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Pro-oxidative, genotoxic and cytotoxic properties of uranyl ions

S.A. Garmash [a,b,1], V.S. Smirnova [a,1], O.E. Karp [a], A.M. Usacheva [a], A.V. Berezhnov [c], V.E. Ivanov [a], A.V. Chernikov [a,b,c], V.I. Bruskov [a,b], S.V. Gudkov [a,b,*]

[a] Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia
[b] Pushchino State University, Pushchino, Moscow Region 142290, Russia
[c] Institute of Cell Biophysics, Russian Academy of Sciences, 3 Institutskaya Street, Pushchino, Moscow Region 142290, Russia

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ABSTRACT

It is demonstrated that hydroxyl radicals and hydrogen peroxide are formed under the action of uranyl ions in aqueous solutions containing no reducing agents. In the presence of uranyl ions, formation of 8-oxoguanine in DNA and long-lived protein radicals are observed *in vitro*. It is shown that the pro-oxidant properties of uranyl at micromolar concentrations mostly result from the physico-chemical nature of the compound rather than its radioactive decay. Uranyl ions lead to damage in DNA and proteins causing death of HEp-2 cells by necrotic pathway. It is revealed that the uranyl ions enhance radiation-induced oxidative stress and significantly increase a death rate of mice exposed to sublethal doses of X-rays.
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1. Introduction

There are three main isotopes of uranium: ^{238}U (99.3%), ^{235}U (0.7%), and ^{234}U (0.005%), and several relevant oxidation states: II, III, IV, V, and VI (Zhengji, 2010). Depleted uranium (DU) is the byproduct of uranium enrichment, containing 0.3–0.4% of ^{235}U and 60% of the radioactivity of natural uranium (Bleise et al., 2003). The high density (19.1 g/cm^3) and pyrophoric properties of DU make it useful in several civilian and military applications. When DU ammunition hits hard targets, it causes formation of aerosols of uranyl ions and solidified molten particles (Sajih et al., 2010). DU was used in armor-piercing shells during international military conflicts and was claimed to contribute to health problems. The soil chemistry converts uranium oxides to uranyl salts, which then enter the plant and animals organisms with water and eventually can reach the human via the food chains. In the soil and water solution, uranium can exist as free uranyl ion or be complexed with hydroxides, carbonates, phosphates, and sulfates (Duquene et al., 2010).

The high toxicity of depleted uranium leads to malignant diseases (Miller et al., 2009) and mass ailments of unknown etiology (Bleise et al., 2003). Particles of uranium oxides, mainly UO_2, have been found in the soil near Kosovo, where depleted uranium shells had been fired (Salbu et al., 2003). Similar particles of enriched ^{235}U polluted the environment after the Chernobyl disaster (Salbu et al., 2003) and could have caused mass morbidity among the liquidators.

The accumulation of uranyl ions in the body leads to various physiological disorders (Barillet et al., 2011) and is associated with a high geno- and cytotoxicity (Heintze et al., 2011). External exposure to DU does not present a significant health hazard, but internal exposure via inhalation of dust particles containing uranyl ions may lead to adverse health effects due to both chemical and radiological toxicity. However, the toxicology of uranyl compounds is poorly studied. It was assumed that toxicity of DU at molecular level is related to its radioactivity (Bleise et al., 2003) and the ability to generate hydroxyl radicals in the presence of reducing agents (Miller et al., 2002a,b). It was suggested that the toxicity of DU at low concentrations is mostly related to physico-chemical properties of uranyl ions (Miller et al., 2002a,b;Smirnova et al., 2005b; Vanhoudt et al., 2011).

Thus, the main objectives of this study were: (1) to investigate the formation of reactive oxygen species (ROS) in the aqueous

Abbreviations: DU, depleted uranium; MN, micronuclei; PCE, polychromatophilic erythrocytes; DMF, dose modification factor; 8-oxoGua, 8-oxoguanine (8-oxo-7,8-dihydroguanine); ROS, reactive oxygen species.

* Corresponding author. Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, 3 Institutskaya Street, Pushchino, Moscow Region 142290, Russia. Tel.: +7 4967 739497; fax: +7 4967 330553.
E-mail address: S_makariy@rambler.ru (S.V. Gudkov).
[1] These authors contributed equally to this work.

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solutions under the influence of uranyl ions at different temperatures in the absence of exogenous reducing agents; (2) to evaluate possible contributions of chemical and radioactive components to the toxicity of uranyl ions at micromolar concentrations; (3) to determine whether uranyl ions aggravate the consequences of oxidative stress.

2. Materials and methods

2.1. Exposure of samples

All solutions were heated in a U10 ultrathermostat (Prufgerate-Werk Medingen, Germany). X-ray-irradiation was carried out on an RUT-15 therapeutical X-ray device (Mosrentgen, Moscow, Russia) at a dose rate of 1 Gy/min (animals) (focal distance 0.375 m, current 15 mA, voltage 200 kV) or 4.5 Gy/min (DNA solution for immunoenzyme analysis) (focal distance 0.195 cm, current 20 mA, voltage 200 kV). Laser irradiation was carried out with a LGN 208A helium–neon laser (MedApparatura, Russia) (1.7 mW, flow density 0.7 mW/mm^2) with an emission band at 632.8 nm in the darkness at room temperature.

2.2. Determination of hydroxyl radicals

Hydroxyl radicals were detected using a highly specific fluorescent probe, coumarin-3-carboxylic acid. Experimental conditions have been described in detail elsewhere (Gudkov et al., 2006). Uranyl nitrate and sodium nitrate solutions in bidistilled water were heated in glass vials for liquid scintillation counter (Beckman, USA). Fluorescence intensity was measured on a Cary Eclipse spectrofluorometer (Varian, Australia) at λ_{ex} = 400 nm and λ_{em} = 450 nm in a mirror quartz cell at room temperature. Solutions of 7-OH-coumarin-3-carboxylic acid of known concentrations were used for the calibration of results.

2.3. Determination of hydrogen peroxide

The highly sensitive method of enhanced chemiluminescence in the luminol–*p*-iodophenol–horseradish peroxidase system was used (Bruskov et al., 2003; Gudkova et al., 2005). The reaction was quantified with a Beta-1 liquid scintillation counter (MedApparatura, Ukraine). The counter was used in single photon counting mode with one photomultiplier and the coincidence scheme disengaged. Uranyl nitrate and sodium nitrate solutions were heated in glass vials for liquid scintillation counter (Beckman, USA). The hydrogen peroxide solutions of known concentrations were used for the calibration of measurements.

2.4. Determination of 8-oxoguanine (8-oxoGua) in DNA

The method of immunoenzyme analysis was described in detail previously (Smirnova et al., 2005a). DNA was immobilized by a simple dry adsorption procedure until the solution had evaporated. The details of the calibration procedure have been published elsewhere (Shtarkman et al., 2008).

2.5. Determination of protein radicals

The method (Gudkov et al., 2007) and chemiluminometer characteristics (Bruskov et al., 2009) were described in detail previously. Protein concentration was 1 g/l, because we found in previous experiments that maximal sensitivity of the method was reached at this concentration (Gudkov et al., 2010a).

2.6. Animals

Males of random-bred white Kv:SHK mice aged 5–6 weeks and weighing 18–22 g (nursery Kryukovo, Russian Academy of Medical Sciences) were used in the experiments. The animals were housed in polypropylene cages with sawdust as bedding material. They were maintained under controlled conditions of temperature (22 ± 3 °C) and were given standard commercial mouse feed (Arno, Russia) and drinking water *ad libitum*. Radiobiological and physiological parameters and parentage of Kv:SHK mice were previously described in detail (Andrievsky et al., 2009; Gudkov et al., 2009). Animal handling was done according to institutional guidelines for animal care. All experiments with mice were approved by the institutional bioethics committee.

2.7. Measurement of protein oxidation levels

To evaluate the intensity of oxidative modification of proteins in the blood serum of mice, we employed a technique based on the reaction of oxidized amino acid residues of proteins (aldehyde and ketone groups) with 2,4-dinitrophenylhydrazine (DNFG) to form its derivatives. The method of protein carbonyls analysis was described previously (Lenz et al., 1989). Level of protein carbonyls was assessed in protein pellets left after preceding centrifugation of blood plasma with trichloroacetic acid. 2,4-Dinitrophenylhydrazine (10 mM in 2 M HCl) was added to the pellets. The reaction mixture was incubated for 1 h at 37 °C and then centrifuged (5000 g, 15 min). The pellets were rinsed triply with ethanol–ethylacetate mix (1:1). Washed pellets were dissolved in 6 M carbamide. Insoluble material was separated by centrifugation. The content of protein carbonyl bonds was estimated in supernatants taking molar extinction coefficient to be 22,000 $M^{-1} \times cm^{-1}$.

2.8. Cell culture

HEp-2 cells (kindly provided by Prof. Vladimir S. Akatov, Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences) were grown on 24-well plates in Dulbecco's modified Eagle's medium (DMEM) (Sigma, USA) supplemented with 2 mM L-glutamine, 30 μg/ml gentamicin and 15% fetal bovine serum (Gibco, USA) in CO_2-incubator at fully humidified atmosphere at 37 °C, 5% CO_2 and 95% room air. The initial cell density was adjusted to 1×10^4 cells per well. At 72 h after seeding, cells were treated with uranyl nitrate and cultivation continued. At 48 h after treatment, cells were washed with Hank's balanced salt solution (HBSS) (PanEko, Russia) and stained with Hoechst 33342 (5 μg/ml), propidium iodide (1 μg/ml) for 20 min at 37 °C. Then cells were washed twice with dye-free HBSS.

2.9. Fluorescent microscopy

Apoptotic and necrotic cells in culture were detected using Aviovert 200M microscope (Carl Zeiss, Germany) equipped with AxioCam HSm monochrome CCD camera. HBO 103 mercury lamp was used as the excitation light source. Hoechst 33342 fluorescence was excited at 365 ± 20 nm and emission was collected at 425 ± 25 nm. Propidium iodide fluorescence was excited at 540 ± 10 nm, emission was collected at 630 ± 40 nm. Five 3-channel (transmitted light, blue channel and red channel) images from random areas within each well were acquired. Cells in each well were divided into 3 groups according to their Hoechst 33342 (blue), propidium iodide (red) fluorescence intensity: (1) viable cells, which showed weak both blue and red fluorescence; (2) apoptotic cells, which showed strong blue fluorescence but weak red fluorescence; (3) necrotic cells, which showed strong both blue

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