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Copper-induced oxidative damage to the prophenoloxidase-activating system in the freshwater crayfish *Procambarus clarkii*

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ABSTRACT

Previous studies have demonstrated copper-induced proteins damage in gill and hepatopancreas of the freshwater crayfish *Procambarus clarkii*, but little information is available about its effects on key component of the innate defense in haemolymph. In the present study, we evaluated the relationship between oxidative carbonylation and prophenoloxidase-activating system (proPO-AS) activity, by exposing *P. clarkii* to sub-lethal concentrations (1/50, 1/12, 1/6 and 1/3 of the 96 h LC_{50}) Cu^{2+} up to 96 h. Six biomarkers of oxidative stress, i.e. reactive oxygen species (ROS), superoxide dismutase (SOD), catalase (CAT), protein carbonyl (PC), malondialdehyde (MDA) and DNA-protein crosslinks (DPCs), and six indicators of immune status, i.e. total hemocyte counts (THCs), differential hemocyte counts (DHCs), hemocyanin (HC), prophenoloxidase (proPO), serine protease (SP) and phenoloxidase (PO), were determined in haemolymph. The results indicated that there was a significant increase ($P < 0.05$) in the levels of ROS, PC, MDA and DPCs accompanied by markedly decreased ($P < 0.05$) activities of proPO, SP, PO and HC in a dose and time dependent manner. The significant and positive correlations ($P < 0.01$) between ROS production and the formation of PC, MDA and DPCs were observed in crayfish at 96 h. There was a significant negative correlation ($P < 0.01$) between the levels of protein carbonyls and the activities of proPO and SP in hemocyte lysate supernatant and PO and HC in haemolymph. Carbonylated proteins may be recognized not merely as a specific signal in oxidative stress pathways but also as a "non-self" molecule in proPO-AS. In crayfish species, copper-catalyzed protein carbonylation may be one of the main mechanisms for immunity dysfunction in proPO-AS.

1. Introduction

Copper (Cu) is one of the most ubiquitous pollutants in aquatic environment. The 2013 Report on the State of the Fishery Eco-Environment in China indicated that Cu contamination has been more and more serious in the important fishery areas [1]. As an essential trace element, Cu is also required for aquatic animals, serving as a catalytic cofactor in enzymes and as a structural component of proteins such as superoxide dismutase (SOD), phenoloxidase (PO) and hemocyanin (HC). It has been well noted that copper plays vitally important role in the immune system of crustaceans [2]. However, the optimal range between essential and toxic concentrations is found to be rather narrow. Even at low concentration, Cu could suppress the host's immune response, thus increasing its susceptibility to pathogens [3–5].

In crustaceans, Cu levels are tightly regulated by complex homeostatic mechanisms. Waterborne Cu is absorbed mainly by haemolymph in gill, whereas the hepatopancreas functions as storage and detoxification organ. Once in excess of cellular needs, Cu could catalyze the generation of reactive oxygen species (ROS) through the Haber-Weiss reaction. Small increase of ROS is considered to be beneficial with respect to increased immunity, but overproduction of ROS could induce oxidative damage to cellular macromolecules, including proteins, lipids and nucleic acids [2,5,6]. Normally, the deleterious effect of ROS can be counteracted by endogenous antioxidants like SOD and catalase (CAT). The two enzymes are not only used as biomarkers of oxidative stress but also implicated in the mediation of innate immunity [7].

The immune system of crayfish is a non-adaptive response, based on both cellular and humoral components. Circulating hemocytes are generally classified into three types, i.e. hyaline, semi-

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granular (SGC) and large granular (GC) cell, and they are responsible for various protective mechanisms ranging from phagocytosis and encapsulation to melanization [8]. The prophenoloxidase-activating system (proPO-AS) is an efficient non-self recognition system which consists of several important proteins including prophenoloxidase (proPO), pattern-recognition proteins (PRPs), serine protease (SP), and serine protease inhibitors (serpins). The proPO, an inactive proenzyme of phenoloxidase (PO), is synthesized in hemocytes, localized in the granules of SGC and GC and released into the haemolymph upon activation. An endogenous SP, the so-called prophenoloxidase activating enzyme (ppA), is at the center of this complex and restricted proteolysis cascade [9–11]. Finally PO catalyzes the production of the melanin and toxic reactive intermediates against invading pathogens. When proPO was knocked down via RNAi, invertebrate animals were easily infected by virulent bacteria and viruses [9]. Some research also showed that PO activity may originate from other sources such as HC, although in contrast to proPO, it is synthesized in the hepatopancreas [4,10]. At present, it is completely clarified that the proPO cascade is set off in a stepwise process with the recognition of microbial cell wall components, such as β-1, 3-glucan, lipopolysaccharide and peptidoglycan, by PRPs [12,13]. However, the mechanisms by which ROS regulates the proPO-AS in the case of pollutants exposure are still not fully understood.

As a redox active metal, Cu can directly catalyze highly reactive free radicals formation. Oxidative stress occurs when there is an imbalance between ROS and antioxidant defense molecules [5,7]. Proteins are the most abundant cell components (70%) and responsible for most functional processes in cells. So they are possibly the primary target for ROS assault, resulting in protein aggregation, inactivation or degradation [14–16]. Protein carbonylation is widely accepted as a biomarker of oxidative damage due to its relatively early formation and stability compared with other oxidative stress-induced protein modifications. Protein carbonyls (PC) groups can be generated directly by amino acids oxidation and α-amidation pathway or indirectly by forming adducts with lipid peroxidation products [17]. Malonaldehyde (MDA) is the main breakdown product of oxidized lipids, which reacts preferentially with Lys residues of proteins. The lipid peroxidation can influence membrane fluidity as well as the integrity of biomolecules associated with the membrane (membrane bound proteins or cholesterol) [18]. Recent reports indicated that there is a fairly strong correlation between the protein carbonylation and DNA-protein crosslinks (DPCs) induction [19,20]. DPCs are bulky, helix-distorting DNA lesions, which are created when cellular proteins become covalently captured on DNA strands upon exposure to various endogenous, environmental and chemotherapeutic agents. DPCs can interfere with DNA replication, transcription, and repair, potentially contributing to mutagenesis, genotoxicity and cytotoxicity [19]. While higher ROS levels could enhance the risk of protein carbonylation, lipid peroxidation and DNA damage, there is little information on oxidative lesions of hemocytes and their constituents in crustaceans.

The red swamp crayfish (*Procambarus clarkii*) is native to northeastern Mexico and south-central USA. Now it has become a commercially important freshwater aquaculture species in China. The farmed production reached 370 000 tons in 2010. Crayfish has been used as a sensitive bioindicator of heavy metals contaminants in aquatic environments [5,18]. Numerous studies conducted so far have shown that copper exposure could induce immunomodulation in crustaceans, possibly leading to a remarkable change in hemocyte numbers, PO activity and other immune-related proteins levels [2,5,11,21–23]. ROS are known to serve as second messengers in cellular signaling cascades that mediate responses to environmental stress. There was also evidence that carbonylated proteins are recognized not merely as a specific signal in stress-response but also as a "non-self" molecule in immunology system [6,15]. So we speculated that protein carbonylation may play an important regulatory role in proPO-AS activation. In the present study, we assessed the relationship between oxidative stress and proPO-AS activity after acute exposure to sublethal concentrations of Cu^{2+}, by determining biomarkers of oxidative stress (i.e. ROS, SOD, CAT, PC, MDA and DPCs) and indicators of immune status (i.e. hemocyte counts, proPO, SP, PO and HC) in haemolymph of the crayfish. The results will provide the cellular and molecular evidence for the possible immunotoxic mechanisms of environmental pollutants.

2. Materials and methods

2.1. Crayfish

Adult crayfish *P. clarkii* (8.5 ± 0.6 cm in length and 24 ± 1.5 g in wet weight) were purchased from a local aquatic product market. They were kept in glass aquaria (45 cm × 30 cm × 30 cm) filled with continuous aerated and dechlorinated tap water (pH 7.2 ± 0.4 and hardness 43.2 ± 1.3 mg $CaCO_3$/L). The water temperature was maintained at 23 ± 1 °C and the photoperiod was set to 12:12 (L:D). The animals were fed with commercial feed and acclimatized for 7 days prior to the experiment. Only apparently healthy males in the intermoult stage were used.

2.2. Chemicals

All chemicals were of analytical grade and purchased from Sigma (St. Louis, MO, USA) unless stated otherwise. Cu was added as $CuSO_4 \cdot 5H_2O$ (99% purity) in deionised water for stock solution. Test solutions were prepared by dilution of this stock solution with dechlorinated tap water to the desired concentration.

2.3. Exposure

Two hundred and twenty-five crayfish were randomly divided into five groups. There were triplicates for each test group with a total number of 45 crayfish (15 per replicate). Four sublethal concentrations of Cu^{2+} (nominal: 0.5, 2.0, 4.0 and 8.0 mg/L) corresponded to approximately 1/50, 1/12, 1/6 and 1/3 of the 96 h LC_{50} were chosen for the exposure. The control group was prepared with no additional copper. The 96 h LC_{50} value (25 mg/L Cu^{2+}) was based on our previous study of acute toxicity in this species. The Cu content in the dechlorinated tap water was 0.003 ± 0.001 mg/L and the actual Cu^{2+} level in each treatment group varied between 93 and 97% of the nominal concentrations, as determined with atomic absorption spectrometer (Varian Spectra AA 220, Palo Alto, CA, USA). The bioassays were carried out under static conditions without aeration and solution replacement for a period of 96 h. Crayfish were not fed during the experiment. All other conditions were kept the same as those used for acclimation.

2.4. Sampling

No crayfish died in any group during the trials. Three crayfish randomly selected in each aquaria were sampled at the interval of each 24 h. They were anaesthetized on ice for about 15 min. Haemolymph (0.9 mL) was withdrawn from the cardiac cavity of each crayfish into a 1.0 mL sterile syringe (25 gauge) containing 0.1 mL ice-cold anticoagulant solution. A drop of haemolymph was immediately placed on a hemocytometer for total hemocyte counts (THCs) and differential hemocyte counts (DHCs) assay.

Hemocyte lysate supernatant (HLS) was prepared following the method described by Pan et al. [24]. The diluted haemolymph