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Copper or/and arsenic induces autophagy by oxidative stress-related PI3K/AKT/mTOR pathways and cascaded mitochondrial fission in chicken skeletal muscle

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ABSTRACT

Autophagy is an ubiquitin proteasome system for degradation of intracellular damaged proteins and organelles. Both as environmental pollutants, flourishing data show arsenic (As) and copper (Cu) as robust oxidative stress inducers. Whether this kind of damage correlates with autophagy through the phosphoinositide-3-kinase/protein kinase b/mammalian target of rapamycin (PI3K/AKT/mTOR) pathway still remains elusive. A 12-week exposures of Cu or/and As to chicken time-dependently displayed significant element residue in the pectoralis. Aligning with previous results, a strong pro-oxidant nature of Cu and As was clearly indicated by enzyme/nonenzyme antioxidants. Fragmented mitochondria induced by oxidative damage were accompanied by over-expressed dynamin related protein-1 and decreased mitochondrial fusion-related genes. Upon comparative analysis, time-dependent conversion of light chain 3 (LC3)-I to LC3-II, increases in autophagy-related genes such as Bcl-2-interacting protein (Beclin-1) and inhibited PI3K/AKT/mTOR pathway firmly supported the fact that Cu or/and As induces autophagy. These results further coincided with ultrastructure showing clusters of vesicles and autophagosome in the skeletal muscle. Interestingly, the time-dependently elevated heat shock proteins observed in Cu or/and As treated chicken suggest the continuous adaptation and physiological acclimation of organisms to this stress responses. Interestingly, the combination of copper and arsenic elicited more serious oxidative damage and its-cascaded injuries than their individuals. Together, our results showed that after Cu or/and As insult and accumulation, inhibited PI3K/AKT/mTOR pathway activated autophagy and disturbed mitochondrial dynamic, forming a positive feedback with redox disorder.

1. Introduction

Water system and soil can contain elevated concentrations of toxic elements, such as arsenic (As), lead (Pb), copper (Cu), and cadmium (Cd) and organochlorine pesticides due to long-term use of soil amendments and agrichemicals [1,2]. These residues have provoked to be a major global environmental problem, threatening in every aspect the health of vegetation, wildlife, and humans. The toxicity of As and Cu has been unambiguously elucidated in a variety of epidemiological and experimental studies. Prolonged arsenic ingestion leads to various pathological conditions, including dermal hypertension, liver disease, lesions, neuropathy, and cancer [3]. On the other hand, Cu^{2+} poisoning also clinically features by intravascular hemolysis, erosive gastropathy, hepatitis, and acute kidney injury [4]. Mechanically, both copper and arsenic are able to elicit the generation of reactive oxygen species (ROS) in cells, resulting in significant oxidative stress and subsequent tissue damage [5–7]. Constitutionally, organisms are protected against oxidative stress by multiple enzyme/non enzyme antioxidants, which includes glutathione (GSH), a vital endogenous antioxidant responses for the detoxification and excretion of heavy metals through its sulfhydryl group; superoxide dismutase (SOD), a cleaner of O^{2-} and inhibitor of H_2O_2 [8]. In the model of chicken, sublethal exposure to Cu or As caused damage in the immune and cardiovascular systems via disturbing oxidative stress and inhibiting total antioxidant capacity (T-AOC) [9,10]. However, individual and mixed effects of these two oxidative stress-inducers have been less studied in skeletal muscle, which tissue characterized by abundant mitochondria [11].

The mitochondrion is organized in a highly dynamic tubular network that is uninterruptedly reshaped by two opposite processes, namely mitochondrial fission and fusion [12]. Defects in either of them

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could limit mitochondrial motility, thereby decreasing energy production and increasing oxidative stress, promoting cell dysfunction and death [13], indicating a negative feedback between oxidative stress and mitochondrial dysfunction. Mitochondrial dysfunction is closely linked to numerous neurodegeneration and muscular-related disorders [14,15]. Minimizing mitochondrial dysfunction does indeed occur naturally at different levels. On the organelle level, dysfunctional mitochondria are recognized or degraded by autophagy. In the human erythroleukemic cell, copper overload-induced ROS stimulate higher rates of mitochondrial turnover. After two uneven daughter mitochondria are generated by fission event, the one depolarized is later degraded by autophagy [16]. On the molecular level, chaperones (e.g., heat shock proteins (Hsps)) and mitochondrial proteases exert an indispensable part in preventing misfold and aggregation of proteins. Various heavy metal/metalloid exposure including arsenic, cadmium, copper, silver, and zinc substantially modulated the expression of Hsp genes to prevent organisms from detrimental oxidative stress, autophagy and inflammation [9,17,18].

Elevated ROS regulate autophagy in many models [19]. During autophagy flux, damaged organelles and proteins are degradated by autolysosome after being sequestered by double-membraned autophagosome [20,21]. This machinery finally generates nucleotides, ATP, fatty acids and amino acids which are fundamental for cell survival [22]. Using genetic screening, > 30 autophagy genes (ATG) have been identified conservatively from yeast to mammals [23]. Among them, Bcl-2-interacting protein (Beclin-1) is anchored in the phosphoinositide 3-kinase (PI3K, vacuolar protein sorting 34 (VPS34)) complex localized to the trans-Golgi network, which controls the initiation of pre-autophagosome structures [24]. As upstream regulators of autophagy, mammalian target of rapamycin (mTOR) inhibits autophagy by blocking the initiation stages of autophagic flux [25]. In addition, PI3K and protein kinase b (AKT) (upstream activators of mTOR) suppress autophagy, whereas phosphatase and tensin homolog deleted on chromosome ten (PTEN), an inhibitor of this pathway, induces autophagy conversely [26]. It was reported that both arsenic and copper are able to elicit oxidative damage in a PI3K-dependent manner in cardiac myocyte [9]. In a mouse-derived myoblast cell, the inhibition of the PI3K-AKT-mTOR pathway also results in an elevation in autophagy flux, which was hypothesized to be compensatory for exercise capacity and proper muscle-mass maintenance [27]. While in vivo studies will be urgent to fully understand these connections.

While as the highest consumption of meat in North America, chicken muscle has been regarded as highly sensitive to oxidative processes [11]. What's worse, the negative impact of genetic selection and intensive breeding makes chicken more susceptible [28,29]. Thus skeletal muscle can be a potential model for evaluating copper and arsenic-induced oxidative stress and its cascaded responses. The present study aimed to perform a comprehensive risk assessment of Cu or/and As on chicken skeletal muscle with regard to pathophysiology. Results showed that Cu or/and As induced oxidative damage, which cascaded autophagy cell death and mitochondrial fusion through PI3K/AKT/mTOR pathway.

2. Materials and methods

2.1. Animals and experimental design

This research was supervised by the Animal Care, Use and Ethics Committee of Northeast Forestry University (approval no. UT-31; 20 June 2014). In according to LD_{50} of arsenic for chicken (50 mg/kg body weight (BW)) [Appendix 1], and the reasonable dose used in subchronic toxicity test (1/20 to 1/5 of LD_{50}) [Appendix 2], As_2O_3 (2.5 mg/kg BW, corresponding 30 mg/kg feed) was mixed into food to make supplements (Table S1). On the other hand, although the addition of 125–250 mg/kg $CuSO_4$ of feed results in increased feed efficiency and body weight [30], that of over 250 mg/kg resulted in reduced feed intake [31] and that of 300 mg/kg causes growth depression in chickens [30]. Thus we confirmed the dose of $CuSO_4$ (300 mg/kg feed) from above references. Seventy-two male chickens (Hy-line strain; 1-day-old; Weiwei Co. Ltd., Harbin, China) were divided into four groups and fed for 12 weeks, which were arranged as (I) Control: basal diet [10]; (II) As group: 2.5 mg/kg As_2O_3 BW (III) Cu group: 300 mg/kg $CuSO_4$ and (IV) As + Cu group: 2.5 mg/kg As_2O_3 BW and 300 mg/kg of $CuSO_4$. Six individuals in each group were sacrificed randomly at the 4th, 8th and 12th week. The skeletal muscles (pectoralis) were quickly collected and kept in −80 °C.

2.2. Determination of copper/arsenic contents

The contents of copper and arsenic in chicken skeletal muscle were determined using inductively coupled plasma mass spectrometry (ICP-MS) (Thermo iCAPQ, American). The instrumental parameters are summarized in Table S2. The mineral element concentrations and digestion conditions for the microwave system were applied as our previous research [32].

2.3. Detection of antioxidant system

The skeletal muscle tissues were homogenized in 0.9% NaCl solution and centrifuged. The antioxidant function were determined using supernatants. Using spectrophotometric analysis, T-AOC, SOD and GSH were measured six copies using the corresponding detection kits (Nanjing Jiancheng Bioengineering Institute, China).

2.4. RNA isolation and qPCR

Total RNA of skeletal muscle tissues were extracted using Trizol (Invitrogen; Thermo Fisher Scientific: Waltham, MA, USA). Subsequently, cDNA was synthesized by the PrimeScript™ RT Reagent Kit (Takara, China). Table S3 shows specific primers used for amplification in this study. The reaction conditions and procedures of qPCR were conducted as previously described [7].

2.5. Western blotting analysis

Protein extracts were prepared by using sodium dodecyl sulfate (SDS) Lysis Buffer then separated by electrophoresis through 15% SDS-polyacrylamide gels and transferred to polyvinylidene fluoride (PVDF) membranes. Detailed information of antibodies is shown in the Table S4.

2.6. Microscopy

The skeletal muscle tissues (1.0 mm^3) were rapidly extracted at the 12th weeks. Specific staining procedure was performed according to [9]. Finally, transmission electron microscope (GEM-1200ES, Japan) was used to take microphotographs.

2.7. Bioinformatics

Firstly, the String online software (http://string-db.org/) was used to generate interaction networks of proteins. Next, Principal Component Analysis (PCA) was performed in SPSS (version 20.0), by which Pearson's correlation coefficients (PCC) were also calculated.

2.8. Statistical analysis

SPSS (version 20.0) were used to statistical analyses. All values were mean ± S.D. and assessed with ANOVA. OmicShare (www.omicshare.com/tools) was performed for data analysis of heat map.