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Late cardioprotection of exercise preconditioning against exhaustive exercise-induced myocardial injury by up-regulatation of connexin 43 expression in rat hearts

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ABSTRACT

Objective: To investigate the expression of myocardium connexin 43 (Cx43) in late exercise preconditioning (LEP) cardioprotection.
Methods: Eight-week-old adult male Sprague Dawley rats were randomly assigned into four groups ($n = 8$). Myocardial injury was judged in accordance with serum levels of cTnI and NT-proBNP as well as hematoxylin basicfuchsin picric acid staining of myocardium. *Cx43* mRNA was detected by *in situ* hybridization and qualified by real-time fluorescence quantitative PCR. Cx43 protein was localized by immunohistochemistry and its expression level was determined by western blotting.
Results: The LEP obviously attenuated the myocardial ischemia/hypoxia injury caused by exhaustive exercise. There was no significant difference of *Cx43* mRNA level between the four groups. Cx43 protein level was decreased significantly in group EE ($P < 0.05$). However, LEP produced a significant increase in Cx43 protein level ($P < 0.05$), and the decreased Cx43 protein level in exhaustive exercise was significantly up-regulated by LEP ($P < 0.05$).
Conclusions: LEP protects rat heart against exhaustive exercise-induced myocardial injury by up-regulating the expression of myocardial Cx43.

1. Introduction

Cardiovascular disease, which is induced by ischemic, is the major cause of death in the industrialized world. Repeated transient ischemia, which is called ischemic preconditioning (IP), have been shown to protect myocardium against a subsequent more sustained ischemic insult [1–3]. Until now, many studies have evidenced that IP shows cardioprotection by enhancing myocardial tolerance to ischemia-reperfusion (I/R) injury and improving myocardial function [4,5]. Myocardium connexin 43 (Cx43) is a predominant protein forming gap junctions and non-junctional hemichannels in ventricular myocardium, through which ions and small molecules diffuse between cells [6].

It plays a key role in IP cardioprotection against I/R injury and IP have been indeed documented to affect the phosphorylation status of Cx43. Jain *et al.* showed that IP attenuates ischemia-induced dephosphorylation of myocardial Cx43 and the resulting electrical uncoupling [7]. The preserved phosphorylation of Cx43 during IP may be related to the enhanced association of Cx43 with PKC and p38 mitogen activated protein kinases [8]. Furthermore, IP cardioprotection disappears in heterozygous Cx43-deficient mice [9,10], indicating that Cx43 is very important in relieving IP cardioprotection against I/R injury. Like IP cardioprotection, exercise preconditioning (EP), which is brief episode regular exercises, is also widely demonstrated to protect heart during an I/R insult [11–15]. At present, many researches about myocardial protection have focused especially on EP, which includes a biphasic protection manner, *i.e.*, early cardioprotection of exercise preconditioning (EEP) occurring immediately after the exercise and late cardioprotection of exercise preconditioning (LEP) developing 24 h post exercise. For example, EP can improve myocardial function and enhance myocardial tolerance to I/R injury. Its endogenous myocardial protection mechanisms may be closely related to channels

opening, attenuation of apoptosis, and proteins activation closely [12,15–17]. EP was also found to markedly attenuate exhaustive exercise-induced myocardial injury, and PKC is probably involved in EP cardioprotection [18].

Although many studies have demonstrated the protective effect of EP against exhaustive exercise-induced myocardial injury, its mechanisms are still unclear. Considering the same powerful cardioprotective effects of both EP and IP as well as the key role of increased expression of Cx43 in IP cardioprotection, we hypothesized that Cx43 may involve in cardioprotective signaling transduction of EP. It has been observed that endurance exercise training preserves a higher expression level of Cx43 in heart under resting conditions [19]. Moreover, the expression level of Cx43 is down-regulated in trained mice during acute exercise [20]. However, to our knowledge, no research has been done to study the regulatory effects of Cx43 on EP cardioprotection against exhaustive exercise-induced myocardial injury. Therefore, this study aimed to determine the relationship between Cx43 and LEP cardioprotection against exhaustive exercise-induced myocardial injury.

2. Materials and methods

2.1. Rats

Eight-week-old adult male Sprague Dawley rats weighting 220–300 g were purchased from Chinese Academy of Sciences (Shanghai, China). All rats were housed in standard rat cages and maintained at a constant temperature and humidity with a 12 h: 12 h light–dark cycle. They were fed and watered *ad libitum*. All animal care and experimental procedures were conducted in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Sciences and approved by the Ethics Committee for Science Research of the Qufu Normal University.

2.2. Experimental protocol

All rats were given an adaptive training on a treadmill for 5 d, with an adaptive training velocity of 15 m/min and a time course of 10–20 min/d. After 1-day rest, all rats were randomly divided into four groups, eight in each group: (i) sedentary control group (group C), the rats of which were placed on the treadmill without any treadmill exercise; (ii) exhaustive exercise group (group EE), rats of which run to exhaustion on the 0% grade treadmill at a speed of 35 m/min to induce myocardial injury; (iii) late exercise preconditioning group (group LEP), rats of which were allowed to run on the treadmill for four periods of 10 min, each at 30 m/min, with intervening periods of rest of 10 min at 0% grade. Exercise began and ended with 5-min 'warm up' and 'cool down' periods at 15 m/min and 0% grade. The rats were sacrificed 24 h after the exercise. (iv) late exercise preconditioning plus exhaustive exercise group (group LEP + EE), rats of which were treated as those in the group LEP, except that they run to exhaustion 24 h after the exercise and were sacrificed 0.5 h after the exhaustion.

Animals were anesthetized with trichloroacetaldehyde monohydrate (400 mg/kg intraperitoneally) and fixed in the dorsal position on an animal operation table. After abdominal cavity was quickly opened and heart was exposed, blood was drawn via inferior caval vein. Partial heart was rapidly excised, and left ventricle (LV) free wall was isolated at the level of the near apex for real-time fluorescent quantitative PCR and western blot analysis. Once the tissue was divided, the pieces were quick frozen in liquid nitrogen. The rest heart was exposed to perfusion fixation for *in situ* hybridization and immunohistochemistry.

2.3. Detection of serum cTnl and NT-proBNP

The serum levels of cTnl and NT-proBNP in the inferior caval vein blood samples were quantified. The serum cTnl levels were determined by automated immunochemiluminescence on Access 2 immunoassay system (Beckman Coulter, USA), while the serum NT-proBNP levels were determined by using rat NT-proBNP ELISA kits (R&D Systems, Minneapolis, MN, USA) in accordance with the manufacturer's instructions. The sensitivity thresholds for cTnl and NT-proBNP were 0.01 μg/L and 1 μg/L, respectively.

2.4. Hematoxylin basic fuchsin picric acid (HBFP) staining

Following deparaffinization and rehydration, tissue section was stained in alum hematoxylin for 5 min and differentiated in 1% acid alcohol for 4–6 s. After being stained in 0.1% basic fuchsin for 3 min, the section was rinsed with absolute acetone for 15–22 s. Then, it was differentiated in 0.1% picric acid in absolute acetone for 5–10 min. Finally, it was cleared with xylene and covered with a coverslip. The ischemic/hypoxic myocardial fibers stain a crimson red color under light microscope.

2.5. In situ hybridization

The tissue section was first deparaffinized and rinsed with distilled water and phosphate-buffered saline (PBS). After that, it was soaked in 3% hydrogen peroxide for 30 min, followed by pepsin digestion for 5–7 min. After the second washing with PBS and distilled water and prehybridization at 42 °C thermostat, the section was hybridized, blocked with serum, incubated, washed with PBS, colorated, counterstained with hematoxylin, dehydrated in graded series of ethanol and mounted. Serial adjacent sections were hybridized, and probe hybridization solution was replaced by PBS as a negative control. Cx43 *in situ* hybridization kit and DAB chromogenic reagents were purchased by Wuhan Boster Biological Engineering (Wuhan, China). The sequences of three intermediate mRNA fragment probes for rat *Cx43* gene are as follows: 5′-TCT CTC ACG TGC GCT TCT GGG TCC TTC AGA TCA TA-3′, 5′-CTC ATC CAG TGG TAC ATC TAT GGG TTC AGC TTG AG-3′, and 5′-AAC AAT TCC TCG TGC CGC AAT TAC AAC AAG CAA GC-3′.

2.6. Real-time fluorescent quantitative PCR

Total RNA was isolated from LV myocardium using a commercial kit (RNeasy, Mini Kit, Qiagen) according to the manufacturer's instructions. Strand cDNA was synthesized with random hexanucleotides from 1.5 μg of total RNA using a reverse transcription system kit (SuperScript™ III Reverse Transcriptase, Invitrogen). Real-time quantitative PCR was done using Biorad's iQ 10000× SYBR Green Supermix (Invitrogen). The primer sequences are as follows: 5′-AAA GGC GCG TTA AGG ATC GCG TG-3′ (the first primer for *Cx43* gene), 5′-GTC

ATC AGG CCG AGG CCT-3′ (the second primer for *Cx43* gene), 5′-TGA CGT GGA CAT CCG CAA AG-3′ (the first primer for *GAPDH* gene), and 5′-CTG GAA GGT GGA CAG CGC GAG G-3′ (the second primer for *GAPDH* gene). The quality of the RT-PCR product was routinely checked by the thermal denaturation curve following RT-PCR reactions. The expression of RNA encoding the channel subunits was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and relative mRNA levels were quantified by the ΔCT method.

2.7. Western blotting

The LV tissue was homogenized in lysis buffer and centrifuged at 14 000× *g* for 15 min at 4 °C. Then the supernatant was collected to get total Cx43. Electrophoresis was performed on 10% SDS-polyacrylamide gels to transfer proteins to 0.2 μm Sequi-Blot PVDF membranes (200 Ma, 4 °C). After incubation with 5% BSA, the membranes were incubated with rabbit anti-rat Cx43 antibodies (1:5 000 dilution, Boster Biological Engineering, Wuhan, China). Rabbit anti-rat GADPH antibodies (1:10 000 dilution, Kang Biological Engineering, Shanghai, China) overnight at 4 °C. After being washed, the membranes were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG antibodies (1:5 000 dilution, Boster Biological Engineering, Wuhan, China). Results were visualized by the enhanced chemiluminescence method and evaluated by Image J software (NIH, Bethesda, MD, USA).

2.8. Immunohistochemistry

The tissue section was deparaffinized, rehydrated, washed, immersed in 3% hydrogen peroxide, and then digested with pepsin for antigen retrieval. After the blocking of unspecific binding by serum, the section was incubated with rabbit anti-rat Cx43 antibodies (1:200, Boster Biological Engineering, Wuhan, China) at 4 °C overnight. Then, it was detected using the streptavidin-biotin complex kit (1:200, goat anti-rabbit IgG, Boster Biological Engineering, Wuhan, China). Diaminobenzidine/peroxidase substrate was used to produce a brown-colored signal. The section was counterstained, dehydrated, cleared, and coverslipped. PBS was used to replace primary antibody and adjacent sections were used as negative control.

2.9. Statistical analysis

Results are expressed as mean ± SD, and values were compared using the one-way analysis of variance (SPSS 10.0; SPSS, Chicago, IL). Upon confirmation of a significant main effect, individual differences were determined with post hoc analysis. A value of $P < 0.05$ was considered significant.

3. Results

3.1. Establishment of animal model

Table 1 shows the serum levels of cTnI and NT-proBNP. The serum levels of both cTnI and NT-proBNP were significantly higher in the group EE than in the group C ($P < 0.05$). However, the serum levels of cTnI and NT-proBNP were significantly lower in the group LEP + EE than in the group EE ($P < 0.05$). No significant difference in the serum cTnI level was found between the group C and group LEP.

HBFP-stained cardiomyocytes of the LV free wall are represented in Figure 1. The ischemic cardiomyocytes were stained vivid crimson red, and the non-ischemic tissues were stained light brown. A light brown color was seen in the cardiomyocytes of the group C, while crimson red displayed in the majority of cardiomyocytes of the group EE. There is less crimson red color and the red patchy stain was scattered in the cardiomyocytes of the group LEP + EE, compared with that of the group EE. Thus, detection of serum cardiac biomarkers and an obvious HBFP staining pattern in cardiomyocytes confirmed the successful establishment of rat model of exhaustive exercise-induced myocardial injury and cardiopretection induced by exercise preconditioning.

3.2. Observation of myocardial Cx43 mRNA by in situ hybridization

The *Cx43* mRNA in the cardiomyocytes of the LV anterior free wall at the level of the near apex was detected by *in situ* hybridization (Figure 2). The cardiomyocytes of the group C displayed a light brown color and granular distribution in the cytoplasm, while those of the group EE showed no obvious changes, and No obvious difference in the *Cx43* mRNA was found between the group EE and group LEP + EE.

3.3. Changes of Cx43 mRNA and protein levels in myocardium

No miscellaneous peak and abnormal broadening of the main peak was found in the myocardial *Cx43* melting curve, indicating that *Cx43* was the only product of PCR amplicon. Table 1 represents the real-time fluorescent quantitative PCR results of myocardial *Cx43* gene. No significant differences in the *Cx43* mRNA level in myocardium were found between the four groups.

As shown by the western blotting (Table 1), the Cx43 protein level in myocardium was significantly lower in the group EE than in the group C ($P < 0.05$), but it was significantly higher in the group LEP and group LEP + EE than in the group EE ($P < 0.05$).

Table 1
Effects of LEP on biomarkers of myocardial injury and Cx43 expression.

Group	Serum cTnI level (μg/L)	Serum NT-proBNP level (μg/L)	*Cx43* mRNA level	Cx43 protein level[c]
C	0.02 ± 0.01	46.99 ± 4.72	2.00 ± 0.34	0.58 ± 0.16
EE	3.86 ± 2.05[a]	50.73 ± 3.42[a]	1.55 ± 0.26	0.30 ± 0.10[a]
LEP	0.02 ± 0.01	42.20 ± 3.64	1.93 ± 0.26	0.57 ± 0.25[b]
LEP + EE	0.83 ± 0.63[b]	45.75 ± 2.86[b]	1.51 ± 0.15	0.52 ± 0.18[b]

[a] $P < 0.05$ *vs.* the group C. [b] $P < 0.05$ *vs.* the group EE. [c] Cx43 protein level is revised using GADPH level.
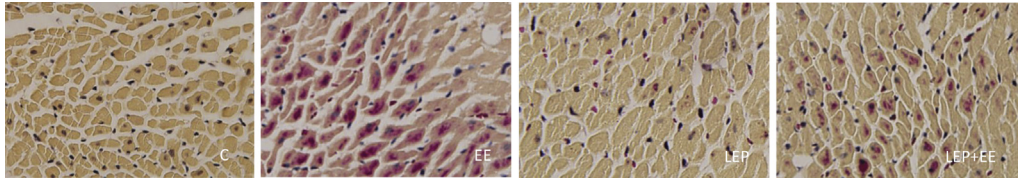
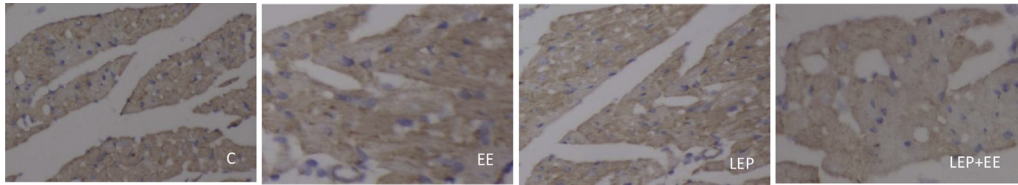**Figure 1.** Myocardial ischemia detected by HBFP staining (×400).

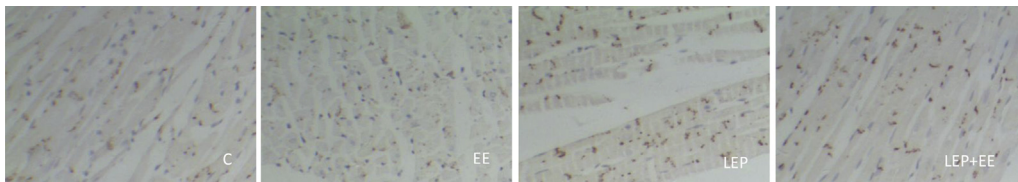**Figure 2.** *Cx43* mRNA in rat myocardium detected by *in situ* hybridization (×400).

Figure 3. Cx43 protein in cardiomyocytes detected by immunohistochemistry (×400).

3.4. Immunohistochemical detection of myocardial Cx43

The Cx43 protein in cardiomyocytes was also detected by the immunohistochemical method (Figure 3). The Cx43 protein in the cardiomyocytes of the group C displayed a light brown color and the cardiomyocytes showed a rod-shaped or punctate distribution pattern in end-to-end connection (intercalated disc) and side to side connection. Similar appearance was found in the rest groups. Compared with the group C, the group EE showed only less rod-shaped or punctate Cx43 in the cardiomyocytes. In contrast, the group LEP and group LEP + EE had considerable more rod-shaped or punctate Cx43 in the cardiomyocytes, compared with the group EE, and more Cx43 can be found in intercalated disc than in side to side connection of cardiomyocytes.

4. Discussion

In the present study, we evaluated the expression of Cx43 in the cardioprotection of LEP against exhaustive exercise induced myocardial injury, by using the established rat model. Exercise training, a way for progressive adaptation of the organism to physical activity, can make the subject to acquire new abilities and increase resistance for better performances. A lot of studies have also proved that endurance exercise is the practical and sustainable countermeasure which can provide cardioprotection against myocardial injury [21–25]. Moreover, some studies have documented that EP which is brief episode regular exercise training, can protect the heart against a subsequent ischemic insult [12,14,16,18,26]. Especially, EP cardiopretection against exhaustive exercise induced myocardial injury have been recently reported in rats trained in a once EP program. Some researchers have been proved that exhaustive exercise renders heart injury in rats, and EP markedly attenuates exhaustive exercise-induced myocardial injury [18,27,28].

Our study showed that the serum levels of NT-proBNP and cTnI, which are biomarkers of myocardial injury, increased when the rats suffered the exhaustive exercise program, but their levels were reduced in the LEP group. In particular, a decrease in the serum levels of NT-proBNP and cTnI appeared in the hearts of rats in the group LEP before the exhaustive exercise induced myocardial injury. The HBFP staining also demonstrated myocardial ischemia in the rat hearts, with the ischemic fibers being stained a vivid crimson color, and crimson red was found in majority of cardiomyocytes in hearts of rats suffering the exhaustive exercise program. However, less crimson red color and red patchy stain were found scattered in the cardiomyocytes of the group LEP before the exhaustive exercise. These results indicated that the successful establishment of animal model of exhaustive exercise-induced myocardial injury and cardiopretection induced by exercise preconditioning. Our results conform to those of Shen *et al*. [18], and similar results have been documented by other researchers [29,30].

In recent years, many studies related to myocardial protection have focused especially on EP, and a few studies have stated the powerful beneficial effect of EP [15,17,31]. Nevertheless, its effectiveness and mechanisms have not yet been clearly demonstrated. Therefore, it is necessary to explore the protective effects of EP on exhaustive exercise-induced myocardial injury.

Functional gap junctions are necessary for the cell-to-cell electrical coupling which is required for synchronized cardiac contraction. Cx43, the main gap junction protein, is predominantly localized at the sarcolemma in myocardium of rats, and it also appears to be one vital element of the signal transduction cascade of the protection by preconditioning. Cx43 in cardioprotection induced by preconditioning to attenuate myocardial I/R injury have been probed in previous studies [8,32–34]. Schwanke *et al*. pointed out that Cx43 knockout mice presented a disappeared cardioprotection by IP [10], which demonstrated that myocardial Cx43 plays a very important role in cardioprotection.

To our knowledge, the effect of EP on Cx43 expression in the cardioprotection against exhaustive exercise-induced

myocardial injury have not been examined yet, and this is the first study to probe into the relationship between Cx43 and cardioprotection which is induced by LEP to alleviate exhaustive exercise-induced myocardial injury.

The RT-PCR results showed that no significant differences in the *Cx43* mRNA levels were found between the groups, as supported by the *in situ* hybridization detection. Obvious decreased expression of *Cx43* mRNA was found in trained mice during acute exercise [20,35], while chronic exercise training has no effect on the expression of *Cx43* mRNA in intact blood vessels *in vivo* [36]. Therefore, we speculated that different exercise intensity has different effects on the expression of *Cx43* mRNA. Further studies are needed to describe the related explicit mechanisms.

As demonstrated by the western blotting, the group EE had less myocardial Cx43 than the group C, while the group LEP and group LEP + EE had more myocardial Cx43 than the group EE. The results demonstrated that LEP could relieve exhaustive exercise-induced myocardial injury by maintaining the spatial distribution of Cx43-formed gap junction channels, through which possibly protecting electrophysiological properties of myocardial tissues. Similar results have been reported [34,37]. Thus, redistribution of Cx43 and remolding of Cx43-based gap junction channels play a very important role in myocardium preconditioning. Different from the variation of *Cx43* mRNA, the Cx43 protein level markedly decreased in the rats with exhaustive exercise-induced myocardial injury, which is consistent with the results of Tiscornia *et al*. [20]. Cx43 expression is regulated by a variety of factors, and the reasons why Cx43 expression was decreased in the rats with exhaustive exercise-induced myocardial injury are unknown.

Cx43 becomes dephosphorylated with prolongation of ischemia [8,38], and its expression area and fluorescence intensity in hypertrophied myocardium are reduced after I/R, compared with non-I/R hypertrophied myocardium [34]. Therefore, dephosphorylation of Cx43 and reduced Cx43 expression in myocardium ischemia injury suggested that modification of gap junction conductance by dephosphorylation of Cx43 induces electrical uncoupling companied with ischemia.

Like I/R injury, exhaustive exercise may render myocardial ischemia injury, and the ischemia injury can decrease Cx43 expression, which suggested that both I/R injury and exhaustive exercise-induced myocardial injury may have the same mechanism to cause myocardial ischemia injury. In addition, LEP alleviated exhaustive exercise-induced myocardial injury, and a remarkable increased Cx43 expression was found in the LEP group. And a higher Cx43 protein level was found in the group LEP + EE than in the group EE. Bellafiore *et al*. also reported similar results, and they found there is a progressive increase of Cx43 expression levels in response to endurance training that reached a peak in ventricular myocardium of mice exercised for 30 d [19].

IP can reduce Cx43 dephosphorylation compared to non-preconditioned myocardium [7,8] and almost completely abolish the Cx43 dephosphorylation-related electrical uncoupling [39] in rat hearts [7]. Moreover, IP can alleviate ischemia/reperfusion-induced arrhythmias by up-regulating Cx43 expression [34]. The same results of Cx43 expression from cardioprotection of IP and LEP indicated the possible presence of similar signal transduction pathways. LEP can preserve phosphorylation of Cx43, and the putative mechanism may be related to an enhanced association of Cx43 with protein kinases such as PKC and PKA. Bao *et al*. showed that the phosphorylation of Cx43 at Ser368 by PKC induces a closure of hemichannels, whereas the inhibition of PKC induces their opening [40]. In contrast, Ek-Vitorin *et al*. pointed out that phosphorylation of Cx43 at Ser368 by PKC increases gap junctional permeability [41] indicating that Cx43 is a target protein of several kinases. Furthermore, Cx43 is associated not only with protein kinases but also with a variety of other proteins [42–44]. The finding that Cx43 can interact with several proteins and exert different functions indicated that Cx43 not only acts as a channel-forming protein but also is involved in intracellular signaling. More recently, the activation of protein kinase C has been reported as an important element in myocardial protection with EP [18]. In addition, we also found Cx43 was involved in myocardial protection with LEP. Hence, the relationship between Cx43 and protein kinase C in myocardial protection with LEP needs further exploration in future work.

In summary, the LEP attenuated the exhaustive exercise-induced myocardial injury in the cardioprotection. Cx43-formed channels are involved in protection against exercise-induced myocardial injury and exercise preconditioning. Cx43 was markedly up-regulated in the LEP rat myocardium. The exact underlying mechanism, how in fact Cx43 mediates protection, remains to be established. Cx43 can quickly translocate between several organelles under pathologic conditions such as ischemia and exercise. The changes in membrane connexin or gap junction plaque density and the relationship between Cx43 and protein kinase C in myocardial protection with LEP should be further elucidated by other techniques.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgments

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