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Increased hydrostatic pressure induces nuclear translocation of DAF-16/FOXO in *C. elegans*

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ABSTRACT

Mechanical stimulation is well known to be important for maintaining tissue and organ homeostasis. Here, we found that hydrostatic pressure induced nuclear translocation of a forkhead box O (FOXO) transcription factor DAF-16, in *C. elegans* within minutes, whereas the removal of this pressure resulted in immediate export of DAF-16 to the cytoplasm. We also monitored DAF-16-dependent transcriptional changes by exposure to 1 MPa pressure for 5 min, and found significant changes in collagen and other genes in a DAF-16 dependent manner. Lifespan was markedly prolonged with exposure to cyclic pressure treatment (1 MPa once a day for 5 min from L1 larvae until death). Furthermore, age-dependent decline in locomotor activity was suppressed by the treatment. In contrast, the nuclear translocation of the yes-associated protein YAP-1 was not induced under the same pressure conditions. Thus, moderate hydrostatic pressure improves ageing progression through activation of DAF-16/FOXO in *C. elegans*.

1. Introduction

The forkhead box O (FOXO) transcription factors are key regulators of cell survival in response to environmental stresses and are evolutionarily well conserved across all metazoans [1–3]. In the nematode *C. elegans*, the FOXO ortholog DAF-16 is a master transcriptional regulator involved in the activation of stress resistance genes in response to heat, oxidative damage, ionizing radiation, starvation, and ageing [4–11]. In addition, hypergravity has also been shown to induce DAF-16 nuclear localization in somatic cells in *C. elegans* via the mechanosensory channels of touch receptor neurons [12].

In humans, mechanical stimuli at the cellular and organ level have important effects that can contribute to the development of heart disease, muscular dystrophy, and cancer metastasis, mainly through remodeling and impaired homeostasis of the extracellular matrix [13–15]. One such mechanical stimulus, hydrostatic pressure, is commonly applied to articular cartilage and occlusal contacts during daily activities such as walking and chewing. Under normal physiological conditions, the hydrostatic pressure can reach to several tens of megapascals, and such mechanical loading is important to maintain tissue and organ homeostasis [16,17]. However, the signaling pathway activated in response to increased hydrostatic pressure remains unclear. This level of pressure or less is insufficient to change protein structure [18–22].

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Here, we visualized the nuclear translocation and export of DAF-16 in response to application and removal of moderate hydrostatic pressure in *C. elegans*. In addition, we evaluated the effect of hydrostatic pressure-induced DAF-16 nuclear translocation on gene expression, lifespan, and motility with ageing in *C. elegans.*

2. Materials and methods

2.1. Caenorhabditis elegans strains and culture methods

N2 (WT strain), CF1038 (*daf-16 (mu86)*, null mutant), and TJ356 (*zIs356* [*daf-16p::daf-16a/b::GFP* + *rol-6 (su1006)*], strain expressing GFP-conjugated DAF-16) were obtained from the Caenorhabditis Genetics Center. IH34 (*lin-15(n765)*; *ihls34* [*yap-1p::yap-1::GFP* + *lin-15(+)*], strain expressing GFP-conjugated yes-associated protein 1: YAP-1::GFP) was used as described [23]. All strains were maintained on NGM agar plates or in NGM liquid with *E. coli* OP50 as the food source at 20 °C.

2.2. Hydrostatic pressure treatments by using a syringe system and SEED system

A syringe system was used to expose the animals to hydrostatic pressures up to 1 MPa (accuracy 0.1 MPa; Supplementary Fig. S1). Synchronized TJ356 younger larvae (approx. 100 animals of L1 and L2) were transferred to a 0.2-mL thin-wall polymerase chain reaction tube (Biotix Inc.) filled completely with M9 buffer, and the tube was placed inside the syringe system. The syringe was then filled with 20 mL of water (20 °C) and 10 mL of N_2 gas. To apply a pressure of 1 MPa, the Luer lock cap on the syringe was closed and the plunger was pushed until the gas space occupied 1 mL. After waiting for 20 min, the pressure was released, and the animals were fixed immediately with 10 mM NaN_3 and observed under a fluorescence microscope (BX51, Olympus). Fluorescence images were recorded with a CCD camera (DP73, Olympus). Live imaging of DAF-16::GFP in response to pressure was performed by using high pressure experimental equipment "SEED" (Syntek Co. Ltd) with fluorescent stereomicroscopy (SMZ18, Nikon).

2.3. Effect of pressure on C. elegans lifespan, locomotor activity, and gene expression

To examine the effects of exposure to moderate hydrostatic pressure on lifespan and thrash rate, approximately 200 WT L1 larvae were exposed to a hydrostatic pressure of 1 MPa for 5 min every day until death by using our syringe system. After exposure, the live animals were counted and transferred to 3.5-cm-diameter plastic dishes containing 2 mL of S medium supplemented with 2 × freeze-dried OP50, and then cultured until death without 5′-fluorodeoxyuridine (FUdR). FUdR has been reported to unexpectedly induce DAF-16 expression [24,25]. To determine age-dependent movement decline, we calculated the average thrashing rate for 5 s after tapping in liquid culture [26].

To examine the effects of increased hydrostatic pressure on gene expression, synchronized, well-fed WT or *daf-16* mutant L1 larvae were exposed to a hydrostatic pressure of 1 MPa for 5 min (syringe system). After exposure, the animals were cultured in liquid medium containing freeze-dried OP50 for 3 h, after which total RNA was extracted with TRIzol Reagent (Invitrogen Corp.) Real-time quantitative reverse transcription polymerase chain reaction analysis was performed with the following primer sets:

abu-14: fw 5′-AGAGACTGTCAAGAACGAGACC, rv 5′-GGAGCATTGGTTGTTGCAGG;

col-107: fw 5′-GTCACATTCAGCAATGAGTACCA, rv 5′-CTTCATTGGCGGTGTTTCTGA;

dod-21: fw 5′-ATTGACTCCCCGCGCTCTTC, rv 5′-CGGTCATTGTCATTGAGCATC;

dod-22: fw 5′-GCCAGCGGTAATAGTGACAAG, rv 5′-AGATGACTTCAGTTTTCCGCC;

pqn-74: *fw* 5′-CGCTTCCTCCTCATTATCGGA, rv 5′-TGGCA-GACTCGCAAAGTTCT; and

act-1: fw 5′-CATGGTCGGTATGGGACAGAA, rv 5′-TCAATTGGGTACTTGAGGGTA.

2.4. Statistical analysis

Each experiment was performed in triplicate with three independent samples. Statistical analysis was performed in Microsoft Excel (Microsoft Corp.). Statistical significance was set at $P < 0.05$ and $P < 0.01$, using Student's two-tailed *t*-test and a two-tailed Fisher's extract test with the use of a 2 × 2 contingency table.

3. Results

3.1. Nuclear translocation of DAF-16 but not YAP-1 in response to moderate hydrostatic pressure

When *C. elegans* (strain TJ356) young larvae (L1 and L2) were exposed to 1 MPa hydrostatic pressure by using a syringe system (Supplementary Fig. 1), nuclear localization of DAF-16::GFP was observed in almost half of the animals within 5 min, and in 80% animals within 20 min (Figs. 1 and 2). Intriguingly, after the pressure was released, the DAF-16 nuclear signal decreased within 20 min, indicating that DAF-16 quickly returned to the cytoplasm (Fig. 1). We also conducted live imaging of DAF-16::GFP by using high pressure experimental equipment "SEED" (Syntek Co. Ltd). Under higher pressure conditions, the nuclear translocation was further accelerated and was completed within 3 min. The movement of larvae completely stopped in this condition and the body curvature was lost (Fig. 1); however, after the pressure was released, locomotor activity immediately returned to normal. Using a novel high-pressure microscope [27], it was found that a pressure of approximately 70 MPa was applied when *C. elegans* stopped moving. Thus, the effect of pressure on *C. elegans* muscular activity was reversible below 70 MPa, even at high pressures.

To investigate whether there is any molecular specificity of DAF-16 in response to hydrostatic pressure, we conducted imaging of YAP-1::GFP (*ihIs34*) in L2 larvae. Like DAF-16, YAP-1 is a transcription co-activator shuttling between cytoplasm and nucleus in response to stimuli such as high temperatures [18]. In particular, YAP-1 nuclear translocation is mainly observed in epithelial cells (18, Fig. 2). Compared to high temperature treatment at 35 °C, hydrostatic pressure of 1 MPa for 20 min caused little change in the distribution pattern of the YAP-1::GFP (Fig. 2). These results indicate that DAF-16 is much more prone to nuclear translocation in presence of moderate hydrostatic pressure.

3.2. Changes in DAF-16-dependent gene transcription in response to increased hydrostatic pressure

We examined whether DAF-16, which translocates to the nucleus in response to short-term exposure to pressure, acts as a transcription factor. Expression changes of some DAF-16 target genes (*abu-14, col-107, pqn-74, dod-21,* and *dod-22*) were monitored 3 h after exposure to 1 MPa for 5 min in WT and *daf-16 (mu86)* mutant larvae. Expression of *abu-1* and *pqn-74* genes, known to be upregulated by DAF-16 and unfolded protein response (UPR) [28,29], were increased two fold in WT but not in the *daf-16* mutant after exposure to 1 MPa pressure (Fig. 3). Similar, a cuticle collagen *col-107* gene, known to be upregulated by DAF-16 [28], was slightly

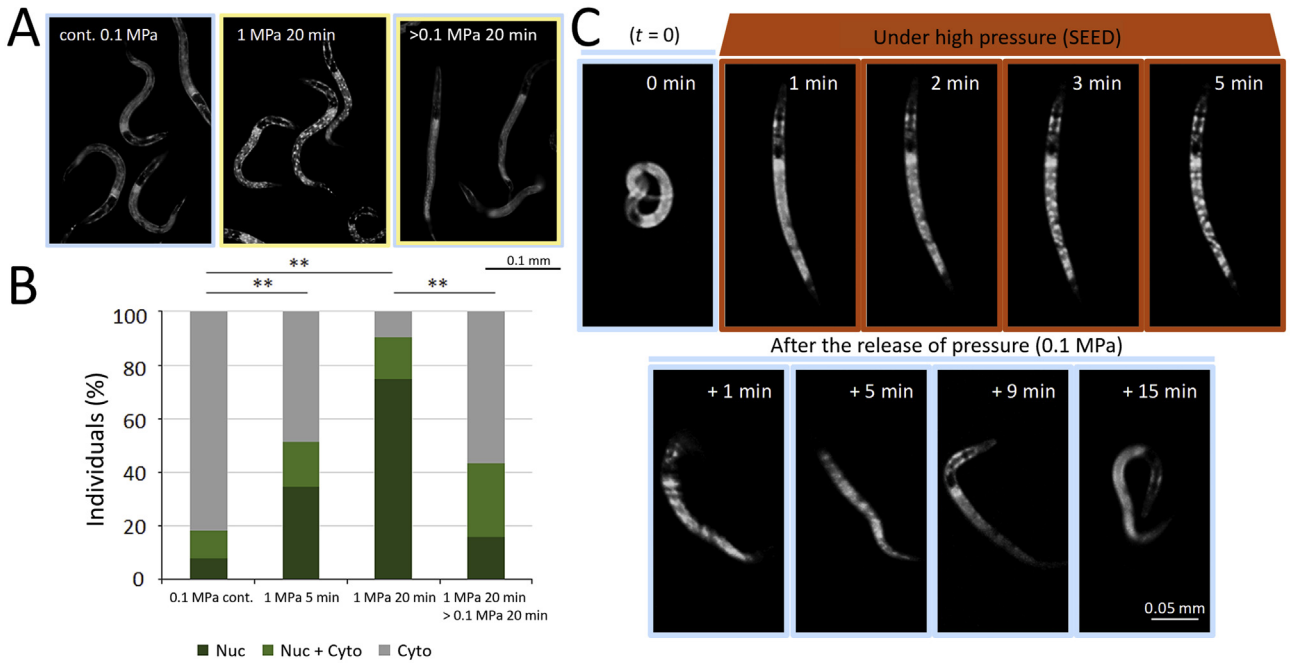

Fig. 1. DAF-16::GFP nuclear translocation in *C. elegans* TJ356 L1 larvae exposed to a hydrostatic pressure. (A) Representative photomicrographs showing DAF-16::GFP localization when TJ356 L1 larvae were exposed to a hydrostatic pressure of 1 MPa for 20 min (yellow-framed panel), and followed by release of the pressure (0.1 MPa) for 20 min (yellow and blue-framed pannel). (B) Percentage of L1 larvae showing complete nuclear translocation of DAF-16::GFP when exposed to a hydrostatic pressure of 1 MPa for 5 and 20 min. After the pressure was released, the DAF-16 nuclear signal significantly decreased within 20 min. Nuc, most DAF-16::GFP was located in the nucleus (dark green); Nuc + Cyto, DAF-16::GFP was distributed in both the nucleus and cytoplasm (green); Cyt, most DAF-16::GFP was located in the cytoplasm (gray). $n > 35$. **P < 0.01 significant difference was calculated in a two-tailed Fisher's extract test with the use of a 2 × 2 contingency table. (C) DAF-16::GFP nuclear translocation was immediately, yet reversibly, induced in *C. elegans* TJ356 L1 larvae in response to increased hydrostatic pressure with "SEED" system. Representative time-series photomicrographs showing DAF-16::GFP localization in TJ356 L1 larvae exposed to a high hydrostatic pressure for 5 min (brown-framed panels) followed by release of the pressure (0.1 MPa) for 15 min (blue-framed panels). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

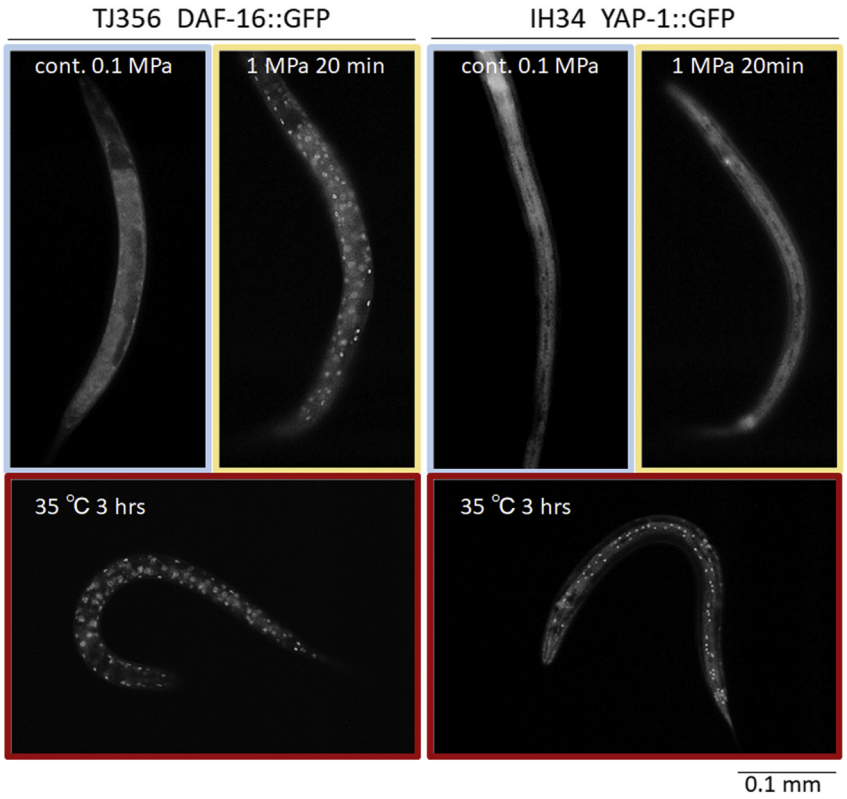

Fig. 2. Comparison between DAF-16 and YAP-1 in nuclear translocation in response to hydrostatic pressure (yellow-framed panels) and high temperature (dark red-framed panels).

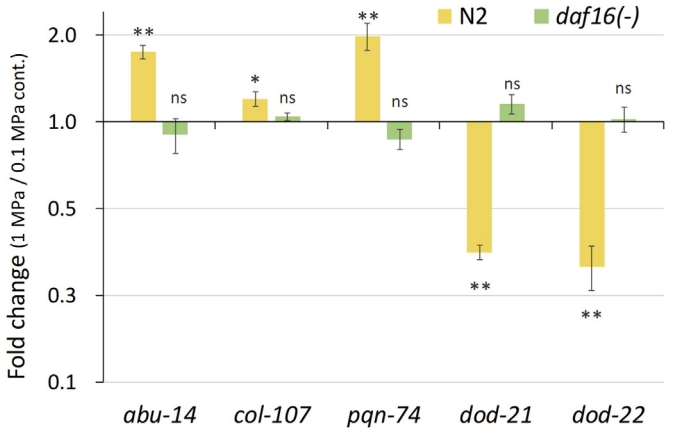

Fig. 3. Real-time quantitative reverse transcription polymerase chain reaction analysis of changes in gene expression induced by exposure to moderate hydrostatic pressure in *C. elegans* wild-type and *daf-16* null mutant. Wild-type (yellow bars) and *daf-16* mutant (green bars) of L1 larvae were harvested after 3 h of exposure at 1 MPa pressure for 5 min. Fold change in gene expression was calculated by comparison of the expression levels between the animals exposed to hydrostatic pressure and mock control. The *act-1* gene was used as the internal standard. **P < 0.01 and * P < 0.05 were taken as significant difference between mock control and pressurized animals. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

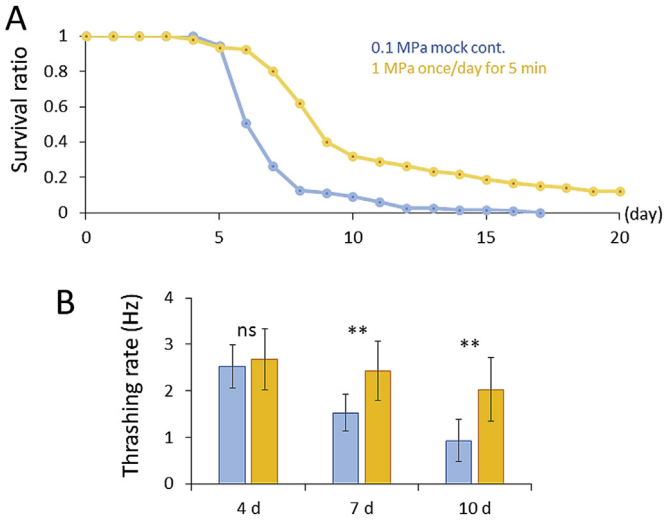

Fig. 4. Effect of increased hydrostatic pressure on lifespan and age-dependent decline in motility in wild-type *C. elegans*. (A) Survival rate (0.1 MPa mock control: blue); L1 larvae were exposed to a hydrostatic pressure of 1 MPa once a day for 5 min until death (orange). $n > 160$. (B) Thrashing rate (Hz) were measured in 4, 7, and 10 days old animals. Blue indicates mock control. Orange indicates cyclic pressure exposed. **P < 0.01 significant difference was calculated in Student's two-tailed *t*-test. $n = 20$, number of survivors examined. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

but significantly induced in WT but not in the *daf-16* mutant (Fig. 3). Two genes, *dod-21* and *dod-22* [30], that are known to be downregulated by DAF-16 were significantly reduced in WT but not in the mutant (Fig. 3). These results indicate that hydrostatic pressure-induced DAF-16 nuclear translocation functions as a transcriptional activator and repressor.

3.3. Pressure-induced prolongation of lifespan and suppression of age-dependent decline in locomotor activity in C. elegans

DAF-16 or FOXO is important downstream regulator that integrates signals from several different pathways, to play a crucial role in ageing and longevity [4,7,10,11]. Here, we examined the effect of exposure to moderate hydrostatic pressure on the lifespan of *C. elegans* in liquid culture by using a syringe system. L1 larvae were exposed to a pressure of 1 MPa once a day for 5 min until death. The pressure treatment did not change the growth rate because at day 3 all L1 larvae had become adults. On the other hand, exposure to cyclic pressure treatment significantly increased the lifespan of the animals (Fig. 4).

We also evaluated the effect of cyclic pressure treatment on age-dependent decline in locomotor activity of the worms. In control animals, the thrash rate gradually decreased with age after 4 days of adulthood (Fig. 4). In 10-day-old animals, the thrash rate was halved. In contrast, cyclic pressure treatment could significantly suppress the decline in locomotor activity (Fig. 4). These results clearly indicate that mechanical stimulation with moderate hydrostatic pressure impaired ageing progression.

4. Discussion

Here, we found that application of hydrostatic pressure immediately induced nuclear localization of DAF-16 in *C. elegans*. Interestingly, nuclear export after releasing pressure also progressed very quickly. A previous report [12] shows that nuclear localization of DAF-16 is induced by exposure to 100 *G* hypergravity; however, under such conditions the complete activation of DAF-16 takes 3 h and the return of DAF-16 to the cytoplasm takes an hour or more. This suggests that the biological response to moderate hydrostatic pressure is different from that to hypergravity. Hypergravity is a unidirectional vector force whereas pressure is an isotopic force. These suggest that there are at least two different pathways in response to mechanical stimuli for DAF-16/FOXO activation.

In general, nuclear–cytoplasmic shuttling of DAF-16/FOXO family proteins are regulated by phosphorylation and dephosphorylation in response to nutrients and stress stimuli [1–3,7,10,11]. Akt/protein kinase B-dependent phosphorylation of DAF-16/FOXO inhibits the activity of the proteins by promoting their export through interaction with 14-3-3 proteins from the nucleus and sequestration in the cytoplasm [1–3,31]. Since both hydrostatic pressure-induced nuclear translocation and pressure-released export of DAF-16 are rapidly progressed, it is important to study the phosphorylation status of DAF-16 for understanding the signal transduction mechanism. In future, we intend to investigate the alterations of the phosphorylation and protein–protein interaction states of DAF-16/FOXO in response to increased hydrostatic pressure.

Physical contact and pressure are important to maintain homeostasis in metazoan tissues and organs. During normal daily activities, the mechanical loading and hydrostatic pressure experienced by human articular cartilage fluctuates between zero and several tens of megapascals [21]. An in vitro analysis has shown that exposure to pressure induces the expression of cartilage matrix proteins such as aggrecan and type II collagen [32]; it also activates the transcription factor Cbp/p300-interacting transactivator 2 (CITED2), which suppresses the expression of matrix-degrading enzymes such as matrix metalloproteinase (MMP)-1 [33]. In chondrocytes, exposure to cyclic tension and pressure upregulates vascular endothelial growth factor (VEGF) [34,35]. A recent transcriptome analysis has also shown that applied pressures regulate the expression of hundreds of genes in cartilage precursor cells [36]. However, details of the signaling pathways involved in these responses to mechanical loading remain unclear. Interestingly, in different approaches, there are many reports that note the relationship between mammalian FOXO transcription factors and the

control of the extracellular matrix [37–40]; for example, FOXO3a regulates CITED2 expression during hypoxia [37], MMP13 in vascular smooth muscle cells [38], and VEGF in breast cancer cells [39]. Furthermore, a recent report using knockout mice has shown that FOXO1 and FOXO3 are important for cartilage homeostasis and regulation in osteoarthritis [40]. Taken together, the discovery of pressure-mediated DAF-16/FOXO nuclear translocation may serve to link the application of pressure and the regulation of matrix protein expression. Thus, DAF-16/FOXO mechanical stimulation through pressure is important to maintain and enhance homeostasis of the highly conserved cuticular and extracellular matrix proteins, which are also known as age-dependent decline molecules in metazoans.

Declaration of competing interest

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2020.01.047.

Representative photomicrographs showing DAF-16::GFP nuclear translocation in TJ356 and YAP-1::GFP little response in IH34 of L2 larvae exposed to mock (0.1 MPa, blue-framed panels), and 1 MPa hydrostatic pressure for 20 min (orange-framed panels). Exposed to high temperatures at 35 °C for 3 h (red-framed panels), nuclear translocation of DAF-16::GFP and YAP-1::GFP are observed in almost all somatic cells and epithelial cells, respectively.

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