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Effects of uranium on crayfish *Procambarus clarkii* mitochondria and antioxidants responses after chronic exposure: What have we learned?

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ABSTRACT

We examined the impacts of Uranium (U) on mitochondria and on the response of antioxidants in the gills and the hepatopancreas of crayfish *Procambarus clarkii* after long-term exposure (30 and 60 days) to an environmentally relevant concentration (30 μg U/L). The expression of mitochondrial genes (*12s, atp6,* and *cox1*), as well as the genes involved in oxidative stress responses (*sod(Mn)* and *mt*) were evaluated. The activities of antioxidant enzymes (SOD, CAT, GPX and GST) were also studied. U accumulation in organs induced changes in genes' expression. The evolution of these transcriptional responses and differences between gene expression levels at high and low doses of exposure were also discussed. This study demonstrated that, after long-term exposure, U caused a decrease in antioxidant activities and induced oxidative stress. A possible ROS-mediated U cytotoxic mechanism is proposed. Expression levels of the investigated genes can possibly be used as a tool to evaluate U toxicity and seem to be more sensitive than the enzymatic activities. However a multiple biomarker approach is recommended as the perturbed pathways and the mode of action of this pollutant are not completely understood.

1. Introduction

Uranium (U) is a ubiquitous environmental trace metal, often found in water supplies as a non-essential inorganic component (Bleise et al., 2003). Concentrations in freshwater ecosystems are highly variable and range from 0.01 μg/L to over 12.4 mg/L, depending on the geological background (Salonen, 1994; WHO, 2001). U may occur in different oxidation states, the major forms being U(VI) in oxic water, and U(IV) in anoxic water (Markich, 2002). In addition to its natural occurrence and distribution in the environment, U has also several civilian and military applications that could cause its dispersion and increase its abundance in environmental compartments (Bleise et al., 2003). These anthropogenic activities include the use of phosphate fertilizers, various mining activities, and the industrial processing of U — including the use of depleted U — for the manufacture of nuclear fuel and other products (ATSDR, 1999). Depleted U (DU) is artificially obtained as a by-product of the U enrichment process and is about 60% less radioactive than natural U, which is considered a weakly radioactive element. Depleted U, however, retains all the chemical properties of natural U (WHO, 2001). Thus, effects from exposure of biota to either natural U or DU are usually attributed to their chemical toxicity (ATSDR, 1999). Nevertheless, the mechanisms by which this metal induces its toxicity have not been sufficiently investigated (Pourahmad et al., 2006; Barillet et al., 2007; Al Kaddissi et al., 2011). A greater knowledge of interactions between U and living organisms is needed in order to select pertinent biomarkers that could be used in ecological risk assessments. Certain studies have shown that this radioelement is able to chemically activate oxygen species in the course of redox reactions via the redox chemistry of transition metals (Miller et al., 2002; Yazzie et al., 2003). Moreover, Jones et al. (2003) stated that since U is an alpha-emitting radioactive element (WHO, 2001; Taulan et al., 2004) it can enhance the production of free radicals via the ionization phenomenon induced by alpha particle emissions. High quantities of free oxygen species (generated within cells) sometimes exceed the cell's protective controllability, resulting in damage to cell proteins, nucleic acids and lipids (Labrot et al., 1996; Barillet et al., 2007; Lourenço et al., 2010). In a previous study, carried out in our laboratory, we demonstrated that U alters the expression of some mitochondrial genes (*atp6*, *cox1*, *12S*) and genes involved in oxidative stress responses (*sod(Mn), mt*) in *Procambarus clarkii* after short-term exposure (4 and 10 days) to 30 μg/L (Al Kaddissi et al., 2011). We supposed that this radioelement can generate oxidative stress. Further experiments were,

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however, required to improve our understanding of the effects of U on *P. clarkii* and to choose potential markers of U toxicity. Thus evaluation of the responses of the selected biomarkers after long term exposure was needed. It was also necessary to investigate the responses of the classic major indicators of oxidative stress such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX) and glutathione S-transferase (GST). Since the genes of *P. clarkii* that encode for these antioxidants have not been sequenced, the investigation of oxidative stress induction had to be done by following the enzymatic activities. It is established that *P. clarkii* is a cosmopolitan species (Gherardi, 2006), relatively static, easily captured, bioaccumulates U (Al Kaddissi et al., 2011) and adults provide sufficient tissue for individual analyses. The use of this species as a biological model is therefore of great interest. In our current work the expression levels of mitochondrial genes (*12s, atp6* and *cox1*) and genes involved in oxidative stress responses (*sod(Mn)* and *mt*) were evaluated and the enzymatic activities of antioxidants (SOD, CAT, GPX and GST) were studied after 30 and 60 day exposure to 30 μg U/L. The concentration of U that we selected is within the range commonly found close to drill wells (Kurttio et al., 2006) and mining sites (e.g. 20 μg/L) (Simon and Garnier-Laplace, 2005). It is also double the World Health Organization provisional drinking water guideline (15 μg/L) (WHO, 2004) but equal to the recommended levels of the USEPA promulgated in 2000 (EPA 2000). Gills and hepatopancreas were collected after various periods of exposure to assess the different biological parameters and U bioaccumulation levels were studied in parallel. Comparisons between genes expression levels after short-term (4 and 10 days) (Al Kaddissi et al., 2011) and long-term exposures, and after high and low levels of contamination, were conducted. This approach helps identify possible changes in the mechanisms of action of U over time, and helps gather information to choose appropriate biomarkers of U contamination. The opportunity was also taken to link the antioxidant responses to the mitochondrial dysfunction.

2. Materials and methods

2.1. General experimental protocol

Adults inter-molt *P. clarkii* males were used in this study (23.55 ± 1 g fresh weight; 8.96 ± 0.11 cm from cephalothorax to telson; $n=30$). These were obtained from the Vigueirat swamp of Camargue, France (GPS coordinates: 43°31.863′N–4°45.417′E).

Crayfish were acclimatized to experimental conditions for 3 weeks under a 12/12 h light/dark photoperiod at 17 ± 1 °C while submerged in synthetic water equilibrated by means of air bubbling (water composition: $Ca^{2+}=1640$; $Mg^{2+}=500$; $Na^{2+}=870$; $K^{+}=80$; $Cl^{-}=4100$; $SO_4^{2-}=509$; $NO_3^{-}=76.5$; $HCO_3^{-}=281$ all in μmol/L; pH 6.6 ± 0.3). The artificial water composition, the pH value (6.5) and the voluntary lack of phosphate were chosen as a compromise to ensure abiotic conditions that satisfied the physiological needs of crayfish (Al Kaddissi et al., 2011) and to optimize U bioavailability (Fortin et al., 2007; Fortin et al., 2004). Speciation of 30 μg/L of U in the synthetic water at pH 6.5 was simulated using the geochemical speciation software J-CHESS (Java Chemical Equilibrium with Species and Surfaces, Van der Lee, 1998). Based on this simulation 0.7% of UO_2^{2+} and 4.6% of UO_2OH^{+} were present in the medium (Table S1). These forms of U are known to be the most bioavailable species to aquatic organisms (Markich, 2002, Fortin et al., 2004, 2007). The dominant species under these conditions were $(UO_2)_2CO_3(OH)_3^{-}$ (42%) and UO_2CO_3(aq.) (25.7%), which can be accumulated by organisms (Denison, 2004). Commercial trout pellets were fed to crayfish, at dosages of 0.1 g/individual, every 2 days. Nourishment continued during exposure conditions. During the acclimation and experimentation phases, animals were kept at a maximum density of 3 crayfish/L. Groups of 10 crayfish were exposed to 0 (control) and 30 μg U/L for 60 days. The stock solution of U used to contaminate water was prepared from depleted uranyl nitrate $UO_2(NO_3)_2 6H_2O$ (Sigma, France) and acidified with nitric acid (0.016 M). 25% of the water column was renewed daily by a continuous flow-through technique. A large volume of each test solution (0 and 30 μg/L) was prepared once a week and maintained at a constant flow through the respective tanks containing crayfish. U concentration in the water column was measured once daily and adjusted, if necessary, to ensure constant contamination pressure. Temperature and pH were monitored daily, whereas NO_3^{-}, NO_2^{-}, Cl^{-}, SO_4^{2-} and PO_4^{3-} concentrations were verified twice a week by ICS-3000, Ion Chromatography System. Crayfish were isolated in individual chambers (plastic netting: 1 cm mesh, 11 cm diameter) to avoid cannibalism. Five crayfish were sampled from each tank at Day 30 (T30) and Day 60 (T60). The hepatopancreas and gills of each individual were collected, split into three parts and stored at −80 °C for further bioaccumulation, gene expression and enzymatic activities' analyses.

2.2. Uranium quantification

Water samples were acidified by nitric acid (3.1 mM of HNO_3) prior to metal quantification by inductively coupled plasma-atomic emission spectrometry (ICP-AES Optima 4300DV, PerkinElmer, Wellesley/USA; detection limit: 10 μg/L ± 10%). Organs were dried at 45 °C for 2 days and, then digestion was performed in a DigiBLOC 3000 digestion system (SCP Science, Champlain, NY, USA). Thereafter, 3 mL of 15.5 M nitric acid was added to each organ and was heated for 90 min at 95 °C after which samples were evaporated in a second heating cycle (60 min, 105 °C). The digestion process was then completed by the addition of 2 mL H_2O_2 (33%) followed by evaporation (20 min, 105 °C). Samples were dissolved in acidified water (3.1 mM of HNO_3) prior to analysis. ICP-AES was used to determine the highest U concentrations and inductively coupled plasma mass spectrometry (ICP-MS Agilent 7500 Cs; detection limit: 10 ng/L ± 7%) to determine the lowest concentrations. Combined tests, using ICP-AES and ICP-MS techniques, were also run to validate the assumption that there was no significant difference between results from these two analytical methods.

Table 1
Genes, accession numbers, specific primers pairs used in the quantitative PCR analysis of the 6 studied genes of *P. clarkii* and their function.

Gene	Primers (5′–3′)	Function and accession number
cox1: Cytochrome C oxydase subunit 1 (complex IV).	AATGGGATACCTCGACGTTATTCA[a] GCAGGAGGATAAGAATGCTGT[b]	Encoding for an enzyme in the inner membrane of mitochondrion that helps establish a transmembrane gradient of protons. AY701195.1
atp6: ATP syntase subunit 6 (complex V).	GGCAGCAAATATAATTGCTGG[a] TTGCAACGGCAGATTCTAATAT[b]	Encoding for an enzyme in the inner membrane of mitochondrion that uses the gradient of protons created by other complexes to synthesize ATP. GU220369.1
12S: Ribosomal RNA 12 S.	ACTAGAATATTAGGAGTTATGTTCTT[a] GCTGCACCTTGATCTAATATAC[b]	Indicator of the amount of mitochondria in cells. EF012280.1
Sod(Mn): Mitochondrial Mn- Superoxyde dismutase.	GCCACCACTAAAATACGAGTA[a] CCATTGAACTTTATAGCTGGTA[b]	Encoding for an enzyme involved in the fight against oxidative stress. EU254488.3
mt: Metallothionein.	CGAGGGCGGGTGCAAGACT[a] CTTGGAGCAGGTCTTGGCAC[b]	Encoding for MT protein involved in detoxifying Cd and in the protection against reactive oxygen species. GU220368.1
18s: 18S ribosomal RNA.	GCAATAACAGGTCTGTGATGCC[a] AGGGACGTAATCAGCGCAA[b]	Housekeeping gene. X90672.1

[a] Forward primer.
[b] Reverse primer.