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Reactive oxygen and nitrogen species are involved in sorbitol-induced apoptosis of human erithroleukaemia cells K562

KATIA AQUILANO[1], GIUSEPPE FILOMENI[1], LIVIA DI RENZO[2], MAURA DI VITO[2], CARLA DI STEFANO[3], PAOLA SINIBALDI SALIMEI[2], MARIA R. CIRIOLO[1], & GABRIELLA MARFÈ[3]

[1]*Department of Biology, University of Rome "Tor Vergata", Via della Ricerca Scientifica, 00133 Rome, Italy,* [2]*Department of Experimental Medicine, University of Rome "La Sapienza", Viale Regina Elena 324, 00161 Rome, Italy, and* [3]*Department of Experimental Medicine and Biochemical Sciences, University of Rome "Tor Vergata", Via Montpellier 1, 00133 Rome, Italy*

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Abstract

In this study, we found that production of both reactive oxygen (ROS) and nitrogen (RNS) species is a very early event related to treatment with hyperosmotic concentration of sorbitol. The production of nitric oxide (NO) was paralleled by the increase of the mRNA and protein level of the inducible form of the nitric oxide synthase (iNOS). ROS and RNS enhancement, process concomitant to the failure of mitochondrial trans-membrane potential ($\Delta\Psi$), was necessary for the induction of apoptosis as demonstrated by the protection against sorbitol-mediated toxicity observed after treatment with ROS scavengers or NOS inhibitors. The synergistic action of ROS and RNS was finally demonstrated by pre-treatment with rosmarinic acid that, by powerfully buffering both these species, prevents impairment of $\Delta\Psi$ and cell death. Overall results suggest that the occurrence of apoptosis upon sorbitol treatment is an event mediated by oxidative/nitrosative stress rather than a canonical hyperosmotic shock.

Keywords: *Sorbitol, ROS, RNS, mitochondrial transmembrane potential, apoptosis, rosmarinic acid*

Introduction

Sorbitol is a hydrogenated form of carbohydrate obtained by the reduction of the carbonyl group of the glucose molecule to the hydroxyl group. It has been demonstrated that sorbitol is able to efficiently and rapidly induce apoptosis when provided at high concentrations, as a part of the mechanisms related to hyperosmotic stress. In fact, hyperosmotic challenge has been shown to induce apoptosis in several cell lines such as human neuroblastoma [1], cardiac myocites [2], Hep-2 cells [3] and human gastric cells [4,5]. Moreover, among the polyhydric alcohols, it has been demonstrated that xylitol (five hydroxyl groups) and erythritol (four hydroxyl groups) are also able to induce chromosomal DNA fragmentation but less efficiently than mannitol (six hydroxyl groups), which behaves as sorbitol. In contrast, neither glycerol (three hydroxyl groups) nor ethylene glycol (two hydroxyl groups) is able to induce DNA fragmentation [6]. These data indicate that polyhydric alcohols, with at least four hydroxyl groups in the molecule, have an increasing ability to induce apoptosis in a manner proportional to the length of the molecule.

Osmotic changes can occur in pathological conditions such as ischemia, septic shock and diabetes, the latter of which is usually associated with increased sorbitol concentration due to alteration of the carbohydrates metabolism [7,8]. In fact, among the multiple mechanisms so far identified as secondary transducers of hyperglycaemia, increased aldose reductase activity [9], non-enzymatic glycation and glyco-oxidation [10,11], activation of protein kinase C [12,13] are some events among the best studied.

Correspondence: M. R. Ciriolo, Department of Biology, University of Rome "Tor Vergata", Via della Ricerca Scientifica, 00133 Rome, Italy. Tel: 39 6 72594369. Fax: 39 6 72594311. E-mail: ciriolo@bio.uniroma2.it

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All these mechanisms contribute to oxidative stress which could derive from increased production of reactive oxygen species (ROS) and/or insufficient up-regulation of antioxidant defence [14]. Growing evidence suggests that superoxide is the primary free radical formed in hyperglycemic conditions and is also the source of other highly reactive free radicals and oxidants, such as hydroxyl radical, hydrogen peroxide and peroxynitrite, known to exert detrimental effects in tissues from diabetic patients [15–20]. In fact, generation of the potent oxidant peroxynitrite has recently been documented in both experimental and clinical diabetic neuropathy [21,22]. Peroxinitrite is formed by the reaction of superoxide anion with nitric oxide (NO), a gaseous and radical molecule endogenously produced from L-arginine by a family of NADPH-dependent enzymes called NO synthases (NOSs). These enzymes exist in a constitutive (endothelial NOS and neuronal NOS) and inducible form (iNOS), which is expressed in many cell types including those of the immune system in response to many stimuli (as bacteria and cytokine) and during inflammation [23,24]. Excessive production of peroxynitrite and/or other reactive nitrogen species (RNS) cause the so-called nitrosative stress [25,26]. As well as oxidative, also nitrosative stress is known to induce a variety of downstream consequences in patho-physiological conditions that ultimately result in the induction of the apoptotic process [27–29]. However, the pathogenetic role of ROS/RNS in apoptosis induced by high concentration of sorbitol remains unexplored. This condition might represent, in a short time, the harmful challenge mediated by hyperglycemia in diabetes.

We previously demonstrated that sorbitol was able to efficiently induce apoptosis in human K562 cells via the mitochondrial pathway [30]. The present study was designed to deeply evaluate the role of oxidative/nitrosative stress in the processes leading to apoptosis upon treatment with high concentrations of sorbitol, examining whether ROS/RNS are critical mediators of sorbitol-induced cell death or the consequence of the apoptotic process. We demonstrated that apoptosis induced by sorbitol was related to the massive production of ROS and RNS, rather than to osmotic stress.

Materials and methods

Cell cultures and treatments

Human chronic myelogenous leukemia cell line K562 was purchased from American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum at 37°C in an atmosphere containing 5% CO_2. Cells were routinely collected by centrifugation at 700*g* and resuspended in fresh medium at a concentration of 2×10^5/ml. Cell viability was assessed by Trypan Blue exclusion.

K562 cells were incubated with different concentration of sorbitol for different time points. The majority of experiments were carried out with sorbitol at 1 M concentration. This condition was selected on the basis of our previous studies were it was able to rapidly and significantly induce apoptosis in K562 cells [30]. The cells not treated with sorbitol were considered controls.

5 mM *N*-acetyl-L-cysteine (NAC), 20 μM dinitrophenil iodonium (DPI), 50 μM L-N6-(1-iminoethyl) lysine hydrochloride (NIL), 100 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME) and 25 μM rosmarinic acid were independently added 1 h before sorbitol and maintained throughout the sorbitol treatment. Successively, cells were seeded in minimum fresh medium and recovered after 2 h.

Analysis of cell viability and apoptosis

Cell counting. For detection of apoptosis, 1 M sorbitol-treated cells were centrifuged at 900*g* for 5 min and viable cells were determined using a hemocytometer by counting the number of cells that excluded Trypan blue dye.

DNA fragmentation assay. Cells were washed twice with phosphate-buffered saline (PBS) and lysed by addition of a hypotonic solution (1% NP-40 in 20 mM EDTA, 50 mM Tris–HCl pH 7.5). After centrifugation at 1600*g* for 5 min, the supernatant was collected and the extraction was repeated with the same lysis buffer. The supernatants was brought to 1% SDS and treated with RNase A (final concentration 5 mg/ml) for 2 h at 56°C followed by digestion with proteinase K (final concentration 2.5 mg/ml) at 45°C for at least 6 h. Before hydrolysis, a further cleaning of DNA was performed by phenol–chloroform extraction, followed by three successive ethanol precipitations in 2 M ammonium acetate. Pellets were dried for 30 min and resuspended in 200 ml Tris–EDTA pH 8.0. Aliquots of 20 μl containing 10 mg DNA were electrophoresed in 1.5% agarose gel. Aliquots of 20 ml containing 10 mg DNA were analyzed by electrophoresis on 1.8% ethidium bromide-containing agarose gels and visualized and quantitized under UV transillumination of apparatus BioRad Gel Doc 1000 (BioRad) with the program Quantity One. Sizes of DNA fragments in the samples were compared with standard size fragments in a 1 Kb DNA ladder (Invitrogen) [30].

Measurement of intracellular ROS. For detection of intracellular ROS, cells were incubated with both sorbitol and 50 μM DCF-DA at 37°C. At each time, cells were pelletted, washed and resuspended in ice-cold PBS. The fluorescence intensities of 2′,7′-dichlorofluorescein, formed by the reaction of DCF-DA with ROS, of more than 10,000 cells from

each sample, were analyzed by recording FL-1 fluorescence by a FACScalibur instrument (Beckton and Dickinson). Prior to data collection, propidium iodide was added to the samples for gating out dead cells. Treatment with 100 μM *tert*-butylhydroperoxyde was used as a positive control. Experiments were repeated at least three times with similar results. The data are given as one representative histogram.

Determination of nitrites and nitrates in culture media. K562 cells were washed and incubated for different times with 1 M sorbitol in Hank's balanced salt solution (HBSS) containing 0.3 mM arginine. Next, the cells were pelletted at 700*g* and the medium was further centrifuged at 10,000*g* to avoid cell contamination. Nitrates produced during sorbitol treatment were previously reduced to nitrites by adding 0.1 U/ml nitrate reductase, 1 mM FAD, 0.2 mM NADPH to each sample. Total nitrites were measured by the Griess reaction, according to Schmidt et al. [31] and their concentration was determined by a standard curve obtained with known amount of sodium nitrite.

Western blot analyses. Cell pellet was resuspended in lysis buffer containing 10 mM Tris–HCl, pH 7.4, 5 mM EDTA, 150 mM NaCl, 0.5% IGEPAL CA-630, and protease inhibitors. After 30 min incubation on ice, cells centrifuged at 14,000*g* for 15 min at 4°C, and supernatants were stored at −80°C. Twenty micrograms of proteins were loaded on 10% polyacrylamide gel and transferred onto a nitrocellulose membrane (Bio-Rad). Monoclonal anti-iNOS was used as primary antibody (1:1000) (Santa Cruz Biotechnology). The specific protein complex, formed upon anti-mouse secondary antibody treatment (1:5000), was identified using Fluorchem Imaging system (AlphaInnotech–Analitica De Mori, Italy) after incubation with ChemiGlow chemiluminescence substrate (AlphaInnotech–Analitica De Mori, Italy).

Detection of protein carbonyls. Carbonylated proteins were detected using the Oxyblot Kit [Intergen (Purchase, NY)]. Briefly, 5 μg of proteins were reacted with 2,4-dinitrophenylhydrazine (DNP) for 15 min at 25°C. Samples were resolved on 10% SDS-polyacrylamide gels and DNP-derivatized proteins were identified by Western blot using an anti-DNP antibody.

Analysis of mitochondrial transmembrane potential (ΔΨm). For ΔΨm analysis, cells were incubated with 200 nM TMRE for 30 min at 37°C before the addition of sorbitol and subjected to cytofluorimetric analysis by a FACScalibur instrument. Experiments were repeated at least three times with similar results. The data are given as percentage of TMRE negative (−) cells.

Reverse transcription-polymerase chain reaction (RT-PCR) for human iNOS RNA. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA). Contaminating DNA was digested using RNAse-free DNAse I (Promega, Madison, WI). RNA was re-extracted with phenol/chloroform, precipitated with ethanol, and diluted in RNAse-free water. After reverse transcription using an oligo d(T)16 primer, human iNOS cDNA was amplified using the primer pairs 5 5 5′-CGG TGC TGT ATT TCC TTA CGA GGC GAA GAA GG-3′ and 5′-GGT GCT GCT TGT TAG GAG GTC AAG TAA AGG GC-3′. Human actin was used as control. Reagents were supplied by Perkin–Elmer. PCR products were electrophoresed in 1.2% agarose gel and bands visualized under ultraviolet (UV) light after staining with ethidium bromide.

Measurement of intracellular calcium. Prior sorbitol addition, cells were pre-loaded with 4 μM Fluo 3-AM (Molecular Probes) for 45 min at 37°C, 5% CO_2 in HBSS containing HEPES 10 mM, Ca++2 mM, and Mg++2 mM. HBSS containing 2 M with or without 6 mM EGTA was added in equal volume to Fluo 3 loaded cell suspension. 2 μM ionomycin was used as positive control. The calcium dependent fluorescence intensity was measured by cytofluorimetric analysis.

Results

Sorbitol induces oxidative stress in K562 cells

Previously we established that 1 M sorbitol was able to significantly induce apoptosis as early as 30 min of treatment via the activation of caspase-9 and -3 [30]. In the present work, we investigated the molecular mechanisms underlying the apoptotic process by focusing on the possible involvement of ROS/RNS. To this aim, we performed cytofluorimetric analysis of sorbitol treated cells stained with 2′,7′dihydro-dichlorofluorescein diacetate (DCF-DA), a probe which freely crosses the plasma membrane and emits fluorescence by reaction with a broad range of ROS and RNS [32]. As shown in Figure 1A, K562 cells treated with 1 M sorbitol exhibited a rapid and time-dependent increase of radical species levels. In fact, a shift of cell population towards higher fluorescence levels was detectable as early as 10 min of treatment. In order to verify whether sorbitol had a dose response effect on ROS/RNS production, we carried out experiments with different concentration of sorbitol in a range

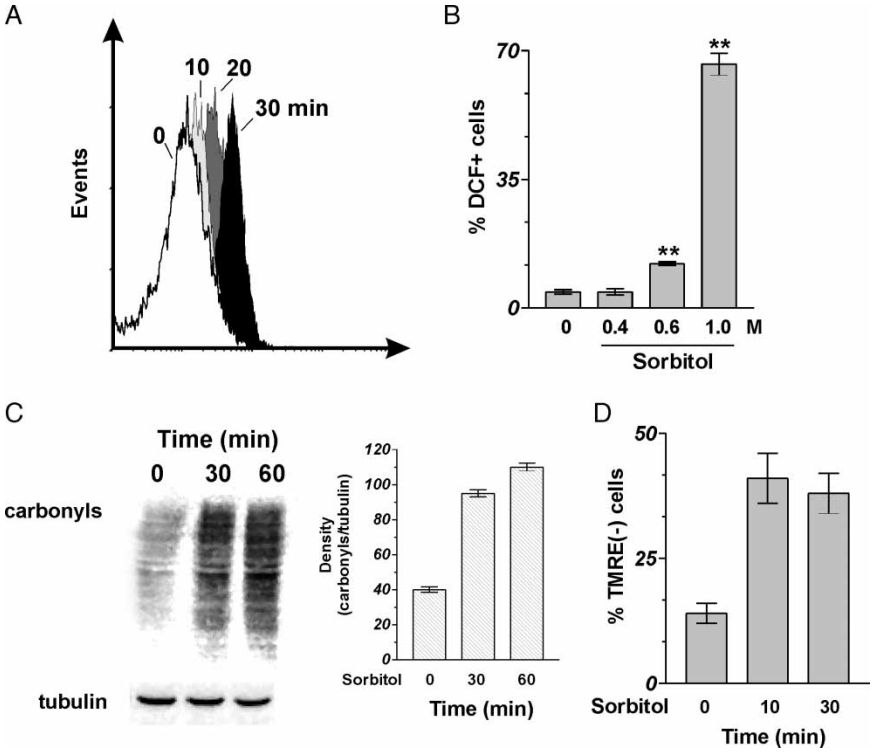

Figure 1. Sorbitol causes oxidative stress and loss of $\Delta\Psi$. A: K562 cells were treated with 1 M sorbitol for the indicated time points. The concentration of intracellular ROS/RNS production was determined by cytofluorimetric analyses following incubation with DCF-DA. Histograms reported are from one experiments of three which gave similar results. B: K562 cells were treated for 30 min at indicated concentration of sorbitol. The concentration of intracellular ROS/RNS production was determined by cytofluorimetric analyses following incubation with DCF-DA. Data are expressed as means of percentage of DCF positive cells ± SD; ($n = 3$), $^{**}p < 0.001$. C: K562 cells were treated with 1 M sorbitol for the indicated time points. Cells were lysed and protein extracts (20 μg) used for carbonyls analysis upon derivatization with DNP followed by Western blot using an anti-DNP antibody. A representative immunoblot of three that gave similar results is shown. Tubulin was used as loading control. *Bottom panel*: densitometric analysis of protein carbonyls was calculated using Quantitiy One Software (Bio-Rad) and data are shown as ratio of protein carbonyls/tubulin and reported as means ± SD; ($n = 3$). The results are statistically significant with respect to untreated cells ($p \leq 0.05$). D: K562 cells were preloaded with TMRE, treated with 1 M sorbitol for 10 and 30 min and used for citofluorimetric analyses. Data are expressed as means of percentage of TMRE negative cells ± SD; ($n = 3$). The results are statistically significant with respect to untreated cells ($p \leq 0.05$).

between 0.4 and 1 M. Figure 1B shows that sorbitol induced a significant amount of ROS/RNS starting at 0.6 M as assessed by cytofluorimetric analyses after 30 min treatment. To further investigate whether ROS/RNS production was a downstream effect of increased extracellular osmolarity we treated cells with mannitol at the same concentrations. We found that mannitol was less effective than sorbitol in inducing ROS/RNS increase at 1 M (− 40%) while no effect was observed at 0.6 M concentration.

Protein oxidation in cells is usually increased as consequence of ROS production since ROS can oxidize specific amino acids leading to the formation of oxidative modifications. We hence tested the level of protein carbonyls, one of the most frequent oxidative modifications, after 30 and 60 min of 1 M sorbitol treatment. Cells were lysed and protein extracts were derivatized with DNP that specifically reacts with carbonyl groups. The Western blot carried out with an anti-DNP antibody revealed that the level of protein carbonyls significantly increased starting from 30 min with a higher degree at 60 min (Figure 1C), suggesting that ROS induced by sorbitol treatment affected protein integrity.

ROS increase can be regarded both as a causative or downstream factor of the impairment of mitochondrial integrity and function, typical of the apoptotic process [33]. Therefore, we investigate the role of ROS production by analyzing the mitochondrial function in terms of transmembrane potential ($\Delta\Psi$m) perturbation. Figure 1D shows cytofluorimetric histograms of K562 cells preloaded with the fluorescent probe tetramethyl rodamine-ethyl ester (TMRE), which can enter the cell and specifically localize within viable mitochondria, thus responding to $\Delta\Psi$m alteration. Figure 1D shows that TMRE fluorescence decreased as early as 10 min after treatment with no further variations, indicating that impairment of $\Delta\Psi$m was concomitant with respect to ROS/RNS increase.

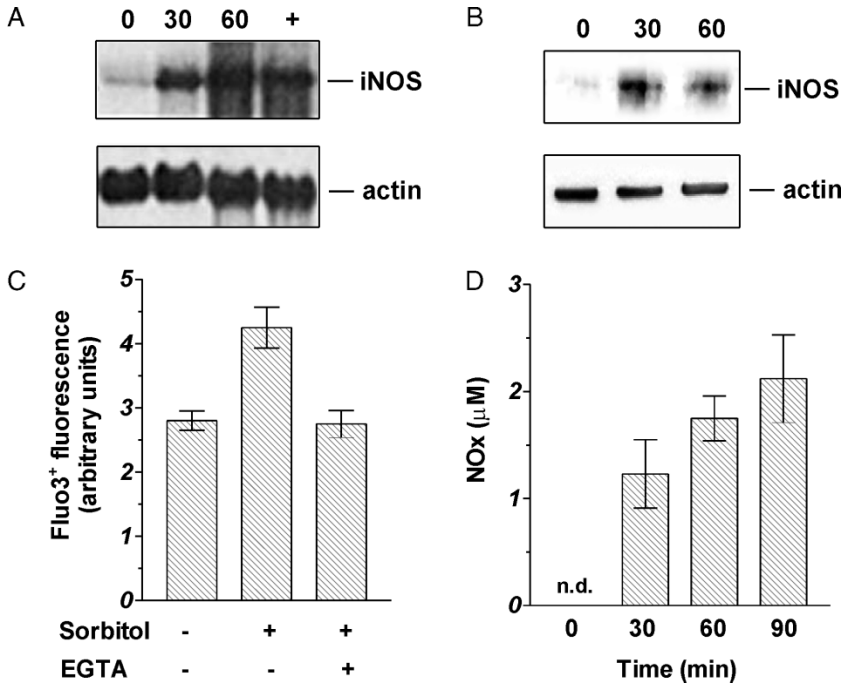

Figure 2. Sorbitol induces the expression of iNOS and the increase of NO production. A: K562 cells were treated with 1 M sorbitol for the indicated time points. iNOS RNA content was evaluated by RT-PCR as described under Materials and methods section. Human actin and total RNA extracted by human A459 cells (+) were used as controls. B: Cells were lysed and protein extracts used for Western blot analysis of iNOS protein with a monoclonal antibody. Actin was used as loading control. Immunoblot reported is representative of three which gave similar results. C: Cells were preloaded with Fluo3, treated with sorbitol and used for citofluorimetric analyses by recording fluorescence after 1 min from sorbitol addition. Data shown are reported as means ± SD; ($n = 3$). The results are statistically significant with respect to untreated cells ($p \leq 0.05$). D: Nitrite and nitrates (NOx) released in the culture media were assayed by Griess reaction as described under Material and Methods section. Data are expressed as means ± SD; ($n = 6$). All the results are statistically significant with respect to untreated cells ($p < 0.001$).

Sorbitol induces nitrosative stress in K562 cells

To assess whether also RNS could contribute to the increase of radical species we investigated whether sorbitol treatment could elicit a raise in the protein level of inducible NOS (iNOS). Using RT-PCR we found an increase in the content of iNOS mRNA already at 30 min from sorbitol treatment (Figure 2A). The up-regulation of iNOS expression was confirmed by the parallel increase in the content of iNOS protein as determined by Western blot analysis (Figure 2B). We then examined whether the up-regulation of iNOS was associated with the increase of intracellular calcium, which usually precedes the raise of iNOS expression and activity [34,35]. Intracellular calcium was determined cytofluorimetrically after labelling the cells with the specific probe FLUO-3. As shown in Figure 2C, sorbitol treatment resulted in a rapid raise in intracellular calcium within 1 min. One hour pre-treatment with EGTA, an extracellular calcium chelator, completely abolish the increase of intracellular calcium suggesting a mobilization of the ion from extracellular milieu. Finally, the effective increase of iNOS activity was checked by measuring nitrites and nitrates in the culture medium. These metabolites were under the detection limit of the method in untreated cells, while they were easily measurable after sorbitol treatment (Figure 2D). In particular, Figure 2D shows that sorbitol induced a time-dependent increase of nitrite and nitrates starting from 30 min of sorbitol treatment. Moreover, mannitol, at the same concentrations, did not increase the level of nitrite and nitrate (data not shown).

Inhibition of ROS and RNS productions limits sorbitol induced apoptosis

As hyperosmotic stress caused by 1 M sorbitol treatment seemed to induce a massive production of both ROS and RNS, we perform experiments in order to clarify whether oxidative and/or nitrosative stress was the cause or a side-effect of sorbitol-induced apoptosis. To this end, K562 cells were pre-treated with different antioxidants before sorbitol addition. Cell viability was checked by direct counts upon Trypan blue staining and production of ROS/RNS was evaluated by cytofluorimetric analyses upon DCF-DA treatment. Figure 3A shows that 5 mM *N*-acetyl cysteine (NAC), which should increase the intracellular antioxidant thiol pool, or 20 μM diphenyl iodonium (DPI), which inhibits the membrane-associated NADPH oxidases, were able to partially decrease the ROS/RNS levels and to significantly protect cells from sorbitol-induced cell death. To investigate the contribute of RNS in sorbitol-induced apoptosis,

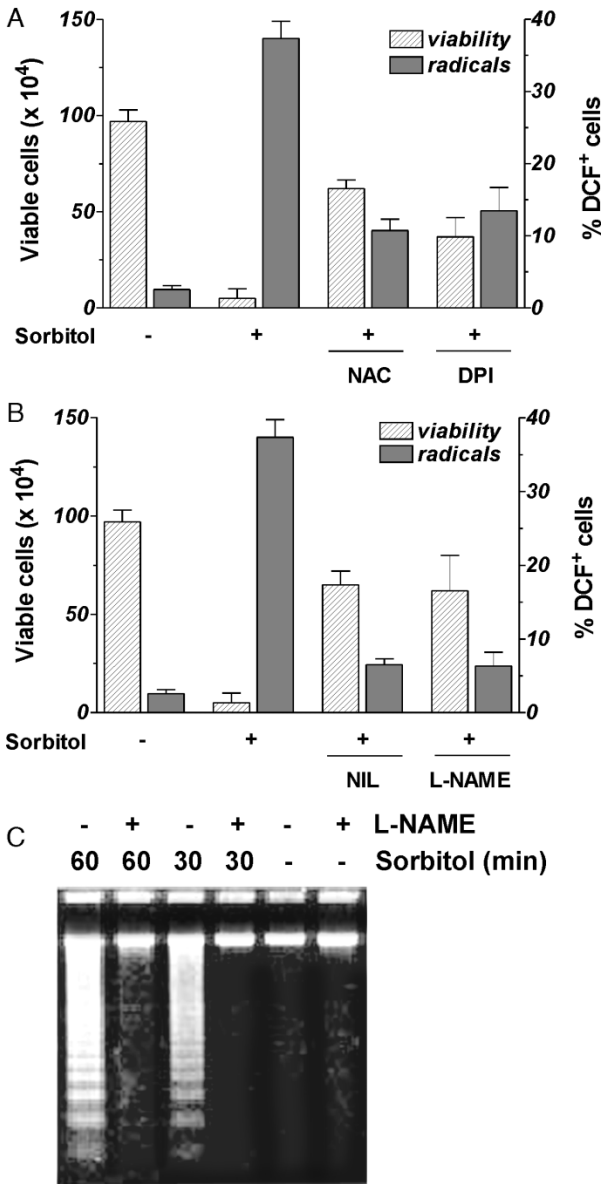

Figure 3. Sorbitol-induced apoptosis was inhibited by antioxidants and NOS inhibitors. K562 cells were treated with 1 M sorbitol for 60 min. NAC or DPI (A) and NIL or L-NAME (B) were added 30 min prior sorbitol addition and maintained throughout the experiment. Viable cells were evaluated by cell counting after Trypan blue staining. The concentration of intracellular ROS/RNS production was determined by cytofluorimetric analyses following incubation with DCF-DA. Data are expressed as means ± SD. Results obtained with anti-oxidants or NOS inhibitors are statistically significant with respect to sorbitol-treated cells ($p \leq 0.05$). C: K562 cells were treated with 1 M sorbitol for the indicated time points. L-NAME was added 30 min prior sorbitol addition and maintained throughout the experiment. DNA fragmentation assay was carried out as described under Materials and methods section. The agarose gel reported is representative of three which gave similar results.

we carried out pre-incubations with two specific NOS inhibitors, L-N6-(1-iminoethyl) lysine hydrochloride (NIL) and 100 μM N^{ω}-nitro-L-arginine methyl ester (L-NAME). Figure 3B shows that both 50 μM NIL and 100 μM L-NAME were able to inhibit cell death more efficiently than DPI and NAC, suggesting a prominent role for RNS in triggering sorbitol-induced apoptosis. This hypothesis was reinforced by the results of experiments carried out with L-NAME, where an almost complete prevention of chromosomal DNA fragmentation was observed at both 30 and 60 min (Figure 3C).

Rosmarinic acid inhibits sorbitol-induced apoptosis

Due to the evidence that ROS and RNS were concomitantly produced in the sorbitol-induced apoptosis, we analyzed whether antioxidants, such as polyphenols were able to counteract sorbitol toxicity. Since rosmarinic acid was found to be a potent inhibitor of both cellular ROS-producing enzymes [36], e.g. lipoxygenase [37] and cyclooxygenase [38,39], and NOS [40,41], as well as to interfere with DNA fragmentation mediated by sorbitol [30], we treated K562 cells with 25 μM rosmarinic acid for 1 h before incubation with 1 M sorbitol. Figure 4A shows that the rate of cell viability under these conditions, almost matched the control values, as well as the percentage of DCF positive cells (Figure 4A), suggesting that this compound was able to completely counteract hyperosmotic-mediated ROS/RNS production and apoptosis. These data were also paralleled by the preservation of $\Delta\Psi m$ in the presence of rosmarinic acid. Figure 4B shows the results of cytofluorimetric analyses of K562 cells stained with TMRE and pre-treated with 25 μM rosmarinic acid, which completely inhibited sorbitol-mediated mitochondrial impairment.

Discussion

The present study attempts to extend our previous observations that sorbitol mediates the induction of apoptosis by a mechanism independent on hypertonicity in cultured chronic myelogenous leukemia cells [30]. We identified ROS/RNS as the upstream mediators of the activation of apoptosis in response to sorbitol treatment and found that exposure of K562 cells to sorbitol induces a rapid increase in the concentration of radical species. The synergistic role played by ROS and RNS in committing cell death was demonstrated by experiments carried out in the presence of general ROS scavengers or NOS inhibitors. In fact, the sulfhydryls-containing compound NAC and the inhibitor of flavo-enzymes DPI, usually considered efficient in lowering ROS production, significantly rescued cells from sorbitol-induced toxicity and buffered the increase of DCF positive cells. The important contribute of ROS in committing cell death was also demonstrated by the increase of oxidative damage to proteins, in terms of carbonyls, after sorbitol treatment. Furthermore, a partial inhibition of sorbitol toxic effects was also observed using NIL

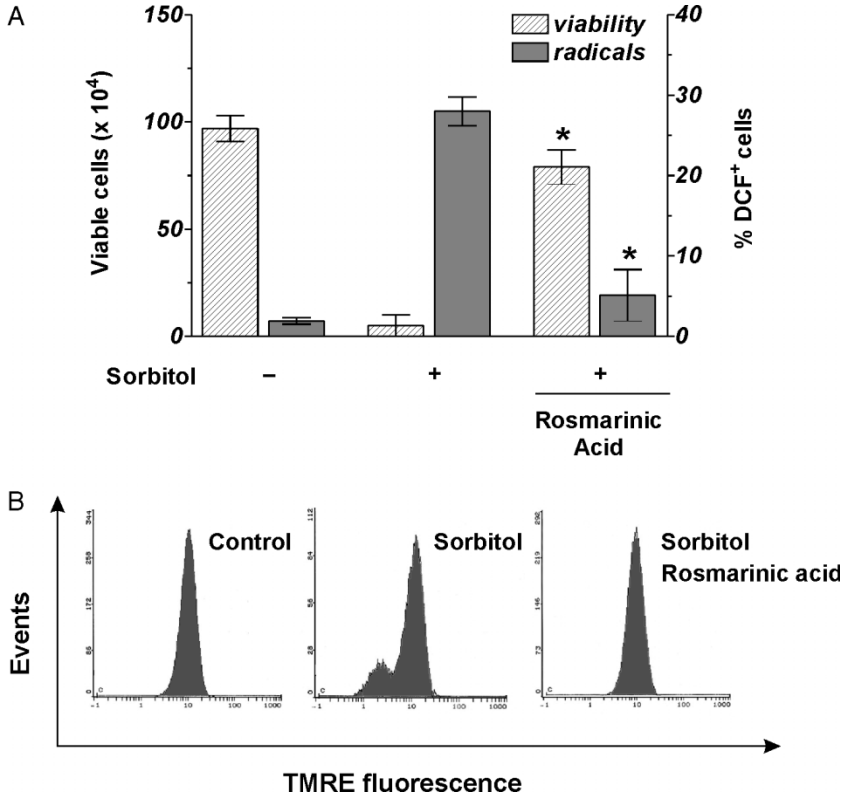

Figure 4. Sorbitol cytotoxicity was inhibited by rosmarinic acid. A: K562 cells were treated with 1 M sorbitol for 60 min. Rosmarinic acid was added 30 min prior to sorbitol addition and maintained throughout the experiment. Viable cells were evaluated by cell counting after Trypan blue staining. The concentration of intracellular ROS/RNS production was determined by cytofluorimetric analyses following incubation with DCF-DA. Data are expressed as means ± SD ($n = 3$). Results obtained with rosmarinic acid are statistically significant with respect to sorbitol-treated cells ($p \leq 0.05$). B: Cells were preloaded with TMRE, treated with sorbitol for 30 min and used for citofluorimetric analyses. Rosmarinic acid was added 30 min prior sorbitol addition and maintained throughout the experiment. Histograms reported are representative of three experiments which gave similar results.

and L-NAME, two specific NOS inhibitors, demonstrating that RNS participates in the process leading to apoptosis. In fact, NOS seems to be highly implicated in sorbitol-induced stress as a significant and rapid increase in the level of nitrites and nitrates was detected after sorbitol supplementation. Such increase reasonably derives from the induction of iNOS expression as stated both by RT-PCR and Western blot analysis. Another important aspect that has emerged is that the intracellular pool of calcium rapidly increases in the first minute after sorbitol addition being mobilized from the extracellular milieu. Our data are in agreement with other works, dealing with hyperosmotic shock, where an increase of intracellular calcium was induced by high dose of sorbitol or glucose [42,43]. Although iNOS has traditionally been considered as calcium independent, some data suggest that the elevation in intracellular calcium regulates iNOS mRNA and end-product synthesis [34,35]. Therefore, the elevation of intracellular calcium may contribute to the up-regulation of iNOS and the increased production of RNS observed after sorbitol supplementation.

Moreover, the data obtained with hyperosmotic concentrations of mannitol, which demonstrate that it was unable to induce nitrite and nitrate formation and less effective in inducing ROS, is further evidence that ROS/RNS production is the crucial event for cell death commitment under sorbitol treatment. On the other hand, although K562 cells were treated with different hyperosmotic concentrations of sorbitol (0.4–1 M), DNA fragmentation occurs only upon treatment with 0.6 M sorbitol and exponentially increases up to 1 M [30], mirroring the ROS/RNS production. These results suggest that the plasma membrane could represent at the same time the sensor and the site of production of ROS/RNS in response to hyperosmotic shock caused, particularly, by sorbitol. The capacity of sorbitol to more efficiently activate production of ROS/RNS than mannitol is currently under investigation in our laboratory.

Among producers of radical species, good candidates could be the NADPH-oxidase and NOS, for which activation and consequent induction of oxidative/nitrosative stress has been demonstrated upon hyperosmotic conditions in human hepatocytes and murine macrophages, respectively [44,45]. Furthermore, from the data obtained in this work mitochondria could also be involved in the production of ROS at least at early times of treatment. In fact, $\Delta\Psi m$ falls early but remains at low values throughout the experimental time,

whereas ROS/RNS proportionally increases, suggesting that $\Delta\Psi m$ loss could be a direct effect of sorbitol-induced osmotic changes. However, upon sorbitol treatment, mitochondria represent the site where the execution of apoptosis takes place. This is in line with our previous results where we demonstrated that the cleavage of procaspase 9 was effective at 60 min after sorbitol treatment [30]. Moreover, unpublished data from our laboratory also demonstrated that Bcl-X_L down-regulation and Bax up-regulation together with the release of cytochrome c from mitochondria were operative in the early phases (30 min) of sorbitol-induced apoptosis.

The final interesting finding of the present work was the identification of rosmarinic acid as a potent scavenger of both ROS and RNS, and an effective inhibitor of sorbitol-induced apoptosis. In fact, pre-treatment with rosmarinic acid totally prevented increase of DCF-positive cells and $\Delta\Psi m$ failure, thus reinforcing the hypothesis of a radical-mediated cell death.

In conclusion, the ability of antioxidants or NOS inhibitor to counteract the apoptotic process in the presence of hyperosmotic conditions points to a causative role for oxidative/nitrosative stress in sorbitol-mediated apoptosis.

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