.1 and 0.5 μmol/L) used in our study are clinically relevant. In our study, the difference of the antagonistic potency of neostigmine between the sham and the septic groups at 0.5 μmol/L was found to be narrowed compared with that at 0.1 μmol/L. Therefore, it is expected that increasing the concentration of neostigmine will further narrow this difference because with the enhanced inhibiting action of neostigmine on AChE in the NMJ, the

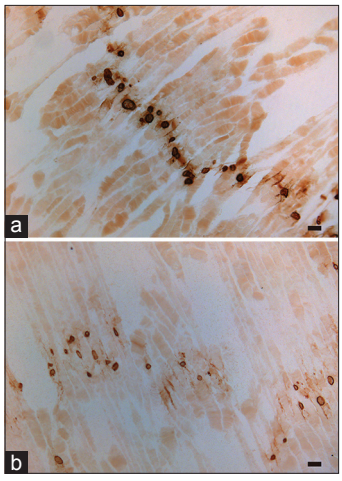

Figure 2: The presence of AChE activity with a modified Karnovsky and Roots method in the neuromuscular junction of rat diaphragm from the sham (a) and the septic (b) groups. Bars = 50 μm. AChE: Acetylcholinesterase.

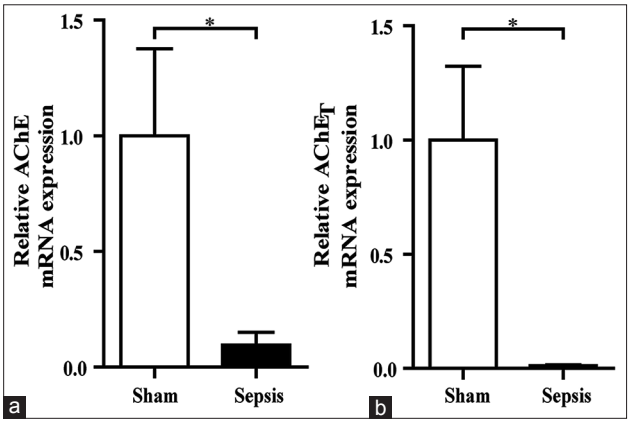

Figure 3: Real-time polymerase chain reaction analysis of both the AChE (a) and $AChE_T$ (b) mRNA expression levels in the rat diaphragm. Values are presented as the mean ± SD, $n = 6$. *$P < 0.01$. AChE: Acetylcholinesterase; SD: Standard deviation.

basic difference of AChE activity between the two groups will be further masked.

In our study, we found that sepsis strengthened the antagonistic actions of neostigmine on Roc-depressed twitch tension, which was contrary to what was found in the previous study.[8] As mid-grade and high-grade sepsis models were utilized in our study and in the previous study, respectively,[9] we speculated that the different extent of inhibition of AChE activity might be one of the reasons for the inconsistence of the two research results. In addition, the two factors (time and dosage) that could influence the antagonistic actions of neostigmine were taken into full consideration in our study. Therefore, we considered our results more accurate. Further studies are needed to illustrate the effects of different grades of sepsis on the actions of neostigmine.

The activity of AChE was found to be decreased during sepsis through the staining method. In view of the strengthened antagonistic actions of neostigmine, we speculated that it was easier for neostigmine to inhibit AChE during sepsis in which the activity had already been partially depressed. The mechanism by which the activity of AChE was inhibited during sepsis was not clearly understood. AChE is an acid-labile enzyme with a pH-optimum at pH 7.6,[18] and the severe metabolic acidosis that often occurs during sepsis might affect its activity. Furthermore, a great amount of hypochlorous acid was generated in sepsis as a potent bactericidal oxidant;[19] meanwhile, it was a potent inhibitor of AChE most likely by oxidizing amino acids, which is critical for enzyme function.[20] In addition, the content of AChE during sepsis might be reduced because the protein synthesis was impaired.[21]

The cellular origin of AChE in NMJ has been an area of long-standing investigation. After reviewing the previously published articles, Rotundo concluded that most, if not all, of the enzyme under normal conditions originates from the skeletal muscle.[22] Afterward, coculture experiments suggested that both motor neuron and skeletal muscle could synthesize and secrete synaptic AChE, with muscle being the dominant contributor.[23] Later, after observing complete absence of AChE in the NMJ of mice in which AChE expression was prevented in the muscle but remained normal in the spinal cord, Camp *et al*. suggested that the muscle alone is the origin of AChE in the motor endplates.[24] Based on the above-mentioned available evidence, we thought that at least the majority of the AChE in the NMJ would come from the muscle. The *AChE* gene produces several types of coding sequences and corresponding catalytic subunits by alternative splicing in the 3' region of the primary transcript.[25,26] However, in adult mammalian muscle, there is only one type of splice subunit, referred to as type T ($AChE_T$).[13] Catalytic subunits of $AChE_T$ produce homo-oligomers as well as heteromeric associations of tetramers with anchoring proteins.[26] Therefore, in this study, we detected both the primary transcript and the type T transcript to more effectively illustrate that the muscular generation of AChE was reduced during sepsis. The AChE activity in the NMJ detected in our study was the sum of all the individual AChE activity, so the decreased activity of AChE in the NMJ of the diaphragm during sepsis might be partially due to its reduced content.

The significance of this study was that the dosage of neostigmine can be reduced properly when used to antagonize the residual neuromuscular block in septic patients. This will consequently decrease the incidence of adverse reactions of neostigmine. There were three limitations in our study. First, we only conducted an *in vitro* experiment, so we do not know whether our conclusion is applicable *in vivo*. Furthermore, we only observed the effects of sepsis at 24 h after surgery, so we do not know what will occur in the context of chronic sepsis. Last but not the least, with the exception of the content, many factors such as pH value, ion concentration, and ambient temperature will affect the overall activity of AChE in the NMJ. Therefore, the reduced content of AChE was only one of the possible causes of the decreased AChE activity in the NMJ.

In conclusion, sepsis could strengthen the antagonistic actions of neostigmine on Roc-depressed twitch tension of the diaphragm by inhibiting the activity of AChE in the NMJ. The reduced content of AChE might be one of the possible causes of the decreased AChE activity in the NMJ.

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Conflicts of interest

There are no conflicts of interest.

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