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Hypoxia Inducible Factor-1α Directly Induces the Expression of Receptor Activator of Nuclear Factor-κB Ligand in MLO-Y4 Osteocytes

Kyunghwa Baek[1], Hyun-Jung Park[2], and Jeong-Hwa Baek[2*]

[1]*Department of Pharmacology, College of Dentistry and Research Institute of Oral Science, Gangneung-Wonju National University, Gangwondo, 210-702, Korea*
[2]*Department of Molecular Genetics, School of Dentistry and Dental Research Institute, Seoul National University, Seoul 110-749, Korea*

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Osteocytes may function as mechanotransducers by regulating local osteoclastogenesis. Reduced availability of oxygen, i.e. hypoxia, could occur during disuse, bone development, and fracture. Receptor activator of nuclear factor-κB ligand (RANKL) is an osteoblast/stromal cell derived essential factor for osteoclastogenesis. The hypoxia induced osteoclastogenesis via increased RANKL expression in osteoblasts was demonstrated. Hypoxic regulation of gene expression generally involves activation of the hypoxia-inducible factor (HIF) transcription pathway. In the present study, we investigated whether hypoxia regulates RANKL expression in murine osteocytes and HIF-1α mediates hypoxia-induced RANKL expression by transactivating RANKL promoter, to elucidate the role of osteocyte in osteoclastogenesis in the context of hypoxic condition. The expression levels of RANKL mRNA and protein, as well as hypoxia inducible factor-1α (HIF-1α) protein, were significantly increased in hypoxic condition in MLO-Y4s. Constitutively active HIF-1α alone significantly increased the levels of RANKL expression in MLO-Y4s under normoxic conditions, whereas dominant negative HIF-1α blocked hypoxia-induced RANKL expression. To further explore to find if HIF-1α directly regulates RANKL transcription, a luciferase reporter assay was conducted. Hypoxia significantly increased RANKL promoter activity, whereas mutations of putative HIF-1α binding elements in RANKL promoter prevented this hypoxia-induced RANKL promoter activity in MLO-Y4s. These results suggest that HIF-1α mediates hypoxia-induced up-regulation of RANKL expression, and that in osteocytes of mechanically unloaded bone, hypoxia enhances osteoclastogenesis, at least in part, via an increased RANKL expression in osteocytes.

Key words: hypoxia, hypoxia inducible factor-1α, osteocytes, RANK Ligand

Introduction

Bone undergoes constant remodeling through a series of bone resorption and bone formation. New bone formation enhances bone strength in regions of high mechanical loads, while when routine loads are removed or reduced, bone resorption and bone loss ensues.

Osteocytes are the most abundant cell type in bone. Osteocytes reside inside lacunae and network to each other or to other types of cells via long cytoplasmic extensions that occupy tiny canals called canaliculi. Osteocytes are well

*Correspondence to: Jeong-Hwa Baek, Department of Molecular Genetics, Seoul National University School of Dentistry, 28 Yeongun-dong, Jongno-gu, Seoul 110-749, Korea.
Tel.: +82-2-740-8688, Fax: +82-2-741-3193
E-mail: baekjh@snu.ac.kr

known to be a mechanosensor that detect mechanical stimuli and transfer mechanical signals to other effector cells, by which regulate bone adaptation process to mechanical loading. Receptor activator of nuclear factor-κB ligand (RANKL) is an essential factor for osteoclastogenesis that is mainly produced by osteoblasts and bone marrow stromal cells [1]. RANKL induces osteoclast differentiation from hematopoietic precursors and stimulates the bone-resorbing activity of osteoclasts [2]. Previous studies have established the role of osteocyte in the regulation of bone resorption. You et al., demonstrated that mechanical stimulation of MLO-Y4s osteocyte-like cells decreases their osteoclastogenic-support potential and soluble RANKL and OPG, which are released from osteocytes, is the signaling axis in the regulation of osteoclastogenesis [3].

Reduced availability of oxygen, i.e. hypoxia, activates a transcriptional response that has an important role in various physiological or pathological conditions, such as tumorigenesis, or in normal development. It's been reported that a reduction in pericellular oxygen tension within bone, which occurs during bone development [4], disuse [5], and fracture [6-8]. Diminished mechanical loading of the skeleton initiates a series of cellular events that induce mediated bone loss. Hypoxia induced enhanced osteoclastogenesis via increased RANKL expression has been well demonstrated in *in vitro* system using osteoblasts or periodontal ligament fibroblasts [9,10] It's been suggested that mechanical unloading induces osteocyte hypoxia [11], but hypoxic regulation mechanism in mediating osteoclastogenesis has remained unclear. Hypoxic regulation of gene expression generally involves activation of the hypoxia-inducible factor (HIF) transcription pathway. Hypoxia activates hypoxia inducible factor 1 (HIF-1), a heterodimeric transcription factor comprised of α(inducible) and β (ubiquitous) subunits [12,13]. Under hypoxic conditions, HIF-1α is stabilized and translocated to the nucleus. In the nucleus, HIF-1α binds to its dimerization partner, HIF-1β, and stimulates the expression of its target genes, such as vascular endothelial growth factor (VEGF). Hypoxia stimulates bone resorption by promoting both the osteoclastic differentiation of hematopoietic precursor cells and the bone-resorbing activity of osteoclasts [14,15]. We previously showed that hypoxia induces RANKL expression in human Periodontal Ligament Fibroblasts and that HIF-1α mediates hypoxia-induced RANKL expression by directly binding the RANKL promoter [10].

In the present study, we investigated whether hypoxia regulates RANKL expression in murine osteocytes. Here we show that hypoxia induces RANKL expression in MLO-Y4s osteocytes and that HIF-1α mediates hypoxia-induced RANKL expression by transactivating RANKL promoter.

Materials and Methods

Materials

Alpha-modified Eagle's medium (α-MEM), fetal bovine serum (FBS) and other cultural reagents were obtained from Hyclone (Logan, UT, USA). A GasPak™ EZ CO_2 Pouch System was purchased from BD (Franklin Lakes, NJ, USA). The easy-BLUE™ and *i-Star*Taq™ reagents and WEST-ZOL (plus) were purchased from iNtRON Biotechnology (Sungnam, Korea). The AccuPower RT-PreMix was from Bioneer (Daejeon, Korea), and the SYBR *Premix Ex Taq*™ was from TaKaRa (Otsu, Japan). The PCR primers were synthesized by CosmoGenetech (Seoul, Korea). Anti-HIF-1α antibody was purchased from NOVUS (Littleton, CO, USA), anti-RANKL antibody was from R&D Systems (Minneapolis, MN, USA), anti-actin antibody was from Santa Cruz Biotechnology (Dallas, TX, USA) and HRP-conjugated secondary antibodies were from Thermo Fisher Scientific (Waltham, MA, USA). LipofectAMINE 2000 reagent was purchased from Invitrogen (Waltham, MA, USA).

Cell culture and hypoxic treatment

MLO-Y4 cells, a murine osteocyte-like cell line, were maintained in α-MEM supplemented with 2.5% fetal bovine serum and 2.5% calf serum. To induce hypoxia, the MLO-Y4s were transferred to a GasPak pouch, where the total oxygen concentration was reduced to less than 1% [16] or incubated for 24 h in the presence of desferoxamine (DFO, 200 μM), a high affinity iron chelator [17].

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Quantitative real-time PCR was performed to evaluate mRNA expression. Total RNA was isolated using easy-BLUE™ RNA Extraction Reagents. Complementary DNA was synthesized from 1 μg of total RNA using the AccuPower RT-PreMix and was subsequently used for PCR amplification. Real-time PCR was performed using the SYBR *Premix Ex Taq*™and an AB 7500 Fast Real-Time system (Applied Biosystems Grand Island, NY,

USA). Each sample was analyzed in triplicate, and target genes were normalized to GAPDH. Fold differences were then calculated for each treatment group using normalized C_T values for the control. The primer sequences used for the real-time PCR were as follows: RANKL-forward (f) 5′ -AGA GCG CAG ATG GAT CCT AA-3′, RANKL-reverse (r) 5′-TTC CTT TTG CAC AGC TCC TT-3′; and GAPDH-f 5′- CCA TCT TCC AGG AGC GAGATC-3′, GAPDH-r 5′-GCC TTC TCC ATG GTG GTG AA-3′.

Plasmid constructs

The constitutively active and dominant negative HIF-1α expression plasmids were generous gifts from Prof. J.-W. Park at Seoul National University [18,19].

The reporter construct containing the mouse RANKL promoter (RANKL-WT-luc) was prepared as described previously [10]: the DNA sequence spanning -2174 to +1 bp was amplified by PCR using the following primers: f 5′- CGA GCT CAG AAT GAG GTG GTG GTC TTG CAG AC-3′ and r 5′- CCA AGC TTG GCG CGG CGC CCG GAG TTC G-3′. The amplicons were ligated into the *Sac*I and *Hind*III sites of pGL3-Basic to generate RANKL-luc. To produce the function-defective reporter construct (RANKL-MT-luc), which contains mutations in the putative HIF-1α binding site, a site-directed mutagenic PCR was performed at -971 to -968 and -965 to -962 bp (CGTG->AAAA) [20]. The PCR primers used for the site-directed mutagenesis were as follows: f 5′-TGT GCG CGC GCG AAA ATG AAA ACT TGC GCA CAT GCC GGA GGA-3′ and r 5′-TCC TCC GGC ATG TGC GCA AGT TTT CAT TTT CGC GCG CGC ACA-3′. PCR products were then used to replace the wild-type counterpart of the reporter vector.

Western blot analysis

For RANKL detection, MLO-Y4s were scraped into lysis buffer (10 mM Tris-Cl [pH 7.5], 150 mM NaCl, 1 mM EDTA [pH 8.0], 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM sodium fluoride, 0.2 mM sodium orthovanadate, 1 mM PMSF, 1 μg/ml aprotinin, 1 μM leupeptin and 1 μM pepstatin) and sonicated briefly. Protein concentrations were determined using a modified Bradford method. The proteins were separated using a 10% SDS-PAGE gel and then electro-transferred onto a PVDF membrane. For HIF-1α detection, confluent cells were lysed in 2×Laemmli sample buffer, and the proteins were separated using a 6% SDS-PAGE gel. The membrane was blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20 and incubated with the relevant primary antibody, followed by incubation with the corresponding HRP-conjugated secondary antibody. Immune complexes were visualized using WEST-ZOL (plus) reagent and luminescence was detected with a LAS1000 (DNR; Jerusalem, Israel).

Luciferase reporter assay

Cells were seeded in a 96-well plate at a density of 5×10^3 cells/well. After overnight culture, the cells were transiently transfected with plasmids using the LipofectAMINE™ reagent. In each transfection, 0.2 μg expression vectors (pcDNA, constitutively active or dominant negative HIF-1α), 0.2 μg reporters (pGL3, RANKL-luc or RANKL-MT-luc) and *Renilla* luciferase plasmid were used as indicated. After 24 h incubation under hypoxic or normoxic conditions, the cells were harvested and the luciferase activity was measured using the Dual-Glo luciferase assay kit. Relative luciferase activity was calculated after normalizing the transfection efficiency to *Renilla* luciferase activity.

Statistical analysis

The data from the real-time PCR and reporter assays were expressed as the mean ± S.D. The statistical significance was analyzed by Student's *t*-test. A p value less than 0.05 was considered statistically significant.

Results

Hypoxia increases the levels of RANKL mRNA and protein in MLO−Y4s

We first examined whether hypoxia regulates the expression of RANKL. To induce hypoxia, MLO-Y4s were incubated in the GasPak pouch system or treated with DFO for 24 h. To confirm that MLO-Y4 cells were exposed to hypoxia, we assessed HIF-1α protein levels by Western blot analysis. As expected, stabilization of HIF-1α was observed in hypoxia-induced MLO-Y4s (Fig. 1A), suggesting that incubation in the GasPak pouch or DFO treatment induced hypoxic responses in the MLO-Y4s, respectively. We then evaluated the effect of hypoxia on

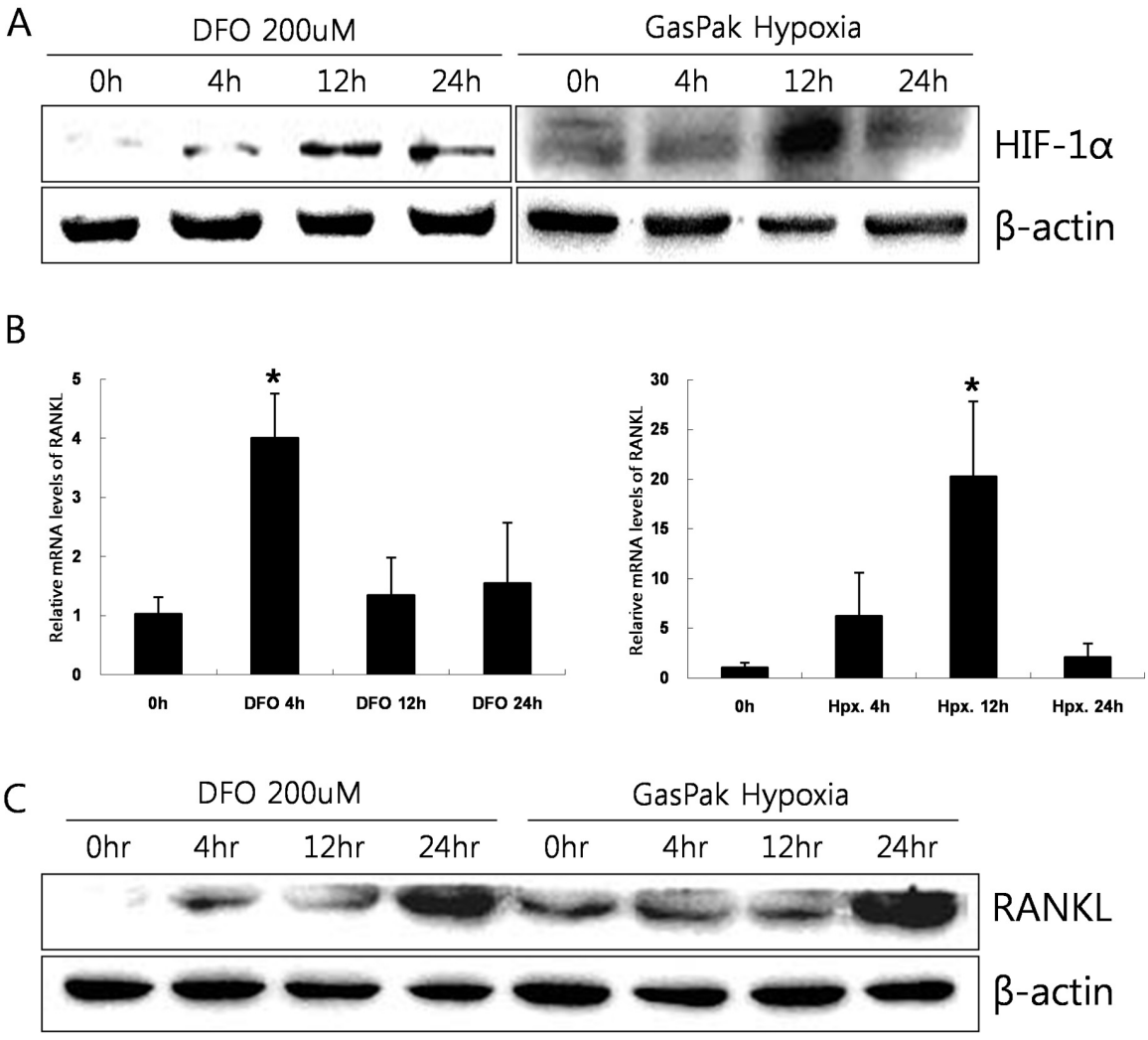

Fig 1. Hypoxia enhances the levels of RANKL mRNA and protein in MLO-Y4s. MLO-Y4s were incubated in the GasPak pouches or treated with DFO for 24 h to induce hypoxia. The Western blot results showed that hypoxia increased the levels of HIF-1α protein (A). Quantitative real-time PCR demonstrated that the level of RANKL mRNA was up-regulated by hypoxia (B) and western blot results showed that hypoxia enhanced RANKL protein levels (C). The data represent the mean ± S.D. of three independent experiments. *$p < 0.05$, compared to 0 h.

RANKL expression in the same culture conditions. The results of quantitative real-time PCR and Western blot analysis showed that both GasPak pouch incubation and DFO treatment enhanced RANKL expression (Fig. 1B, C).

Hypoxia-induced RANKL expression is HIF-1α dependent

Given that HIF-1α is an important regulator of the cellular responses to hypoxia [21,22], we next investigated whether HIF-1α mediates hypoxia-induced RANKL expression in MLO-Y4s. MLO-Y4s were transiently transfected with constitutively active HIF-1α, and the expression levels of RANKL mRNA and protein were examined. Transfection efficiencies were confirmed by western blotting using anti-HIF-1α antibody. Constitutively active HIF-1α significantly increased the levels of RANKL mRNA in MLO-Y4s (Fig. 2A). Consistent with the real-time PCR results, constitutively active HIF-1α enhanced RANKL protein levels, suggesting that RANKL is the target gene of HIF-1α (Fig. 2B).

Next, to further verify the role of HIF-1α in hypoxia-induced RANKL expression, MLO-Y4s were transiently transfected with dominant negative HIF-1α and subjected to hypoxic conditions. Hypoxia increased mRNA

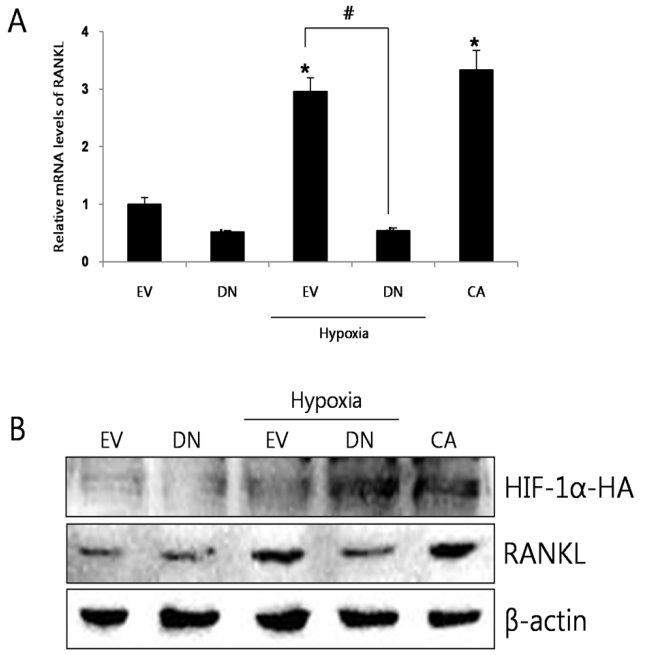

Fig. 2. Hypoxia-induced RANKL expression is HIF-1α -dependent.
MLO-Y4s were transiently transfected with pcDNA (EV), constitutively active HIF-1α (CA) or dominant negative HIF-1 α (DN), and incubated for 24h under the normoxic or hypoxic conditions (GasPak pouches). Then real-time PCR (A) and western blot analysis (B) were performed. Hypoxic treatment or CA overexpression increased the expression levels of RANKL, whereas DN overexpression blocked hypoxia-induced RANKL expression. *p<0.05, compared to EV-transfected cells under normoxia. #p< 0.05,compared to EV-transfected cells under hypoxia.

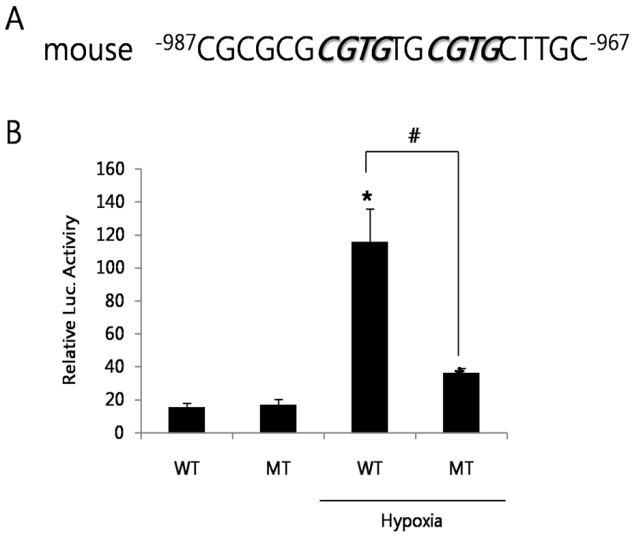

Fig. 3. Hypoxia enhances HIF-1α-dependentRANKL promoter activity.
(A) DNA sequence alignments of mouse RANKL promoter region containing the putative HIF-1α binding site (CGTG). (B) Reporter plasmids containing the mouse RANKL promoter sequence (RANKL-WT-luc) and mutated HIF-1α binding site (RANKL-MT-luc) were prepared. MLO-Y4s were transiently transfected with reporter plasmids and incubated for 24 h under the conditions of normoxia or hypoxia (GasPak pouches). Hypoxia significantly increased reporter activity, whereas HIF-1α binding site mutation blocked hypoxia -induced reporter activity. Data are shown as activity relative to *Renilla* luciferase activity and represent the mean ± S.D. of six independent experiments. *p < 0.05, compared to RANKL-WT-luc alone under normoxic conditions. #p < 0.05, compared to RANKL-WT-luc plus hypoxia.

levels of RANKL in empty vector-transfected cells (Fig. 2A). When dominant negative HIF-1α was overexpressed, the hypoxia induced increment of RANKL mRNA was abolished (Fig. 2A). The hypoxia-induced increase in RANKL protein levels was also attenuated by dominant negative HIF-1α (Fig. 2B). These results indicate that stabilized HIF-1αenhances RANKL expression under hypoxic conditions.

HIF−1α directly stimulates RANKL transcription

As described above, hypoxia induces RANKL expression in MLO-Y4s. Therefore, we examined whether HIF-1α directly regulates RANKL transcription. In silico analysis of the mouse RANKL promoter using the Transcription Element Search System showed that two putative HIF-1α binding elements(CGTG) resides at -971 to -968 and -965 to -962 bp within 2 kb of the RANKL promoter region (Fig. 3A) [23]. Using the reporter constructs containing wild-type and mutant mouse RANKL promoter sequences (RANKL-WT-luc, RANKL-MT-luc), we performed a luciferase reporter assay. Hypoxia induced by GasPak pouch system significantly increased RANKL-WT-luc reporter activity (Fig. 3B). Mutations in the HIF-1α binding elements (CGTG→ AAAA) prevented hypoxia- induced increase in reporter activity. These results suggest that hypoxia-induced HIF-1α transactivates the RANKL promoter.

Discussion

Previous reports have shown that hypoxia induces osteoclastogenesis via direct action on osteoclast precursor cells or via indirect action on osteoblasts and bone marrow cells [15]. Riddle et al. recently elucidated the role of

Hif-1α in regulation of skeletal mechanotransduction in osteocytes, demonstrating Hif-1α impinges Wnt signaling pathway associated with bone anabolic activity [11]. However, there have been no reports on hypoxia-induced RANKL expression in osteocytes to our knowledge. In this study, we demonstrated that hypoxia induces RANKL expression in murine MLO-Y4s and that RANKL is a target gene of HIF-1α. This result is consistent with our previous reports showed that hypoxia induces RANKL expression in human PDLFs and that HIF-1α mediates hypoxia-induced RANKL expression by directly binding the RANKL promoter [10].

Given that mechanical unloading may induce osteocyte hypoxia [5], osteocytes may regulate bone resorption through HIF-1α induced RANKL expression, creating the bone microenvironment favorable conditions for osteoclastogenesis in mechanically unloaded or disused bone. Our data cannot address whether the RANKL derived from osteocytes could actually localize to the hematopoietic cells distant from lacunae via long cytoplasmic extensions, canaliculi. Further investigation on the effect of hypoxia on expression of RANKL and its potential localization to the cytoplasmic target cells is justified.

In conclusion, our results suggest that RANKL is a target gene of HIF-1α and that hypoxia plays a role in enhanced osteoclastogenesis in osteocytes, at least in part, through the induction of RANKL expression in MLO-Y4s.

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Conflict of interest

All of the authors declare no conflicts of interest related to this study.

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