УДК 577.15.612.438

PHOTOACTIVATED FULLERENE C_{60} INDUCES STORE-OPERATED Ca^{2+} ENTRY AND CYTOCHROME *c* RELEASE IN Jurkat CELLS

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The values of endoplasmic reticulum Ca^{2+}-pool and store-operated Ca^{2+} entry (SOCE) were estimated in rat thymocytes and Jurkat cells loaded with indo-1 and treated with thapsigargin. It was shown that the relative value of SOCE in thymocytes was substantially lower than in Jurkat cells. Significant increase of SOCE in Jurkat cells preincubated with 10^{-5} M C_{60} and exposed to uv/visible light irradiation was detected at 1-3 h after exposure. At this time FCCP-induced Ca^{2+}-release from mitochondria was shown to be reduced, while cytochrome c level into the cytoplasm of Jurkat cells, detected by Western blot analysis, to be increased. It is supposed that Ca^{2+} flux remodulation induced by photoexcited fullerene C_{60} in Jurkat cells might be involved in the initiation of signalling events leading to cell apoptosis.

K e y w o r d s: thymocytes, Jurkat, Indo-1, fullerene C_{60}, store-operated Ca^{2+} entry, cytochrome c, apoptosis.

Ca^{2+} is highly regulated within cellular compartments to achieve precise control over multiple processes in the cell. The increase of cytosolic Ca^{2+} level ($[Ca^{2+}]i$) mediated by Ca^{2+} entry through the plasma membrane and cation release from endoplasmic reticulum (ER) or mitochondria provide calcium signals of different kinetics, amplitude and subcellular localization, which in integration with other signal-transduction cascades control not only cell proliferation, but also apoptosis and cell transformation. The role of Ca^{2+} in signalling pathways involved in carcinogenesis is well established, and altered expression of specific Ca^{2+} channels and pumps is shown in cancer cells of different tissue origin [1]. Though the question still remains how cancer cells can increase Ca^{2+} cycling to drive cell proliferation and to avoid Ca^{2+}-dependent apoptosis.

In non-excitable cells Ca^{2+} entry from extracellular space is mainly supported by the mechanism known as store-operated calcium entry (SOCE). This mechanism is capable of monitoring ER Ca^{2+} filling enabling influx through plasma membrane only when ER content is essentially decreased. It is assumed that mitochondria, which are central to cell death, do not accumulate Ca^{2+} that enters across the plasma membrane as efficiently as Ca^{2+} that is released from ER. Privileged transport of Ca^{2+} between ER and mitochondria sensitises mitochondria to opening of permeability transition pore (PTP) and releasing of apoptotic factors [2, 3]. It is thought that remodelling of Ca^{2+}-buffering system and, in particular, reduced basal filling of endoplasmic reticulum Ca^{2+}-stores, decreased SOCE. In this way, ER-mitochondrial Ca^{2+} fluxes in cancer cells to bypass Ca^{2+}-dependent apoptotic pathways [1, 4].

Taking into account that many Ca^{2+} transporters and signalling proteins are sensitive and directly exposed to reactive oxygen species (ROS) [2], redox regulation of their activity seems to be one of the ways to modulate Ca^{2+} compartmentalization and calcium homeostasis in cancer cells.

Recent progress in nanobiotechnology has arisen interest to biomedical application of fullerenes C_{60} – spherical carbon nanostructures, able to accommodate hydrophobic regions of cell membrane, to penetrate into cytosol and to generate superoxide anion and singlet oxygen with almost 100% quantum yield after UV/Vis irradiation [5]. In previous investigations we have demonstrated that pristine C_{60} fullerenes, when combined with UV/Vis light irradiation, exhibit cytotoxic effect and induce increased ROS production in transformed T lymphocytes but not in rat thymocytes [6]. Photoinduced apoptosis of Jurkat cells was confirmed by DNA fragmentation and caspase-3 activation [7].

The aim of this study was to estimate parameters of Ca^{2+} homeostasis (Ca^{2+}-pool of ER, SOCE, Ca^{2+} influx from mitochondria) and cytochrome *c* release from mitochondria in transformed T cells

(Jurkat cell line) in control or after treatment with fullerene C_{60} following light exposure.

Materials and Methods

Rat thymocytes were isolated from Wistar rats (150-180 g). Thymus was removed and passed through nylon mesh into RPMI 1640 medium. The isolated cells were washed by centrifugation at 600 g. The T leukemia Jurkat cell line was obtained from Bank of Cell Line from human and animal tissue of the Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology (NAS of Ukraine). Jurkat cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin and 100 μg/ml streptomycin.

Fullerenes C_{60} were synthesized in Technical University of Ilmenau (Germany) [8]. A stable water colloidal solution of 10^{-4} M fullerene C_{60} (purity > 99.5%), prepared by C_{60} transfer from toluene to water using ultrasound sonication, contains C_{60} clusters with the size of 12-50 nm.

Cells were incubated for 1 h with or without fullerene C_{60} (10^{-5} M). Fullerene C_{60} photoactivation was done by UV/Vis irradiation of probes with mercury-vapor lamp (320-600 nm light, irradiance 200 mW/cm^2, distance 2 cm).

The concentration of free cytosolic Ca^{2+} was measured using fluorescent probe Indo-1 (Sigma, USA). Cells (3×10^7/ml) in buffer A consisting of (mM): KCl – 5, NaCl – 120, $CaCl_2$ – 1, glucose – 10, $MgCl_2$ – 1, $NaHCO_3$ – 4, HEPES – 10, pH 7.4 were loaded with Indo-1AM in the presence of 0.05% Pluronic F-127 (Sigma, USA) for 40 min at 25 °C, then washed twice from excessive fluorescent probe by centrifugation (600 g, 10 min) and resuspended in a Ca^{2+}-free buffer A, containing 0.1 mM EGTA. Cells, loaded with Indo-1, were incubated at 37 °C for 1 h with or without fullerene C_{60} (10^{-5} M). Indo-1 fluorescence in cells was recorded using spectrophotometer (Shimadzu RF-510, Japan), λ excitation – 350 nm, λ emission – 410 and 495 nm. The concentration of free cytosolic Ca^{2+} was calculated as described in [9].

Release of cytochrome *c* from mitochondria was measured by Western blotting. Cells were harvested by centrifugation and gently lysed for 15 min in ice-cold buffer containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 50 mM NaF, 1 mM PMSF and the Complete Protease Inhibitor Cocktail Tablet (Roche, Germany). Lysates were centrifuged for 15 min at 14,000 g. Cell lysates (about 30 μg of total cell protein) were run in 15% SDS-polyacrylamide gel. After electrophoresis the proteins were transferred onto PVDF membranes. The PVDF membranes were then incubated in blocking solution (5% skim milk in PBST) for 1 h, followed by overnight incubation with antibody against cytochrome *c* (Sigma, USA) at a dilution of 1 : 2,500 at 4 °C. The membranes were washed with PBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (1 : 5,000 dilution) for 1 h. Immunoreactive bands were detected by enhanced chemiluminescence with an ECL plus Western blotting detection system (Amersham, USA). Equal loading of samples was confirmed by using anti-β-actin antibodies (Sigma, USA). Protein concentration was determined using DC Protein Assay Kit (Bio-Rad, USA).

Data processing and plotting were performed by IBM PC using specialized applications Excel 2003 and Origin 7.0.

Results and discussion

Ca^{2+}-content in endoplasmic reticulum (ER) of cells was measured indirectly using thapsigargin (TG) which inhibits ER Ca^{2+} pump allowing Ca^{2+} to release from the ER pool. Indo-1 loaded cells in Ca^{2+}-free medium were first depleted of ER Ca^{2+} with 1 μM TG. In the absence of Ca ions in the extracellular medium calcium pool of ER cannot be replenished, so the increase of calcium probe fluorescence corresponds to Ca^{2+} released from the endoplasmic reticulum. It was shown that 1 μM TG caused a gradual increase in $[Ca^{2+}]_i$, which was hold on the plateau within 10 min, indicating that calcium pool of ER was depleted (Fig. 1). The value of TG-induced $[Ca^{2+}]_i$ increase above the basal level was considered as the relative value of ER Ca^{2+}-pool.

Store-operated Ca^{2+} entry was examined by adding 1 mM $CaCl_2$ to cells, which were treated with TG in Ca^{2+}-free medium. The value of Ca^{2+}-induced $[Ca^{2+}]_i$ increase above the $[Ca^{2+}]_i$ level in TG-treated cells was considered as the relative value of SOCE. The corresponding traces of Ca^{2+} probe fluorescence changes are presented at Fig. 1, and quantitative data calculations - in a Table.

We found that dynamics of TG-induced Ca^{2+} release from ER was slower and the relative value of ER Ca^{2+} - pool was lower in Jurkat cells than in thymocytes (Fig. 1, Table).

When the medium was supplemented with 1 mM $CaCl_2$ 15 min after TG addition, the sustained plateau phase of $[Ca^{2+}]_i$ increase caused by SOCE was observed in cells. Detection of SOCE both in thymocytes and Jurkat cells indicates the presence of Ca^{2+}-channels in plasma membrane that are activated in response to ER depletion - store-operated Ca^{2+} channels (SOCC). These results correspond to the literature data about SOCC

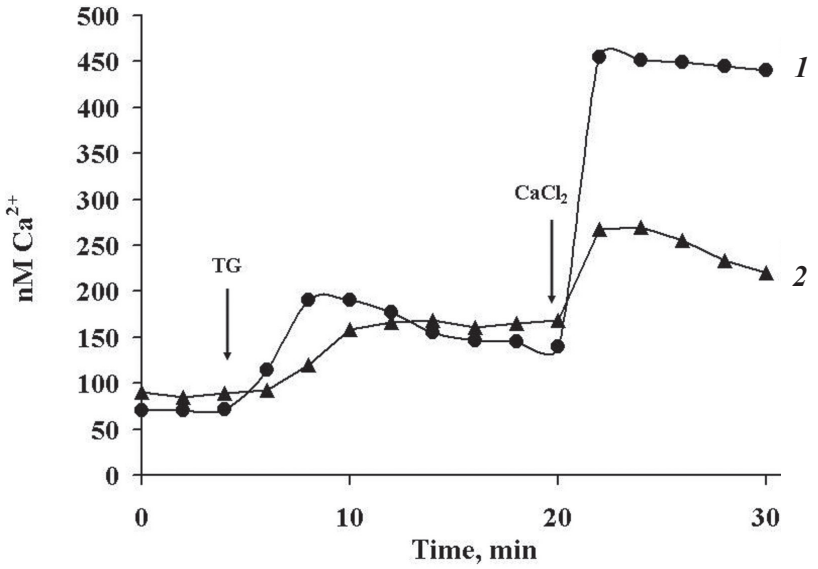

Fig. 1. Dynamics of thapsigargin-induced Ca^{2+} release from ER and store-operated Ca^{2+} entry in thymocytes (1) and Jurkat cells (2) loaded with Indo-1

expression in B- and T-lymphocytes and Jurkat cell line [10, 11].

Further, we demonstrated a significant difference in the relative value of SOCE in cells of two types. It was 3.5 times lower in Jurkat cells than in thymocytes (Fig. 1, Table). The obtained data agree with the assumption about genetically determined remodulation of calcium homeostasis: underfilling of ER calcium pool and reducing of SOCE in cancer cells is directed at prevention of Ca^{2+}-dependent way of apoptosis induction [1, 4].

The next task of the study was to determine whether photoexcited fullerene C_{60} affects calcium homeostasis in transformed T-cells. It was shown that separate action of fullerene C_{60} or light irradiation have no evident effect on dynamics of Indo-1 fluorescence signals in treated cells as compared to control cells (data not presented).

It was shown that combined action of 10^{-5} M C_{60} and light irradiation in the range of 320-600 nM results in time-dependent enhancement of store-operated Ca^{2+} entry in Jurkat cells. Ca^{2+}-induced an increase of $[Ca^{2+}]_i$ in cells incubated with fullerene C_{60} was characterized by the appearance of a net peak which was 4.5 and 7.8 times higher than those obtained in the control cells at 1 h and 3 h after fullerene C_{60} photoexcitation, respectively (Fig. 2).

Quantification of ER Ca^{2+} pool and SOCE in thymocytes and Jurkat cells

$\Delta[Ca^{2+}]_i$, nM	Thymocytes	Jurkat cells
Thapsigargin induced Ca^{2+} release from ER	130 ± 10	81 ± 7*
Store-operated Ca^{2+} entry	350 ± 15	102 ± 8*

*$P < 0.05$ in comparison with thymocytes

In our previous study we have demonstrated that transformed T-cells responded to a combined action of fullerene C_{60} and light irradiation by the increase of ROS production in a time period of 3 hours [6]. We assume that the increase of store-operated Ca^{2+} entry induced by photoactivated fullerene C_{60} in Jurkat cells is associated with intensification of ROS production and modulation of the activity of the components of calcium homeostasis-maintaining system – SERCA pumps, IP_3 and ryanodine receptors, mitochondrial PTP, which are the direct molecular targets of ROS [12, 4].

In Jurkat cells treated with photoexcited fullerene C_{60} the concentration of cytosolic $[Ca^{2+}]_i$ after reaching a peak induced by SOCE has been gradually reduced (Fig. 2). Since the accumulation of cation by endoplasmic reticulum was depressed due to the presence of thapsigargin, an inhibitor of ER Ca^{2+}-ATPase, the decrease of $[Ca^{2+}]_i$ was likely to be caused by mitochondrial accumulation. Recent studies have shown that pseudosynaptic contacts exist between ER and mitochondrial Ca^{2+} pools, so the release of Ca^{2+} from ER stores can result in rapid Ca^{2+} uptake by mitochondria [3, 14].

To estimate mitochondrial Ca^{2+} content we used FCCP - proton ionophore and uncoupler, which completely releases the mitochondrial Ca^{2+} pool. It was shown that subsequent addition

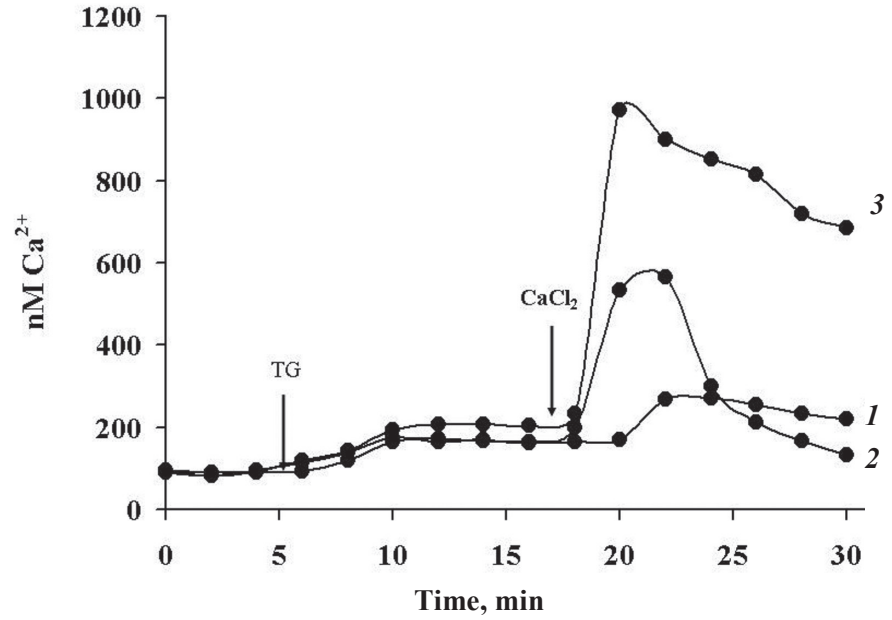

Fig. 2. Dynamics of store-operated Ca^{2+} entry in control Jurkat cells (1) and at 1 h (2) or 3 h (3) after fullerene C_{60} photoexcitation

of FCCP to thapsigargin-treated control Jurkat cells induced sustained $[Ca^{2+}]_i$ increase indicating that mitochondrial Ca^{2+} pool was filled enough (Fig. 3, 1). FCCP induced weaker Ca^{2+} release from mithochondria in the cells exposed to combined action of fullerene C_{60} and light irradiation than in the control cells indicating that mithochondrial Ca^{2+} pool had been already partially depleted. So, in Jurkat cells treated with photoexcited fullerene C_{60} SOCE is increased and mitochondria do not retain accumulated Ca^{2+}, possibly because of transient fall of $\Delta\mu$.

When Ca^{2+} released from ER is localized in microenvironment of mitochondria and when high local concentrations of Ca^{2+} and ROS are combined, mitochondria could respond by depolarization, PTP opening and release of apoptotic factors. To confirm this possibility we estimated the

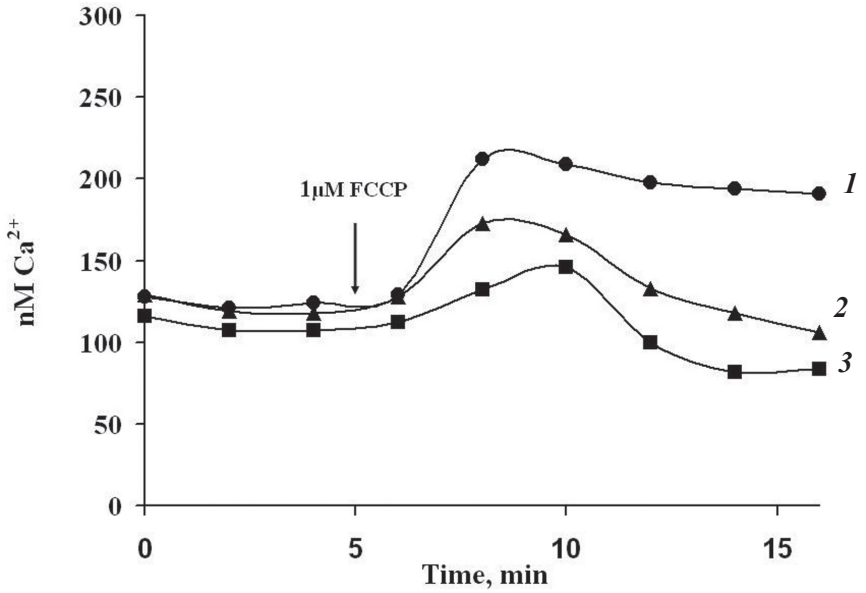

Fig. 3. FCCP-induced Ca^{2+} release from mitochondria of TG-treated control Jurkat cells (1) and at 1 h (2) or 3 h (3) after fullerene C_{60} photoexcitation

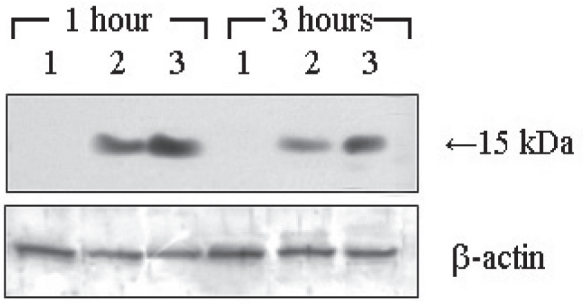

Fig. 4. Cytochrome c release into cytoplasm of control Jurkat cells (1), after irradiation (2) or fullerene C_{60} photoexcitation (3)

content of cytochrome *c* in the cytosolic fraction which corresponds to the release of cytochrome *c* from mitochondria as the marker of Jurkat cell apoptosis induced by photoexcited C_{60}.

As can be seen from Fig. 4, cytochrome *c* was detectable within cytosolic fraction prepared 1 h after exposure of Jurkat cells to light irradiation, but 3 h later its level was decreased (Fig. 4). Combined action of fullerene C_{60} and light irradiation was followed by increase of cyt *c* release from mitochondria. Its content in cytosolic fraction was substantially higher in cells irradiated in the presence C_{60} both at 1 h and 3 h after irradiation. Cytochrome *c* was also shown to appear in the cytosol of HeLa cells treated with photodynamic therapy immediately (1-2 h) following light activation of the photosensitizer verteporfin and before caspase-8 activation [15].

The similar dynamics of SOCE increase and cyt *c* release from mitochondria (Fig. 2 and 4) induced by photoactivated fullerene C_{60} indicates that Ca^{2+} fluxes are involved in early events of apoptosis induction in Jurkat cells. Remodulation of Ca^{2+} fluxes precedes caspase-3 activation in Jurkat cells, that was not detected during 6 h after combined action of fullerene C_{60} and light irradiation [7].

In the present study the effect of photoexcited fullerene C_{60} on ER and mitochondrial Ca^{2+}-pools and store-operated Ca^{2+} entry in Jurkat cells loaded with Indo-1, as well as on cytochrome *c* content in cell cytosol were estimated. Together, it was demonstrated that under light irradiation C_{60} induces the increase of SOCE, depletion of mitochondrial Ca^{2+}-pool and cytochrome *c* release from mitochondria. Our data suggest that Ca^{2+} flux remodulation induced by photoexcited fullerene C_{60} might be involved in the initiation of Jurkat cells apoptosis.

This work was supported by grants from National Academy of Science of Ukraine (№ 5.18.5.16, State Programm «Nanotechnologies and Nanomaterials») and BMBF (Ukr10/012).

ІНДУКЦІЯ ЄМНІСНОГО ВХОДУ Ca^{2+} І ВИВІЛЬНЕННЯ ЦИТОХРОМУ *c* В КЛІТИНАХ Jurkat ЗА ДІЇ ФОТОЗБУДЖЕНОГО ФУЛЕРЕНУ C_{60}

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З використанням флуоресцентного зонда Індо-1 оцінено відносну величину кальцієвого пулу ЕПР, ємнісного входу Ca^{2+} і ступеня наповнення кальцієвого пулу мітохондрій в тимоцитах і клітинах Jurkat. Показано, що ємнісний вхід катіона в тимоцити значно нижчий, ніж у клітини Jurkat. Відмічено значне посилення ємнісного входу Ca^{2+} у клітини Jurkat, преінкубовані з 10^{-5} М C_{60} і піддані дії видимого опромінення. Через 1–3 години після його впливу було зафіксовано зниження FCCP-індукованого вивільнення Ca^{2+} з мітохондрій і підвищення вмісту цитохрому *c* у цитоплазмі клітин Jurkat. Припускається, що ремодуляція системи Ca^{2+}-сигналювання може бути залучена до початкових етапів індукованого фотозбудженим фулереном C_{60} апоптозу в клітинах Jurkat.

Ключові слова: тимоцити, Jurkat, Індо-1, фулерен C_{60}, ємнісний вхід цитозольного Ca^{2+}, цитохром *c*, апоптоз.

ИНДУКЦИЯ ЕМКОСТНОГО ВХОДА Ca^{2+} И ВЫСВОБОЖДЕНИЯ ЦИТОХРОМА *c* В КЛЕТКАХ Jurkat ПРИ ДЕЙСТВИИ ФОТОВОЗБУЖДЕННОГО ФУЛЛЕРЕНА C_{60}

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С использованием флуоресцентного зонда Индо-1 дана оценка относительной величины кальциевого пула ЭПР, емкостного входа Ca^{2+} и степени наполнения кальциевого пула митохондрий в тимоцитах и клетках Jurkat. Показано, что емкостный вход катиона в тимоциты значительно ниже, чем в клетки Jurkat. Отмечено значительное увеличение емкостного входа Ca^{2+} в клетки Jurkat, преинкубированные с 10^{-5} М C_{60} и подверженные действию видимого облучения. Через 1–3 часа после его воздействия зафиксировано снижение FCCP-индуцированного высвобождения Ca^{2+} из митохондрий и повышение содержания цитохрома *c* в цитоплазме клеток Jurkat. Предполагается, что ремодуляция системы Ca^{2+}-сигнализации может быть задействована в начальных этапах индуцированного фотовозбужденным фуллереном C_{60}.апоптоза в клетках Jurkat

Ключевые слова: тимоциты, Jurkat, Индо-1, фуллерен C_{60}, емкостной вход цитозольного Ca^{2+}, цитохром *c*, апоптоз.

1. *Llewelyn Roderick H., Simon J. Cook.* // Nat. Rev. Cancer. – 2008. – **8**. – P. 361–375.
2. *Csordas G., Renken C., Varnai P. et al.* // J. Cell Biol. – 2007. – **174**. – P. 915–921.
3. *Franzini-Armstrong C.* // Physiology. – 2007. – **22**. – P. 261–268.
4. *Flourakis M., Prevarskaya N.* // Biochim. Biophys. Acta. – 2008. – **7**. – P. 295–306.
5. *Da Ros T., Spalluto G., Prato M.* // Croatica Chemica Acta. – 2001. – **74**. – P. 743–755.
6. *Grebinyk S., Prylutska S., Grynyuk I. et al.* // 36th FEBS CONGRESS Torino, June 25-30. – 2011. – P. 35–36.
7. *Palyvoda K., Grynyuk I., Prylutska S. et al.* // Укр. біохім. журн. – 2010. – **82**, № 4. – С. 121–128.
8. *Scharff P., Risch K., Carta-Abelmann L. et al.* // Carbon. – 2004. – **42**. – P. 1203–1206.
9. *Grynkiewicz G., Poenie M., Tsien R. Y.* // J. Biol. Chem. – 1985. – **260**. – P. 3440–3450.
10. *Kerschbaum H. H., Cahalan M. D.* // Science. – 1999. – **283**. – P. 836–839.
11. *Fomina A. F. Fomina A. F.* // J. Cell Biol. – 2000. – **150**. – P. 1435–1444.
12. *Hidalgo C., Donoso P.* // Antioxid. Redox Signal. – 2008. – **10**. – P. 1275–1312.
13. *Brini M.* // Cell. Calcium. – 2003. – **34**, N 4–5. – P. 399–405.
14. *Szabadkai G., Bianchi K., Varnai P. et al.* // J. Cell Biol. – 2006. – **175**. – P. 901–911.
15. *Graville D., Saw J., Leong C.* // Am. J. Pathol. – 1999. – **55**. – P. 1021–1025.

Received 13.09.2012