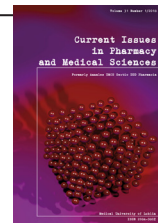


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Evaluation of the impact of the proteasome inhibitor on calcium channel expression in cardiomyocytes treated with doxorubicin

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ABSTRACT

One of the less known mechanisms of doxorubicin action is the effect on the functioning of the ubiquitin-proteasome degradation system (UPS). So far, the role of impaired proteasome activity in the development of anthracycline cardiomyopathy has not been clarified. It has been shown, however, that doxorubicin decreases the expression of proteins, including the expression of the calcium channel. However, it has not been established whether the observed disturbances are due to the activation of the UPS system by doxorubicin, or due to inhibition of translation or transcription. Therefore, the aim of the study was to evaluate the mRNA and protein expression of plasmalemmal (NaCaX, L-type) and sarcoplasmic reticulum (SERCA2, RyR2) channels in rat embryonic cardiomyocytes treated with doxorubicin and the proteasome inhibitor – bortezomib. The study was conducted utilizing the rat cardiomyocyte H9C2 line that was treated with doxorubicin and bortezomib in different concentrations. After 24 hours incubation, mRNA and protein expression analysis followed. The study did not show any universal mechanism of doxorubicin influence on calcium channel expression. With regard to the Na/Ca exchanger, we saw that DOX decreased the protein level in a proteasome activity-dependent manner. Moreover, we noted that the SERCA2 protein expression level was regulated by degradation intensity, however at the same time, no significant effect of doxorubicin on the level of this protein was demonstrated.

INTRODUCTION

Doxorubicin (DOX), a anthracycline antibiotic, was first isolated from the culture of *Streptomyces peucetius* in the early 1960s. It is a drug with an exceptionally broad spectrum, including use in the chemotherapy of solid tumors in children, in soft tissue sarcoma, lymphoma and breast cancer. The primary factor limiting the use of DOX, as well as other anthracyclines is their cardiotoxicity. This can lead to diastolic cardiomyopathy and congestive heart failure [1].

It is generally accepted that the mechanism of cardiotoxicity of anthracyclines is dependent on the occurrence of oxidative stress [1]. At the same time, mitochondrial respiration disorders are observed [2,3], as are changes in regulation of synthesis and the function of molecules responsible for maintaining calcium balance in cardiomyocyte [4,5], and consequently, in disorders of myocardium contraction and diastole [6].

The Ca²⁺ transport system plays an important role in the intracellular regulation of the flow of calcium ions responsible for contractility of the myocardium. Of notable relevance is the ryanodine receptor (RyR2), responsible for the transport of calcium ions from the endoplasmic reticulum of cardiomyocytes, to the cytoplasm. This activity is essential to induce muscle fibre contraction and ATPase (SERCA2) transportation of Ca²⁺ in the opposite direction (the basis of diastolic physiology) [7,8,9,10]. The cell membrane transport of calcium ions is connected with the L-type calcium channel and the Na/Ca exchanger.

Doxorubicin affects the functioning of calcium channels directly or through the free radicals which are produced in excess in the presence of the drug. It has also been shown that it affects the expression of mRNA [4] and channel building proteins [11,12]. The mechanism of transcription blockage by doxorubicin is known [13], however, the mechanism of lowering of protein expression is not fully

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understood. There have been reports that doxorubicin activates the proteasome-ubiquitin (UPS) system of protein degradation in the cell [14], but it is unclear how this phenomenon influences anthracycline cardiotoxicity. For this reason, the aim of this study was to evaluate the expression of membrane and sarcoplasmal calcium channels in cardiac cells after simultaneous treatment with doxorubicin and a proteasome inhibitor – bortezomib.

METHOD

Cell culture

The study was conducted on an adherent H9C2 line of rat embryonic cardiomyocytes (ATCC, USA). Cultures were grown on Dulbecco's Modified Eagle Medium (DMEM, PAA Laboratories, Austria), supplemented with 10% foetal bovine serum (Life Technologies, USA). Cells were cultured at 37°C in 5% CO₂-air.

The cells were first treated with bortezomib and after 30 min, doxorubicin were added. The drug concentrations are presented in table 1.

Table 1. Concentrations of compounds used in the study

	Control	B1	B0.1	A1	A5	B1 A1	B1 A5	B0.1 A1	B0.1 A5
Doxorubicin [μM]	-	-	-	1	5	1	5	0.1	0.1
Bortezomib [μM]	-	1	0.1	-	-	1	5	1	5

Gene expression analysis were conducted after 24 hours incubation with drugs.

mRNA expression

Total RNA was extracted with TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Subsequently, cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Life Technologies), following the manufacturer's instructions. Quantitative real-time PCR was performed in triplicate by using TaqMan® Gene Expression Assays: Rn00568762_m1 – SERCA2, Rn01470303_m1 – RyR2, Rn00570527_m1 Na⁺/Ca²⁺ exchanger, Rn00709287_m1 CanalC and Rn00667869_m1 – β-actin (Life Technologies, USA) in a 7500 Fast Real-Time PCR system (Applied Biosystem, USA). Relative gene expression levels were determined using the ΔΔCt method and presented as % of expression in control cells. β-actin was used as reference gene.

Protein expression

The cells were first washed with PBS and then were incubated in lysis buffer (150 mM NaCl, 1% Triton X-100, 0.1% SDS, 50 mM Tris pH=8) for 30 min at 4°C. The lysates were then centrifuged (20 min, 12000 rpm) and protein concentration in supernatants were determined via the Bradford method. After this, 20 μg of protein was loaded in a polyacrylamide gel and 50-minute electrophoretic separation was performed under reducing conditions utilizing a XCellSureLock apparatus (Invitrogen, USA) at constant voltage of 200V. After separation, the gel and the nitrocellulose membrane were placed between two layers of filter paper and put between the two electrodes of the XCell II

Blot Module electrotransfer apparatus (Invitrogen, USA). The transfer was conducted for 60 minutes at the constant voltage of 30V. Blots were developed using a Western Breeze Chromogenic Detection Kit (Invitrogen, USA). Nonspecific antibody-binding sites were blocked and the membrane was incubated in the solution of the primary antibody: monoclonal mouse antibody against rat SERCA2 protein (clone 2A7-A1; Affinity BioReagents, USA), rat RyR2 protein (clone 34C; Affinity BioReagents, USA), L-type Ca²⁺ CP L1C (A-20), sc-16230 goat polyclonal IgG (Santa Cruz Biotechnology), NCX (H-300) sc-32881 rabbit polyclonal IgG (Santa Cruz Biotrchnology) and against β-actin: Rb pAb to beta Actin (Abcam, GB). After washing, the membrane was incubated with the secondary antibody combined with alkaline phosphatase. The last step was to mark the sites of reaction with antigen by the reaction of alkaline phosphatase and chromogen. The relative intensities of bands were quantified using the 1D Image Analysis Software program (Kodak, USA), and all the values were normalized to the intensities of the respective beta-actin signal that was employed as a loading control (Abcam, USA).

RESULTS

We saw that RyR2 expression (Fig. 1) showed slight variation in each culture compared to the control. The highest expression was observed in culture A5, B100A1 and B1A1. RNA expression changes were not, however, reflected in the level of protein expression. No differences were observed in most of the studied cultures. In cells treated with 1 μM of DOX alone and 1 μM of DOX with 0.1 μM bortezomib, there were even slight decreases in the RyR2 protein level.

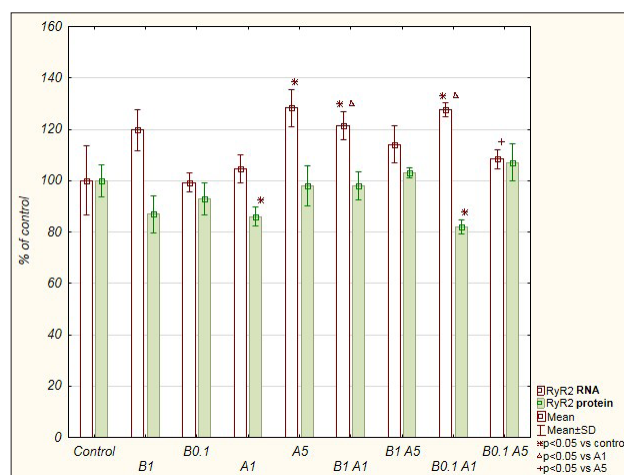


Figure 1. Expression at the mRNA and protein level (mean ± SD) of RyR2 content seen as percent changes with respect to the control group (which was established at 100%)

Furthermore, we noted that the expression of mRNA for the SERCA2 gene (Fig. 2) increased in cells cultured in the presence of bortezomib at both concentrations. It also increased in cells treated with doxorubicin at 1 μM. A slight increase was observed in 5 μM doxorubicin culture with 1 μM bortezomib. Of note, the levels of the SERCA2 protein were also significantly increased in cultures treated

with bortezomib at both concentrations, as well as in cells treated with bortezomib and 1 μM of DOX. Only in cultures treated with 5 μM of DOX were there slight decreases in the tested protein level.

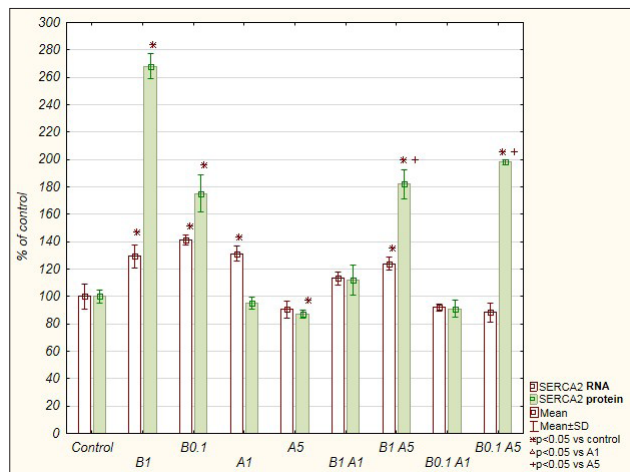


Figure 2. Expression at the mRNA and protein level (mean ± SD) of SERCA2 content seen as percent changes with respect to the control group (which was established at 100%)

We saw higher mRNA expression of the L-type calcium channel (Fig. 3) after bortezomib administration at a lower concentration, in the 1 μM DOX and B1A5 culture. No significant changes in protein expression were observed when compared to the control.

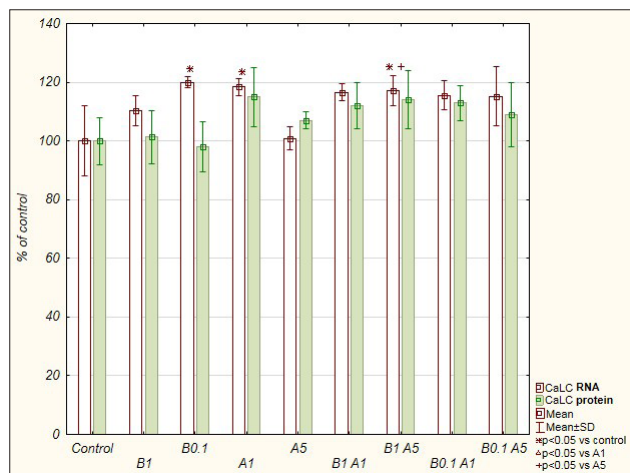


Figure 3. Expression at the mRNA and protein level (mean ± SD) of CaLC content seen as percent changes with respect to the control group (which was established at 100%)

In our study, we observed that mRNA expression for the Na/Ca exchanger gene (Fig. 4) was increased only in cells cultured in the presence of bortezomib at both concentrations. In other cultures, it did not change in relation to the control. We also saw that doxorubicin, no matter the concentration, significantly reduced the level of this protein. This effect is exacerbated by the concurrent use of bortezomib at a concentration of 0.1 mM. Finally, co-administration of bortezomib at higher concentration resulted in increased expression of the tested protein above the control level.

DISCUSSION

One of the less known mechanisms of doxorubicin action is the effect on the functioning of the ubiquitin-proteasome degradation system. So far, the role of impaired proteasome activity in the development of anthracycline cardiomyopathy has not been clarified. It has been shown, however, that doxorubicin administration does decrease protein expression, including calcium channel expression. The level of proteins that form the calcium channels affect the efficiency of contraction and relaxation of cardiomyocytes [15,16]. The influx of calcium ions into the cell is initiated by passage through the L-type calcium channel. In this way the calcium ions from the outside of the cell enter into the endoplasmic reticulum. Muscle fibre contraction is initiated by the flow of calcium from the sarcoplasmic reticulum to the cytoplasm through the ryanodine receptor. Relaxation of the muscle fibres is a result of the transport of Ca²⁺ ions in the opposite direction – from the cytoplasm, to the endoplasmic reticulum. This is possible through active transport by an ATP-dependent pump (SERCA2). Excess calcium ions from the cell are removed by the Na/Ca exchanger.

So far, however, it has not been established whether the observed disturbances in protein expression are due to the activation of the UPS system by doxorubicin, or whether the disorder is due to inhibition of translation or transcription. For this reason, the expression of calcium channels in the sarcoplasmic reticulum (SERCA2 and RyR2), as well as the cell membrane L-type calcium channel and the Na/Ca exchanger, under the influence of doxorubicin at both mRNA and protein levels was investigated in combination with the proteasome inhibitor, bortezomib, to determine the effect of doxorubicin on the ubiquitin proteasome system.

Bortezomib is a dipeptide derivative of boric acid, and is a chemotherapeutic drug used in the treatment of multiple myeloma or mantle cell lymphoma. It acts as a strong and reversible inhibitor of chymotrypsin-like proteasome activity [17]. Studies by Lyu and *co-workers* show that bortezomib has the ability to reduce DOX cardiotoxicity. This may indicate that proteasome activation is responsible for the toxic effect of the drug [18]. In the present study, bortezomib was used as a factor to prevent potential doxorubicin-induced degradation of proteins.

The present study did not show any universal mechanism of doxorubicin influence on calcium channel expression.



Figure 4. Representative Western blot analysis for RyR2, LCaC, NaXCa, SERCA2 proteins in the rat cardiomyocyte H9C2 line. Beta-actin is shown as a loading control

However, with regard to the Na/Ca exchanger, we noted that DOX decreased the protein level in a proteasome activity-dependent manner. Interestingly, we saw that SERCA2 protein expression level in cells is regulated by degradation intensity, however, at the same time, no significant effect of doxorubicin on the level of this protein was demonstrated. In the case of the other calcium channels, no significant decrease in protein expression was observed after the incubation of cells with doxorubicin.

This is different from the effect observed in the work of Arai *et al.* [4], where doxorubicin was administered chronically to rabbits, and there was a selective inhibition of mRNA expression for these proteins in cardiomyocytes of animals that was accompanied by a significant decrease in the ejection fraction. Olson *et al.* [11], conducted studies in rabbits via the chronic model. They saw a decrease in mRNA expression and protein content for RyR2 and a decrease in synthesis of the SERCA2 protein, with an unmodified amount of mRNA. However, in the same study, a decreased expression of the Na/Ca exchanger protein was found. In our earlier *in vivo* study [12] conducted on rat, there was no effect of doxorubicin on the protein levels of calcium ATPase, but RyR2 level was reduced by approx. 60%.

The diverse results of *in vitro* and *in vivo* studies may be due to the fact that the animal organism is a very complex model and the effect on cardiomyocytes depends on other signals from the body as well as doxorubicin metabolites produced in the liver. Such effects are not seen in a simple *in vitro* system. In addition, the cited study included a chronic model, where doxorubicin was administered repeatedly at weekly intervals. In our experiment, the chemotherapeutic agent was administered once and affected the cells for 24 hours. It is therefore likely that this was too short a time to induce significant decreases in calcium channel protein expression. The significance of the duration of doxorubicin interaction in the context of the expression of calcium channels is noted by Boucek *et al.* [19]. In studies in rabbits given 2 mg/kg b.w., doxorubicin differences were found between the early and late cardiotoxicity. Moreover, after 4 days, no changes in mRNA and RyR2 and SERCA2 were observed, while at the time cardiac hypertrophy was revealed. After 10 weeks of DOX administration, expression of mRNAs of the studied genes decreased.

CONCLUSIONS

In conclusion, Our results confirmed the hypothesis that doxorubicin administration brings about a decrease in protein level by activating the UPS system. However, this was seen only in the case of the Na/Ca exchanger. Moreover, this is not the only mechanism in operation, thus the effect of doxorubicin on calcium homeostasis in cardiomyocytes is more complex. For this reason, it can be assumed that compounds belong to the proteasome inhibitors will not be fully effective as cardioprotective agents.

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