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- Note -

Copper Regulates Apelin Expression in L6 Skeletal Muscle Cells

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In this experiment, we aimed to investigate the role of copper in regulating the biosynthesis of a myokine called apelin in mammalian skeletal muscle cells. Our approach involved culturing skeletal muscle cells and subjecting them to treatments with copper sulfate or a copper chelator known as bathocuproinedisulfonic acid (BCS). We employed standard techniques, such as reverse transcription-polymerase chain reaction (RT-PCR) and Western blotting, to assess the synthesis of apelin at different stages, including transcription, translation, and post-translational modifications. Our findings demonstrated that copper had an inhibitory effect on apelin biosynthesis at all three stages: transcription, translation, and post-translation. However, when we treated the cells with BCS, the biosynthesis of apelin was restored to its original state. This finding suggests that copper is required for the synthesis of apelin in mammalian skeletal muscle cells. This study represents the first documented evidence of the inorganic copper-dependent regulation of apelin biosynthesis, shedding light on potential strategies for the prevention and treatment of sarcopenia induced by copper imbalances.

Key words : Apelin, bathocuproinedisulfonic acid (BCS), copper sulfate, skeletal muscle cells

Introduction

Copper sulfate, denoted by the chemical formula $CuSO_4$, represents a crystalline solid of vivid blue coloration, exhibiting a significant degree of solubility in aqueous solutions. This compound finds extensive application across diverse domains owing to its multifaceted characteristics. Numerous metalloenzymes depend on the critical micronutrient copper to function [12, 16]. In some diseases, the copper's homeostatic equilibrium may be off. It has been demonstrated that various types of cancer are related to the high serum copper levels [12]. According to some researches, copper may have anticancer effects when combined with other substances [7]. At high concentrations, copper becomes toxic to the organism; this toxicity is mostly exhibited by an excess production of reactive oxygen species [13, 24], interference with methionine metabolism, and DNA methylation changes [18]. Copper has been reported to be directly involved in many biological phenomena. For example, copper induces oxidative stress, autophagy and apoptosis [8, 14] and is also involved in spermatogenesis [19], superoxide dismutase like activity [17], cholesterol metabolism and steroidogenesis [9, 10], insulin-like growth factor 1 (IGF-1) and transforming growth factor beta (TGF-beta) gene expression [28]. The WHO daily copper requirement (1996) is 12.5 μg/kg body weight for adults, but because of its low cost, copper sulfate is widely used as a food additive, fungicide, and preservative [20].

The aging-related loss of skeletal muscle mass, strength, and functionality is known as sarcopenia. People's muscular mass and strength naturally decrease as they age, which can restrict mobility, increase frailty, and raise the risk of falls and fractures. Multiple factors, such as hormonal changes, decreased physical activity, poor diet, and chronic diseases, might contribute to sarcopenia [4, 5]. A worldwide pandemic of sarcopenia has emerged. Sarcopenia has become more common, affecting more than one-third of elderly persons [19]. Skeletal muscle cells create and release myokines, which are signaling molecules or cytokines, during contraction and exercise. They serve as chemical messengers that facilitate communication among the body's many tissues and

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organs. In addition to its anti-inflammatory properties, myokines also control metabolism, increase insulin sensitivity, and support muscle growth and repair. Interleukin-6, brain-derived neurotrophic factor, and irisin are a few well-known myokines [1, 21]. Even though the precise mechanisms and interactions between myokines and sarcopenia are still being studied, new data indicates that myokines may be crucial in maintaining muscle mass, encouraging muscular growth, and reducing the symptoms of sarcopenia. Additional research is required to examine the particular myokines implicated, their methods of action, and any possible therapeutic benefits for sarcopenia prevention or treatment [11].

The regulatory effects of copper on gene expression in muscle, particularly with respect to the IGF-1 and TGF-beta, have been established in previous studies [8, 19]. However, the specific role of copper in skeletal muscle regarding sarcopenia remains unexplored. This current investigation presents novel findings, elucidating the influence of copper on apelin gene expression, biosynthesis, and secretion in skeletal muscle cells [30].

Materials and Methods

L6 rat myoblast-derived cells were cultured under specific conditions consisting of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 μg/ml of penicillin-streptomycin antibiotic, and maintained at a controlled environment of 37℃ with 5% CO_2. To induce the formation of myotubes, the cells were cultured for a period of 2-3 days while maintaining their confluence between 80% and 90%, without altering the composition of the culture medium. The copper chelator, bathocuproinedisulfonic acid (BCS), and $CuSO_4$ were procured from Sigma Aldrich. To quantify and isolate total RNA from L6 cells, the NanoDrop Lite UV-spectrophotometer (Thermo Scientific, Waltham, MA, USA) and the SV Total RNA Isolation System (Promega, Madison, WI, USA) were employed, respectively.

For reverse transcription of mRNA in the samples, the Superscript II™ First Strand Kit (Invitrogen, Carlsbad, CA, USA) was employed. RT-PCR (Reverse Transcription-Polymerase Chain Reaction) was performed using the following settings: Taq DNA polymerase along with specific primers were used, and the amplification was carried out through 30 cycles. The cycling conditions included denaturation at 94℃ for 30 sec, annealing at 58℃ for 30 sec, and extension at 72℃ for 1 min (with a final extension of 10 min in the last cycle). The resulting RT-PCR bands were quantified using the ImageJ tool developed by the National Institutes of Health (NIH). Following lysis of L6 cells using RIPA buffer [25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), and 1 mM phenylmethylsulfonyl fluoride], Western blotting was conducted with certain modifications. The total protein concentration was determined using the Pierce BCA Protein Assay Kits (Thermo Fisher Scientific, Cleveland, OH, USA). SDS-Polyacrylamide Gel Electrophoresis (PAGE) was utilized to separate the proteins, which were subsequently transferred to a nitrocellulose membrane. The resulting membrane was subjected to overnight incubation with the primary antibody at 4℃, followed by incubation with the appropriate secondary antibody. The chemiluminescence Western blotting detection system Kit was employed in accordance with the manufacturer's instructions (Amersham, Sweden). The anti-apelin and goat anti-actin antibodies were procured from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Results and Discussion

We screened L6 rat myoblast-derived cells for genes whose expression was regulated (negatively or positively) by copper sulfate for 30 myokines: Apelin, BDNF (Brain-derived neurotrophic factor), CTSB (cathepsin B), CX3CL1 [chemokine (C-X3-C) ligand 1] (fractalkine), Decorin, DPP4 (dipeptidyl peptidase 4), FGF21 (fibroblast growth factor 21), Follistatin (myostatin-binding protein), FSTL-1 (Follistatin-like protein 1), GDF11 (Growth differentiation factor 11), GDF-8 (Myostatin), IL-12 (Interleukin-12), IL-6, IL-7, Irisin (FNDC5), LIF (leukemia Inhibitory factor), Metrnl (Meteorin like protein1), MG53 (Mitsugumin 53), MIF (macrophage miration inhibitory factor), Musclin, Myonectin (C1q/TNF-related protein, CTRP15), OSM (Oncostatin M), PEDF (pigment epithelium derived factor), RARRES1 (Retinoic Acid Receptor Responder 1), Sclerostin, SDF1 (Stromal Cell-Derived Factor 1), Sestrin 1, SPARC (Osteonectin, secreted protein acidic and rich in cysteine), VEGF (vascular endothelial growth factor), LTBP2 (Latent-transforming growth factor beta-binding protein 2). Among them, we found that the expression of the apelin gene decreased in a copper sulfate dose-dependent manner. Apelin is a peptide hormone that is involved in a number of physiological functions in the body of an individual. It was initially found in 1998, and its name comes from the Greek word ‘apela’, which means ‘to prepare’ [26]. For the G-protein coupled apelin receptor, apelin serves

as the endogenous ligand [25, 29]. The cardiovascular system, central nervous system, kidneys, lungs, and gastrointestinal tract are only a few of the tissues that have the apelin receptor [3, 15]. Numerous processes, including as fluid balance, energy metabolism, and cardiovascular control are regulated by apelin [10]. Another classification of signaling molecule known as a myokine, which is created and released by skeletal muscle cells, has been assigned to apelin [6]. Myokines have a variety of physiological functions and aid in the communication between muscle tissue and other organs [22]. Age-related decrease of muscle mass, strength, and function is referred to as sarcopenia [5]. It is a prevalent condition among older persons and is linked to a number of detrimental health effects. Apelin has been studied in relation to sarcopenia as a myokine, and some studies indicate that it might be involved in the onset and progression of the condition [27]. More investigation is required to properly understand how apelin and sarcopenia are related, while their precise relationship is currently under investigation. According to certain research, older people with sarcopenia had lower apelin levels than older people without sarcopenia [31]. This implies that apelin levels may decrease with aging and aid in the onset or advancement of sarcopenia. In preclinical experiments, apelin was found to support muscle regeneration and repair [23]. It might promote the activation and differentiation of satellite cells, two crucial steps in muscle regeneration. As a result, it is speculated that decreased apelin levels in sarcopenic people may hinder muscle regeneration and speed up the onset of sarcopenia.

Fig. 1A. shows the results of apelin gene expression by different concentrations (1 μM-4 μM) of copper sulfate treatment. Especially, it showed a significant decrease in apelin gene expression of about 50% at 3 μM copper sulfate concentration and about 40% at 4 μM concentration. To demonstrate that apelin gene expression is copper-dependent, we treated the copper chelator, BCS, with copper sulfate. As shown in Fig. 1B, apelin gene expression was restored to control levels by chelation of copper by BCS. The results in Fig. 1. indicate that copper specifically negatively regulates the apelin gene. The result in Fig. 1. is the transcriptional level of apelin gene by copper, and the effect of copper on the translation step of apelin was investigated by western blotting method to examine the biosynthesis of apelin protein. As shown in Fig. 2A, the biosynthesis of apelin protein was inhibited by copper sulfate treatment in a concentration-dependent manner, almost identical to the pattern of transcriptional level. Of course, this was also restored by BCS treatment (Fig. 2B), which was identical to the results in Fig. 1B. The results in Fig. 1. and Fig. 2. demonstrate that apelin is copper-specifically regulated in both step transcription & translation, respectively. However, the results in Fig. 1. and Fig. 2. only show that copper sulfate inhibits apelin gene expression and apelin protein biosynthesis. In fact, the apelin used *in vivo* is the apelin protein secreted extracellularly after

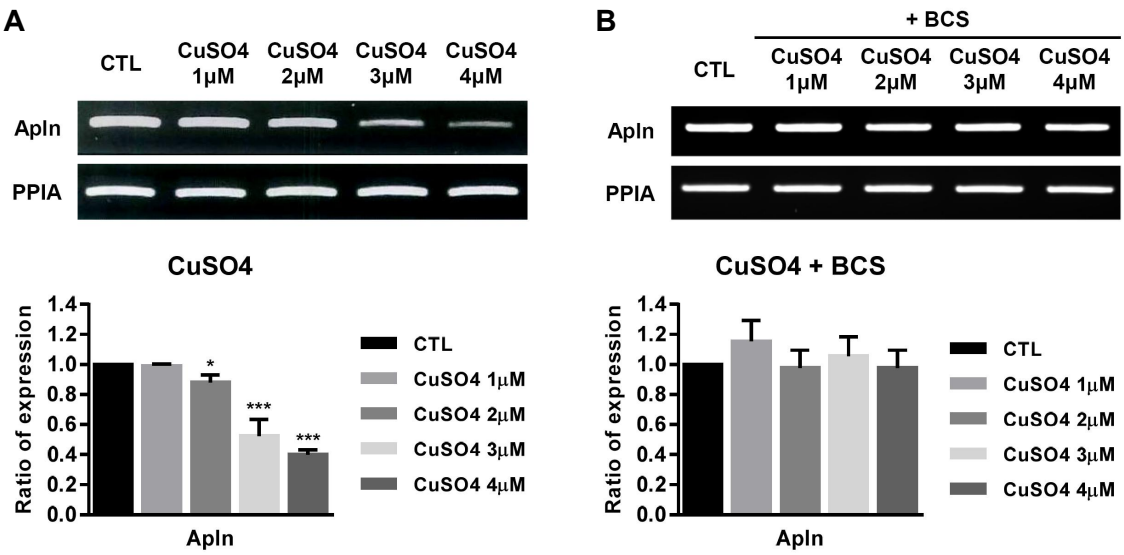

Fig. 1. Gene expression of apelin by copper sulfate. Changes in apelin gene expression after 24 hr exposure of various concentrations of copper sulfate. BCS; copper chelator bathocuproinedisulfonic acid. Mean ± SEM. n=4. *p<0.05 ***p<0.001. Statistical significance between multiple groups: one-way analysis of variance (ANOVA) test. GraphPad Prism 6 software (GraphPad Software Inc.).

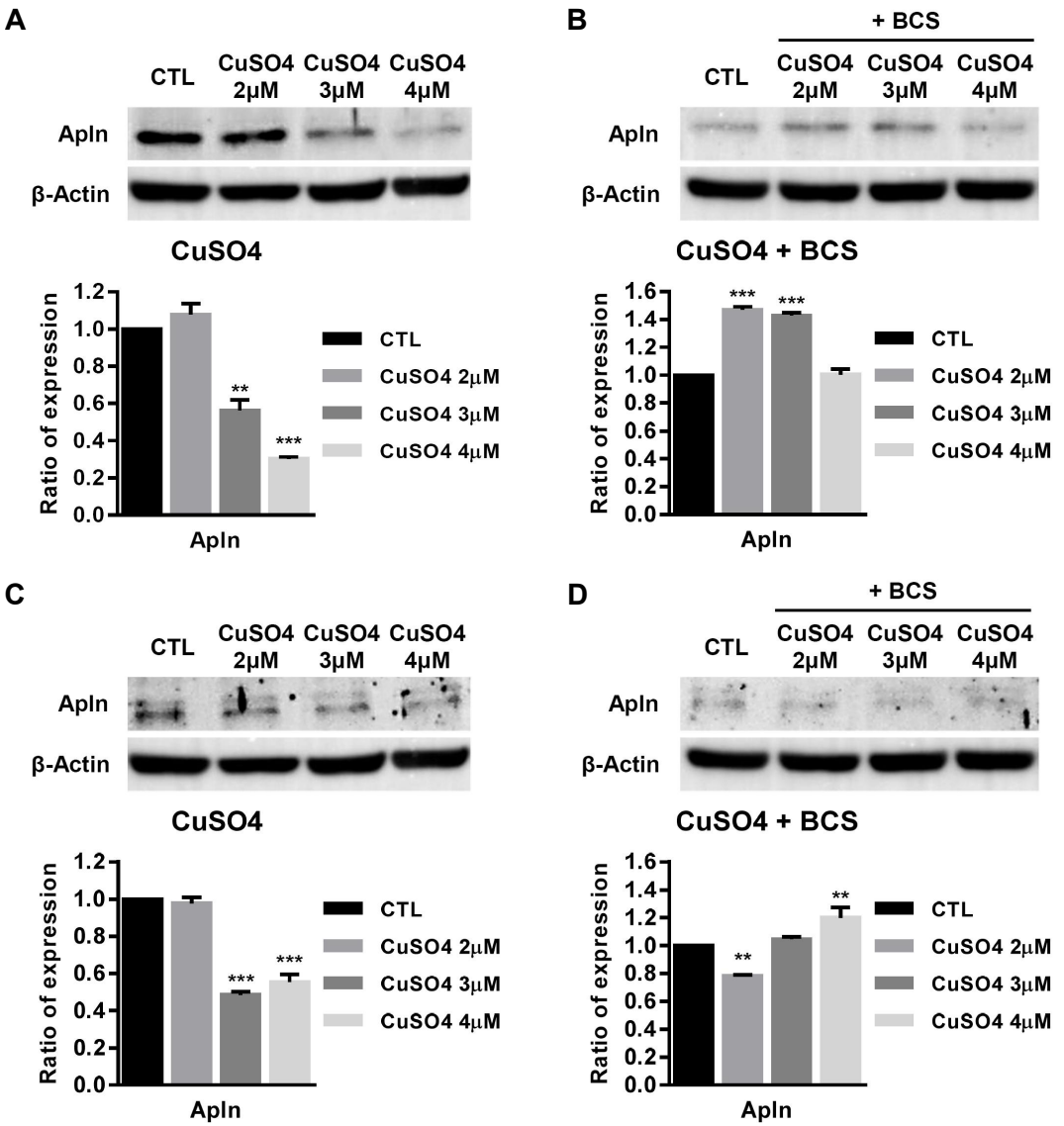

Fig. 2. Translation and secretion of apelin by copper sulfate. Results of apelin protein expression (A, B) and apelin protein secretion (C, D) by both treatment of copper sulfate and copper sulfate + BCS treatment, respectively. Mean ± SEM. n=4. $^{**}p<0.005$ $^{***}p<0.001$. Statistical significance between multiple groups: one-way analysis of variance (ANOVA) test. GraphPad Prism 6 software (GraphPad Software Inc.).

the posttranslational step, so it is necessary to quantify the apelin secreted extracellularly after copper sulfate treatment. L6 cells were subjected to copper sulfate treatment (2 μM, 3 μM, and 4 μM) for 24 hr and then washed 2-3 times with DMEM to fully remove copper sulfate. After 6 hr in fresh DMEM, each 200 μl medium was collected for Western blotting and the results of copper sulfate treatment only is shown in Fig. 2C and the result of BCS + copper sulfate treatment are shown in Fig. 2D, respectively. The results in Fig. 2 showed the same pattern as those in Fig. 1, i.e., the amount of apelin protein secreted decreased in a copper sulfate dose-dependent manner and was restored by BCS treatment.

In conclusion, this study provides evidence that apelin, a myokine found in mammalian skeletal muscle, is effectively suppressed by copper at multiple stages: transcription, translation, and posttranslation. Given that the inhibition of apelin protein synthesis directly contributes to the development of sarcopenia, it is crucial to give serious consideration to preventing sarcopenia by controlling copper intake through various pathways. The investigation of novel substances, such as BCS, that can fulfill biochemical functions holds promise for the treatment of copper-dependent sarcopenia.

The Conflict of Interest Statement

The authors declare that they have no conflicts of interest with the contents of this article.

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초록 : 골격근세포에서 구리에 의한 마이오카인 apelin의 발현

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본의 연구 결과는 무기질 구리가 전사, 번역 및 번역 후의 여러 단계에서 아펠린 생합성에 억제 효과를 발휘한다는 것을 명확하게 보여준다. 그러나 바토쿠프로인디설폰산의 구리 킬레이터로 처리하면 구리의 억제 영향이 효과적으로 역전되어 포유류 골격근 세포에서 아펠린 생합성의 구리 의존적 특성이 확인되었다. 이러한 결과는 구리가 마이오카인 아펠린의 생합성 조절에 중요한 역할을 한다는 최초의 보고이며, 구리 관련한 근육감소증의 예방 및 치료의 전략 개발에 유용한 실마리를 제공할 수 있다.