Intracellular pH in Thymocytes at the Early Stages of Apoptosis and Necrosis

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Dynamics of intracellular pH during apoptotic and necrotic death of lymphocytes was studied with the help of fluorescent pH-sensitive probe BCECF. Change in intracellular pH is an early differential marker of apoptotic and necrotic types of death in thymus lymphocytes.

Key Words: *apoptosis; necrosis; intracellular pH; Na/H-exchange; lymphocytes*

Apoptosis and necrosis are two types of cell death with different morphofunctional characteristics. Necrosis is based on permanent and progradient hydrolysis, oxidation, and catabolic changes [6,10].

Apoptosis is genetically programmed cell death initiated by active energy-consuming processes requiring integrity of the protein-synthesizing and transcriptional cell systems. Suppressor proteins (p53, p21) produced during the first stage arrest cell cycle and initiate the suicidal program [12]. Inhibitors of ATP and protein synthesis and kinase reactions inhibit *in vitro* apoptosis [13]. The second stage of apoptosis is characterized by irreversible morphofunctional changes including nucleus destruction and dramatic enhancement of endonuclease activity. Cell shrinkage, chromatine fragmentation, and formation of apoptotic bodies occur against the background of preserved barrier function of the plasma membrane.

Some chemicals, in particular antineoplastic drugs cause both apoptosis and necrosis. Opposite responses of various clones of FM3A mouse mammary gland cells to 5-fluoro-2-deoxyuridine (5-FDU) were reported [9]. Treatment of F28-7 clone with 5-FDU induced necrotic changes such as cell swelling and formation of 100,000-200,000 b.p. DNA fragments. Cells of F28-7-A clone responded to 5-FDU by apoptotic reaction associated with the formation of typical apoptotic bodies and nucleosomic DNA loops of about 200,000 b.p.

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Necrotic agents cause lysis of tumor cells, thus inducing inflammatory reaction, which promote tumor dissemination. In contrast to necrosis, apoptotic death of transformed cells induces no local inflammatory reaction which allows to localize and eliminate neoplastic foci.

Intracellular pH (pH_i) is an important integral parameter of cell homeostasis. In resting cells, constant pH_i is maintained due to buffer capacity of cell proteins, nucleotides, inorganic phosphates, and carbonates. An active component of H^+-exchange, Na/H antiporter, localized in the plasma membrane exchanges extracellular sodium ion for intracellular proton. Na^+ ions are transported by electrochemical gradient (resting potential of the lymphocyte plasma membrane is about -60 mV [2]).

In the present study we examined the dynamics of pH_i in thymus lymphocytes using *in vitro* models of necrotic and apoptotic cell death.

MATERIALS AND METHODS

Rat thymocytes were isolated as described elsewhere [4]. Cytoplasmic pH in thymocytes was measured with fluorescent probe BCECF serving as the indicator of intracellular pH.

Hydrophobic BCECF tetraacetoxymethyl ester (BCECF/AM) penetrates through plasma membrane and is quickly hydrolyzed in the cytoplasm by highly active unspecific intracellular esterases to tetraacid, which prevents its penetration into mitochondrial matrix or reticulum vesicles. The conformation of this

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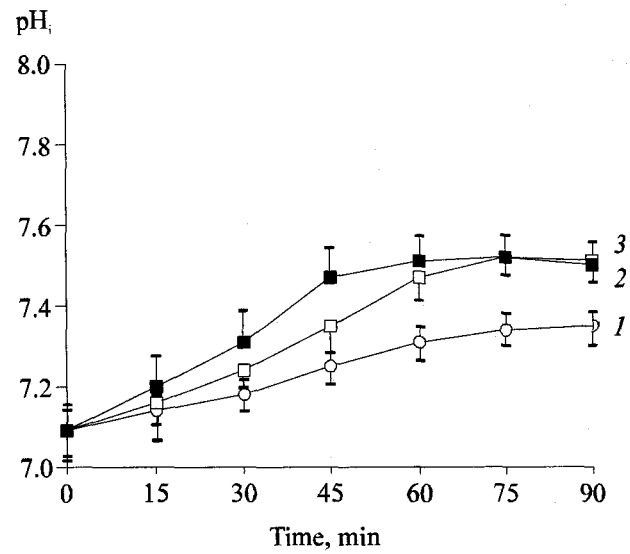

Fig. 1. Dynamics of pH$_i$ during necrotic lymphocyte death (oxidative stress): *1*) in the presence of 100 μM butyl hydroperoxide (BHP; pH$_o$ 7.35), *2*) at pH$_o$ 7.5, *3*) in the presence of 100 μM BHP, 30 mM deoxyglucose, and 50 μM trifluoperazine.

probe depends on pH and modulates the fluorescence parameters, thus allowing to measure pH$_i$.

Cells were loaded with the probe as described previously [3]. Isolated lymphocytes (1-2×10^7/ml) were incubated with 1 mM BCECF/AM in DMSO (final concentration 3 μM) for 20 min at 37°C, washed, and resuspended in HEPES buffer.

Lymphocyte suspension (2 ml, 3×10^6/ml) was placed into a cuvette of a Hitachi MPF-4 spectrofluorimeter at 37°C. Fluorescence excitation and emission spectra were recorded at 500 and 530 nm, respectively.

Levels of pH were calculated by calibration curves of BCECF/AM fluorescence [7], and the balance between pH$_o$ (pH of the incubation medium) and pH$_i$ was adjusted with nigericin in MES (6.5-6.8), HEPES (7.1-7.4), and Tricine (7.4-8.8) buffers.

The data were analyzed statistically using Pharmacological Basic Statistics software. Significance of differences was estimated by Student's *t* test.

RESULTS

Necrotic death of lymphocytes was induced by oxidative stress [11]. The cells were incubated with t-butyl hydroperoxide (BHP) which caused oxidative destruction within 8-12 h. Changes in pH$_i$ were studied during the first 1.5 h, because further incubation impaired cell integrity.

In control untreated cells (control 1, n=6) pH$_i$ was 7.12±0.05 and did not change significantly during observation. After 1.5-h incubation, pH$_i$ in 3 control samples decreased to 7.03±0.05.

In BHP-treated cells, the initial pH$_i$ did not differ significantly from the control (7.09±0.06, n=4). During incubation with BHP pH$_i$ gradually increased and reached a plateau after 60-75 min of observation (Fig. 1). Final pH$_i$ value (7.35±0.04) approximated pH in the incubation medium (pH$_o$). We assumed that pH$_i$ tended to extracellular pH. To verify this assumption we used incubation medium with pH$_o$ 7.5. These experimens revealed similar dynamics of pH$_i$ changes (Fig. 1), but final pH$_i$ was 7.51±0.07 (n=3). It should be noted that in control thymocytes incubated at pH$_o$ 7.5 (control 2), pH$_i$ varied from 7.02 to 7.16 and did not differ significantly from control 1.

To exclude possible leakage of the fluorescent probe into extracellular space, the cells after 1.5-h incubation with BHP were sedimented, resuspended in fresh incubation medium, and fluorescence was recorded. Total fluorescence was no less than 85% of the initial level (before washing) indicating primarily intracellular localization of the probe.

Thus, necrotic cell death is characterized by impaired homeostatic function of the plasma membrane and leveling of pH$_i$ and pH$_o$ after 60-min incubation with BHP. The increase in pH$_i$ seems to be independent on activation of Na/H-exchange, because selective Na/H antiporter inhibitor amiloride did not affect the dynamics of pH$_i$.

The inhibitor ATP synthesis 2-deoxyglucose (30 mM) and kinase inhibitor trifluoperazine (50 μM) added to BHP-containing incubation medium aggravated homeostasis disturbances leveling pH$_i$ and pH$_o$ within 45 min of incubation (Fig. 1, *3*).

Apoptotic death of thymocytes was induced by 1 μM dexamethasone. Changes in the cell shape and ionic homeostasis typical of this apoptotic model were described earlier [1.4]. Dexamethasone induced intracellular acidification, significant changes were noted after 45-min incubation (pH$_i$ 6.95±0.04, n=4). Thereafter, pH$_i$ gradually decreased reaching 6.87±0.04 by the end of observation (Fig. 2). Addition of 2-deoxyglucose and trifluoperazine to the incubation medium abolished dexamethasone-induced pH$_i$ changes, which suggests participation of energy-dependent reactions in the pH response of lymphocytes.

Molecular mechanisms underlying these phenomena can be connected with previously reported role of Na/H antiport in changes of lymphocyte volume and dexamethasone-induced apoptosis [4].

Amiloride-sensitive voltage-independent Na/H antiport is present in the plasma membranes of all mammalian cells and plays a crucial role in maintaining intracellular pH, transepithelial ion transport, and induction of mitogenesis [5].

In resting lymphocytes, only weak ionic currents are necessary for maintaining physiological pH. Mi-

togene activation of lymphocytes is characterized by rapid and sustained increase in cytoplasmic pH. The action of concanavalin A (con A) is mediated by a receptor mechanism and includes activation of phospholipase C and formation of the second messengers inositol trisphosphate and diacylglycerol. Diacylglycerol-activated protein kinase C phosphorylates the antiporter, thus increasing the rate of Na/H-exchange.

Phorbol esters, structural analogues of diacylglycerol, also sharply enhance antiporter activity. Their primary targets are Ca^{2+}- and phospholipid-dependent protein kinases C. Several facts point to protein kinase C-mediated stimulation of Na/H antiporter by phorbol ester: 1) Na/H antiporter and protein kinase C are activated by the same concentration of phorbol myristate acetate (PMA); 2) only kinase-activating phorbol derivatives affect Na/H antiporter; 3) the antiporter is stimulated by diacylglycerol, a natural protein kinase C activator.

In lymphocytes, as well as in other cells, Na/H antiporter can be activated at normal pH by a decrease in osmotic cell volume. Under these conditions Na/H antiporter plays a role in increasing cell volume to normal. Osmotic and phorbol-dependent antiporter activation are similar: both responses modulate transmembrane ionic currents and intracellular pH. Both mechanisms can be blocked by ATP depletion induced by trifluoperazine or sulfhydryl group blocker N-ethylmaleimide. However, activation of osmotic antiport does not depend on protein phosphorylation by protein kinase C [8].

Molecular mechanisms underlying the effect of dexamethasone on Na/H-exchange were studied by evaluating its action on Con A-, PMA- and osmotic stimulation of Na/H-exchange in lymphocytes.

Independently on the applied agent, pH_i rapidly (within 5-10 min) increased to 7.28-7.32, and these changes were blocked by 200 μM amiloride. In all experimental models, dexamethasone (1 μM) significantly inhibited pH_i increase without preincubation (glucocorticoid was added to cells 3-5 min prior to Con A, PMA, or hyperosmotic shock).

Thus, the inhibitory action of dexamethasone can be refered to early nongenomic effects of glucocorticoids, while inhibition of Na/H-exchange is an early manifestation of dexamethasone-induced apoptotic cell death.

These experiments showed that necrotic lysis of thymocytes induced by oxidative stress is characterized by early impaiment of pH_i-maintaining mechanisms and equilization of pH_i and pH_o as soon as after 60-min incubation with BHP. The experiments on the model of apoptosis revealed significant intracellular acidification (pH_i 6.87±0.04) 1 h after addition of dexamethasone. Inhibition of Na/H-exchange is a molecular mechanism of lymphocyte pH response. Intracellular pH can serve as an early differential marker of apoptosis and necrosis of thymus lymphocytes.

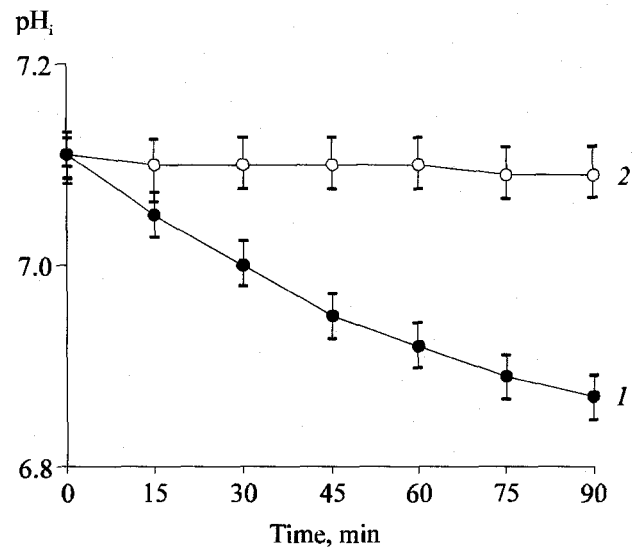

Fig. 2. Dynamics of pH_i during dexamethasone-induced apoptotic death of lymphocytes. *1*) 1 μM dexamethasone; *2*) 1 μM dexamethasone, 30 mM deoxyglucose, and 50 μM trifluoperazine.

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