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Apoptotic death in erythrocytes of lamprey *Lampetra fluviatilis* induced by ionomycin and tert-butyl hydroperoxide

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ABSTRACT

The work examined the effects of Ca^{2+} overload and oxidative damage on erythrocytes of river lamprey *Lampetra fluvialtilis*. The cells were incubated for 3 h with 0.1–5 μM Ca^{2+} ionophore ionomycin in combination with 2.5 mM Ca^{2+} and 10–100 μM pro-oxidant agent tert-butyl hydroperoxide (tBHP). The sensitivity of lamprey RBCs to studied compounds was evaluated by the kinetics of their death. Both toxicants induced dose- and time dependent phosphatidylserine (PS) externalization (annexin V-FITC labeling) and loss of membrane integrity (propidium iodide uptake). Highest doses of ionomycin (1–2 μM) increased the number of PS-exposed erythrocytes to 7–9% within 3 h, while 100 μM tBHP produced up to 50% of annexin V-FITC-positive cells. Caspase inhibitor Boc-D-FMK (50 μM), calpain inhibitor PD150606 (10 μM) and broad protease inhibitor leupeptin (200 μM) did not prevent ionomycin-induced PS externalization, whereas tBHP-triggered apoptosis was blunted by Boc-D-FMK. tBHP-dependent death of lamprey erythrocytes was accompanied by the decrease in relative cell size, loss of cell viability, activation of caspases 9 and 3/7, and loss of mitochondrial membrane potential, but all these processes were partially attenuated by Boc-D-FMK. None of examined death-associated events were observed in ionomycin-treated erythrocytes except activation of caspase-9. Incubation with ionomycin did not alter intracellular K^+ and Na^+ content, while exposure to tBHP resulted in 80% loss of K^+ and 2.8-fold accumulation of Na^+. Thus, lamprey erythrocytes appear to be more susceptible to oxidative damage. Ca^{2+} overload does not activate the cytosolic death pathways in these cells.

1. Introduction

Although the programmed self-destruction of cells is an extensive area of research, the evolutionary aspects of the mechanisms leading to cell death are explored insufficiently. In this respect, red blood cells (RBCs) are quite interesting objects because they underwent considerable morphological and molecular changes during evolution from lower to higher vertebrate animals. However, the majority of investigations in this field have been performed on mammalian, mostly human, erythrocytes (Berg et al., 2001; Bratosin et al., 2001; Lang et al., 2006; Lang and Lang, 2015). The works on RBCs of animals phylogenetically more ancient than mammals are scarce. The available data are limited by a few studies on fish *Cyprinus carpio* (Li et al., 2013, 2015, 2016), chicken (Weil et al., 1998), and amphibian species such as *Rana esculenta* (Bratosin et al., 2004), *Rana catesbiana* (Krauter et al., 1987) and *Xenopus laevis* (Sakamoto et al., 1997). Investigations on lamprey *Lampetra fluviatilis* (Petromyzontidae, Petromyzontiformes) can greatly contribute to understanding the evolution of the death pathways in erythrocytes, since it belongs to Cephalaspidomorphi class, the most ancient of presently living vertebrate animals which blood contains RBCs.

The efforts to understand intracellular molecular processes leading to the death of erythrocytes have unraveled a few related chains of events. Among them are oxidative and post-translational modifications of hemoglobin accompanied by its aggregation and conformational changes, which result in formation of hemichromes with oxidized and denatured hemoglobin (Waugh et al., 1986) and shedding of hemoglobin-containing vesicles (Willekens et al., 2008). Erythrocytes of the animals of different phylogenetic levels subjected to oxidative stress *in vitro* develop apoptotic signals including translocation of death receptor Fas into rafts, formation of Fas-associated complexes, activation of caspases 8, 9 and 3, and impairment of flippase activity leading to disturbances in membrane phospholipid asymmetry (Mandal et al., 2002, 2003, 2005; Roy and Sil, 2012; Li et al., 2015, 2016). Moreover, oxidative stress activates Ca^{2+}-dependent protease calpain (Roy and Sil, 2012; Li et al., 2015, 2016).

Abbreviations: RBCs, red blood cells; IO, ionomycin; tBHP, tert-butyl hydroperoxide; PS, phosphatidylserine; PI, propidium iodide; FAM-LEHD-FMK, carboxyfluorescein analog of benzyloxycarbonyl-Leu-Glu-His-Asp(OMe) fluoromethyl ketone (z-LEHD-fmk); FAM-DEVD-FMK, carboxyfluorescein analog of benzyloxycarbonyl-Asp(OMe)-Glu(O-Me)- Val-Asp(O-Me) fluoromethyl ketone (z-DEVD-fmk); MMP, mitochondrial membrane potential; TMRM, tetramethyl rhodamine methyl ester; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone.

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Another process believed to trigger the mechanisms of erythrocyte self-destruction is accumulation of free cytosolic Ca^{2+}, which activates Ca^{2+}-sensitive K^+ channels (Maher and Kuchel, 2003). Efflux of K^+ accompanied by anion permeability and loss of water exceeding that of hemoglobin plays an important role in the shrinkage of apoptotic and old RBCs (Lang et al., 2003b; Clark, 1988). In *in vitro* experiments, Ca^{2+} stimulates scramblase but inhibits flippase thus inducing externalization of phosphatidylserine (PS) at the outer membrane surface (Bassé et al., 1996; Daleke and Lyles, 2000), and activates calpain resulting in the degradation of cytoskeletal proteins (Berg et al., 2001; Lang et al., 2006).

The present study was designed to examine the effects of Ca^{2+} overload and oxidative stress on RBCs of river lamprey *Lampetra fluviatilis*. The erythrocytes were treated with Ca^{2+} ionophore ionomycin, widely used to load the cells with Ca^{2+} (Berg et al., 2001; Lang et al., 2003a), and tert-butyl hydroperoxide, low molecular pro-oxidant compound which easily enters the cell and induces oxidation of hemoglobin to its methemoglobin form (Ataullakhanov et al., 1986). The goals of work were to confirm an ability of lamprey erythrocytes to trigger apoptosis-like death program in response to treatment with ionomycin and tBHP and partially characterize the signaling mechanisms underlying the toxicity of studied compounds. Lamprey RBCs contain nuclei and mitochondria playing a crucial role in the induction and execution of apoptosis in cells of other types (Green and Llambi, 2015). Therefore, we made an attempt to establish the potential of these cells to activate mitochondria-dependent death pathway, starting from disruption of mitochondrial membrane, and to recruit the caspases of intrinsic apoptotic pathway. Lamprey erythrocytes were incubated for 3 h with different doses of ionomycin in combination with 2.5 mM external Ca^{2+} (further referred to as ionomycin) and tert-butyl hydroperoxide (tBHP). The cells were analyzed for early apoptotic markers (exposure of phosphatidylserine at outer membrane assessed by annexin V-FITC labeling), late apoptotic/necrotic processes (damage of cellular membrane evaluated by uptake of propidium uptake), viability (calcein assay), activation of caspases 9 and 3/7, and loss of mitochondrial membrane potential. The changes in cellular content of monovalent cations and possible involvement of Ca^{2+}-dependent protease calpain in the death of lamprey RBCs were evaluated as well.

2. Materials and methods

2.1. Chemicals

Buffer salts, tert-butyl hydroperoxide (Luperox® TBH70X, catalog # 458139), leupeptin (# L0649), propidium iodide (PI, # 81845), calcein-AM (# 17783), tetramethyl rhodamine methyl ester (TMRM, # T5428), Fura-2 AM (# F0888), carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP, # C2920) and dimethyl sulfoxide (DMSO, # D4540) were obtained from Sigma-Aldrich (USA). Ionomycin (# 124222) and FITC-conjugated annexin V solution (# A13199) were purchased from Molecular Probes/ThermoFisher Scientific (USA). Pan-caspase inhibitor Boc-D-FMK (# 1160) and calpain inhibitor PD150606 (# 1906) were procured from BioVision (USA). Caspase 3/7 FAM FLICA™ assay kit (# 93) and Caspase 9 FAM FLICA™ assay kit (# 912) were from ImmunoChemistry Technologies, LLC (USA). Romanowsky–Giemsa dye solution and buffer were purchased from Abris company (Russia). Stock solutions of tert-butyl hydroperoxide, propidium iodide and leupeptin were prepared on distilled water. Ionomycin, Fura-2, calcein-AM, TMRM, FCCP, Boc-D-FMK and PD150606 were dissolved in DMSO. The final volume of solvents in the samples did not exceed 1%.

2.2. Animals

The experiments were performed on erythrocytes of lampreys *Lampetra fluviatilis* of both sexes. The animals were caught in December in the delta of the Neva River during their pre-spawning migration from Baltic Sea and kept at 3–5 °C in aquaria with dechlorinated and constantly aerated tap water, which was periodically replaced with fresh water. The experiments were carried out from December to April. All procedures were in accordance with the European Communities Council Directive (86/609/EEC).

2.3. Preparation of RBC suspension

The lampreys were sacrificed by rapid decapitation. The blood was immediately collected into heparinized tubes with the cold washing solution containing (mM) 130 NaCl, 4 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 HEPES/NaOH (pH 7.4 at 4 °C). The erythrocytes were washed three times by centrifugation (3000 *g* for 5 min at 4 °C). The supernatant and upper layer of white cells were carefully removed by aspiration. The final suspension of washed RBCs (hematocrit of 20-30%) was prepared on incubation medium containing (mM) 130 NaCl, 4 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 10 HEPES/NaOH (pH 7.4 at room temperature).

2.4. Experimental procedures

All experiments were performed at room temperature. Washed RBCs were allowed to acclimate to experimental temperature for 30 min. Then the aliquots of erythrocyte suspension were placed in 500 μl of incubation medium to a final hematocrit of 1%. For time-course experiments the increasing concentrations of ionomycin and tBHP were added to the samples, and 100 μl of cell suspensions were taken from the same sample each hour for annexin V-FITC and propidium iodide labeling. To study an involvement of proteases in apoptotic processes, the erythrocytes were pre-incubated for 15 min with 50 μM of caspase inhibitor Boc-D-FMK, 10 μM of calpain inhibitor PD150606 or 200 μM of broad protease inhibitor leupeptin, incubated for 90 min with 1 μM ionomycin or for 30 min with 50 μM tBHP, and 100 μl of cell suspensions were taken for parallel labeling with fluorescent dyes.

2.5. Annexin V-FITC and propidium iodide labeling

The kinetics and different phases of the death of lamprey erythrocytes induced by ionomycin and tBHP were detected using the method of dual labeling with annexin V-FITC and propidium iodide, which discriminates apoptotic and necrotic cells (Vermes et al., 1995). Following treatment with ionomycin or tBHP the erythrocytes were washed and resuspended at 1% hematocrit in 100 μL of annexin-binding buffer containing (mM) 140 NaCl, 2.5 $CaCl_2$, 10 HEPES/NaOH (pH 7.4 at room temperature). Then 5 μl of annexin V-FITC solution and 1 μl of PI solution (250 μg/ml stock) were added to each sample. The cells were stained in the dark for 15 min. After labeling the aliquots of erythrocytes were diluted with annexin-binding buffer to 0.01% hematocrit, mixed gently, placed on ice and immediately subjected for flow cytometry biparametric analysis. Flow cytometry was carried out on EPICS XL cytometer (Beckman Coulter Inc., Brea, CA, USA) equipped with SYSTEM II (Version 3.0) software for acquisition and analysis. The fluorescence of annexin V-FITC and propidium iodide was excited by argon laser (488 nm) and registered at 530 nm and 620 nm, respectively. The data were first plotted using FS (forward scatter) vs SS (side scatter) to reveal cell size and structure and remove debris along the axes. Then the data were re-plotted in the fluorescence channels FL-1 and FL-3 for annexin V-FITC and propidium iodide, respectively. Fluorescence channels were set on logarithmic scale. Quadrant analysis of annexin V-FITC and propidium iodide fluorescence was applied to distinguish the cells with different fluorescence intensities. For each sample 30,000 events were collected. The values are expressed as percentage of the cells to untreated control. The images were processed using WinMDI2.9 program.