Hypoxia-inducible factor-1 modulates the expression of vascular endothelial growth factor and endothelial nitric oxide synthase induced by eccentric exercise

Paula Rodriguez-Miguelez, Elena Lima-Cabello, Susana Martínez-Flórez, Mar Almar, María J. Cuevas, and Javier González-Gallego

Institute of Biomedicine (IBIOMED), University of León, León, Spain

Submitted 28 August 2014; accepted in final form 12 February 2015

Rodriguez-Miguelez P, Lima-Cabello E, Martínez-Flórez S, Almar M, CuevS MJ, González-Gallego J. Hypoxia-inducible factor-1 modulates the expression of vascular endothelial growth factor and endothelial nitric oxide synthase induced by eccentric exercise. J Appl Physiol 118: 1075–1083, 2015. Submitted March 6, 2015; doi:10.1152/japplphysiol.00780.2014.—The present study investigated the effects of acute and chronic eccentric exercise on the hypoxia-inducible factor (HIF)-1α activation response and the concomitant modulation of vascular endothelial growth factor (VEGF) and endothelial nitric oxide synthase (eNOS) expression in rat skeletal muscle. Twenty-four male Wistar rats were randomly assigned to three experimental groups: rested control group, acutely exercised group after an intermittent downhill protocol for 90 min, and acutely exercised group with a previous eccentric training of 8 wk. HIF-1α activation, VEGF and eNOS gene expression, protein content, and promoter activation were assessed in vastus lateralis muscle biopsies. Acute eccentric exercise induced a marked activation of HIF-1α and resulted in increased VEGF and eNOS mRNA level and protein concentration. The binding of HIF-1α to the VEGF and eNOS promoters, measured by a chromatin immunoprecipitation assay, was undetectable in rested rats, whereas it was evident in acutely exercised animals. Acute exercise also increased myeloperoxidase, toll-like receptor-4, tumor necrosis factor-α, and interleukin-1β protein content, suggesting a contribution of proinflammatory stimuli to HIF-1α activation and VEGF overexpression. All of these effects were partially abolished by training. Moreover, training resulted in an increased capillary density. In summary, our findings indicate that eccentric exercise prompts an HIF-1α response in untrained skeletal muscle that contributes to the upregulation of VEGF and eNOS gene expression and is attenuated after an eccentric training program.

Address for reprint requests and other correspondence: J. González-Gallego, Institute of Biomedicine (IBIOMED), Univ. of León, 24071 León, Spain (e-mail: jgonza@unileon.es).

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1075-7587/15

First published March 6, 2015; doi:10.1152/japplphysiol.00780.2014.
In the O2 delivery to the tissues (38) and, moreover, with a consideration of other markers involved in the vascularization process. The expression of HIF-1 concerning the effect that an acute eccentric bout could have on the acute bout.

**MATERIALS AND METHODS**

**Animals and procedures.** Male Wistar rats weighting 200 ± 10 g were used for the study. They were fed with standard laboratory chow and allowed free access to water in an air-conditioned room with a 12:12-h light-dark cycle. All experiments were approved by the Institutional Animal Care Committee of the University of León and conducted in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Initially and to accustom the animals to the locomotion involved in the acute exercise protocols without resulting in training adaptation in skeletal muscle, all rats were acclimated for 7 consecutive days to a running on a motor-driven treadmill (model LI8706, Letica, Barcelona, Spain) with 10 min of walking (8 m/min) at 0° incline. Twenty-four animals were randomly assigned to three different groups. The first group (control) kept a normal daily routine without extra physical activity. A second group of rats performed an acute bout of exercise, which consisted of an intermittent downhill protocol (−16° incline) at 16 m/min for a total of 90 min [5 min/bout (18 bouts) separated by 2 min of rest] (31). The third experimental group completed an eccentric training protocol with 40 sessions over 8 wk (5 sessions/wk). Each training session included a downhill protocol (−16°) during which the incline was changed every 5 min. This protocol was repeated 8 wk (5 sessions/wk). Each training session included a downhill protocol (−16°) during which the incline was changed every 5 min. This protocol was repeated two times over 8 wk (5 sessions/wk).

**Tissue preparation.** All animals were anesthetized with pentobarbital sodium (50 mg/kg). After exsanguination, the skeletal muscle deep portion of the vastus lateralis (VL) muscle was immediately excised from both legs, freeze-clamped between aluminum tongs precooled with liquid nitrogen, and stored at −80°C for later analysis.

**RNA extraction and real-time quantitative reverse transcription PCR analysis.** Total RNA was isolated from VL muscle using SV Total RNA Isolation Kit (Promega, Madison, WI) and quantified by the fluorescent method Ribogreen RNA Quantiﬁcation Kit (Molecular Probes, Leiden, The Netherlands). DNase I (RNase-free) (Ambion, Austin, TX) was used to remove residual genomic DNA. RNA integrity was confirmed by formaldehyde gel electrophoresis. First-standard cDNA was amplified using High-Capacity cDNA Archive Kit (Applied Biosystems, Paisley, UK). Real-Time PCR was carried out using SYBER Green PCR master mix (Applied Biosystems) and the appropriate forward and reverse specific primers. The sets of PCR primers used were as follows: HIF-1α, 5′-TCCAGATTGACCATGATCA-3′ and 5′-TTTGAG-GACGTGGGTCTTCA-3′; VEGF, 5′-TCTACTCACCACATGC-CAAGT-3′ and 5′-GATATTGTGCGTCTCCTTCT-3′; eNOS, 5′-TGAGCAAGAACAGGTACAAATC-3′ and 5′-GGCAGCAGA-GAGATTACCA-3′; IL-1β, 5′-ATTGCCAAGTGCCTTCTC-GTCTC-3′ and 5′-ATTAGTTATCGATGATGC-3′; and the housekeeping hypoxanthine phosphoribosyltransferase-1 (HPRT-1), 5′-CTCATG-GACTGATTATGGACAGG-3′ and 5′-CGAGGTGCAGCAA-GACCTTATGCC-3′. Relative gene expression changes were determined using the 2−ΔΔCT method, as described previously (32). The cycle number at which the transcripts were detectable (CT) was normalized to the cycle number of HPRT-1 gene detection, referred to as ΔCT.

**Western blot.** Samples of cytosolic and nuclear fraction containing 50 μg of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (9–14% polyacrylamide) and transferred to polyvinylidene difluoride membranes. Non-specific binding was blocked by preincubation of the polyvinylidene difluoride membranes in PBS containing 5% bovine serum albumin for 1 h. Then membranes were incubated overnight at 4°C with appropriate antibodies. Antibodies against VEGF (42 kDa), eNOS, (133 kDa), HIF-1α (120 kDa), and myeloperoxidase (MPO) (84 kDa) were purchased from Abcam (Cambridge, UK), and antibodies against IL-1β (17 kDa), toll-like receptor-4 (TLR4) (95 kDa) and TNFα (17 kDa) were purchased from Santa Cruz (Santa Cruz, CA). Bound primary antibody was detected using a peroxidase-conjugated secondary antibody (Dako, Glostrup, Denmark) by chemiluminescence using the ECL kit (Amersham, Arlington Heights, IL). The density of the specific bands was quantified with an imaging densitometer. The blots were stripped in 6.25 mM Tris, pH 6.7, 2% SDS, and 100 mM mercaptoethanol at 50°C for 15 min and probed again for anti-β-actin (42 kDa) or lamin-B (67 kDa) antibodies (Sigma-Aldrich, St. Louis, MO) to verify equal protein loading (10).

**EMSA.** Binding activity of HIF-1α was determined in nuclear extracts of VL skeletal muscle by means of EMSA (1). Oligonucleotides were end-labeled with [γ-32P] ATP to a specific activity >5 × 107 cpm/μg. DNA-HIF-1α consensus: 5′-TCTGTACGTGACCA-CACCTACCC-3′ and 3′-AGACATGCACTGGTTGAGTGGAG-5′. Nuclear extract (40 μg) was incubated 20 min at room temperature in binding buffer in the presence of ~1 ng labeled oligonucleotide (~250 μCi; GE Healthcare Bio-sciences AB, Uppsala, Sweden). To verify that the results from EMSA analysis did not arise from nonspecific binding, competition experiments were carried out using a negative control (cold probe) and a nonspecific competitor probe. The cold probe containing all reagents without sample was loaded into the gel. The nonspecific competitor reaction used muscle sample with 32P-labeled HIF-1α oligo plus unlabeled AP2 oligonucleotide (Promega, Madison, WI) with a different sequence to that previously described (11). Protein-DNA complexes were separated from the free DNA probe by electrophoresis through 6% native polyacrylamide gels containing 10% ammonium persulfate and 0.5 X Tris-borate-EDTA buffer. Gels were dried under vacuum on Whatmann DE-81 paper and exposed for 48–72 h at Amersham Hyperfilms at ~80°C.

**Chromatin immunoprecipitation assay.** Chromatin in VL muscle was fixed and immunoprecipitated (IP) according to Lima-Cabelo et al. (31). Briefly, ~100 mg of VL muscle were treated with 1% (vol/vol) formaldehyde for 12 min to cross-link the chromatin, and the reaction was stopped by adding glycine to a final concentration of 1.025 M. After centrifugation at 1,500 g for 5 min, the cell pellet was incubated in cell lysis buffer (85 mM KCl, 0.5% Nonidet P-40, 5 mM HEPES, pH 8.0), supplemented with protease inhibitor cocktail (Sigma-Aldrich) for 15 min and then centrifuged at 3,500 g for 5 min to pellet the nuclei. The pellet was resuspended in nuclear lysis buffer (10 mM EDTA, 1% SDS, 50 mM Tris-HCl, pH 8.1) at a ratio 1:1 (vol/wt).
relative to the initial tissue weight, incubated on ice for 10 min, aliquoted in 1 ml fractions, and stored at −80°C until use for chromatin immunoprecipitation assay. Then cross-linked chromatin (1 ml of each sample) was sonicated on ice at 29% amplitude in a Vibra-Cell (Sonics & Materials, Newtown, CT). The average size of the chromatin fragments obtained (~100–500 bp) was checked using agarose gel electrophoresis. The sonicated chromatin was centrifuged at 14,000 g, 4°C for 10 min, and the supernatant, containing soluble chromatin fragments, was diluted 10-fold with dilution buffer (165 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, pH 8.0) supplemented with protease inhibitor cocktail. The diluted chromatin fractions were prepared by adding 30 μl/ml protein A/G-agarose (GE Healthcare Bio-sciences), previously blocked for 1 h with 100 μg/ml λ-DNA, 500 μg/ml transfer RNA, and 1 mg/ml BSA, and kept for 4 h at 4°C on a rotating plate. Then the suspension was centrifuged at 14,000 g for 30 s to remove nonspecifically bound chromatin fragments. Aliquots from the supernatant (equivalent to 50 μg of DNA) were incubated with 2 μg of specific antibodies against HIF-1α (Abcam) and RNA polymerase II (Santa Cruz) and left overnight at 4°C under rotation. Then the samples were incubated with 50 μl of protein A/G-agarose under rotation for an additional period of 4 h. The immunocomplex was recovered by centrifugation at 14,000 g for 30 s and washed, as described previously (49). An aliquot of the cross-linked chromatin was treated in the absence of the antibody (No Ab fraction); the first supernatant, after preclearing with protein A/G-agarose, was saved as the input fraction. The immunoselected chromatin was eluted from the protein A/G-agarose in two consecutive steps by adding 100 μl of elution buffer (1% SDS, 100 mM NaHSO₃) each time, with 30 s of vigorous vortexing. The two supernatants were combined (IP fraction) and incubated at 65°C overnight to reverse formaldehyde cross-links. The DNA from all the...
samples was purified with a PCR purification kit (Qiagen, Germantown, MD) and used for PCR analysis of the target genes.

**PCR analysis of the IP chromatin.** After DNA purification, the input, IP, and No Ab fractions were analyzed by PCR with appropriate primers pairs to amplify products of 200–300 bp in length, corresponding to either the promoter or the coding regions of the target genes. Primers for VEGF 5'-CAGGAAAAGGCCCTCTGTCT-3' and 5'-TGTCCTCTCGACAAATGTCGACC-3', eNOS 5'-AAGACGGTCTGTGGGATCC-3' and 5'-GGCGGCTCTGTAACCTCT-3', and HPRT-1 5'-CAGCAGGGCATTACATT-3' and 5'-AAATTGGCCTGAAAATT-3' were used. Primers selected for the analysis of the coding regions were as follows: VEGF, 5'-TGATGCAAGGAGCAGAG-3' and 5'-ATTACAGGGGTCTTTCC-3'; eNOS, 5'-CTCCAGAGCATACCCGACT-3' and 5'-CTTGTGTCCACCCGCTCGAG-3'; and HPRT-1, 5'-GCTGAGATGCATTAGGAGAT-3' and 5'-AGCAGGTTCCAAAGAACTTATGC-3'. PCR fragments were size-fractionated by 2% (wt/vol) agarose gel electrophoresis and stained with ethidium bromide.

**Immunohistochemistry.** The muscle biopsies were embedded in Tissue-Tek II optimal cutting temperature compound (Sakura Finetek USA, Torrance, CA), frozen, and stored at -80°C. Sections of 6-μm thickness were cut on a cryostat and fixed with methanol. After the inactivation of the endogenous peroxidase by methanol/hydrogen peroxide, the slides were washed in Tris-buffered saline (TBS) for a further 10 min. Then the tissue specimens were incubated with an anti-platelet endothelial cell adhesion molecule (PECAM)-1 mouse monoclonal antibody (Abcam). At the completion of the overnight incubation, the slides were then rinsed in TBS twice and washed for a further 10 min, and Dako antimon-enous (Dako, Glosstrup, Denmark) was added. After a 30-min incubation at 25°C, the slides were washed quickly twice in TBS, given a final 5-min wash, and were immersed in diaminobenzidine to develop color (2–10 min; the reaction was terminated when the positive control included for each sample gave obvious color development). At the completion of incubation, slides were rinsed in distilled water, counterstained with hematoxylin (HD Scientific), washed in tap water, dehydrated, and coverslipped. All images were reviewed under light microscope Leica DM1000 (Leica Microsystems, Barcelona, Spain).

**Statistical analysis.** Results are expressed as means ± SE. Data are presented as percentages from control values. Data were analyzed using a one-way ANOVA. Post hoc comparisons were carried out by the Newman Keuls test. The level of significance was set at 5% (P < 0.05). All of these calculations were performed by using the SPSS 18.0 statistical software (SPSS, Chicago, IL).

**RESULTS**

To determine whether HIF-1α activates and translocates to the nucleus following acute eccentric exercise, an EMSA assay was carried out. Figure 1 depicts results for the various groups of animals. The acute eccentric bout employed in this study induced a significant activation of HIF-1α (P < 0.05) in the VL skeletal muscle of rats. This response was significantly reduced in trained rats which have completed an 8-wk training protocol (P < 0.05) (Fig. 1A). Western blot data also confirmed a significant increase in the nuclear presence of HIF after the acute exercise (P < 0.05), which was lower in rats previously trained (Fig. 1B).

VEGF is paramount for exercise-induced angiogenesis. To determine whether acute exercise modulates VEGF expression, RT-PCR was performed. VEGF mRNA levels were examined 2 h after the acute bout of eccentric exercise and clearly showed a significant increase (P < 0.05) compared with control animals (Fig. 2A). Likewise, the protein content of VEGF, studied by Western blot (Fig. 2B), followed the same pattern than the mRNA expression, showing a significant increase compared with untrained rats (P < 0.05). Effects of acute exercise on VEGF mRNA levels and protein content were partially prevented in trained rats (P < 0.05).

The eNOS isoform was assessed due to its key role in blood flow regulation during exercise. Figure 2, C and D, depicts eNOS mRNA and protein values for the three experimental groups. Rats from the acute exercise group showed a significant (P < 0.05) increase in both eNOS mRNA level and protein (P < 0.05). However, data from the trained group indicated a significant (P < 0.05) lower eNOS gene and protein expression compared with the acute exercised group (P < 0.05).

To establish the contribution of HIF activation to VEGF and eNOS overexpression, chromatin immunoprecipitation assays were carried out with an affinity-purified antibody against HIF-1α. DNA was extracted from the input and immunoprecipitated, and No Ab fractions and equal amounts of each
fraction were amplified using specific primers to the VEGF and eNOS promoter regions. Binding was determined by the relative intensity of ethidium bromide fluorescence compared with the input control. Results elicited from the acute exercised group clearly showed that the binding of HIF-1α to the promoters of both VEGF (Fig. 3A) and eNOS (Fig. 3B) was evident in the acute exercised group, whereas it was undetectable in control nonexercised rats (Fig. 3, A and B). However, this effect was partially abolished in the skeletal muscle of trained rats (P < 0.05). The negative control used was the binding to the HPRT promoter, which was not observed in any of the cases, indicating specific binding of HIF-1α to VEGF and eNOS.

To characterize the extent of muscle damage in both the acute and trained group, several markers of inflammation were measured. The acute eccentric bout triggered a significant (P < 0.05) increase in MPO protein concentration (Fig. 4A). Such upregulation was partially reversed in the experimental group, which completed the eccentric training protocol (P < 0.05). The acute exercise also induced an increase of TLR4 protein content that was significant after 2 h (P < 0.05); however, after the training program, no significant change was detected following the eccentric bout (Fig. 4B). The proinflammatory cytokines IL-1β and TNF-α exert many biological effects; furthermore, several studies suggest that IL-1β can increase HIF-1α DNA binding and VEGF production, which suggests a link between this cytokine and HIF-1α regulation. Protein content of IL-1β is depicted in Fig. 4C. VL skeletal muscle from acute exercised rats showed a marked (P < 0.05) increase in IL-1β; this effect was partially prevented (P < 0.05) in trained rats. In the same line as IL-1β, a single bout of eccentric exercise caused a significant increase of TNF-α expression (P < 0.05), which was significantly attenuated by training (P < 0.05) (Fig. 4D).

Finally, to assess whether acute exercise alone or in combination with training affected vasculature, muscle capillary density was analyzed by immunohistochemistry of PECAM-1 (Fig. 5). Acute exercise did not affect muscle microvasculature. However, an increase in capillary density was detected in the VL skeletal muscle of trained animals.

DISCUSSION

Acute eccentric exercise is recognized for its ability to damage skeletal muscle, resulting in multiple physiological modifications (31). In fact, to support the work carried out by locomotor muscles, the metabolic demand increases (29). Consequently, O₂ availability is lowered, and muscles have to match metabolic O₂ requirement by an increased extraction of O₂ from blood (47). This situation is compensated by the expansion of skeletal muscle vascularity and the development of additional capillaries (34). Cell adaptations to hypoxia are largely regulated by the transcription factor HIF-1 (50). During normoxic conditions and in contrast with other tissues, the O₂-dependent component HIF-1α is expressed in skeletal muscle (54). Nevertheless, when cellular O₂ tension drops, HIF-1α is rapidly accumulated and becomes an important stimulus for the expression of multiple target genes (34). In our study, under resting conditions, HIF-1α showed a low activation, while the acute eccentric exercise protocol carried out elicited a high translocation into the nuclei, enhancing the HIF-1α-dependent DNA binding activity. However, this effect of the acute bout of exercise was markedly decreased in nuclear extracts from the group that completed the training protocol. Our data support...
findings by Lundby and colleagues (35), who reported how regular exercise training reduces the transient increase in HIF-1α mRNA induced in untrained human skeletal muscle in response to an acute exercise bout.

Although O₂ tension may be sufficiently low following the acute bout of exercise to result in HIF-1α stabilization and nuclear translocation, HIF activation may also be triggered by muscle damage involving inflammation and oxidative stress. During acute eccentric exercise, neutrophils recruited to skeletal muscle release ROS, NO, and proinflammatory cytokines (52), which in turn activate the NF-κB signaling pathway, resulting in a further increase of inflammatory molecules (9). Among mediators in inflammation, IL-1β possesses an important role in endothelial and smooth muscle cell proliferation (48), and it is known that IL-1β increases both HIF-1α protein levels and HIF-1α DNA binding through a NF-κB-dependent pathway (18, 31). Other proinflammatory cytokines, such as TNF-α, have been reported to induce expression of HIF-1α mRNA and protein (56). Moreover, it is known that TLR4 downstream signaling could induce HIF-1α expression and activation (60). Supporting this notion, in the present study the acute bout of exercise induced a marked increase of MPO levels in muscle, which was accompanied by an overexpression of inflammatory mediators, such as IL-1β, TNF-α, and TLR4. All of these effects were attenuated in skeletal muscle of trained rats after completing the 8 wk of physical activity, results that are in accordance with previous investigations that have described how training programs may exert analogous inhibitory effects on NF-κB-DNA binding (5, 12, 31).

Fig. 5. Photomicrographs of immunohistochemistry for platelet endothelial cell adhesion molecule (PECAM)-1 in vastus lateralis skeletal muscle for the three experimental groups: C, A, and T. Tissue Tek-embedded sections were immunostained with a PECAM-1 antibody. Original magnification: ×100.
Among the multiple genes that HIF-1α transcription factor may control, VEGF has a critical importance. Certainly, its deletion results in a remarkable drop of muscle capillarity density (55). Previously, other studies have described extensively the relationship between the levels of HIF-1α and VEGF in skeletal muscle (15, 16, 23, 40, 43). In addition, and as indicated by Tang and colleagues (55), increases in VEGF mRNA content are commensurate with HIF-1α protein levels, but only during specific exercise conditions. Our report provides evidence that both mRNA and protein levels of VEGF were significantly upregulated after an acute bout of eccentric exercise, and this effect was lower in rats that previously followed a 8-wk training program. Previous reports available in the literature show that VEGF muscle levels return to baseline following an exercise training in both humans (20) and rodents (45). In addition, the VEGF gene possesses an HRE promoter region, behaving similarly in response to exercise as HIF-1α (44). Actually, muscle HIF-1α knocked out mice show attenuated VEGF mRNA increase in response to acute exercise (36). Our data revealed that binding of HIF-1α to the VEGF promoter region was undetectable in controls rats, whereas it was evident following a single bout of exercise. However, binding was significantly lower in the skeletal muscle of trained animals compared with the acute group. Capillary density, as measured by PECAM-1 immunohistochemistry, was also higher following the training period. Hence, it is plausible that the metabolic stress induced by the physical activity was satisfied by the increase of muscle capillarity, so then, after cumulative training, VEGF levels diminish to return to initial values. In fact, training-induced increase in skeletal muscle capillarization is a well-known phenomenon and is associated with an improved oxidative capacity of the muscle (19, 30).

The key factor in vascular remodeling, VEGF binds tyrosine kinase receptors, resulting in a signaling cascade, which increases NO production through calcium mobilization and phosphorylation of eNOS (28). However, it is worth noting that NO production can be also stimulated independently of VEGF due to increase in flow and the resulting shear stress, enhancing the expression and activity of eNOS (2). Previous studies have already reported that acute exercise may be associated with the induction of eNOS in skeletal muscle (7, 31), being necessary for maintaining a suitable physical capacity (39). Consequently, defects in the activation of eNOS result in a decrease in NO production and contribute to impaired angiogenesis and blood flow (58). Data from the present investigation showed that eNOS mRNA level and protein increase after completing an acute bout, while this exacerbated response was mitigated following the 8 wk of training exercise. Moreover, following an acute bout of eccentric exercise, HIF-1α binds to the eNOS promoter region, which also possesses an HRE, demonstrating that HIF-1α activation modulates the expression of eNOS. As a result, our data support the idea that HIF-1α contributes to a larger release of NO (57) and promotes changes in the blood flow and vascular tone (14). It should be highlighted that, previously, Lima-Cabello and coworkers described a similar relationship among the expression of the three isoforms of NOS and the transcription factor NF-κB after eccentric exercise in rats (31).

In summary, our study suggests that an acute bout of eccentric exercise promotes a significant activation of HIF-1α in rat skeletal muscle, which in turn contributes to the increased expression of mediators, such as VEGF and eNOS, involved in the regulation of the vascular response to enhance O2 delivery. Nevertheless, eccentric training is able to blunt the greater acute vascularization response shown after an acute eccentric exercise in skeletal muscle. Although contribution of other transcription factors cannot be ruled out, data obtained suggest that modulation of VEGF and eNOS expression by HIF-1α may play an important role in the skeletal muscle response to eccentric exercise.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


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