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Role of Hydrogen Sulfide in the Regulation of Cell Apoptosis

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We studied the effect of a gas transmitter hydrogen sulfide (H_2S) on the realization of apoptosis in Jurkat cells and mononuclear leukocytes from healthy donors. Treatment with H_2S donor NaHS was accompanied by a dose-dependent intensification of cell death via apoptosis and necrosis. T-cell leukemia cells were more sensitive to H_2S than mononuclear leukocytes from healthy donors. H_2S-induced cell apoptosis was accompanied by activation of caspase-3 and caspase-9.

Key Words: *apoptosis; hydrogen sulfide; caspases*

For a long time, hydrogen sulfide (H_2S) was known because of its toxic properties realized via blockade of cytochrome C oxidase. Recent studies showed that considerable amounts of H_2S are produced in mammalian cells and affect a variety of physiological processes. It was hypothesized that H_2S serves as a regulatory transmitter. H_2S is the third (after NO and CO) endogenous gas transmitter. H_2S serves as a signal molecule in the regulation of cardiovascular and nervous functions and realization of the inflammatory process [2,8].

H_2S is produced by nearly all cells in the body. It is interesting to evaluate the effect of H_2S on various processes in the cell, including the molecular mechanisms for apoptosis. Apoptosis is a mechanism for regulation of cell homeostasis during normal embryogenesis of a multicellular organism, as well as in adult organism [3]. Abnormalities in apoptosis serve as one of the pathogenetic mechanisms for disturbances in cell homeostasis under various pathological conditions. Therefore, studying the molecular mechanisms for its dysregulation is an urgent problem.

There is no general agreement regarding the influence of H_2S on apoptosis. Some authors showed that H_2S inhibits apoptosis due to its protective effect on mitochondrial function [5]. Others reported that H_2S produces a proapoptotic effect, which is realized during activation of the mitochondrial pathway of apoptosis induction, caspase-3, and MAP kinase family [4].

Here we evaluated the role of H_2S in the system of intracellular transduction of the apoptotic signal.

MATERIALS AND METHODS

Experiments were performed on Jurkat cells (T-lymphoblast leukemia) and mononuclear leukocytes (MNL). MNL were isolated from the blood of 10 healthy donors by centrifugation in Ficoll layer (Pharmacia) with a density of 1.077. The cells were incubated in complete nutrient medium at 37°C. To evaluate the role of H_2S in the molecular mechanisms of apoptosis, H_2S donor sodium hydrosulfide (NaHS, Sigma) was

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added to the nutrient medium. The number of necrotic cells was estimated by light microscopy after staining with 0.5% trypan blue (Serva).

TUNEL method (Webstain) was used to evaluate the presence of apoptotic cells in the test culture. This method is based on the ability of an enzyme TdT (terminal deoxynucleotidyl transferase) to recognize specifically the 3'-OH end of the chromosome break and to attach a fluorescent probe to this site. The cells were placed on a polylysine-coated glass, dried, and fixed in vapors of 10% formalin (pH 7.4). The sample was treated with phosphate buffered saline (500 µl) and incubated at 37°C for 30 min. The buffer was removed. Protein kinase C solution (250 µl) necessary for permeabilization of cell membranes) was added. Incubation was performed at 37°C for 30 min. The samples were washed 4 times with distilled water and treated with TdT buffer for 5-10 min. The buffer was removed. The sample was treated with 50 µl TdT solution (TdT buffer, FITC label, and TdT enzyme) and incubated at 37°C for 60 min. The solution of TdT was removed. A glass with the sample was maintained in TB solution (stop reagent of 300 mM NaCl and 30 mM sodium citrate; obtained from the manufacturer) at room temperature for 15 min and washed 4 times with distilled water. The samples were prepared and subjected to a fluorescent microscopic study for 10 min.

Activities of caspase-3 and caspase-9 were measured spectrophotometrically using Abcam reagents. This method is based on the detection of chromophore *p*-nitroanilide (pNA) released after cleavage of DEVD-pNa and LEHD-pNA (substrates) by caspase-3 and caspase-9, respectively. The cells were resuspended in 50 µl lysing buffer, incubated in cold, and centrifuged. Protein content and caspase activity were measured in the supernatant (cytosolic extract). The protein (50-200 mg) was diluted with 50 µl lysing buffer. The reaction buffer (50 µl) and caspase substrate (5 µl) were added. Incubation was performed at 37°C for 24 h. The dilution buffer was added. Caspase activity was measured on a spectrophotometer at 400-405 nm (against the corresponding cytosolic extract and buffers for each sample). Extinction of intact Jurkat cells was taken as one unit.

The data were analyzed by statistical tests and testing of statistical hypotheses. The mean values were calculated for each sample. The normality of the distribution of quantitative parameters was evaluated by Kolmogorov–Smirnov test. When the data conformed to the normal distribution, hypothesis testing for the equality of mean values was conducted with Student's *t* test. When the data did not conform to the normal distribution, the differences between dependent samples were evaluated by nonparametric Wilcoxon test. The differences between independent samples were evaluated by Mann–Whitney rank test. The differences were significant at $p<0.05$.

RESULTS

The study was performed in 2 series. In series I, we compared the dose-dependent effects of H_2S on death of intact and transformed tumor cells. Various donors of H_2S were used to evaluate the role of this gas in physiological functions and pathological processes. NaHS is extensively used in these researches. NaHS dissociates into Na^+ and HS^- in an aqueous solution. HS^- binds to hydrogen ion with the formation of H_2S, which easily crosses the cell membrane due to lipophilic properties [6].

For evaluation of the role of H_2S in apoptosis, Jurkat cells and MNL were cultured with NaHS in various concentrations (from 10 mM to 1 M) for 15 min and 2 h. Incubation of cells with 1 M and 500 mM H_2S donor resulted in cell necrosis. These concentrations were not used to study the effect of H_2S on apoptosis. Incubation of Jurkat cells with 10 mM NaHS for 15 min was followed by a 2.2-fold increase in the number of apoptotic cells. However, the count of these cells was significantly reduced after incubation of Jurkat cells with 50 mM NaHS. NaHS in a concentration of 100 mM intensified apoptotic death (by 1.5 times compared to the control; $p<0.05$). Addition of 100 mM NaHS to the culture medium was accompanied by necrotic death of more than 50% cells. NaHS in a concentration of 50 mM increased the number of necrotic cells by 5 times. The count of necrotic lymphocytes after treatment with 10 mM NaHS was 1.7-fold higher than that in the intact culture. Two-hour treatment with 10 mM induced a decrease in the number of apoptotic Jurkat cells (as compared to that observed after 15-min

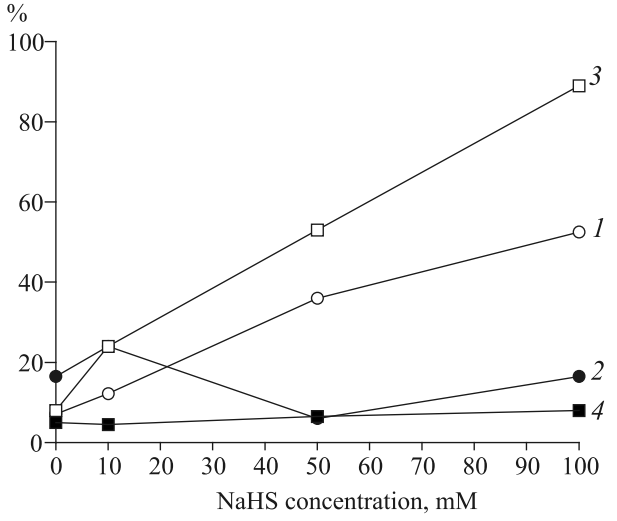

Fig. 1. Number of necrotic (*1*, *3*) and apoptotic Jurkat cells (*2*, *4*) after treatment with NaHS for 15 min (*1*, *2*) and 2 h (*3*, *4*).

TABLE 1. Number of Necrotic and Apoptotic Cells in MNL Suspension from Healthy Donors after 15-min Treatment with H_2S in Various Concentrations (Me(Q1-Q3)

Cell number, %	Intact cells	H_2S-treated cells, mM			
		10	50	100	500
Necrotic	1.79 (0-3.2)	3.6 (1.1-5.8)	2.8 (0-5.3)	9.6 (4.3-11.2)*	92.3 (85-100)*
Apoptotic	2.1(0.5-3.7)	0.7 (0-1.5)	1.1 (0.5-2)	0.3 (0-0.5)	–

Note. Here and in Table 2: *p<0.05 compared to the intact culture.

TABLE 2. Activities of Caspase-3 and Caspase-9 in Jurkat Cells and MNL after Treatment with 10 mM NaHS (arb. units)

Cells		Caspase-3 activity, arb. units	Caspase-9 activity, arb. units
Intact Jurkat cells		1	1
Jurkat cells after treatment with 10 mM NaHS	15 min	1.6*	2.7*
	2 h	0.45*	1.7*
MNL from healthy donors		0.72*	1.4

incubation), which was related to rapid dissociation of the donor in an aqueous solution (Fig. 1).

Our results indicate that NaHS in a dose of 10 mM produces a proapoptotic effect on Jurkat cells, but does not modulate apoptosis in MNL from healthy donors. Moreover, the increase in the number of necrotic cells in the culture of MNL from healthy donors was observed only after treatment with NaHS in a dose of 10 mM. The count of TUNEL-positive cells remained unchanged under these conditions (Table 1). It can be hypothesized that tumor-transformed cells of T-cell leukemia are more sensitized to the effect of H_2S than lymphocytes and monocytes from healthy donors.

Series II was performed to evaluate the molecular mechanisms for H_2S-induced dysregulation of apoptosis. The apoptotic process is realized due to induction of caspases that belong to a family of cysteine proteases. Depending on the structure and role in apoptotic signal transduction, caspases are divided into initiator and effector enzymes [7]. Initiator caspases (caspase-2, caspase-8, caspase-9, and caspase-10) transactivate effector caspases (caspase-3, caspase-6, caspase-7, and caspase-14) that hydrolyze the cell substrates [1].

Induction of apoptosis with 10 mM NaHS for 15 min was accompanied by a significant increase in caspase-3 activity in T-cell leukemia cells (as compared to the intact culture). Activity of this enzyme after 2-h incubation with 10 mM NaHS was lower compared to that observed after 15-min incubation and in the intact culture. Caspase-3 activity in MNL from healthy donors was much lower than in Jurkat cells (Table 2). Caspase-3 is induced after cleavage of the corresponding zymogene with initiator caspase-2, caspase-8, caspase-9, and caspase-1 [3]. The data indicate that treatment of Jurkat cells with 10 mM NaHS for 15 min is accompanied by an increase in caspase-9 activity (as compared to that in the intact culture). Enzyme activity after 2-h incubation with 10 mM NaHS was higher than in the control (Table 2).

We conclude that intracellular H_2S is involved in the regulation of apoptosis. H_2S has a selective effect on normal and transformed cells. The molecular mechanisms for proapoptotic action of H_2S are associated with activation of caspase-3 and caspase-9.

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