Vol. 216, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
November 2, 1995 Pages 313-320

NON-OXIDATIVE LOSS OF GLUTATHIONE IN APOPTOSIS VIA GSH EXTRUSION

L. Ghibelli, S. Coppola, G. Rotilio^, E. Lafavia, V. Maresca, and M.R. Ciriolo*

Dipartimento di Biologia, Università di Roma Tor Vergata
via della Ricerca Scientifica, 00133 Roma -ITALY

*Istituto di Scienze Biochimiche, Università di Chieti G. D'Annunzio
via dei Vestini, 66013 Chieti -ITALY

Received September 22, 1995

Summary: Reduced glutathione (GSH) has been hypothesized to play a role in the rescue of cells from apoptosis, by buffering an endogenously induced oxidative stress. We correlated GSH levels and apoptosis in U937 human monocytic cells induced to apoptosis by different agents. All treatments led to depletion of GSH concomitant with the onset of apoptosis. The loss was due to extrusion of GSH outside the cell, while GSSG was not accumulated in the apoptosing cells, nor was it found in the extracellular medium. Modulation of intracellular GSH level did not influence the overall extent of apoptosis. We conclude that glutathione loss in apoptosis is not necessarily preceeded by an oxidative stress, and that GSH depletion alone is not sufficient to lead cells to apoptosis.

Cell death under physiological conditions usually occurs by apoptosis. This mode of cell death allows the cell to control its own demise. The process of "self" regulated death involves a series of biochemical and morphological changes including DNA digestion, cell shrinkage, chromatin condensation, nuclear and cell fragmentation into membrane-bound "apoptotic" bodies [1-4]. Many treatments that are capable of inducing apoptosis are also known to imply an oxidative stress. For example exposure to low doses of H_2O_2 induces apoptosis in a variety of cell types [5-8]; apoptogenic agents such as Tumor Necrosis Factor [9], cycloheximide [10], natural killer cells [11] are known to elicit oxidative stress. Moreover, the product of the oncogene *bcl-2*, a protein which is able to block the onset of apoptosis upon many stimuli [12] has been shown to act through a radical-scavenging mechanism [13]. All this evidence led to hypothesize that oxidative stress might be a universal trigger for apoptosis, also where the inducing stimuli are apparently unrelated to redox modulation.

^To whom correspondence should be addressed (FAX 39 6 72594311).

0006-291X/95 $12.00

Glutathione is the most abundant bulk antioxidant in the cell, where it is found in two redox forms, reduced (GSH) and oxidized (GSSG). Its protective action is based on the maintenance, by a variety of mechanism, of high GSH/GSSG ratio [14]. Upon oxidative stress GSSG may recycle to GSH through recruiment of the cell reducing power, or can exit from the cells leading to overall glutathione depletion [15]. GSH has been shown to prevent apoptosis and maintain viability in cells lacking bcl-2 [16], and to decrease upon induction of cells to apoptosis [17], adding support to a causative role of oxidative stress in apoptosis.

In this work we show that apoptosis is associated to glutathione depletion independently of the inducing stimulus, and that the depletion, which occurs by extrusion of reduced glutathione, is not preceeded by an oxidative stress.

Materials and Methods

Cell culture: U937 cells have been cultured in RPMI 1640 medium supplemented with 10% FCS, 2mM L-Glutamine, 100 IU/ml penicillin and streptomycin, and kept in a controlled atmosphere (5% CO_2) incubator at 37°C. Cell viability has been assessed by trypan blue exclusion.

Analysis and quantification of apoptosis - Apoptosis has been characterized by DNA fragmentation to give a ladder-like pattern, and nuclear fragmentation in several smaller fragments, ranging in number from 2 up to >20 per cell, detectable by optical microscopy on slides of hematoxylin-stained cells. Preparation and staining of slides: $2x10^5$ cells, fixed in 4% paraformaldeide, are loaded on a gelatinized slide, stained with hematoxylin, and analyzed for direct optical microscopy. Quantification of apoptosis: the fraction of cells with a fragmented nucleus among the total cell population, is calculated on the hematoxylin-stained slides, counting at least 300 cells in at least 10 random selected fields as described in [6].

Apoptogenic treatments: Oxidative stress: U937 cells have been treated with freshly prepared hydrogen peroxide, at the concentration of 1mM (unless otherwise specified) for 1 hour; the stress has been stopped by the addition of catalase, that has been immediately removed by changing medium; the cells have then been incubated for recovery. Protein synthesis inhibitors: 10 or 100ug/ml Cycloheximide (CHX); 1 or 10 µg/ml Puromycin (PMC). Topoisomerase II inhibitor: 100µg/ml etoposide (VP16).

Glutathione determination
Cells were harvested by centrifugation, washed with PBS (20 mM sodium phosphate, 140 mM NaCl pH 7.4) and resuspended in the same buffer. Cells were then lysated by repeated cycles of freezing and thawing. Proteins were precipitated by adding sodium metaphosphoric acid to a final concentration of 5% (w/v). The clear supernatant obtained after centrifugation at 22,000 g for 15 min was utilized for GSH and GSSG determination by HPLC according to Reed et al. [18]. Mixed disulfides were assayed by HPLC according to Brigelius et al. [19]. In each case results are expressed as nmol of GSH per mg of protein in the original cell extract. GSH and GSSG of the cell culture medium were determined as described above after acidification and concentration of the media. Results were expressed as nmol of GSH per ml.

GSH modulation
Cells have been cultured in 20mg/ml N-Acetyl-Cysteine (NAC) or 100µg/ml Cystamine for 48 hrs. For GSH depletion, cells were cultured for 24 hrs in Buthionine Sulfoximine (BSO).

LDH determination
The activity of lactic dehydrogenase was determined at 25°C as the change in absorbance at 340 nm, using as substrate 0.18 mM NADH and 0.72 mM pyruvate in 50 mM phosphate buffer, pH 7.4.

Results

Induction to apoptosis of U937 cells

In this study, apoptosis has been induced on U937 cells by several, well known apoptogenic agents, which act through different mechanisms: direct oxidative stress with hydrogen peroxide [6]; protein synthesis inhibition with compounds (cycloheximide, CHX, and puromycin, PMC) that block the synthetic machinery at two different steps [20]; and topoisomerase II inhibition by etoposide (VP16) [21]. Fig. 1 shows the morphology (a) and DNA pattern (b) of apoptotic U937; in (c) the time course of the induction to apoptosis is shown. Among these apoptogenic agents, PMC at 10μg/ml and VP16 displayed a very fast apoptogenic action, so that the apoptotic cells have no time to undergo secondary necrosis (leakage of the plasma membrane of old apoptotic cells). In fact, they remain totally unpermeable to Trypan blue until the end of the treatment, and the extent of LDH release, compared to completely lysed cells, is only twice that of untreated cells (23% for PMC and 19% for VP16, vs. 10% of untreated cells).

Apoptosis is not affected by GSH modulation

In order to establish a possible cause-effect relationship between GSH and apoptosis, U937 cells were pre-treated with agents that modulate GSH intracellular content: 48hrs pre-treatment with N-acetyl-cystein (NAC) or cystamine (Cys) increased GSH content from 31.18±4.8 to 41.78±3.86 and 49.05±4.07 nmol/mg prot. respectively, whereas pretreatment with buthionine sulfoximine (BSO) led to nearly full GSH depletion within 24hrs (1.11±0.11 nmol/mg prot.). Cells depleted of GSH were viable and able to undergo at least two rounds of duplication, showing that the absence of GSH *per se* does not lead cells to apoptosis. Moreover, GSH modulation did not affect the extent (fig. 2a) nor the kinetics (fig. 2b) of apoptosis, besides a slight effect observed for treatments which imply an oxidative stress (H_2O_2 and CHX).

GSH depletion occurs concomitantly with the onset of apoptosis

All apoptogenic treatments led to a depletion of GSH in the apoptotic cells. Fig 3a shows the extent of apoptosis compared with the residual concentration of GSH measured at the end of each apoptogenic treatment.

The time course of GSH decrease was compared with the accumulation of apoptotic cells upon treatment with PMC and VP16. Fig. 3b shows that a temporal relationship between GSH loss and onset of apoptosis could not be determined, since the two phenomena were not separable.

The treatments that led to values close to 100% of apoptotic cells caused a total GSH depletion, which indicates that each single apoptotic cells has lost its GSH content. Treatments that caused lower extent of apoptosis, or samples taken at earlier time points, left some residual GSH in the cell population. Normalization of GSH

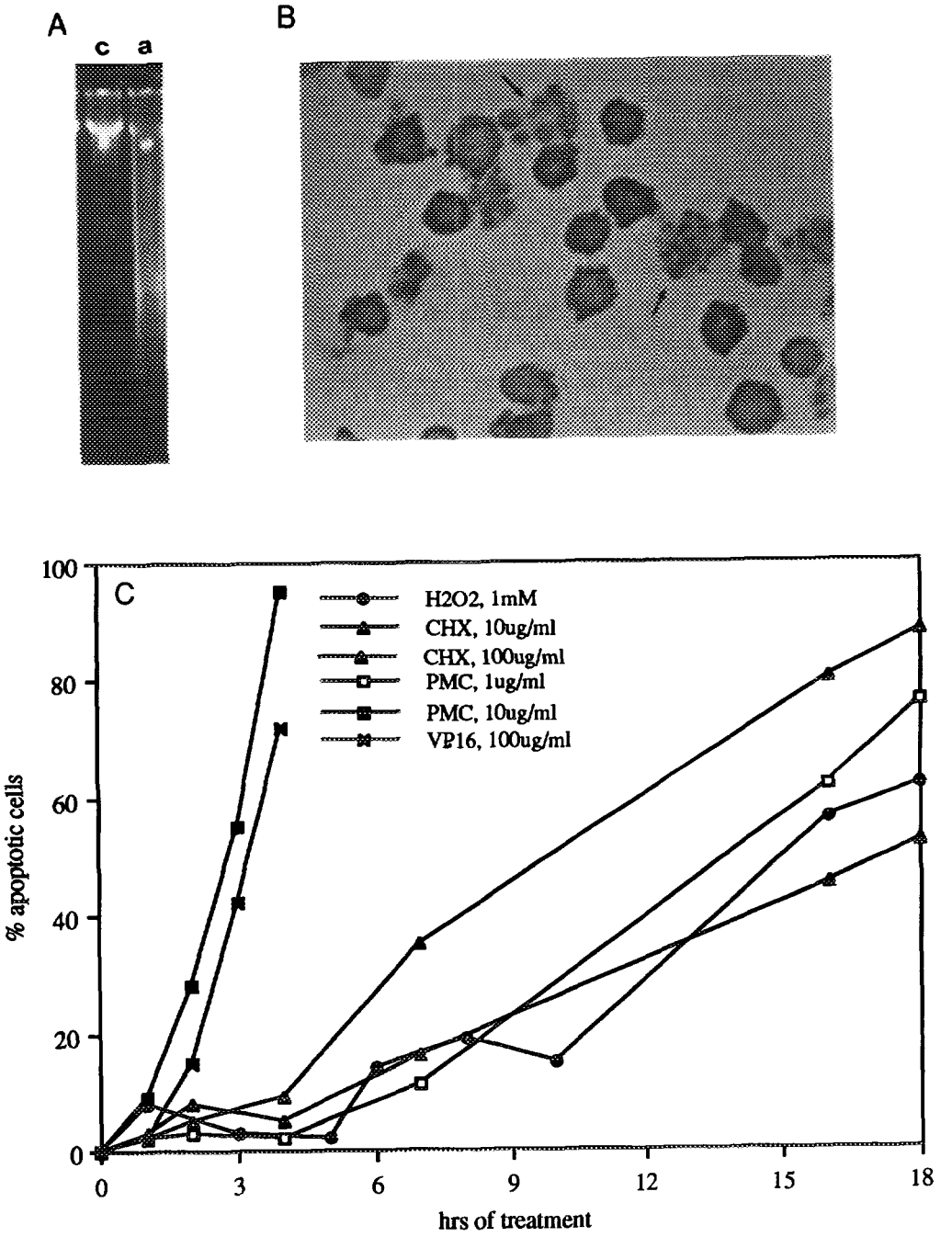

Fig. 1. Apoptosis on U937 cells
B: morphology of apoptotic U937, indicated by the arrows; A: DNA electrophoretic analysis (a=apoptotic cells; c=control cells); C: time course of induction, quantified by morphological criteria as described in [6]: background values of apoptosis (1 to 4%) have been subtracted. One experiment among >10 is shown for each treatment.

values for the viable residual cells upon each treatment, led to values close to control cells, which suggests that the apoptotic process caused GSH depletion, while the treatment itself did not. However, treatments that either directly (H_2O_2) or indirectly

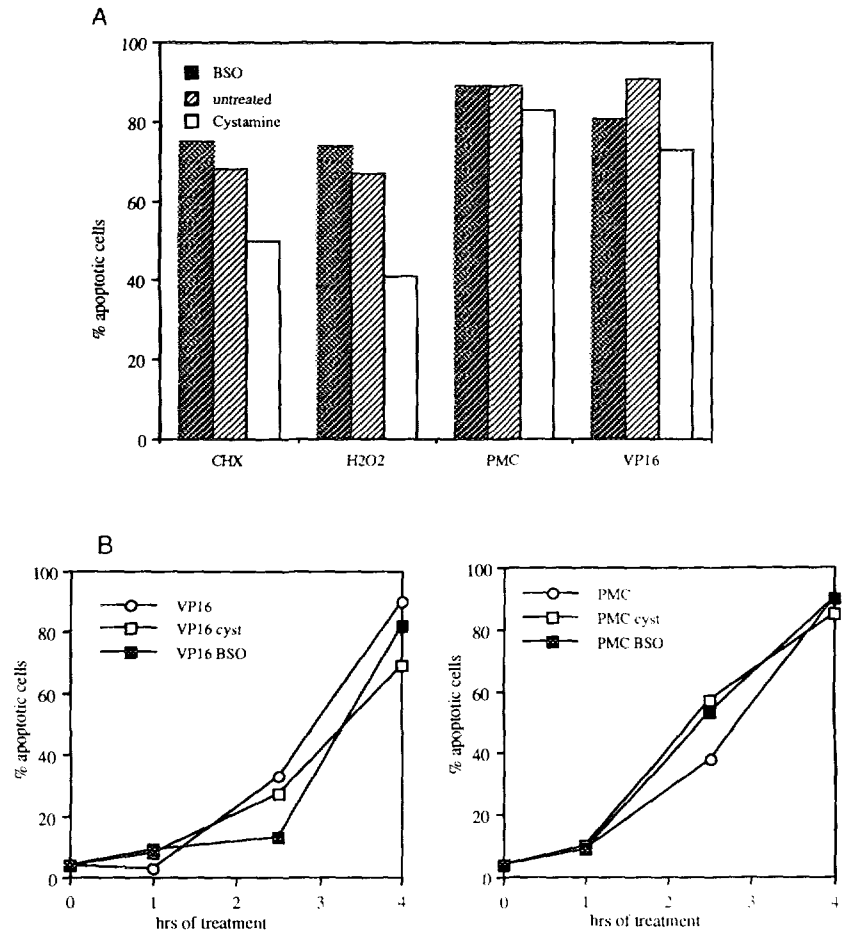

Fig. 2. Effect of GSH modulation on apoptosis
U937 have been pre-incubated with BSO or Cys as described under materials and methods; one of three to four experiments is shown
A: extent of apoptosis at the end of each treatment (CHX, H_2O_2: 18hrs; PMC, VP16: 4hrs)
B: kinetics of apoptosis upon PMC and VP16 treatment.

(CHX) implicate an oxidative stress, led to a lower GSH content in the surviving cells (Table I).

Glutathione is extruded as GSH outside the apoptotic cells

The fate of GSH was investigated in PMC and VP16-treated cells. Tab. II shows that the loss of intracellular GSH was paralleled by an increase of GSH in the culture medium. GSSG was not accumulated in the apoptotic cells, nor was it found in the extracellular medium. Since the culture medium of both control and apoptotic cells did not reduce added GSSG within 5 hours of incubation, it is concluded that glutathione is extruded in its reduced form during the apoptotic process. Mixed disulfides were formed in cells induced to apoptosis by PMC, but they were undetectable in VP16-treated cells (Table II).

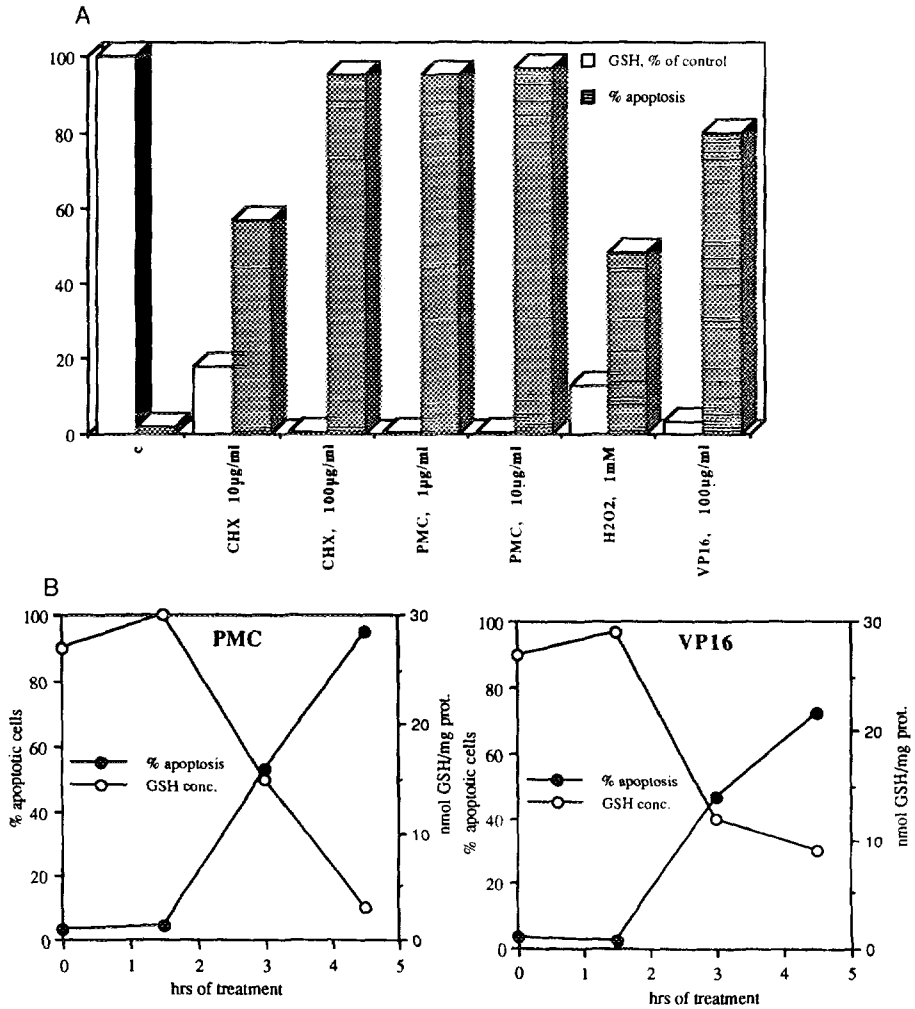

Fig. 3. Effect of apoptosis inducers on intracellular GSH content
A: comparison between the extent of apoptosis (% apoptotic cells measured as in Fig. 1) and residual GSH, indicated as % of untreated cells, measured at the end of each apoptogenic treatment. Average GSH value was 23.70±2.79 .
B: time course of GSH level and extent of apoptosis.

Table I. Normalization of GSH content according to the fraction of residual viable cells

	untreated	CHX (18h)	H_2O_2 (18h)	PMC (3h)	PMC (4h)	PMC (4.5h)	VP16 (3h)	VP16 (4.5h)
% viable cells	97	41	50	48	12	4	53	28
GSH, % of control/viable cells	100	43	28	110	95	-	85	110

The value of GSH in the treated cell populations has been normalized for the residual viable (=non-apoptotic) cells in the culture, at the indicated times and for the indicated treatments.

Table II. Glutathione status of U937 cells treated with PMC or VP16

	Cells			Extracellular Medium	
	GSH	GSSG	Mixed disulfides	GSH	GSSG
	nmol/mg prot.	nmol/mg prot.	nmol/mg prot.	nmol/ml	nmol/ml
Untreated (n>10)	23.70±2.79	0.99±0.11	n.d.	1.18±0.13	n.d.
PMC, 10ug/ml (n>10)	2.53±0.79	n.d.	3.51±1.05	5.12±0.56	n.d.
VP16, 100ug/ml (n=6)	9.22±1.84	0.50±0.06	n.d.	2.36±0.20	n.d.

Intracellular GSH, GSSG, and mixed disulfides have been measured after 4hrs treatment with PMC and VP16, as described under materials and methods (n.d.=not detectable).

Discussion

The results reported above show that there are, as far as GSH loss is concerned, differences between various types of apoptogenic treatments. GSSG is formed only upon oxidative stress (e.g., H_2O_2 treatment). Moreover, the intracellular GSH level affects the extent of apoptosis only in the case of oxidative stress (Fig. 2). However, it was found that GSH was lost by the apoptosing cells independently of the inducing stimulus, and that this depletion was due to the apoptotic process itself. Glutathione loss in PMC and VP16 treatment was not a consequence of an oxidative stress, since no GSSG was accumulated in the apoptotic cells or in the extracellular medium. GSH was found outside the cells: since no significant LDH leakage is associated to complete GSH loss, this suggests that a specific extrusion has taken place.

Cells deprived of GSH may be more prone to undergo an oxidative stress even under normal conditions, since their ability to scavenge or detoxify the various reactive oxygen intermediates which form in the normal cell metabolism is impaired. Even though we cannot exclude that GSH loss in apoptosis is only circumstantial, these considerations suggest that this phenomenon may have a physiological significance, since GSH loss could favour the onset of apoptosis by passively allowing an oxidative stress to take place. While glutathione loss may be necessary for apoptosis, it is not suffucient [this work and 22], since its direct deprivation with BSO does not affect cell viability for a long time.

Acknowledgments This work was supported in part by Consiglio Nazionale delle Ricerche, grant CT 04. V.M. is a recipient of a fellowship from "Scuola di Specializzazione in Biotecnologie" by Montedison, whose grant has partially supported the cost of this research.

References

[1] Wyllie,A.H., Ker,J.F.R., and Currie, A.R. (1980) Int. Rev. Cytol. 68, 251-273

[2] Kerr,J.F.R., and Harmon,B.V. (1991) in Apoptosis: The Molecular Basis of Cell Death (Tomei,L.D., and Cope,F.O., Eds.), pp. 5-29, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

[3] Wyllie,A.H. (1987) J. Pathol. 153, 313-316
[4] Buttke,T.M., Sandstrom,P.A. (1994) Immunol. Today 15, 7-10
[5] Lennon,S.V., Martin,S.J., Cotter,T.G. (1991) Cell Prolif. 24, 203-214
[6] Nosseri,C., Coppola,S., Ghibelli,L. (1994) Exp. Cell Res. 212, 367-373
[7] Coppola,S., Nosseri,C., Maresca,V., Ghibelli,L. Exp. Cell Res., in press
[8] Ghibelli,L., Nosseri,C., Coppola,S., Maresca,V., Dini,L. Exp. Cell Res., in press
[9] Feng,L., Xia,Y., Garcia,G.E., Hwang,D., Wilson,C.B. (1995) J. Clin. Inv. 95, 1669-1675
[10] Zuckerman,S.H., Evans,G.F., Guturie,L. (1991) Immunol. 73, 460-465
[11] Malorni,W., D'Ambrosio,A., Rainaldi,G., Rivabene,R., Viola,M. Immunol. Lett. (1994) 43, 209-214
[12] Korsmeyer,S.J. (1992) Immunol. Today 14, 131-136
[13] Hockenbery,D.M., Oltvai,Z.N., Yin,X.N., Milliman,C.L., Korsmeyer,S.J. (1993) Cell 75, 241-251
[14] Meister,A., Anderson,M.E. (1983) Annu. Rev. Biochem. 52, 711-760
[15] Reed,D.J. (1990) Annu. Rev. Pharmacol. Toxicol. 30, 603-631
[16] Kane,D.J., Sarafian,T.A., Anton,R., Hahn,H., Butler Gralla,E., Selverstone Valentine,J., Ord.T., Bredesen,T.E. (1993), Science 262, 1274-1277
[17] Slater,A.F.C., Nobel,C.S.I., Maellaro,E., Bustamante,J., Kimland,M. Orrenius,S. (1995) Biochem. J. 306, 771-778
[18] Reed,D.J., Babson,J.R., Beatty,P.W., Brodie,A.E., Ellis,M.W., Potter,D.W. (1980) Anal. Biochem. 106, 55-62
[19] Brigelius,R., Muckel,C., Ackerboom,T.P.M., Sies,H. (1983) Biochem. Pharmacol. 31, 1637-1641
[20] Chow,S.C., Peters,I., Orrenius,S. (1995) Exp. Cell Res. 216, 149-159
[21] Walker,P.R., Smith,C., Youdale,T., Leblanc,J., Whitfield,J.F., Sikorska,M. (1991) Cancer Res. 51, 1078-1085
[22] Sato,N, Iwata,S., Nakamura,K., Hori,T., Mori,K., Yodoi,J. (1995) J. Immunol. 154, 3194-3203