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Fluoride-induced death of rat erythrocytes *in vitro*

Natalia I. Agalakova *, Gennadii P. Gusev

Sechenov Institute of Evolutionary Physiology and Biochemistry, Russian Academy of Sciences, Sankt-Petersburg, Russia

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ABSTRACT

Although fluoride (F) in low concentrations is essential for teeth and bone development, its excessive consumption causes numerous deleterious abnormalities in cellular metabolism and physiology often leading to cell death. The present study was performed to establish the toxic F effects inducing the death of rat erythrocytes *in vitro*. The cells were cultured in the presence of 0.5–16 mM NaF for 1, 5 and 24 h. The progression of erythrocyte death was monitored by cell viability (calcein assay), membrane integrity (hemolysis assay), alterations in the cell morphology (light microscopy) and size (flow cytometry forward scatter), plasma membrane scrambling (annexin V binding). To elucidate the molecular mechanisms underlying F-induced cell death, the cytosolic Ca^{2+} activity (Fluo-3 fluorescence) and ceramide formation (binding of FITC-labeled antibodies) were determined. Exposure of the rat erythrocytes to NaF considerably suppressed their viability and caused partial cell hemolysis within 24 h. The cells underwent dramatic morphological alterations resulted in appearance of shrunken echinocytes after 1 h and swollen spherocytes within 24 h. The development of NaF-induced erythrocyte death was accompanied by progressive PS externalization at the outer cell membrane, ~45% of the cells were annexin V-positive in response to 16 mM NaF within 24 h with a small cell population exhibiting necrotic features. The cell death was preceded by considerable accumulation of the free cytosolic Ca^{2+}, with statistically significant increase in the number of Fluo-3-positive erythrocytes observed as early as during 1-h incubation with 0.5 mM NaF. NaF also induced moderate ceramide formation. Overall, exposure of the rat erythrocytes to NaF triggers rapid progression of their death in a dose- and time-dependent manner, with appearance of apoptotic cells after 1 and 5 h and transition to necrosis within 24 h. An increase in intracellular [Ca^{2+}] appears to be crucial mechanism implicated in development of NaF-induced apoptosis in rat erythrocytes.

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1. Introduction

Fluoride (F) is ubiquitous natural compound and widespread industrial pollutant released in the environment through a combination of natural and anthropogenic processes. In low concentrations F has been proven to be beneficial for teeth and bone development, therefore, the prophylactic supplementation of drinking water sources with F is widely used in many countries over several decades (WHO, 2006; Pizzo et al., 2007). However, in the developed countries a wide range of F-containing topical agents and supplements designed to reduce dental caries became widely available what makes the consumption of F by humans uncontrolled often exceeding its therapeutic window. In addition, F compounds are widely used in industry and agriculture, they are present in domestic chemicals such as cleaning products, insecticides, rodenticides. As a result, each year there are thousands of reports related to acute or lethal poisoning due to excessive ingestion of F-containing dental products and accidental or suicidal exposure to F-containing chemicals at home, industrial workplaces and laboratories. Chronic consumption of high F doses results in adverse health effects such as dental and skeletal fluorosis, arthritis, osteoporosis, infertility, mental retardation (Aoba and Fejerskov, 2002; Tang et al., 2008; Bronckers et al., 2009; Dhar and Bhatnagar, 2009). Endemic fluorosis is a serious national problem in many counties affecting millions of people using ground waters with high F content for their daily needs (Krishnamachari, 1986; Reddy, 2009).

The F toxicity is associated with numerous deleterious effects on cell metabolism and physiology such as inflammatory reactions, oxidative stress, leading to the generation of reactive oxygen species and lipid peroxidation, inhibition of protein synthesis and cell cycle progression, alterations in the gene expression and DNA damage (Barbier et al., 2010). Most of these processes ultimately lead to apoptosis. The F ability to trigger apoptosis has been demonstrated *in vivo* and *in vitro* in cells from different organs including kidneys (Xu et al., 2006; Bai et al., 2010), liver (Ha et al., 2004; Wang et al., 2004; Zhan et al., 2006), lung (Thrane et al., 2001; Refsnes et al., 2003), pancreas (Elliot et al., 2001), brain (Ge et al., 2006; Zhang et al., 2007), as well as in fibroblasts (Lee et al., 2008),

* Corresponding author.
E-mail addresses: agalak@iephb.ru, nagalak@mail.ru (N.I. Agalakova).

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ameloblasts (Kubota et al., 2005; Yan et al., 2007), odontoblasts (Karube et al., 2009), osteoblasts (Qu et al., 2008; Yan et al., 2009), thymocytes (Matsui et al., 2007), endometrium (Guney et al., 2007), oral mucosa (He and Chen, 2006; Tsai et al., 2008) and HL-60 leukemic cells (Anuradha et al., 2000, 2001; Otsuki et al., 2005).

Blood is a major tissue participating in the distribution of F (Whitford, 1994), however, F ability to induce red blood cells (RBCs) death, as well as the molecular mechanisms underlying this process, have not been sufficiently investigated. Two studies described the development of anemia in cattle afflicted with fluorosis (Hillman et al., 1979) and in mice exposed to sublethal F doses (Bhaskara Rao and Vidyunmala, 2010) what might indicate the premature erythrocyte death. Susheela (2001) reported that intoxication of the human body with F was associated with severe anemia due to shorter erythrocytes lifespan caused by membrane degeneration that turns them into echinocytes. Recently, we shown that exposure of the rat erythrocytes to NaF induced pronounced inhibitory impact on the transport of monovalent cations across plasma membrane associated with the Na^+–K^+-pump inhibition and Ca^{2+}-dependent K^+ loss (Agalakova and Gusev, 2008). The loss of cellular K^+ was not compensated by Na^+ accumulation indicating erythrocyte shrinkage. Moreover, the observed Ca^{2+}-dependent changes in Na^+ and K^+ concentrations might be the early events involved in development of F-induced apoptosis.

The present experiments were designed to investigate the toxic F effects leading to cell death on a model of isolated rat erythrocytes *in vitro*. Low NaF doses (0.5–4 mM) applied in our study give the values of ionic F concentrations determined in the human plasma in the clinical cases of sublethal and lethal F poisoning (Kaa et al., 1986; Baselt, 2004; Lech, 2011). The early cellular apoptotic responses to toxic NaF action were examined during 1-h incubation. High NaF concentrations (8–16 mM) and prolonged incubations (5–24 h) were applied to monitor the cell transition from apoptotic to necrotic state.

2. Materials and methods

2.1. Erythrocyte suspension

The experiments were conducted on erythrocytes of healthy 8–12 weeks-old male Wistar rats weighting 150–200 g. The animals were housed in a temperature-controlled room at 20–23 °C under a 12-h light/dark cycle with free access to the standard rat chow and nominally F-free tap water. All experimental procedures were performed in accordance with the Animal Welfare act and the Institute Guide for Care and Use of Laboratory Animals. The blood from 7 to 12 animals was used in each experimental series.

The rats were anesthetized with ether and the blood from tail vein was collected into tubes containing cold washing solution and heparin. The blood was immediately centrifuged (3000 rpm at 4 °C for 5 min), plasma and upper layer of white cells were discarded, and erythrocytes were washed three times with the same solution. The resulting erythrocytes were suspended in the standard incubation medium to 10% hematocrit.

The cell suspension aliquots (100 μl) were added to 900 μl of incubation medium (final hematocrit of 1%) and preincubated for 1 h at 37 °C under 5% CO_2/95% O_2 atmosphere. Then the cells were incubated under the same conditions with increasing NaF concentrations (0.5–16 mM) for 1, 5 and 24 h.

To confirm the validity of analysis, in some experiments we employed Ca^{2+} ionophore ionomycin as a positive control. In human erythrocytes ionomycin has been shown to be a potent inducer of cell shrinkage and phosphatidylserine (PS) externalization, the characteristic apoptotic features (Berg et al., 2001; Lang et al., 2003). Rat erythrocytes were treated with 0.5 μM ionomycin for 1 h in parallel with NaF.

2.2. Solutions and chemicals

Washing solution contained (mM) 137 NaCl, 4 KCl, 1 $MgSO_4$, 1 $CaCl_2$, 5 $NaHCO_3$, 0.3 Na_2HPO_4, 0.4 KH_2PO_4 (pH 7.4 at 5 °C). Incubation medium contained (mM) 137 NaCl, 4 KCl, 1 $MgSO_4$, 1 $CaCl_2$, 5 $NaHCO_3$, 0.3 Na_2HPO_4, 0.4 KH_2PO_4, 10 glucose (pH 7.4 at 37 °C). The incubation medium was supplemented with 1% (w/v) penicillin–streptomycin. Annexin-binding buffer contained (mM) 140 NaCl, 2.5 $CaCl_2$, 10 HEPES/NaOH (pH 7.4 at room temperature).

NaF, calcein-AM, monoclonal mouse anti-ceramide antibodies (clone MID 15B4), anti-mouse IgG-FITC and all buffer salts were obtained from Sigma Aldrich (St. Louis, USA). Fluo-3 AM, FITC-conjugated annexin V and ionomycin were purchased from Molecular Probes/Invitrogen (Eugene, USA).

2.3. Determination of cell viability and membrane integrity

The viability of rat erythrocytes was assessed using calcein-AM (calcein acetoxymethyl ester) according to procedure described by Bratosin et al. (2005). Calcein, non-fluorescent membrane-permeable dye, rapidly enters viable cells where it is converted by the cytosolic esterases into green fluorescent calcein retained in the cells with intact membranes but extruded from dying or damaged cells. Calcein-AM was prepared as a 10 mM stock solution in DMSO, aliquoted and stored at −20 °C. The stock solution was diluted by incubation medium to 100 μM working solution before each experiment. The aliquots of control and NaF-treated RBCs (100 μl, 1% hematocrit) were incubated with 5 μM calcein-AM for 45 min at 37°C in the dark. Then the samples were diluted in 1 ml of incubation medium for immediate flow cytometry. Flow cytometric analysis was performed on EPICS XL cytometer (Beckman Coulter Inc., Brea, CA, USA) using SYSTEM II (Version 3.0) software for acquisition and analysis. The fluorescence channel FL-1 was set on logarithmic scale. The viability of 3×10^4 cells was analyzed in each experiment.

The integrity of erythrocyte plasma membrane was determined in hemolysis assay. After incubation with NaF the samples were sedimented (3000 rpm at 4 °C for 5 min) and the supernatants were collected. The cell hemolysis was measured photometrically at 405 nm based on the hemoglobin (Hb) content in the supernatant. The absorption of the supernatant of erythrocytes lysed in distilled water was taken as 100% hemolysis.

2.4. Detection of erythrocyte morphology and size

Changes in the rat erythrocyte shape were monitored using H605T Trinocular Microscope (WPI Inc., USA), objective ×100, with the digital camera Leica DFC300 FX and Leica IM50 software. Alterations in the relative erythrocyte size were determined by flow cytometry using forward scatter (FSC) correlating with cell volume and size. Forward scatter was set on the linear scale.

2.5. Annexin V-FITC labeling

PS exposure at the outer plasma membrane leaflet was measured in annexin-binding assay introduced by Vermes et al. (1995). Following NaF treatment, the erythrocytes were washed twice with the standard solution and resuspended (1% hematocrit) in annexin-binding buffer. Then the cells were stained with FITC-annexin V (1:20) in the dark for 15 min, diluted with the same buffer to 0.01% hematocrit and subjected for flow cytometry. Annexin V fluorescence intensity was measured in FL-1 with 488 nm excitation wavelength and 530 nm emission wavelength.

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