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Ursolic acid regulates aging process through enhancing of metabolic sensor proteins level

Soroush Alaghehband Bahrami, Nuredin Bakhtiari*

Department of Biochemistry, Faculty of Basic Sciences, Sanandaj Branch, Islamic Azad University, Sanandaj, Iran

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ABSTRACT

We previously reported that Ursolic Acid (UA) ameliorates skeletal muscle performance through satellite cells proliferation and cellular energy status. In studying the potential role of the hypothalamus in aging, we developed a strategy to pursue UA effects on the hypothalamus anti-aging proteins such as; SIRT1, SIRT6, PGC-1β and α-Klotho.

In this study, we used a model of aging animals (C57BL/6). UA dissolved in Corn oil (20 mg/ml) and then administrated (200 mg/Kg i.p injection) to mice, twice daily for 7 days. After treatment times, the mice *perfused* and the hypothalamus isolated for preparing of tissue to Immunofluorescence microscopy.

The data illustrated that UA significantly increased SIRT1 (~3.5 ± 0.3 folds) and SIRT-6 (~1.5 ± 0.2 folds) proteins *overexpression* ($P < 0.001$). In addition, our results showed that UA enhanced α-Klotho (~3.3 ± 0.3) and PGC-1β (~2.6 ± 0.2 folds) proteins levels ($P < 0.01$). In this study, data were analyzed using SPSS 16 (ANOVA test).

To the best of our knowledge, it seems that UA through enhancing of anti-aging biomarkers (SIRT1 and SIRT6) and PGC-1β in hypothalamus regulates aging-process and attenuates mitochondrial-related diseases. In regard to the key role of α-Klotho in aging, our data indicate that UA may be on the horizon to forestall diseases of aging.

1. Introduction

Ageing is characterized by the gradual and overall loss of various physiological functions, leading to the end of lifespan [1]. Researchers have now uncovered an area in the brain about the size of an almond in humans that wields powerful control over the body's aging process [2]. The hypothalamus is a critical anatomical site in which cells monitor changes in energy status of the body and trigger responses aimed at maintaining metabolic homeostasis as well as controls a number of hormones that influence development, growth, metabolism and reproduction [3]. Previous research has also shown that an unhealthy hypothalamus can lead to disorders associated with aging such as glucose intolerance and hypertension [1]. It has been reported that many aspects of aging are controlled by the hypothalamus. They excitingly declared that, it in addition to the increase longevity, enhancing of Sirt1 protein levels might also one day be a strategy to combat neurodegenerative diseases associated with aging [4].

With regard to the role of small molecules in enhancing of sirtuin activities, including Caloric Restriction (CR) mimetics and NAD^+ derivatives, which promising strategies to ameliorate age-related diseases [5]. Hence, according to our previous study; UA, a triterpenoid compound which found in apple peels and has many biological effects such as; anti-diabetic, anti-inflammatory, anti-HIV and hepatoprotective [6], led to decrease in cellular energy status, up-regulating of Sirt1 and PGC-1α genes *overexpression* [7]. Furthermore, rejuvenating effects of UA on the skeletal muscle tissue through satellite cells proliferation, enhancing of myoglobin protein level, switching of fiber typing toward fast-oxidative IIA and neomyogenesis [7] made a new avenue to appraise metabolic sensor proteins as like as Sirt1 and Sirt-6 in hypothalamus. Besides, based on the critical role of hypothalamus in maintaining of energy homeostasis and crosstalk between peripheral organs and the central nervous system and because of Glucose, lipids and amino acids are metabolized in the mitochondria and defects in mitochondrial function may contribute to nutrient over load causing cellular damage as typically associated with obesity, insulin resistance, cardiovascular disease, neurodegeneration or accelerated aging [8]. Accordingly, we devised an experiment to evaluate Peroxisome-proliferator-activated-receptor γ coactivator

* Corresponding author.
E-mail address: Nuredin.bakhtiary@modares.ac.ir (N. Bakhtiari).

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1 beta (PGC-1β) which regulates mitochondrial biogenesis and function through its coactivating effects on specific nuclear receptors [9]. Finally, we examined an anti-aging hormone as well, α-Klotho, which restrains the aging-like phenotypes [10].

Consequently, in this survey we used aged-mice C57BL/6 and evaluated UA on the metabolic sensor proteins. Due to the prominent role of hypothalamus in managing of aging process, our decision was to appraise the anti-aging biomarkers with Immunofluorescence (IF) microscopy, a robust and broadly applicable method generally used by researchers to assess both the localization and endogenous expression levels of proteins of interest.

2. Material and methods

2.1. Material

UA was purchased from SIGMA (U6753) with high purity (≥90%). Antibodies specific for Sirt1(Ab110304), Sirt-6 (S4322), PGC-1β (Ab176328) and α-Klotho (MAB1819) were provided from Santa Cruz Biotechnology (santa cruz) and Abcam. Goat- anti Rabbit FITC (Ab6717), donkey-anti Rabbit (SC-2095), Goat-anti mouse FITC (Ab97022) and Goat-anti mouse (ab6787) were purchased from Santa Cruz Biotechnology (santa cruz) and Abcam. Paraformaldehyde, Triton X-100, DAPI, Tris-HCl, NaCl were purchased from Sigma Aldich Company and all other chemicals were purchased from Merck Company.

2.2. Methods

2.2.1. Animal study

In this research, we used 20 inbred males, aged-mice C5BL/6 (20 months old) prepared from Iranian Institute Pasteur. Within 3 weeks of onset treatment, the mice housed in the colony cages with 12 h light/12 h dark cycles to adopt the new condition, and kept on standard chow (Harlan Teklad formula 7013). UA was dissolved in Corn Oil (20 mg/ml) and administrated 200 mg/kg intra-peritoneal injection (i.p) [11]. The mice were classified into 3 groups, one group was received UA, another just obtained placebo (Corn Oil) and last as a group control which received distill water. UA was administrated twice daily for 7 days [12]. Finally, for Immunofluorescence (IF) and histochemical tests, all animals were weighted and anaesthetized by an i.p injection of ketamine/xylazine and then fixed with 4% paraformaldehyde and 2.5% glutaraldehyde. Moreover, all animal procedures were approved by the Institutional Animal Care and Committee of the Endocrinology and Metabolism Research Center, Tehran University of Medical Sciences.

2.2.2. Tissue preparing for immunofluorescence test

After anaesthetizing and perfusing of mice, the hypothalamus was properly isolated and in order to fix them, the tissue immersed in 4% paraformaldehyde and 2.5% glutaraldehyde overnight [13]. Then, the samples were impregnated with 30% sucrose and stored in 4 °C for IF tests. Serial cross-sections (8 μm thicknesses) were performed using a cryostat microtome at −25 °C, mounted onto the glass slides, and then stained for cytoplasm and nucleus detection. In this experiment, prior to staining, slides should be blocked in room temperature for 10 min. Next, the cytoplasm staining performed by Hematoxyline for 5 min, washed with PBS and water, the nuclei staining performed by Eosine for 5 min and washed with PBS and water, dehydrated by descending alcohol, then mounted onto glass slides and they were visualized using a bright-field microscope (Nikon, TE2000-S) and captured by camera (TCH-1.4CICE).

2.2.3. Immunofluorescence microscopy

To identify the mentioned anti-aging proteins in the hypothalamus tissue, at first, cryosection slides were dehydrated in Room Temperature (RT) for 10 min. Then, the slides were embedded in PBS for 10 min (rehydration), and exposed in HCl (Normal) for 20 min. After that, it was replaced by Borate Buffer for 5 min. Next, they were washed by PBS (2 × 5 min). To evaluate nucleus antigen, tissue were permeable by Triton X-100 (3% in PBS) for 30 min and then washed by PBS (2 × 5 min). In the next step, the semi prepared tissues were blocked by goat-serum (500 μl goat-serum in 4.5 cc PBS) for 45 min in RT. Addition of first antibodies were based on optimized protocol in 4 °C overnight. Likewise, SIRT1 (1–1000 diluted in blocking buffer), SIRT6 (2–4 μg/ml diluted in blocking buffer), α-Klotho(8-25 μg/ml diluted in blocking buffer) and PGC-1β(1–1000 diluted in blocking buffer). Then, the slides were washed in PBS (2 × 5 min). Following, the secondary antibodies were included for 2 h in 37 °C, FITC-conjugated goat anti-mouse IgG1 (1–500 diluted in blocking buffer) and FITC-conjugated goat anti-rabbit IgG1 (1–1000 diluted in blocking buffer). Next, the slides were washed in PBS (2 × 5 min). In the last step, to identify tissue nucleus, 50 μl of DAPI (4′, 6-diamidino-2-phenylindole) which diluted in PBS was included to each slide for 2 min in dark, and then removed from tissue surface and washed by PBS (2 × 5 min) [14]. Finally, the slides were embedding in PBS visualized by bright-field microscope (Nikon, TE2000-S) and captured by camera (TCH-1.4CICE) and the images were analyzed with the LSM 510 image browser software.

2.3. Statistical analysis

The results of this study were analyzed by one a way ANOWA test. Each experiment was done at least three times, and the data are presented as the mean ± SEM, where applicable.

3. Results

3.1. UA enhanced metabolic sensor proteins (SIRT1 and SIRT6) levels in aged-mice hypothalamus

Based on our previous studies about rejuvenation effects of UA in the mice skeletal muscle, we promoted to more confirm this phenomenon in the aged mice hypothalamus. Therefore, we planned a new strategy to evaluate anti-aging biomarkers (SIRT1 and Sirt6). Interestingly, as shown in Figs. 1 and 2 the results illustrated that UA significantly increased SIRT1 (∼3.5 ± 0.3) and SIRT6 (∼1.5 ± 0.2) proteins level, $p < 0.001$.

3.2. UA Up-regulated PGC-1β protein in Hypothalamus

With regarding to the key roles of mitochondrial in regulation of aging and with respect to the age-associated diseases in related to the mitochondrial dysfunction. In this study, our thought was to examine the main factor which regulates aging-process. For this reason, we evaluated PGC-1β in hypothalamus. Surprisingly, our data showed that UA remarkably increased PGC-1β (∼2.6 ± 0.2) protein level (Fig. 3), $p < 0.001$.

3.3. UA resulted in α-Klotho Protein Overexpression in Hypothalamus

Since some neuroproductive factors prevent from developing of many neurodegenerative disorders including Alzheimers, Parkinson and Amyotrophic lateral sclerosis (ALS), we induced to know whether UA might regulate α-Klotho in aged-mice hypothalamus as well. Obviously, our findings demonstrated that UA enhanced α-Klotho (∼3.3 ± 0.3) protein level in comparison with control mice (Fig. 4), $p < 0.01$.