Ghrelin Alleviates Angiotensin II-Induced H9c2 Apoptosis: Impact of the miR-208 Family

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Background: Ghrelin is a novel peptide with abundant cardioprotective effects. The miR-208 family, consisting of cardiac-specifically expressed microRNAs, are not only involved in hypertrophy and fibrosis, but are also closely related with myocyte apoptosis. This study explored the role of the miR-208 family in the protective effect of ghrelin on angiotensin II (Ang II)-induced apoptosis.

Material/Methods: H9c2 cells were exposed to Ang II with or without ghrelin. Cell viability was detected by MTT assay and the percentage of apoptotic cells was confirmed by flow cytometry. miRNAs expression levels were measured by qRT-PCR. Then, cells transfected with miR-208 negative control, mimics, and inhibitors were treated with Ang II and ghrelin, followed by flow cytometry. PCR array was performed to explore the pathways affected by miR-208.

Results: The miR-208 level was reduced in Ang II-treated H9c2 cells, accompanied with increased cell apoptosis, which were both reversed by ghrelin administration. Flow cytometry revealed that miR-208 inhibitors clearly upregulated the apoptotic percentage, whereas miR-208 mimics showed the opposite effects in the Ang II group. miR-208a further alleviated apoptosis when treated with ghrelin. miR-208 mainly affected caspase, inflammatory-related genes, and several signaling pathways.

Conclusions: We provide new evidence that the miR-208 family is regulated by Ang II and ghrelin. Overexpression of miR-208 family alleviated Ang II-induced cell apoptosis and miR-208a assisted in the protective effect of ghrelin. Several apoptosis pathways affected by miR-208 family were found. These findings suggest the pathogenesis of cardiomyocyte apoptosis and the protective mechanism of ghrelin.

MeSH Keywords: Angiotensin II • Apoptosis • Ghrelin • MicroRNAs

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Background

Heart failure (HF) has a high fatality rate and poor prognosis is the terminal stage of various kinds of cardiovascular diseases [1–4] and imposes a cumulative burden on society. Myocardial infarction (MI) is one of the most common initiating factors resulting in heart failure. MI starts with myocyte apoptosis in the ischemic region, followed by a progressive cell loss in the non-infarcted region, and then leads to endothelium inflammation, collagen deposition, hypertrophy, and fibrosis, which all lead to cardiac dysfunction [5]. The molecular mechanisms underlying myocyte apoptosis, the initial factor of HF, and its therapeutic target have been studied for several years.

Ghrelin, first discovered by Kojima et al. in 1999 [6], is an endogenous ligand specific for growth hormone secretagogue receptor (GHS-R). This peptide consists of 28 amino acids and stimulates the release of growth hormone (GH), thus regulating food intake and metabolism. Although isolated from the rat stomach, ghrelin was found to be secreted by cardiomyocytes through either autocrine or paracrine pathways [7]. Ghrelin confers abundant cardioprotective effects such as raising left ventricular ejection fraction, ameliorating myocardial remodeling, alleviating cardiac dysfunction [8–10], and inhibiting cardiomyocyte and endothelial cell apoptosis through the ERK1/2 and PI3-kinase/AKT signaling pathways [11,12], making it a promising treatment in HF and inspiring researchers to further investigate the mechanism behind these effects.

MicroRNAs (miRNAs), a group of small non-coding regulatory RNAs, have a unique structure called the “seed region” (2–7 or 2–8nt, from the 5’ end) that can recognize and pair to the 3’ untranslated region (UTR) of the target gene to repress mRNA transcription or trigger mRNA degradation [13–15]. Over 30% of mammalian functional genes are under the control of miRNAs through complementary ways [15,16], making them indispensable in the post-transcriptional regulation of cardiovascular diseases such as HF.

miR-208 is characterized by strict spatial, temporal, and chamber-specific expression patterns [17,18]. It has 2 subfamilies, miR-208a and miR-208b, which are encoded by Myh-6 and Myh-7 gene, respectively (4). As a cardiac-specific miRNA, miR-208 is required for cardiomyocyte hypertrophy and fibrosis in the first miRNA deletion animal model [19]. Abundant evidence proves that miR-208 is closely related to cardiac remodeling [17,18,20]. Although in previous studies miR-208 was always associated with hypertrophy, an increasing number of researchers have investigated its role in apoptosis. Some of these studies showed that miR-208a reduced cell apoptosis [21,22] while others found that silencing miR-208a could protect the heart from myocardial cell loss and dysfunction [23]. miR-208b downregulation was reported to alleviate ischemia/reperfusion(RR)-induced myocardial injury [24]. We found the miR-208 family significantly changed in the rat pathological model of postinfarction heart failure, indicating that the miR-208 family is probably involved in HF [25]. In conclusion, under different circumstances miR-208 may show opposite effects, which reflects the complexity of miRNA regulation and shows the need to explore the role of miR-208 in other cases such as under insult by angiotensin II, a key mediator of HF genesis and development. In clinical application, the miR-208 family is a sensitive biomarker for AMI and, with the correlation with myocardial enzyme, it can also reflect the state of diseases and the prognosis [26,27].

Renin-angiotensin system (RAS) activation is a contributing factor in many cardiovascular diseases, especially HF. In this study, as the core part of RAS, angiotensin II (Ang II) was used to establish H9c2 cell apoptosis model while ghrelin was given as a therapeutic option for apoptosis. qRT-PCR was performed to detect the mRNA level of miR-208 family. Additionally, miR-208 family mimics and inhibitors were transfected into H9c2 cells followed by treatment of Ang II and ghrelin to investigate their impact on the cardioprotective effect of ghrelin on Ang II-induced apoptosis. PCR array was performed to further explore the role of miR-208 family in apoptosis.

Material and Methods

Materials

The rat myocardial cell line H9c2 was obtained from ATCC (Manassas, VA, USA). Dulbecco’s Minimal Essential Medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone Laboratories (Logan, UT, USA). The annexin V – fluorescein isothiocyanate (FITC) apoptosis detection kit was from KeyGen Biotech (Nanjing, China). The FuGENE transfection reagent was purchased from Promega (Madison, WI, USA). PrimeScript™ RT reagent Kit and SYBR Premix Ex Taq™ (Tli RNaseH Plus) were provided by Takara (Kusatsu, Shiga, Japan). The Rat Apoptosis RT² Profiler™ PCR Array and other related reagents were from QIAGEN Bioinformatics (Redwood City, CA, USA). Ghrelin, Ang II, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl- tetrazolium bromide (MTT) were provided by Sigma-Aldrich (St Louis, MO, USA).

Cell Culture and treatments

The DMEM with 10% fetal bovine serum was used to culture H9c2 cells (the culture medium also contained 100 U/mL penicillin, and 100 mg/mL streptomycin). A 220-nanometer filter agent was used for the filtration. After culturing in a 5% CO₂/95% air humidified incubator at 37°C, the cells were digested and seeded in 6-well plates for the following experiments. Culture
Table 1. Primers used in quantitative RT-PCR.

| Primers                  | Sequence 5’→3’          |
|-------------------------|-------------------------|
| miR-208a RT primer      | CTCAACTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCTTTTTG |
| miR-208a forward primer | ACACCTCCAGCTGGGATAAGACGAGCA |
| miR-208a reverse primer | TGGTGTCGTGGAGTCGGCAATTCAGTTGAGATAACGCG |
| miR-208b RT primer      | CTAACCTGGTGTCGTGGAGTCGGCAATTCAGTTGAGGCTTTTTG |
| miR-208b forward primer | ACACCTCCAGCTGGGATAAGACGAGCA |
| miR-208b reverse primer | TGGTGTCGTGGAGTCGGCAATTCAGTTGAGATAACGCG |
| U6 forward primer       | GCTTCGAGAGATTTTGCTCAT    |
| U6 reverse primer       | CGCTTCAGAATTITGCTCAT    |

medium (control), ghrelin (0.1 μM), Ang II (0.1 μM), or both were added to the plates for 24 h, and total RNA was extracted afterwards.

**MTT method**

Cell viability was measured by MTT method, which is based on the transformation of exogenous MTT into an insoluble formazan crystal by active mitochondria. After culturing, the 96-well plates for 24 h, H9c2 cells were treated with Ang II for different lengths of times (from 0 h to 48 h) or Ang II and ghrelin of different concentration (from 0 nM to 1000 nM). Then, MTT (5 mg/mL) was added to the cells (2 μL per well) and incubated for 4 h. Finally, the culture medium was removed from the wells, followed by addition of dimethyl sulfoxide (150 μL per well) to resolve the crystals. After agitation at room temperature for 30 min, the absorbance at 490 nm of each well was measured by a spectrophotometer microplate reader. The viability of the treated cells was calculated as the percentage of the untreated control cells.

**Annexin V-FITC/podipidium iodide (PI) staining**

The H9c2 cells were harvested following the common digestion procedure, except for using Tryplete (Gibco, Grand Island, NY, USA) instead of trypsin. Then, the cell pellets were washed with PBS 3 times and resuspended by 500 μL binding buffer mixed with 5 μL annexin V-FITC and PI in the dark. Cell apoptosis was examined by flow cytometry within 1 h. The cells stained with both PI and annexin V were considered as late apoptosis.

**Transfection**

The miRNA mimics, inhibitors, and negative control (NC) were synthesized and labeled with 5-carboxyfluorescein (5-FAM) by Gene Pharma (Shanghai, China), and FuGENE HD reagents used for transfection were purchased from Promega (Madison, WI, USA). When the cells reached 80% confluence, the DNA and transfection reagents mentioned above were added into cells at the ratio of 1: 2.5. Fluorescence microscopy was used to preliminarily verify the transfection efficiency. Then, total RNA was extracted for qPCR to further confirm whether the miRNA level changed.

**RNA extraction and real-time quantitative PCR**

The total RNA was extracted using the RNeasy® Mini Kit (QIAGEN, Shanghai, China) followed by reversion into cDNA using the Takara RT reagent kit. Then, real-time quantitative PCR was carried out on the Stratagene Mx3005P Real-Time PCR System (Agilent Technologies) using SYBR Green. The PCR program including 3 phases. First, pre-degeneration phase, 1 cycle of 95°C for 10 min. Second, PCR phase, 40 cycles of 95°C for 30 s, and 60°C for 1 min. Third, melting curve phase, 1 cycle of 95°C for 1 min, 55°C for 30 s, and 95°C for 30 s. Primers used in qRT-PCR were synthesized by Sangon Biotech (Shanghai, China) and are listed in Table 1. The primer design is guided by stem-loop method (miRNA) and the original miRNA sequences were from miRBase (http://www.mirbase.org/). The mRNA relative expression level of the miR-208 family (normalized to U6) was calculated using ΔΔCt, method.

**RT² profiler PCR array**

Total RNA of cells transfected with miR-208 NC and miR-208 inhibitors were extracted and processed using the following procedures. First, the RNA samples were converted into first-strand cDNA using the RT² First-Strand Kit. Next, the cDNA was mixed with RT² SYBR Green Mastermix and aliquoted into the wells of the RT² Profiler Apoptosis PCR Array. The PCR program was the same as before and relative mRNA expression was determined using data from the real-time cycler and the ΔΔCt method. The RT² Profiler PCR Array Gene Expression Analysis Report was obtained from the QIAGEN analysis website (https://www.qiagen.com/cn/shop/genes-and-pathways/data-analysis-center-overview-page).
To testify the effect of ghrelin on Ang II-induced cell apoptosis, H9c2 cells were treated with different concentration of Ang II and the cell viability measured by MTT assay. (B) H9c2 cells were incubated with 100 nM Ang II from 0 h to 48 h. (C) H9c2 cells were treated with different concentration of Ghrelin, * P<0.05; ** P<0.01 versus control group (0 nM group or 0 h group) (n=3).

Results

Cell viability decreased notably after exposure to 100 nM Ang II for 24 h

To identify the best treatment time and concentration of Ang II, the cell viability of the H9c2 cells treated for different times (from 0 h to 48 h) and with different concentrations (from 0 nM to 1000 nM) of Ang II was measured by MTT method. The results showed that, compared with the control group (0 h group or 0 nM group), cell viability gradually decreased over time or the concentration increased and descended to its lowest point when exposed to 100 nM Ang II or for 24 h (Figure 1A, 1B). The viability of the cells treated with different concentrations of ghrelin was also identified. There was no significant difference between the groups, which means ghrelin had no effect on cell viability (Figure 1C). Thus, we chose the same concentration as that of Ang II for the next experiments.

Ghrelin inhibited Ang II-induced apoptosis of H9c2 cells

To testify the effect of ghrelin on Ang II-induced cell apoptosis, flow cytometry was performed to analyze the apoptotic percentage of H9c2 cells stained with annexin V FITC and propidium iodide (PI). As shown in Figure 2, compared with the control group, Ang II remarkably increased apoptotic cells from 4.97±0.30% to 14.69±0.54%, while this deleterious effect was partially inhibited by ghrelin treatment (8.65±0.28%).

Statistical analyses

All experiments were performed at least 3 times. The data are presented as mean± SEM.

Differences between multiple groups were analyzed by one-way ANOVA followed by Dunnett post-tests (comparing all groups with the control group) or Bonferroni post-tests (compared all pairs of groups). Difference between 2 groups were analyzed by the 2-tailed t test. A value of P<0.05 was considered as statistically significant.

The impact of Ang II and Ghrelin on the expression of miR-208 family

To investigate the mechanism underlying the fatal effect of Ang II and the protective role of ghrelin, the relative level of miR-208 family was detected by qRT-PCR to determine whether miRNA is involved. Data normalized to U6 were calculated by 2^-ΔΔCT method and shown in Fig. 3. Compared with the control group, the miR-208 family was significantly downregulated after exposure to Ang II, and this effect was reversed when simultaneously treated with ghrelin. Ghrelin itself also upregulated the levels of miR-208.

Overexpression of miR-208a alleviated Ang II-induced cell apoptosis and enhanced the cardioprotective effect of Ghrelin

The role of miR-208 family in Ang II-induced cell apoptosis and the cardioprotective effect of ghrelin is still unclear. To further explore this, H9c2 cells were transfected with miR-208a negative control (NC), mimics, and inhibitors (synthesized by Gene Pharma) followed by incubation in culture medium with 100nM Ang II with or without 100 nM ghrelin for 24 h. The transfection efficiency was examined after 12 h of transfection by fluorescence microscopy and qRT-PCR. Cells transfected successfully appear green (Figure 4Aa, 4Ac) and the level of miR-208a rises in the mimics group and drops in the inhibitors group (Figure 4Ab, 4Ad). Finally, the apoptotic percentage of H9c2 cells was calculated by flow cytometry. Compared with NC groups and mimics groups, all the inhibitors groups

Figure 1. The impact of Ang II and Ghrelin on cell viability. (A) H9c2 cells were treated with different concentration of Ang II and the cell viability was measured by MTT assay. (B) H9c2 cells were incubated with 100 nM Ang II from 0 h to 48 h. (C) H9c2 cells were treated with different concentration of Ghrelin. * P<0.05; ** P<0.01 versus control group (0 nM group or 0 h group) (n=3).
had significantly increased cell apoptosis, indicating the vital role of miR-208a in myocyte survival. Furthermore, overexpression of miR-208a observably inhibited apoptosis in the Ang II group and ghrelin-cotreated groups in comparison with the control groups (Figure 4B, 4C).

Overexpression of miR-208b suppressed Ang II-induced cell apoptosis

The role of miR-208b, as another member of the miR-208 family, was investigated in the same way as in the previous section. The transfection efficiency is shown in Figure 5A and the cell apoptosis is exhibited in Figure 5B, 5C. Similar results were obtained in the miR-208b inhibitors groups. Moreover, miR-208b mimics significantly reduced the apoptotic percentage of cells treated with Ang II when compared with NC groups.

Figure 2. Ang II-induced H9c2 cell apoptosis was inhibited by Ghrelin. (A, B) H9c2 cells incubated in culture medium (control), Ghrelin (100 nM), Ang II (100nM) or both for 24 hours were stained with Annexin V-FITC/propidium iodide (PI) and analyzed by flow cytometry. ** P<0.01 versus control group; ## P<0.01 versus Ang II group(n=3).

Figure 3. The expression of miR-208 family was regulated by AngII and Ghrelin (A, B). Cells were incubated with culture medium, Ang II with or without Ghrelin. After 24 h treatment, total RNA was extracted and reversed followed by qPCR. Primers used in this section were listed in Table 1. The relative mRNA levels of miR-208a and miR-208b were measured by Mx7500 Real-Time PCR System and normalized to U6. * P<0.05, ** P<0.01 versus control group (culture medium group); ## P<0.01 versus Ang II group(n=3).
Figure 4. The role of miR-208a in Ang II-induced apoptosis and the cardioprotective effect of Ghrelin. (A) MiR-208a negative control (NC), mimics and inhibitors with 5-carboxyfluorescein (5-FAM) were transfected into H9c2 cells by Fugene reagent. Whether the transfection is successful was detected using fluorescence microscope and confirmed by qRT-PCR. (B, C) After transfection for 24 hours, the cells were then treated with Ang II or Ang II+Ghrelin for another 24 hours. The percentage of apoptotic cells was measured by flow cytometry. * P<0.05, ** P<0.01 versus NC group; # P<0.05, ## P<0.01 versus mimics group (n=3).
**Figure 5.** The role of miR-208b in Ang II-induced apoptosis and the cardioprotective effect of Ghrelin. (A) H9c2 cells were transfected with FAM-labeled miR-208b NC, mimics and inhibitors. The transfection efficiency was confirmed followed the same procedure as above. (B, C) The apoptotic percentage was measured by flow cytometry after 48 h. * P<0.05, ** P<0.01 versus NC group; # P<0.05, ## P<0.01 versus mimics group (n=3).
MiR-208 family influenced multiple apoptosis pathways

In our study, downregulation of the miR-208 family, which also occurred when exposed to Ang II, notably induced H9c2 cell apoptosis. To reveal the specific mechanism of miR-208 in cardiomyocyte apoptosis, the Rat RT² Profiler PCR Array was used to determine which apoptosis signaling pathways and/or which apoptosis-related genes were affected by the miR-208 family. According to the analysis website provided by QIAGEN, inhibition of miR-208a upregulated the expression of Casp1, Casp3, Casp12, Fas, Fasl, Tp53bp2, Abl1, and Anxa5 and downregulated the expression of Mapk8ip1, Nfkβ1, and Sphk2. Inhibition of miR-208b upregulated the expression of Casp3, Casp4, Fas, Fasl, Cideb, and Gadd45a and downregulated the expression of Akt1, Mapk8ip1, Nfkβ1, and Sphk2 (Figure 6).

Discussion

Cardiac apoptosis, a vital part of cardiomyocyte loss, is the initial factor of MI and a key contributor to HF [5,28]. Ang II, which has been associated with regulating apoptosis and
cardiac remodeling [29], was used to induce H9c2 apoptosis. Ghrelin, a cardioprotective peptide hormone [8–10], was proved to inhibit Ang II-induced cell apoptosis by flow cytometry in this study. We sought to elucidate the mechanism underlying cardiomyocyte apoptosis and the protective effect of ghrelin.

With the rapid development of sequencing techniques, an increasing number of miRNAs are known to be involved in cardiovascular pathology by the differential miRNA expression pattern [30]. To date, miRNAs have been proved to regulate apoptosis, cardiac fibrosis, hypertrophy, cardiac electrophysiology, and many other aspects of cardiovascular diseases. In addition, miRNAs can serve as HF biomarkers to diagnose and predict the prognosis of HF [4,30–32]. In our previous study [25], we used high-throughput sequencing to develop a differential miRNA profile in the rat model of postinfarction heart failure. The detection was performed after 10 weeks of myocardial infarction when HF was stable. The results showed that the miR-208 family (including miR-208a and miR-208b) was highly changed in the pathological model and the 2 members of this family had different variation trends, indicating that miR-208a and miR-208b may play disparate or even opposite roles in heart failure, which needs to be clarified by further research.

miR-208, encoded by Myh-6 and Myh-7 genes, is a cardiac-specific microRNA. Callis et al. [17] and et al. [33] found that overexpression of miR-208a increased the Myh7 expression and hypertrophic growth and induced arrhythmias, while miR-208a knock-out mice presented defective cardiac conduction. Yu Kakimoto et al. [18] reported that the miR-208 family restrains the expression of Thrap1 and myostatin, and thus participates in the regulation of hypertrophy. It was also discovered that miR-208b attenuated post-MI fibrosis through targeting GATA4 [34]. Furthermore, in a hyperthyroidism-induced cardiac hypertrophy rat model, miR-208 was increased by thyroid hormone (TH) mediated by angiotensin II type 1 receptor (AT1R) [20]. Interestingly, in our previous study [35], we found that one of the anti-heart failure mechanisms of ghrelin was through downregulating AT1R. Despite previous relations with hypertrophy and fibrosis, miR-208 was recently found to be involved in cell apoptosis. Lingdong Meng et al. [21] showed that miR-208a reduced cardiomyocyte apoptosis through inhibiting the mitochondrial fission process, whereas Hasahaya Tony et al. [23] reported the opposite results, showing that silencing miR-208a could suppress Doxorubicin-induced myocardial apoptosis and dysfunction. Finally, we found that Luteolin protected the heart from IR insult through downregulation of miR-208b [24].

Based on the above evidence, we hypothesized that ghrelin could inhibit Ang II-induced myocyte apoptosis by regulating miR-208. To verify this, the relative levels of miR-208a and miR-208b were measured by qRT-PCR. In contrast to from previous high-throughput sequencing results, both miR-208 family member decreased when exposed to Ang II for 24 h, which may be due to the complexity of biological conditions and the detection time of the rat model. More in vivo research is in progress to further investigate the role of miR-208. In an in vitro study, miR-208a and miR-208b levels were upregulated by ghrelin and further raised during co-treatment, indicating that miR-208 was the target miRNAs of ghrelin, and this positive correlation was enhanced when cells were under Ang II insult. The mechanism underlying this combined effect and whether angiotensin II type 1 receptor is involved need more investigation. Since miR-208 might be involved in the protective effect of ghrelin on Ang II-induced apoptosis, miR-208 NC, mimics, and inhibitors were then transfected into H9c2 cells followed by administration of Ang II and ghrelin. The flow cytometry analysis showed that all the miR-208 inhibitors group gave rise to more apoptosis compared with the NC group, demonstrating that miR-208 is indispensable to cardiomyocytes. miR-208 mimics slightly suppressed apoptosis in culture medium-incubated group, which means that increase of miR-208 might have no effect on cell survival. Moreover, when cells were exposed to Ang II and/or treated with ghrelin, overexpression of miR-208a alleviated Ang II-induced cell apoptosis and enhanced the protective effect of ghrelin, the former of which was also seen in overexpression of miR-208b. Although miR-208b mimics reduced the apoptotic percentage in the Ang II+ghrelin group, the result showed no significant difference when compared with miR-208b NC. This indicates that miR-208b possibly acts indirectly in ghrelin’s protection of cells from Ang II insult.

Since miR-208 inhibitors induced H9c2 apoptosis, genes from the miR-208 NC group and miR-208 inhibitors group were compared by RT2 Profiler Apoptosis PCR Array. miR-208a mainly affected inflammatory caspases such as Casp1 and Casp12; caspase cascade reaction such as Casp3; Fas signaling pathway including Mapk8ip1; and NF-KAPPA B signaling pathway including NFKB1. miR-208b largely influenced caspase cascade reaction, Fas signaling pathway, MAPK signaling pathway, NF-KAPPA B signaling pathway, and Akt1 expression. miR-208a also downregulated the expression of Akt1 (fold regulation=–1.57). Akt1 is an important molecule in the PI3K/AKT signaling pathway through which ghrelin prevents against cardiomyocyte death. This gives us the possibility of exploring the relationship of miR-208 with the PI3K/AKT pathway in Ang II-induced cell apoptosis. Further studies are needed to investigate whether other pathways are involved in the protective effect of ghrelin and the target gene of miR-208 in cardiomyocyte apoptosis.
Conclusions

The newly discovered miR-208 family is regulated by Ang II and ghrelin. Overexpression of the miR-208 family alleviates cell apoptosis when exposed to Ang II, and miR-208a assists in the cardioprotective effect of ghrelin. Several apoptosis pathways are affected by the miR-208 family. Our findings suggest the pathogenesis of cardiomyocyte apoptosis and the protective mechanism of ghrelin, which may help in treatment of cardiovascular diseases.

Conflicts of interest

None.

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