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Elucidating the Anti-aging Ursolic Acid through Evaluation of SIRT1 and SIRT6 in the Mice Skeletal Muscle Tissue

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ABSTRACT

The process of skeletal muscle aging is linked to the development of age-related diseases such as metabolic syndrome, diabetes, cancer and Alzheimer's disease. Hence, to further investigate the effect of Ursolic Acid (UA) endorses previous studies on skeletal muscle rejuvenation, we tested whether UA alters two prominent anti-aging biomarkers such as SIRT1 and SIRT6 in skeletal muscle. UA is a triterpenoid compound widely found in natural plants and fruits, with known effects on skeletal muscle. To conduct this experiment, UA dissolved in Corn Oil (200 mg ml^{-1}) was administrated (200 mg kg^{-1} IP injection) twice daily for 7 days to male aged-mice C57BL/6. Then, mice skeletal muscle tissues were isolated and proteins were examined through immunofluorescence microscopy. Our findings clearly illustrated that UA enhances SIRT1 (~3.5 folds) and SIRT6 (~4 folds) expression levels, $p < 0.001$. To the best of our knowledge, UA seems to imitate the effects of exercise by increasing the SIRT1 and SIRT6 proteins level in the skeletal muscle tissue. Finally, UA can be recommended as a superior candidate to improve the diseases associated with skeletal muscle aging.

Keywords: Ursolic acid, Skeletal muscle, SIRT1, SIRT6

INTRODUCTION

Aging is more perceptible in non-proliferating cells, like muscle and nerve cells, which have been arrested in the G° phase [1]. Skeletal muscle tissue forms 40-50% of the body. After the age 25, 3-10% of the skeletal muscle mass decreases every 10 years, which significantly affects the quality of life [2]. Epidemiological observations in humans indicate that aging in skeletal muscle is a risk factor in the development of several age-related diseases, such as metabolic syndrome, cancer, Alzheimer's disease, Parkinson's disease and *etc.* [3]. There are several metabolic directions that control longevity in mammals, for instance: insulin/insulin-like growth factor (IGF-1), tuberous sclerosis complex (TSC)/mammalian target of rapamycin (mTOR) and the sirtuins [4-8]. Many studies have shown that sirtuins are known to be a strong aging regulator compared with other regulatory factors, ranging from single cell organisms to mammals [7]. Sirtuins are highly conserved NAD^+-dependent protein deacetylases. There are seven mammalian sirtuins, SIRT1-7, which regulate metabolisms in most tissues [9]. Recent studies have proven that sirtuins are anti-aging proteins that are sensitive to diet [10]. Previous studies have shown that Caloric Restriction(CR) [11], resveratrol [12], NAD^+ derivatives [13], fasting [14] and exercise [15] increase the activity of the sirtuins. Since the modulation of sirtuins can beneficially effects the age-related diseases, there are more interest to modify the activity of the sirtuins by discovering small molecules. In recent studies, Ursolic Acid (UA) has been identified as a sirtuins activator. UA is a 5-cyclic triterpenoids [16] found in fruits such as apple (mostly apple peel), cranberry, grape skin, plum, blueberry lavender, lemon balm, hawthorn, and in plants such as rosemary, thyme, basil, and mint [17]. UA has protective effects on body organs such as brain [18,19], skeletal muscle [20,21], liver [22,23], heart [24,25], kidneys [26,27] and *etc.* also UA has other pharmacological activities, such as anti-cancer effects in different body organs [28-30], anti-inflammatory [31,32], anti-diabetic [33,

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34], anti-bacterial [35-37], anti-fungal [38,39], anti-HIV [40,41], anti-HPV [42,43], anti-hepatitis B and C [44,45], anti-aging [46,47] and also leads to increase the skeletal muscle hypertrophy and reduced skeletal muscle atrophy [21,48]. Hence, according to our previous studies, UA results in up-regulation of PGC1-α and SIRT1 genes overexpression, decrease cellular energy status, myoglobin overexpression, replace the type of muscle fiber into oxidative [49,50], induce neomyogenesis through hyperplasia and increase of satellite cells number in mice skeletal muscle [50,51]. These findings provide a new insight for the evaluation of sirtuins in skeletal muscle tissue. Among the 7 sirtuins in mammals, the effect of SIRT1 on extending lifespan has been reported [52,53]. SIRT1 has effects such as tumor suppressor [54], positive regulation on insulin secretion [55], maintaining lipid homeostasis [55], controls mitochondria biogenesis [56], neuroprotective actions on mouse models of the Parkinson's disease and the Huntington's disease [57-59], and anti-inflammatory action [60,61]. Studies have also shown that SIRT6 increases longevity in male mice [62]. SIRT6 plays a key role in DNA repair, genome stability and metabolic homeostasis [63,64]. SIRT6 acts as a strong tumor suppressor protein [65-67]. SIRT6 also has an anti-inflammatory effect [68,69]. In skeletal muscle, aging is also associated with metabolic syndrome, increased DNA damage, insulin resistance, mitochondrial function inhibition, cancer, chronic heart failure, reduced energy metabolism and muscle performances [70,71]. Accordingly, we designed an experiment to study the effects of UA on the levels of SIRT1 and SIRT6 in the skeletal muscle tissue of gastrocnemius in old C57BL/6 mice. In this study, immunofluorescence (IF) microscopy was used to evaluate anti-aging biomarkers, which is a powerful and secure method for evaluating protein levels, widely used by researchers.

MATERIALS AND METHODS

Material

UA was purchased with high purity (≥90%) from SIGMA (U6753). Primary antibodies specific for SIRT1 (Ab110304), SIRT6 (S4322), were obtained from Abcam. Secondary Goat-anti mouse FITC (Ab97022), Goat-anti Rabbit FITC (Ab6717) and Goat-anti mouse (Ab6787) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz) and Abcam. DAPI, Paraformaldehyde, Triton X-100, NaCl, Tris-HCl were purchased from Sigma Aldrich Company and whole other chemicals were obtained from Merck Company.

Methods

Animal study. Male C57BL/6 mice (n = 20), aged 20 months were prepared from Pasteur Institute, Iran. Within 3 weeks of beginning treatment, the mice housed in the colony cages with 12 h light/12 h dark cycles, and maintained on standard chow (Harlan Teklad formula 7013). The mice were divided into 3 groups, the first group received distilled water and considered as a control group (C), and the second group merely received placebo (Corn Oil) as a control group (CO) and the last group as a treatment group received UA. UA was dissolved in Corn Oil at a concentration of 20 mg ml^{-1} and administrated 200 mg kg^{-1} *via* intra-peritoneal (i.p) injection twice daily for 7 days. Finally, for Immunofluorescence (IF) tests, all animals were weighted and anaesthetized by an i.p injection of ketamine/xylazine then perfusing and muscles were harvested and fixed with 4% paraformaldehyde and 2.5% glutaraldehyde [21,72,73]. All animal procedures were approved by the Institutional Animal Care and Uses Committee of the Endocrinology and Metabolism Research Center, Tehran University of Medical Sciences.

Tissue preparing for immunofluorescence test. After anaesthetizing and perfusing of the mice, the gastrocnemius muscles were properly harvested in order to fix them, the tissue submerged in 4% paraformaldehyde and 2.5% glutaraldehyde overnight [74]. Then, the samples were infused with 30% sucrose and stored in 4 °C for IF tests. Next, the tissue sectioned with a cryostat microtome at -25 °C, mounted onto the glass slides. After that, they were stained for nucleus detection. In fact, they should be blocked in room temperature for 10 min prior to staining. Then, nucleus staining performed by Eosin for 5 min and washed with PBS and water, dehydrated by descending alcohol, then mounted onto glass slides and they were imaged using a bright-field microscope (Nikon, TE2000-S) and recorded by camera (TCH-1.4CICE) [72,73].

Immunofluorescence microscopy. Cryosection slides

were initially dehydrated in Room Temperature (RT) for 10 min in order to identify the mentioned anti-aging proteins in the skeletal muscle tissue. Then, the slides were fixed in PBS for 10 min (rehydration), and impregnated in HCl (normal) for 20 min. After that, it was substituted by Borate Buffer for 5 min. Next, they were washed by PBS (2 × 5 min). To assess nucleus antigen, tissues were made penetrable by Triton X-100 (3% in PBS) for 30 min and then washed by PBS (2 × 5 min). Then, the semi prepared tissues were blocked by goat-serum (500 µl goat-serum in 4.5 cc PBS) for 45 min in RT. The first antibodies were added based on optimized protocol in 4 °C overnight. Similarly, SIRT1 (1-1000 diluted in blocking buffer), SIRT6 (2-4 µg ml^{-1} diluted in blocking buffer). Afterwards, the slides were washed in PBS (2 × 5 min). Then, 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). After that, the slides were washed in PBS (2 × 5 min). In order to recognize tissue nucleus, 50 ml of DAPI (4',6-diamidino-2-phenylindole) diluted in PBS, was added to each slide for 2 min in dark, and then removed from tissue surface and washed by PBS (2 × 5 min). Finally, the slides were embedded in PBS and visualized by bright-field microscope (Nikon, TE2000-S) and then captured by camera (TCH-1.4CICE) and where the images were analyzed with the LSM 510 image browser software [72,73,75].

Statistical Analysis

The results findings were analyzed by one away ANOWA test. Each experiment was done at least three times, and the data were presented as the mean ± SEM, where applicable.

RESULTS

UA Enhanced Sirt1 Protein Level in Aged-mice Skeletal Muscle

Known as a nuclear protein SIRT1 is also exist in cytosol where its levels were measured only at the nucleus in this study. The results indicated (Figs. 1A-C) that UA significantly increases the SIRT1 protein level in aged-mice treated with UA in comparison with the C group (~3.3 folds) and CO group (~3.5 folds), $p < 0.001$.

UA Increased SIRT6 Protein Level in Aged-mice Skeletal Muscle

SIRT6 is localized in the nucleus. Therefore, in this study, it was measured at the nucleus. Our results showed that UA significantly increased SIRT6 protein level (Figs. 2A-C) in aged-mice treated with UA in comparison with group C (~4.3 folds) and the CO group (~4 folds), $p < 0.001$.

DISCUSSION

Several studies have shown that the early onset of age-related damages to the muscle may affect aging in other tissues and increase the chance that the muscle acts as a "sentinel tissue" [3]. Recent studies in yeast, worms, flies, mice, and mammals have revealed that sirtuins are key regulators for aging and longevity [76-79]. In accordance with recent studies on the beneficial effects of UA on skeletal muscle SIRT1, aging and longevity studies, we decided to evaluate expression of SIRT1 in skeletal muscle in old mice. Our findings demonstrate that UA significantly increases protein levels in treated mice muscle cells. As shown in Figs. 1A-C, UA has increased SIRT1 expression level. Sirtuins are classified as class III histone deacetylases which are dependent on NAD^+ for their activities and mammals have 7 sirtuins (SIRT1 to7) [80]. Sirtuins have demonstrated activities such as DNA damage repair, genomic stability, cancer prevention, neuroprotection, gluconeogenesis, anti-inflammatory, anti-obesity, lifespan extension and *etc.* [81]. Exercise [15], fasting [14] and CR [11] are among external factors that can be used to activate sirtuins and several small activating molecules involving resveratrol, SRT_{1720}, Oxazolo [4,5b] pyridines derivative, imidazo [1,2-b] thiazole derivative, 1,4-dihydropyridine (DHP) derivatives also have been identified [82]. The inhibition of SIRT1 results in the loss of cancer cells [83], the suppression of the gene silencing in fragile x mental retardation syndrome [84]. Sirtuins are also considered as target in the treatment of Parkinson's disease [83], Human Immunodeficiency Virus (HIV) [85] and Leishmaniasis [86]. As a result, searches for controlling sirtuins are also important and splitomicin, HR_{73}, sirtinol, AGK2, cambinol,

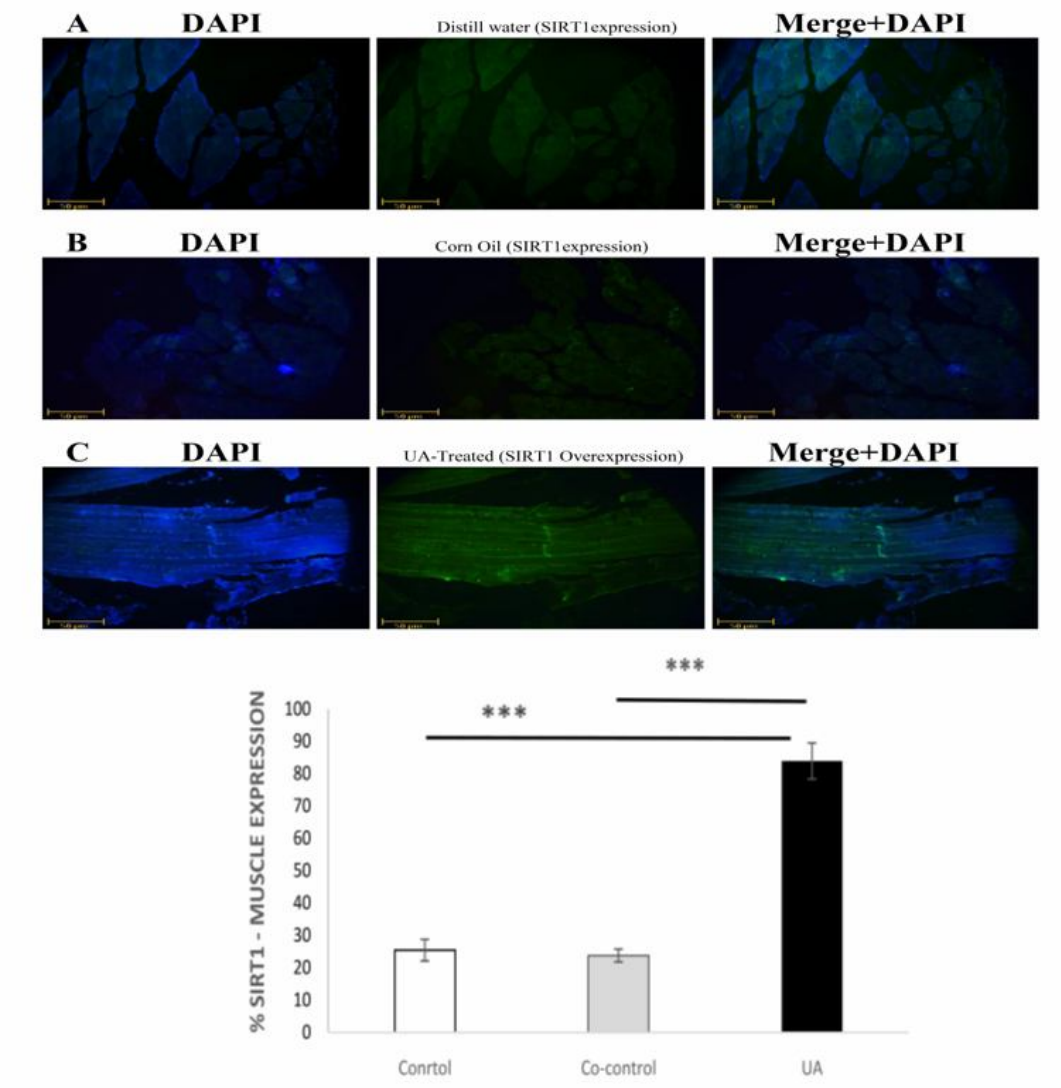

Fig. 1. UA enhances SIRT1 protein in aged-mice skeletal muscle. Nuclear SIRT1 expression in aged-mice skeletal muscle. The mice (C57BL/6, 20 aged-month, n = 20) were treated with 200 mg kg^{-1} of UA which dissolved in Corn Oil (20 mg ml^{-1}) previously. Mice were then administrated with UA + Corn Oil or vehicle alone (Corn Oil) or distilled water *via* i.p injection twice daily for seven days. On day 7, gastrocnemius muscles were harvested for analysis. The tissue was cryosectioned (8 µm) and stained with FTTC-anti-SIRT1 monoclonal antibody; (A) the control mice which just received distilled water; (B) the control mice which received vehicle alone (Corn Oil = Co); (C) UA + Corn Oil treated mice (UA). P value was determined by one away ANOVA test. Data have been presented as mean ± SEM (*P ≤ 0.05, **P < 0.01, ***P < 0.001). Scale bar 50 µm. DAPI staining shows entire cell populations.

salermide, tenovin, suramin have been reported as sirtuin inhibitors [82]. Specific studies on UA effects on skeletal muscle showed the decreased fasting-induced muscle atrophy, reduced denervation-induced muscle atrophy, induced skeletal muscle hypertrophy, reduced atrogin-1 and MURF-1 mRNA expression (atrophy-related) and enhanced

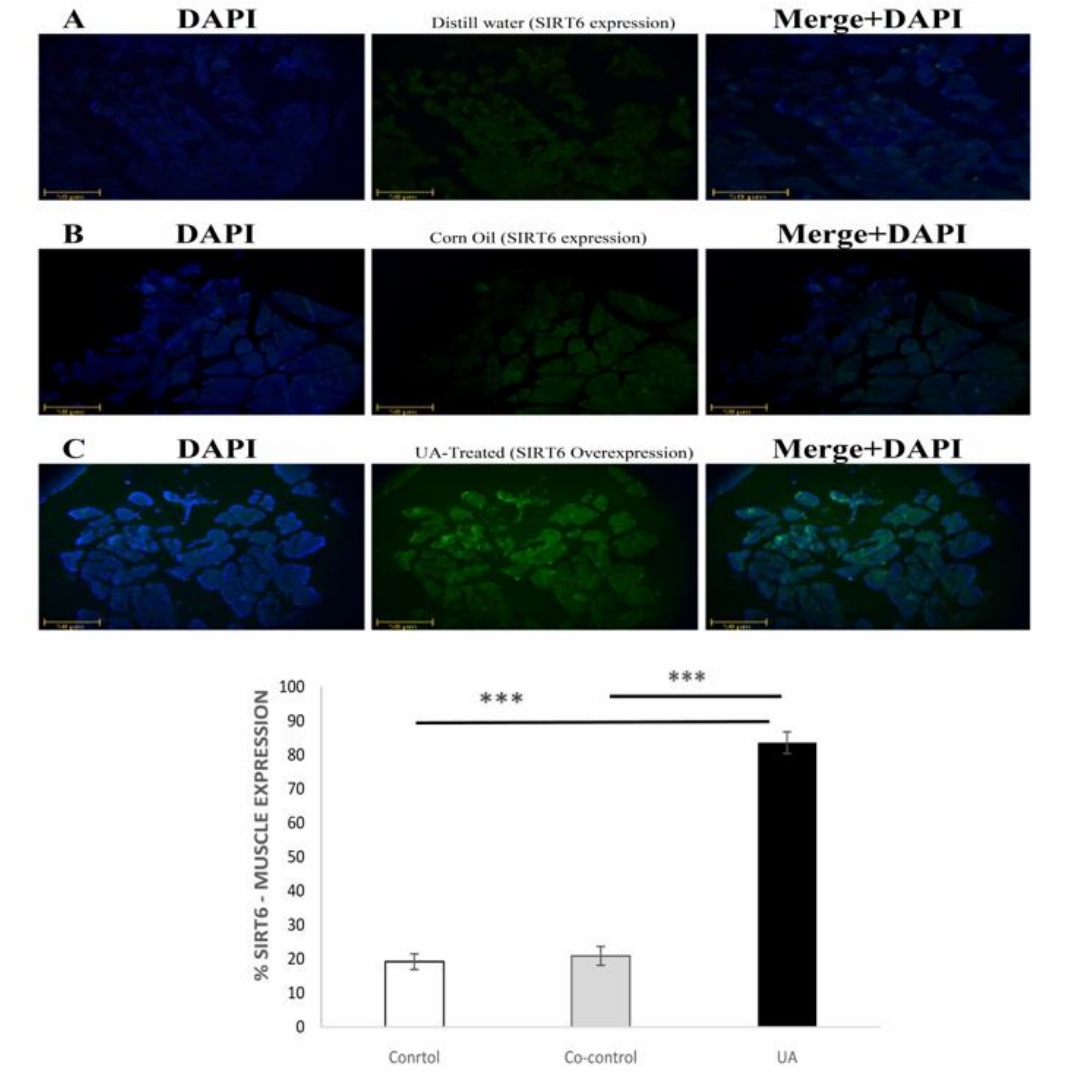

Fig. 2. UA increases SIRT6 protein level in aged-mice skeletal muscle. Expression of nuclear SIRT6 on the skeletal muscle. The mice (C57BL/6, 20 aged-month, n = 20) which received, (A) distilled water, (B) Corn Oil (Co); (C) UA + Corn Oil (200 mg kg^{-1}) twice daily for seven days. On days 7, gastrocnemius muscles were harvested for analysis. The tissue was processed by cryostat microtome (8 µm) and stained with FIIC-anti-SIRT6 monoclonal antibody). P value was determined by one away ANOVA test. Data have been presented as mean ± SEM (*P ≤ 0.05, **P < 0.01, ***P < 0.001). Scale bar 50 µm. DAPI staining shows entire cell populations.

skeletal muscle insulin/IGF-1 signaling (hypertrophic-related) [21], Additonally, UA increased skeletal muscle Akt activity and exercise capacity in a mouse model of diet-induced obesity [48]. In addition, we have recently shown that UA results in myoglobin overexpression, changes anaerobic glycolytic muscle fibers into oxidative fibers, up-regulates SIRT1 and overexpression of PGC-1α in skeletal muscle satellite cells and decreases the cellular energy status (ATP and ADP) in skeletal muscle (Fig. 3) [49]. Induced neomyogenesis in mice skeletal muscle [51,87], up-regulates SIRT1, SIRT6, PGC1-β and Klotho proteins in aged-mice hypothalamus [72] and liver [73]. Also we have

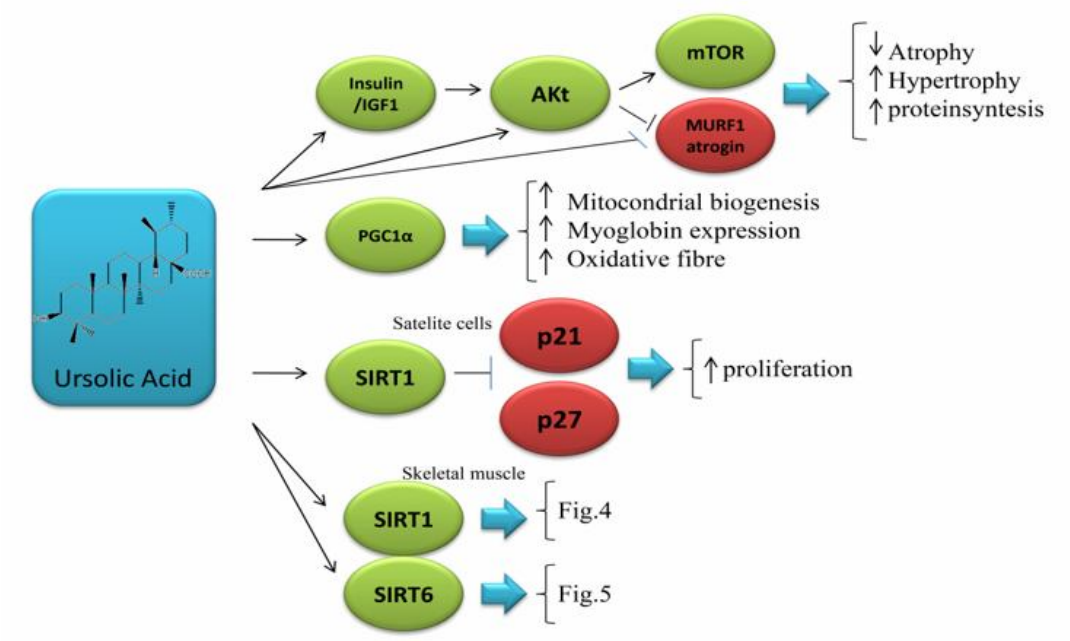

Fig. 3. Schematic representation of a number of known mechanisms of UA on skeletal muscle. UA decreased the cellular energy status (ATP and ADP) that leads to activated AMPK, which in turn leads to increase in SIRT1 expression in satellite cells. UA also increased PGC1-α in satellite cells and PGC1-α drives oxidative metabolism and the conversion from fast IIB fibers to fast IIA and slow-twitch fibers, and in addition, elevates mitochondrial biogenesis and myoglobin expression. SIRT1 inhibits the expression of cell cycle inhibitors (p21 and p27) and promotes its proliferation in satellite cells. Activating the insulin/IGF1 and AKt pathways, UA causes the hypertrophy of the muscle, and on the other hand, by inhibiting MURF1 and atrogin1, inhibits the atrophy. Our findings showed that UA also increases the levels of SIRT1 and SIRT6 in skeletal muscle.

shown that UA directly activates SIRT1 [88] and significantly has increased protein levels in treated mice. As given in Fig. 4, when the level of SIRT1 increase, PGC1-α is deacetylased by SIRT1 to activate genes that regulate fatty acid oxidation, mitochondrial biogenesis [89], and fibers conversion from glycolytic to oxidative ones [89]. Studies have also shown that AMPK activates PGC1-α either in direct way or through activation of SIRT1 [90]. It has been recently proven that the activation of Akt stimulates hypertrophy of the muscle, and subsequently, reduced Akt activity is accompanied by atrophy [91]. Studies have shown that SIRT1 deacetylases and activities Akt and activating mTOR, Akt leads to protein synthesizes [92]. SIRT1 promotes insulin sensitivity by suppressing PTP1B gene in skeletal muscle [93]. Deactivating foxO1 and subsequently MURF1 and MAFbx, SIRT1 prevents atrophy [91,92]. Moreover, the activity of SIRT1 on FoxO4 induced during oxidative response and the oxidative metabolism in muscle cells [94]. SIRT6, like SIRT1 (Fig. 5), increases longevity as mice lacking SIRT6 survived only a few weeks after birth. SIRT6 depletion leads to telomere dysfunction with premature cellular senescence (resemble defects observed in Werner syndrome) [95], tumor formation [96], increased insulin resistance, hepatic steatosis [69], enhanced appetite and obesity, growth attenuation [62], genomic instability, a curved spine, lymphopenia, and loss of subcutaneous fat [66]. In contrast, SIRT6 overexpression induced cardiac hypertrophy [66], apoptosis in a cancer cell lines [97], decreased LDL-cholesterol and triglycerides in mice fed

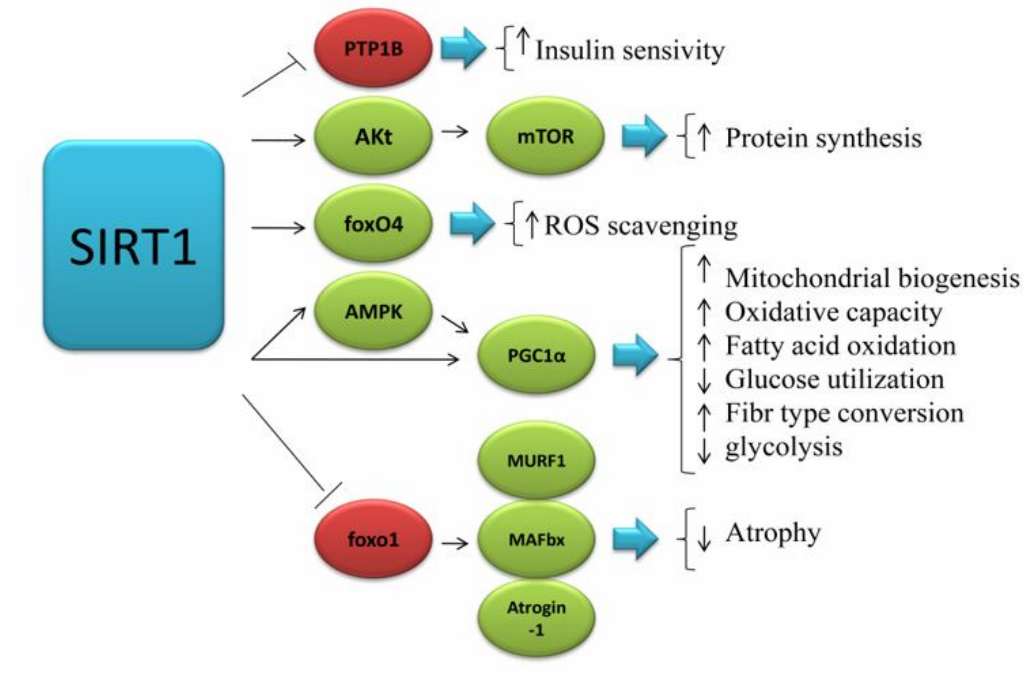

Fig. 4. Schematic illustrating some of the major effectors and regulators (no means an exhaustive description) of SIRT1 activity and their relevance to skeletal muscle. Through deacetylation and activation of PGC1-α and AMPK, SIRT1 causes oxidation of fatty acids, mitochondria biogenesis, alters glycolytic fibers to oxidation. It also causes hypertrophy by activating the Akt, and increases insulin sensitivity by suppressing the PTP1B gene and prevents oxidative damage by activating FoxO4. Deactivating foxO1 and subsequently MURF1 and MAFbx, Sirt1 prevents atrophy.

with a high-fat diet [97]. Previous studies have revealed that exercise alters the levels of SIRT1 and SIRT6 in skeletal muscle of aged rats [15]. We attempted to determine whether the UA might also alter the levels of SIRT6 protein where the overexpression of SIRT6 observed in skeletal muscle in old mice. SIRT6 is a key regulator of glucose metabolism by suppressing the expression of HIF-1α and other glycolytic genes. SIRT6 knockout mice show hypoglycemia, a diminished mitochondrial respiration and enhanced glycolysis [64].

CONCLUSIONS

In summary, the data presented in this paper show that the short-term administration of UA increases the levels of SIRT1 and SIRT6 in skeletal muscle of old mice. In other words, SIRT1 and SIRT6 by activating downstream genes in muscle tissues demonstrates activities such as increased mitochondrial biogenesis, increased fatty acid oxidation, fiber type conversion, decreased glycolysis, increased insulin sensitivity, increased mitochondrial respiration, decreased atrophy and increased hypertrophy. It appears that UA postpones aging or enhances aging through improvement of anti-aging biomarkers in skeletal muscle. Thus, it might be assumed as a suitable candidate for treatment of pathological conditions related to dysfunction such as skeletal muscle atrophy, ALS, sarcopenia and metabolic diseases associated with muscle aging.

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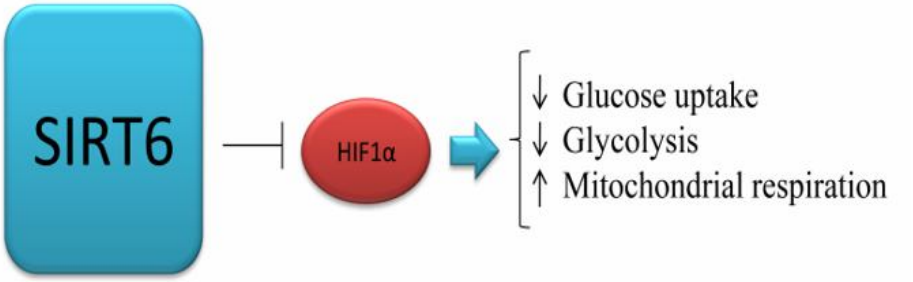

Fig. 5. Schematic illustrating some of the major effectors and regulators (no means an exhaustive description) of SIRT1 activity and their relevance to skeletal muscle. SIRT6 shows its function by suppressing the expression of HIF1-α in the skeletal muscle tissue which reduces glucose uptake and glycolysis and also increases mitochondrial respiration.

CONFLICT AND INTEREST

The authors verify that there are no any conflict and interest.

LIST OF ABBREVIATIONS

UA = ursolic acid
SIRT1= sirtuin (Silent mating Type Information Regulation 2 homolog) 1.
PGC1-α = peroxisome proliferator-activated receptor gamma co-activator 1-alpha
PGC1-β = peroxisome proliferator-activated receptor gamma co-activator 1-beta
MURF-1 = muscle ring finger-1.
IGF-1 = insulin growth factor receptor-1.
Akt = protein kinase B.
AMPK = AMP-activated protein kinase.
mTOR = mammalian target of rapamycin.
PTP1B = protein-tyrosine phosphatase 1B.
FoxO1 = forkhead box protein O1.
HIF-1α = hypoxia-inducible factor 1-alpha.
ROS = reactive oxygen species
ALS = amyotrophic lateral sclerosis

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