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Proteasome inhibitors eliminate protective effect of postconditioning in cultured neonatal cardiomyocytes

Участь протеасомного протеолізу в реалізації феномену посткондиціювання вивчалася на первинній культурі неонатальних кардіоміоцитів, яку піддавали впливу аноксії–реоксигенації. Посткондиціювання відтворювали за допомогою трьох циклів реоксигенації тривалістю 1 хв, що чергувалися з 1 хв аноксії. Встановлено, що трипсиноподібна, хімотрипсиноподібна та пептидил-глутаміл пептидгідролазна (PGPH) активність протеасоми знижується після аноксії, а реоксигенація призводить до підвищення трипсиноподібної та хімотрипсиноподібної активності порівняно з аноксією, але ці показники ніколи не сягали вихідних значень. PGPH-активність після реоксигенації відновлювалася до контрольного рівня. Посткондиціювання призводило до підвищення кількості живих кардіоміоцитів за рахунок зменшення кількості некротичних, апоптотичних та аутофагічних клітин. Парадоксально, але інгібітори протеасоми, попереджуючи некротичну та апоптотичну клітинну смерть кардіоміоцитів при аноксії–реоксигенації, у тій самій концентрації запобігали ефекту посткондиціювання. Отримані результати свідчать про те, що протеасомний протеоліз має велике значення в реалізації феномену посткондиціювання, а інгібітори протеасоми можуть застосовуватися для фармакологічного посткондиціювання.

INTRODUCTION

Recently, more and more attention is paid to study ubiquitin-dependent proteasomal proteolysis in the pathogenesis of ischemia-reperfusion [5, 18, 20, 26, 33]. According to certain data, ischemia-reperfusion leads to the decrease in proteasome activity in ischemic zone [6, 11]. On the other hand, proteasome inhibitors prevent ischemic injury. So, this phenomenon seems to be of practical importance. At present, proteasome inhibitors are regarded as potential drugs for prevention of negative consequences of heart and brain ischemia [13]. In the experiments by Zhang L. et al., it was shown that the use of proteasome inhibitors decreases significantly the infarct size, resulting from embolism of the middle cerebral artery in rats [38]. In the experiments carried out on isolated heart, which was per-

fused with the solution containing polymorphonuclear leucocytes, the use of a proteasome inhibitor also decreased a degree of myocardial damage in ischemia-reperfusion [7]. This effect was explained by the proteasome-dependent inhibition of adhesion molecule expression on leucocytes [7, 9]. This process is associated with the formation of NF-kappa B, the molecule playing a critical role in cardioprotection, especially in the realization of preconditioning [28]. It was shown that NF-kappa B activation due to proteasomal hydrolysis of its endogenous inhibitor results in translocation of NF-kappa B as a transcription factor to a nucleus [29]. Thus, importance of the proteasome in ischemia-reperfusion injury is clear to a certain extent, but its role in postconditioning remains to be practically unexplored.

Also, it is known that proteasomal proteolysis plays an ambiguous role in apoptosis –

low concentrations of proteasome inhibitors prevent the cell death, but higher concentrations trigger the apoptotic programme [15, 21, 24, 27, 36]. In our study, we showed that proteasomal proteolysis plays an important role in realization of the phenomenon of postconditioning in the cultured rat neonatal cardiomyocytes. It was established that proteasome inhibitors prevent the necrotic and apoptotic cell death of cardiomyocytes in anoxia-reoxygenation, but in the same concentration they abolish the effects of postconditioning.

METHODS

Isolated neonatal cardiomyocytes were obtained from ventricular myocardium of 2-day old rats by enzymatic digestion by Reinecke H. et al. [30] with some modifications. The numbers of living and necrotic cells were 90-95 % and 5–10 %, respectively, as determined by staining with 0.2 % trypan blue solution. The cells were placed in dishes covered with 2 % gelatin solution with density 120 000 per cm^2. The cultivation was carried out for 1–2 days in the nutrient medium of such composition: DMEM, medium 199 (DMEM/199 – 4: 1), 15 % calf serum, Na_2CO_3 – 4.2 mM/l, HEPES – 15 mM/l and antibiotics (streptomycin 0.1 mg/ml, gentamycin 0.05 mg/ml and penicillin 100 U/ml) at 37^oC in an atmosphere with 5 % CO_2, 20 % O_2 and 75 % Ar (standard gas mixture). Anoxia was reproduced by aeration of the cells with a gas mixture containing 5 % CO_2 and 95 % Ar for 30 minutes. Reoxygenation was realized by exchanging fresh medium and by its aeration with the standard gas mixture for 60 minutes. Postconditioning after anoxia was performed by 3 cycles of 1-minute reoxygenation followed by 1-minute anoxia, respectively, before reoxygenation. To determinate the concentration of proteasome inhibitors, which does not cause any significant increase in apoptotic, necrotic and autophagic cell death, but affects effectively the cell ratio in anoxia-

reoxygenation, we carried out additional experiments. Firstly, clasto-lactacystin β-lactone (1.5–10 μM) was added to the cardiomyocyte culture and incubated under the above indicated conditions. After these experiments, clasto-lactacystin β-lactone in the dose that does not cause cell death (2.5 μM) was added after anoxia in anoxia-reoxygenation. Consequently, proteasome inhibitors, clasto-lactacystin β-lactone (2.5 μM), and MG132 (5 μM), were added just before cycles of postconditioning.

After sonication, the remaining unlysed cells and nuclei were removed by centrifugation at 5 000 g for 10 min. The supernatant was incubated in a buffer consisting of 25 mM Tris-HCl (pH 7.5) and 1 mM dithiothreitol. The fluoropeptide substrate Suc-Leu-Leu-Val-Tyr-7-amydo-4-methylcoumarin was used to measure the chymotrypsin-like activity of the proteasome; Boc-Leu-Ser-Thr-Arg-7-amydo-4-methylcoumarin was used for the trypsin-like activity and CBZ-Leu-Leu-Glu-AMC for the peptidyl-glutamyl peptide-hydrolyzing (PGPH) activity. After a 30-min (for trypsin-like activity) or a 1-h (for other) incubation with 6 μM of one of these fluorogenic peptides, the fluorescence of the reaction products was monitored at 380 nm excitation and 440 nm emission using free 7-amino-4-methylcoumarin (AMC) as a standard on Hitachi 4000 spectrofluorimeter. The reaction was carried out in the absence and presence of selective proteasome inhibitors – clasto-lactacystin beta-lactone (2.5 μM) or MG132 (5 μM) to differentiate between nonproteasome and proteasome-mediated peptide hydrolysis. Percent inhibition of the hydrolysis of respective substrates under the action of indicated inhibitors was evaluated as the proteasome activity and was expressed as nM AMC per 10^6 cells per 1 min.

The numbers of living, necrotic and apoptotic cells were determined by staining with 8.75 μM/l bisBenzimide (Hoechst 33342) and propidium iodidum [12] and examined by

fluorescent microscopy. The destruction of autophagic cardiomyocytes was evaluated by monodansyl cadaverine (50 µM) staining of vacuolar structures in vivo [1, 25]. In support of specificity of autophagic cells staining, 3-methyladenine (100 µM), autophagy inhibitor, was used.

For statistics Student's t test and χ^2 test were used. In tables and figure, values are presented as mean ±SE. All P-values are given as exact values except, when P<0.001 or P>0.2. Data analysis and graph generation were performed using Origin 7.0.

RESULTS AND DISCUSSION

The study of trypsin-like, chymotrypsin-like and PGPH proteasomal activities in isolated cardiomyocytes under anoxia-reoxygenation showed that the activity of proteasome decreased significantly during anoxia and partially restored to the initial level after reoxygenation. Trypsin-like activity decreased after anoxia by 39.8 % (P=0.006), chymotrypsin-like activity – by 34.7 % (P=0.004), and PGPH activity decreased 4.8-fold (P=0.004). Reoxygenation led to an increase in trypsin-like and chymotrypsin-like activities comparing to anoxia (by 8.7 %; P=0.75 and 46.0 %; P=0.03, respectively), but these parameters never reached the control level. PGPH activity restored up to the initial level (fig. 1) and increased 5.4 times (P=0.01) comparing to anoxia. Clasto-lactacystin β-lactone, a specific proteasome inhibitor, inhibited significantly all the activities of the proteasome comparing to control: trypsin-like – by 28.3% (P=0.016), chymotrypsin-like – by 71.1% (P=0.02), PGPH – by 51.7% (P=0.038).

Anoxia – reoxygenation increased significantly the number of cells with necrosis, apoptosis and autophagy features (tabl. 1 and outputting). Postconditioning reduced the number of all types of cell death. The number of living cardiomyocytes increased significantly by 12.6% (P<0.0001) compared with anoxia-reoxygenation. In contrast, the numbers of necrotic, apoptotic and autophagic cardiomyocytes decreased 2.0-fold (P=0.004), 2.1-fold (P<0.0001) and 2.84-fold (P<0.0001),
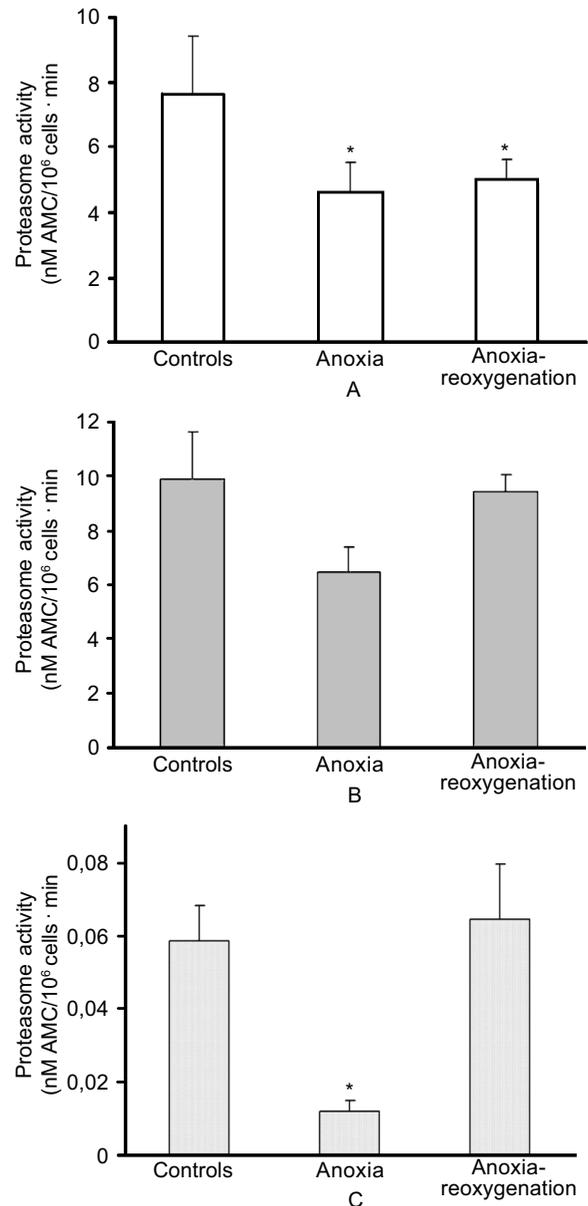
Trypsin-like (A), chymotrypsin-like (B) and peptidyl-glutamyl peptide-hydrolyzing (C) activities of proteasome in isolated cardiomyocytes in anoxia-reoxygenation. Data are expressed in nM 7-amyno-4-methylcoumarin on 10^6 cells per min and represent the mean ± S.E. of seven independent experiments *P < 0.05 vs. control.

respectively. The number of autophagic cardiomyocytes in control, anoxia-reoxygenation (A-R), postconditioning and in proteasome inhibitors - clasto-lactacystin -lactone (2.5 μM) and MG132 (5 μM) – application is described in outputting:

Experimental groups	Percentage of autophagic cells
Control n = 10	4.3 ± 0.23
Anoxia-reoxygenation (30-60min) n = 10	14.2 ± 0.96 $P_1 < 0.001$
A-R + Postconditioning (1min x 3) n = 10	5.0 ± 0.37 $P < 0.001$ $P_1 = 0.14$
A-R + Postconditioning + clasto-lactacystin β-lactone (2.5 μM) N = 7	16.0 ± 1.17 $P = 0.25$ $P_2 < 0.001$
A-R + Postconditioning + MG132 (5 μM) N = 7	18.8 ± 0.43 $P = 0.002$ $P_2 < 0.001$
Clasto-lactacystin β-lactone (2.5 μM) before reoxygenation n = 7	17.52 ± 1.89 $P = 0.11$ $P_1 < 0.001$

Proteasome inhibitors (clasto-lactacystin β-lactone and MG132) significantly and in dose-dependent way affected a ratio between the living, necrotic, apoptotic and autophagic cardiomyocytes. Clasto-lactacystin b-lactone (5 μM) and MG132 (10 μM) induced the de-

Table 1. The numbers of living, necrotic and apoptotic cardiomyocytes in control, anoxia-reoxygenation (A-R), postconditioning and in proteasome inhibitors – clasto-lactacystin β-lactone (2.5 mM) and MG132 (5 mM) – use. Data are expressed as the percentage of general number of cells and represent the mean ±S.E. P – comparing to anoxia-reoxygenation, P_1 – comparing to control, P_2 – comparing to postconditioning

Experimental groups	Percentage of the cells		
	Living	Necrotic	Apoptotic
Control n =10	89.8 ± 0.90	3.2 ± 0.33	5.8 ± 0.73
Anoxia-reoxygenation (30–60min) n = 10	79.1 ± 1.31 $P_1 < 0.001$	7.7 ± 1.11 $P_1 < 0.001$	11.9 ± 1.04 $P_1 < 0.001$
A–R + Postconditioning (1 min x 3) n = 10	89.1 ± 0.75 $P < 0.001$ $P_1 = 0.56$	3.8 ± 0.42 $P = 0.004$ $P_1 = 0.28$	5.7 ± 0.61 $P < 0.001$ $P_1 = 0.88$
A–R + Postconditioning + clasto-Lactacystin β-lactone (2.5 μM) n = 7	82.6 ± 1.53 $P = 0.11$ $P_2 < 0.001$	4.4 ± 0.45 $P = 0.029$ $P_2 = 0.37$	9.7 ± 0.72 $P = 0.14$ $P_2 < 0.001$
A–R + Postconditioning + MG132 (5 μM) n = 7	86.1 ± 1.27 $P = 0.002$ $P_2 = 0.048$	3.6 ± 0.61 $P = 0.011$ $P_2 = 0.76$	7.6 ± 0.59 $P = 0.006$ $P_2 = 0.049$
Clasto-lactacystin β-lactone (2.5 μM) before reoxygenation n = 7	85.2 ± 1.99 $P = 0.018$ $P_1 = 0.035$	4.7 ± 0.61 $P = 0.051$ $P_1 = 0.034$	7.2 ± 0.65 $P = 0.004$ $P_1 = 0.21$

velopment of apoptotic and autophagic cell death; at the same time, proteasome inhibitors did not effect significantly the number of necrotic cardiomyocytes. In the lower concentrations, these inhibitors did not cause any significant increase in cell death of cardiomyocytes during incubation for 90 minutes. Based on these data, we applied clasto-lactacystin β-lactone in the concentration of 2.5 μM to prevent of anoxia-reoxygenation injuries. MG132, which is less effective a proteasome inhibitor, was applied in the concentration of 5 μM.

Administration of clasto-lactacystin β-lactone after anoxia (just before reoxygenation) abolished damaging effects of anoxia-reoxygenation – number of living cells increased by 7.7 % (P=0.018), but the numbers of necrotic (by 38.9 %, P=0.051) and apoptotic (by 39.5 %, P=0.004) cardiomyocytes decreased (tabl. 1 and outputting). The number of autophagic cells did not differ significantly, comparing to anoxia-reoxygenation (P=0.11).

Thus, the inhibition of proteasome activity during anoxia-reoxygenation of cardiomyocytes prevented the cell death through necrosis and apoptosis, but increased to a certain extent the number of autophagic cardiomyocytes. It is important that the general number of living cells by proteasome inhibitors increased significantly independent of changes in a ratio between the cells, which died through various pathways.

At the same time, the inhibition of proteasome activity by clasto-lactacystin β-lactone and MG132 prevented the protection (decrease of apoptotic and autophagic cell death) afforded by postconditioning (tabl. 1, outputting). In postconditioning, clasto-lactacystin β-lactone increased the numbers of apoptotic and autophagic cells 1.7-fold (P<0.001) and 3.2-fold (P<0.001), respectively. The number of necrotic cells did not differ significantly that in postconditioning. Thus, our data allow a suggestion to be made of an important role for proteasomal proteolysis in realization of

this phenomenon in endogenous cardioprotection.

The study of mechanisms of postconditioning realization has been mainly carried out in context of the well known mechanisms of preconditioning [14, 19, 31, 34, 35, 37, 39]. The majority of scientists suggest the participation of analogous systems in the realization of these processes (tabl. 2). According to the data in table 3, the only one proteasome-dependent mechanism of preconditioning has been demonstrated till now. It is related to the formation of NF-kappa B [28]. At the same time, a participation of the ubiquitin-dependent proteasomal proteolysis as a universal system of gene transcription regulation, post-translational quality control of the proteins and degradation of cytoplasmic and membrane proteins in the pathogenesis of ischemia-reperfusion and realization of programmes of apoptosis and autophagic cell death can not be disregarded. It is well known that proteasome is intracellular multicatalytic macromolecular proteolytic complex carrying out the ubiquitin- and ATP-dependent proteolysis. Specificity of proteasomal degradation is provided by the ubiquitination – successive addition of ubiquitin to the protein to be degraded. Polyubiquitinated proteins are subjected to proteasomal proteolysis [3, 13].

Rapidity of the apoptosis programme execution, the necessity for rapid inactivation of certain proteins and principal reversibility of this process define a need for proteasome participation in the realization of apoptotic signal of different nature. The proteasome ability to function in any of the cellular compartments (in submembrane area and in the nucleus) and high specificity of the proteolysis executed permits to suggest the ubiquitin-dependent proteasomal proteolysis as an important link in apoptosis regulation [15, 24, 27].

It should be mentioned that the role of proteasomal proteolysis in apoptosis is ambiguous. The role of proteasome in realization of apoptotic programme as well as its participa-

Table 2. Factors involved in realization of preconditioning and postconditioning

FACTORS	PRECONDITIONING	POSTCONDITIONING
Phosphatidylinos-itol 3-kinase (PI3K)- protein kinase B	PI3K inhibitor wortmannin and LY 294002 abolished the protective effects of preconditioning. [Hausenloy DJ., 2004; Oudit GY., 2004; Germack R., 2004]	PI3K inhibitor wortmannin and LY 294002 abolished the protective effects of postconditioning. Post increased Akt phosphorylation [Tsang A., 2004; Yang XM., 2005; Chiari PC., 2005]
Extracellular signal-regulated kinase 1/2 (ERK 1/2)	Phosphorylation of ERK1/2 in preconditioning. Mitogen-activated protein kinase-ERK inhibitor U-0126 and PD98059 blocked preconditioning [Huang YF., 2003; Reid EA., 2005; Toma O., 2004]	PD98059 (inhibitor of ERK 1/2) abrogate protection [Yang XM, 2003, 2005]
P38 mitogen-activated protein kinase (MAPK)	Phosphorylation of cytosolic p38 MAPK. p38 MAPK inhibitor SB203580 abolished cardioprotection in preconditioning [Weber NC., 2005; Ballard-Croft C., 2005; Marais E., 2005]	p38 MAPK inhibitor SB203580 not abolished cardioprotection [Reuβner C., 2004]
Protein kinase C (PKC)	Phosphorylation of PKC-epsilon in preconditioning. Calphostin C blocked preconditioning [Toma O., 2004]	Calphostin C not blocked postconditioning [Reuβner C., 2004]
Glycogen syn-thase kinase-3beta	Phosphorylation inactivates GSK-3beta in precondi-tioning. Inhibitors of GSK-3beta, lithium and SB 216763 mimicked the protective effects of precondi-tioning [Tong H., 2002]	No data
Adenosine receptors	Adenosine, CPA and AMP-579 (A(1) selective ago-nists) and Cl-IB-MECA (A(3) selective agonist) involved in adenosine-induced cardioprotection [Germack R., 2004 Ballard-Croft C., 2005]	Non-specific adenosine receptor blocker 8-p-(sulfophenyl) theophyl-line (SPT) blocked postconditioning [Yang XM., 2005]
K$_{ATP}$-channels	Preconditioning abolished by 5-hydroxydecanoate (5-HD) and glibenclamide, but not selective sarcolem-mal K(ATP) channel antagonist HMR-1098. Specific mitochondrial K(ATP) activator diaxozide induce cardioprotection [Kristiansen SB., 2005] HMR-1098, but not 5-HD abolished preconditioning [Patel HH., 2005]	Glibenclamide or 5-hydroxydecanoate blocked the protection afforded by postconditioning. Diaxozide decrease quantity of autophagic cardiomyo-cytes in anoxia-reoxygenation [Yang XM., 2004]
Mitochondrial permeability transition pore (mPTP)	Carbonyl cyanide m-chlorophenylhydrazone (CCCP) abolished preconditioning protection [Ganote CE., 2003]. Transient mPTP opening triggered by preconditioning [Hausenloy DJ & Yellon DM., 2004]. Diaxozide and 5-HD prevents mPTP opening [Piriou V., 2004; Ytrehus K., 2004]	Specific inhibitor of the mPTP (NIM811) limited infarct size [Argaud L et al., 2005]
NO-synthases	NO-releasing agents induced cardioprotection. NO activates the epsilon isoform of PKC [Dawn B. & Bolli R, 2002]. L-NAME and L-NNA and the guanylyl cyclase inhibitor ODQ attenuated the protective effect of preconditioning [Lochner A., 2002]	Phosphorylation of eNOS in post; NOS inhibitor L-NAME and guanylyl cyclase inhibitor 1H-[1,2,4]oxadiazole[4,3-a]quinoxalin-1-one (ODQ) blocked postconditioning [Yang XM, 2003, 2005; Tsang A., 2004]
12-lipoxygenase (12 LO)	12-LO inhibitor baicalein abolished preconditioning protection. 12-LO metabolite 12-(S)-hydroxyeicosa-5Z,8Z,10Z,14Z-tetraenoic acid mimicked precondition-ing [Gross ER., 2004]. Mice lacking 12-lipoxygenase is not protected by preconditioning [Gabel SA., 2001]	No data
Nuclear factor kappa B (NF-kB)	Specific proteasome inhibitor lactacystin blocked preconditioing-induced NF-kB activation [Pradillo JM., 2005]	No data

tion in prevention or reverse development of the cell suicide has been proved. It could be explained by ability of proteasome to cleave the antiapoptotic proteins, on the one hand, and by well-timed degradation of endogenous proapoptotic proteins or degradation of the proteins involved in the realization of apoptotic programme, on the other (tabl. 3). Apoptosis of certain cells, for example lymphocytes, polymorphonuclear (PMN) leucocytes, thymocytes, involves the proteasome [15, 21, 27, 36] and disfunction of ubiquitin-dependent proteolysis in this case interrupts the development of apoptosis. At the same time, it was shown in many papers that proteasome inhibitors provoke apoptosis [3, 10, 13, 24].

According to our data, selective proteasome inhibitors (clasto-lactacystin β-lactone and MG132) in high doses trigger the programmed cell death (apoptosis and autophagy) of isolated neonatal cardiomyocytes. This is caused by accumulation of ubiquitinated proteins in the cells, impaired regulation of transcription of certain genes (e.g. genes dependent from NF-kappa B). The development of autophagy is a result of diminution of free amino acids in the cells, which are formed during protein breakdown by proteasome under normal conditions [1].

The level of inhibition of all three proteasomal activities by clasto-lactacystin β-lactone in high dose is comparable to the inhibition of proteasomal activity during anoxia (our unpublished data). Thus, a significant decrease in proteasomal activity is a damaging factor under the anoxia-reoxygenation conditions. In contrast, lower concentrations of proteasome inhibitors have a protective effect under the anoxia-reoxygenation conditions. This can be related to the fact, that proteasome inhibitors prevent the restoration of proteasome activity during reoxygenation as was observed in our experiments.

In anoxia-reoxygenation, occurs disequilibrium between antiapoptotic and proapoptotic proteins in the process of realization of apoptotic programme [3]. It has been shown that this results from proteasomal degradation caused by phosphorylation and subsequent ubiquitination of some proteins (bcl2, bclX$_L$, IAP, XIAP etc) [3, 23]. Therefore, the inhibition of proteasome activity prevents

Table 3.Role of proteasomal proteolysis in regulation of apoptosis

Proteasome substrate	Factor/cause of ubiquitination	Result	References
Caspase	IAP	Survival	Suzuki Y., 2001
Inhibitor of apoptotic proteins (IAP)	Smac	Apoptosis	Yang Q.H., 2004
Second mitochondria derived activator of caspase (Smac)	IAP	Survival	MacFarlane M., 2002
Bcl-2	Dephosphorylation on Ser$_{87}$	Apoptosis	Breitschopf K., 2000 Brichese L., 2002
Bax, t-Bid	Phosphorylation or dephosphorylation	Survival	Breitschopf K., 2000
P53	mdm2	Survival	Haupt Y., 1997
P27	Phosphorylation on Thr$_{187}$	Survival	An B., 1998
Inhibitor of NF-kappaB	Phosphorylation on Tyr$_{42}$	Activation of pro- or antiapoptotic genes by NF-kappaB	Bui N.T., 2001
C-myc	Phosphorylation on Thr$_{58}$	Differentiation, apoptosis	Welcker M., 2004 Gregory M.A., 2000
c-myb, c-jun, c-fos, E2F	Phosphorylation	Differentiation, apoptosis	Bossis G., 2003 Ferrara P., 2003

apoptotic cell death. According to the data by Stangl K. group, proteasome inhibitor strigger expression of heat shock proteins (HSP) in cardiomyocytes 32]. The HSP-dependent cardioprotection, especially due to prevention of apoptosis, is a well known fact. In addition, the inhibition of the proteasome results in depression of cellular adhesion molecules, expression that depends on NF-kappa B; and in vivo this leads to a decrease in leucocyte infiltration of the ischemic zone [38].

Proteasome inhibitors prevent also necrotic death of cardiomyocytes in anoxia-reoxygenation. While the basic factor of necrosis triggering in oxidative stress is the augmentation of free radical production simultaneously with a reduction in the capacity of antioxidant systems, a decrease in the rate of necrosis could be due to a shift in the mentioned ratio. In certain papers, it has been shown that proteasome takes part in degradation of superoxide dismutase and catalase [8, 16, 17]. Thus, the inhibition of proteasome activity can directly affect the number of the antioxidant protein content and, in this way, decrease the rate of free-radical processes in the cell.

At the same time, autophagic cell death, according to our data, is not prevented by proteasome inhibitors. Vice versa, the number of autophagic cells is increased comparing to anoxia-reoxygenation.

More interesting, as it seems to us, is the fact that proteasome inhibitors prevent the protection induced by postconditioning. This paradox is not simple to explain. First of all, it should be mentioned that one of the cytoprotection mechanisms in postconditioning is the inactivation of proapoptotic proteins of bcl2 family (bax, bad etc.) [34]. To our opinion, this can be explained by phosphorylation of bad protein with protein kinases involved in the realization of postconditioning (PI3K, Akt, Erk 1/2). The inhibition of proteasome activity in this case disrupts realization of cardioprotection induced by postconditioning. Furthermore, this effect of proteasomal inhibitors can be executed due to influence on the

endothelial NO-synthase (eNOS) activity, which plays an important role in realization of postconditioning [35]. According to our data, the certain endogenous negative regulator of eNOS exists, and its degradation by proteasome fraction II results in the augmentation of eNOS activity [1]. Thus, proteasome inhibitors can prevent proteasomal degradation of the mentioned factor and thus decrease the eNOS activity in postconditioning.

As a hypothesis, preconditioning and postconditioning as damaging factors "train" the cell by launching the apoptotic cell death programme due to release of certain proapoptotic factors from mitochondria. However, the numbers of these factors is not sufficient to realize this programme, and antiapoptotic proteins prevent the cell death. The interaction between many of pro- and antiapoptotic proteins is based on the principles of reciprocal ubiquitination (tabl. 3). For example, inhibitor of apoptotic proteins (IAP) and X-linked inhibitor of apoptotic proteins (XIAP) are the factors of second mitochondria derived activator of caspase (Smac), and the latter is ubiquitination factor for IAP and XIAP [23]. The ubiquitination (with subsequent proteasomal degradation) or activation of caspases depends on interrelations between IAP and Smac. In postconditioning, such an interaction will surely resulted in ubiquitination and degradation of proapototic factors, thereby permitting the cell to cleave more effectively these specific proteins during prolonged anoxia-reoxygenation. Proteasomal inhibitors disrupt this process, and protective effect of postconditioning is eliminated. Some of mentioned apoptotic factors take part also in realization of the autophagic programme [22]. However, this question is studied insufficiently especially in anoxia-reoxygenation.

The data obtained, show the important role for proteasomal proteolysis in anoxia-reoxygenation, as well as in realization of the phenomenon of postconditioning. Damaging effects of proteasome inhibitors, which result

in an increase in the population of living cells in anoxia-reoxygenation, permit to suppose that these substances can be used in pharmacological postconditioning.

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PROTEASOME INHIBITORS ELIMINATE PROTECTIVE EFFECT OF POSTCONDITIONING IN CULTURED NEONATAL CARDIOMYOCYTES

A role of proteasomal proteolysis in the pathogenesis of ischemia-reperfusion is being actively studied. To evaluate the participation of the proteasome in postconditioning phenomenon, we used primary culture of neonatal cardiomyocytes. 30 minutes of anoxia followed by 60 minutes of reoxygenation was undergone. Postconditioning was modeled by 3 cycles of 1-minute reoxygenation followed by 1-minute anoxia, respectively. Clasto-lactacystin b-lactone, a specific proteasome inhibitor, in the dose that does not cause cell death (2.5 mM) was added to the culture medium just before the cycles of postconditioning. Percentages of living, necrotic, and apoptotic cells were determined by staining with bisBenzimide and propidium iodide. Autophagy was demonstrated by staining vacuolar structures with monodansyl cadaverine. Proteasomal activity was determined by cleavage intensity of specific fluorogenic substrates. Trypsin-like, chymotrypsin-like and peptidyl-glutamyl peptide-hydrolyzing (PGPH) activities were decreased after anoxia. Reoxygenation led to an increase in trypsin-like and chymotrypsin-like activities comparing to anoxia, but these parameters never reached the control levels. PGPH activity was restored up to the initial level. Postconditioning increased numbers of living cells and decreased that of necrotic, apoptotic and autophagic cells. Paradoxically, it was established, that proteasome inhibitors prevented the necrotic and apoptotic cell death of cardiomyocytes in anoxia-reoxygenation, but in the same concentration abolished the effects of postconditioning. The data obtained permit to suppose that proteasome inhibitors can be used for pharmacological postconditioning.

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