Effects of Ascorbic Acid on Calcium Signaling in Tumor Cells

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Effects of ascorbic acid on calcium homeostasis of human laryngeal carcinoma cells were studied. Intracellular concentration of free calcium and intracellular pH were measured by fluorescent analysis. Ascorbic acid in concentrations of 3-10 mM caused pH drop and sharply increased concentrations of free Ca ions in HEp-2 cells. Intracellular concentration of free Ca ions resulted from Ca ion release from the thapsigargin-sensitive Ca depots.

Key Words: *calcium; intracellular signaling; ascorbic acid; laryngeal carcinoma*

The possibility of using ascorbic acid (AA) as an antitumor drug is now actively discussed. In concentrations of 1-10 mM AA induces the death of prostatic cancer, hepatoma, gastric cancer cells, U937 promyelocytic cells and other cultured tumor cells [8,13,15]. *In vitro* experiments on 10 tumor cell strains and 4 normal cell strains showed that AA in concentrations above 4 mM induced death of exclusively tumor cells [2].

The cytotoxicity of high AA doses can be mediated through H_2O_2, forming in solutions containing AA in the presence of metals of alternating valency [1]. It is known that H_2O_2 induces tumor cell death. Generation of H_2O_2 is an important stage in induction of tumor cell apoptosis by some drugs (cisplatin, etoposide, bleomycin, rituximab, bortezomid) [9]. However, the cytotoxic effect is not the only biological effect of H_2O_2 and AA. It was found that H_2O_2 stimulated the growth of vascular smooth-muscle cells, HeLa cells, mesenchymal stem cells, aortic epithelial cells, *etc.* In addition, H_2O_2 induces differentiation of HD-11EM precursor cells into osteoclasts [11]. It was shown that AA induced differentiation of HL-60 promyelocytic leukemic cells [5] and stem cells [14]. Hence, H_2O_2 formation in the presence of AA does not prove the cytotoxic effect of AA, because H_2O_2 produces not only toxic, but also regulatory effects. It is noteworthy that intravenous injection of AA in high (up to 8 mM) concentrations is not followed by the formation of H_2O_2 and AA radicals in the blood [3]. In the plasma, AA prevents lipid peroxidation even in the presence of metals of alternating valency and H_2O_2 [12]. Despite ample published data on AA cytotoxicity towards tumor tissues, the molecular and cellular mechanisms underlying this phenomenon remain unknown.

One of the stages of apoptosis activation in various cells is an increase of intracellular concentration of free calcium ions ($[Ca^{2+}]_{cyt}$). However, the effects of AA on calcium signaling processes were never studied.

We studied the mechanisms regulating calcium signaling in laryngeal carcinoma cells by AA.

MATERIALS AND METHODS

The study was carried out on HEp-2 human laryngeal carcinoma cells. This cell culture was obtained from Institute of Epidemiology and Microbiology,

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Ministry of Health of the Republic of Belarus. Measurements were carried out in balanced buffered saline (BBS) of the following composition: 131 mM NaCl, 5 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, 20 mM HEPES, and 6 mM glucose; pH 7.4. Buffered saline free from Ca ions contained 1 mM EGTA instead of $CaCl_2$. Intracellular concentration of free Ca ions and intracellular pH were measured by fluorescent analysis. Fluorescent probes fura 2-AM and 2,7-bicarboxyl-5(6)-carboxyfluorescein (BCECF) (Sigma) were used. The fluorescence parameters were measured using LSF 1211A spectrofluorometer (SOLAR).

RESULTS

Addition of AA and H_2O_2 changes $[Ca^{2+}]_{cyt}$ in HEp-2 human laryngeal carcinoma cells. AA induces a sharp increase in intracellular concentration of free calcium (Fig. 1, curves *1*, *2*), while H_2O_2 causes a long-lasting increase in $[Ca^{2+}]_{cyt}$ (Fig. 1, curve *3*).

The increment of $[Ca^{2+}]_{cyt}$ depends on AA concentration. The increase in intracellular concentration of free calcium is induced by AA in concentrations of 3-10 mM. The effect of AA in concentrations below 5 mM is reversible: replacement of extracellular buffer solution with AA-free BBS restores cell reactivity to external stimuli. In concentrations above 5 mM AA induces irreversible changes in HEp-2 cell homeostasis disturbing Ca homeostasis and cell reactivity to external stimuli.

According to published data, the effect of AA in high concentrations on functional activity of cells can be mediated through H_2O_2 forming in the presence of AA [2,3]. However, we showed that catalase in a concentration of 500 U/ml did not modify the increase in $[Ca^{2+}]_{cyt}$ (data not presented). Hence, the mechanism of $[Ca^{2+}]_{cyt}$ regulation by AA does not depend on the formation of extracellular H_2O_2.

As we know, intracellular concentration of free calcium increases as a result of Ca^{2+} release from intracellular depots and Ca^{2+} entry from extracellular environment through the plasma membrane channels. In order to evaluate the role of each of these mechanisms in the increase in intracellular concentration of free calcium, experiments were carried out in buffered saline with and without Ca^{2+}. It was shown that AA increases intracellular concentration of free calcium even in the absence of Ca^{2+} ions in extracellular solution. Hence, the increase of $[Ca^{2+}]_{cyt}$ under the effect of AA is a result of Ca^{2+} release from intracellular depots.

Calcium depots in non-excitable cells are intricate structures of great variety. It is hypothesized

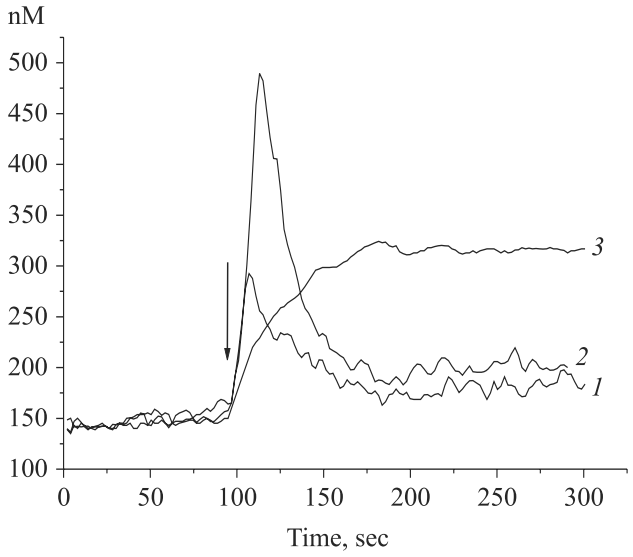

Fig. 1. Effect of AA in concentrations of 5 mM (*1*), 10 mM (*2*), and of H_2O_2 (*3*) on $[Ca^{2+}]_{cyt}$ in HEp-2 cells. Concentration of H_2O_2 in BBS was 4.5 mM. Here and in Figs. 2, 3: concentration of HEp-2 cells: $2.5*10^6$/ml. Arrow shows the moment of AA and H_2O_2 addition.

that one of the main mechanisms of Ca^{2+} increase in non-excitable cells is the so-called depot-dependent or "capacitance" Ca^{2+}entry [10]. The capacitance model of Ca^{2+} entry in cells is based on the hypothesis according to which Ca^{2+} entry from outside is regulated by the degree of Ca^{2+} depot filling: empty depots activate Ca^{2+} entry. It is hypothesized (by analogy with the role of the capacitor in an electric circuit) that Ca^{2+} depot prevents Ca^{2+} entry when it is full ("charged") and activates it when the depot is exhausted ("discharged").

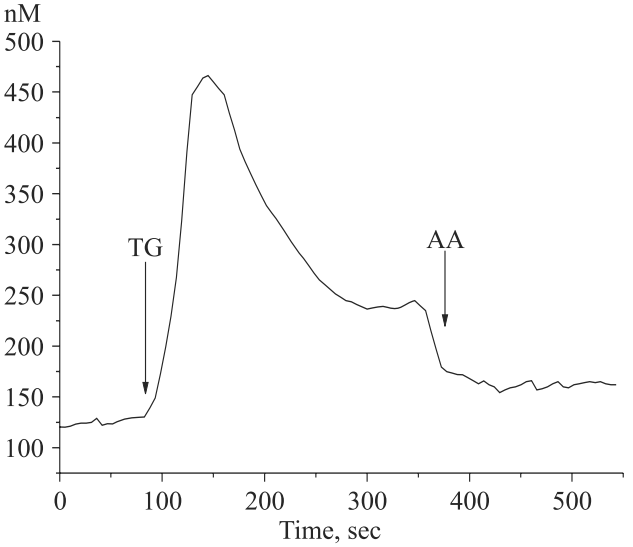

Fig. 2. Effect of AA on thapsigargin-induced elevation of $[Ca^{2+}]_{cyt}$ in HEp-2 cells. Thapsigargin (TG) concentration of BBS: 400 nM; AA in BBS: 5 mM. Arrow shows the moments of TG and AA addition.

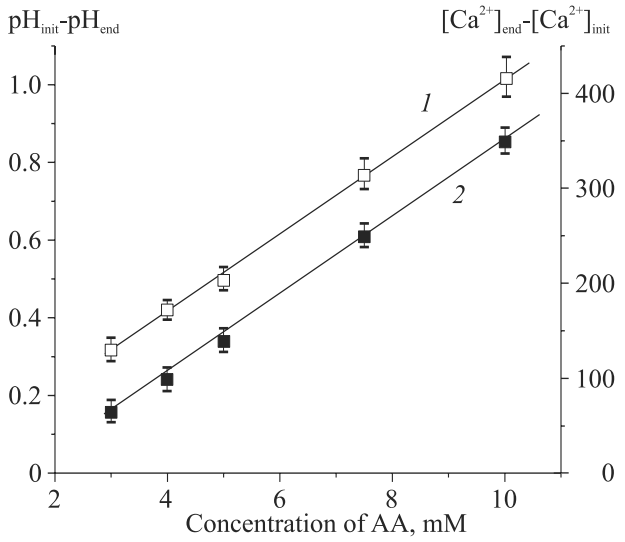

Fig. 3. Intracellular pH (*1*) and $[Ca^{2+}]_{cyt}$ (*2*) in HEp-2 cells as a function of extracellular AA concentration.

The capacitance Ca^{2+} entry is stimulated by various agents with a common characteristic (to promote Ca^{2+} release from the depot). We found that AA induced the release of Ca^{2+} ions from thapsigargin-sensitive Ca^{2+} depot. During thapsigargin treatment, the release of Ca^{2+} from intracellular Ca^{2+} depot stimulates entry of Ca^{2+} ions into the cell by activating plasma membrane Ca^{2+} channels after depot exhaustion (Fig. 2). After thapsigargin exhaustion of intracellular Ca^{2+} depots, AA causes no increase in the intracellular concentration of free calcium. Addition of AA to cell suspension at stage 2 of thapsigargin-induced elevation of intracellular concentration of free calcium causes a decrease of $[Ca^{2+}]_{cyt}$ (Fig. 2).

These facts suggest the following mechanism of AA effect. As we know, thapsigargin induces Ca^{2+} release from mitochondria and from inositol-1,4,5-triphosphate (IP_3)-sensitive Ca^{2+} depots. Calcium ions are released from HEp-2 cell mitochondria under the effect of AA. Thapsigargin-induced release of Ca^{2+} ions from depots after AA addition was reduced significantly in comparison with the control (data not presented). In addition, we found that AA caused a dose-dependent reduction of pH in HEp-2 cells. The increase of $[Ca^{2+}]_{cyt}$ depends on the degree of changes in pH (Fig. 3). Greater changes in pH are associated with greater changes in $[Ca^{2+}]_{cyt}$. The decrease in intracellular pH causes an increase in the electrochemical proton potential gradient on the inner mitochondrial membrane, leading to subsequent opening of high conduction pores, through which Ca^{2+} ions enter the intracellular space. It is noteworthy that hyperpolarization of the mitochondrial membrane, caused by a decrease in intracellular pH, is the initial stage of apoptosis activation in mammalian cells [7].

Reduction of intracellular pH, in turn, can modify the Ca-transporting systems on the plasma membrane. For example, it was shown that the increase in intracellular concentration of hydrogen ions inhibits Ca^{2+} flow through potential-dependent L-type Ca^{2+} channels [6]. It was also shown that the drop of extracellular pH inhibited capacitance entry of Ca^{2+} into the cell [4]. Hence, AA-induced Ca^{2+} release from depot blocks the subsequent Ca^{2+} entry through membrane channels.

In addition, we showed that reduction of the extracellular medium pH potentiated the effect of AA on calcium homeostasis. Intracellular calcium concentration increased 2-fold in the presence of AA in a concentration of 5 mM in BBS with pH of 6.9 in comparison with AA effect in BBS with pH 7.4. It is known that extracellular pH is reduced significantly in tumor tissues, as a result of which AA more effectively modulates calcium homeostasis of tumor cells in comparison with normal cells. The results of this study suggest that selective effect of AA on tumor cells *in vivo* is mediated through modification of the acid-base balance of tumor tissues. Further studies will provide more detailed data on the above mechanisms and will serve as the basis for the search and creation of new antitumor agents and new methods of antitumor therapy.

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