Journal of Cellular Biochemistry 78:578–587 (2000)

Glutathione Is A Factor of Resistance of Jurkat Leukemia Cells to Nitric Oxide-Mediated Apoptosis

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Abstract We have previously reported that nitric oxide (NO) stimulates apoptosis in different human neoplastic lymphoid cell lines through mitochondrial damage (including degradation of cardiolipin, a major mitochondrial lipid) followed by activation of caspases. Here we demonstrate that Jurkat human leukemia cells which survive after 24 h treatment with NO form subpopulations with higher and lower cardiolipin content (designated as NAO^{high} and NAO^{low}, respectively). Sorted NAO^{high} cells were found to survive in culture whereas sorted NAO^{low} cells died. Moreover, NAO^{high} cells acquired an increased resistance to the exposure to NO donors which remained unchanged during long-term culture. These cells showed a similar cardiolipin content and expressed the same level of anti-apoptotic proteins Bcl-2 and Bcl-x_L as APO-S unsorted cells but contained significantly higher concentration of the antioxidant glutathione. Depletion of glutathione in these cells with buthionine-sulfoximine (BSO) correlated with a significant stimulation of NO-mediated apoptosis whereas the exposure of NO-sensitive APO-S cells to the glutathione precursor N-acetylcysteine (NAC) resulted in a substantial suppression of this effect. Our data suggest a complex mechanism of the resistence to NO-induced apoptosis in Jurkat human leukemia cells in which glutathione plays an important role. J. Cell. Biochem. 78:578–587, 2000.

Key words: nitric oxide; glutathione; neoplastic lymphoid cells; apoptosis; mitochondria

Apoptosis (programmed cell death) is a distinct form of cell death occuring during development and differentiation, in tumor cell deletion, and in response to different stimuli like TNF, CD95 (FAS/APO-1) ligand, TRAIL (APO-2 ligand), reactive oxygen species (ROS), and shortage of growth factors or certain metabolites [Thompson, 1995]. Apoptosis involves an initial commitment phase followed by an execution phase characterized by the activation of a cascade of cytoplasmic cystein proteases (caspases) [Henkart et al., 1996] and by structural changes including externalization of phosphatidylserine, cell shrinkage, nuclear condensation, DNA fragmentation, and the breakdown of the cell into small fragments (apoptotic bodies) that are then phagocytosed [Earnshaw, 1995]. Recently, it has been found that alterations in mitochondrial functions play an important role in the effector phase of apoptosis induced by different agents [Kroemer et al., 1997]. Moreover, apoptotic cells were shown to accumulate oxidized proteins and lipids [Buttke et al., 1994], and antioxidants were reported to protect against different forms of apoptosis [Slater et al., 1995].

Nitric oxide (NO), generated from the conversion of L-arginine to L-citrulline by at least three distinct isoforms of NO synthase (NOS), plays a key role in different physiological and pathological processes [Moncada et al., 1991; Nathan et

Abbreviations used: BSO, buthionine-sulfoximine; γ-GCS, γ-glutamylcysteine synthetase; GTN, glycerol trinitrate; mAb, monoclonal antibody; mBB, monobromobimane; NAC, N-acetylcysteine; NAO, 10-N-nonyl-3,6-bis(dimethylamino)acridine orange; NO, nitric oxide; PI, propidium iodide; ROS, reactive oxygen species.

Grant sponsor: Dr. Mildred Scheel Stiftung; Grant number: 10-0980-Schi2, V.U.

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Received 30 September 1999; Accepted 29 February 2000

al., 1994]. The high level of NO produced by inducible NOS in activated macrophages and endothelial cells for long periods of time was found to be associated with apoptosis in normal and tumor cells [Fehsel et al., 1995; Geng et al., 1996; Hortelano et al., 1997; Umansky et al., 1997;]. It was also shown that NO-mediated cytotoxicity involved persistent inhibition of the mitochondrial respiratory chain complexes in different types of cells [Clementi et al., 1998; Richter et al., 1999]. In particular, NO blocked cytochrome c oxidase (complex IV) activity via binding to its heme moiety and complex I activity due to S-nitrosylation of this enzyme [Brown et al., 1994; Clementi et al., 1998; Richter et al., 1999].

Glutathione, the tripeptide L-γ-glutamyl-L-cysteinylglycine, is an ubiquitous cellular nonprotein sulfhydryl which plays an important role in maintaining intracellular redox balance and in cellular defence against oxidative stress [Dröge et al., 1994]. Glutathione is synthesized by two enzymes, γ-glutamylcysteine synthetase (γ-GCS), which is the rate limiting enzyme, and glutathione synthetase. It has been reported that glutathione inhibits mitochondrial damage induced by NO [Clementi et al., 1998]. In addition, glutathione can suppress apoptosis mediated by different agents including NO [Kane et al., 1993; Ghibelli et al., 1998]. Glutathione depletion with buthionine-sulfoximine (BSO) or diethyl maleate leads to stimulation of apoptosis which may be reversed by N-acetylcysteine (NAC) [Watson et al., 1996]. An anti-apoptotic effect is also provided by the inhibition of glutathione efflux via bcl-2 gene overexpression [Bojes et al., 1997] or via pretreatment with methionine [Ghibelli et al., 1998]. Taken together, these data suggest that modification of intracellular glutathione content can play an important role in apoptosis.

Investigating mitochondria-dependent mechanisms of NO-mediated apoptosis in human leukemia cells, we have previously found: 1) a significant decrease in the concentration of cardiolipin, a major mitochondrial lipid; 2) a downregulation in the respiratory chain complex activities; and 3) a release of mitochondrial protein cytochrome c into the cytosole [Ushmorov et al., 1999]. Here we studied the mechanisms of human leukemia cell resistance to NO-mediated apoptosis. We provide evidence that Jurkat APO-S cells, treated with NO at the apoptosis-inducing concentration and sorted for high cardiolipin content (NAO^{high} cell population), acquire increased resistance to NO which correlates with the elevated concentration of glutathione. In addition, the apoptotic effect of NO in these cells is substantially increased by BSO, the inhibitor of γ-GCS, and is suppressed by glutathione precursor NAC.

MATERIALS AND METHODS

Cell Culture

Human leukemic T cell line Jurkat APO-S was maintained in 5% CO_2 at 37°C in RPMI 1640 medium (Gibco-BRL, Eggenstein, Germany) containing 5% fetal calf serum (FCS).

Antibodies and Other Reagents

The monoclonal antibody (mAb) against human Bcl-x_L (clone 2H12) and human ERK-1 (clone MK12), an ubiquitously expressed serine/threonine kinase, were purchased from PharMingen (Hamburg, Germany). The polyclonal rabbit Ab against human Bcl-2 and the secondary horseradish peroxidase-conjugated anti-rabbit Ab were purchased from Santa Cruz (Heidelberg, Germany). The secondary horseradish peroxidase-conjugated goat anti-mouse mAb was from Dianova (Hamburg, Germany). Glycerol trinitrate (GTN; Merck, Darmstadt, Germany) and DETA/NONOate (NOC-18; Alexis, Gruenberg, Germany) were used as NO donors at the concentration 0.2 mM and 0.5 mM, respectively. The following reagents were from Molecular Probes, Inc. (Eugene, OR): 10-N-nonyl-3,6-bis(dimethylamino)acridine orange (NAO), monobromobimane (mBB), and propidium iodide (PI). The glutathione precursor NAC and the inhibitor of γ-glutamylcysteine synthetase BSO were from Sigma (Munich, Germany). All other chemicals used were of analytical grade and also purchased from Sigma.

Apoptosis Assay

Apoptosis was assessed by measuring the binding FITC conjugated annexin V protein to the phospholipid phosphatidylserine which is present on the external membrane surface of apoptotic cells [Koopman et al., 1994]. 10^6 cells were incubated with annexin V-FITC and with 1 μg/ml PI following manufacturer's instruction (Beckman-Coulter, Krefeld, Germany). In addition, apoptotic response was evaluated with the mAb APO2.7 which reacts with a 38-kDa mitochondrial membrane protein in apoptotic cells [Koester et al., 1997]. 10^6 cells were permeabilized in digitonin (100 μg/ml) and stained with

APO2.7-PE mAb following manufacturer's instruction (Beckman-Coulter). Typically, 10^4 cells per sample were analysed by flow cytometry using a FACScan with CELLQuest software (Becton-Dickinson, Heidelberg, Germany).

Cell Sorting and Culture

APO-S Jurkat cells were treated with 0.2 mM GTN or 0.5 mM NOC-18 for 24 h. Then cells were incubated with NAO (25 nM) for 10 min at 37°C in DMEM medium containing 1% FCS. After treatment with 1 μg/ml PI for 5 min, the cells were sorted using a FACS Vantage sorter (Becton-Dickinson). Windows for sorting were defined in the cell populations with higher or lower NAO expression (NAO^{high} and NAO^{low}, respectively) excluding PI-positive (dead) cells. Flow rate was 3,000–5,000 cells and sorted cells were collected in sterile tubes containg RPMI medium with 20% FCS. After washing, NAO^{high} and NAO^{low} sorted cells were cultured in RPMI medium at the same conditions as parental APO-S cells. For the second round of sorting, NAO^{high} cells were incubated with 25 nM NAO for 10 min, and PI-negative (live) cells were sorted for high and low NAO expression and put in culture as described above.

Cytofluorometric Analysis of Cardiolipin and Glutathione Concentration

To measure the content of a main mitochondrial phospholipid cardiolipin, cells (7×10^5/ml) were incubated with NAO (100 nM) for 30 min at 37°C [Ushmorov et al., 1999]. Intracellular glutathione was analysed after incubation with a specific dye mBB (40 nM) for 10 min at 37°C [Hedley et al., 1994]. One μg/ml PI was added to the samples for 5 min before FACS analysis and measured at red fluorescence (FL3). Recordings were made only on PI negative (viable) cells at green fluorescence (FL1) for NAO and at blue fluorescence (FL4) excited by He-Ne laser for mBB. Typically, 10^4 cells per sample were measured using a FACScan with CELLQuest software (Becton-Dickinson).

Determination of Intracellular Glutathione Content

Jurkat APO-S parental cells or APO-S cells sorted for high NAO expression (NAO^{high}) were lysed with sulfosalicylic acid, sonified and kept on ice for 20 min followed by centrifugation for 10 min at 12,000*g*, 4°C. Supernatants were used for determination of total glutathione concentration according to Tietze et al. [1969] and glutathione disulfide (GSSH) as described [Griffith, 1980].

RT-PCR

Total RNA was isolated from both parental and NAO^{high} sorted cells incubated for different periods of time with 0.2 mM GTN using guanidium isothiocyanate. The first strand cDNA was synthesized in 20 μl reaction mixture from 2 μg total RNA using a cDNA preamplification kit (Gibco-BRL). PCR was performed using specific primers for glutathione S-transferase, glutathione reductase, glutathione peroxidase, and γ-glutamylcysteine synthetase as described [Dubrovskaya et al., 1998; Rahman et al., 1999].

Measurement of Bcl-2 and Bcl-x_L Expression

After incubation with 0.2 mM GTN for 24 h, APO-S parental and sorted NAO^{high} cells were treated with a lysis buffer (20 mM Tris-HCl at pH 7.5, 1% NP-40, 150 mM NaCl, 25 mM NaF, 1 mM $MgCl_2$, 1 mM sodium orthovanadate, 10% glycerol, 10 μg/ml leupeptin, and 10 μg/ml aprotinin). Equal amounts of total protein were subjected to 12.5 % SDS-polyacrylamide gel electrophoresis followed by Western blot analysis. The proteins were transfered onto a polyvinylidene fluoride membrane (Millipore, Bedford, MA) using a semi-dry blotting apparatus (Bio-Rad, Munich, Germany). The membrane was blocked with 5% solution of non-fat dry milk in TTBS buffer (10 mM Tris-HCl at pH 8.0, 150 mM NaCl, 0.05% [v/v] Tween 20) and incubated overnight at 4°C with Abs against Bcl-x_L (dilution 1:1,000) or Bcl-2 (dilution 1:1,000) proteins. The same blots were incubated with mAbs to ERK-1 (dilution 1:2,500) to show that equal amounts of protein were loaded. Blots were extensively washed with TBST buffer and treated with goat anti-mouse Abs (dilution 1:10,000) for Bcl-x_L or with anti-rabbit Abs (dilution 1:100,000) for Bcl-2. The immunoreactive bands were visualized by enhanced chemiluminescece (ECL) method following the manufacturer's protocol (Amersham, Braunschweig, Germany).

RESULTS

Jurkat Cells Sorted for High Concentration of Cardiolipin (NAO^{high} Cells) Acquire Resistance Against NO-Induced Apoptosis

We have previously shown that the treatment of APO-S Jurkat cells with increasing concentrations of the NO donor GTN caused a significant degradation of major mitochondrial phospholipid cardiolipin (measured by staining with fluorescent dye NAO) followed by apoptosis in about 40% of cells [Ushmorov et al., 1999]. After 24 h of GTN treatment, live (PI-negative) APO-S cells were found to form two subpopulations: one with lower cardiolipin content (NAO^{low}) and another with the higher concentration of cardiolipin (NAO^{high}; Fig. 1A). NAO^{low} and NAO^{high} cells were found to represent around 20% and 40% of total APO-S cells, respectively. Incubation of APO-S cells with another NO donor NOC-18 for 24 h also caused the formation of NAO^{low} and NAO^{high} subpopulations (data not shown). To study the significance of cardiolipin for the resistance of leukemia cells against NO, we sorted these two subpopulations using FACS Vantage sorter. Then we put them in culture medium and measured the number of apoptotic cells at the different time points after the onset of the culture testing the binding of FITC-conjugated annexin V to these cells. Annexin V preferentially binds to phosphatidylserine, a phospholipid component of the inner leaflet of the plasma membrane that is rapidly externalized during apoptosis [Koopman et al., 1994]. As shown in Figure 1B, NAO^{low} cells tested at day 2 after sorting were already dead (98% annexin V^+PI^+ cells), whereas NAO^{high} cells cultured in the same conditions showed at this time point the similar amount of annexin V^+PI^- early apoptotic cells and annexin V^+PI^+ dead cells as in unsorted APO-S population. This level of early apoptotic cells in NAO^{high} subpopulation (around 2%) was remained constant during more than 6 months in culture. Moreover, NAO^{high} cells did not differ from parental cells in growth kinetics.

Next we tested the sensitivity of these sorted NAO^{high} Jurkat cells to NO-mediated apoptosis. Preliminary experiments revealed that the sorting procedure by itself does not affect the capacity of NO to induce apoptosis since unsorted APO-S cells showed the same level of NO-mediated apoptosis as in intact APO-S cells after FACS sorter (data not shown). Figure 2 shows the proportion of annexin V^+PI^- early apoptotic and annexin V^+PI^+ dead cells in both parental unsorted and NAO^{high} cell population after treatment with 0.2 mM GTN for 24 h. There is a significant decrease in the numbers of both annexin V^+PI^- and annexin V^+PI^+ NAO^{high} cells as compared to the respective values in unsorted cells. For evaluation of apoptosis in these cells, we used also the staining with APO2.7 mAb. It has been recently described that APO2.7 binds to a 38-kDa mitochondrial membrane protein in apoptotic but not in normal cells [Koester et al., 1997]. We found that after incubation with GTN for 24 h, the number of apoptotic cells in NAO^{high} population was substantially lower than in unsorted cells (34 ± 4% and 71 ± 8%, respectively; $P < 0.01$). Application of another NO donor NOC-18 (0.5 mM) for 24 h also revealed the increased resistance of NAO^{high} sorted cells to NO-mediated apoptosis as compared to parental cells (38 ± 5% and 78 ± 9%, respectively; $P < 0.01$). Furthermore, after more than 6 months in culture, NAO^{high} cells maintained the similar level of resistance to NO-induced apoptosis as compared to freshly sorted cells (data not shown).

Next we addressed the question whether the sorted NAO^{high} cells were able to increase their resistance to NO after more rounds of selection. Treatment of NAO^{high} cells with NO donor GTN for 24 h also resulted in the formation of subpopulations with higher and lower cardiolipin content. However, NAO^{high} cells showed after the second sorting the same level of resistance to NO-mediated apoptosis as NAO^{high} cells isolated after the first selection (data not shown).

Cardiolipin and Bcl-2 are not Responsible for the Resistance of NAO^{high} Cells to NO-Induced Apoptosis

Since Jurkat cells sorted for high cardiolipin concentration became more resistent to NO-mediated apoptosis, it was logic to suggest an important role for cardiolipin in this phenomenon. We therefore compared the basic content of cardiolipin in unsorted APO-S and NAO^{high} cells (2 days after sorting) using staining with NAO (FL1 fluorescence) and found that the level of cardiolipin was similar in both cell population tested (mean FL1 fluorescence 922 ± 38 in APO-S cells and 887 ± 29 in NAO^{high}

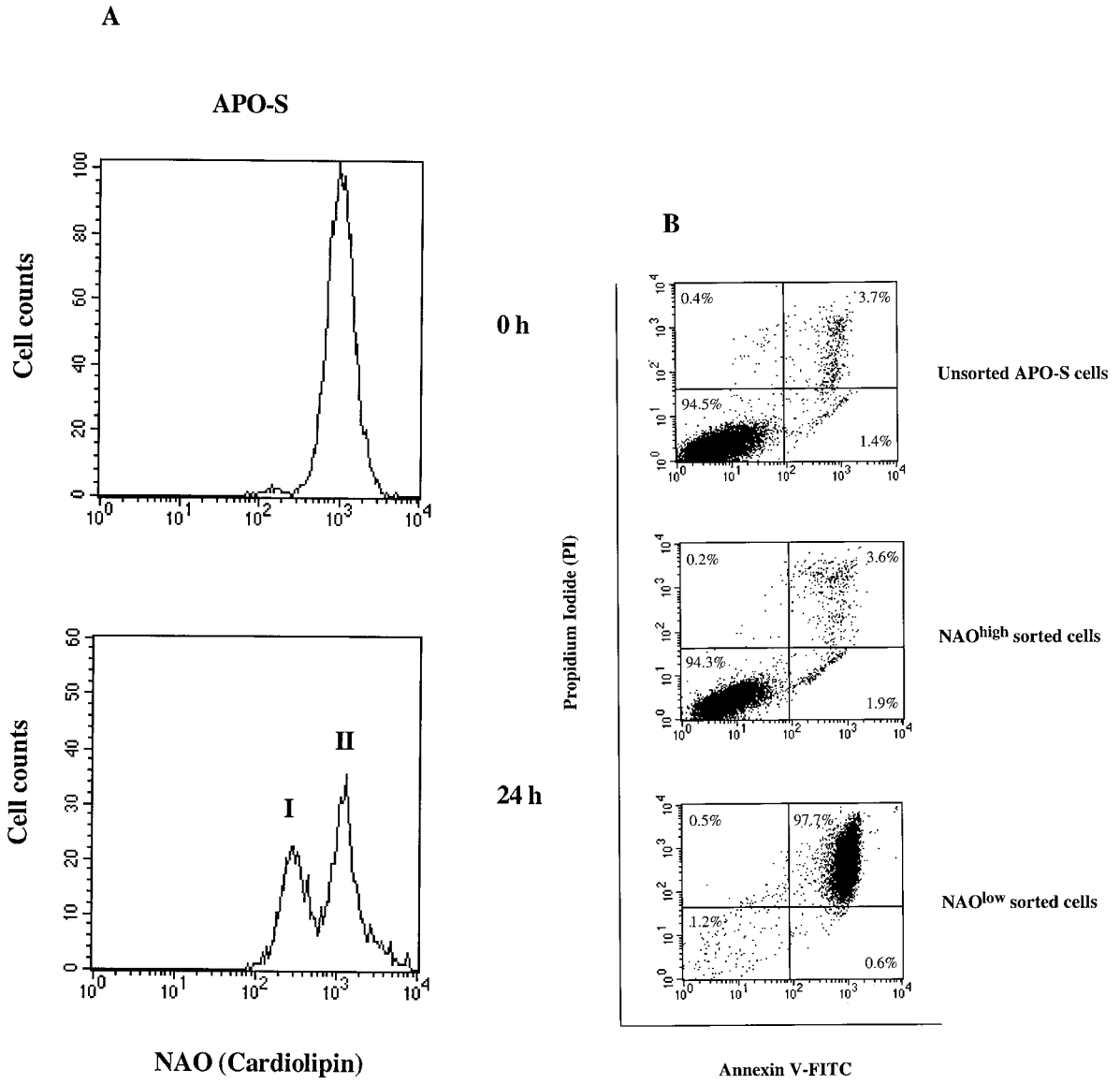

Fig. 1. NO-mediated changes in cardiolipin content in APO-S Jurkat cells (A) and viability of APO-S cells sorted for higher and lower cardiolipin concentration (B). **A**: APO-S Jurkat cells were treated with 0.2 mM NO donor GTN for 24 h. The content of cardiolipin was analysed after incubation with 100 nM NAO for 30 min at 37°C. Recordings were made only on PI negative (viable) cells at green fluorescence (FL1) using FACScan and CELLQuest software. I: Cell population with lower cardiolipin content (NAO^{low}) and II: Cells with higher cardiolipin concentration (NAO^{high}). **B**: APO-S Jurkat cells were incubated with 0.2 mM GTN for 24 h followed by staining with 25 nM NAO for 10 min at 37°C and sorting using a FACS Vantage sorter. Windows for sorting were defined in the cell populations with higher or lower NAO expression (NAO^{high} and NAO^{low}, respectively) excluding PI positive (dead) cells. Sorted cells were collected and incubated in RPMI medium at the same conditions as parental APO-S cells. After two days in culture, the cells were stained with annexin V and PI to distinguish annexin V^+PI^- early apoptotic and annexin V^+PI^+ dead cells. Recordings were made at green fluorescence (FL1) for annexin V and at red fluorescence (FL3) for PI using a FACScan with CELLQuest software. A representative experiment of four is shown.

cells; $P > 0.05$). During the whole period of cell culture and after the second round of cell sorting, the concentration of cardiolipin in NAO^{high} cells did not differ from this value in APO-S parental cells (data not shown).

Therefore, the content of cardiolipin in NAO^{high} cells cultured for 2 days returns back to the level observed in unsorted APO-S cells and remains unchanged during further cell culture.

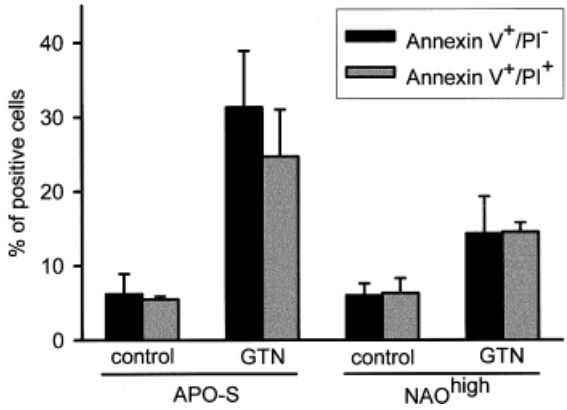

Fig. 2. Effect of NO on apoptosis in NAO^{high} sorted and parental APO-S Jurkat cells. Cells were incubated with 0.2 mM GTN for 24 h. Apoptosis was assessed by staining with annexin V and PI followed by flow cytometric analysis. Each bar represents the mean ± SD of three independent experiments.

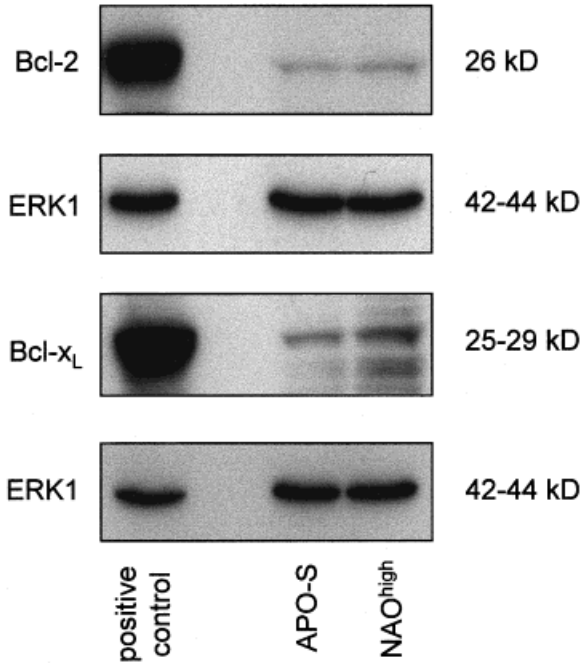

Fig. 3. The expression of Bcl-2 and Bcl-x_L proteins in NAO^{high} sorted and APO-S parental cells. Cells were treated with a lysis buffer and extracted total protein was subjected to 12.5% SDS-polyacrylamide gel electrophoresis followed by Western blot analysis. The membranes with transfered proteins were incubated with Abs against Bcl-2 and Bcl-x_L proteins. Then the blots were treated with anti-rabbit Abs and goat anti-mouse Abs, respectively, and visualized by ECL method. The same blots were washed and incubated with Abs against the ERK-1 protein to show that equal amounts of protein were loaded. As positive controls, bcl-2 transfected Jurkat cells were used for Bcl-2 blots and bcl-x_L transfected MCF-7 cells for Bcl-x_L blots. A representative experiment out of three is shown.

It was demonstrated that Bcl-2 and Bcl-x_L overexpression could block apoptosis induced by a number of stimuli including NO, and that this anti-apoptotic effect involved the normalization of mitochondrial functions [Zamzami et al., 1998]. We investigated the expression of Bcl-2 and Bcl-x_L proteins by Western blot analysis and found that the expression of these two anti-apoptotic proteins was equally low both in Jurkat parental and sorted NAO^{high} cells (Fig. 3).

Modulation of the Intracellular Glutathione Concentration Affects the Sensitivity of Jurkat Cells to NO-Mediated Apoptosis

It was reported that glutathione inhibited apoptosis induced by different agents [Singh et al., 1998; Mayer et al., 1994]. As shown in Table 1, the level of total glutatione in NAO^{high} cells is significantly higher than in APO-S cells (48 ± 17 and 30 ± 5 nmol/mg protein; $P < 0.05$). When measuring glutathione concentration at the single cell level using mBB and flow cytometry, we found that, after 24 h of GTN treatment, the content of glutathione in live NAO^{high} cells was substantially higher than in respective population of parental APO-S cells (FL4 fluorescence 389 ± 19 and 350 ± 4, respectively; $P < 0.05$).

To investigate whether accumulation of intracellular glutathione is responsible for the resistance of leukemia cells to NO-mediated apoptosis, we used BSO to deplete the intracellular glutathione. Preliminary experiments showed that overnight treatment of Jurkat cells with 30 μM BSO (that was not toxic for these cells) caused a dramatic reduction of intracellular glutathione level (to 2 nmol/mg protein or less). It was demonstrated that the preincubation of NAO^{high} cells with 30 μM BSO for 18 h followed by treatment with GTN for next 24 h stimulated NO-mediated apoptosis (from 14 ± 3% to 25 ± 3% of annexin V^+ PI^- early apoptotic cells; $P < 0.05$; Fig. 4A). The quantity of annexin V^+PI^+ cells in the same culture was only moderately increased (from 9 ± 1% to 11 ± 1%; $P > 0.05$). Parental APO-S cells showed much more intensive stimulation of NO-induced cell death under BSO pretreatment (an increase from 21 ± 3% to 72 ± 11% annexin V^+PI^+ cells; $P < 0.05$; Fig. 4A). A decrease in annexin V^+PI^- cell number (from 31 ± 11% to 11 ± 3%; $P < 0.05$; Fig. 4A) can be explained by accelerated transition from early

TABLE I. Glutathione Content in APO-S and NAO^{high} Cells[a]

Cell	Total glutathione	GSH	GSSG	GSH/GSSG
APO-S	28.6 ± 2.8	28.3 ± 2.7	0.4 ± 0.1	80.1 ± 29.9
NAO^{high}	53.2 ± 28.1*	52.8 ± 27.9*	0.4 ± 0.1	127.5 ± 24.7

[a]The content of glutathione in NAO^{high} sorted and in APO-S parental cells. 1×10^7 cells were treated with sulfosalicyclic acid to precipitate proteins. The concentration of total, reduced (GSH), and oxidized glutathione (GSSG) was measured in supernatants. Data are expressed in nmol of glutathione per mg protein and represent the mean ± SD of three independent experiments.
*Significantly different from concentration in APO-S cells ($P < 0.05$).

apoptotic phase (annexin V^+PI^- cells) to the late apoptotic/secondary necrotic phase (annexin V^+PI^+ cells) [Koester et al., 1997]. Thus, glutathione depletion with BSO caused a significant stimulation of NO-mediated apoptosis both in NAO^{high} and APO-S cells.

Next we tested whether the incubation with the glutathione donor NAC could downregulate NO-mediated apoptosis in Jurkat cells. Addition of 100 μM NAC twice, 24 h and 4 h before the beginning of GTN treatment, to APO-S unsorted cells resulted in the elevation of the glutathione concentration as compared to untreated cells (from 31 ± 1 to 39 ± 4 nmol/mg protein; $P < 0.05$). Interestingly, this increased level of glutathione was maintained in APO-S cells during the whole period of incubation with NO donor GTN (data not shown). As shown in Figure 4B, NAC pretreatment caused a significant decrease in the numbers of annexin V^+PI^- and annexin V^+PI^+ cells induced by NO (from 31 ± 8% to 21 ± 3% and from 25 ± 6% to 10 ± 2% of cells respectively; $P < 0.05$). To investigate whether NAC provide its anti-apoptotic effect through its own antioxidant activity or via induction of glutathione synthesis, we incubated APO-S cells with GTN and NAC together with BSO to deplete produced glutathione (Fig. 4B). We observed a similar stimulation of apoptosis as in the cells exposed only to GTN and BSO suggesting thereby that the anti-apoptotic effect of NAC occured through glutathione production.

Effect of NO on Glutathione Synthesis in NAO^{high} and APO-S Cells

Since glutathione seems to play an important role in the protection against NO-mediated apoptosis, we studied the expression of mRNA of main enzymes involved in the glutathione synthesis and degradation as well as the ability of NO to regulate their mRNA expression. PCR analysis showed no clear difference in the mRNA expression of glutathione peroxidase, glutathione S-transferase, γ-glutamylcysteine synthetase, and glutathione reductase in unsorted APO-S and sorted NAO^{high} Jurkat cells (data not shown). Furthermore, the incubation of both cell lines with GTN caused no apparent modulation of the expression of these genes at the mRNA level (data not shown).

To check whether higher glutathione level and the acquisition of resistance to NO-induced apoptosis in NAO^{high} Jurkat cells requires protein synthesis de novo, we incubated these cells with protein synthesis inhibitor cycloheximide (CHX) in combination with GTN. We observed no increase in the number of annexin V^+PI^- apoptotic cells in this culture as compared to the cells treated with GTN only (18% and 17% cells, respectively; $P > 0.05$).

Therefore, the synthesis of proteins and, in particular, of enzymes regulated glutathione metabolism is not required for the resistance of sorted Jurkat cells to NO-dependent apoptosis.

DISCUSSION

It was reported that NO induced apoptosis in different cell types [Fehsel et al., 1995; Geng et al., 1996; Hortelano et al., 1997; Umansky et al., 1997]. In our recent publication, we described the NO-mediated decrease in the concentration of cardiolipin (measured by staining with fluorescent dye NAO) in Jurkat cells undergoing apoptosis [Ushmorov et al., 1999]. It is known that a decrease in NAO fluorescence ultimatively indicates the cardiolipin degradation and mitochondrial damage [Macho et al., 1995]. This major mitochondrial lipid plays a crucial role in the attachment of cytochrome *c* to the inner mitochondrial membrane [Choi et al., 1995].

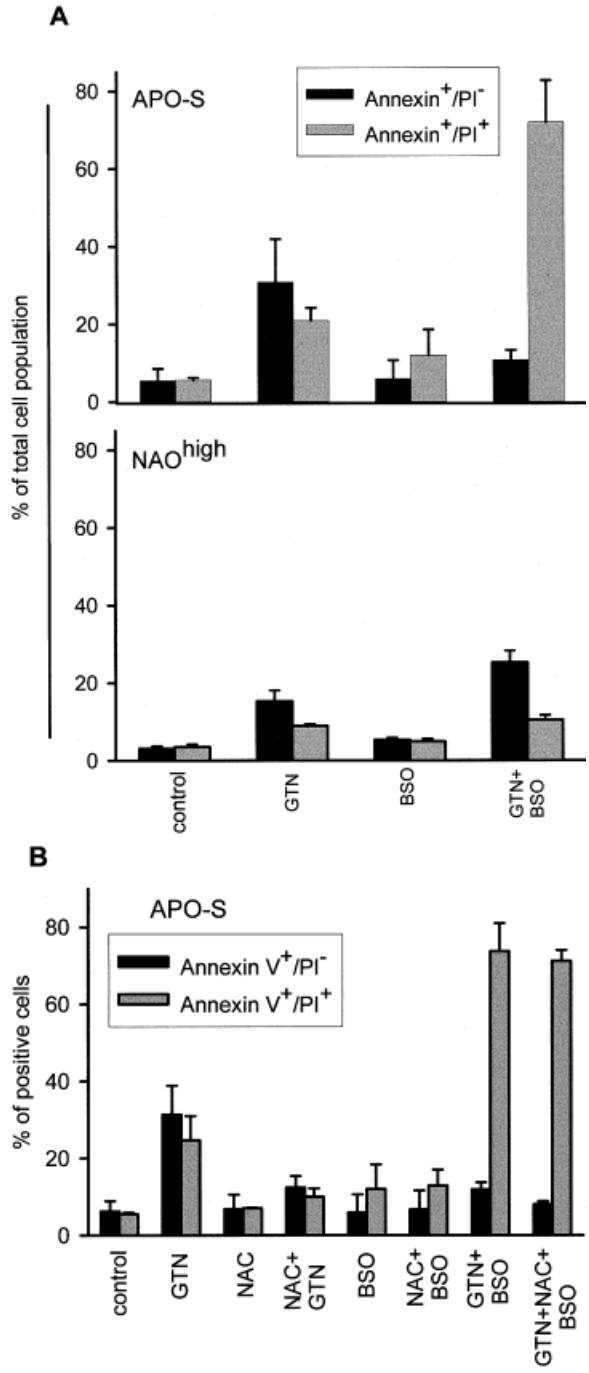

Fig. 4. Effect of BSO (A) and NAC (B) on NO-mediated apoptosis in NAO^{high} and APO-S unsorted Jurkat cells. **A**: APO-S and NAO^{high} cells were pretreated with 30 μM BSO for 18 h followed by incubation with 0.2 mM GTN for 24 h. **B**: 100 μM NAC was added to APO-S cell culture twice, 24 h and 4 h before the onset of the treatment with 0.2 mM GTN for the next 24 h. Thirty μM BSO was added once together with NAC (24 h before GTN). Apoptosis was assessed using the staining with annexin V and PI followed by flow cytometric analysis. Each bar represents the mean ± SD of three independent experiments.

Here we studied the mechanisms of human leukemia cell resistance to NO-mediated apoptosis. From the population of the cells which survived after exposure to NO donors for 24 h, we sorted the cells with higher and lower cardiolipin concentration (NAO^{high} and NAO^{low}, respectively). We demonstrated that the NAO^{high} population contained 95% live cells whereas the NAO^{low} cells were dead cells. NAO^{high} cells were substantially more resistant to NO-induced apoptosis than unsorted APO-S cells and maintained this level of resistance after more than 6 months in culture. Interestingly, NAO^{high} cells does not significantly differ from parental cells in the sensitivity to other apoptotic agents like CD95 ligand and Newcastle disease virus (unpublished observations).

It has been previously reported that the cell resistance to some apoptotic stimuli is associated with an elevated cardiolipin concentration [Lieser et al., 1998]. However, in NAO^{high} cells, the increased cardiolipin content quickly returned back to the level observed in parental APO-S cells and remained unchanged during the whole period of culture. Furthermore, the second round of selection of NAO^{high} cells caused no increase in the resistance of these cells to NO-mediated apoptosis. We thus suggest that the stimulation of cardiolipin synthesis is associated with Jurkat cell survival after NO treatment but does not play a crucial role in this process.

Bcl-2 and Bcl-x_L proteins have been shown to suppress NO-induced apoptosis via mitochondrial effects [Zamzami et al., 1998; Ushmorov et al., 1999]. In this study, we observed a similar low level of Bcl-2 and Bcl-x_L expression in NAO^{high} and unsorted cells (Fig. 3). It is known that the activation of the nuclear factor kappa B (NF-κB) results in the protection from apoptosis [Wang et al., 1996]. However, we found no NF-κB activation under treatment of NAO^{high} cells with NO (unpublished observation). This suggests that Bcl-2, Bcl-x_L, and NF-κB are not responsible for the increased resistance of NAO^{high} cells to NO.

Antioxidants have been reported to protect against the cytotoxicity induced by many different agents [Slater et al., 1995]. Glutathione as a physiological antioxidant plays an important role in cellular defenses against oxidative stress [Meister et al., 1994; Rahman et al., 1999]. Moreover, a depletion of intracellular glutathione has been demonstrated to occur in

several different apoptotic systems [Van den Dobbelsteen et al., 1996; Ghibelli et al., 1998] and has been considered as an early apoptotic event [Macho et al., 1997]. In addition, exposure of the cells to NO resulted in a decrease in the content of the intracellular glutathione [Clementi et al., 1998; Bolanos et al., 1996]. In contrast, increased glutathione concentration could downregulate NO-induced apoptotic cell death [Zhao et al., 1997]. The intracellular glutathione content was found to be significantly higher in NAO^{high} cells resistant to NO as compared to APO-S NO-sensitive cells (Table 1). In addition, the depletion of glutathione in NAO^{high} cells with BSO resulted in an increased NO-mediated apoptosis suggesting thereby that the low sensitivity to NO can be, at least partially, explained by higher glutathione concentration in these cells. Our data are in agreement with the paper reporting that the inhibitor of glutathione synthesis BSO stimulated cell susceptibility to different apoptotic inducers [Luperchio et al., 1996]. To increase the concentration of intracellular glutathione, we used NAC. This glutathione precursor could decrease the level of NO-induced apoptosis in unsorted APO-S cells, an observation that confirms recently published results [Zamora et al., 1997]. The effect of NAC was due to the glutathione production, since BSO added together with NAC could completely reverse the observed inhibition of apoptosis (Fig. 4A).

Taken together, our results demonstrate that Jurkat cells sorted for high cardiolipin content acquire increased resistance to NO treatment. These NAO^{high} cells have an elevated glutathione content as compared to unsorted cells and show a significant stimulation of NO-induced apoptosis, when the inhibitor of glutathione synthesis BSO is added. Conversely, the glutathione precursor NAC substantially blocks NO-induced apoptosis in unsorted Jurkat cells. These data suggest an important role of glutathione in the protection against NO-induced apoptosis in human leukemia cells.

ACKNOWLEDGMENTS

We thank Prof. V. Schirrmacher for support and critical reading of the manuscript.

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