

Original Article

α -Klotho is an acute phase protein and altered by restraint stress in mice

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Abstract: Objective: To identify whether α -Klotho is a kind of acute phase protein and alternation in the level of protein and mRNA under restraint stress. Method: 48 mice were divided into three groups of 16: 1h, 10h and 20h treat time group. Each group included restraint stress and control subgroup with same number animals (n = 8). ELISA was utilized in the assay of serum α -Klotho and corticosterone, RT-PCR was occupied in the detection of the expression of α -Klotho mRNA in renal tissue. Result: α -Klotho protein concentration of control group 1h, 10h and 20h was 757.71 ± 333.93 pg/ml, 687.38 ± 342.79 pg/ml and 912.90 ± 337.8 pg/ml, respectively. While the concentration of restraint stress group 1h, 10h and 20h was 726.40 ± 342.79 pg/ml, 1261.54 ± 442.71 pg/ml, and 1696.18 ± 404.11 pg/ml. There was no significant difference among 1h, 10h and 20h control groups ($P > 0.05$) as well as 1h treat time subgroup. Compared with respective control group, the difference of restraint stress group in 10h and 20h treat time group was significant ($P < 0.05$). The expression of α -Klotho mRNA was slightly downregulated even when the mice underwent a 1 hour restraint stress, though without significance ($p > 0.05$). Prominent fold change, 2.02 and 2.46, happened in 10h and 20h restraint group with significance ($P < 0.05$ and $P < 0.01$), respectively. Conclusion: α -Klotho is a kind of acute phase protein. The serum α -Klotho protein is promoted while the α -Klotho mRNA is downregulated under the constraint stress.

Keywords: α -Klotho, acute phase protein (APP), corticosterone, restraint stress

Introduction

α -Klotho was identified as an anti-aging gene in 1997 [1]. From then on, lots of researches have been done to clarify its function. Now it is generally known that membrane α -Klotho protein is a coreceptor of FGF23 and the other type, soluble α -Klotho independent of FGF23 considered as an endocrine hormone [2, 3]. On the year 2004, Takeshita etc conducted a research on the α -Klotho knockout mouse with restraint stress. The result shows that α -Klotho is indispensable for the normal function of sinoatrial node because of the reality that α -Klotho knockout mouse died of sinus block or arrest [4]. Nevertheless, the mechanism underlying remains unclear up to now. On the basis of that study, we reckon that, for the wild type mouse, it is the stress that alters the concentration of soluble or membrane α -Klotho, then with the change of α -Klotho, a waterfall alternation happened in the downstream signal transduction

to neutralize the adverse change in internal environment. But for the α -Klotho deficiency mouse, lack of this benignant effect to alleviate the deadly change, then the mice died because of sick sinus syndrome [5, 6]. So the prerequisite of the hypothesis is that stress changes the concentration of soluble or membrane α -Klotho at the level of protein or mRNA under restraint stress. This study is to identify whether α -Klotho alters under the restraint stress.

Materials and methods

All animal experiments were conducted according to the guidelines of the Chinese law for the welfare of animals and were approved by local authorities.

Animals and tissue sampling

The experiment was conducted on 48 Five-week-old male C57BL6/J mice (~17 g, provided

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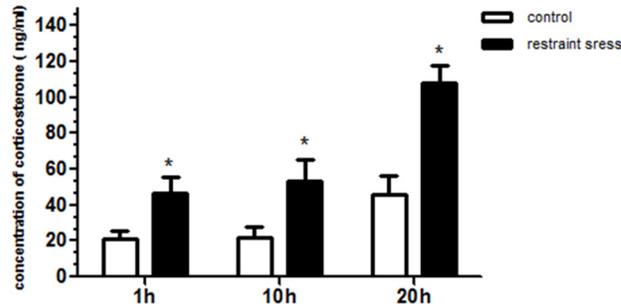


Figure 1. Concentration of corticosterone in different treat time groups. * $p < 0.05$ vs. control in each group.

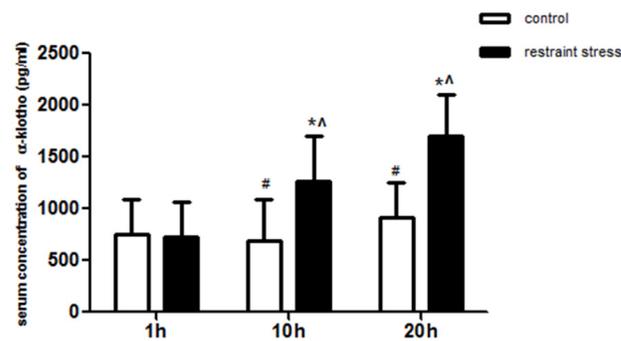


Figure 2. Concentration of α -klotho in different groups. * $p < 0.05$ vs. control in the group. [#] $p < 0.05$ vs. 1h restraint stress group. # $p > 0.05$ vs. 1h control group.

by animal center of Xi'an Jiaotong University, Xi'an, China). The mice were randomly divided into six groups and housed in a group of 8 in a well air-conditioned room ($16 \pm 1^\circ\text{C}$, $55 \pm 5\%$ humidity) under a 12-h light/12-h dark cycle, with the light on from 07:00 to 19:00. The mice were given food and water ad libitum. All the mice were acclimated for 1 week before initiation of restraint stress at six weeks (~20 g). During the restraint period (20h group: from noon on the first day to 8 AM on the second day; 10h group: from 8 AM to 6 PM; 1h group: from noon to 1 PM), all the mice were away from water and food.

Right after the restraint stress, blood plasm samples were collected from the supra orbital veins within 2 minutes and then centrifuged (Eppendorf 5415R centrifuge, NY, U.S.A) with 1500 g at 4°C for 15 minutes. At last the supernatant was collected and stored at -20°C for later use.

Immediately, after the blood sampling, the animals were killed by exsanguination under

anaesthesia, then both of the two kidney were excised and washed by normal saline before snapp frozen in liquid nitrogen and storing at -80°C for subsequent analysis.

Restraint stress

All the mice were secured in well-ventilated, horizontal, 50-mL conical centrifuge tubes away from food and water [7].

Quantitative RT-PCR

Total RNA was isolated from mouse kidney by using the RNAiso plus (Takara Biotechnology, Dalian, China). Reverse transcription of 0.8 μg RNA was performed using Prime Script TM RT reagent Kit with gDNA Eraser (Takara Biotechnology, Dalian, China). The cDNA fragment contains the internal splicing site in exon 3 of the kl gene. To determine Klotho mRNA transcript levels, quantitative real-time PCR with the BioRad iCycler iQ5TM Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) was applied using the following primers: α -Klotho primer1: 5'-CGCAAAGTGCTCAACTGGCTAA-3'; α -Klotho primer2: 5'-AAGGTTGATGTCGT-CCAACACGTA-3'. Gapdh primer1: 5'-TGT-GTCCGTCGTGGATCTGA-3'; Gapdh primer2: 5'-TTGCTGTTGAAGTCGCAGGAG-3'.

PCR reactions were performed in a final volume of 25 μl containing: 2 μl cDNA, 0.4 mM of each primer, 10 μl SYBR Premix Ex TaqTM II (Tli RNaseH Plus) (Takara Biotechnology, Dalian, China) and sterile water up to 25 μl . qPCR conditions were 95°C for 30 s, followed by 40 cycles of 95°C for 5 s and 60°C for 30 s. The specificity of the PCR products was confirmed by analysis of a melting curve and in addition with a 4% agarose gel. Calculated mRNA expression levels were normalized to the expression levels of GAPDH of the same cDNA sample. Relative quantification of gene expression was performed using the delta delta CT method.

Serum α -Klotho and corticosterone determination

Serum Klotho and corticosterone level was determined with the enzyme-linked immunosorbent assay kit (USCN, Wuhan, China). The

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Table 1. The expression of α-Klotho mRNA in different groups

group	ΔCT ₁ (kl ^a -GAPDH)	ΔCT ₂ (kl ^b -GAPDH)	ΔΔCT (ΔCT ₁ -ΔCT ₂)	Expression decreased in restraint stress group (folds)
1h	4.91 ± 0.38	4.74 ± 0.50	0.17	1.13
10h	6.58 ± 0.20	5.77 ± 0.30	1.01	2.02
20h	6.59 ± 0.59	5.29 ± 0.36	1.30	2.46

GAPDH served as the loading control and the relative cycle of threshold (ΔCt) in the restraint stress group (kl^a) and control group (kl^b) were obtained respectively. The relative expressions between restraint stress and control group were calculated by the comparative Ct (ΔΔCt) method, and then, the relative expression levels ($2^{-\Delta\Delta Ct}$) were calculated. ΔΔCt = ΔCt (kl^a-GAPDH)-ΔCt (kl^b-GAPDH). ΔCt results are the Mean ± SE.

optical density was measured with spectrophotometer (FLUOstar OPTIMA, Offenburg, Germany) at a wavelength of 450 nm ± 10 nm. The concentration of Klotho and corticosterone in the samples was then determined by comparing the O.D. of the samples with the standard curve.

Statistical analysis

Statistical analysis was computed with SPSS for Windows Release 19.0 (SPSS, Chicago, IL). The mRNA levels presented in this article are the mean ± SE unless otherwise specified. Statistical significance was evaluated by student's *t*-test and ANOVA when appropriate. We repeated the experiments three times before taking an average Ct, and then calculated the target gene expression relative to the *GAPDH* gene by calculating the difference between the Ct of the target gene and the Ct of the house-keeping gene. $2^{-\Delta\Delta Ct}$ formula was used in calculating relative expression. A probability value *P* < 0.05 was considered statistically significant.

Results

To determine the effect of stress on the serum α-Klotho protein and alternations happened at the gene level, we use a typical stress model-restraint stress, which is a kind of most commonly used neural stimulation and pure frustration stress model [8].

To evaluate the stress model, corticosterone which is the primary stress hormone in rodents [9], was measured in both restraint stress and control group. Comparing with controls, if there is a two to three fold change happens then the model is meeting the requirements [10]. The following **Figure 1** is the concentration of corticosterone in each group, respectively. To get rid of the effect of Circadian Rhythm of Cor-

ticosterone in Mice, control group was set. The distinguishing experimental conditions between restraint and control group was just restraint stress. Though the concentration of each group is lower than the existing study [11], the fold change is significant. Concentration of all the restraint stress groups (1h, 10h and 20h) was 2.03, 2.52, and 2.34 times compared with its control group (*p* < 0.05), respectively.

Restraint stress increases the serum α-Klotho protein

Based on the estimate of animal model, serum α-Klotho protein was measured with the same mice blood samples. There remains no consolidated point of view on the concentration of serum α-Klotho protein, as different study has different data [12]. Partial data in our study is accordance with the existing literature 10~50 Nm [13]. α-Klotho concentration of control group 1h, 10h and 20h was 757.71 ± 333.93 pg/ml, 687.38 ± 342.79 pg/ml and 912.90 ± 337.8 pg/ml, respectively. While the concentration of restraint stress group 1h, 10h and 20h was 726.40 ± 342.79 pg/ml, 1261.54 ± 442.71 pg/ml, and 1696.18 ± 404.11 pg/ml. There was no significant difference among 1h, 10h and 20h control groups, though the concentration of 20h group was little higher than the other two. There was no statistic difference between 1h restraint stress and control group, while it is not the case in the other two groups (10h and 20h). Compared with the 1h restraint stress group, striking significant difference has been shown in **Figure 2**.

α-Klotho mRNA is downregulated by the restraint stress

As is shown in the **Table 1** and **Figure 3**, the expression of α-Klotho mRNA is slightly downregulated even when the mice undergo a 1 hourrestraint stress, though without significance (*p* > 0.05). Prominent fold changes, 2.02 and 2.46, happen in 10h and 20h restraint group, respectively. The specificity of the PCR amplification product verified by 4% agarose gel, the results are shown in **Figure 4**.

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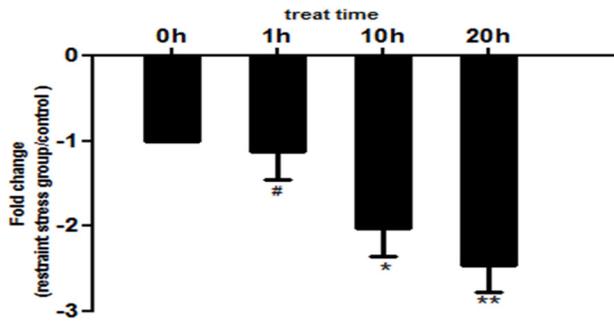


Figure 3. Expression of α-klotho mRNA in different treat time groups determined by qRT-PCR. The y-axis represents the relative mRNA expression level. The average value of mRNA expression in control group is designated -1 (0 h), and the level of mRNA expression in restraint stress group is calibrated to obtain the folds change. #p > 0.05 vs. 0h group; *p < 0.01 vs. 0 h group.

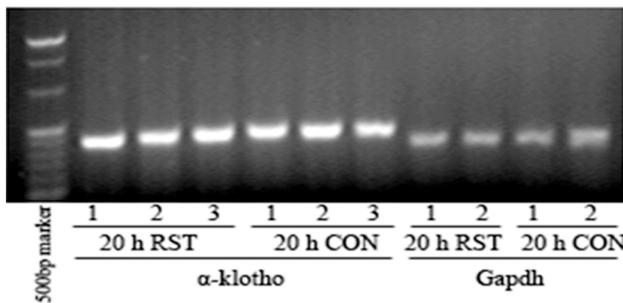


Figure 4. Identification of the amplification fragments of qrt-pcr products. A 4% agarose gel was used in the study. RST: Restraint stress group; CON: Control group; The amplification fragment of klotho and gapdh is 171 bp and 150 bp, respectively.

Discussion

Our study reveals that α-Klotho is a kind of acute phase protein, for acute restraint stress induces an increase of the serum concentration. While it is contrary to our expectation, as decrease happened in the expression of α-Klotho mRNA. The underlying mechanism of the seemingly contradictory result should be as follows: 1) there exists two producing way of soluble α-Klotho protein, one is the mRNA alternative splicing way, and the other is ectodomain shedding of the membrane α-Klotho [14, 15]. So if there is only the former producing way, the change should be unanimous. That is to say when the serum protein is up, the expression of mRNA should also increase. However, butting in of the other way, makes it complicated. If restraint stress has a binary effect: down-regulation of the expression of α-Klotho mRNA together with the increasing of shedding of the

membrane α-Klotho protein, the contradictory result above could be reality. 2) According to literature data, FGF-23 may increase α-Klotho in blood and directly or indirectly suppress α-Klotho mRNA expression [16]. So the further study should elucidate whether the binary effect of restraint stress is true and what the upstream regulators of α-Klotho shedding are. Stress function through hypothalamic-pituitary-adrenal axis, the sympatho-adrenomedullary axis and the opioid system. Takeshita and his colleagues' research shows striking increase of Plasma norepinephrine in wild type mouse under restraint stress and a slightly decrease in Plasma norepinephrine of α-Klotho knockout mouse [4]. A great many of literatures focused on the relationship between restraint stress and corticosterone have reported notable increase of corticosterone, the primary stress hormone in rodents [11, 17, 18]. So more research should put on whether there is certain relation between the alteration of α-Klotho and this stress hormone. In conclusion α-Klotho is a kind of acute phase protein and altered by restraint stress, even though the mechanism remains unknown and many interesting problems should be settled.

Acknowledgements

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Disclosure of conflict of interest

None.

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References

- [1] Kuro-o M, Matsumura Y, Aizawa H, Kawaguchi H, Suga T, Utsugi T, Ohyama Y, Kurabayashi M, Kaname T, Kume E, Iwasaki H, Iida A, Shirakiiida T, Nishikawa S, Nagai R, Nabeshima YI. Mutation of the mouse Klotho gene leads to a syndrome resembling ageing. *Nature* 1997; 390: 45-51.
- [2] Urakawa I, Yamazaki Y, Shimada T, Iijima K, Hasegawa H, Okawa K, Fujita T, Fukumoto S,

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- Yamashita T. Klotho converts canonical FGF receptor into a specific receptor for FGF23. *Nature* 2006; 444: 770-774.
- [3] Ohyama Y, Kurabayashi M, Masuda H, Nakamura T, Aihara Y, Kaname T, Suga T, Arai M, Aizawa H, Matsumura Y, Kuro-o M, Nabeshima Yi, Nagail R. Molecular cloning of rat Klotho cDNA: markedly decreased expression of Klotho by acute inflammatory stress. *Biochem Biophys Res Commun* 1998; 251: 920-925.
- [4] Takeshita K, Fujimori T, Kurotaki Y, Honjo H, Tsujikawa H, Yasui K, Lee JK, Kamiya K, Kitai-chi K, Yamamoto K, Ito M, Kondo T, Iino S, Inden Y, Hirai M, Murohara T, Kodama I, Nabeshima Y. Sinoatrial node dysfunction and early unexpected death of mice with a defect of Klotho gene expression. *Circulation* 2004; 109: 1776-1782.
- [5] Rubenstein JJ, Schulman CL, Yurchak PM, De-Sanctis RW. Clinical Spectrum of the Sick Sinus Syndrome. *Circulation* 1972; 46: 5-13.
- [6] Watt AH. Sick sinus syndrome: an adenosine-mediated disease. *Lancet* 1985; 325: 786-788.
- [7] Yamamoto K, Takeshita K, Shimokawa T, Yi H, Isobe K, Loskutoff DJ, Saito H. Plasminogen activator inhibitor-1 is a major stress-regulated gene: implications for stress-induced thrombosis in aged individuals. *Proc Natl Acad Sci U S A* 2002; 99: 890-895.
- [8] Posevitz V, Vizler C, Benyhe S, Duda E, Borsodi A. Restraint stress and anti-tumor immune response in mice. *Acta Biol Hung* 2003; 54: 167-176.
- [9] Hardy MP, Gao HB, Dong Q, Ge R, Wang Q, Chai WR, Feng X, Sottas C. Stress hormone and male reproductive function. *Cell Tissue Res* 2005; 322: 147-153.
- [10] Horan MP, Quan N, Subramanian SV, Strauch AR, Gajendrareddy PK, Marucha PT. Impaired wound contraction and delayed myofibroblast differentiation in restraint-stressed mice. *Brain Behav Immun* 2005; 19: 207-216.
- [11] Malisch JL, Saltzman W, Gomes FR, Rezende EL, Jeske DR, Garland T Jr. Baseline and stress-induced plasma corticosterone concentrations of mice selectively bred for high voluntary wheel running. *Physiol Biochem Zool* 2007; 80: 146-156.
- [12] Massy TB, Massy ZA. Circulating Klotho levels clinical: clinical relevance and relationship with tissue Klotho expression. *Kidney Int* 2013; 83: 13-15.
- [13] Bloch L, Sineshchekova O, Reichenbach D, Reiss K, Saftig P, Kuro-o M, Kaether C. Klotho is a substrate for alpha-, beta- and gamma-secretase. *FEBS Lett* 2009; 583: 3221-3224.
- [14] Matsumura Y, Aizawa H, Shiraki-Iida T, Nagai R, Kuro-o M, Nabeshima Y. Identification of the human Klotho gene and its two transcripts encoding membrane and secreted Klotho protein. *Biochem Biophys Res Commun* 1998; 242: 626-630.
- [15] Imura A, Iwano A, Tohyama O, Tsuji Y, Nozaki K, Hashimoto N, Fujimori T, Nabeshima Y. Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane. *FEBS Lett* 2004; 565: 143-147.
- [16] Marsell R, Krajisnik T, Goransson H, Ohlsson C, Ljunggren O, Larsson TE, Jonsson KB. Gene expression analysis of kidneys from transgenic mice expressing fibroblast growth factor-23. *Nephrol Dial Transplant* 2008; 23: 827-833.
- [17] Burton CL, Chatterjee D, Chatterjee-Chakraborty M, Lovic V, Grella SL, Steiner M, Fleming AS. Prenatal restraint stress and motherless rearing disrupts expression of plasticity markers and stress-induced corticosterone release in adult female Sprague-Dawley rats. *Brain Res* 2007; 1158: 28-38.
- [18] Flores CM, Hernandez MC, Hargreaves KM, Bayer BM. Restraint stress-induced elevations in plasma corticosterone and beta-endorphin are not accompanied by alterations in immune function. *J Neuroimmunol* 1990; 28: 219-225.