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Postconditioning prevents apoptotic, necrotic and autophagic cardiomyocyte cell death in culture

У роботі представлено результати щодо можливості відтворення феномену постkондиціонування в культурі кардіоміоцитів. Після 30 хв аноксії (5% CO_2 та 95% Ar) до початку 60-хвилинної реоксигенації моделювали постkондиціонування за допомогою трьох циклів реоксигенації тривалістю 1, 3 або 5 хв, що чергувалися з 1, 3 або 5 хв аноксії. Встановлено, що кількість ушкоджених клітин поступово зменшується при скороченні періодів реоксигенації та аноксії. При постkондиціонуванні в режимі 3 рази по 1 хв досягається найбільш виражений протективний ефект – кількість живих клітин збільшується порівняно з аноксією-реоксигенацією на 11,5 % (P=0,002), а кількість апоптотичних, некротичних та аутофагічних зменшується на 42 % (P=0,04), 40 % (P=0,05) та 70 % (P<0,001) відповідно. Отримані результати свідчать про те, що постkондиціонування є одним з ефективних методів кардіопротекції, що дозволяє значно зменшити кількість кардіоміоцитів з ознаками запрограмованої чи незапрограмованої клітинної смерті.

INTRODUCTION

In the work performed in the laboratory of Vinten-Johansen J. (2003) postconditioning, a new phenomena of endogenous cardioprotection was described [20]. Postconditioning similar to preconditioning significantly decreases the infarct area after ischemia-reperfusion and intensity of PMN infiltration, attenuates PMN adhesion to the endothelial surface, particularly due to inhibition of P-selectin expression, decreases myeloperoxidase activity and contents of malondialdehyde in blood serum of dogs. This phenomena has at once attracted attention of many investigators, because comparing to preconditioning, postconditioning is more relevant to clinical situation. Further study of postconditioning on different species (rabbits, rats) established, that this effect is abolished by inhibitors of ATP-sensitive K-channels (K_{ATP}), inhibitors of endothelial NOS and inhibition of Akt [8, 9, 18, 19, 20]. Therefore, there is sufficient basis to suppose that postconditioning mechanisms are similar to those of preconditioning. On the other hand, it was shown that on the contrary to preconditioning, postconditioning effect does not disappear when MAP-kinases and protein kinase C are inhibited [14]. This means, that mechanisms of postconditioning have some peculiarities. The use of cellular cultures is the most prospective in the investigation of molecular mechanisms of myocardium postconditioning. Particularly, this method gives the possibility to estimate interrelation of living cardiomyocytes and cells, which have died through programmed or not programmed pathway [1, 4, 10, 11, 16]. Programmed cell death can be realized by apoptosis and autophagic cell death (ACD). While there is enough information about the role of apoptosis in pathogenesis of ischemia-reperfusion, the role of ACD of cardiomyocytes in cardiac pathology (cardiomyopathies, heart failure) was mentioned only in a few works [7, 10, 15]. In our previous investigations using monodansyl cadaverine, a specific dye and electron microscopy was shown that ACD was observed after anoxia-reoxygination in cardiomyocyte culture. Fur-

thermore the quantity of cells with features of ACD is comparable with the quantity of apoptotic cells [4]. Autophagy can be defined as a biologically programmed process of organelle and protein structures elimination that provides the cell with nutrients in the case of switch to endogenous nutrition. In some cases autophagy can be finished by auto digestion of the cell (autophagic cell death), and debris is eliminated through phagocytosis by macrophages or other cells. Morphologic characteristics of autophagic cell death (ACD) are: sequestration of cytoplasm and organelles; formation of vacuoles; lysosomes hyperplasia; condensation of chromosomes and others [5, 7, 10, 11]. Taking into account all mentioned above, we suppose, that reproduction of postconditioning phenomena on isolated cardiomyocytes will give a possibility to effect the processes of necrotic, apoptotic and ACD at modelling of anoxia-reoxygenation.

METHODS

Experiments were carried out on the primary culture of isolated neonatal cardiomyocytes, which were received from ventricular myocardium of 2-days old rats by enzyme digestion as shown previously by Reinecke H. et al. with our modification [13]. The yield of living and necrotic cells was 90–95 % and 5–10 % respectively that was proved by staining with 0,2 % trypan blue solution. The cells were placed in the dishes covered with 2 % gelatin solution with density 120 000 per cm^2. Cell were cultivated during 1–2 days in the nutrient medium of such composition: DMEM, medium 199 (DMEM/199 – 4 : 1), calf serum 15 %, Na_2CO_3 – 4,2 mM/l, HEPES – 15 mM/l and antibiotics (streptomycin 100 g/ml, gentamycin 0,05 mg/ml and penicillin 100 U/ml) at 37°C in the atmosphere, which included 5 % CO_2, 20 % O_2 and 75 % N_2. Anoxia was reproduced by aeration of the cells with a gas mixture containing 5 % CO_2 and 95 % Ar for 30 minutes. For reoxygenation nutrient medium was replaced and cell culture was aerated with a standard gas mixture for 60 minutes. Postconditioning after anoxia was performed by 3 cycles of short reoxygenation (1, 3 or 5 minutes) followed by 1, 3 or 5 minutes of anoxia respectively before final reoxygenation. The amount of living, necrotic and apoptotic cells was determined by staining with 8.75 microM/l bisBenzimide (Hoechst 33342) and propidium iodidum [3]. The autophagic cardiomyocytes destruction was proved by specific staining of vacuolar structures with monodansyl cadaverine (50 mM) in vivo (fig. 1) [12]. Monodansyl cadaverine is a well-known inhibitor of receptor-mediated endocytosis and transglutaminase activity, binds to late endosomes, which active formation is typical for ACD. Specificity of autophagic cell determination was proved by using 3-methyladenine (100 mM), which prevents autophagy. For electron microscopic study a routine method of tissue embedding in epoxide resin with species fixation in 2,5 % glutaraldehyde on cacodylate buffer and postfixation by 1 % osmium acid was used. Ultrathin microscopic sections were counterstained with uranylacetate and lead citrate. Material was investigated using electron microscope Jem – 100 CX (Japan). All reagents were produced by "Sigma" (USA).

Student's t test and χ^2 test were used to determine statistical significance. Data analysis and graph generation were performed using Origin 7.0.

RESULTS AND DISCUSSION

Obtained results witnesses, that postconditioning significantly decreases the quantity of necrotic, apoptotic and autophagic cardiomyocytes. Taking into account the conditions of cardiomiocytes' cultivating and gas mixture diffusion rate we decreased gradually the duration of short periods of reoxygenation and anoxia from 5 to 3 minutes and 1 minute, looking for an optimal variant with a maximal cy-

toprotective effect. It was established, that cytoprotection distinctly depends on duration of postconditioning intervals (fig. 2). The quantity of damaged cells gradually decreased at contraction of periods of reoxygenation and anoxia. If the quantity of living, necrotic and apoptotic cells after anoxia-reoxygenation was 78,5 ± 1,53, 7,76 ± 0,88, 12,8 ± 1,48 % correspondingly, at postconditioning in the regime three times for 5 minutes (3 x 5) when the quantity of living cells significantly risen (on 6,8 %, P<0,05 compared to anoxia-reoxygenation) while necrotic and apoptotic cells quantity decreased. At postconditioning in regime three times for 3 min (3 x 3), quantity of living cells grown on 8,7 % (P<0,05). The maximal effect was reached at postconditioning in regime three times for 1 min (3 x 1), interrelation of living, necrotic and apoptotic cells did not differ significantly from control values (87,64 ± 0,33, 4,66 ± 0,45, 7,42 ± 0,35 correspondingly). Moreover postconditioning also resulted in decrease the quantity of cells with signs of ACD (fig. 2 D). In control experiments the quantity of such cardiomyocytes did not exceed 5 %, anoxia-reoxygenation stimulated autophagy significantly – the quantity of autophagic cells increased 3,2 times and was about 13,9 %. When reproducing postconditioning in regime 5 x 3, the quantity of autophagic cells decreased on 46 % (P<0,001), in regime 3 x 3 – on 57 % (P<0,001), and at regime 1 x 3 – on 70 % (P<0,001).

While the role of necrosis and apoptosis is well elucidated in anoxia-reoxygenation, the role of ACD and its mechanisms in heart damage remains poorly studied [1, 4]. We are the first who have established, that this variant of cell death emerges during anoxia-reoxigenation of cardiomyocytes and that postcon-

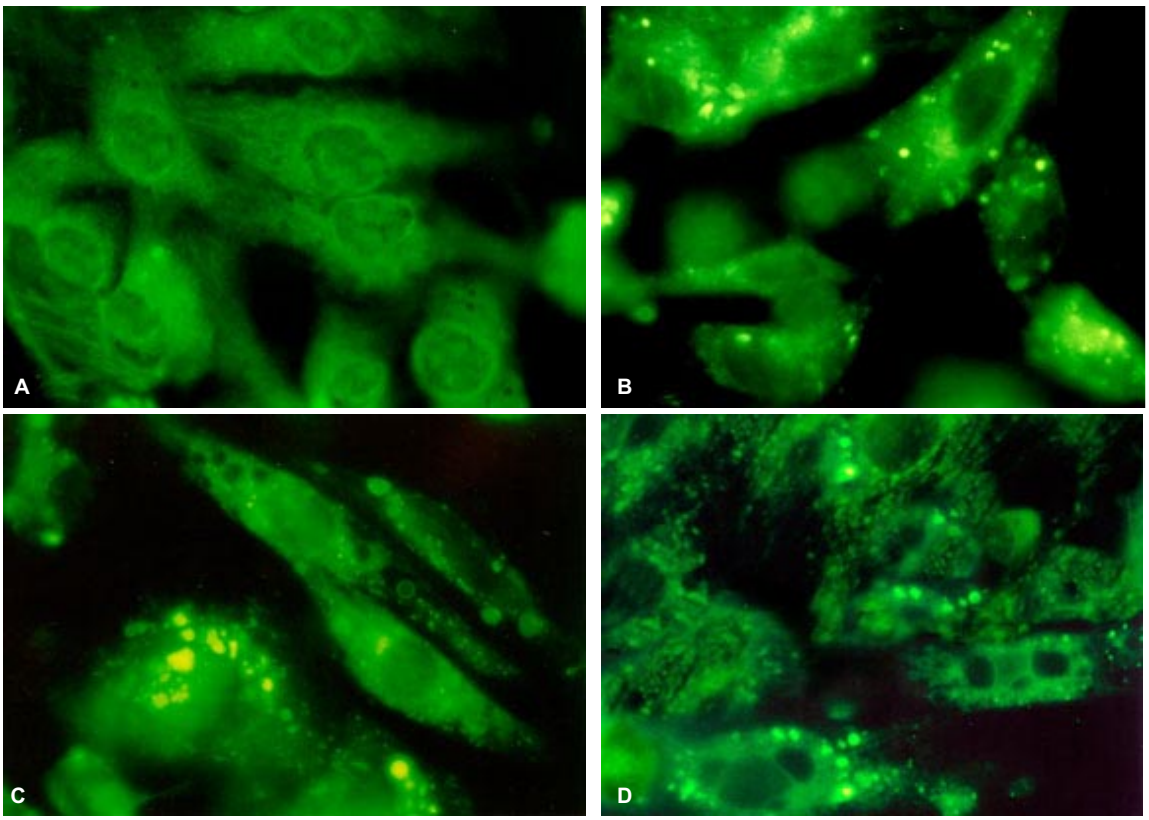

Fig. 1. Neonatal cardiomyocytes stained by monodansyl cadaverine. A – control, B, C, D – different variants of autophagic destruction

ditioning prevents increasing of quantity of cells with autophagic features. It was proved earlier, that nutrient deprivation of cells under conditions of amino acids deficiency leads to activation of autophagic processes. Further it was shown, that ACD is regulated by protein kinase Tor (target of rapamycin). This serine-threonine proteinase in presence of amino acids and growth factors coordinates activity of transcription, translation, ribosome biogenesis, formation of tRNA and has a row of functions in regulation of cellular growth [4, 7, 10, 17]. Under conditions of amino acids deprivation or under rapamycin action Tor activity decreased and this leads to arrest of cell cycle in G1 period, inhibition of protein synthesis through hypophosphorylation of p70S6-kinase, which is necessary for ribosome association with endoplasmic reticulum and, in the end, autophagy. The latter is a multi-step, strictly regulated process, which occurs with involvement of more, then 20 proteins, which form a so called APG (autophagosomal) cascade [10]. It should be mentioned, that this system is very conservative – the majority of yeast proteins, involved in autophagy are homologous to mammalian proteins. Other mechanisms of ACD are also

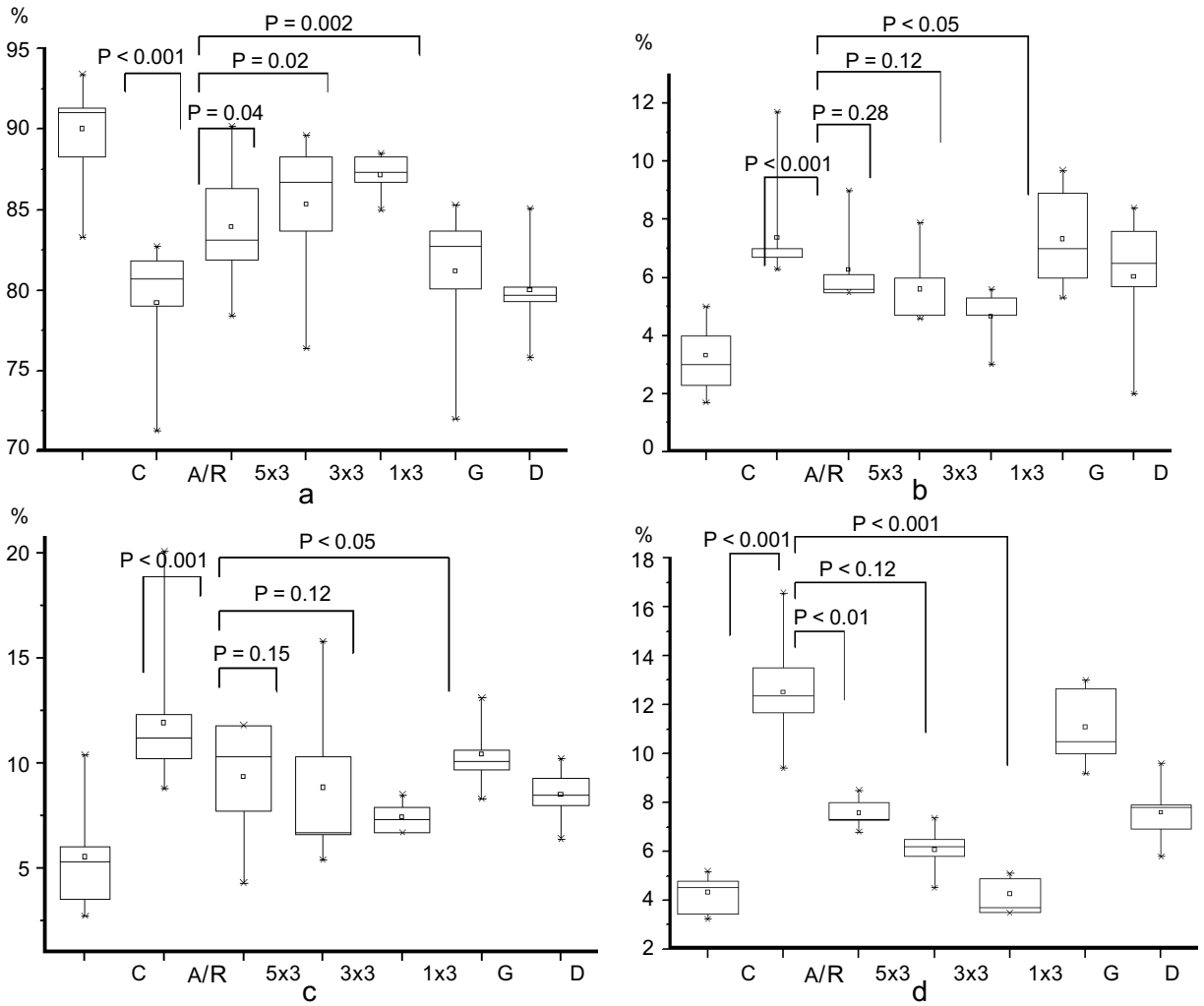

Fig. 2. Quantity of living (A), necrotic (B), apoptotic (C) and autophagic cells (D). C – control, A/R – anoxia-reoxygenation (30/60 min), 5x3, 3x3, 1x3 – regimes of postconditioning. Are represented means, standard deviations, maximal and minimal ranges

described. Particularly, mutant protein *ras* can induce ACD through inositol-3-phosphate protein kinase (PI3K) [5]. In the work of Tsang et al. it was shown, that postconditioning activates PI3K – Akt system, and PI3K inhibitor Wortmannin eliminates protective effect of postconditioning at ischemia reperfusion in rat isolated heart [18]. An important factor of autophagy stimulation is thought to be inhibition of proteasomal proteolysis [11]. According to our previous data, application of NO-synthase inhibitor (L-NNA) during myocardial ischemia-reperfusion in dogs also leads to activation of autophagy [2]. As far as mechanisms of development of autophagic processes in cardiovascular pathology are insufficiently studied, it is difficult to explain this phenomena and emerging of signs of autophagy at anoxia-reoxygenation in isolated cardiomyocytes. It can be proposed that disruptions of amino acid transport through cytoplasmic membrane as a result of oxidative damage of transport proteins and insufficient energy provision of this process can provoke ACD through depression of Tor. It is not improbable, that disturbances of proteasomal proteolysis in cardiomyocytes at ischemia-reperfusion also play a certain role in triggering of ACD. According to the data of Belteau A-L., at ischemia-reperfusion chymotrypsin-like activity of 26S proteasome is decreased [6]. According to our own data in isolated neonatal cardiomyocytes this activity is decreased almost for 50 % during anoxia and is almost restored during reoxygenation [1]. It is possible, that development of autophagy is a result of diminution of free amino acids quantity, which under normal conditions are formed during proteins breakdown by proteasome [7].

Therefore anoxia-reoxygenation of cardiomyocytes launches not only the apoptotic program but also the program of ACD. This fact provides more perspectives for further investigations and testing of substances, which can prevent apoptosis as well as autophagic cell destruction, together with the study of physiological methods of endogenous cardioprotection, particularly postconditioning.

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POSTCONDITIONING PREVENTS APOPTOTIC, NECROTIC AND AUTOPHAGIC CARDIOMYOCYTE CELL DEATH IN CULTURE

In the paper the data concerning the possibility of the reproduction of the postconditioning phenomena in the cardyomicyte culture are presented. Primary cultures of cardiomyocytes from neonatal rats underwent 30 minutes of anoxia followed by 60 minutes of reoxygenation. Three different models of postconditioning were used: 3 cycles of 1, 3, or 5 minutes of reoxygenation followed by 1, 3, or 5 minutes of anoxia, respectively. The percentage of living, necrotic, and apoptotic cells were determined by staining with Hoechst 33342 and propidium iodide. Autophagy was demonstrated by the staining of vacuolar structures in vivo by monodansyl cadaverine. After anoxia and reoxygenation the amount of living, necrotic and apoptotic cells were 79±1.5, 7.8±0.9 and 13±1.5 %, respectively (in unstimulated cell culture 90±0.8, 3.3±0.3, and 5.5±0.7, $P<0.0001$ for all). Postconditioning with 1 min anoxia 3-fold increased the amount of living cells and decreased the number of necrotic and apoptotic cells ($P=0.002$, $P=0.02$ and $P=0.043$ respectively). Postconditioning with cycles of 3 and 5 minutes had a gradually reduced effect compared to cycles of 1 minute. The percentage of autophagic cells in control cell culture was 4.3±0.3%. This number increased after anoxia-reoxygenation to 14±0.8%, and was reduced by postconditioning ($P<0.001$). The data obtained indicate that postconditioning is one of the effective methods of cardioprotection and could effectively decrease the amount of cardiomyocytes with traits of programmed or non-programmed cell death.

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