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EFFECT OF DALARGIN ON APOPTOSIS OF L929 FIBROBLASTS DURING COLD STRESS

Nataliia Moisieieva, Olga Gorina* and Yuliia Akhatova

Institute for Problems of Cryobiology and Cryomedicine of National Academy of Sciences of Ukraine, 23, Pereyaslavska Str., Kharkiv 61016, Ukraine.
*Corresponding author's E-mail: ogorina2603@gmail.com

Abstract

BACKGROUND: The search for compounds that can prevent cold stress-attributed apoptosis is of immediate interest. In this regard, the use of neuropeptides, in particular synthetic leu-enkephalin, as protectors is promising, due to their ability to prevent the development of apoptosis under some stresses. **OBJECTIVE:** To study apoptotic phenomena after cold stress and to evaluate the protective effect of dalargin on these processes. **MATERIALS AND METHODS:** The study was performed on a L929 fibroblast line. The impact of cold stress and the protective effect of dalargin on apoptosis against cold stress were evaluated using morphological parameters, distortion of cell membrane asymmetry and release of cytochrome C into the cell cytoplasm. To assess the proliferative potential of fibroblasts, mechanical damage to the monolayer was modeled as a scratch wound. **RESULTS:** The study showed that cold stress induced apoptosis in L929 fibroblasts and reduced proliferation in the fibroblast monolayers. Conspicuous apoptotic changes were found to develop only after a certain time after cold exposure, when the cells were returned to normothermia. Dalargin was demonstrated to exert a protective effect on proliferation and against apoptosis during cold stress. Using the opioid receptor antagonist naloxone, we revealed that the protective mechanism of dalargin appeared to be due to activation of δ-opioid receptors of L929 fibroblasts, which affected the development of apoptosis. **CONCLUSION:** In addition to their fundamental value, these findings are of practical importance since neuropeptides, in particular dalargin, added to perfusion solutions and media for hypothermic preservation of organs and cells, can improve their efficiency.

Keywords: apoptosis; cell proliferation; cold stress; L929 fibroblasts.

INTRODUCTION

Numerous researchers have reported that temperature deviations below the physiological range cause apoptosis in cells both in vivo and in vitro (1, 2, 3, 4, 5). In particular, it was demonstrated that lowering the temperature below 30°C initiated apoptosis in cells, which was manifested as impaired morphology, chromatin and DNA fragmentation, release of cytochrome C, appearance of acylation stimulating protein (ASP), activation of the pro-apoptotic transcription factor p53, etc. (1, 4). Given the above, as well as other numerous investigations, apoptotic phenomena are to be expected in cells, not only during hypothermia but also after their warming to physiological temperatures (6, 7). However, the latter has been poorly studied. Moreover, we found few publications describing apoptosis after cooling and exposure of cells at 2-4°C followed by warming to normal temperature (37°C) (7, 8). This extreme situation, commonly occurring in nature, can be described as cold stress, which requires further comprehensive studies. Numerous studies showed that apoptosis was

induced by an increase in reactive oxygen species under hypoxia, which led to disruption of plasmatic and mitochondrial membranes and, consequently, to ionic disbalance and metabolic disorders with congruous outcomes (2, 4, 5). Therefore, at present, the search for compounds capable of preventing these processes is extremely important. In this regard, our attention has been drawn to neuropeptides and, in particular, to a synthetic analogue of leu-enkephalin (dalargin), which is known to have anti-hypoxic effects as a result of interactions with opioid receptors and, through this mechanism, to be capable of correcting the structure and functions of plasmatic and mitochondrial membranes (9, 10). Data on the antiapoptotic effect of dalargin were reported in our previous publication, where we demonstrated that the agent prevented the development of apoptotic processes in cells of homeothermic animals after cold stress, which was evidenced by a decrease in DNA fragmentation and stimulated cell proliferation (11, 12). Thus, the purpose of this study was to investigate apoptotic phenomena in a culture of mouse fibroblasts (L929) after cold stress and to evaluate the protective effect of dalargin.

MATERIALS AND METHODS

Cell culture

The L929 cell line (culture of mouse fibroblasts) was kindly provided by D.K. Zabolotny, Institute of Microbiology and Virology of the NAS of Ukraine (Kyiv, Ukraine). The study was performed on L929 fibroblasts passaged four times. Prior to the experiments, the culture was stored in liquid nitrogen (LN) in the low-temperature bank of the Institute for Problems of Cryobiology and Cryomedicine of the National Academy of Sciences of Ukraine. Cells were cultured in DMEM/F12 nutrient medium (Biowest, France) supplemented with 10% fetal bovine serum (Biowest, France), 200 U/mL benzylpenicillin (Arterium, Ukraine) and 200 μg/mL streptomycin (Arterium, Ukraine), in a humidified incubator with 5% CO_2 at 37°C. For trypsinization, cells were incubated in 0.25% trypsin (Sigma, USA) in Versene solution (PAA, USA) for 5 min and washed from the enzyme with DMEM/F12 containing 10% fetal bovine serum.

Cold stress

After trypsinization, cells were transferred to 24-well plates (SPL Life Sciences, Korea) at a concentration of 0.5×10^6 cells/mL. L929 cells were cultured for 7 days. After formation of the monolayer on day 7, the plates were transferred to an ice bath and kept for 20 min, and then the culture was placed in a CO_2 incubator at 37°C for 60 min. The temperature of the samples was monitored with an OMRON E5CSV and TCViews software. In parallel, the culture without cold exposure was prepared (control).

To study the protective effect of dalargin (CJSC "Biolik", Ukraine) on fibroblasts subjected to cold stress, the agent was added at a concentration of 100 μg/L to culture medium 15 min prior to cold stress. An equivalent volume of saline solution was added to the control wells. To explore the mechanism of dalargin action, we used a competitive opioid receptor antagonist, naloxone (LLC, Kharkiv Pharmaceutical Enterprise "Zdorovia Liudyny", Ukraine), at a concentration of 100 μg/L. It was added to the incubation medium simultaneously with dalargin prior to cold stress.

Hematoxylin and eosin staining

After cold stress, the plates were returned to the culture conditions (5% CO_2, 37°C). The following groups were analyzed: 1 - control (cell culture not subjected to cold stress); 2 - culture subjected to cold stress; 3 - culture pre-incubated with dalargin and subjected to cold stress; 4 - culture pre-incubated with naloxone and subjected to cold stress; 5 - culture pre-incubated with dalargin and naloxone and subjected to cold stress. After 60 min, the medium was removed from each well and the cells were washed with PBS and fixed in methanol. Then 1% hematoxylin (Sigma-Aldrich, USA) was added with subsequent washing with PBS and staining with 1% eosin (Sigma-Aldrich, USA). Cell morphology was assessed using an inverted microscope. Apoptotic changes in cells were analyzed by the following indicators: cell pyknosis, nucleus fragmentation and blebbing. A total of 200 cells per sample were counted. The portion of cells with apoptotic signs was expressed as a percentage of the total number of counted cells.

Annexin V and 7-AAD double staining

After cold stress, the plates were returned to the culture conditions (5% CO_2, 37°C). After 60 min, cells of three groups (1 - control, 2 - culture

subjected to cold stress, 3 - culture pre-incubated with dalargin and subjected to cold stress) were trypsinized, washed with PBS, centrifuged and incubated in 100 μL of buffer [0.1 M Hepes/NaOH (pH 7.4), 1.4 M NaCl, 25 mM $CaCl_2$) containing 5 μL FITC-conjugated annexin V (BD Biosciences, USA) and 5 μL (2.5 μg/mL) 7-AAD (BD Biosciences, USA)] for 10 min at room temperature (23-25°C) in the dark. Cells were analyzed using a FACS Calibur flow cytometer and Cell Quest software (BD Biosciences, USA). We took 10,000 cells as 100%. Simultaneous staining with vital DNA-specific dye 7-AAD and annexin V (the latter has a high affinity for phosphatidylserine) allows differentiating apoptotic cells (Annexin V+/7AAD–) from necrotic (dead) ones (Annexin V+/7AAD+) (13).

Cytochrome C release into cytoplasm

Cells at a concentration of 0.5×10^6 cells/mL were transferred to 96-well plates and cultured for 48 h. After the monolayer formation, they were exposed to cold, as described above. Then the plates were cultured (5% CO_2, 37°C) again for 15 min or 60 min, depending on the experimental conditions. The culture medium was removed from the wells; the cells were washed once with PBS and fixed in BD Cytofix buffer (BD Biosciences, USA) for 10 min. Cell permeabilization and subsequent washing steps were performed using 1 x Perm / Wash buffer (BD Biosciences, USA) in accordance with the manufacturer's instructions. Afterwards, 50 μL anti-cytochrome C monoclonal antibody (6H2.B4) conjugated to Alexa Fluor® 488 (BD Biosciences, USA) were added to each well and incubated in the dark. Antibody was removed by three-time washing with 1 x Perm / Wash buffer. After the final wash, cell nuclei were stained by adding 2 μg/mL Hoechst 33342 solution (Sigma-Aldrich, USA) in accordance with the manufacturer's instructions. The fluorescence intensity was recorded using a confocal laser scanning microscope (Carl Zeiss, Germany). LSM 510 and LSM Image Examiners were used to visualize and count the number of stained cells. Two hundred cells were taken as 100%.

Proliferative potential of fibroblasts

To assess the proliferative potential of fibroblasts, mechanical damage to the monolayer was modeled as a scratch wound (14). After cold stress, a cell monolayer was scratched with a plastic tip of 0.8 mm diameter (for a 200 μL pipette dispenser) and then the monolayer was washed with culture medium to remove cell debris and then was returned to culture (5% CO_2, 37°C). The "wound" surface was assessed immediately after scratching as well as after 24 and 48 h by inverted microscopy; the photomicrographs were analyzed with a Zeiss LSM Image Examiner. The results are presented as the ratio of the wound area at hour 24 or 48 to that after immediately scratching and are expressed as a percentage.

To study the effect of dalargin on fibroblast proliferation after cold stress and mechanical damage to the monolayer (wound model), the agent was added at a concentration of 100 μg/L to the culture medium before cold stress and after 24 h. An equivalent volume of saline solution was added to the control groups.

Statistical analysis

The data were statistically processed using the non-parametric Mann-Whitney test in Statgraphics plus for Windows 2.1 (“Manugistics Inc.”, USA). All experiments were repeated five times. The results are expressed as mean ± SEM (standard error of the mean). Differences at $p < 0.05$ were considered significant.

RESULTS

The study of signs of apoptosis on fibroblasts subjected to cold stress showed that the number of apoptotic cells significantly ($p < 0.05$) increased to 44.86 ± 1.33% compared to the control (9.0±0.1%) (Fig. 1). Pre-incubation with 100 μg/L dalargin significantly ($p < 0.05$) decreased the number of cells with morphological signs of apoptosis compared to those subjected to cold stress without dalargin treatment. Incubation of cells with naloxone prior to cold stress did not affect the number of apoptotic cells.

To study apoptotic changes in L929 fibroblasts after dalargin treatment and cold stress, we conducted cytofluorimetric analysis using cell labeling with annexin V. The results of these experiments are summarized in Table 1, which shows that in the control group the relative number of Annexin+7AAD– - cells was 15.2 ± 1.4% of the total population, while after cold stress it was 55.1 ± 1.9%. Pre-incubation with dalargin significantly ($p < 0.05$) reduced

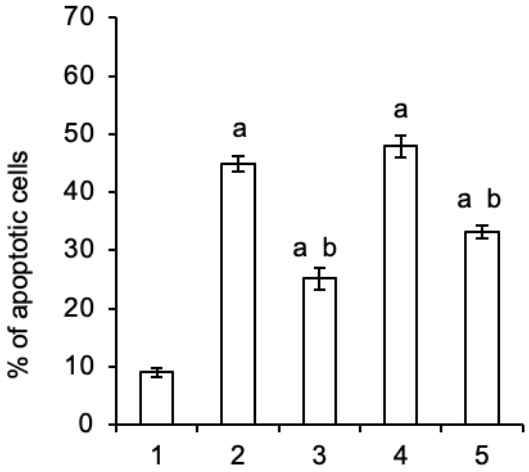

Figure 1. L929 fibroblasts with morphological signs of apoptosis: 1 – control (cells under normothermia [5% CO_2, 37°C]); 2 – cells after cold exposure; 3 – cells incubated with dalargin at 37°C for 15 min prior to cold exposure; 4 – cells incubated with naloxone at 37°C for 15 min prior to cold exposure; 5 - cells incubated with dalargin and naloxone at 37°C for 15 min prior to cold exposure. Two hundred cells were taken as 100%. Results are represented as mean ± SEM (n = 5). (a) - differences are significant (p<0.05) in comparison with "1"; (b) - differences are significant (p<0.05) compared to "2".

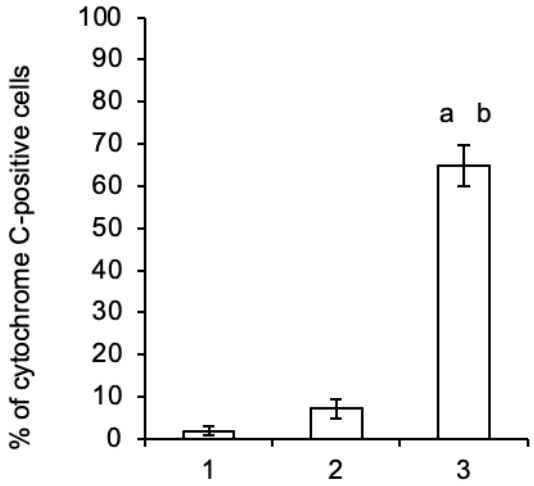

Figure 2. L929 fibroblasts with cytoplasmic cytochrome C: 1 – control (cells under normothermia [5% CO_2, 37°C]); 2 - cells, which after 20-min exposure in an ice bath, were incubated at 37°C for 15 min; 3 - cells, which after 20-min exposure in an ice bath, were incubated at 37°C 60 min. Two hundred 200 cells were taken as 100%. Results are represented as mean ± SEM (n = 5). (a) - significant difference (p<0.05) in comparison with "1"; (b) - significant difference (p<0.05) in comparison with "2".

this parameter to 27.9 ± 0.9%, although it did not reach the control level 15.2 ± 1.4%.

To assess changes in the plasmatic membrane permeability after cold stress in order to detect necrotic cells and those at final stages of apoptosis, we used 7-AAD. The results summarized in Table 1 indicate that the normal number of Annexin+7AAD+-cells did not exceed 7.3 ± 0.6%, but cold stress caused its rise to 18.2 ± 1.4%. In case of pre-incubation with dalargin, the number of Annexin+7AAD+-cells significantly ($p < 0.05$) decreased (to 8.3±0.2%).

We evaluated the number of fibroblasts with cytoplasmic cytochrome C 15 and 60 min after cells were returned to normothermia from cold. It was found that after cold stress and 15-min incubation of fibroblasts under the physiological conditions (5% CO_2, 37°C) the number of cells with cytoplasmic cytochrome C amounted to 7.13 ± 2.32%, but did not significantly ($p < 0.05$) exceed the control value (1.92 ± 0.95%). The extension in the post-cold incubation of cells under the physiological conditions (CO_2 incubator, 37°C) up to 60 min led to a significant ($p < 0.05$) rise in the percentage of cells with cytoplasmic cytochrome

Table 1. Effect of dalargin on the numbers of apoptotic (AnnexinV$^+$7AAD$^-$) and necrotic (AnnexinV$^+$7AAD$^+$)

Status	Control	Cold stress	Dalargin + cold stress
Annexin$^+$7AAD$^-$, %	15.2±1.4	55.1±1.9^a	27.9±0.9$^{a\ c}$
Annexin$^+$7AAD$^+$, %	7.3±0.6	18.2±1.4^b	8.3±0.2^d

(a) - significant difference (p<0.05) compared with the number of Annexin$^+$7AAD$^-$-cells in the control; (b) - significant difference (p<0.05) compared with the number of Annexin$^+$7AAD$^+$-cells in the control; (c) - significant difference (p<0.05) compared with the number of Annexin$^+$7AAD$^-$-cells after cold stress; (d) - significant difference (p<0.05) compared with the number of Annexin$^+$7AAD$^+$-cells after cold stress. 10,000 cells were taken as 100%. Results are represented as mean ± SEM (n = 5).

C compared to the control (64.82 ± 4.77% vs. 1.92 ± 0.95%) (Fig. 2).

Taking into account these results, we assessed the protective effect of dalargin on cold-stressed fibroblasts as the number of cells with cytoplasmic cytochrome C 60 min after returning to the normal conditions. Fig. 3 shows that pre-incubation with dalargin significantly ($p < 0.05$) decreased (by 19%) the number of cells with cytoplasmic cytochrome C.

In further experiments, we assessed the delayed effect of dalargin on "wound" healing of the fibroblast monolayer (mechanical damage to the L929 fibroblast monolayer) after cold stress. The results graphed in Fig. 4 show that the damaged area of the monolayer was 48.92 ± 3.78% and 10.50 ± 3.71% when cells were cultured at 37°C for 24 and 48 h, respectively. At the same time, "wound healing" in the cold-stressed culture was significantly ($p < 0.05$) slower, as the damaged area of the monolayer was 73.86 ± 5.21% and 40.02 ± 2.8% after 24- and 48 h incubation at 37°C, respectively (Fig. 4). When dalargin was added to the incubation medium prior to cold stress, there was a significant ($p < 0.05$) reduction in the damaged area of the monolayer: 60.08 ± 3.37% and 17.95 ± 3.13% after 24 and 48 h incubation at 37°C, respectively (Fig. 4).

DISCUSSION

Programmed cell death or apoptosis is a natural process of eliminating damaged cells. Upon apoptosis, in contrast to necrosis, there is no inflammatory response of adjacent cells to degradation products, because membranes in apoptotic cells remain intact until the final stages of the process, and then such cells are phagocytosed by macrophages (15). Exposure of living cells to temperatures below the physiological range is associated with structural injuries and can eventually lead to cell death via apoptosis (1, 2, 3, 5). Heat and cold shocks as well as hypoxia activate the transcription factor p53, which is involved into the expression of pro-apoptotic proteins such as Bax, Puma, and Noxa. These proteins trigger the mitochondrial pathway of apoptosis and the corresponding caspase cascade (15).

Apoptosis is most often identified through morphological signs, namely blebbing, pyknosis and nucleus fragmentation (15). In our experiments, cold stress resulted in a significant ($p < 0.05$) increase in the number of cells with morphological signs of apoptosis compared to the control.

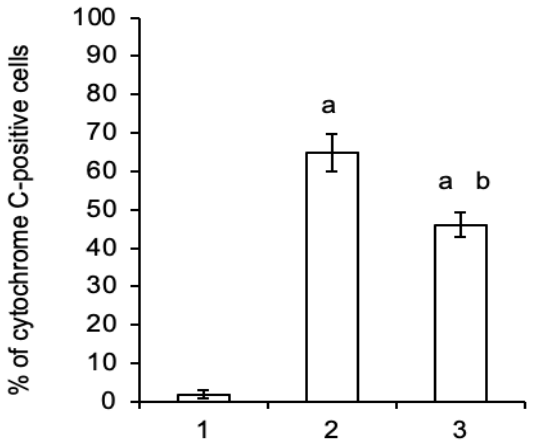

Figure 3. Effect of dalargin (100 μg/L) on the number of fibroblasts with cytoplasmic cytochrome C: 1 – control (cells under normothermia [5% CO_2, 37°C]); 2 – cells after cold stress; 3 - cells, which were incubated with dalargin 37°C for 15 min prior to cold stress. Two hundred cells were taken as 100%. Results are represented as mean ± SEM (n = 5). (a) - significant difference ($p<0.05$) in comparison with "1"; (b) - significant difference ($p<0.05$) in comparison with "2".

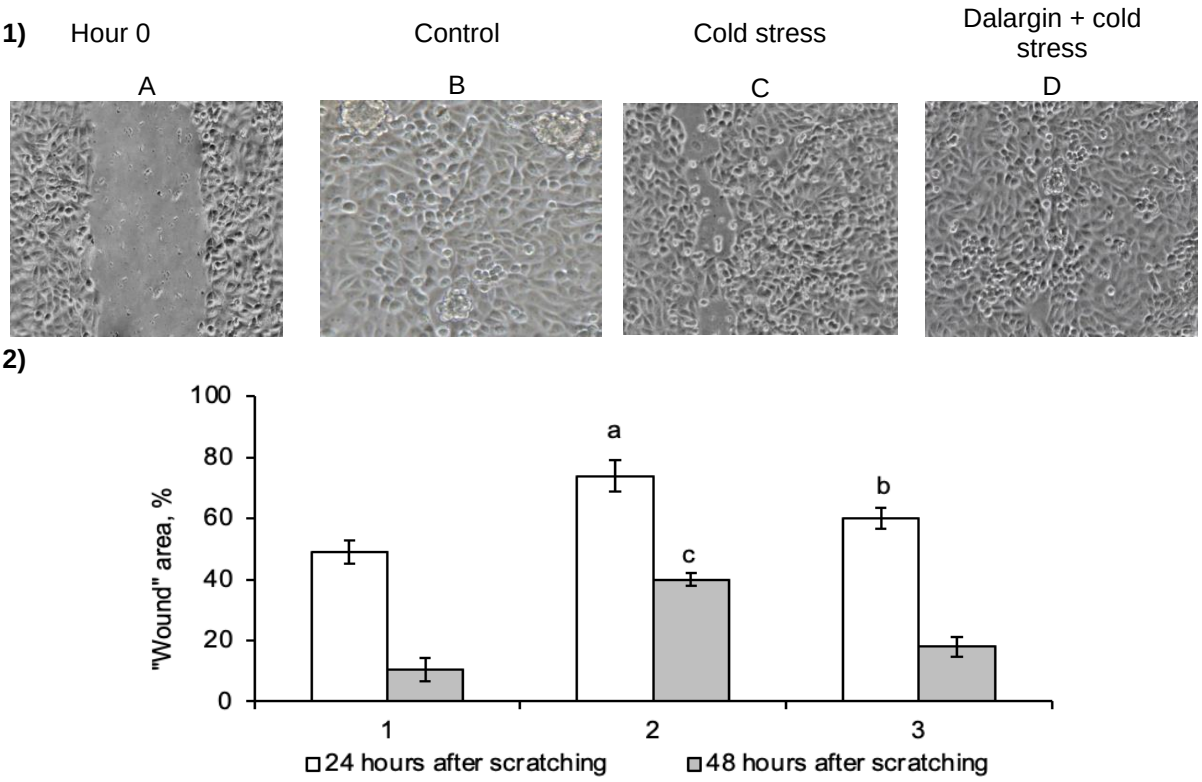

Figure 4. Protective effect of dalargin on the "wound healing" in the L929 fibroblasts monolayer: 1) Photomicrographs (×200) of the L929 fibroblast monolayer after mechanical damage. A - Monolayer immediately after scratching; B – Control (48 h at 37°C after scratching (5% CO_2); C - Pre-exposed to cold monolayer, 48 h after sctatching; D - Pre-incubated with dalargin and then pre-exposed to cold monolayer, after 48 h after scratching. 2) "Wound" area: 1 – control ("wound" in the fibroblast monolayer under normothermia); 2 – cells were incubated under normothermia for 60 min after 20-min exposure in an ice bath; 3 - pre-incubated with dalargin cells were incubated under normothermia for 60 min after 20-min exposure in an ice bath. Results are represented as mean ± SEM (n = 5). (a) - significant difference ($p<0.05$) in comparison with corresponding "1"; (b) - significant difference ($p<0.05$) in comparison with corresponding "2"; (c) - significant difference ($p<0.05$) in comparison with corresponding "1".

Phospholipids are known to be asymmetrically distributed between the plasmatic membrane layers. In particular, phosphatidylserine is normally located in the inner layer of the plasmatic membrane, but after apoptosis initiation it migrates to the outer surface of the membrane. So, the presence of phosphatidylserine in the impaired membrane is a sign of apoptosis (13). A marker protein, annexin V is able to bind to phosphatidylserine, and this feature is used to assess the cell membrane status during apoptosis. Our experiments demonstrated that, after cold stress, there were significant changes in the distribution of phosphatidylserine between the inner and outer layers of the plasmatic membrane compared to the control (not exposed to cold stress fibroblasts). In particular, cold stress can lead not only to the redistribution of phosphatidylserine in the plasmatic membrane, but also to its complete destruction. In this regard, we evaluated the percentages of cells at different stages of apoptosis, using for this purpose another dye, a fluorescent probe 7-AAD, which stains DNA of cells with damaged membranes. Our findings revealed that under cold stress (20 min exposure of cells in an ice bath with subsequent return to normothermia) the L929 cells showed signs of necrotic process activation, as evidenced by a significant ($p < 0.05$) increase in the number of 7-AAD-positive cells.

The mitochondrial pathway of apoptosis initiation in cells is known to be associated with the release of cytochrome C into the cytosol as a result of impaired permeability of mitochondrial membranes (15, 19, 20). We revealed that cold stress initiated the mitochondrial pathway of apoptosis, as evidenced by a significant increase of the number of cells in the cytoplasm of which

cytochrome C was detected. We also demonstrated that after cold stress a certain time was required for cytochrome C to be released and accumulated in the cytoplasm. In particular, we found that extension of exposure at normothermia from 15 to 60 min after cold stress led to increase in the number of cells with cytoplasmic cytochrome C.

We experimentally proved that pre-incubation of L929 fibroblasts with dalargin reduced the number of cells with apoptosis signs after cold stress. Opioid peptides, including dalargin, are known to be able to bind and interact with opioid receptors, in particular δ-receptors, and to a much lesser extend with μ-receptors of plasmatic membranes (16, 17, 18). Of particular interest are the results on the role of opioid receptors in the effect of dalargin on cold-induced apoptosis, where opioid receptors were blocked with naloxone. We found that simultaneous incubation of cells with naloxone and dalargin prior to cold stress compromised the anti-apoptotic effect of this synthetic analogue of leu-enkephalin, indicating that its anti-cold protective mechanism involves opioid receptors. Since the anti-apoptotic protective effect of dalargin on cold-stressed fibroblasts was not completely abrogated by naloxone blocking opioid receptors, we assume that mechanisms other than interaction with receptors may exist.

The findings on annexin V and 7-AAD staining as well as on anti-cytochrome C antybody binding prove that cold stress initiates the mitochondrial pathway of apoptosis.

Assessments of the dalargin effect on the apoptotic processes in cold-stressed cells suggest that dalargin is beneficial to fibroblasts under these conditions. In particular, we showed that incubation of cells with dalargin prior to cold stress reduced the number of cells with morphological signs of apoptosis (impaired asymmetry of phospholipid distribution in the plasmatic membrane and cells with cytoplasmic cytochrome C) in comparison with no dalargin culture. Based on Tang et al. (21), we hypothesized that the anti-cold protective effect of dalargin might be associated with the activation of δ-opioid receptors, which stabilizes the potential of mitochondrial membranes and prevents the release of cytochrome C into cytoplasm.

The proliferation intensity is a hallmark metabolic activity of cells (3). Our experiments simulating "wound" damage to the monolayer of L929 fibroblasts showed that cold stress significantly compromised the cell proliferation. This may be attributed to apoptosis, which we observed. Or the response could be due to other cell-damaging factors such as free radical reactions, hypoxia and metabolic disorders reducing the energy capacity of cells and, thuswise, resulting in their necrosis. Cold stress was found to slow down recovery of the damaged monolayer for 24 and 48 h of cultivation. Dalargin in the culture medium made the post-cold "wound" significantly ($p < 0.05$) smaller after 24 and 48 h of cultivation in comparison with no dalargin culture. It is noteworthy that, after 48 h of cultivation, the "wound" area did not differ from the control value, indicating the protective effect of dalargin on cells under cold stress.

Thus, our findings demonstrated that cold stress initiated apoptosis via the mitochondrial pathway in L929 fibroblasts. The protective effect of dalargin on cells under cold stress was revealed, as evidenced by a reduced number of cells with morphological signs of apoptosis, distorted asymmetry of plasmatic membrane and cytochrome C released in cytoplasm. The experiments with the opioid receptor antagonist, naloxone, showed that the mechanisms of anti-apoptotic and proliferation-stimulating effects of dalargin were partially related to binding to opioid receptors. Inhibition of cold-induced apoptosis after pre-incubation of cells with dalargin may be attributed to activation of δ-opioid receptors and prevention of mitochondrial membrane permeability impairments, which decreases the number of apoptotic cells under cold stress.

Currently, there are many biologically active supplements to preservation and perfusion solutions, which are well described (22). However, they all act directly during perfusion of organs. The neuropeptide we used can exert its protective effect at very low concentrations both before and after perfusion. This is a remarkable feature and an advantage over proposed compositions of storage and perfusion media. In addition, given data about the dominant role of apoptosis in cell death during ischemia-reperfusion (23), the anti-apoptotic effect of dalargin under cold stress is another important reason to add this synthetic analogue of leu-enkephalin to reperfusion media and preservative solutions.

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