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Role of mitochondrial calcium in hypochlorite induced oxidative damage of cells

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ABSTRACT

Hypochlorite (HOCl) is one of the most important mediators of inflammatory processes. Recent evidence demonstrates that changes in intracellular calcium pool play a significant role in the damaging effects of hypochlorite and other oxidants. Mitochondria are shown to be one of the intracellular targets of hypochlorite. But little is known about the mitochondrial calcium pool changes in HOCl-induced mitochondrial dysfunction. Using isolated rat liver mitochondria, we showed the oxidative damage of mitochondria (GSH oxidation and mixed protein-glutathione formation without membrane lipid peroxidation) and alterations in the mitochondrial functional parameters (decrease of respiratory activity and efficiency of oxidative phosphorylation, NADH and FADH coenzyme levels, and membrane potential) under hypochlorite action (50–300 μM). Simultaneously, the mitochondrial calcium release and swelling were demonstrated. In the presence of EGTA, the damaging effects of HOCl were less pronounced, reflecting direct involvement of mitochondrial Ca^{2+} in mechanisms of oxidant-induced injury. Furthermore, exposure of HeLa cells to hypochlorite resulted in a considerable increase in cytoplasmic calcium concentrations and a decrease in mitochondrial ones. Applying specific inhibitors of calcium transfer systems, we demonstrated that mitochondria play a key role in the redistribution of cytoplasmic Ca^{2+} ions under hypochlorite action and act as mediators of calcium release from the endoplasmic reticulum into the cytoplasm.

1. Introduction

Many diseases are associated with the development of inflammatory processes in body tissues [1,2]. Hypochlorite is considered to be one of the most important mediators of inflammation. It is produced by activated neutrophils at the inflammation sites via the myeloperoxidase-catalyzed reaction of chloride ions with hydrogen peroxide [3]. The presence of HOCl in the injured tissues is confirmed by accumulation of chlorinated tyrosine residues, and its concentrations can reach the values of 20–400 μM during an hour [3,4]. As was previously shown, HOCl is a cytotoxic agent affecting not only microorganisms and damaged cells in the inflammation foci, but also the surrounding normal cells. In particular, chlorinated tyrosine residues have been found in many septic and periseptic tissues of human cirrhotic liver [5–7].

Hypochlorite is known to penetrate rapidly into mammalian cells, achieving intracellular targets [8]. Significant alterations in cellular physiology have already been observed within the first minutes of hypochlorite action, confirming its high cytotoxicity. In addition, hypochlorite compared to other reactive oxygen species is not a substrate for antioxidative enzymes and, at the same time, is able to interact with multiple subcellular structures [9,10]. Cell exposure to hypochlorite results in oxidation of various

Abbreviations: CysA, cyclosporine A; Dnt, dantrolene; HOCl, hypochlorite; ER, endoplasmic reticulum; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid; FCCP, carbonyl cyanide-4-trifluoromethoxy)phenylhydrazone; GSH, reduced form of glutathione; GSSP, mixed glutathione protein disulfides; RR, ruthenium red; ROS, reactive oxygen species; mPTP, membrane permeability transition pore; MDA, malonyldialdehyde; IP_3R, inositol 1,4,5-trisphosphate receptor; RyR, ryanodine receptor; MCU, mitochondrial calcium uniporter.

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biomolecules including membrane lipids, proteins, DNA, and low weight molecules (ascorbate, nucleotides, thiol groups, thioethers) [6,9,10]. Moreover, HOCl causes protein damage due to the formation of chloramines and chlorotyrosines as well as protein thiol group oxidation [11]. Hypochlorite at low physiological concentrations (up to 400 μM) generated by neutrophils acts on specific cellular targets and induces various mechanisms of cell death depending on the cell type [3,8,12].

Mitochondria are essential for cell viability, supplying the cell by energy and participating in cell signaling, particularly in apoptosis [13]. It was demonstrated that mitochondrial dysfunction plays a critical role in the pathogenesis of many diseases, and application of the mitochondria directed drugs is effective in the treatment of these diseases [13]. Mitochondria are shown to be one of the intracellular targets of hypochlorite [9,14]. Using human HepG2 cells, it was demonstrated that HOCl at concentrations of 15–125 μM reduced the cellular ATP level, induced the membrane permeability transition pore (mPTP) formation, mitochondrial swelling, membrane potential decrease, and release of cytochrome C, thereby causing the caspase 3 activation and cell death by apoptosis [9]. In human macrophages, HOCl (50–200 μM) brought about a rise in cytoplasmic calcium level via the plasma membrane L- and T-type calcium channels and endoplasmic reticulum RyR channels as well as cellular ATP and mitochondrial membrane potential loss, and rapid necrotic cell death [15].

Currently, a number of papers have demonstrated that changes in the intracellular calcium pool play a significant role in the damaging effects of oxidants and, in particular, hypochlorite [8,16–21]. Ca^{2+} is known to be involved in the regulation of many cellular signals [8,13,22]. Mitochondrial Ca^{2+} influx controls both the oxidative phosphorylation and cellular ROS generation. Whereas dysregulation of cellular Ca^{2+} homeostasis leads to Ca^{2+} elevation in the mitochondrial microenvironment, causes cell death via an impairment in ATP and mitochondrial redox homeostasis, and promotes the membrane protein thiol cross-linking, inducing mitochondrial permeability transition [23]. Ca^{2+} ions activated specific ATP independent proteases that resulted in damage of many cell structures including mitochondria [8]. Moreover, Ca^{2+} is able to activate ROS generation in mitochondria [24,25]. Simultaneously with the protein thiol groups oxidation under hypochlorite action, this stimulates mPTP opening [26]. Interactions between the ROS and calcium signaling can be considered as bidirectional [18]. Mitochondria can regulate intracellular Ca^{2+} level by forming contact sites between the mitochondria and endoplasmic reticulum (MAMs). At these sites, a rapid and significant Ca^{2+} release out of the endoplasmic reticulum followed by its accumulation in mitochondria occurs [27]. Although mitochondria play an important role in the regulation of cell Ca^{2+} homeostasis [13], little is known about hypochlorite-induced changes in mitochondrial calcium level at the inflammation sites. Similarly, the mechanisms of hypochlorite-induced mitochondrial dysfunction and cell death are still unclear.

Thus, due to the great importance of mitochondria in the cell response to hypochlorite exposure and regulation of many cellular processes, the aim of the work was to study the functional state and involvement of mitochondria in the calcium pool changes during hypochlorite-induced cell damage.

2. Materials and methods

2.1. Materials

Succinic acid disodium salt hexahydrate (succinate), L-glutamic acid sodium salt (glutamate), L-malic acid sodium salt (malate), sodium hypochlorite, sucrose, tris(hydroxymethyl)aminomethane (Tris-HCl), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), adenosine diphosphate sodium salt (ADP), safranin O, valinomycin, carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), 5,5′-dithiobis(2-nitrobenzoic acid), calcium chloride dehydrate, trichloroacetic acid, cyclosporine A (CysA), ruthenium red (RR), thiobarbituric acid (TBA), gentamicin, Fura-2-acetoxymethyl ester (Fura-2AM), rhod calcium indicator acetoxymethyl ester (X-Rhod-1-AM), JC-1 dye and dimethyl sulfoxide were purchased from Sigma-Aldrich, St. Louis, MO, USA or Steinheim, Germany. All other reagents of analytical grade were purchased from POCh (Gliwice, Poland) and Reakhim (Moscow, Russia). All solutions were made with water purified in the Milli-Q system.

HeLa cells were grown in the Dulbecco's modified Eagle's medium with 4.5 g/l glucose supplemented with 10% fetal bovine serum and 100 μg/ml gentamicin at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. When the cell monolayer reached 75–90% of confluence, the culture medium was removed, and monolayer was washed with Hepes-buffer containing 131 mM NaCl, 5 mM KCl, 1.3 mM $MgSO_4$, 6 mM glucose, and 20 mM Hepes, pH 7.3. The cells were dissociated using 0.2% trypsin with 0.02% EDTA and resuspended by Hepes-buffer.

2.2. Mitochondria isolation

Mitochondria were prepared from the liver of Wistar male rats weighing 200–250 g as previously described [28]. The animals were fasted for 12 h until killing. The animals were killed by decapitation according to the rules of the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes, and the study was approved by the Ethics Committee of the University. The liver was removed into cold (0–4 °C) isotonic phosphate buffer (150 mM NaCl, 10 mM Na_2HPO_4, pH 7.4), dried with filter paper, weighed to provide the required quantitative yield of mitochondria and homogenized in a glass-teflon Potter-Elvehjem homogenizer with the ice-cold isolation medium containing 250 mM sucrose, 20 mM Tris-HCl and 1 mM EGTA, pH 7.2. The homogenate was centrifuged at 600 g for 10 min (4 °C) for nuclei precipitation. Then supernatant was centrifuged at 8,500 g for 10 min (4 °C). The mitochondrial pellet was washed twice with the isolation medium (with and without 1 mM EGTA) and centrifuged at 8,500 g for 10 min (4 °C) after each washing again. The mitochondrial pellet was resuspended by pipetting with the isolation medium without of EGTA to the protein concentration of 40 mg/ml. All procedures were performed at 4°C. The quality of the mitochondrial fraction was confirmed by electron microscopy (JEM-1011, Japan) at magnifications of 5,000x to 40,000x, and the purity of the isolated mitochondrial fraction was at least 98%. The protein concentration of the mitochondrial suspension was determined using Folin-Ciocalteu reagent [29].

2.3. Biochemical measurements

Before biochemical measurements mitochondrial suspension was subjected to 3 cycles of freeze-thawing, mixed with 25% trichloroacetic acid, and centrifuged to precipitate of proteins at 6000 g for 3 min.

The reduced mitochondrial glutathione, GSH, was determined spectrophotometrically in the mitochondria-free supernatant using 5,5′-dithiobis(2-nitrobenzoic acid) [30]. Glutathione level in mitochondria was calculated using the molar extinction coefficient $\varepsilon_{412} = 13.6 \cdot 10^3\ M^{-1}\ cm^{-1}$ and expressed as nmol GSH per mg of protein. Mixed glutathione-protein disulfides, GSSP, were measured spectrophotometrically in the mitochondrial pellet using

5,5′-dithiobis(2-nitrobenzoic acid) and expressed as nmol GSSP per mg of protein [31]. Glutathione release from disulfides with protein thiol groups was performed by incubating the mitochondrial pellet with 0.5 M sodium phosphate buffer, pH 7.4, at 25°C for an hour. The content of mixed glutathione-protein disulfides was calculated in the mitochondria using the molar extinction coefficient $\varepsilon_{412} = 13.6 \cdot 10^3$ M^{-1} cm^{-1}.

Lipid peroxidation of mitochondrial membranes was characterized by accumulation of thiobarbituric acid reactive substances (TBARS). After precipitation with trichloroacetic acid, the mitochondria-free supernatant was incubated with 0.2% thiobarbituric acid in a boiling-water bath for 20 min [32]. After cooling under tap water, the level of TBARS in probes was monitored spectrophotometrically by measuring the difference in absorption between 532 nm and 600 nm wavelengths, and calculated using the molar extinction coefficient $\varepsilon_{532} = 1.56 \cdot 10^5$ M^{-1} cm^{-1}. The content of membrane lipid peroxidation products was expressed as nmol TBARS per mg of protein.

The NAD(P)H and FAD levels in mitochondria were estimated by their autofluorescence in the buffer containing 125 mM KCl, 50 mM sucrose, 10 mM Tris-HCl, 2.5 mM KH_2PO_4, 5 mM $MgSO_4$, and 5 mM succinate (or 5 mM glutamate/2.5 mM malate in the case of NAD(P)H), pH 7.5, as previously described [33–36]. NAD(P)H and FAD fluorescences were measured at $\lambda_{ex/em}$ = 340/465 nm or $\lambda_{ex/em}$ = 454/530 nm, respectively. The coenzyme levels were expressed as fluorescence arbitrary units (FAU).

EGTA at a concentration of 0.5 mM was added to experimental samples immediately before the addition of hypochlorite (25–300 μM) when effects of calcium chelator were necessary to be studied.

2.4. Respiratory activity measurement

Mitochondrial respiration was determined polarographically at 25°C using the Clark-type electrode incorporated in a thermostatic hermetic cell [37]. The mitochondrial suspension (1 mg/ml) was placed into the cell with the buffer containing 125 mM KCl, 50 mM sucrose, 10 mM Tris-HCl, 2.5 mM KH_2PO_4, 5 mM $MgSO_4$, pH 7.2 with or without 0.5 mM EGTA added. HOCl (25–300 μM) was added directly before measurement. Then the substrate succinate (5 mM) and ADP (180 μM) were introduced into the mitochondrial suspension sequentially. The duration of sample measurement was 10 min. To characterize the respiratory activity, the rate of oxygen consumption by mitochondria was determined in different metabolic states: V_2 is substrate-dependent respiratory rate (in the presence of the substrate and the absence of ADP), V_3 is ADP-dependent respiratory rate (in the presence of the substrate and ADP). The parameters of coupling of the mitochondrial oxidation and phosphorylation processes were determined: the acceptor control ratio (ACR) (V_3/V_2) and the coefficient of phosphorylation (ADP/O). V_2 and V_3 were expressed as ngatom oxygen per mg of protein for a minute.

2.5. Mitochondrial membrane potential

Membrane potential of isolated rat liver mitochondria was measured by fluorescence of positively charged lipophilic fluorescent probe safranin O [38]. The buffer for measurement consisted of 125 mM KCl, 50 mM sucrose, 10 mM Tris-HCl, 2.5 mM KH_2PO_4, 5 mM $MgSO_4$, and 5 mM succinate, pH 7.5. The safranin O fluorescence was recorded at $\lambda_{ex/em}$ = 495/586 nm (25°C). In experimental samples, mitochondria (0.3 mg/ml) were incubated with ruthenim red (1 μM), cyclosporine A (0.15 μM), or EGTA (0.5 mM) for 2 min before HOCl (50 μM or 200 μM) was added.

To express in millivolts (mV), the mitochondrial membrane potential values ($\Delta\Psi$) were determined in the medium containing 250 mM sucrose, 20 mM Tris-HCl, pH 7.2 [38]. The calibration plot, which represented the dependence of safranin O fluorescence intensity on the mitochondrial membrane potential value, was calculated according to the Nernst equation:

$$\Delta\Psi = 60 \log [K^+]_{out}/[K^+]_{in} \text{ (mV)},$$

where $[K^+]_{in}$ is the intramitochondrial potassium concentration (120 mM), and $[K^+]_{out}$ is the extramitochondrial potassium concentration in the medium that varies from 0 to 20 mM [38,39]. For calibration, the membrane potential values were changed by varying extramitochondrial potassium concentrations in the medium (0–20 mM) in the presence of ionophore valinomycin (0.28 μM). Complete depolarization of mitochondria to calibrate the dye fluorescence was achieved by addition of FCCP (0.5 μM).

Mitochondrial membrane potential of HeLa cells was measured by fluorescence of cationic dye JC-1 that exhibit potential-dependent accumulation in mitochondria indicated by a fluorescence emission shift from green (~530 nm) to red (~590 nm) [40]. Mitochondrial depolarization is indicated by a decrease in the fluorescence intensity ratio (590 nm/530 nm). The buffer for measurement consisted of 131 mM NaCl, 5 mM KCl, 1.3 mM MgSO4, 6 mM glucose, and 20 mM Hepes, pH 7.3. The cell suspension contained 10^6 cells per ml. HeLa cells were incubated with JC-1 (1 μM) for 15 min at 37 °C. The fluorescence intensity of JC-1 was measured spectrofluorimetrically or by epifluorescence microscopy. In the latter case, BK6000 Fluorescence Microscope with a 520 nm longpass optical filter was used. Regions of high mitochondrial polarization are indicated by orange fluorescence due to the J-aggregate formation by the concentrated dye. Depolarized regions are indicated by green fluorescence of the JC-1 monomers. Complete depolarization of mitochondria was achieved by addition of FCCP (0.5 μM) after JC-1 loading and before hypochlorite in the case of the corresponding experiment.

2.6. Mitochondrial swelling

Rat liver mitochondria swelling was measured spectrophotometrically by the decrease in optical density (D_{520}) of mitochondrial suspension (0.4 mg/ml) at 520 nm on the time (25°C) using a buffer containing 125 mM sucrose, 60 mM KCl, 10 mM Tris-HCl, 1 mM KH_2PO_4, and 5 mM succinate, pH 7.2 [41]. Ruthenium red (1 μM) or cyclosporine A (0.15 μM) were added 2 min before HOCl (200 μM). Mitochondrial swelling was determined as maximal rate of decrease in optical density of the mitochondrial suspension [42].

2.7. Calcium measurements

Calcium concentration in cell cytoplasm was measured using the Ca^{2+} probe Fura-2AM [43]. HeLa cells (10^6 per milliliter) in Hepes-buffer containing 131 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.3 mM $MgSO_4$, 6 mM glucose, and 20 mM Hepes, pH 7.3 were incubated with Fura-2AM (2 μM) at 37°C for 30 min. Thereafter the cells were centrifuged at 150 g for 5 min, washed and resuspended with Hepes-buffer containing 1 μM EGTA and no $CaCl_2$. The amount of cells was determined using an automated cell counter. The fluorescence of Fura-2 was assessed at 510 nm using two wavelengths of excitation: 340 nm that corresponded to the Ca^{2+}-bound form of Fura-2 and 380 nm that corresponded to the Ca-free form of the probe. For each experiment, 0.1% Triton X-100 and 1.3 mM $CaCl_2$ were added into control sample to obtain the values of the maximum fluorescence level, and 5 mM EGTA to obtain the minimum fluorescence level at a given concentration of the Fura-2. The concentration of cell calcium was calculated using the equation:

$[Ca^{2+}]_{cyt} = K_d * [(R-R_{min})/(R_{max}-R)] * S_f/S_b$,

where R is the ratio of fluorescence intensities at two excitation wavelengths (I_{340}/I_{380}); R_{max} is the I_{340}/I_{380} ratio at Ca^{2+}-saturating conditions in the presence of 0.1% Triton X-100 and 1.3 mM $CaCl_2$; R_{min} is the I_{340}/I_{380} ratio in the presence of 5 mM EGTA that corresponds to the Ca^{2+} -free form of the probe; S_f and S_b are the baseline fluorescence intensities of Fura-2 at the excitation wavelength of 380 nm under Ca^{2+}-free and -bound conditions, respectively; K_d for Ca^{2+} binding to Fura-2 was assumed to be equal to 220 nM [44]. The figures represent the results of calcium change under hypochlorite action as a difference of the calcium levels before and after hypochlorite addition ($\Delta[Ca^{2+}]$, nM).

The changes of mitochondrial calcium level in HeLa cells under hypochlorite action were determined using the fluorescent dye X-Rhod-1-AM [45,46]. HeLa cells (10^6 per milliliter) were incubated in Hepes-buffer, pH 7.3, with X-Rhod-1-AM (2 μM) at 37°C for 15 min. Then they were centrifuged at 150 g for 5 min, washed and resuspended with Hepes-buffer containing 1 μM EGTA and no $CaCl_2$. The fluorescence of X-Rhod-1 was measured at $\lambda_{ex/em} = 580/602$ nm. The Ca^{2+} concentration inside the mitochondria was proportional to the fluorescence intensity of X-Rhod-1. The figures represent the Ca^{2+} level decrease in hypochlorite-treated mitochondria as a difference of the fluorescence intensities before and after hypochlorite addition (ΔF).

In experimental samples, cells were incubated with each of the inhibitors, ruthenium red (RR, 1 μM), cyclosporine A (CysA, 1 μM), and dantrolene (Dnt, 10 μM) for 2 min before HOCl (50 μM or 200 μM) addition.

The determination of Ca^{2+} level in isolated rat liver mitochondria was carried out with Fura-2AM as previously described [47]. Initially, mitochondria (40 mg/ml) were incubated in the isolation buffer with Fura-2AM (6 μM) at 25°C for 10 min. Then mitochondria were washed, centrifuged at 8,500 g for 10 min (4 °C), and resuspended by pipetting with the same volume of isolation buffer without probe. The measurement of Fura-2 fluorescence was performed after addition of mitochondria (0.4 mg/ml) to the medium containing 125 mM sucrose, 60 mM KCl, 10 mM Tris-HCl, 1 mM KH_2PO_4, and 5 mM succinate, pH 7.4. The fluorescence of Fura-2 in isolated mitochondria was assessed at 510 nm using two excitation wavelengths of 340 nm and 380 nm. After that the ratio of fluorescence intensities was calculated (I_{340}/I_{380}). The figures represent the mitochondrial calcium level decrease under hypochlorite exposure (50–300 μM) as a difference of the ratio (I_{340}/I_{380}) before and after hypochlorite addition (ΔF).

2.8. Statistical analysis

The results of the experiments were expressed as the means of four or five replicates ± SEM. The differences between the values of parameters measured in groups were analyzed using the Student's t-test or nonparametric Mann-Whitney test depending on the normality of values distribution in the groups. The normality of distribution was determined by Shapiro-Wilk test. Statistical analysis was conducted using the GraphPad Prism 6.0 software package. The results were assumed to be statistically significant compared to the group in the absence of hypochlorite when P value was less than 0.05 (*), 0.01 (**) or 0.001 (***).

3. Results

3.1. Functional activity, antioxidant system and level of calcium of rat liver mitochondria exposed to hypochlorite

In our experiments, we evaluated the effects of hypochlorite on the mitochondrial respiratory activity, antioxidant system, NADH and FADH levels, mPTP formation, mitochondrial membrane potential, and Ca^{2+}-ion level.

The antioxidant system of mitochondria is very important for maintaining the redox balance and, therefore, the functional activity of these organelles. In the absence of calcium chelator EGTA in the incubation medium, the exposure of isolated rat liver mitochondria to hypochlorite (25–300 μM) for 10 min decreased the GSH level in a concentration-dependent manner (Supplementary Fig. 1). In addition, HOCl caused a significant increase in the mitochondrial GSSP level (by 1.5 fold at a HOCl concentration of 100 μM) (Supplementary Fig. 3). This kind of oxidative modifications can alter protein functions. It is interesting that we did not observe any changes in the content of lipid peroxidation products (TBARS) during HOCl exposure (Supplementary Fig. 2). Similar results were demonstrated on macrophages and red blood cells [12,48,49]. In the presence of EGTA, we noticed less pronounced GSH oxidation and GSSP formation without a change in the content of lipid peroxidation products in hypochlorite-treated mitochondria (Supplementary Figs. 1, 2 and 3). One of the possible mechanisms of the EGTA effects can be the binding of calcium ions by chelator and, thereby, preventing of ROS generation in mitochondria. It was shown that calcium uptake of mitochondria led to an increase in the generation of mitochondrial ROS regardless of the metabolic state of the mitochondria [50]. Since hypochlorite also oxidizes glutathione, the protective effect of EGTA on the mitochondria glutathione level was not full.

Mitochondria exposure to HOCl (25–300 μM) in EGTA-free medium resulted in a considerable impairment in mitochondrial respiratory activity within 10 min (Fig. 1, Supplementary Figs. 2 and 5). Starting from a concentration of 50 μM, hypochlorite considerably decreased the ADP-dependent respiratory rate (V_3) and the phosphorylation coefficient (ADP/O), leading to a decline in the efficiency of mitochondrial oxidative phosphorylation and ATP synthesis (Fig. 1 A and B). It should be noted that hypochlorite at all the tested concentrations did not cause a full cessation of ATP synthesis. These changes contributed to the increase in the substrate-dependent respiratory rate (V_2) and decrease in the acceptor control ratio (ACR) (Supplementary Figs. 2 and 5). We showed that in the presence of EGTA in the medium significant alterations in the mitochondrial respiratory activity parameters studied were observed only at high concentrations of HOCl (200–300 μM). At the same time, EGTA attenuated the effects of hypochlorite on the mitochondrial respiratory activity at low concentrations (25–100 μM) (Fig. 1, Supplementary Fig. 2). Therefore, the involvement of Ca^{2+} ions in the hypochlorite-induced damage of mitochondrial respiratory activity was indirectly confirmed by the influence of EGTA on this process.

Also, hypochlorite caused a concentration-dependent increase in the mitochondrial NAD and FAD levels within 10 min which reached maximum values at a hypochlorite concentration of 200 μM (Fig. 2 A, B). The effect of hypochlorite was more pronounced in the case of NAD, for which a significant effect was observed already at 25 μM of hypochlorite (data not shown). It should be noted that in the presence of EGTA the effects of hypochlorite were absent (Fig. 2 A, B).

The HOCl effects on the mitochondrial membrane potential were assessed. HOCl was shown to induce the mitochondrial depolarization in a dose-dependent manner (Fig. 3, Supplementary Fig. 3). To study the mechanisms of the HOCl effect, inhibitory analysis was performed using ruthenium red, an inhibitor of mitochondrial calcium uniporter, and cyclosporine A, a common inhibitor of mPTP, and EGTA. All of them decreased the level of mitochondria depolarization, completely restoring the membrane potential at a hypochlorite concentration of 50 μM (Supplementary

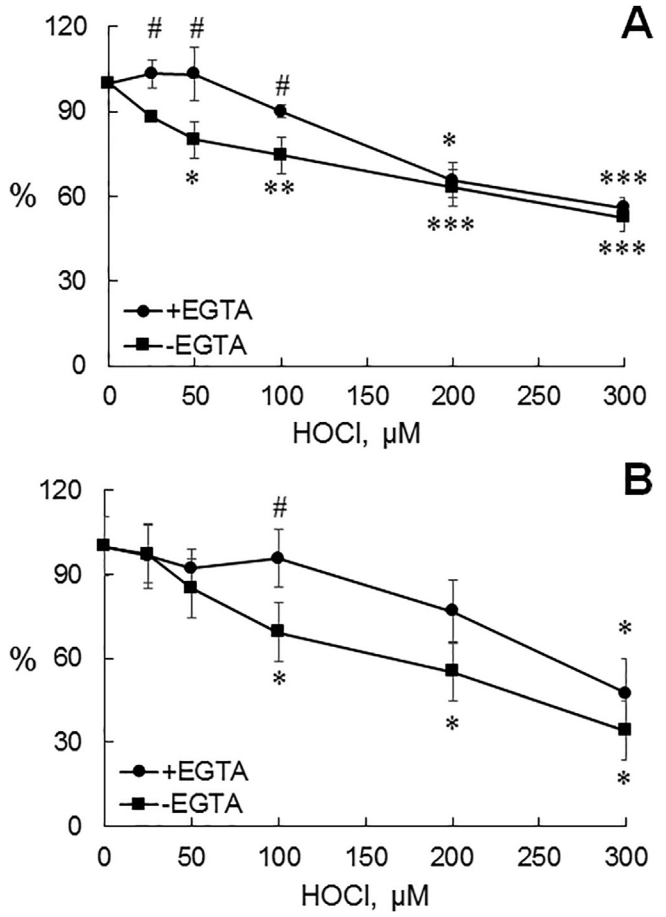

Fig. 1. Effects of hypochlorite on the respiratory activity of rat liver mitochondria. A, ADP-dependent respiratory rate (V_3); B, phosphorylation coefficient (ADP/O). Mitochondria were incubated without or with 0.5 mM EGTA in the medium (125 mM KCl, 50 mM sucrose, 10 mM Tris-HCl, 2.5 mM KH_2PO_4, 5 mM $MgSO_4$, pH 7.2) at 25°C. Succinate (5 mM) as oxidation substrate and ADP (180 μM) were introduced into the mitochondrial suspension. HOCl was added directly before measurement. The duration of sample measurement was 10 min. Mitochondria protein concentration was 1 mg/ml. *p < 0.05, **p < 0.01, ***p < 0.001 vs group in the absence of HOCl; #p < 0.05 vs group without EGTA.

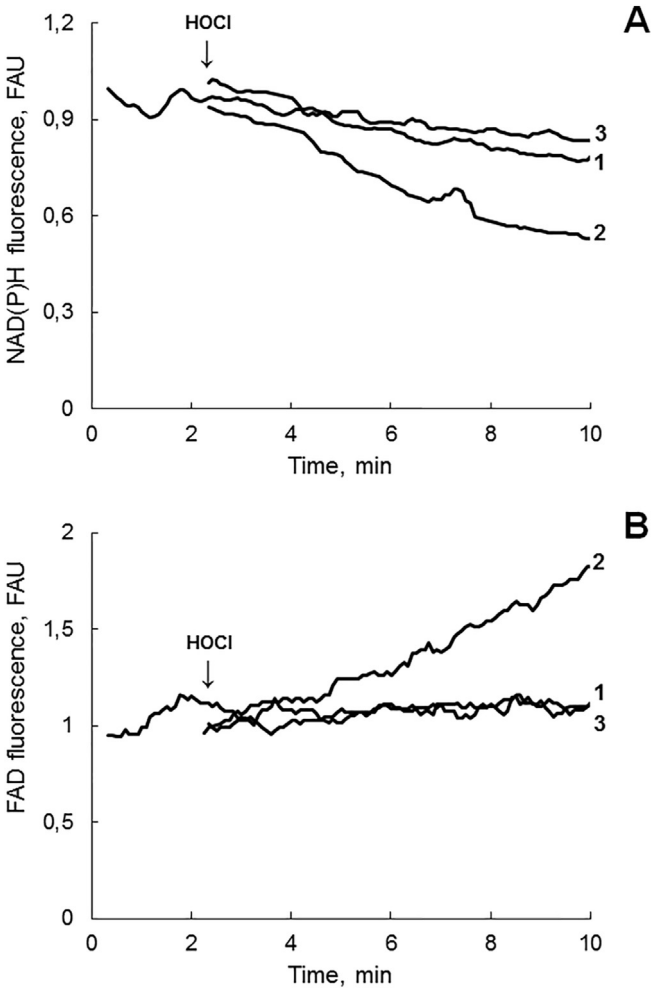

Fig. 2. Mitochondrial NAD(P)H and FAD levels under hypochlorite action. The substrates used were: A, glutamate (5 mM)/malate (2,5 mM); B, succinate (5 mM). 1 – control, 2–200 μM hypochlorite, 3–200 μM hypochlorite plus 0.5 mM EGTA. Mitochondria were incubated in the medium (125 mM KCl, 50 mM sucrose, 10 mM Tris-HCl, 2.5 mM KH_2PO_4, 5 mM $MgSO_4$, pH 7.5) at 25°C. The duration of sample measurement was 10 min. Mitochondria protein concentration was 0.3 mg/ml. FAU, fluorescence arbitrary units.

Fig. 3), and partially prevented the depolarizing action of hypochlorite at a concentration of 200 μM (Fig. 3).

In order to confirm the direct involvement of mitochondrial calcium ions in the mechanisms of hypochlorite action, we studied the permeability transition pore formation and alterations in the content of mitochondrial calcium ions during hypochlorite exposure. In the absence of externally added Ca^{2+}, HOCl at a high concentration of 200 μM slowly decreased the optical density of the mitochondrial suspension as a result of mitochondrial swelling which indicates mPTP formation (Fig. 4 A). Ruthenium red and cyclosporine A, which were added 2 min before HOCl, completely blocked this process. Since there was no externally added calcium, the redistribution of mitochondrial calcium might participate in the mitochondria swelling process under hypochlorite action. This is due to the fact that the mitochondrial suspension derived from the tissue can contain mitochondrial fractions having different thresholds of sensitivity to an oxidizing agent, and a calcium concentration [42]. Therefore, the mPTP formation under the hypochlorite action occurs only in those mitochondrial fractions which are most sensitive to the oxidant. The released calcium is immediately redistributed among other fractions of mitochondria. As a result, some of them reach their calcium threshold at which mPTP opens. Addition of ruthenium red and cyclosporine A prevented the calcium distribution between mitochondria by inhibiting the MCU activity and mPTP formation, which are main pathways of calcium intake and release in mitochondria. At the same time, the almost complete decrease of mitochondrial membrane potential observed in our experiments at a hypochlorite concentration of 200 μM was associated not only with the mPTP formation, but also with the alterations in the functional activity of mitochondrial respiratory complexes as a result of oxidative stress. This is confirmed by the absence of complete blocking of the decrease in membrane potential at high concentrations and its restoration at low concentrations (namely 50 μM) of hypochlorite when using inhibitors (Fig. 3, Supplementary Fig. 3).

A direct release of mitochondrial calcium during HOCl exposure was shown by using the fluorescent probe Fura-2AM (Fig. 4 B). As the concentration increased, hypochlorite was observed to elevate the amount of calcium released out of mitochondria.

Thus, our findings demonstrate that alterations in the mitochondrial functional state under hypochlorite action were associated with the reduction of respiratory activity, increase in the levels of the oxidized forms of NAD and FAD coenzymes, and decrease of

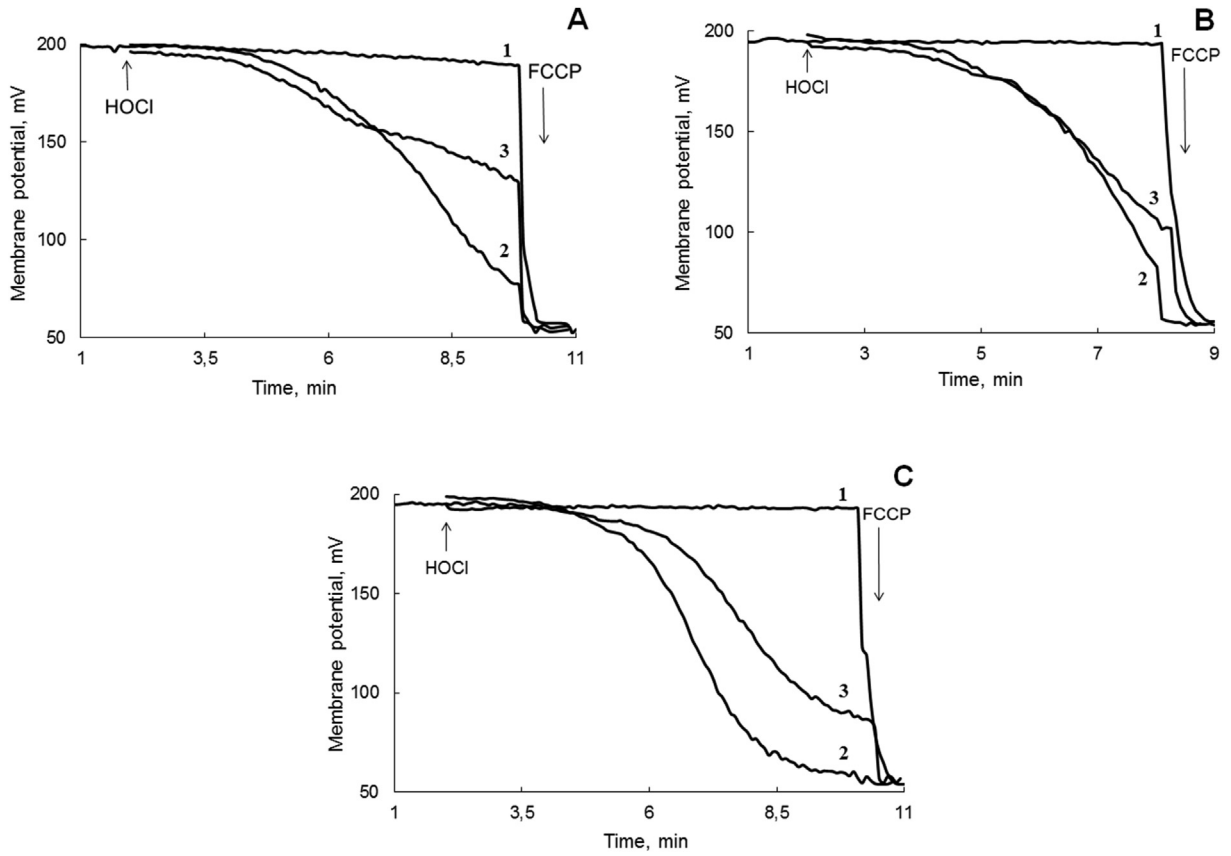

Fig. 3. Membrane potential of rat liver mitochondria under hypochlorite action. A. EGTA -containing medium (0.5 mM). B, addition of 1 μM ruthenium red (RR). C, addition of 0.15 μM cyclosporine A (CysA). 1 – control, 2–200 μM hypochlorite, 3–200 μM hypochlorite plus EGTA (RR or CysA). Mitochondria were incubated with the inhibitors for 2 min before HOCl was added. Mitochondria protein concentration was 0.3 mg/ml.

mitochondrial membrane potential. On the one hand, these disorders may be caused by changes in the mitochondrial antioxidant system (reduced glutathione oxidation) and the oxidative damage (glutathionylation) of mitochondrial enzymes. On the other hand, the demonstrated effects of EGTA and inhibitors on the mitochondrial functional activity indicate an essential role of the mitochondrial Ca^{2+} and membrane permeability transition in the mechanisms of hypochlorite effects, especially at low concentrations. Earlier similar effects as in a case of hypochlorite we demonstrated on the mitochondria exposed to another oxidative agent tert-butyl hydroperoxide [19].

3.2. Cytoplasmic and mitochondrial calcium levels in HeLa cells exposed to hypochlorite

At the next stage, we evaluated the intracellular calcium content and the role of mitochondria in the formation of cytoplasmic calcium pool under hypochlorite action using HeLa cells suspension (10^6 cells per milliliter) in Hepes-buffer with EGTA.

To study the cytoplasmic calcium content in the cells, we used a Fura-2AM probe, which gets the ability to interact with calcium after its transforming by cytoplasmic esterases into the unesterified form. The resting level of cytosolic calcium measured was about 100 nM that corresponded to the literature data [51]. Hypochlorite dose-dependently caused a pronounced rise in the cytoplasmic calcium content (Fig. 5 A). This is in line with other observations [8,52]. The calcium content in the cytoplasm reached nearly 200 nM at high concentrations of HOCl (200–300 μM) (delta [Ca^{2+}] was equal about to 120 nM). It is noteworthy that because of the absence of exogenous calcium in the incubation medium (in the presence of extracellular EGTA), the increase of calcium content in cell cytoplasm was only due to its release from intracellular stores namely the endoplasmic reticulum and mitochondria. Moreover, the plasma membrane calcium ATPase (PMCA) and sarco/endoplasmic reticulum calcium ATPase (SERCA) have very similar structure, and both are sensitive to oxidative damage due to the protein thiol groups oxidation [53,54]. As a result, the activity of both calcium pumps are decreased because of HOCl-induced cellular SH- groups oxidation. That prevents the removal of excess calcium from the cell cytoplasm.

To assess the dynamics of the mitochondrial calcium changes in HeLa cells under hypochlorite action, we used a X-Rhod-1-AM probe having a net positive charge that promotes its sequestration into mitochondria [46]. A significant decrease of X-Rhod-1 fluorescence caused by the release of mitochondrial Ca^{2+} was shown after the exposure of cells to hypochlorite at concentrations of 25 μM and more (Fig. 5 B). At the same time, the mitochondrial membrane potential of HeLa cells was almost unchanged at the given concentrations of hypochlorite that helps to keep unesterified form of the positively charged X-Rhod-1 within organelles (Supplementary Fig. 4). The Ca^{2+} release from mitochondria was enhanced as the HOCl concentration was elevated.

We applied an inhibitory analysis to distinguish the contribution of the endoplasmic reticulum and mitochondria to the increase in cytoplasmic Ca^{2+} level under hypochlorite action (Fig. 6). In this experiment, we studied the effects of inhibitors on the cytoplasmic and mitochondrial Ca^{2+} pools of HeLa cells exposed to HOCl at concentrations of 50 μM and 200 μM. That was due to a difference

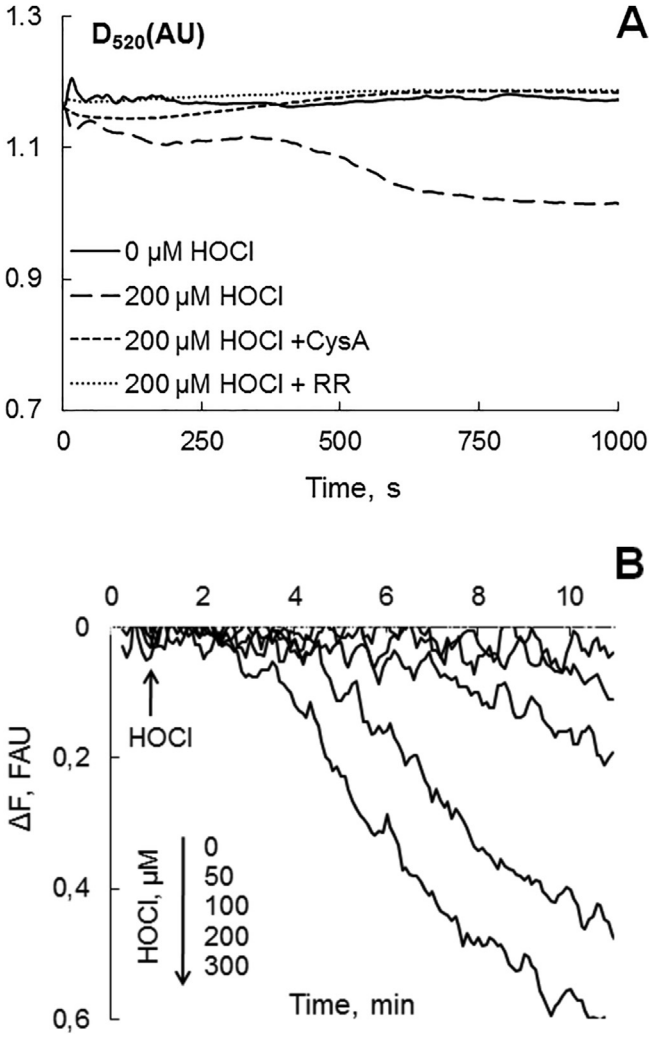

Fig. 4. Swelling and calcium content in hypochlorite-exposed rat liver mitochondria. A. Mitochondrial swelling in the absence of externally added calcium. 1 μM Ruthenium red (RR) and 0.15 μM cyclosporine A (CysA) were added 2 min before HOCl. B. Mitochondrial calcium release at different concentrations of hypochlorite. ΔF represents the amount of calcium released out of mitochondria. Mitochondria protein concentration was 0.4 mg/ml. AU, absorbance units, FAU, fluorescence arbitrary units.

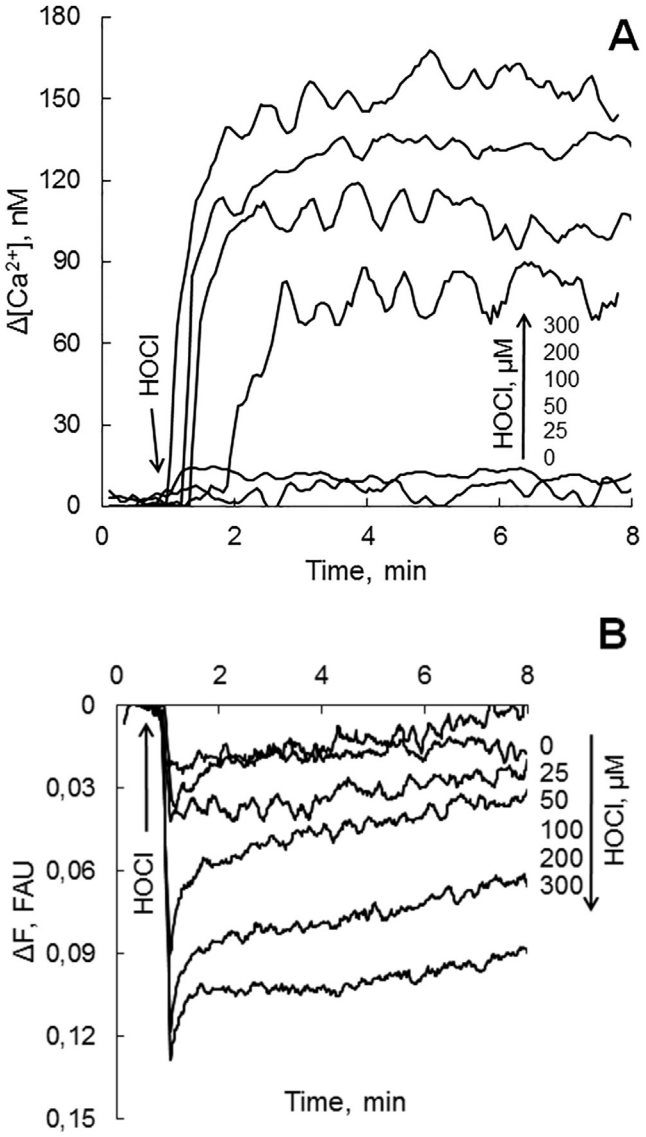

Fig. 5. Calcium level in HeLa cells (10^6 cells per ml) under hypochlorite action. A. Cytoplasmic Ca^{2+} measurements with Fura-2AM. B. Mitochondrial Ca^{2+} measurements with X-Rhod-1-AM. Arrow indicates HOCl addition. FAU, fluorescence arbitrary units.

in the degree of mitochondria oxidative damage under hypochlorite action at these concentrations.

A feature of HeLa cells is the presence of two types of calcium channels in the endoplasmic reticulum: IP_3R and RyR2 receptors [55,56]. Both channels are shown to interact with the calcium release out of the endoplasmic reticulum [55,57].

Since ER is considered to be the main store of intracellular calcium [58], we used dantrolene to evaluate its contribution to the formation of the cytoplasmic Ca^{2+} pool under HOCl action. Dantrolene is known to be an inhibitor of RyR receptors responsible for calcium release out of the ER [59]. In addition, it was shown that dantrolene is capable to block IP_3R receptors as well [60,61]. Both RyR and IP_3R receptors are very similar in their molecular structure and share a high sequence homology in their ion-conducting pore [62,63]. Dantrolene can bind to the IP_3R and RyR receptors by interacting with the IP_3-binding domain occured in the structure of both receptors [60]. Furthermore, it is well-known that RyR2 is activated by cytosolic Ca^{2+} via the process of Ca^{2+}-induced Ca^{2+} release (CICR) [64]. While IP_3Rs activity is suppressed by supramicromolar concentrations of calcium [57]. Opening of the RyR receptors releases about 20 times more Ca^{2+} ions than IP_3Rs [65].

In our studies, dantrolene (10 μM) was observed to prevent the HOCl-induced enhancement of cytoplasmic Ca^{2+} level in HeLa cells at hypochlorite concentrations of 50 μM and 200 μM (Fig. 6 A and C). We also used cyclosporine A to assess the involvement of HOCl-induced calcium leakage out of mitochondria (due to mPTP opening) in cytoplasmic Ca^{2+} pool. Mitochondria were demonstrated to be able to form mPTPs under HOCl action [9]. In our studies, cyclosporine A (1 μM) was shown to reduce the cytoplasmic Ca^{2+} level in HeLa cells after exposure to both HOCl concentrations by preventing the mPTPs formation and Ca^{2+} leakage out of mitochondria (Fig. 6 A and C). Interestingly, cyclosporine A and dantrolene reduced the cytoplasmic Ca^{2+} level in HeLa cells almost to the same degree (Fig. 6 A and C). It should also be noted that the effects of inhibitors were less pronounced at a hypochlorite concentration of 200 μM rather than 50 μM (Fig. 6 A and 6 C, the corresponding curves before and after HOCl addition in the presence of dantrolene or cyclosporine A). To study the role of mitochondria in the cytoplasmic Ca^{2+} pool formation, we also applied

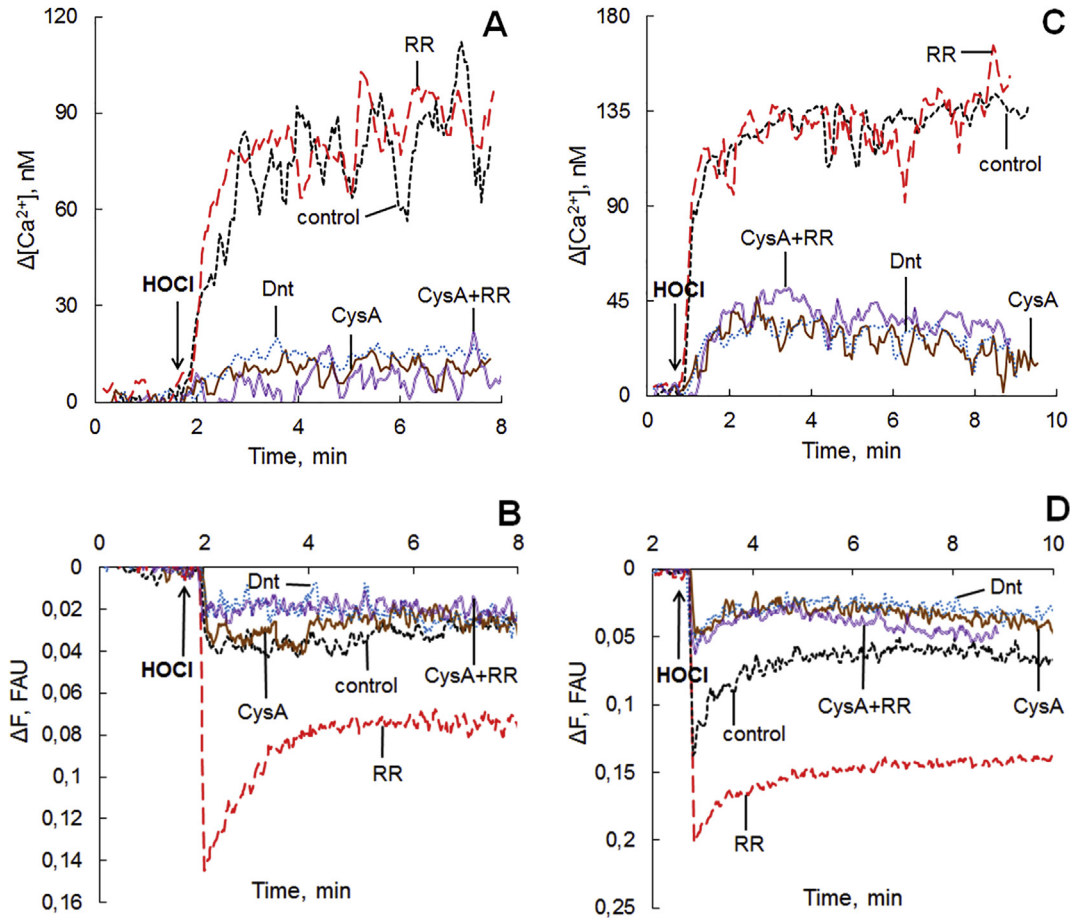

Fig. 6. Effects of inhibitors on the Ca^{2+} levels in the cytoplasm and mitochondria of HeLa cells (10^6 cells per ml) exposed to hypochlorite at concentrations of 50 μM (A, B) and 200 μM (C, D). A, C, cytoplasmic Ca^{2+} measured by Fura-2AM. B, D, mitochondrial Ca^{2+} measured by X-Rhod-1-AM. The addition of HOCl was indicated by arrows. No inhibitors were added to control samples. The cells were incubated with each of the inhibitors, ruthenium red (RR, 1 μM), cyclosporine A (CysA, 1 μM), and dantrolene (Dnt, 10 μM) for 2 min before HOCl addition. FAU, fluorescence arbitrary units.

ruthenium red, which prevents Ca^{2+} entry into the mitochondria. Ruthenium red (1 μM), an inhibitor of MCU, did not change the cytoplasmic Ca^{2+} level in HeLa cells under HOCl action (Fig. 6 A and C). The results of ruthenium red and cyclosporine A co-administration were the same as in the case of using cyclosporine A alone (Fig. 6 A and C). Furthermore, aiming to confirm the participation of mitochondria in the alterations of cytoplasmic calcium under hypochlorite action, we carried out the full mitochondrial depolarization of HeLa cells with FCCP (Supplementary Figs. 5 and 9). Addition of hypochlorite at concentrations of 50 and 200 μM to HeLa cells treated by FCCP resulted in a significant increase in the level of cytoplasmic calcium compared to untreated cells (Supplementary Figs. 10 and 11).

In our studies, the same inhibitors were also applied to estimate changes in the mitochondrial Ca^{2+} pool of HeLa cells under hypochlorite action. Interestingly, both cyclosporine A (1 μM) and dantrolene (10 μM) decreased Ca^{2+} leakage out of mitochondria at hypochlorite concentrations of 50 μM and 200 μM (Fig. 6 B and D) that prevented the cytoplasmic Ca^{2+} increase. It is noteworthy that more significant HOCl-induced Ca^{2+} leakage out of mitochondria was found in the presence of ruthenium red (1 μM) at both hypochlorite concentrations. It is likely, ruthenium red by blocking of MCU prevented the mitochondrial uptake of Ca^{2+} ions released from endoplasmic reticulum and enhanced the leakage of calcium out of mitochondria. The inhibition of MCU was previously shown to alter local Ca^{2+} feedback near IP_3Rs and promote the release of Ca^{2+} by mitochondria [66,67]. At the same time, co-administration of cyclosporine A (1 μM) and ruthenium red (1 μM) eliminated the effect of ruthenium red alone, indicating a key role of mPTPs in the Ca^{2+} release from mitochondria (Fig. 6 B and D). It should be noted that all these inhibitors did not completely prevent the leakage of calcium out of mitochondria (the curves with dantrolene or cyclosporine A before and after HOCl addition, Fig. 6 B and D). This is likely to be caused by occurrence of non-mPTP associated leakage of Ca^{2+} ions under HOCl action.

4. Discussion

Hypochlorite is an important mediator of inflammatory processes in many pathological conditions [68]. HOCl causes the death of damaged cells and healthy cells surrounding inflammation sites [5–7]. The mechanisms of hypochlorite effects in cells remain to be confirmed by more detailed studies. Reactions of HOCl with biological molecules include oxidation and chlorination [10,11]. In our experiments, we studied the mechanisms of cell damage under hypochlorite action, focusing on the role of mitochondria and cellular calcium. Our attention to the study of mitochondrial functional state was stipulated by the fact that mitochondria play a key role in many cellular physiological processes and contain a variety of signaling molecules including Ca^{2+} ions [13].

Earlier we used human erythrocytes to show that active forms of chlorine (hypochlorite or chloramine) in a concentration-dependent manner (50–200 μM) inhibited membrane Na^+-, K^+- and Mg^{2+} - ATP-ases as well as active glutathione S-conjugate

efflux, oxidized cellular reduced glutathione and membrane protein thiols and induced complex morphological transformations of cells: swelling, echinocytosis, and haemolysis, but did not induce lipid peroxidation [49,69].

In the present study, hypochlorite at physiologically relevant concentrations induced significant alterations in mitochondrial functional state that was accompanied by variations in respiratory activity, oxidation of GSH, increase of coenzymes NAD and FAD levels, and decrease of membrane potential which stimulates the mPTP opening. It was demonstrated earlier that the exposure of isolated mitochondria to HOCl *in vitro* in EDTA-containing medium inhibited the rat liver mitochondria respiratory activity and caused the uncoupling of the respiratory and phosphorylation processes without appreciable changes in the phosphorylation coefficient [70]. The decreased respiration rate at V_3 state was related to reduced levels of the mitochondrial protein sulfhydryl groups (PSH) and mitochondrial GSH. HOCl significantly inhibited a key enzyme in the Krebs cycle, α-ketoglutarate dehydrogenase. This is in line with the conclusions that alterations in the mitochondrial antioxidant system led to damage of enzyme complexes of the respiratory chain and Krebs cycle, cell bioenergetics disturbances, ROS production, and release of proapoptotic factors by mitochondria [13]. Similar effects of hypochlorite on the GSH level have already been demonstrated in different types of human cells [6,71,72]. Earlier we concluded that the membrane pore formation and disturbance in passive ion permeability are responsible for the HOCl-induced haemolysis [72]. Previously, it was assumed that the main cause of these disorders was largely hypochlorite-induced oxidative stress [10]. At the same time, we demonstrated that in the presence of EGTA the damaging effects of HOCl on the functional state of isolated mitochondria were less pronounced and fully eliminated at low concentrations of the oxidant. This reveals the importance of mitochondrial Ca^{2+} in the mechanisms of hypochlorite effect. One of the possible mechanisms of inhibition of the respiratory activity of rat liver mitochondria, especially at low concentrations of hypochlorite, is direct inhibition of cytochrome C oxidase activity by specific interaction of calcium ions with the cation binding site. It was previously demonstrated that calcium at a concentration of 0.5 μM suppressed the activity of liver mitochondria cytochrome oxidase by 50% [73]. And it is well established that such high levels of calcium can occur in cells only in the space of MAM contacts [74]. Since the calcium uptake by mitochondria leads to the intensification of ROS production, and in the case of calcium overload to the depolarization of mitochondrial membranes and mPTP opening [75–78], the protective effect of EGTA on isolated mitochondria exposed to hypochlorite could be due to the binding of calcium released out of mitochondria, and, therefore, decreasing of ROS production in mitochondria and preventing of the mitochondrial depolarization and cytochrome oxidase inhibition by Ca^{2+} ions. Because the calcium-induced ROS increase and ROS-mediated calcium vulnerability can create a self-amplifying loop [50,79]. It is known that in mitochondria Ca^{2+} transport is realized due to the mitochondrial permeability transition pore, mitochondrial calcium uniporter, and sodium/calcium exchanger [80]. Using Ca^{2+} indicator Fura-2AM, we demonstrated HOCl-induced Ca^{2+} release out of the isolated liver mitochondria that might be mainly due to mPTP opening. Our present findings pointed out that in the absence of exogenous Ca^{2+} the mitochondrial swelling was caused by HOCl-induced redistribution of intramitochondrial Ca^{2+} as a result of its re-accumulation by other intact mitochondria. That is confirmed by the ruthenium red and cyclosporine A prevention of the isolated mitochondria swelling and membrane depolarization. In addition, mitochondria are able to accumulate a significant amount of cellular Ca^{2+} [13,81].

Using HeLa cells, we observed a considerable increase in cytoplasmic Ca^{2+} level and a decrease in mitochondrial one under hypochlorite action. It was earlier shown that the HOCl-induced elevation of cytoplasmic Ca^{2+} was driven mainly by its entry into the cells from the intercellular space [8]. We can explain the increase in cytoplasmic Ca^{2+} level only by its release from the cellular stores in EGTA-containing medium (exogenous Ca^{2+} - free medium) used in our studies. It is assumed that the ER is the main cellular calcium store [58]. Ca^{2+} leakage from ER cisternae occurs mainly through Ca^{2+} release channels such as IP_3R and RyR receptors [18]. As was mentioned above, both channels are contained in HeLa cells. Since dantrolene considerably prevented an increase in cytoplasmic Ca^{2+} level in our experiments, we confirmed that the ER is the main source of cytoplasmic calcium released under HOCl action. It is well known that RyR2 receptors are very sensitive to the cell redox status because of having many cysteine residues in a reduced free thiol state per tetrameric channel [64]. In our studies, S-glutathionylation and shifts in the ratios of GSH/GSSG and NADH/NAD+ pairs can increase the ion-conducting ability of RyRs that explains the inhibitory effect of dantrolene [82–84]. Also, IP_3Rs are up-regulated under oxidative stress by stabilizing the active conformation of the receptor, and they are not inactivated by high intracellular Ca^{2+} concentrations [20,21]. As stated above, dantrolene is also capable to inhibit IP_3Rs. But dantrolene also prevented calcium release out of mitochondria in HeLa cells. This is due to the fact that because dantrolene prevents the Ca^{2+} release out of the endoplasmic reticulum, the mitochondria will not be overloaded by Ca^{2+}, and mPTPs will not activate. In its turn, cyclosporine A also prevented the decrease of mitochondrial calcium as well as the increase of cytoplasmic calcium levels in HeLa cells exposed to HOCl. And the effects of cyclosporine A on the cytoplasmic and mitochondrial Ca^{2+} levels were similar to dantrolene. Moreover, in the case of fully depolarized mitochondria hypochlorite induced more pronounced increase of Ca^{2+} level in cytoplasm.

These results demonstrated that mitochondria might accumulate Ca^{2+} released from ER. Mitochondria release calcium by mPTP opening, thereby participating in the formation of the cytoplasmic calcium pool. Since, in the presence of ruthenium red, HOCl-induced Ca^{2+} release from mitochondria is enhanced and the cytoplasmic Ca^{2+} level in HeLa cells does not change, we can assume that there is a redistribution of cytoplasmic Ca^{2+} between mitochondria. Additionally, it was shown that mitochondria could allow the propagation of Ca^{2+} signals in their network, independently to the cytoplasm [85].

Our conclusions are supported by the finding that major amount of RyRs and IP_3Rs, which are responsible for Ca^{2+} release from ER, is concentrated in the area of ER-mitochondria contacts (MAMs) [86–88]. The formation of MAMs, which are characterized by high [Ca^{2+}] microdomains, involves 5–20% of the mitochondrial surface [51]. There is a macromolecular complex containing mitochondrial VDAC proteins, 75 kDa glucose-regulated protein (grp75) and IP_3R receptors in the area of MAMs. This complex facilitates Ca^{2+} transfer between the mitochondria and ER [88]. In addition, blocking of mitochondrial Ca^{2+} uptake with uncouplers in HeLa cells was demonstrated to inhibit Ca^{2+} release and oscillations because of the increased feedback inhibition by Ca^{2+} of IP_3Rs in the absence of Ca^{2+} uptake by neighbouring mitochondria [89]. Also, MCU is known to be the main transporter of calcium ions into mitochondria. However, MCU has a low affinity for calcium (Kd ~ 15–20 μM) and a high rate of calcium transfer [51,74,90]. To transfer calcium ions by MCU into mitochondria, significant concentrations of Ca^{2+}, which arise only in the area of MAM contacts, are required [91]. Furthermore, in the area of such contacts a direct transfer of Ca^{2+} ions from ER into mitochondria was demonstrated [90]. Also, it was shown that large fraction of the Ca^{2+} released via RyRs or IP_3Rs can be taken up by mitochondria [92,93].

4.1. Conclusions

Hypochlorite impairs the antioxidant system and functional state of mitochondria. In the presence of EGTA and inhibitors of calcium transport, the effect of hypochlorite on the functional state of mitochondria is decreased. The present study provides an evidence that the changes in mitochondrial Ca^{2+} level are important part of the mechanisms of HOCl-mediated damage, especially at low hypochlorite concentrations. Our findings show that in cells exposed to HOCl mitochondria play a key role in the redistribution of cytoplasmic Ca^{2+} and act as mediators of Ca^{2+} release from ER into the cellular cytoplasm.

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Author contributions

V.T. Cheshchevik conceived of the conception of the work, planned the experiments and wrote manuscript.

N.G. Krylova contributed to the design and implementation of the studies of calcium level in HeLa cells, collected and analyzed the data.

G.N. Semenkova contributed to the design and implementation of the studies of calcium level in HeLa cells, collected and analyzed the data.

N.G. Cheshchevik contributed to the implementation of the experiments with the animals and isolated rat liver mitochondria, collected and analyzed the data.

E.A. Lapshina contributed to the design of the experiments with the isolated rat liver mitochondria, collected and analyzed the data.

I.B. Zavodnik took part in writing the manuscript, discussing the results and substantiating key ideas.

All authors provided critical feedback and helped shape the research, analysis and manuscript.

All authors have approved the final version of manuscript submitted to the Biochimie.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biochi.2021.02.009.

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