Biochemical Effects of Some Endoplasmic Reticulum Stressors and pre-B Lymphocytes Apoptosis

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Tunicamycin is a powerful endoplasmic reticulum stressor. Endoplasmic reticulum stress reflects the accumulation of unfolded proteins and is followed by the activation of Unfolded Protein Response (UPR). The effects of tunicamycin are related to its capacity to inhibit the protein N-glycosylation. This moment, there are few data concerning the importance and mechanisms of pre-B cells apoptosis induced by tunicamycin. In our study, tunicamycin induced apoptosis in a large number of isolated murine pre-B cells. Its apoptotic effects were less extensive than those induced by valinomycin, affecting almost all cultured pre-B cells. On the other hand, thapsigargin, a well known endoplasmic reticulum Ca^{2+} disruptor and stressor had no significant apoptotic effects in pre-B cells. The mechanisms underlying the apoptotic effects of tunicamycin in pre-B cells remain to be studied in depth by future approaches.

Keywords: tunicamycin, thapsigargin, pre-B cells, apoptosis

The cell organelles are including endoplasmic reticulum (ER), featuring synthesis of transmembrane proteins and proteins. Hypoxia, starvation, infections and alterations in secretory functioning induce ER stress, modifying its protein folding capacity. Numerous diseases are associating as pathologic mechanism the ER stress, playing an important role in neurodegenerative conditions, stroke, cancer, metabolic diseases and inflammation. The cellular response is aiming to restore homeostasis through the activation of multiple sensors. Such responses are known as the Unfolded Protein Response (UPR), which might cross with the Integrated Stress Response (ISR), represented by protein synthesis reduction through inactivation of the initiation factor eIF2á. The basic sensors involved in UPR are: PERK, IRE1, TF6, JNK, but also transcription factors XBP-1, ATF4 and CHOP. Trying to restore homeostasis, these proteins might also induce cell death through necroptosis or, obviously, through mitochondrial apoptosis. Furthermore, ER as well as proteotoxic stress are demonstrated to induce TRAIL receptors and further activation of caspase-8. If we will be able to completely understand how cells react to ER stress we'll discover the treatment for the above mentioned diseases [1].

Many intracellular signaling pathways are included in UPR as a very complex network. The stress intensity and duration, type of insults, as well as the cell type, all are related to homeostasis restoration or apoptosis induction. Several regulators and effectors are shared in the cells by inflammatory signaling and ER stress in a broad range of biological processes. In many of the pathological conditions the relationships between inflammation and ER stress. However, the interaction between ER stress and inflammation in many of these diseases are far from being at least clarified. Countering these pathologic mechanisms needs the best understanding of their biochemistry, cell biology, and physiology [2].

This moment it is clearly demonstrated that mitochondria is involved in pathologic conditions as degenerative and hyperproliferative ones (e.g., hypertriglyceridemia, nonalcoholic fatty liver disease, and metabolic syndrome) [3].

Mitochondria is also initiating apoptosis as a result of photosensitizers activation in tumor cells, destroying them through the increased release of reactive oxygen species [4].

Enhancement of respiratory function (OXPHOS state) in isolated liver mitochondria from mice with murine melanoma is achieved after the treatment with with betulinic acid, an anti-tumoral agent with antiinflammatory, antiangiogenic, and immunomodulatory effects [5].

Interleukin-24 (IL-24) is a unique member of IL-10 family cytokine which might destroy in a selective way the cancer cells through activating apoptosis, without any kind of effects on normal ones. These antitumor effects are characteristic only for intracellular IL-24, inducing an ER stress response. Intracellular administration of IL-24 using a recombinant fusion protein TAT-IL-24-KDEL (where TAT

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is the targeting domain of transactivator of transcription; and KDEL is the ER retention four-peptide sequence Lys-Asp-Glu-Leu) blocked the development of bladder cancer cells, non-small cell lung cancer cell line and breast cancer cell line in vitro. On the other side, the TAT-IL-24-KDEL protein did not affect the normal human lung fibroblast cell line, indicating its high efficiency against tumor cells [6].

The human ovarian cancer cells cisplatin resistance is suggested to be related to ER stress tolerance. These effects are evident in cisplatin resistant SKOV3/DDPcells, whereas SKOV3cells are cisplatin-sensitive. The pro-apoptotic pathways were amplified in the cisplatin-sensitive SKOV3cells by the overload of mitochondria with Ca^{2+} released from ER as result of its stress. Such effects were multiplied by more ER-mitochondria contacts in the cisplatin-treated SKOV3cells. Thus, cisplatin resistance in ovarian cancer cells is induced through the enhancement of cell survival [7].

The highest incidence among all cancers is owned by small cell lung cancer (SCLC). Although chemotherapy is the election treatment for SCLC, the currently used drugs are having a very low effectiveness. Evodiamine (EVO) might transform in a promising potent antitumor drug candidate for SCLC. When investigated in both human SCLC cell lines NCI-H446 and NCI-H1688, EVO induced apoptosis by the upregulation of caspase-12, cytochrome C, and Bax mRNA, as well as the down-regulation of Bcl-2 mRNA expression, at the same time with cell cycle arrest at G2/M phase. Apoptosis is dependent on mitochondria and endoplasmic reticulum stress (intrinsic pathway) and not on extrinsic death receptor-induced pathway [8].

Retinitis pigmentosa (RP) development is associated with the deleterious effects of TULP1 gene mutations. Cellular apoptosis might be the result of UPR complex activation, following the accumulation of misfolded proteins in ER. In hTERT-RPE-1 cells the wt-TULP1 localized in its majority to the cytoplasm and plasma membrane. In contrast, TULP1 missense mutations (I459K, R420P and F491L) were evidenced as cytoplasmic punctate staining which co-localized with the ER. The downstream targets of the ER-UPR complex are BiP/GPR-78, phosphorylated-PERK (Thr980) and CHOP [9].

C1q/TNF-Related Protein (CTRP) 9, the closest paralog of adiponectin, could counteract the diet-induced obesity and non-alcoholic fatty liver disease (NAFLD). Human recombinant CTRP9 significantly ameliorated palmitate- or tunicamycin-induced hepatic steatosis and apoptosis and decreased ER stress markers, such as eIF2alpha, CHOP and IRE-1, in HepG2 cells. The expression of autophagy markers like LC3 conversion, P62 degradation, Beclin1 and ATG7 was increased through AMPK phosphorylation in human primary hepatocytes under CTRP9 treatment [10].

The hepatic manifestation of metabolic syndrome is the so-called non-alcoholic fatty liver disease (NAFLD). The development of steatosis involves ER stress, whereas reactive oxygen species (ROS) might be involved in further transformation in non-alcoholic steatohepatitis (NASH). There is also an important cross talk between ER and oxidative stress, suggesting the possibility to develop drugs influencing both statuses [11].

ER stress is involved also in type 2 diabetes altered beta cell function and death. Some small molecules were able to reduce the mouse beta TC6 cell death induced by ER stressors tunicamycin and palmitate, as well as to restore impaired glucose-stimulated insulin secretion responses, triggered by ER stress. These effects are mediated through reducing the expression of key genes of the unfolded protein response and apoptosis. Thus, ER stress is removed [12].

Hyperthermia is known to reduce cell viability. Vitexin might be a potent protector against hyperthermia lesions and its effects seem to be mediated by the induction of Hsp90, reduction of oxidative stress, as well as the increasing of MAPKs via ER stress-induced autophagy [13].

Some malignancies are resistant to currently used proteasome inhibitors. The 19S DUB inhibitor b-AP15 is inducing a strong apoptotic response, elicited by enhanced oxidative stress and ER stress [14].

It is also to be mentioned that one modern approach for heart failure treatment is represented by ER stress-induced apoptosis [15].

Many chronic inflammatory (e.g., the three facets of atopic asthma: inflammation, airway fibrosis, and airways hyperresponsiveness) and autoimmune diseases are including as pathologic mechanism the endoplasmic reticulum (ER) stress response. House Dust Mite (HDM) was able to induce ER stress transducers in epithelial cells. HDM extract induced activation of caspase-3, along with ATF6 alpha (activating transcription factor 6 alpha) and ERp57 (protein disulfide isomerase, inducing oligomerization of Bak). Inflammation, airway hyper-responsiveness and airway fibrosis were dramatically reduced by siRNA-mediated knockdown of ATF6 alpha and ERp57. Thus, it was demonstrated that the inhibitors or ER stress could represent promising agents for the treatment of sub-epithelial fibrosis associated with loss of lung function and chronic bronchial asthma [16].

This study aimed the effects of endoplasmic reticulum stressors as thapsigargin and tunicamycin on apoptosis of pre-B lymphocytes.

Experimental part

Mouse pre-B cells were isolated from bone marrow using peanut agglutination and fractionation by fluorescence-activated cell sorter after slight adaptation of the method previously described [17]. High levels of pre-B cells are obtained using this method. The growth of mouse pre-B cells required RPMI 1640 medium (Sigma-Aldrich), with 2 mM L-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 10% heat-inactivated FBS, incubated with 5% CO_2 and at 37°C, as previously described [18, 19].

The density of pre-B cells was maintained at 5×10^5 per ml during the growth. For some experiments (in triplicate), pre-B cells were treated with 1μM thapsigargin or 0.05 mg/mL tunicamycin for 9 h. As control we used the effects of valinomycin 10μM, a good inducer of apoptosis through mitochondrial permeability transition pore opening, also in triplicate. To study the mitochondrial membrane potential we used 1μM of the very sensitive marker JC-1 (Sigma-Aldrich), at 37°C for 30 min.

The Microradiance setup for the laser confocal microscopy included an inverted Nikon Eclipse TE-300 microscope, oil-immersion objectives (x60), as well as the LaserSharp software. HQ515/530 was used as emission filter for 488 nm excitation and HQ530/560 for 514 nm excitation, respectively.

The collected images (having a standard imposed resolution of 1280 x 1024) were analyzed using free ImageJ (National Health Institute, U.S.A.).

Results and discussions

The treatment of pre-B cells with 1 μM thapsigargin did not significantly dissipate the mitochondrial membrane potential (Ψ_{mt}, fig. 1) as compared to 0.05 mg/mL

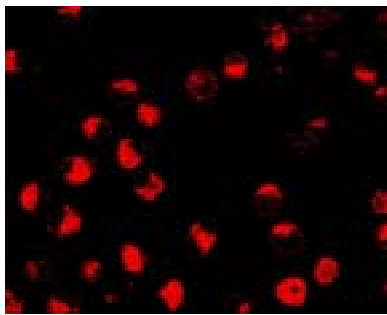

Fig. 1. Laser confocal microscopy and JC-1 reveal high ψ_{mt} for 89.19±4.53% of pre-B cells treated with 1 μM thapsigargin for 9 h. Representative image of many acquired from triplicate experiments (60x).

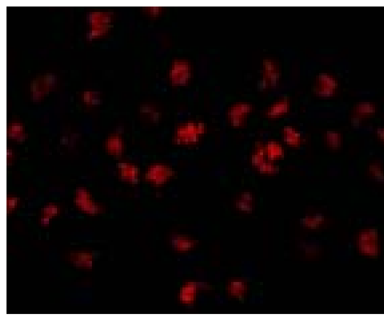

Fig. 2. Pre-B cells, receiving 0.05 mg/mL tunicamycin for 9 has treatment, associate high ψ_{mt} for 61.07±3.93% of them. Representative image of many acquired from triplicate experiments (60x)

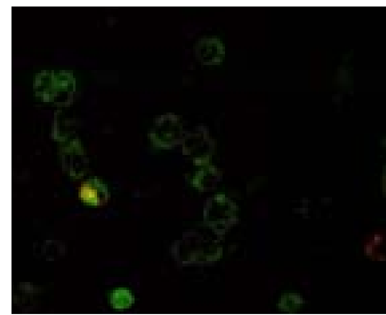

Fig. 3. In the presence of 10 μM valinomycin for 9 h only 6.57±1.17% of pre-B cells associate high ψ_{mt}, showing very good control experiments. There is a large green emission of JC-1, evident for mitochondrial membrane potential dissipation. Representative image of many acquired from triplicate experiments (60x).

tunicamycin for 9 h (fig. 2). The high Ψ_{mt} was associated with almost 89.19±4.53% of the cells in the case of thapsigargin, in contrast to 61.07±3.93% in the case of tunicamycin and 6.57±1.17% for valinomycin (fig. 3). The high Ψmt is always mirrored by bright intensity of red emission of JC-1. The above results clearly suggest that ER stressor thapsigargin is not inducing massive pre-B cells apoptosis. Thapsigargin is interacting rapidly, in a stoichiometric and irreversible manner with all isoforms of SERCA (Ca-ATPase family). In contrast, tunicamycin, an inhibitor of protein N-glycosylation, induced important pre-B cells apoptosis. Cycloheximide, an inhibitor of protein synthesis, blocked tunicamycin effects in pre-B cells (personal observation, data not shown). Anyway, the control apoptotic effects induced by valinomycin were the highest ones, almost all pre-B cells being apoptotic.

ER stress is representing the incriminated inductor of apoptosis through aberrant autophagy of T lymphocytes in systemic lupus erythematosus (SLE). Both biological processes, anomalous regulation of ER stress and apoptosis, were involved in the pathogenesis of autoimmune disorders. Thapsigargin (TG) is a well known disruptor of intracellular free Ca^{2+} levels, and, thus, a ER stressor and apoptosis inducer. Lupus T lymphocytes exhibited reduced autophagy and increased apoptosis in response to ER stress induced by thapsigargin administration. Furthermore, ER stress transducers as PERK, p-eIF2 alpha, IRE1, and ATF6, as well as GRP78, were reduced, in contrast to increased CHOP concentrations, as result of thapsigargin administration. On the other hand, the anti-apoptotic molecules (Bcl-2 and Bcl-X-L) decreased, whereas the pro-apoptotic molecules (Bax and caspase 6) were amplified in lupus T cells. It is thus to mention that SLE is associating an abnormal T cell homeostasis related to increased apoptotic T cell death [20].

Extremely important from the point of view of the survival or death pathways following UPR activation, the chosen one is dependent on the duration and type of stressors, that means the duration and type of UPR exposure. In ovarian cancer cells diindolylmethane (DIM) treatment enhanced the expression of the regulators of ER stress (e.g., Bip, IRE1, CHOP) and of UPR related apoptotic proteins, same time with the decreasing of EpCAM and activity of AP-1 transcription factor. The clear conclusion is that UPR mediated JNK/Ap-1 signal transduction is extremely important in apoptosis regulation in human ovarian cancer cells [21].

Extensive lymphocyte apoptosis induced by sepsis is an abnormal phenomenon. ER stress involvement through UPR activation was experimentally demonstrated in C57BL/6 mice with cecal ligation and puncture (CLP, septic mice). Their splenocytes presented amplified induction of GRP78 (glucose-regulated protein 78) and spliced XBP1 mRNA amassment, indicating UPR activation. Furthermore, CHOP (C/EBP homologous protein), as well as its mRNA, were both found in higher amounts, suggesting the switching of ER stress response into an pro-apoptocic one [22].

Apoptotic changes in T lymphocytes could be induced by atrazine (ATR), with important immunotoxicity in the immune system. Dysregulation of immune system induced by ATR can be attributed to activation of ER stress as shown by eIF2á phosphorylation and CHOP induction in apoptotic human Jurkat T-cells. Mitochondrial cytochrome C release was not affected, whereas caspase-3 was activated and caspase-8 and PARP were cleaved. When mice were exposed to ATR for 4 weeks, was observed an important fall in number of spleen $CD3^{+}$ T lymphocytes, in contrast to no changes of the number of $CD19^{+}$ B lymphocytes and nonlymphoid cells [23].

ER homeostasis might be the basic phenomenon underlying the autophagy (macrophagy)-regulated mobilization of Ca^{2+} in T lymphocytes. Activation of T lymphocytes means T cell receptor (TCR) engagement and triggering of calcium-signaling flux. One of the above mentioned fluxes is begun through the release of ER Ca^{2+} stores, after the specific IP_3 receptor activation. This pathway might be regulated also by autophagy, maintaining its homeostasis in a developmental manner. Deleting the genes related to autophagy generates defective Ca^{2+} fluxes in autophagy-deficient T cells. Thus, ER is expanded, including higher calcium stores in the ER. The treatment with thapsigargin [ER sarco/ER Ca^{2+}-ATPase pump inhibitor] reverses the calcium influx defect in autophagy-deficient T cells [24].

In contrast, B cells are subjected to an apoptotic program, AICD (activation-induced cell death), induced by surface B-cell receptor (BcR), with importance in restricting and focusing the immune response. De novo produced C16 ceramide in Ramos B cells is induced by BcR-activated cell death, as demonstrated by the use of specific inhibitor for ceramide synthase, fumonisin B1 (FB1) and palmitate. There was no evidence for sphingomyelinases activation or hydrolysis of sphingomyelin. Apoptotic characteristic processes as poly(A)DP-ribose polymerase cleavage and DNA fragmentation were also not evidenced. On the other side, mitochondria could be involved in BcR-triggered apoptosis, as underlined by morphological electron

microscopy changes and FB1 inhibition, as well as by the oss of mitochondrial membrane potential. Caspases might not be involved since the broad spectrum inhibitor benzyloxycarbonyl-Val-Ala-dl-Asp does not alter the BcR-induced mitochondrial membrane permeability transition, although it blocked DNA fragmentation. Thus, the mitochondrial damage induced by AICD execution involves de novo generation of C16 ceramide and consecutive activation of caspases and apoptosis [25].

Emodin is a pro-apoptotic inducer, firstly being considered an inhibitor of human T cells growth. It activates ER stress and, consecutively, elevates intracellular Ca^{2+}. Moreover, emodin disrupts mitochondrial membrane potential, induces the release of high amounts of mitochondrial cytochrome C in lymphocytes cytosol, and, thus, further activates caspase-3, -4, and -9. In addition, it increases the ROS and MDA levels, meanwhile inhibiting SOD and decreasing GSH/GSSG ratio. All the above mentioned effects are inhibited by the administration of ROS scavenger N-acetylcysteine. Thus, ROS-mediated ER stress and mitochondrial dysfunction in human T cells form the pathophysiologic basis for emodin immunosuppressive actions [26].

Conclusions

In our study, thapsigargin, a sesquiterpene lactone extract, the endoplasmic reticulum stressor through the inhibition of Ca^{2+}-ATPase activity, is not inducing massive apoptosis of isolated murine pre-B cells. In contrast, tunicamycin, an inhibitor of protein N-glycosylation, induced important pre-B cells apoptosis. Anyway, the apoptotic effects of tunicanycin were not so important as those induced by valinomycin, a K^+ ionophore, affecting almost all pre-B cells. The mechanisms underlying the apoptotic effects of tunicamycin in pre-B cells remain to be studied in depth by future approaches.

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