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ARTICLES

The Response of Neurons and Glial Cells of Crayfish to Photodynamic Treatment: Transcription Factors and Epigenetic Regulation

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Abstract—Photodynamic therapy (PDT) based on photoproduction of highly toxic singlet oxygen, which causes oxidative stress and death of stained cells, is used for treatment of cancer including that of brain tumors. The study of signaling and epigenetic mechanisms of photodynamic damage of normal neurons and glial cells was carried out on isolated crayfish mechanoreceptors consisting of single sensory neurons enveloped by glial cells. PDT effect caused necrosis of neurons and glial cells and apoptosis of glial cells. Application of specific inhibitors or activators of transcription factors: NF-κB (betulinic acid, parthenolide, CAPE), AP-1 (SR11302), STAT-3 (cucurbitacin, stattic), HIF-1 (KG-548, FM19G11, DMOG), and p53 (RITA, WR1065, nutlin-3, pifithrin-α) or those of epigenetic processes, such as DNA methylation (5-azacytidine, decitabine) or histone deacetylation (sodium valproate, trichostatin A, SBHA) demonstrated that PDT-induced death of neurons and glial cells is regulated by transcription factors and epigenetic regulators. Epigenetic processes did not influence PDT-induced necrosis of neurons. Among the transcription factors studied, only STAT-3 was involved in PDT-induced necrosis of neurons. DNA methylation and histone deacetylation, which suppress transcription, mediated PDT-induced necrosis of glial cells. The transcription factor NF-κB had antinecrotic effects on glia. All transcription factors studied and histone deacetylase were involved in apoptosis of glial cells. Their modulators might serve as potential glia- and neuroprotective agents.

Keywords: neuron, glia, necrosis, apoptosis, transcription factors, epigenetics

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INTRODUCTION

Photodynamic effect is a damage and destruction of cells upon photoexcitation of a photosensitizer dye, transfer of photoexcitation energy to oxygen, transformation of oxygen into cytotoxic singlet form, generation of other reactive oxygen species, development of oxidative stress in the cells, and apoptotic death of the cells in the end. It is used for destruction of tumors, including brain tumors [1–4].

Under the effect of external factors or controlling signaling molecules (hormones, neuropeptides, cytokines, growth factors), cells change their functional state and either divide and differentiate or die. These processes are implemented by different effector proteins, with their functioning being initiated and regulated by intracellular signaling cascades [5, 6]. The role of signaling processes in the regulation of the survival and death of photosensitized cells is described in a number of reviews [1, 7–10].

If there is a lack of effector and signaling proteins in the cell, transcription factors activate expression of the corresponding genes and synthesis of necessary proteins. A higher level of regulation is epigenetic one, which controls the total level of gene expression and biosynthetic processes. Epigenetic processes include methylation and demethylation of promotor regions of DNA and covalent histone modifications, such as methylation and demethylation, acetylation and deacetylation, and phosphorylation and dephosphorylation. They regulate availability of promoters for transcription factors and RNA polymerase II [11]. Abnormal DNA methylation and histone modifications are involved in the regulation of different neuronal functions, e.g., synaptic plasticity and memory formation [12, 13] and in pathogenesis of a number of neurological diseases, such as Alzheimer and Parkinson diseases [13–16], epilepsy and stroke [17], and responses of the nervous system to acute and chronic stress [18]. Using protein microarrays, it has been shown recently that photodynamic effect increases the expression of proteins involved in epigenetic regulation in the mouse cortex: histone deacetylases HDAC-1 and HDAC-11, transcription repressor Kaiso, dimethylated histone H3, transcription factors AP-1/C-Jun

and FOXC2, importins α5/7, and others [19]. However, brain tumors contain different cell types: neurons, glial cells, blood vessels, and connective tissue. Biochemical analysis of the tissue does not make it possible to study regulatory processes in different cell types.

Simple but informative object to study the role of signaling and epigenetic processes in interacting neurons and glial cells is an isolated crayfish mechanoreceptor surrounded by glial cells. The role of different signaling processes in regulation of the PDT-induced death of the isolated crayfish mechanoreceptor and satellite glial cells is described in [1, 10, 11, 20–25]. This work presents the data on the role of a number of transcription factors (NF-κB, AP-1, STAT-3, HIF-1, p53) and proteins involved in epigenetic regulation (DNA methyltransferases and histone deacetylases) in the PDT-induced inactivation and necrosis of crayfish mechanoreceptor neurons and necrosis and apoptosis of the surrounding glial cells.

MATERIALS AND METHODS

The objects of the study were mechanoreceptor neurons and satellite glial cells of isolated stretch receptors of *Astacus leptodactilus* crayfish. After isolation, they were placed into a 2-mL chamber filled with van Harreveld saline. Action potentials were recorded extracellularly using suction glass electrodes. After amplification, signals were digitized by the L-761 analog-to-digital converter (L-Card, Russia) and their frequency was recorded by a computer. The impulse activity was initially recorded for 30 min. Then, sulfonated aluminium phthalocyanine Photosens ($AlPcS_n$, average $n = 3.1$; NIOPIK, Russia) was added to the chamber at a concentration of 75 nM, and 5 min later pharmacological agents were added. After 25 min, the preparation was irradiated for 30 min by a diode laser (670 nm, 0.4 W/cm^2). Then, the preparation was incubated for 8 h in the dark (the time necessary for apoptosis to develop). For identification of necrosis and apoptosis, the preparations were double stained with propidium iodide, which penetrates only necrotic cells with damaged membranes and makes nuclei fluoresce red, and Hoechst-33342, which imparts blue fluorescence to nuclear chromatin. The preparations were photographed using fluorescence microscope Nikon Ecliplse FN1. These methods are described in detail in [20–22].

Pharmacological modulators were: betulinic acid, an activator of the transcription factor NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells); inhibitors of NF-κB, parthenolide and CAPE (caffeic acid phenethyl ester); an inhibitor of the transcription factor AP-1 (activating protein 1), SR11302; inhibitors of STAT-3 (signal transducer and activator of transcription), cucurbitacin and static; p53 activators RITA, WR-1065, and nutlin-3; p53 inhibitor pifithrin-α; inhibitors of HIF-1 (hypoxia-inducible factor), KG-548 and FM19G11; activator of HIF-1, DMOG; inhibitors of DNA methyltransferases, 5-azacytidine and decitabine; and inhibitors of histone deacetylases (HDAC), sodium valproate, trichostatin A, and suberohydroxamic acid (SBHA).

RESULTS AND DISCUSSION

Four experimental groups were studied: (1) control; (2) effect of the activator or inhibitor in the dark; (3) effect of the photodynamic treatment, and (4) combined effect of the photodynamic treatment and inhibitor or activator of the protein studied. After isolation, all mechanoreceptors fired regularly with a frequency of 6–10 Hz. The photodynamic effect led to an irreversible cessation of the neuronal activity within 20–30 min and in 8 h, to the development of necrosis of the neurons and glial cells and apoptosis of glial cells but not of neurons, as was described earlier in [1, 20–25]. In all cases, changes in the level of the PDT-induced necrosis of the mechanoreceptor neurons and glial cells and in that of apoptosis of the glial cells in the presence of the modulators of the transcription factors and epigenetic proteins were studied. As an example of such experiment, Fig. 1 gives microphotographs of a crayfish stretch receptor stained with Hoechst-33342 and propidium iodide after a 30-min incubation in the dark (control), the effect of decitabine in the dark, photodynamic treatment without decitabine and in its presence. Figure 2 illustrates the effect of decitabine on PDT-induced changes in the level of necrosis of the mechanoreceptor neuron and glial cells and apoptosis of the glial cells. The experiments with other modulators of the transcription factors and epigenetic proteins were performed similarly. The results are concisely summarized in the table, in which only statistically significant changes ($p < 0.05$) in these parameters are indicated.

The Involvement of Transcription Factors in PDT-Induced Inactivation and Death of Neurons and Glial Cells

NF-κB. Transcription factor NF-κB is a central integrator of cellular responses to stress. In response to external factors it initiates expression of a number of anti-apoptotic proteins: survivin, IAP, Bcl-2, and others [26, 27].

To find out the role of NF-κB in photodynamic damage of neurons and glial cells, we used a number of its modulators [28, 29]. In the presence of the NF-κB activator betulinic acid, the PDT-induced necrosis of the mechanoreceptor neurons was enhanced. Quite opposite, the NF-κB inhibitors parthenolide and CAPE decreased the necrosis of the neurons (table). This indicates that NF-κB plays a pronecrotic role in neurons. In contrast, in the glial cells NF-κB plays an antinecrotic role. In fact, its activator betulinic acid decreased PDT-induced necrosis, while the inhibitor

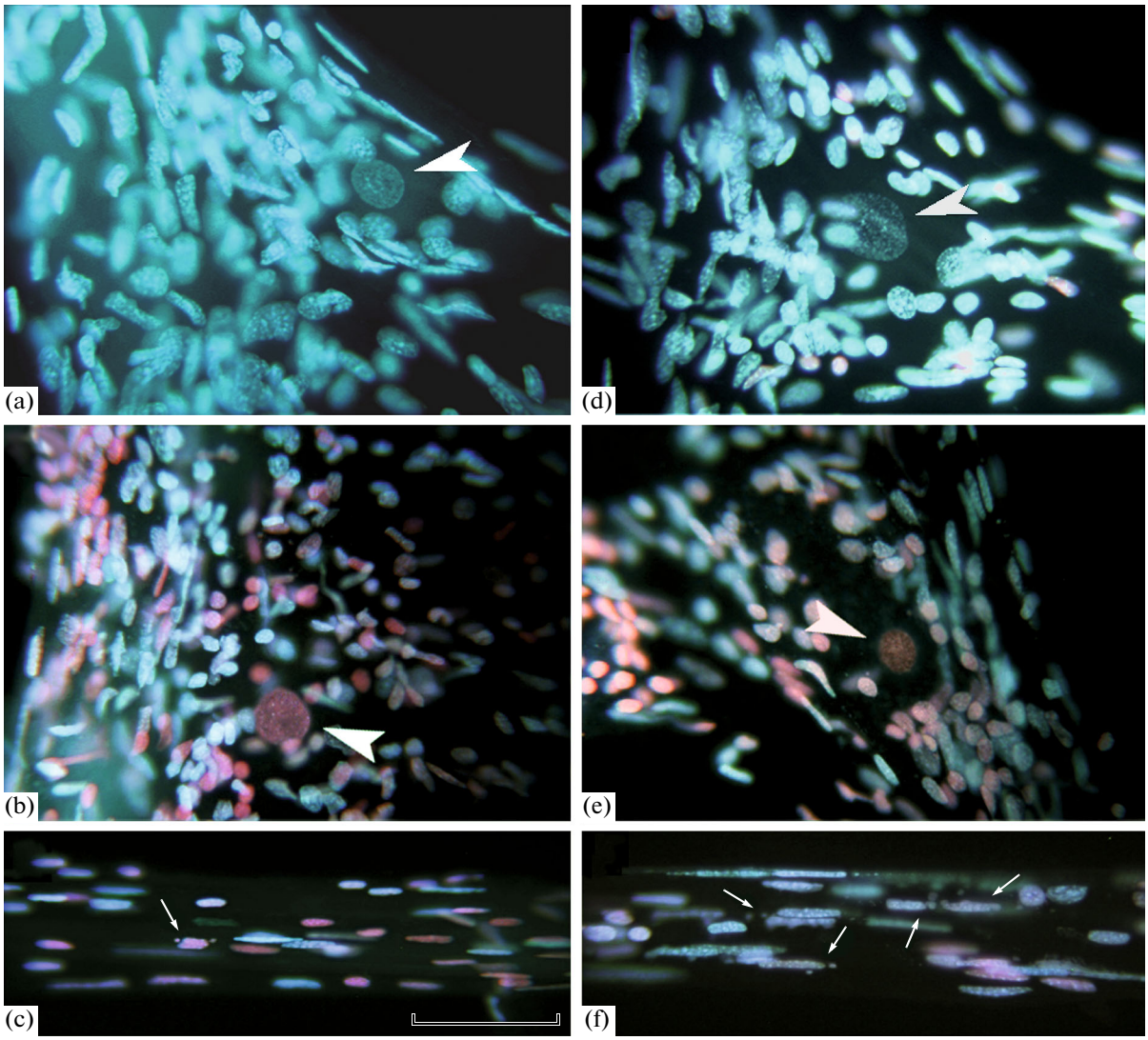

Fig. 1. An example of the effect of photodynamic treatment and decitabine, an inhibitor of DNA methylation, on a sensory neuron and surrounding glial cells in an isolated crayfish stretch receptor stained with propidium iodide, which imparts red fluorescence to the nuclei of necrotic cells, and Hoechst 33342, which imparts blue fluorescence to the nuclear chromatin. (a) Control receptor; (b) effect of decitabine in the dark; (c) photodynamic treatment; (d) photodynamic treatment in the presence of decitabine. Panels (e) and (f) demonstrate fragmented nuclei of apoptotic glial cells surrounding a proximal part of the axon of the mechanoreceptor neuron (*small arrows*). *Large arrows* in (a)–(d) indicates neuronal nuclei. Scale bar, 50 μm.

parthenolide increased the level of necrosis. Another inhibitor, CAPE, had no effects in this regard (table). Together with this, NF-κB exerted a proapoptotic effect on the photosensitized glial cells. The evidence of this is an enhancement of the PDT-induced apoptosis of the glial cells upon NF-κB activation by betulinic acid and reduction in the apoptosis in the presence of CAPE, the NF-κB inhibitor (table).

Normal neurons are known to have a low activity of NF-κB due to binding to the inhibiting protein IκB [30]. In many cell types, the NF-κB activation prevents apoptosis [31]. However, there are data indicating that in glioblastoma cells the photodynamic treatment activates NF-κB and induces apoptosis, which agrees well with our data [32].

Thus, NF-κB mediates PDT-induced necrosis of the mechanoreceptor neurons and apoptosis of the glial cells but, at the same time, in glial cells it exerts protective antinecrotic effects.

AP-1. Transcription factor AP-1, like NF-κB, is involved in early cellular response to stress. It controls the expression of genes involved in differentiation, proliferation, apoptosis, and other cell functions [33]. In our earlier experiments [28] its inhibitor SR11302 did not affect PDT-induced necrosis of the neurons and glial cells. In its presence, however, the level of PDT-induced apoptosis of the glial cells was significantly lowered (table). Therefore, AP-1 is involved in the PDT-induced apoptosis of glial cells. This result agrees with the data on the proapoptotic role of AP-1

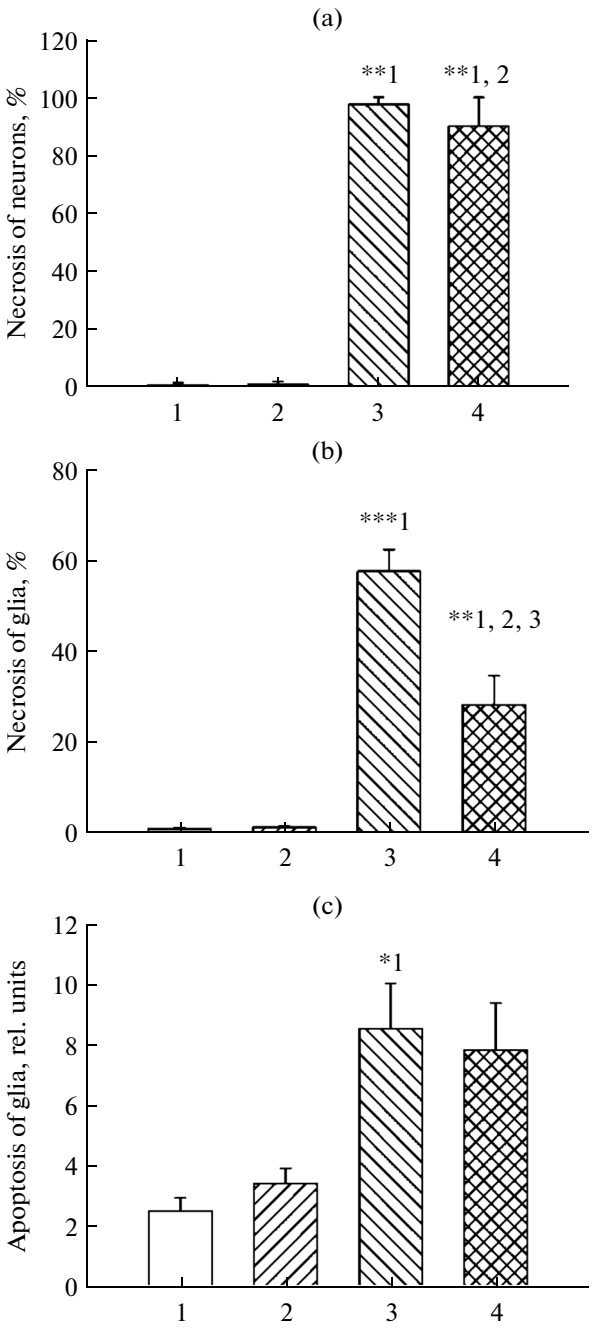

Fig. 2. The effect of decitabine (10 μM), an inhibitor of protein kinase G, on necrosis of neurons (a) and necrosis (b) and apoptosis (c) of glial cells in the dark and upon photodynamic treatment. 1, Control; 2, decitabine in the dark; 3, photodynamic treatment; 4, decitabine combined with photodynamic treatment. Significant changes are labeled with asterisks: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

during development of oxidative stress in oligodendrocytes [34]. The photodynamic effect induces strong and prolonged expression of the AP-1 components c-jun and c-fos [35].

STAT-3. STAT-3 is a component of the Jak/STAT signaling pathway, which controls division, survival, apoptosis, and different functions of cells. This pathway is activated upon binding of growth factors to their receptors, with the Jak tyrosine kinase phosphorylating both its own tyrosines and those of the receptor. Phosphotyrosines recruits STAT-3, and after phosphorylation it forms dimers, which move to the nucleus and regulates gene expression [36]. The role of STAT-3 in PDT-induced damage of neurons and glial cells is poorly studied. It was shown only that the photodynamic treatment induces cross-links in protein STAT-3. This prevents its binding to DNA and expression of the genes under its control [37].

In our study, the STAT-3 inhibitor stattic significantly decreased PDT-induced necrosis of the neurons and necrosis and apoptosis of the glial cells (table). This indicates the involvement of STAT-3 in the PDT-induced necrotic and apoptotic deaths of neurons and glial cells of crayfish.

HIF-1. The transcription factor HIF-1 activated upon hypoxia stimulates transcription of genes providing cell survival under the conditions of low oxygen content. As a result, the cells acquire resistance not only to hypoxia, but also to other external factors as well [38]. Under normal conditions, HIF-1 is hydroxylated and degraded in proteasomes. Hypoxia and reactive oxygen species (ROS) inhibit prolyl hydroxylases stabilizing and activating HIF-1 [39]. PDT induced hyperexpression of HIF-1 [40]. Sublethal photodynamic treatment increased by a factor of 2 the expression of HIF-1α in mouse brain tissues [41].

In our study, the HIF-1 activator DMOG protected glial cells from PDT-induced apoptosis and necrosis (table) [42]. This could indicate the protective role of HIF-1. However, the inhibitors of HIF-1, KG-548 and FM19G11, also demonstrated an antiapoptotic (but not antinecrotic) effect on the photosensitized glial cells, which could be evidence of the involvement of HIF-1 in the photo-induced apoptosis but not necrosis of the glial cells. These contradictory results do not allow definite conclusions on the role of HIF-1 in photodynamic damage of the mechanoreceptor neuron and glial cells.

p53. Protein p53, also known as the guardian of the genome, is a key regulator of the cell response to stress, DNA reparation, cell cycle, and apoptosis. As a transcription factor, it regulates expression of more than 100 genes involved in these processes. It also could function independently of the transcription. Moving to the mitochondria, p53 promotes a release of cytochrome *c* and the apoptosis onset. In normal cells, its concentration is low; it increases upon DNA damage, oxidative stress, and ionizing radiation impact. In neurons, p53 level noticeably increases upon ischemia and neurodegenerative diseases [43, 44].

In [45, 46], the p53 activators RITA and nutlin-3 significantly enhanced PDT-induced apoptosis of glial cells (table). This indicates the involvement of p53 in these processes. It is of interest that the p53 inhibitor pifithrin-α also demonstrated proapoptotic

Involvement of transcription factors and epigenetic proteins in necrosis of neurons and necrosis and apoptosis of glial cells subjected to the photodynamic treatment. Arrows indicate only significant ($p < 0.05$) decrease or increase; dash shows the absence of changes

Modulator	Activator or inhibitor, concentration	Necrosis		Glia apoptosis	References
		neuron	glia		
NF-κB					
Activator	Betulinic acid (5 μM)	↑	↓	↑	[28, 29]
Inhibitors	Parthenolide (20 μM)	↓	↑	–	
	CAPE (30 μM)	↓	–	↓	
AP-1					
Inhibitor	SR11302 (10 μM)	–	–	↓	[28]
STAT-3					
Inhibitors	Cucurbitacin (50 nM)	–	–	↓	[29]
	Stattic (10 μM)	↓	↓	↓	
HIF-1					
Inhibitors	KG-548 (53 μM)	–	–	↓	[42]
	FM19G11 (3.7 μM)	–	–	↓	
Activator	DMOG (1 mM)	–	↓	↓	
p53					
Activators	RITA (10 μM)	–	–	↑	[45, 46]
	Nutlin-3 (1 μM)	–	–	↑	
	WR-1065 (100 μM)	–	↑	–	
Inhibitor	Pifithrin-α (PFT, 500 nM)	↓	↓	↑	
Histone deacetylases					
Inhibitors	Sodium valproate (0.5 mM)	–	↓	↓	[50, 52]
	Trichostatin A (100 nM)	–	↓	↓	
	SBHA (5 μM)	–	↓	↓	
DNA methylation					
Inhibitors	Decitabine (10 μM)	–	↓	–	[50, 52]
	5-azacytidine (5-Aza, 10 μM)	–	↓	–	

activity. Herewith, it decreased the PDT-induced necrosis of the mechanoreceptor neuron and glial cells. The p53 activator WR-1065 had an opposite pronecrotic effect on the glial cells (table), suggesting the involvement of p53 in the PDT-induced necrosis of the glial cells.

Most photosensitizers are not localized in the cell nucleus; therefore, photodynamic treatment does not affect DNA. Nevertheless, the involvement of p53 in the PDT-induced death of cells is observed in different cell types [47–49]. In these cases, p53 probably functioned independently on the transcription.

The Involvement of Epigenetic Processes in the PDT-Induced Inactivation and Death of Neurons and Glial Cells

DNA methylation. DNA methylation, during which DNA methyltransferases transfer a methyl group from S-adenosylmethionine to cytosine in CpG dinucleotides, is a key epigenetic mechanism controlling the transcription processes. Hypermethylation of cytosines in CpG islands in promoter regions of genes prevents binding of transcription factors and RNA polymerases and thus inhibits the transcription. In contrast, demethylation facilitates binding of transcription factors to promoters and stimulates gene expression [11, 51].

We did not observe any significant effect of the DNA methyltransferases inhibitor 5-azacytidine and decitabine on the PDT-induced apoptosis of the glial cells and necrosis of the neurons. However, the inhibition of DNA methyltransferases using 5-azacytidine and decitabine significantly decreased PDT-induced necrosis of the glial cells (table) [52]. It indicates the involvement of DNA methyltransferases in PDT-induced necrosis of glial cells. Decitabine and 5-azacytidine are known not only to inhibit DNA methyl-

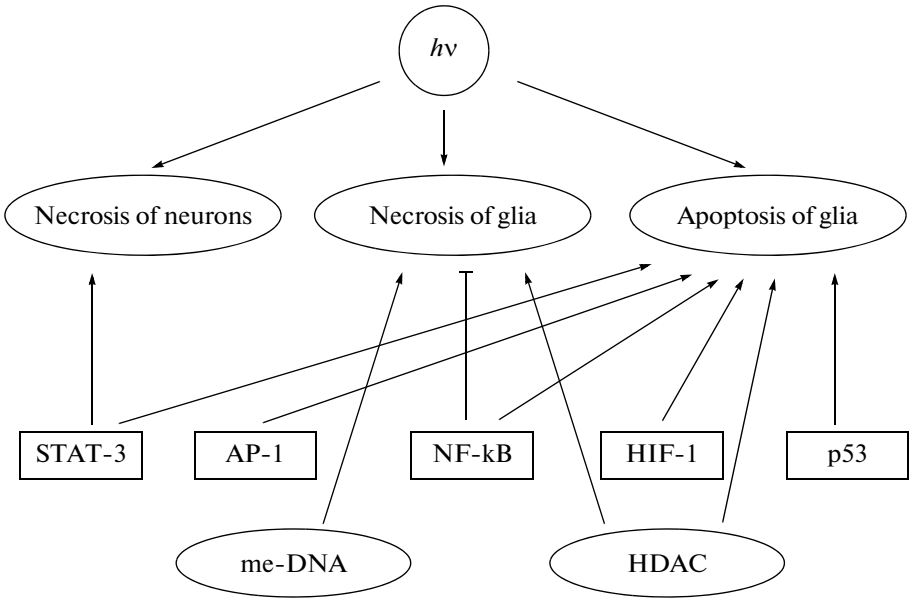

Fig. 3. A scheme illustrating the involvement of transcription factors in PDT-induced necrosis of neurons and glial cells and apoptosis of glial cells.

transferases but also to activate the expression of silent genes [53]. Thus, epigenetic suppression of the transcription promotes PDT-induced necrosis of glial cells. In neurons, this effect is insignificant.

Histone deacetylation. Histone acetylation leads to chromatin decondensation and increases promoter availability for transcription factors. This increases the total level of gene expression. In contrast, histone deacetylation suppresses gene expression. Histone acetylation depends on the balance between the activity of histone acetyltransferases and histone deacetylases [54]. The HDAC inhibitors sodium valproate, trichostatin A, and sodium butyrate demonstrated a neuroprotective effect in neurodegenerative diseases, ischemic stroke, glutamate excitotoxicity, and oxidative stress [16, 55, 56].

In our experiments, inhibitors of histone deacetylases sodium valproate, trichostatin A, and SBHA did not affect PDT-induced necrosis of the mechanoreceptor neurons but significantly decreased the level of necrosis of the surrounding glial cells by a factor of 1.5–2.3. They also protected the glial cells from the PDT-induced apoptosis by decreasing its level by a factor of 2.1–2.7 (table) [51].

Thus, the inhibitors of DNA methylation protected the crayfish glial cells but not neurons from photoinduced necrosis, and the inhibitors of histone deacetylases also protected them from apoptosis. Therefore, such epigenetic processes as DNA methylation and histone deacetylation are involved in the regulation of PDT-induced necrosis of glial cells but not neurons. Apoptosis of glial cells is regulated by histone deacetylation but not by DNA methylation. The insusceptibility of sensory neurons of the crayfish stretch receptor to the inhibitors of DNA methylation and HDAC deacetylation probably suggests an insignificant role of epigenetic processes in regulation of their survival.

CONCLUSIONS

Our data demonstrate that the transcription factors NF-κB, AP-1, STAT-3, HIF-1, and p53 and epigenetic processes of DNA methylation and histone deacetylation are involved in the regulation of PDT-induced death of neurons and surrounding glial cells (Fig. 3).

Upon different physical and chemical impacts these neurons die by necrosis, but not apoptosis [1, 20, 21]. In contrast to the glial cells, DNA fragmentation typical for apoptosis was not observed in the mechanoreceptor neuron. In the crustacean nervous system, mechanoreceptor neurons are not duplicated. They play an important role in controlling animal movements and, thus, are vitally important. They are likely to have not only apoptosis inhibited, but the entire epigenetic regulation as well [51].

Various involvement of these signaling proteins in the PDT-induced death of neurons and glial cells and their different sensitivity to pharmacological modulators suggests the probability of selective regulation of survival of neurons and glial cells using pharmacological agents.

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