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Low-concentration uranium enters the HepG2 cell nucleus rapidly and induces cell stress response

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ABSTRACT

This study aimed to compare the cell stress effects of low and high uranium concentrations and relate them to its localization, precipitate formation, and exposure time. The time-course analysis shows that uranium appears in cell nuclei as a soluble form within 5 min of exposure, and quickly induces expression of antioxidant and DNA repair genes. On the other hand, precipitate formations began at the very beginning of exposure at the 300-μM concentration, but took longer to appear at lower concentrations. Adaptive response might occur at low concentrations but are overwhelmed at high concentrations, especially when uranium precipitates are abundant.

1. Introduction

Uranium is a radionuclide but also a heavy metal to which humans can be exposed due to its natural presence or human activities. Numerous studies of cells from different species (rats, pigs, and humans) have established not only its cytotoxicity but also the dependence of this toxicity on cell type and on uranium isotope composition and speciation (Carriere et al., 2004; Carriere et al., 2006; Milgram et al., 2008b; Rouas et al., 2010). The mechanisms of toxicity have not been fully elucidated, however, especially at low concentrations.

Mechanisms proposed to explain its cytotoxicity, including oxidative stress, genotoxicity, and apoptosis have been observed at high concentrations and at only a few time points (Miller et al., 2002; Shaki et al., 2013; Thiebault et al., 2007; Vicente-Vicente et al., 2010). The potential mechanism investigated most often to explain uranium toxicity is oxidative stress induction, explored both *in vitro*, in cell cultures and *in vivo* after acute or chronic exposure (Banday et al., 2008); (Poisson et al., 2014) (Shaki et al., 2012); (Taulan et al., 2006); (Taulan et al., 2004); (Thiebault et al., 2007). The results of these studies suggest that uranium administered at high concentrations to cells or animals induces a RedOx imbalance, with increased production of reactive oxygen species (ROS) and depletion of endogenous cellular antioxidants.

The relation between uranium penetration into and distribution within cells and its toxicity has been analyzed in different cell types. Some authors have proposed that uranium does not need to penetrate cells to exert its toxic effects (Leggett, 1989); (Muller et al., 2006), while others have argued the opposite (L'Azou et al., 2002); (Mirto et al., 1999) and showed that uranium speciation influences its toxicity (Carriere et al., 2006). Recently, our group proposed that the physical form of uranium (soluble or precipitate) and its intracellular localization play a role in cell toxicity (Rouas et al., 2010), after we demonstrated that soluble uranium localizes mainly in the nucleus in kidney, hepatocyte, and neuron cell cultures, as observed after 24 h of exposure to concentrations less than 100 μM. SIMS (secondary ion mass spectrometry) analysis made it possible to observe soluble uranium within the nuclei of cultured cells for the first time (Rouas et al., 2010) and, more recently, *in vivo* in chronically exposed rats (Poisson et al., 2014; Tessier et al., 2012). High concentrations of uranium lead to the formation of uranium needles, initially named uraniosomes when they were first observed by Ghadially with transmission electronic microscopy (TEM) (Ghadially

Abbreviations: APEX1, apurinic/apyrimidinic (AP) endodeoxyribonuclease; BAX, BCL2-associated X protein; BID, BH3 interacting domain death agonist; CAT, catalase; DHE, dihydroethidium; DU, depleted uranium; HO-1, heme oxygenase; ICP-MS, inductively-coupled plasma mass spectrometry; LDH, lactate dehydrogenase; NEIL, endonuclease VIII-like DNA glycosylase; NRF2, Nuclear Factor Erythroid 2-Related Factor; OGG1, 8-oxoguanine DNA glycosylase; ROS, reactive oxygen species; SIMS, secondary ion mass spectrometry; SOD, superoxide dismutase; TEM, transmission electronic microscopy.

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et al., 1982). Endocytosis may be responsible for forming these precipitates, composed mainly of phosphate, potassium, and calcium, in lysosomes (Carriere et al., 2008; Mirto et al., 1999; Muller et al., 2008). Uranium can thus be present in cells in either a soluble form or a precipitate, depending on concentration and probably exposure time. Nevertheless, it remains unclear whether the presence of soluble uranium in the nucleus at low concentrations produces cell stress and whether the formation of uranium precipitates increases uranium toxicity. To investigate the mechanism of toxicity, especially at the low concentrations that are closer to real exposure situations, it is necessary to study the relation between the localization/distribution of uranium and the cell stress effect.

In this study, for the first time, we investigate the time course of uranium uptake, precipitation, and output simultaneously with cell stress response after exposure to 10–1000 μM of uranium for periods of 5 min to 24 h. A hepatic cell model is used, as previously, both to study uranium localization and cell stress response (Guéguen et al., 2014; Miller et al., 2004; Pourahmad et al., 2006; Rouas et al., 2010) and as a reference model for the toxicological study of exposure to xenobiotics or heavy metals.

2. Materials and methods

2.1. Materials

A solution of uranyl nitrate hexahydrate ($UO_2(NO_3)_2.6H_2O$) (AREVA-COGEMA, France) was prepared to a depleted uranium (DU) concentration of 10 mM by dissolving the powder in 100 mM sodium bicarbonate (HCO_3^-). The radioactive specific activity of DU is $1.4.10^4$ Bq/g and its isotopic composition is $^{238}U = 99.74\%$, $^{235}U = 0.255\%$, and $^{234}U = 0.0055\%$ (AREVA-COGEMA).

Roswell Park Memorial Institute medium (RPMI 1640, ref 21875034), penicillin/streptomycin 10 000 U/mL (PS, ref 15140), fetal bovine serum (FBS, ref 10270-106), and L-glutamine (ref 25030-024) were purchased from Life Technologies (Cergy-Pontoise, France).

HepG2 cells were obtained from ATCC (Molsheim, France). Plastics used for cell cultures were purchased from VWR (Fontenay-sous-bois, France).

2.2. Cell culture

HepG2 cells were grown in a monolayer culture in RPMI supplemented with 10% FBS, 1% PS in an incubator with a humidified atmosphere (*i.e.*, 37 °C, 5% CO_2) to a confluence of 80%.

2.3. Uranium exposure

The DU stock solution (10 mM, pH 6.8) was prepared immediately before use by dissolving 0.5% (w/v) of uranyl nitrate in $NaHCO_3$ solution (100 mM). The exact concentration was verified by ICP-MS. DU solutions used for experiments were prepared by diluting stock solution in cell culture media. Cells were incubated with 8 increasing DU concentrations (10–1000 μM) for 15 min to 24 h for cell stress studies, 4 DU concentrations (10–300 μM) for 5 min to 24 h for SIMS microscopy, and one concentration (100 μM) for 15 min to 24 h for uranium measurement by ICP-MS. Experiments were repeated at least three times for each exposure condition. Because uranium precipitates in cell culture media at high concentrations, 1000 μM was set as the maximal concentration.

2.4. Preparation of the biological samples for SIMS and ICP-MS analyses

After the DU-exposure phase (5 min to 24 h), the culture medium was removed and the cells underwent a standard chemical fixation procedure. Cells were fixed on their culture plates with a solution containing 2.5% glutaraldehyde for 1 h at room temperature and then dehydrated in ethanol baths for SIMS and ICP-MS analyses.

For the SIMS analysis, cells were then permeabilized with an ethanol/Epon mixture and finally embedded in pure EPON-type resin. Serial thin sections (0.5 μm) embedded in resin were cut and laid on polished ultrapure silicon holders for SIMS analysis (to avoid relief effects and minimize charge effects) or on glass slides for histological controls with an optical microscope. This procedure allows the cells to be observed in their physiologic state.

For ICP-MS analysis, cells were then scraped with 1 mL of 2% nitric acid (Aristar quality grade, VWR Prolabo) and put in tubes containing 4 mL of 69% nitric acid. Before this, 5 mL of the supernatant (cell culture media) was collected in a tube containing 300 μL of 69% nitric acid to quantify the uranium remaining in the medium.

2.5. Cellular distribution of uranium with SIMS

The aim of SIMS (secondary ion mass spectrometry) microscopy is to analyze the elemental and isotopic composition of a solid surface by an ion beam coupled with a mass spectrometer. The principle of this technique has been previously described (Rouas et al., 2010). The SIMS analyses were performed with a CAMECA IMS 4F E7 instrument. For this study, O_2^+ beam bombardment was used to enhance the ionization field, composed of electropositive species such as uranium. In this scanning microscope, the primary beam is focused into a small shaft (around 0.5 μm), which scans the sample surface. The secondary ions collected after mass filtering can be measured with an electron multiplier and also sequentially converted into an image. Mass resolution can reach $M/\Delta M = 10\ 000$ and the lateral resolution of the imaging is only 0.5 μm. For each area analyzed, mass spectra at around the mass of isotope 238 of uranium and ionic images were obtained. $^{40}Ca^+$ and $^{23}Na^+$ images show the histological structure of the cells and $^{238}U^+$ images the uranium microdistribution within these structures.

2.6. Quantification of uranium accumulation with ICP-MS

Uranium was quantified in the monolayer cultures and in the supernatant media by inductively-coupled plasma mass spectrometry (ICP-MS) (ICP-MS, PQ, Excell, Thermo Electron, France). The ICP-MS instrument used to perform the analyses was a 7700× series (Agilent Technologies, Les Ulis, France), calibrated with a SPEX CertiPrep uranium standard solution (Jobin Yvon, Longjumeau, France).

Before analysis, supernatant was diluted in 2% nitric acid 1/1 000°000 for cell cultures exposed to DU for 15 min to 24 h. Each cell layer was diluted in 2% nitric acid 1/1000 for cell cultures exposed to DU for 15 min to 8 h, and 1/100°000 for cell cultures exposed to DU for 12, 16, and 24 h.

2.7. Cell viability tests

DU cytotoxicity was determined by using LDH (lactate dehydrogenase) to calculate cell viability or cell death compared with that of untreated cells. The LDH test (Cytotox detection kit, Roche Diagnostic; Meylan, France) was used according to the manufacturer's instructions.

2.8. Real time RT-PCR

Reverse transcription (RT) and real-time PCR were performed with the Power SYBR Green Cells-to-Ct kit in accordance with the manufacturer's instructions (Life Technologies, France). Cultured cells were washed with cold phosphate-buffered saline (PBS), mixed with the lysis solution from the kit, and incubated for 5 min. Cell lysates were reverse-transcribed to synthesize cDNA with the RT enzyme. Finally we used the Power SYBR Green Master Mix kit and the PCR primers listed in Table 1 to amplify the cDNA by real-time PCR. Samples were normalized to glyceraldehyde 3-phosphate dehydrogenase