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Differential sensitivity to resveratrol-induced apoptosis of human chronic myeloid (K562) and acute lymphoblastic (HSB-2) leukemia cells

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Abstract

The in vitro effects of resveratrol (RES) on apoptotic pathway in human chronic myeloid (K562) and acute lymphoblastic (HSB-2) leukemia cells were investigated. RES treatment of both cell types significantly and irreversibly inhibited their growth, associated with extensive apoptosis and increase in hypodiploid cells. Cell cycle analysis showed accumulation in G_1 phase in HSB-2 drug exposed cells, while only K562-treated cells exhibited a marked accumulation in S phase with a concomitant decrease in G_1 and G_2/M at 24 h. Moreover, RES caused internucleosomal DNA fragmentation, even if K562 cells were found less sensitive to the drug, as compared to HSB-2 cells, which also reacted earlier to the treatment. RES-induced apoptosis was associated with an increase of Bax expression and a marked release of cytochrome *c* from mitochondria. Interestingly, K562 cells exhibited a basal content of glutathione 10-fold that of HSB-2 cells, which increased after 24–48 h RES exposure, together with increment of glutathione reductase and peroxidase activities. However, the major resistance to apoptosis of K562 cells cannot be attributed to their higher pool of reducing power, since neither the inhibition of glutathione synthesis by buthionine sulphoximine nor glutathione depletion by diethylmaleate, sensitized these cells. In addition, glutathione enrichment of HSB-2 cells by *N*-acetylcysteine did not prevent the apoptotic effects of RES. Our data indicate that RES commitment to apoptosis in both cell lines is independent from the intracellular content of glutathione, while it is associated with either the enhanced expression of Bax and cytochrome *c* release.

Keywords: Resveratrol; Glutathione; Apoptosis; K562 cells; HSB-2 cells; Caspases

1. Introduction

Resveratrol (*trans*-3,5,4′-trihydroxystilbene; RES) is a natural polyphenol present in high concentration in a wide variety of plant species, red grapes, red wines, peanuts and pines [1]. In these plants RES is synthesized in response to stress conditions such as trauma, UV irradiation, exposure to ozone and fungal infection, and thus it can be considered to be a phytoalexin [2,3].

Since it is only synthesized in the skin of the grape berries, red wine contains more RES than white wine. It has been hypothesized that RES may be the main active principle involved in the ''French Paradox'', the inverse correlation between the red wine consumption and the incidence of cardiovascular diseases [4]. RES has been shown to exhibit estrogenic activity in mammals and therefore is classified as a phytoestrogen [5,6]. RES has also been reported to inhibit platelet aggregation and blood coagulation, alter eicosanoid synthesis, prevent lipoprotein

Abbreviations: Ac-DEVD-AMC, acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin; Ac-IETD-AMC, acetyl-Ile-Glu-Thr-Asp-aminomethylcoumarin; Ac-LEHD-AMC, acetyl-Leu-Glu-His-Asp-aminomethylcoumarin; BSO, buthionine-L-sulphoximine; DCFH-DA, 2′,7′-dichlorofluorescein diacetate; DEM, diethyl maleate; ECL, enhanced chemiluminescence; NAC, *N*-acetyl-L-cysteine; RES, resveratrol; ROS, reactive oxygen species; *t*-BHP, *tert*-butylhydroperoxide

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oxidation and possess anti-inflammatory effects [7–9]. Besides these effects, RES has been identified as one of the most potent chemoprotective compounds able to block tumor initiation and progression [10].

The mechanisms by which RES exerts its antitumoral action are not yet clarified but may include inhibition of ribonucleotide reductase [11], DNA polymerase [12], protein kinase C [13], as well as cyclooxigenase-2 activities [14]. The observation that RES is an efficient oxygen radical scavenger suggests a further mode of action of its anticarcinogenic activity [15]. In addition, it has to be mentioned also the inhibition of cell division as an alternative pathway to block tumor progression in RES-treated cells [16]. Finally, several papers have reported that cancer chemoprotective activity of RES is related to the triggering of apoptosis [17–19]. Therefore, great interest in RES as a cancer therapeutic agent or even tumor chemopreventive has evolved in the last years [20].

However, a recent paper reported that RES exhibits a genotoxic activity in some cell lines by inhibiting microtubule assembly and thus causing mitotic alterations [21]. In this paper, we investigated the in vitro effects of RES on the apoptotic cascade in human chronic myeloid (K562) and acute lymphoblastic (HSB-2) leukemia cells. After incubation with 25 μM RES for up to 72 h, the cells were analysed for their growth rate, nuclear alterations, cell cycle phase distribution, caspase activities, expression of Bcl-2 family proteins, mitochondrial damage. Total glutathione and GSH/GSSG ratio, as well as some GSH-related enzymatic activities, were also determined.

2. Materials and methods

2.1. Materials

RPMI 1640 medium, foetal calf serum and proteinase K were from Labtek–Eurobio. Acridine orange, buthionine-L-sulphoximine, 1-chloro-2,4-dinitrobenzene, diethyl maleate, 5,5′-dithiobis(2-nitrobenzoic acid), ethidium bromide, reduced (GSH) and oxidized (GSSG) glutathione, glutathione reductase, *N*-acetyl-L-cysteine, NADPH, Nonidet P-40, propidium iodide, RES (*trans*-3,5,4′-trihydroxystilbene), RNase A, sulfosalicilic acid, *tert*-butylhydroperoxide and 2-vinylpyridine were purchased from Sigma Chemical Co. Fluorogenic caspase substrates Ac-DEVD-AMC (acetyl-Asp-Glu-Val-Asp-aminomethylcoumarin), Ac-IETD-AMC (acetyl-Ile-Glu-Thr-Asp-aminomethylcoumarin) and Ac-LEHD-AMC (acetyl-Leu-Glu-His-Asp-aminomethylcoumarin) were from Alexis Biochemicals. Monoclonal anti-actin, anti-Bax and anti-cytochrome *c* antibodies were purchased from Roche Molecular Biochemicals, Santa Cruz Biotechnology and PharMingen, respectively. Reagents for enhanced chemiluminescence (ECL) detection were obtained from Amersham Life Science. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was purchased from Molecular Probes, Inc. All other chemicals were reagent grade. Stock solutions of RES were prepared in DMSO and stored in the dark at −20 °C. The DMSO concentration in all drug-treated cells was always less than 0.01% (v/v).

2.2. Cell cultures

Human chronic myeloid (K562) and human acute lymphoblastic (HSB-2) leukemia cells were obtained from the American Type Culture Collection (ATCC), maintained in exponential growth in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum, 2 mM glutamine, streptomycin and penicillin and kept at 37 °C in a humidified atmosphere of 5% CO_2 in air.

2.3. Analysis of cell proliferation and viability

Cells were seeded at a density of 1×10^5 per mL and incubated in the absence or in the presence of 25 μM RES. After 24, 48 and 72 h cells were counted and viability determined by trypan blue exclusion assay.

2.4. Assays for detection of apoptosis

Cells were monitored for apoptosis during an extended time interval (12–72 h) and the best points for detection of apoptosis for each cell line were determined. In some assays, apoptosis was induced by treatment with 25 μM RES, and followed for up to 48 h in HSB-2 cells and for up to 72 h in K562 cells. In fact, HSB-2 cells exposed for 72 h to RES showed a marked cellular debris formation that made apoptotic cell count doubtful.

2.5. Chromatin condensation

Nuclear morphology was analysed by double-acridine orange and ethidium bromide staining. After mixing with an equal volume of a solution containing 100 μg/mL ethidium bromide and 100 μg/mL acridine orange, the cell suspension was examined with a fluorescence microscope [22].

2.6. Apoptosis and cell cycle analysis by flow cytometry

Control and RES-treated cells were collected, washed twice with ice-cold PBS and fixed in 70% ethanol at 4 °C for at least 30 min. The fixed cells were then washed twice with ice-cold PBS and stained with 50 μg/mL of propidium iodide in the presence of 25 μg/mL of RNase A. Cell cycle phase-distribution was analysed in three different experiments using flow cytometry (FACScan flow cytometry, Becton Dickinson Immunocytometry System, San Jose, USA). Data from 10,000 events per sample were collected and analysed using the cell cycle analysis

software (Modfit LT for Mac V 3.0). To evaluate apoptosis, the DNA contents of stained cells were analysed using Cell Quest software.

2.7. Internucleosomal fragmentation of chromatin

Cells (1.0×10^6) were washed with PBS and resuspended in 100 μL of lysis buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 0.25% (v/v) Nonidet P-40, 1 mg/mL RNase A). After 1 h incubation at 37 °C the suspension was supplemented with 1 mg/mL proteinase K and further incubated at 37 °C for 1 h. DNA was analysed by 2% (w/v) agarose gel electrophoresis.

2.8. Caspase activity

The activities of caspase-3, caspase-8 and caspase-9 were determined as previously described [23]. Briefly, cells were collected by centrifugation and suspended in extraction buffer (50 mM Tris–HCl, pH 7.4, 10 mM EGTA, 1 mM EDTA, 10 mM DTT, 1% (v/v) Triton X-100). The supernatants were incubated with 20 μM fluorogenic peptide substrates, Ac-DEVD-AMC (caspase-3), Ac-IETD-AMC (caspase-8) and Ac-LEHD-AMC (caspase-9), in reaction buffer for 30 min at 37 °C.

Fluorescence was monitored on a Perkin–Elmer LS-50B spectrofluorimeter, setting excitation at 380 nm and emission at 460 nm. The increase of protease activities was determined by comparing the levels of the RES-treated cells with untreated controls.

2.9. Western blot analysis

Proteins were extracted by suspending cells for 30 min at 4 °C in a buffer containing 10 mM Hepes, pH 7.2, 0.142 M KCl, 5 mM $MgCl_2$, 1 mM EDTA, 0.2% (v/v) Nonidet P-40 and a suitable cocktail of protease inhibitors. Of each sample, 60 μg of protein per lane were separated on a 12.5% SDS–PAGE and electroblotted onto polyvinylidene difluoride (PVDF) membranes. The membranes were then probed with a monoclonal anti-Bax antibody (1:100 dilution) or with a monoclonal anti-actin antibody (1:7000 dilution), followed by an anti-mouse peroxidase-conjugated secondary IgG antibody. Immune complexes were detected by ECL and exposed to a Kodak X-OMAT film.

2.10. Cytochrome c release

Cells were washed twice with cold PBS and pellets were resuspended in permeabilization buffer (0.25 M Sucrose, 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM Na-EGTA, 1.5 mM Na-EDTA, 1 mM $MgCl_2$, 1 mM DTT, 50 μg/mL digitonin, containing a cocktail of protease inhibitors) for 10 min at 4 °C. Permeabilized cells were centrifuged (800 × *g*, 15 min) at 4 °C. The clear supernatants were centrifuged again (18,000 × *g*, 30 min) at the same temperature. Proteins were separated on a 15% SDS–PAGE, transferred to PVDF membranes, probed with anti-cytochrome *c* antibody (7H8.2C12) and visualized with an ECL detection system.

2.11. Glutathione determination

Cells were washed with PBS and resuspended in 5 mM EDTA and 5% (w/v) sulfosalicilic acid.

Total glutathione and GSSG concentrations were measured as previously reported [24]. An aliquot of cell extract was added to 0.7 mL of a solution of 0.143 M sodium phosphate buffer (pH 7.5), containing 6.3 mM EDTA and 0.2 mM NADPH and to 0.1 mL of 6 mM 5,5′-dithiobis(2-nitrobenzoic acid). The assay was initiated by addition of 5 μL of glutathione reductase (470 U/mL). The rate of formation of 5-thio-2-nitrobenzoic acid, proportional to total glutathione concentration, was followed at 412 nm for 3 min at 25 °C in a Perkin–Elmer LS5 spectrophotometer. GSH concentration values were calculated from a standard curve and expressed as nmol/10^6 cells. GSSG concentration was determined under the same condition after adjusting pH to 6–7 with triethanolamine and GSH derivatization by 2-vinylpyridine.

2.12. Enzymatic assays

K562 cells were washed with cold PBS, resuspended in 50 mM Tris–HCl, pH 7.4, 1 mM EDTA, 1% (v/v) Triton X-100 and then disrupted by three consecutive cycles of freeze–thawing. After centrifugation at 17,000 × *g* for 10 min at 4 °C, the supernatant was collected for protein determination [25] and subsequently analysed.

The glutathione reductase activity of cell extracts was assayed spectrophotometrically by following NADPH oxidation at 340 nm at 25 °C [26]. The assay mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH and the appropriate amount of the extracted proteins.

Total (Se-dependent and Se-independent) glutathione peroxidase activity was evaluated spectrophotometrically by measuring the NADPH oxidation at 340 nm. The reaction mixture contained 50 mM potassium phosphate buffer (pH 7.0), 1 mM EDTA, 0.2 mM NADPH, 1 mM GSH, 0.01 U/mL GSH reductase, 70 μM *tert*-butylhydroperoxide and the appropriate amount of the extracted proteins [27].

The glutathione transferase activity was assayed by measuring the rate of GSH conjugation to 1-chloro-2,4-dinitrobenzene at 340 nm. The reaction mixture contained 0.1 M potassium phosphate buffer, pH 6.5, 2 mM GSH, 1 mM 1-chloro-2,4-dinitrobenzene and aliquots of supernatant [28].

The enzymatic activities were expressed as nmol/min/mg protein.

2.13. Determination of reactive oxygen species (ROS)

The generation of reactive oxygen species was detected by DCF fluorescence [29]. Cell suspensions were loaded with DCFH-DA to a final concentration of 10 μM and incubated at 37 °C for 30 min.

After the addition of RES (or *t*-BHP as positive control), or RES plus *t*-BHP, cells were incubated at 37 °C for short (10–120 min) or more prolonged (12–72 h) incubation times, washed with PBS and the fluorescence intensity was monitored with a Perkin–Elmer LS-50B spectrofluorometer, setting excitation at 485 nm and emission at 530 nm.

2.14. Statistical analysis

Data are reported as means ± S.D. Statistical differences between control and RES-treated cells were calculated using the Student's *t*-test. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Cell growth and viability

In preliminary experiments aimed at assessing the best concentration of RES to be used in our two cell lines, only a mild cytotoxicity was observed in either HSB-2 and K562 cells exposed to 1–10 μM RES for 24–48 h, while an extensive cell death was detected in cells treated with 50–100 μM RES, especially in HSB-2 cells (data not shown). A concentration of 25 μM RES was then selected in all the experiments here reported because it caused a significant inhibition of the growth in both cell lines (Fig. 1). Fig. 2 shows the percentage of viable cells (evaluated by the trypan blue exclusion method described in Section 2) upon 24, 48 and 72 h treatment of HSB-2 and K562 cells with 25 μM RES. As can be seen, only after 48–72 h K562-treated cells exhibited a significant death rate, while exposed HSB-2 cells appeared much more sensitive even at earlier incubation time (Fig. 2). This result is in agreement with a recent publication that reports the different sensitivity of two human melanoma cell lines to RES [30].

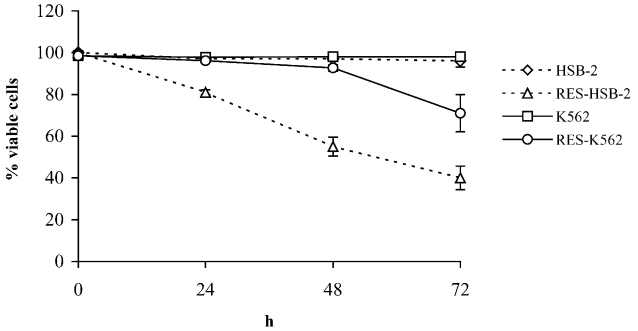

Fig. 2. Kinetics of cell death in untreated or 25 μM RES-treated HSB-2 and K562 cells (RES–HSB-2, RES–K562). Viability was determined by trypan blue exclusion at the indicated time points. Results represent the mean ± S.D. of triplicate experiments.

3.2. Resveratrol induces apoptosis in HSB-2 and K562 cells

To determine the mechanism by which RES inhibits the cell growth and exert its cytotoxic effects, we examined whether the drug induces a programmed cell death in HSB-2 and K562 cells.

Apoptosis in both cell lines, untreated or treated with 25 μM RES for up to 72 h, was evaluated by measuring cellular sub-G_1 populations, cell cycle kinetics, nuclear morphology and DNA fragmentation. As shown in Fig. 3, RES-treated HSB-2 cells displayed a significant increase of the hypodiploid cells in sub-G_1 phase in a time-related manner. At 48 h, 55% of the treated cells were in the sub-G_1 phase, at 72 h being impossible to perform the measure because of the extensive cell death (secondary necrosis).

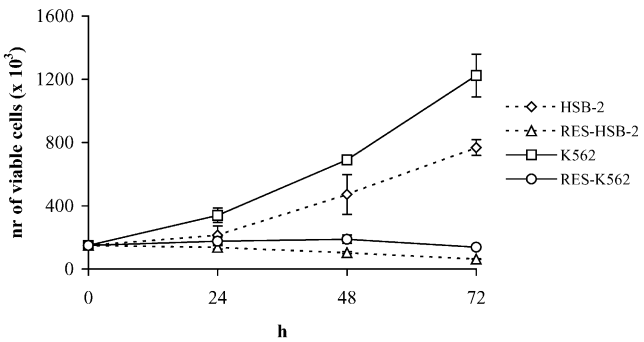

Fig. 1. Effects of resveratrol on cell growth. HSB-2 and K562 cells, seeded at a density of 1.5×10^5 per mL, were treated with 25 μM resveratrol (RES–HSB-2, RES–K562) or left untreated and counted in a Burker chamber at the indicated times. Results represent the mean ± S.D. of triplicate experiments.

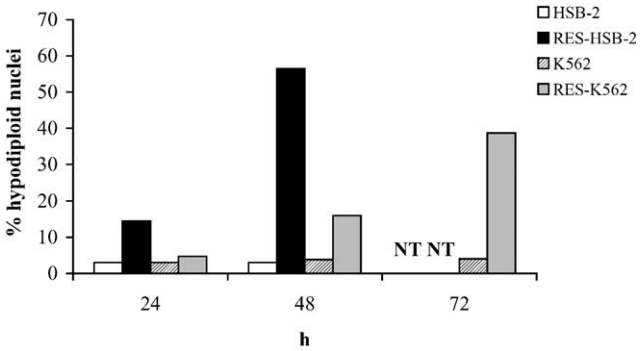

Fig. 3. Flow cytometric DNA analysis of untreated or RES-treated HSB-2 and K562 cells (RES–HSB-2, RES–K562). After drug exposure nuclei were stained with propidium iodide and analysed for hypodiploid DNA content at the indicated times. The histogram is representative of several experiments with similar results. NT: not tested.

Table 1
Cell cycle analysis by propidium iodide staining and flow cytometry after treatment with 25 μM resveratrol (RES) for 6, 24 and 48 h

Time	HSB-2			K562		
	G_1	S	G_2/M	G_1	S	G_2/M
6 h						
Control	46.58 ± 3.9	41.66 ± 3.6	11.76 ± 1.0	41.32 ± 3.9	38.37 ± 3.7	20.31 ± 1.9
RES	60.49 ± 5.8	30.39 ± 2.7	9.11 ± 0.8	54.91 ± 5.0	38.02 ± 3.6	7.08 ± 0.6
24 h						
Control	41.6 ± 3.7	37.14 ± 3.3	21.25 ± 2.0	39.63 ± 3.7	42.51 ± 4.0	17.86 ± 1.6
RES	64.23 ± 5.8	5.67 ± 0.4	30.1 ± 2.7	18.80 ± 1.0	79.64 ± 7.7	1.55 ± 0.1
48 h						
Control	45.29 ± 1.2	36.9 ± 2.5	17.79 ± 1.0	38.0 ± 1.9	52.0 ± 2.5	9.97 ± 0.5
RES	68.6 ± 2.3	4.45 ± 0.01	27.0 ± 1.45	17.0 ± 0.4	72.0 ± 1.4	9.8 ± 0.02

The results are expressed as mean percent ± S.D. of three different experiments.

By contrast, in K562 cells, RES caused only a mild hypodiploidy until 48 h exposure, which reached a value of 40% at 72 h, comparable to that exhibited by HSB-2 cells at 48 h (Fig. 3).

To examine the mechanism(s) underlying the cell proliferation inhibition, viable cell cycle distribution was evaluated using flow cytometric analysis. RES caused accumulation (about 30% increase with respect to control cells) of HSB-2 cells in G_1 phase of the cycle when exposed to 25 μM RES at 6, 24 and 48 h (Table 1). Interestingly, the accumulation in G_1 phase was evident at 6 h and this effect was retained up to 48 h. The increase of the cell population in G_1 phase was associated to a concomitant decrease of cells in S and G_2/M phases at 6 h (approximately 20–25% reduction). At 24 and 48 h, a persistent G_1 phase arrest was evident and a dramatic decrease of viable cells in S phase, together with a consequent relative increase of cell population in G_2 phase, was observed.

Concerning the effect of RES on K562 cell cycle phase distribution (Table 1), the results indicated that also these cells accumulated in the G_1 phase after 6 h treatment (>30% increase as compared to control cells), showing even a remarkable decrease of G_2/M peak (about 60% reduction with respect to control cells). A much more dramatic and different effect was observed after 24 h treatment being evident a significant increase of the cell population in the S phase (80–90% increase as compared to control cells), which was associated to a concomitant reduction of the cell population in G_1 (50% reduction, or even more, with respect to control cells) and G_2/M phases (90% decrease as compared to control cells). The S phase arrest was observed also after 48 h treatment, even if the % increase of viable cells appeared to be lower than that observed after 24 h (Table 1). This result could be likely due to the massive apoptotic death occurred at 72 h. To further confirm the observation of a different RES sensitivity of these two cell lines, the number of apoptotic cells was evaluated by fluorescence microscopy analysing chromatin condensation and fragmentation. Fig. 4 shows that HSB-2 cells exhibited a significant chromatin condensation (30%) upon 24 h of RES treatment, which reached a 60% value after 48 h. On the contrary, in K562-treated cells, chromatin condensation appeared almost unaffected at 24 h, showing a 20% value at 48 h and reaching a 45–50% level only after 72 h of RES exposure.

These data are in accordance with the different kinetics of the hypodiploid cells increase in the two cell types shown in Fig. 3, and strengthen the finding of a higher sensitivity of HSB-2 cells to RES as compared to K562 cells. A prominent feature of apoptosis is the degradation of chromatin DNA at internucleosomal linkages. To determine whether RES induces DNA fragmentation, HSB-2 and K562 cells were treated with 25 μM RES for 24, 48, 72 h and DNA was fractionated by agarose gel electrophoresis. A typical ladder pattern of internucleosomal fragmentation was detected at 48 h in HSB-2-treated cells, but its appearance was also evidentiated, even if to a minor degree, at 24 h (Fig. 5). On the contrary, K562-treated cells failed to show any significant apoptotic DNA fragmentation ladders until 48–72 h, thus paralleling the results obtained for cell cycle phase-distribution and for chromatin condensation.

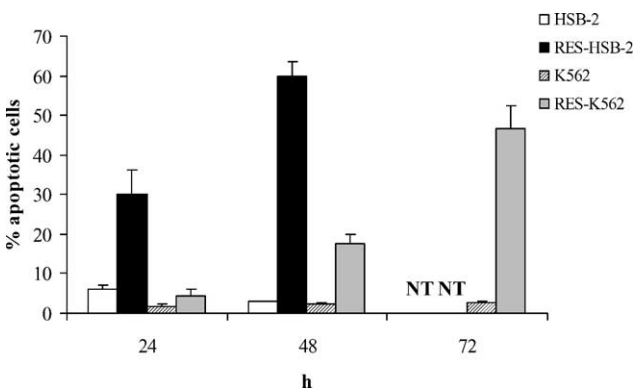

Fig. 4. Time course of nuclear condensation and fragmentation in HSB-2 and K562 cells. The percentage of condensed and fragmented nuclei was estimated on acridine orange and ethidium bromide double-stained cells examined in the fluorescence microscope at the indicated times. At least 400 cells were counted for each determination. Results represent the mean ± S.D. of triplicate experiments. RES-treated HSB-2 and K562 cells (RES–HSB-2, RES-K562); NT: not tested.

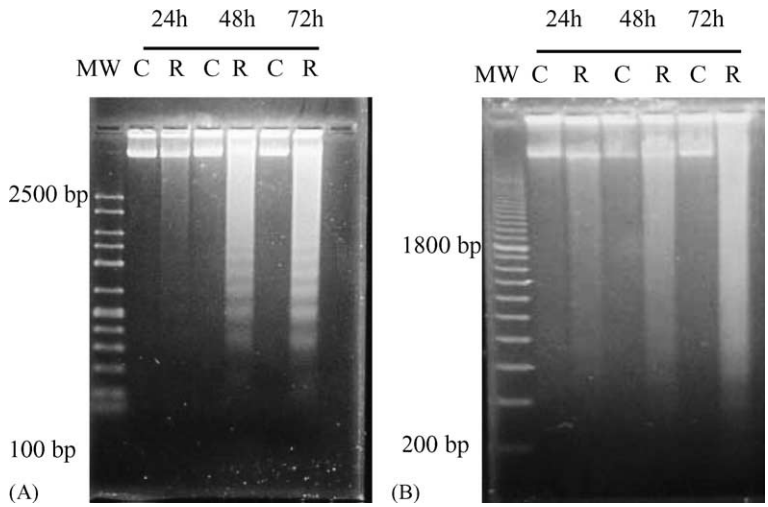

Fig. 5. Effects of RES on induction of DNA fragmentation. Genomic DNA was isolated from untreated or 25 μM RES-treated HSB-2 (A) and K562 (B) cells and assayed for its fragmentation by a 2% agarose gel electrophoresis as described under Section 2. Cell preparations were analysed after 24, 48 and 72 h of incubation. C: control cells; R: RES-treated cells; MW, DNA molecular weight markers.

3.3. Caspase activation, increase of Bax expression and cytochrome c release induced by resveratrol

The effects of RES on caspase-3, caspase-8 and caspase-9 activities were then studied in HSB-2 and K562 cells. As shown in Fig. 6A and B, caspase-8 (IETD-dependent) activity was scarcely affected in both cell lines exposed to RES for 24–48 h, a significant activity being found only in K562 cells treated for 72 h. It has to be noticed that caspase-8 is reported as an early marker of the apoptotic pathway and its activation is dependent from the ligation of death receptors. This ''initiator'' caspase-8 activates, in turn, downstream or ''effector'' caspases such as caspase-3 [31,32]. However, caspase-3 (DEVD-dependent) activity showed a four- to five-fold increase, as compared to control untreated cells, at 24 h and a 20 to 25-fold increase after 48 h RES exposure in HSB-2 cells (Fig. 6A). In K562 cells caspase-3 activity was almost unaffected at 24 h and increased only by a factor 5–7 at 48 h then reaching a 20 to 25-fold increase after 72 h of RES treatment (Fig. 6B). It has to be underlined that caspase-3 is not included in the early events associated to the apoptotic process but it is responsible for the DNA fragmentation at the internucleosomal linkages. When caspase-9 (LEHD-dependent) was assayed in RES-treated HSB-2 cells, the peak of activity (four- to five-fold increase) was found after 24 h incubation that decreased at 48 h (Fig. 6A). By contrast, in K562-treated cells caspase-9 activity was barely detectable at 24 h, showing a mild (two- to three-fold) increase at 48 h, then reaching a 15 to 20 fold increase at 72 h incubation (Fig. 6B). This enzymatic activity is likely reported as an early apoptotic marker that is activated when apoptosis follows the mitochondrial pathway. In this route, various forms of cellular stress cause mitochondrial alterations, like mitochondrial membrane depolarization and release of cytochrome *c*, which in turn activates caspase-9 and effector caspases [33,34]. Moreover, literature data confirm that RES induces a dose-dependent increase of caspase activity in several hematopoietic and lymphoblastoid cell lines [35,36].

It has been previously reported that RES can increase the steady-state levels of pro-apoptotic Bax protein and induce a mitochondria-mediated apoptosis in human colon carcinoma cells [37]. Bax is a member of the Bcl-2 family and Bax–Bax homodimers act as apoptosis inducers while Bcl-2–Bax heterodimers act as anti-apoptotic elements for cells. Contrarily to previous observations that RES decreased the expression of the anti-apoptotic protein Bcl-2 [19,38], we failed to detect changes in the steady-state level of this protein and of its similar Bcl-X_L in RES-treated cells (data not shown). By contrast, in agreement with other authors [39] we detected a significant stimulation of Bax expression in response to RES, mainly in HSB-2 cells where reached the major increase after 24 h (Fig. 7A). In K562-treated cells, instead, the highest expression of Bax was delayed to 72 h, thus paralleling the caspase-9 time-course profile of activity. Fig. 7B shows the effect of 25 μM RES on mitochondrial cytochrome *c* release in HSB-2 and K562 cells. It is evident that HSB-2-treated cells exhibit the cytochrome *c* immunoblot band at 24 h and even better at 48 h, while in K562-treated cells the release of this protein is retarded at 48 h, with a peak at 72 h. This pattern is in line with the above reported data, which show the different sensitivity of our two cell lines to RES. Until now, all the data reported indicate a higher sensitivity of HSB-2 cells to apoptosis induced by RES, with respect to K562 cells.

3.4. Resveratrol causes alterations of glutathione content and of glutathione-related enzymatic activities

To verify if the major resistance of K562 cells to RES was due to a higher content of reducing power, intracellular

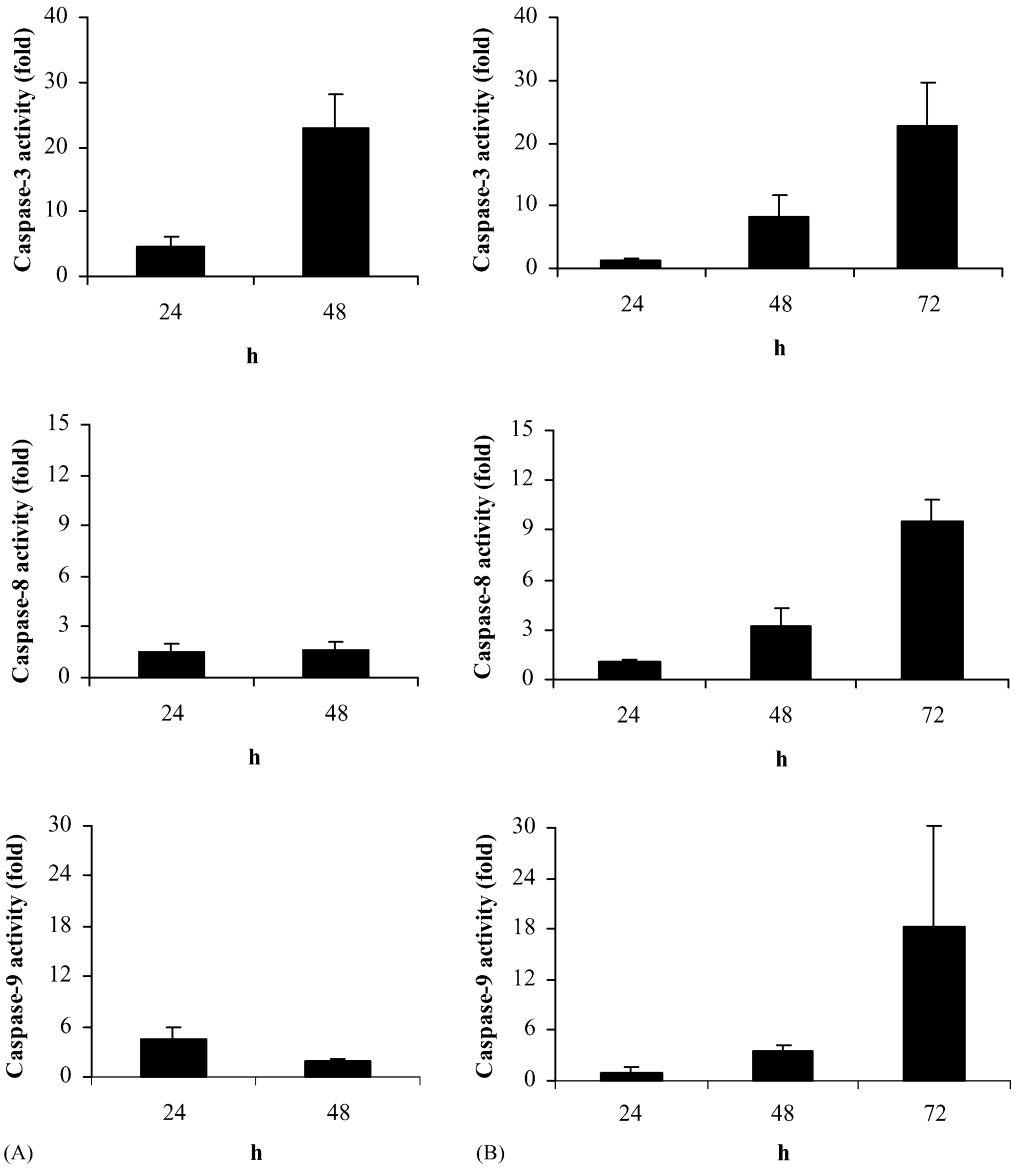

Fig. 6. Time course of caspase activities. The caspase-3, caspase-8 and caspase-9 activities were measured in the cytosol of untreated or 25 μM RES-treated HSB-2 (A) and K562 (B) cells by using DEVD-AMC, IETD-AMC and LEHD-AMC as substrates, respectively. Results represents the mean of fold increase, with respect to control, ± S.D. of triplicate experiments.

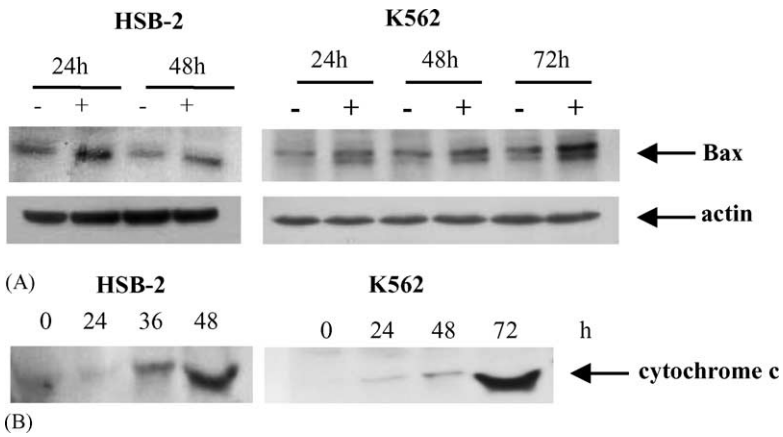

Fig. 7. Alterations of Bax expression (A) and induction of cytochrome *c* release (B). Lysates from untreated (−) or RES-treated (+) HSB-2 and K562, at the indicated times, were subjected to immunoblot analysis with anti-Bax and anti-cytochrome *c* antibodies. No cytochrome *c* band could be detected in control cells at any time of incubation (data not shown).

Table 2
Levels of total, reduced (GSH) and oxidized (GSSG) glutathione in untreated (K562) and resveratrol-treated (RES–K562) cells determined at the indicated times

Incubation time (h)	Total glutathione (nmol/10^6 cells)		GSH (nmol/10^6 cells)		GSSG (nmol/10^6 cells)	
	K562	RES–K562	K562	RES–K562	K562	RES–K562
12	9.05 ± 4.50	19.50 ± 6.06	8.96 ± 4.50	19.40 ± 6.06	0.045 ± 0.02	0.068 ± 0.017
24	13.0 ± 3.24	26.38 ± 2.63*	12.92 ± 3.24	26.18 ± 2.65*	0.043 ± 0.01	0.10 ± 0.072
48	7.50 ± 2.68	20.63 ± 2.68*	7.44 ± 2.68	20.34 ± 7.36*	0.034 ± 0.01	0.17 ± 0.049*
72	6.13 ± 2.02	13.75 ± 6.41	6.06 ± 2.02	12.11 ± 8.15	0.037 ± 0.01	0.11 ± 0.054

Results represents the mean ± S.D. of triplicate experiments.
* Values are significantly different from untreated control ($P < 0.05$).

Table 3
Glutathione reductase and peroxidase activity in untreated (K562) and RES-treated (RES–K562) cells

Incubation time (h)	GSH reductase activity (nmol/min/mg protein)		GSH peroxidase activity (nmol/min/mg protein)	
	K562	RES–K562	K562	RES–K562
24	42.70 ± 2.87	53.47 ± 3.88	27.70 ± 4.06	34.27 ± 8.32
48	37.30 ± 2.55	69.10 ± 6.22*	21.30 ± 2.31	36.50 ± 2.29*
72	41.80 ± 7.24	62.40 ± 6.81*	19.50 ± 5.63	32.93 ± 4.68*

The activities were evaluated after 24, 48 and 72 h of incubation. Results represents the mean ± S.D of triplicate experiments.
* Values are significantly different from untreated control ($P < 0.05$).

glutathione concentration and glutathione reductase, peroxidase and transferase activities were assayed in both cell types treated (or untreated) with 25 μM RES as a function of time. Glutathione is involved in signal transduction, cell proliferation and apoptosis [40,41]. We found a marked difference in the basal content of glutathione in the two cell lines, K562 cells exhibiting a glutathione concentration almost 10-fold higher than HSB-2 cells, irrespective of RES treatment. It has to be recalled that total glutathione practically identifies reduced glutathione, being this intracellular metabolite almost completely in the reduced state (GSH). In HSB-2 cells, RES treatment did not induce significant alterations of glutathione content and of glutathione reductase, peroxidase and transferase activities (data not shown). Instead, RES-treated K562 cells exhibited a substantial (about two-fold) increase of total glutathione in the time interval of 24 to 48 h upon drug exposure, with the maximum increase at 24 h then slowly declining between 24 and 48 h (Table 2). This change in total glutathione concentration reflected variations mainly in GSH concentration. The GSSG content was always less than 1% of GSH concentration but increased significantly between 24 and 48 h of incubation and declined from 48 to 72 h (Table 2). In K562 cells the glutathione transferase activity appeared practically unaffected (data not shown), whereas glutathione reductase and peroxidase displayed enhanced activities especially upon 48–72 h exposure to RES, with the maximum increase at 48 h (Table 3). In particular, after 48 h of incubation RES-treated cells exhibited the glutathione reductase and peroxidase activities about 85 and 71% higher than control cells, respectively ($P < 0.005$). When K562 cells were incubated 48 h with 50 μM BSO (a powerful and specific inhibitor of γ-glutamyl cysteine synthase activity), the glutathione content decreased to concentrations lower than that of the control cells (Fig. 8A). The simultaneous addition of BSO and RES caused a drastic reduction of the glutathione increment induced by RES. However, the lowered concentration of GSH caused by the inhibition of its synthesis, did not change the amount of apoptotic cells (Fig. 8B). It has to be mentioned that also diethylmaleate (a well-known agent used for intracellular GSH depletion) alone or together

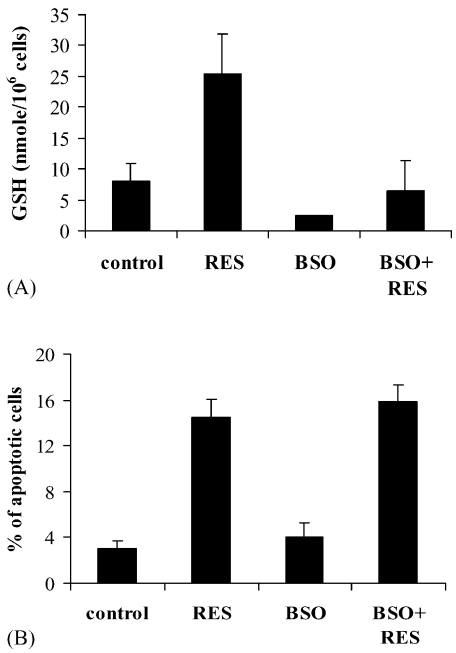

Fig. 8. Variations of total glutathione (A) and chromatin fragmentation and condensation (B) in K562 cells untreated (Control), or exposed 48 h to 25 μM resveratrol (RES), 50 μM buthionine-L-sulphoximine (BSO), and resveratrol plus buthionine-L-sulphoximine (RES + BSO). The histograms represent the mean ± S.D. of triplicate experiments.

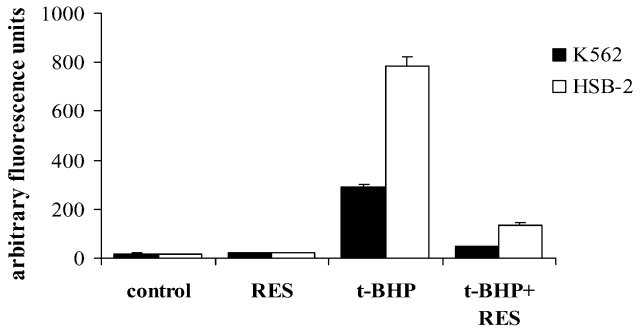

Fig. 9. Generation of ROS in K562 and HSB-2 cells. Cells were loaded with 10 μM DCFH-DA for 30 min at 37 °C, then incubated 60 min in the presence of fresh culture medium (Control), 25 μM resveratrol (RES), 100 μM *tert*-butylhydroperoxide (*t*-BHP), and resveratrol plus *tert*-butylhydroperoxide (RES + *t*-BHP). DCF fluorescence was determined by spectrofluorimetric analysis. Results represent the mean ± S.D. of three independent experiments.

with BSO, failed to sensitize K562 cells to RES-induced apoptosis (data not shown). In addition, even the glutathione enrichement of HSB-2 cells, upon incubation with NAC (the freely permeable form of cysteine, an essential amino acid residue for glutathione synthesis), failed to show an enhanced resistence to apoptosis induced by RES (data not shown).

3.5. Resveratrol is unable to generate ROS but is an efficient ROS scavenger

To verify if RES caused the generation of ROS, K562 and HSB-2 cells were exposed to the drug for brief (10–120 min) and more prolonged (12–72 h) incubation times and then analysed for DCF fluorescence. Surprisingly, not only RES did not produce ROS up to 72 h, but instead greately inhibited their formation, as detected in cell samples treated with the oxidizing agent *t*-BHP (Fig. 9). This result indicates that RES exhibit, in these cell lines, more antioxidant than pro-oxidant properties.

4. Discussion

The present study shows that RES, at a concentration of 25 μM, causes a marked inhibition of both HSB-2 and K562 cells growth as well as a higher cytotoxicity on HSB-2 cells. The anti-proliferative effect is possibly related to the ability of the drug to induce programmed cell death.

Many biological effects of RES, including anti-inflammatory, anti-proliferative, and chemopreventive activities have been previously described. Some authors reported that RES inhibits most of the T cell mediated immune responses in vitro [42] and selectively inhibits the clonogenic growth of numerous human and murine leukemic cell lines but not normal hematopoietic progenitor cells [43]. RES reported contradictory results on the induction of apoptosis. For example, it was claimed that RES is a powerful [17] and a moderate [16] apoptotic agent, but also that RES is able to prevent apoptosis [44]. Our results support the previously reported apoptosis-inducing activity of RES against tumor cell lines and demonstrate that RES triggers apoptosis in either HSB-2 and K562 cells, even if with a different time-course and a different intensity. In fact, all the apoptotic markers monitored in our study, chromatin condensation, DNA laddering, caspases activation, Bax expression and cytochrome *c* release showed an anticipation of 24 h in HSB-2 cells incubated with RES, as compared to K562-treated cells. In addition, the relative amount of apoptotic cells was found significantly higher in HSB-2 than in K562 cells, irrespective of the duration of the treatment (Figs. 3 and 4). The analysis of cell cycle by flow cytometry evidentiated an accumulation of HSB-2 cells in G_1 phase when the cells were treated with 25 μM RES, which was evident at 6 h and was maintained at the same extent at 24 and 48 h. A similar result was observed when K562 cells were treated with RES for 6 h. Nevertheless, the G_1 phase arrest appeared to be transitory since the flow cytometric analysis of K562 cell cycle after a 24 and 48 h treatment with RES indicated the accumulation of cells mainly in S phase which was associated with a significant decrease of cell percentage in G_1 phase and the almost total disappearance of G_2/M peak; this pattern being particularly evident at 24 h. These data well correlated with the results obtained when apoptosis analysis was performed. In fact, the G_1 phase arrest induced by RES in HSB2 cells was associated with the appearance of time-increasing levels of hypodiploid cells at 24 and 48 h, thus suggesting that the RES-treated cells were early induced to arrest in G_1 phase and then undergo apoptosis. As far as the RES effects on K562 cells concerns, the results suggest that the molecular target(s) of the drug could be different in these cells. Indeed, even if at 6 h treatment the G_1 phase arrest extent was comparable to that observed with HSB2 cells, after 24–48 h a very relevant S phase arrest was evidentiated. The appearance of a significant percentage (35–40%) of hypodiploidy in RES–K562 cells could be observed only after 72 h.

Several pathways of the apoptotic cell death program, including direct caspase-2 and caspase-6 activation, upregulation of p-53 and p-21, activation of Fas–Fas ligand route, and even direct cleavage of DNA have been proposed for programmed cell death induced by RES [17,45].

More recently, a novel mechanism of the RES-induced apoptosis in metastatic breast cancer cells, via de novo ceramide signaling, has been reported [46]. Moreover, RES has been demonstrated to cause apoptosis in leukemia cell lines through a mitochondrial pathway regulated by Bcl-2, an anti-apoptotic protein associated with the mitochondrial membrane [19]. Although the results of these studies clearly demonstrated the induction of apoptosis by RES in leukemia cell lines, the exact pathway of the programmed death process remains to be established. Recent papers demonstrated that Bax levels do not always increase

following apoptotic stimuli but that N-terminal domain of Bax, upon a precise unfolding, become exposed and the protein may then co-localize with the mitochondria inducing their damage [47,48]. The different kinetics and intensity of the apoptotic signals observed in our two RES-treated cell types, was further confirmed by monitoring Bax expression. We found indeed the higher stimulation of this pro-apoptotic protein in HSB-2 cells after 24 h RES exposure, while K562 cells, in the same conditions, showed a mild appearance of Bax that become significant only upon 72 h treatment (Fig. 7A). This finding, together with the lack of any effect exerted by RES on Bcl-2/Bcl-X_L expression in both cell lines, suggest that RES could modulate the Bcl-2/Bax ratio which is critical for the differences in the time course and in the intensity of the apoptotic markers exhibited by the two drug-treated cell lines. Furthermore, we also detected a different release of cytochrome *c* from HSB-2 and K562 RES-treated cells as a function of exposure time (Fig. 7B). Because it has already reported that the mitochondrial cytochrome *c* release involves opening of the mitochondrial membrane permeability transition (MPT), which is associated with dissipation of the mitochondrial membrane potential [49], a similar mechanism is likely to be in action also in our experimental conditions.

However, we used a relatively low concentration (25 μM) of RES instead of 100 μM used by these Authors. All together, our data indicate that RES induces apoptosis in HSB-2 and, even if to a minor extent and with a delayed time course, in K562 cells by a mechanism likely involving the interaction of the drug with the mitochondrial MPT complex, leading to cytochrome *c* release into the cytosol. Our findings are in agreement with similar results previously reported in in vivo and in vitro studies on RES-treated pancreatic cancer cells [49]. In the attempt to clarify the reason(s) of the different sensitivity to RES exhibited by HSB-2 and K562 cells, we determined the intracellular glutathione content in both cell lines, incubated in the absence and in the presence of RES. In fact, it has been previously reported that, upon apoptotic stimuli, cytochrome *c* release from mitochondria occurs concomitantly with a massive extrusion of reduced glutathione (GSH) via specific membrane carriers [50,51]. Contrarily to these literature data, we found a significant increase of either total and reduced glutathione in RES-treated K562 cells after 12–24 h exposure that slowly declined at 48 h, and then approaching the value of the control untreated cells at 72 h (Table 2). On the other hand, HSB-2 treated cells, failed to show any significant alteration of glutathione concentration. Our results indicate that RES commitment to cell death is dependent from the mitochondrial loss of cytochrome *c* but is independent from the simultaneous intracellular glutathione depletion, as already reported [52]. There is still debate among researchers, some of them claiming that RES can prevent oxidative damage as result of its ability to protect from reactive oxygen species. By contrast, the pro-oxidant properties of RES could contribute to tumor cell apoptosis and cancer chemoprevention induced by this agent [53].

Alterations of GSH/GSSG (usually ranging between 30 and 100), may modulate several cellular reactions involved in signal transduction, cell cycle regulation and many other cellular processes [42,54,55]. Upon oxidative stress, GSH/GSSG ratio decreases due both to raised GSSG concentration and to diminished GSH amount. However, in response to oxidative stimuli, cells try to maintain glutathione redox balance by different mechanisms, like the enhancement of glutathione reductase activity or the energy-dependent extrusion of GSSG. Concomitantly, γ-glutamylcysteine synthase can be activated to synthesize new glutathione, or thiol disulphide exchange may be promoted. By contrast, the intracellular glutathione increase detected in RES-treated K562 cells, lead us to hypothesize that RES, in our experimental model, does not behave like an oxidizing agent. In fact, its presence abolished almost completely the ROS production in both cell lines exposed to the strong oxidizing agent *t*-BHP (Fig. 9). These data are in agreement with a recent report on protection from free radical-induced peroxidative damage of rat liver microsomes [56].

On the other hand, the enhanced activity of glutathione reductase and, to a minor extent, of glutathione peroxidase in RES-treated K562 cells, supports the hypothesis of RES as an agent able to induce oxidative stress. In spite of the altered pattern of either glutathione concentration and of its related enzymatic activities, GSH cannot be considered responsible for the major resistance of K562 cells to RES-induced apoptosis, since even after blocking glutathione synthesis with BSO or after glutathione depletion with DEM, these cells showed the same resistance. Moreover, the enrichment in glutathione of HSB-2 cells, after incubation with NAC, failed to increase their resistance to apoptosis induced by RES. In conclusion, in RES-treated K562 cells, GSH does not seem to be directly involved neither in the prevention and/or protection from apoptosis, nor in the delay of the appearance of the apoptotic markers.

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