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Dysregulated Calcium Homeostasis and Oxidative Stress in Chronic Myeloid Leukemia (CML) Cells

ROBERTO CIARCIA,[1] DANILA d'ANGELO,[1] CARMEN PACILIO,[3] DAVID PAGNINI,[4] MASSIMILIANO GALDIERO,[2] FILOMENA FIORITO,[2] SARA DAMIANO,[1] ELISEO MATTIOLI,[7] CHIARA LUCCHETTI,[6] SALVATORE FLORIO,[1]* AND ANTONIO GIORDANO[6,7]

[1] *Department of Structures, Functions and Biological Technologies, School of Veterinary Medicine, University of Naples "Federico II", Naples, Italy*

[2] *Department of Pathology and Animal Health, School of Veterinary, Medicine University of Naples "Federico II", Naples, Italy*

[3] *Department of Medical Oncology C INT, Foundation G. Pascale, Naples, Italy*

[4] *Director U.O. Haemathology, ASL Caserta (CE), Hospital Praesidium, San Felice a Cancello, Caserta, Italy*

[5] *Department of Experimental Medicine, Section of Microbiology and Clinical Microbiology, Second University of Naples, Naples, Italy*

[6] *Sbarro Institute for Cancer Research and Molecular Medicine, College of Science and Technology, Temple University, Philadelphia, Pennsylvania*

[7] *Department of Human Pathology & Oncology, University of Siena, Siena, Italy*

Chronic myeloid leukemia (CML) is a hematopoietic stem cell disorder caused by the oncogenic activity of the Bcr-Abl protein, a deregulated tyrosine kinase. Calcium may act directly on cellular enzymes and in conjunction with other cellular metabolites, such as cyclic nucleotides, to regulate cell functions. Alteration in the ionized calcium concentration in the cytosol has been implicated in the initiation of secretion, contraction, and cell proliferation as well as the production of reactive oxygen species (ROS) has been correlates with normal cell proliferation through activation of growth-related signaling pathways. In this study we evaluated in peripheral blood leukocytes from CML patients the role of the balance between intracellular calcium and oxidative stress in CML disease in order to identify possible therapeutic targets in patients affected by this pathology. Our results demonstrated that peripheral blood mononuclear cells derived from CML patients displayed decreased intracellular calcium $[Ca^{2+}]_i$ fluxes both after $InsP_3$ as well as ATP and ionomycin (IONO) administration. CML cells showed lower levels of superoxide dismutase (SOD) activity and significantly higher malondialdehyde levels (MDA) than peripheral blood mononuclear cells derived from control patients. Finally we showed that resveratrol is able to down-regulate InsP3 and ATP effects on intracellular calcium $[Ca^{2+}]_i$ fluxes as well as the effects of ATP and IONO on oxidative stress in CML cells.

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Chronic myeloid leukemia (CML) is characterized by the balanced reciprocal translocation between chromosomes 9 and 22 t(9:22)(q34:q11) giving rise to the Philadelphia chromosome (Deininger et al., 2000). The Bcr-Abl chimeric protein is thought to play a central role in the pathogenesis of Philadelphia (Ph) chromosome-positive leukemias (Sawyers, 1999; Deininger et al., 2000). Three major mechanisms have been implicated in the malignant transformation by Bcr-Abl, namely altered adhesion to stroma cells and extracellular matrix (Gordon et al., 1987), constitutively active mitogenic signaling and reduced apoptosis (Bedi et al., 1994). A fourth possible mechanism is the recently described proteasome-mediated degradation of Abl inhibitory proteins (Dai et al., 1998). Attempts at designing therapeutic tools for CML based on our current knowledge of the molecular and cell biology of the disease have concentrated on three main areas: the inhibition of gene expression at the translational level by "antisense" strategies, the stimulation of the immune system's capacity to recognize and destroy leukemic cells, and the modulation of protein function by specific signal transduction inhibitors (STI). Perhaps the most exciting of the molecularly designed therapeutic approaches was brought about by the advent of STI, which block or prevent a protein from exerting its role in the oncogenic pathway.

It has been reported that calcium plays an important role in the regulation of growth and functions of many kinds of cells (Hennings et al., 1980; Ljchtman et al., 1983). Among the processes in which calcium plays a significant role two opposite determinants of cell life are found: proliferation and apoptosis. Cellular calcium homeostasis is an important factor regulating the switch points of the cell cycle (Whitaker and Patel, 1990). Particularly the ER calcium homeostasis is crucial for cell growth (Waldron et al., 1997). The abnormal proliferation of

*Correspondence to: Salvatore Florio, Department of Structures, Functions and Biological Technologies, School of Veterinary Medicine, University of Naples "Federico II", Via Delpino 1, 80137 Napoli, Italy. E-mail: florio@unina.it

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some cancer cell lines is connected with strongly elevated $InsP_3$ levels and over expression of PLC which must influence calcium signaling, especially calcium release from the ER stores (Berridge, 1995). Being connected with the nuclear envelope, ER can relay the changes of calcium balance to the nucleus. As a result, changes of calcium level in the ER and cytosol are closely tied to nuclear calcium homeostasis (Roche and Prentki, 1994). Strong evidence can be found for store-operated calcium entry (SOCE) importance in calcium signaling regulating the cell division (Golovina et al., 2001). The involvement of SOCE in cell proliferation has been studied in detail in Jurkat T cells. In these cells, T cell receptor stimulation induces sustained SOCE through the Ca^{2+} release-activated current (CRAC) resulting in sustained activation of the nuclear factor of activated T cells (NFAT) that promotes proliferation (Lewis, 2001). Many observations in this and other cell models relate SOCE and cell proliferation: (1) entry in cell cycle is preceded by SOC activation (Sugioka and Yamashita, 2003); (2) growth factor stimulation of cell proliferation is accompanied by increased activity and/or expression of transient receptor potential (TRP) channels that are related to SOCE (Golovina et al., 2001); (3) SOCE inhibition by different means (Ca^{2+} removal, inorganic channel blockers, and CRAC antagonists) abolishes tumor cell proliferation (Weiss et al., 2001; Zitt et al., 2004). Two decades after the discovery SOCE (Putney, 1986) the link between emptying of intracellular Ca^{2+} stores and the increased Ca^{2+} influx is still unknown (Parekh and Putney, 2005).

Calcium may act directly on cellular enzymes and in conjunction with other cellular metabolites, such as cyclic nucleotides, to regulate cell functions. Alteration in the ionized calcium concentration in the cytosol has been implicated in the initiation of secretion, contraction, and cell proliferation (Rasmussen and Goodman, 1977; Kretsinger, 1979) as well as the production of reactive oxygen species (ROS), natural by-products of aerobic metabolism, has been correlates with normal cell proliferation through activation of growth-related signaling pathways (Murrell et al., 1990). Indeed, exposure to low levels of ROS can increase growth of many types of mammalian cells, whereas scavengers of ROS suppress normal cell proliferation in human and rodent fibroblasts (Pluthero and Axelrad, 1991; Sundaresan et al., 1995; Bae et al., 1997). Furthermore, growth factors trigger hydrogen peroxide (H_2O_2) production that leads to mitogen-activated protein kinase activation and DNA synthesis, a phenomenon inhibited by antioxidant molecules (Gupta et al., 1999; Jackson and Loeb, 2001). Several observations suggest that ROS also participate in carcinogenesis. First, ROS production is increased in cancer cells and an oxidative stress can induce DNA damages that lead to genomic instability and possibly stimulate cancer progression (Suh et al., 1999). Second, elevated ROS levels are responsible for constant activation of transcription factors, such as nuclear factor κB and activator protein 1, during tumor progression (Arnold et al., 2001). Finally, the transforming capacity of ROS is illustrated by the oncogenic transformation of NIH 3T3 cells by the NADPH oxidase homologue MOX-1, which induces the production of superoxide anions O^{2-} (Putney, 1990; Putney et al., 2001). Recently Chakraborty et al. (2008) demonstrated that resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) a potent antioxidant non-toxic, plant-derived polyphenol, could considerably enhance the apoptosis induction in K562 cells by 17-allylamino-17-demethoxygeldanamycin, an anticancer agent that inhibits Hsp90 but augments Hsp70 levels. Our previous studies demonstrated in chronic lymphatic leukemia cells that a nitric oxide–calcium overload may play a key role to modulate antitumoral activity of anthracyclines (Pagnini et al., 2000) and, in rat cardiomyocytes, that Cyclosporin A toxicity is due to a calcium overload, which in turn induce oxidative stress-induced cell injury (Florio et al., 2003)

In this study we evaluated in peripheral blood leukocytes from CML patients the role of the balance between intracellular calcium and oxidative stress in CML disease in order to identify possible therapeutic targets in patients affected by this pathology.

Materials and Methods

Chemicals

Adenosine triphosphate (ATP), ionomicyn (IONO), resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) (RESV), phitoemoagglutinine (PHA), RPMI-1640, fetal calf serum (FCS), hystopaque, propidium iodide (PI) were obtained from SIGMA (Milan, Italy).

Leukocytes preparation

Heparinized blood was obtained from patients affected by CML of first diagnosis. Peripheral blood samples were obtained from five patients (three males and two females; mean age 54 ± 15 years; range 29–62 years) diagnosed with CML and five control subjects (three males and two females; mean age 41 ± 9 years; range 27–51 years) were selected. Eligible patients have been diagnosed with Ph^{+} CML-CP (cytogenetic analysis showed that Ph chromosome were 100% of positive). Chronic phase was defined by the presence of $<15\%$ blasts, $<20\%$ basophils, and $<30\%$ blasts plus promyelocytes in the peripheral blood or bone marrow. Leukocytes were isolated using the modification of a method of Boyum as described (Bass et al., 1978). Briefly, 10 ml blood samples were layered over hystopaque (density 1.077) gradient centrifugation at 600*g* per 30 min at room temperature. The ring of leukocytes at the interface was collected and washed two times with 5 ml of PBS. The preparation was found to be 89% pure (range 84–91%; red blood cells 6.1%; polymorphonucleates 2.6%; platelets 2.1%). Cells were cultured in RPMI 1640 containing 10% FCS, 1% penicillin and streptomycin, 2 mM glutamine, at 37°C in humidified atmosphere of 5% carbon dioxide in air. Leukocytes were stimulated with 10 nM L-PHA and observed daily used Nikon Diaphot Inverted microscope. The culture medium was changed every 3 days and the cultured cells replaced with newly/freshly isolated CML cells every 12 days. Viable cells (as assayed by means of the Trypan blue exclusion test) were seeded at 3×10^5 cells in 6-well tissue culture plates.

Intracellular calcium measurement

Intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ were measured by using the radiometric fluorescent indicator dye FURA-2/AM, the membrane-permeant form of FURA-2/AM as previously described and opportunely modified (Pagnini et al., 2000). Briefly, CML cells were washed twice in PBS in 15 ml polypropylene tubes (Falcon/Becton Dickinson Labware, Lincoln Park, NJ) resuspended in Krebs Ringer (KRH) medium (1×10^7 cells/ml) with 3.0 μM Ca^{2+}-sensitive dye FURA-2/AM for 60 min at 30°C. We also added the non-ionic detergent pluronic acid F-127 (0.02%, w/v) to each incubation batch a dispersing agent. Cell were subsequently centrifuged and resuspended in KRH containing 1 mM $CaCl_2$ or not. The samples were then transferred to a thermostat-equipped cuvette (37°C), maintained under continuous stirring and analyzed in a SPEX spectrophotofluorimeter (two wavelengths excitations, 340 and 380 nm and emission intensities at 505 nm). The experiments were initiated by incubation of cell aliquots ($0.5–1 \times 10^6$ cells) with several drugs for 5 min. After stimulation with ATP or IONO or $InsP_3$, the recording was continued until the end of the $[Ca^{2+}]_i$ peak. At the end of the experiment calibration was performed determining minimal fluorescence induced by 0.1% Triton X-100 in presence of 5 mM EGTA (F_{min}) and maximal fluorescence induced by 3 mM $CaCl_2$ (F_{max}). Intracellular calcium concentration was calculated according to the method of

Grynkiewicz et al. (1985) of the equation:

$$[Ca^{2+}]_i = 224 \times [(R - R_{min})/(R_{max} - R)]$$

where R is the experimentally determined fluorescence ratio. Graphs show the mean ± standard deviation values of 3–5 experiments in duplicate.

Nitrite and nitrate assay

During the experiments performed to evaluate NO and MDA production and SOD activity cells were treated for 24 h with medium containing ATP (at a final concentration of 1–150 μM), IONO (at a final concentration of 1–150 μM), InsP3 (at a final concentration of 1–10 μM) and RESV (at a final concentration of 1–150 μM) alone or with 20 μM RESV associated to 100 μM ATP or 100 μM IONO or 5 μM InsP3. Control cultures received fresh medium and were incubated in parallel.

The production of nitrite (NO_2) and nitrate (NO_3), stable metabolites of nitric oxide (NO) production, was determined in the supernatant of cells incubated by Griess reagent (Chandler et al., 1995), which contained 1 part 0.75% sulphanilamide in 0.5 N H_3PO_4 to 1 part 0.75% *N*-(1-naphtyl)-ethylendiamine dihydrochloride in water. Nitrate was reduced at nitrite by addition nitrate reductase 0.4 U/ml in the presence of 10 mM NADPH and 2.5 mM FAD and then assayed as Nitrite. The plates were incubated with the Griess reagent at 25°C under reduced light for 20 min. Absorbance was read at 550 nm using a Perkin Elmer U.V. Spectrophotometer. The concentration of NO_2 was calculated on a calibration curve (range 0.125–16γ/ml), prepared using dilutions of sodium nitrate in the plating medium. 1×10^5 cells were treated or non-treated for 24 h with ATP (range 10–150 μM) or IONO (range 1–100 μM) or RESV (range 1–100 μM). The data were expressed as pmol of nitrite/mg proteins/24 h. The sensitivity of the assay was 0.07γ/ml.

Lipid peroxidation assay

Lipid peroxidation as index of the oxidative stress was determined by assaying the malondialdehyde (MDA) production by means of the thiobarbituric acid (TBA) test (Esterbauer and Cheeseman, 1990). Briefly: 1 ml LMC cells suspension containing 1×10^6 cells treated or non-treated for 24 h with ATP or IONO or RES (range 2–150 μM) was mixed with 0.5 ml of cold 30% (w/v) trichloroacetic acid to precipitate proteins. The precipitate was pelletted by centrifugation and 1 ml of the supernatant was reacted with 1.3 ml of 0.5% (w/v) TBA at 85°C for 40 min. In TBA test reaction, one molecule of MDA reacts with two molecules of TBA with the production of a pink pigment having maximal absorbance at 532–535 nm. Therefore, after cooling, the fluorescence was read at an excitation of 536 nm and an emission of 557 nm wavelengths in a SPEX Fluoromax spectrophotofluorimeter. The concentration of MDA was calculated respect to a calibration curve (range 0.5–2 pmol/ml), and results were expressed as pmol of MDA/mg proteins/24 h.

SOD assay

This assay for superoxide dismutase (SOD) activity involves inhibition of nitroblue tetrazolium (NBT) reduction with xanthine–xanthine oxidase used as a superoxide generator (Sun et al., 1988). SOD is one of the most important antioxidative enzymes. It catalyzes the dismutation of the superoxide anion into hydrogen peroxide and molecular oxygen.

SOD activity was determined by the sensitive SOD assay that utilizes a product a water-soluble formazan dye upon reduction with superoxide anion SOD Activity with absorbance at 450 nm (SOD Assay Kit-WST, Fluka Analytical, Sigma-Aldrich). Briefly, 1 ml control or CML cells suspension containing 1×10^6 cells treated or not treated for 24 h with 1–150 μM of ATP or IONO or RESV was mixed. At the end of incubation cells were lysed and centrifuged at 14,000*g* for 5 min at 4°C and on cytosol was determined cytosolic and mitochondrial SOD activity. The starter of reaction by adding xanthine solution. The absorbance readings every minute for 10 min at room temperature. The rate of the reduction with a superoxide anion is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD. The SOD activity was expressed as % of inhibition respect to activity control. The % inhibition of the rate of increase in absorbance is calculated as follows: % Inhibition = (Slope of Activity Control − Slope of Sample) × 100. The amount of SOD that inhibited NBT reduction 50% was defined to be 1 unit of enzyme activity.

Total protein assay

Total proteins were determined by the method of Lowry et al. (1951).

Statistical analysis

Data are expressed as mean ± standard deviation of 3–5 independent determinations performed in duplicate. An error probability with $P < 0.05$ was selected as significant. All experiments were performed in duplicate and the mean was used for analysis. ED_{50} was calculated according to Cheng and Prusoff (1973). ED_{50} = concentration with 50% efficacy.

Results

Intracellular calcium

In a first series of experiments we evaluated intracellular calcium homeostasis in leukocytes from CML or healthy patients. Thus we used InsP3, a selective receptorial stimulus for depletion of intracellular Ca^{2+} store, or ATP, a selective purinergic stimulus for extracellular elicit intracellular Ca^{2+} mobilization or ionomycin (IONO), a potent and selective Ca^{2+} ionophore caused by direct stimulation of store-regulated cation entry and not by a direct action at the plasma membrane.

Figure 1 shows the results obtained using $InsP_3$ on intracellular calcium concentration $[Ca^{2+}]_i$. Peripheral blood mononuclear cells (10^5 cells) from both CML as well as healthy patients were loaded with FURA-2/AM and balanced for 10 min in a calcium-free medium (part A) or in a 1 mM $CaCl_2$ medium (part B) then treated with $InsP_3$ (0.1–10 μM). $InsP_3$ increased intracellular calcium concentration $[Ca^{2+}]_i$ both trough the depletion of the intracellular calcium stores (A) then Ca^{2+} influx (B) in a dose-dependent manner with a significant lower effect in CML than Control cells.

In fact, InsP3 at the dose of 5 μM produced the maximum effect on $[Ca^{2+}]_i$ with a concentration of 410.2 ± 31.2 nM and 661.6 ± 45.9 nM in CML and Control cells, respectively, when balanced in a calcium-free medium and of 567.2 ± 52.7 and 833.7 ± 87.4 nM in CML and Control cells, respectively, when balanced in a 1 mM $CaCl_2$ medium.

The ED_{50} of InsP3 obtained with calcium-free medium was 3.9 ± 0.63 μM in CML cells and 1.8 ± 0.22 μM in Control cells (*P* vs. control cells <0.05). In cells balanced with 1 mM $CaCl_2$ medium the ED_{50} was 4.5 ± 0.65 and 2.1 ± 0.19 μM in CML and Control cells, respectively (*P* vs. control cells <0.05).

The results obtained using extracellular ATP at doses ranging between 5 and 150 μM on intracellular calcium concentration $[Ca^{2+}]_i$ are shown in Figure 2. Peripheral blood mononuclear cells (10^5 cells) from both CML as well as healthy patients were loaded with FURA-2/AM and balanced for 10 min in a calcium-free medium (part A) or in a 1 mM $CaCl_2$ medium (part B) then treated with ATP (5–150 μM).

Our results show that, in cells balanced in a calcium-free medium, ATP increased intracellular calcium concentration $[Ca^{2+}]_i$ only starting from the concentration 50 μM both in CML and Control cells (A) whereas, in cells balanced with 1 mM $CaCl_2$ medium, ATP was able to increase intracellular calcium

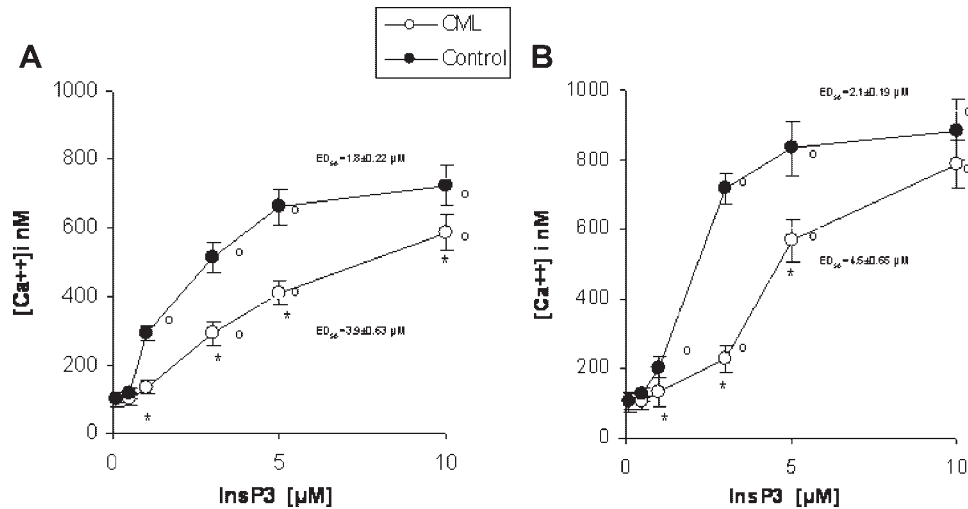


Fig. 1. Effect of increasing concentration of InsP3 used alone on calcium release from intracellular stores (KRH medium without 1 mM Ca^{2+} in presence of 10 µM EGTA) (part A) or calcium entry (KRH medium containing 1 mM Ca^{2+} from the extracellular space) (part B) of lymphocytes of CML or Control patients. Data represent the $[Ca^{2+}]_i$ values (mean ± SD) obtained 3–5 distinct experiments performed in duplicate. *$P < 0.05$ versus control cells. °$P < 0.05$ versus $[Ca^{2+}]_i$ basal levels.

concentration starting from the concentration 10 and 5 µM in CML and Control cells, respectively (B).

The percentage of increase in $[Ca^{2+}]_i$ obtained in cells balanced with a calcium-free medium, using the maximum dose of 150 µM of ATP was of 98.7% in CML cells and 147.3% in Control cells (*P* vs. control cells < 0.05) and 197.3% in CML cells and 273.3% in Control cells (*P* vs. control cells < 0.05) in cells balanced with 1 mM $CaCl_2$. The DE_{50} of ATP in cells balanced with calcium-free medium was 58.9 ± 11.2 µM in CML cells and 39.2 ± 5.8 µM in Control cells (*P* vs. control cells < 0.05) and 31.7 ± 3.1 µM in CML and 9.8 ± 1.6 µM in Control cells in presence of 1 mM $CaCl_2$ medium (*P* vs. control cells <0.05).

Figure 3 shows the results obtained using IONO on intracellular calcium concentration $[Ca^{2+}]_i$. Peripheral blood mononuclear cells (10^5 cells) from both CML as well as healthy patients were loaded with FURA-2/AM and balanced for 10 min in a calcium-free medium (part A) or in a 1 mM $CaCl_2$ medium (part B) then treated with IONO at doses ranging between 5 and 150 µM. IONO was able to mobilize intracellular calcium both trough the depletion of the intracellular calcium stores (A) then Ca^{2+} influx (B) in a dose-dependent manner with a similar effect in CML and in control cells. In fact, the DE_{50} of IONO obtained in cells incubated in a calcium-free medium (A) was 11.4 ± 2.4 µM in CML cells and of 8.7 ± 2.7 µM in Control cells. The results reported in part B show that IONO has a similar

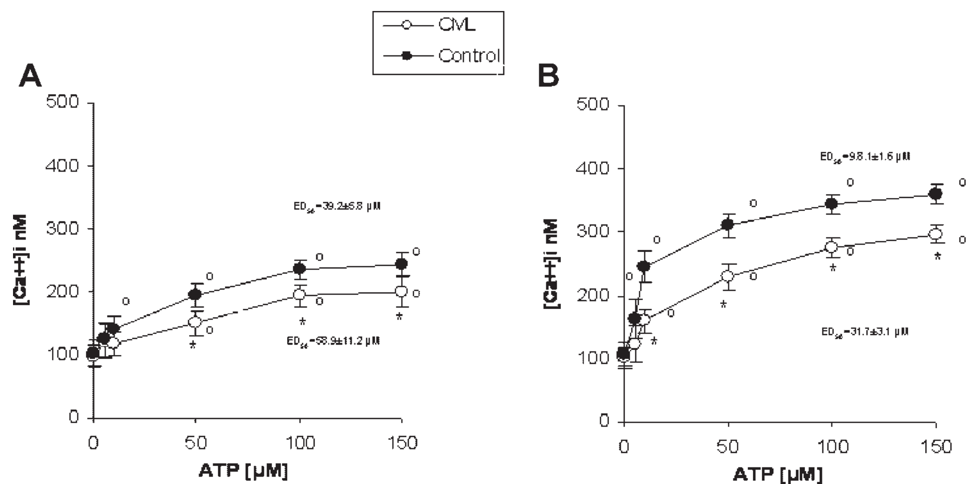


Fig. 2. Effect of increasing concentration of ATP used alone on calcium release from intracellular stores (KRH medium without 1 mM Ca^{2+} in presence of 10 µM EGTA) (part A) or calcium entry (KRH medium containing 1 mM Ca^{2+} from the extracellular space) (part B) of lymphocytes of CML or Control patients. Data represent the $[Ca^{2+}]_i$ values (mean ± SD) obtained 3–5 distinct experiments performed in duplicate. *$P < 0.05$ versus control cells. °$P < 0.05$ versus $[Ca^{2+}]_i$ basal levels.

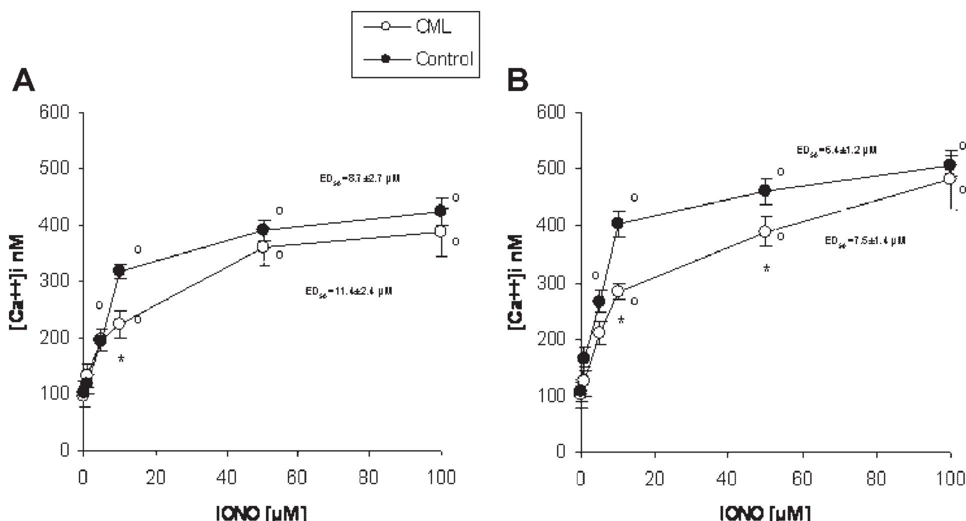


Fig. 3. Effect of increasing concentration of IONO used alone on calcium release from intracellular stores (KRH medium without 1 mM Ca^{2+} in presence of 10 μM EGTA) (part A) or calcium entry (KRH medium containing 1 mM Ca^{2+} from the extracellular space) (part B) of lymphocytes of CML or Control patients. Data represent the $[Ca^{2+}]_i$ values (mean ± SD) obtained 3–5 distinct experiments performed in duplicate. *$P < 0.05$ versus control cells. °$P < 0.05$ versus $[Ca^{2+}]_i$ basal levels.

activity in CML cells and in control with a ED_{50} of 7.5 ± 1.4 and 6.4 ± 1.2 μM in CML and Control cells, respectively.

In order to verify the correlation between $[Ca^{2+}]_i$ modifications and the effects on nitric oxide production and oxidative stress we used RESV, a potent antioxidant which has been demonstrated to be able to promote differentiation and apoptosis in several tumor cell lines (Wang et al., 2003), alone or in association with $[Ca^{2+}]_i$ mobilizer molecules such as ATP, $InsP_3$, and IONO.

The results obtained using extracellular RESV alone at doses ranging between 5 and 100 μM on intracellular calcium concentration $[Ca^{2+}]_i$ are shown in Figure 4. Peripheral blood mononuclear cells (10^5 cells) from both CML as well as healthy patients were loaded with FURA-2/AM and balanced for 10 min in a calcium-free medium (part A) or in a 1 mM $CaCl_2$ medium (part B) then treated with RESV. Our results showed that RESV was not able to mobilize calcium at any of the used concentrations both in CML as well as in Controls cells.

Table 1 shows the percentage of inhibition obtained by using 20 μM of RESV on the intracellular calcium mobilization induced by 3 μM of InsP3 or 100 μM of ATP or 50 μM of IONO. The part A shows the results obtained using peripheral blood mononuclear cells loaded with FURA-2/AM and balanced for 10 min in a calcium-free medium, while the part B shows the results obtained peripheral blood mononuclear cells balanced in a 1 mM $CaCl_2$ medium.

RESV was able to significantly inhibit the depletion of calcium stores induced by $InsP_3$ or by ATP treatment both in CML and in control cells. In cells balanced in a calcium-free medium (A) RESV reduced the depletion of calcium stores induced by $InsP_3$ and ATP of −32.8% and −22.7% in control cells and −37.7% and −45.2% for CML, respectively (P vs. $InsP_3$ or ATP alone <0.05).

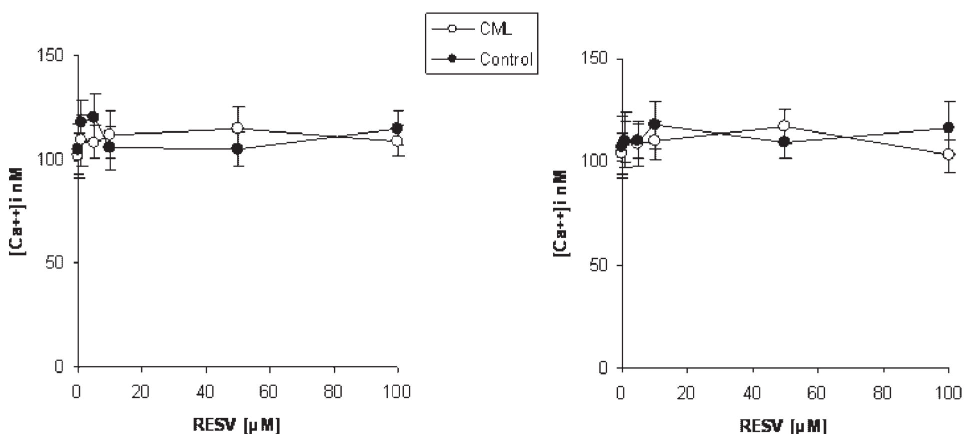


Fig. 4. Effect of increasing concentration of RESV used alone on calcium release from intracellular stores (KRH medium without 1 mM Ca^{2+} in presence of 10 μM EGTA) (part A) or calcium entry (KRH medium containing 1 mM Ca^{2+} from the extracellular space) (part B) of lymphocytes of CML or Control patients. Data represent the $[Ca^{2+}]_i$ values (mean ± SD) obtained 3–5 distinct experiments performed in duplicate. *$P < 0.05$ versus control cells. °$P < 0.05$ versus $[Ca^{2+}]_i$ basal levels.

TABLE 1. Effect of 20 μM RESV in lymphocytes of CML or Control patients treated with InsP3, ATP, or IONO in KRH medium without 1 mM Ca^{2+} in presence of 10 μM EGTA (calcium release from intracellular stores) (part A) or KRH medium containing 1 mM Ca^{2+} from the extracellular space (calcium influx) (part B)

		$[Ca^{2+}]_i$ nM			
Part	Drugs	CML	Δ%	Control	Δ%
A	$InsP_3$ 3 μM	376.4 ± 38.1		613.2 ± 53.9	
	$InsP_3$ 3 μM + RESV 20 μM	**234.5 ± 22.1	−37.7	**411.9 ± 37.8	−32.8
	ATP 10 μM	154.2 ± 12.4		215.5 ± 19.7	
	ATP 10 μM + HESV 20 μM	**84.5 ± 9.1	−45.2	**166.1 ± 15.6	−22.7
	IONO 50 μM	339.6 ± 41.4		390.7 ± 41.8	
	IONO 50 μM + RESV 2 μM	312.7 ± 29.8	−8.1	**362.8 ± 45.9	−7.2
B	$InsP_3$ 3 μM	567.7 ± 52.9		833.4 ± 73.9	
	$InsP_3$ 3 μM + RESV 20 μM	**320.9 ± 28.4	−43.4	**487.3 ± 45.9	−41.5
	ATP 10 μM	275.4 ± 26.1		344.3 ± 31.4	
	ATP 100 μM + RESV 20 μM	**139.7 ± 11.4	−49.2	**241.4 ± 19.6	−29.8
	IONO 50 μM	**395.7 ± 49.2		466.5 ± 51.6	
	IONO 50 μM + RESV 2 μM	371.4 ± 41.5	−6.1	410.2 ± 44.8	−12.1

Data represent the values and at % of $[Ca^{2+}]_i$ value in presence of 20 μM of RESV versus single treatment of InsP3 at 3 μM, ATP at 100 μM or IONO at 50 μM expressed at 100%.
**$P < 0.05$ versus $[Ca^{2+}]_i$ levels obtained whit InsP3 or ATP alone.

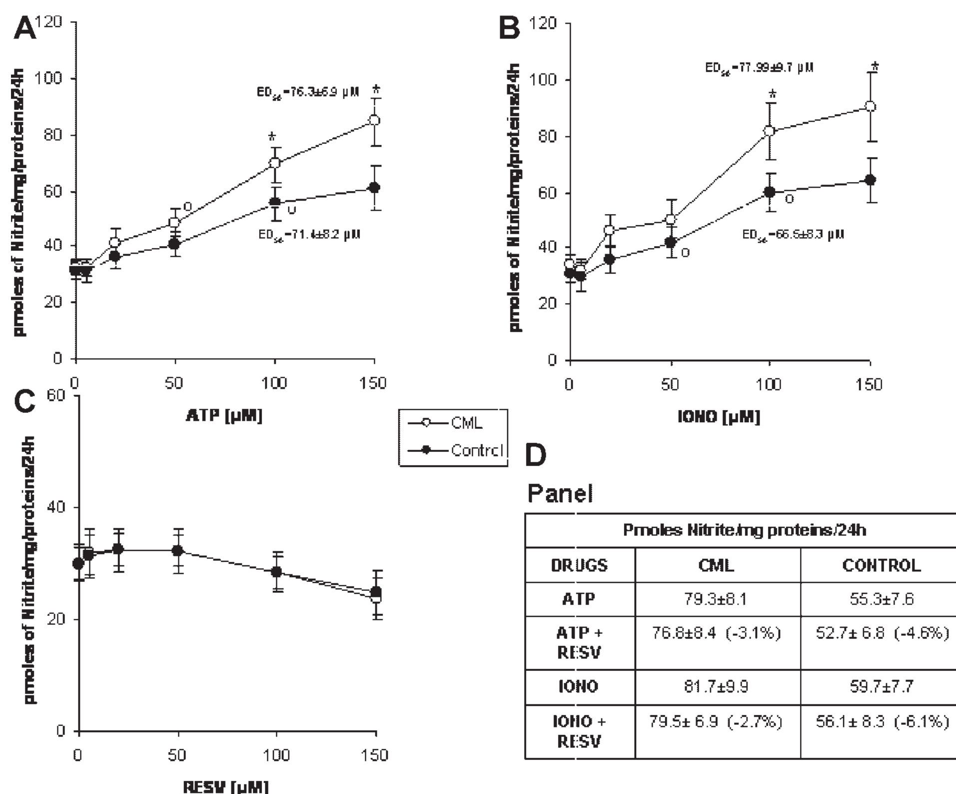
RESV produced no effect on the increase $[Ca^{2+}]_i$ induced by IONO.

Part B of Table 1 shows the results obtained on the intracellular calcium mobilization in cells balanced in medium containing 1 mM $CaCl_2$. Even under these experimental conditions, RESV was able to inhibit the effect induced by InsP3 or ATP of −43.4% and −49.2% in CML and −41.5% and −29.8% in Control cells, respectively (P vs. $InsP_3$ or ATP alone <0.05).

RESV produced no effect on the increase $[Ca^{2+}]_i$ induced by IONO.

NO production

Figure 5 shows the results obtained using ATP, IONO, or RESV on nitric oxide production. Peripheral blood mononuclear cells (10^5 cells) from both CML as well as healthy patients were



Pmoles Nitrite/mg proteins/24h		
DRUGS	CML	CONTROL
ATP	79.3±8.1	55.3±7.6
ATP + RESV	76.8±8.4 (-3.1%)	52.7± 6.8 (-4.6%)
IONO	81.7±9.9	59.7±7.7
IONO + RESV	79.5± 6.9 (-2.7%)	56.1± 8.3 (-6.1%)

Fig. 5. Effect of increasing concentrations of ATP (A) or IONO (B) and RESV (C) used alone, on nitric oxide (NO) production in lymphocytes of CML or Control patients. Part D represents the effect of 20 μM RESV in lymphocytes of CML or Control patients treated with ATP or IONO at 100 μM concentration. The lymphocytes were treated for 12 h at 37°C in humidified atmosphere of 5% carbon dioxide with increasing concentration of ATP or IONO or RESV. After 24 h, nitric oxide ($NO_2 + NO_3$) levels were assayed by the Griess reagent. Data represent the $NO_2 + NO_3$ values (mean ± SD) obtained in 3–5 distinct experiments performed in duplicate. *P < 0.05 versus NO control cells. °P < 0.05 versus nitrite basal levels.

treated for 24 h at 37°C in humidified atmosphere of 5% carbon dioxide with ATP, IONO, or RESV at concentrations ranging between 1 and 150 μM.

After 24 h of incubation nitric oxide (NO) production, was determined in the supernatant of cells was assayed by Griess reagent. ATP and IONO increased the NO production in a dose-dependent manner while RESV did not induce any significant effect.

In particular, extracellular ATP (A) induced a significant increase in maximum levels of nitrite from 32.5 ± 4.2 to 84.5 ± 11.8 and 31.3 ± 4.1 to 60.8 ± 8.3 pmol of nitrite/mg of protein/24 h in CML and Control cells, respectively (P vs. control cells < 0.05) with a DE_{50} of 76.3 ± 6.9 and 71.4 ± 8.2 μM in CML and Control cells, respectively. IONO induced a significant increase (P vs. control cells <0.05) of nitrite levels (B) from 34.1 ± 5.7 to 90.4 ± 12.5 and from 30.9 ± 3.4 to 64.1 ± 8.2 pmol of nitrite/mg of protein/24 h for CML and Control cells, respectively, with a DE_{50} of 77.9 ± 9.7 and 66.5 ± 8.3 μM, respectively.

RESV (C) induced a significant decrease in the production of nitrite starting only from the concentration of 150 μM. InsP3 did not produce any significant effect on NO production (data not shown).

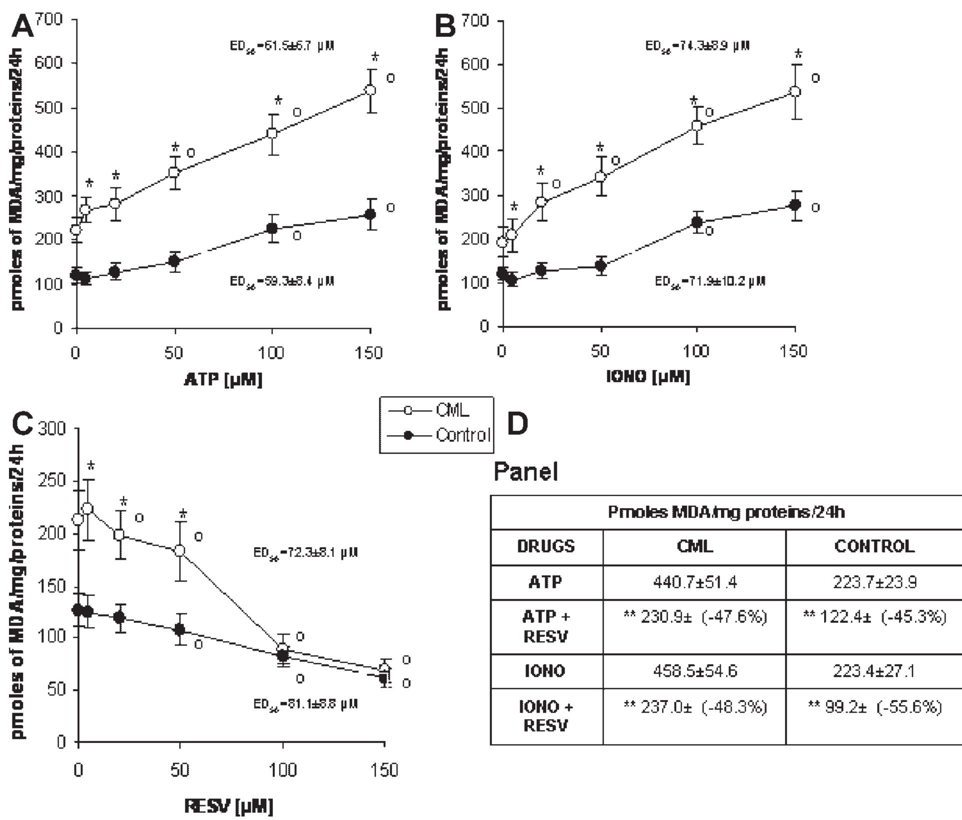
Part D shows the effect of RESV (20 μM) on NO production induced by ATP or IONO (100 μM) after 24 h of incubation. RESV did not produce a significant reduction in nitrite levels in both the lymphocytes from CML than control cells.

MDA production

Figure 6 shows the results obtained using ATP, IONO, or RESV on the MDA production. Peripheral blood mononuclear cells (10^5 cells) from both CML as well as healthy patients were treated with ATP, IONO, or RESV for 24 h at 37°C in humidified atmosphere of 5% carbon dioxide at concentrations between 1 and 150 μM ATP or IONO or RESV, then MDA production was assayed as reported in the Materials and Method Section.

The basal levels of MDA in CML cells were significantly higher than those found in Control cells (P vs. control cells <0.05) being 222.8 ± 33.4 and 120.6 ± 21.1 pmol of MDA/mg of protein/24 h for CML and Control cells, respectively. ATP and IONO increased the MDA production in a dose-dependent manner, while RESV induced a significant reduction (P vs. Basal levels <0.05) of the intracellular MDA levels both in healthy and in CML subjects.

In particular, ATP (A) induced a maximum increase in the MDA levels both in lymphocytes of CML patients than in controls at the dose of 150 increasing MDA levels from 222.8 ± 33.4 to 538.3 ± 67.5 pmol of MDA/mg of protein/24 h (P vs. Basal <0.05) and 120.6 ± 13.8 to 257.2 ± 34.6 pmol of MDA/mg of protein/24 h (P vs. Basal <0.05), with a DE_{50} of 59.3 ± 8.4 and 61.5 ± 6.7 μM in Control and CML cells, respectively. The same effect was observed using IONO (B) at the dose of 150, which induced a significant increase in the MDA



Pmoles MDA/mg proteins/24h		
DRUGS	CML	CONTROL
ATP	440.7±51.4	223.7±23.9
ATP + RESV	** 230.9± (-47.6%)	** 122.4± (-45.3%)
IONO	458.5±54.6	223.4±27.1
IONO + RESV	** 237.0± (-48.3%)	** 99.2± (-55.6%)

Fig. 6. Effect of increasing concentrations of ATP (A) or IONO (B) and RESV (C) used alone, on MDA production in lymphocytes of CML or Control patients. Part D represents the effect of 20 μM RESV in lymphocytes of CML or control patients treated with ATP or IONO at 100 μM concentration. The lymphocytes were treated for 24 h at 37°C in humidified atmosphere of 5% carbon dioxide with increasing concentration of ATP or IONO or RESV. After 24 h, MDA level were assayed by thiobarbituric acid reagent. Data represent the MDA values (mean ± SD) obtained in 3–5 distinct experiments performed in duplicate. *P < 0.05 versus MDA control cells. **P < 0.05 versus ATP or IONO alone. °P < 0.05 versus MDA basal levels.

levels, from 192.6 ± 25.3 to 538.3 ± 66.7 pmol of MDA/mg of protein/24 h (P vs. Basal <0.05) and 121.5 ± 15.4 to 276.4 ± 31.8 pmol of MDA/mg of protein/24 h (P vs. Basal <0.05) with an DE_{50} of 71.9 ± 10.2 and 74.3 ± 8.9 µM for Control and CML cells, respectively. The addition of $InsP_3$ did not produce any significant results on MDA production (data not shown).

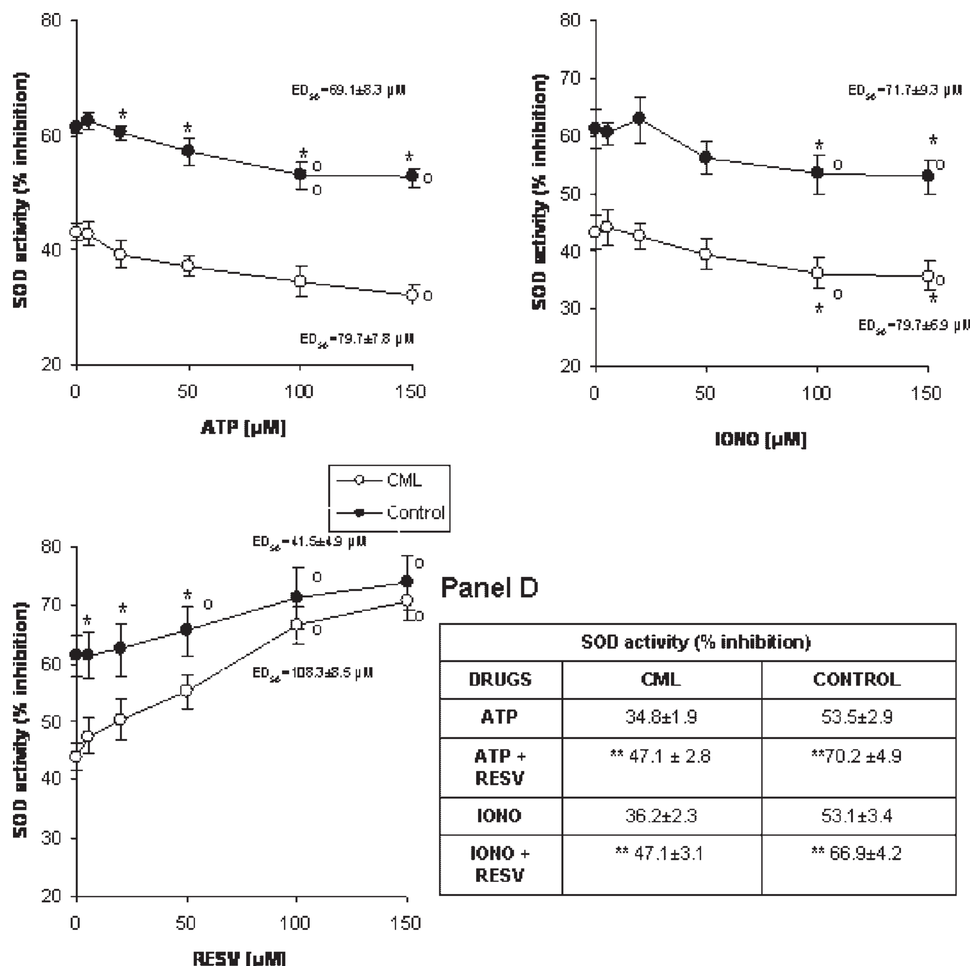
RESV (C) induced a significant inhibition of MDA production in dose-dependent manner both in CML cells than in healthy subjects. At a dose of 150 µM, RESV decreased MDA levels from 212.8 ± 27.3 to 68.4 ± 9.5 pmol of MDA/mg of protein/24 h (P vs. Basal <0.05) in CML and 125.8 ± 16.1 to 62.3 ± 9.7 pmol of MDA/mg of protein/24 h in control cells (P vs. Basal <0.05) with a DE_{50} of 72.3 ± 8.1 and to 81.1 ± 8.8 µM, respectively.

Part D of Figure 6 shows the effect of RESV (20 µM) on the MDA production in cells treated with 100 µM of ATP or IONO for 24 h at 37°C in humidified atmosphere of 5% carbon dioxide. RESV was able to significantly reduce the effect of ATP or IONO both in lymphocytes from CML than in control patients of −47.6% and −45.3% for ATP and −48.3% and −55.6% for IONO in CML and Control cells, respectively (P vs. ATP or IONO alone <0.05).

SOD activity

Figure 7 shows the results obtained using ATP, IONO, or RESV on SOD activity. Peripheral blood mononuclear cells (10^5 cells) from both CML as well as healthy patients were treated with ATP, IONO or RESV for 24 h at 37°C in humidified atmosphere of 5% carbon dioxide at concentrations between 1 and 150 µM ATP or IONO or RESV, then SOD activity was assayed as reported in the Materials and Method Section.

SOD activity, significantly lower in leukocytes form CML patients than from healthy patients, was found to be 43.1 ± 2.3 and 61.4 ± 3.7 in CML and Control cells, respectively (P vs. control cells <0.05). ATP decreased SOD activity (A) in leukocytes form both CML than healthy patients. SOD activity decreased in ATP-treated control cells from 61.4 ± 3.7 to 52.7 ± 2.9 (P vs. Basal levels <0.05) and from 43.1 ± 2.3 to 32.1 ± 3.6 in CML cells (P vs. Basal levels <0.05), with an DE_{50} of 69.1 ± 8.3 and 79.7 ± 7.8 µM respectively. Also IONO (B)



SOD activity (% inhibition)		
DRUGS	CML	CONTROL
ATP	34.8±1.9	53.5±2.9
ATP + RESV	** 47.1 ± 2.8	**70.2 ±4.9
IONO	36.2±2.3	53.1±3.4
IONO + RESV	** 47.1±3.1	** 66.9±4.2

Fig. 7. Effect of increasing concentrations of ATP (A) or IONO (B) and RESV (C) used alone, on SOD activity in lymphocytes of CML or Control patients. Part D represents the effect of 20 µM RESV in lymphocytes of CML or control patients treated with ATP or IONO at 100 µM concentration. The lymphocytes were treated for 24 h at 37°C in humidified atmosphere of 5% carbon dioxide with increasing concentration of ATP or IONO or RESV. After 24 h, SOD activity were assayed by water-soluble formazan dye upon reduction with superoxide anion. Data represent the MDA values (mean ± SD) obtained in 3–5 distinct experiments performed in duplicate. *P < 0.05 versus MDA control cells. **P < 0.05 versus ATP or IONO alone. °P < 0.05 versus MDA basal levels.

significantly decreased SOD activity from 61.3 ± 3.7 to 52.9 ± 4.1 in control cells (P vs. Basal levels <0.05) and from 43.3 ± 2.3 to 35.7 ± 2.2 in CML cells (P vs. Basal levels <0.05), with a DE_{50} of 71.7 ± 9.3 and 79.7 ± 6.9 μM, respectively. $InsP_3$ did not produce any significant results on SOD activity (data not shown). The addition of RESV (C) instead led to an increased SOD activity both healthy and in CML patients. In fact, SOD activity increased from 61.4 ± 3.7 to 73.9 ± 4.6 in Control cells (P vs. Basal levels <0.05) and from 43.8 ± 2.3 to 70.8 ± 5.2 in CML cells with an DE_{50} significantly lower in CML of 108.3 ± 8.5 μM than in control cells of 41.5 ± 4.9 μM.

Part D of Figure 7 shows the effect combined use RESV (20 μM) and ATP or IONO (100 μM) on SOD activity. RESV was able to significantly inhibit ($P < 0.05$) the effect of ATP or IONO both in lymphocytes from CML than healthy patients of 35.3% and 31.2% for ATP and 30.1% and 25.9% for IONO, respectively.

Discussion

Intracellular calcium plays an important role in the regulation of the growth and the functions of many kinds of cells. Cellular calcium homeostasis is an important factor regulating the switch points of the cell cycle (Whitaker and Patel, 1990) and is crucial for cell growth (Waldron et al., 1997). The abnormal proliferation of some cancer cell lines is connected with strongly elevated $InsP_3$ levels and over expression of PLC which must influence calcium signaling, especially calcium release from the ER stores (Berridge, 1995). In this study we investigated in peripheral blood leukocytes from CML patients the role of the balance between intracellular calcium and oxidative stress.

Our data demonstrated that $InsP_3$ increased intracellular calcium concentration $[Ca^{2+}]_i$ both trough the depletion of the intracellular calcium stores then Ca^{2+} influx in a dose-dependent manner with a significant lower effect in CML than Control cells. Similar effects were obtained using ATP, mainly through an increase of Ca^{2+} influx with a lower effect in CML than Control cells. ATP is the ubiquitous energy source in all living organisms, and also plays other important roles in several physiological processes. In animal systems, extracellular ATP (eATP) is well-established as a signal molecule implicated in a number of cellular responses such as neurotransmission, the immune response, and apoptosis (Bours et al., 2006). The role of eATP as a signal agent in plant cells had not drawn much attention until recently, however, it was first proposed by Demidchik et al. (2003) based on the finding that exogenous ATP applied to Arabidopsis roots induced rapid and transient increase in the cytosolic Ca^{2+} concentration.

Our data significantly differs from those obtained by Wiley and Dubyak (1989) in chronic lymphatic leukemia cells, which demonstrated that ATP at the concentration of 100 μM induced no elevations in intracellular calcium concentration $[Ca^{2+}]_i$ but at 3 mmol/L even though produced no significant change in the cytosolic Ca^{2+} in normal monocytes, induced a very rapid and a significant increase in the cytosolic Ca^{2+} on of cell preparations derived from five out of nine CLL patients (Wiley and Dubyak, 1989). Our results, instead, are consistent with the results of Piwocka et al. (2006) which demonstrated that BCR/ABL-expressing cells, such as CLL cells, exhibit a decreased amount of free release-able calcium in the ER as well as a weaker capacitative calcium entry response. This effect was demonstrated to be independent of BCL2, which is a known modulator of ER calcium homeostasis. The reduction in ER releasable calcium resulted in inhibition of the ER/mitochondrial coupling process and mitochondrial calcium uptake (Buggin et al., 2001).

It is widely known that physiological membrane-receptor agonist typically stimulates oscillations, of varying frequencies, in cytosolic Ca^{2+} concentration $[Ca^{2+}]_i$. Whether and how $[Ca^{2+}]_i$ oscillation frequency regulates agonist-stimulated downstream events, such as gene expression, in non-excitable cells remain unknown. Cytosolic Ca^{2+} concentration, the ubiquitous intracellular second messenger, is profoundly modulated by a variety of (patho)physiological conditions and is very important in decoding Ca^{2+}-signal-dependent downstream events. In an artificial $[Ca^{2+}]_i$-oscillation model generated using cell-permeant caged $InsP_3$, it was found that the NFAT activation was optimized by the frequency of $[Ca^{2+}]_i$ oscillations (Li et al., 1998). Moreover, Liping et al. (2008) found that, in endothelial cells cultured from human aorta, intracellular H_2O_2 cooperates with $[Ca^{2+}]_i$ signal to regulate agonist-stimulated gene expression *VCAM1* and contributes to $[Ca^{2+}]_i$ oscillation-frequency-optimized gene expression during agonist stimulation.

It has long been thought that Ca^{2+} and NO (nitric oxide) work together in the control of cell homeostasis and NO could have appeared as a step in the signaling cascade initiated by the cation. However, the interaction between the two messengers does not exist as a dependence but as a true, bi-directional cross-talk. In fact currently, almost all aspects of Ca^{2+} homeostasis have been reported to involve modulation by NO (Meldolesi et al., 1991).

That is why we investigated on the production of NO and ROS, in CML and Control cells following administration of $[Ca^{2+}]_i$ mobilizer molecules.

It has been demonstrated that the leukemic and tumoral pathology are under a higher oxidative stress than normal cells (Szatrowski and Nathan, 1991; Kato et al., 2003) and in this report, we confirmed that also CLL cells differ from control cells in the oxidative stress status and nitric oxide production. The basal levels of MDA in CML cells were significantly higher than those found in Control cells with significantly lower SOD activity levels in CLL than in Control cells. ATP and IONO treatment significantly decrease SOD activity while increased both the MDA and NO production more in CLL than in Control cells. Thus, in order to verify the correlation between $[Ca^{2+}]_i$ modifications and the effects on nitric oxide production and oxidative stress we used RESV, a potent antioxidant which has been demonstrated to be able to promote differentiation and apoptosis in several tumor cell lines, alone or in association with $[Ca^{2+}]_i$ mobilizer molecules such as ATP and IONO (Wang et al., 2003).

Although numerous studies have described intracellular changes leading to cell-cycle arrest or apoptosis in response to resveratrol treatment, the effects are often cell-type specific, and these studies have not yet identified the underlying mechanism of drug action (Aggarwal et al., 2004; Fulda and Debatin, 2006). Resveratrol is a non-toxic, plant-derived polyphenol. Different studies have established the beneficial effects in cancer therapy, showing inhibition of proliferation and induction of apoptosis in various tumor cell lines (of different origin). Data concerning resveratrol activity on $[Ca^{2+}]_i$ appear controversial, in fact RESV (100 μM) seem to act at mitochondrial inducing apoptosis in human HepG2 hepatoma cells by a sustained elevation of intracellular $[Ca^{2+}]_i$ (Ma et al., 2007) while in ventricular myocytes of rats, at 40 μM, decreased the peak amplitude of L-type calcium current (Zhang et al., 2006) and in human platelets inhibited Ca^{2+} influx in thrombin-stimulated platelets as well as thapsigargin-mediated Ca^{2+} influx (Dobrydneva et al., 1999). Recently, Li-Jun et al. (2008) demonstrated, in medulloblastoma cell lines (UW228-2 and UW228-3), that the expression of STAT3 downstream genes, survivin, cyclin D1, Cox-2, and c-Myc, was suppressed but Bcl-2 was enhanced by resveratrol and that the production and secretion of leukemia inhibitory factor, a STAT3 activator, became active in resveratrol-treated cells.

In a recent publication Hassan-Khabbaar et al. (2008) showed that RESV at a low dose (0.2 mg/kg) has antioxidant properties

and that by the contrary at dose of 20 mg/kg shows a pro-oxidant effect and exacerbates the injury caused by ischemia/reperfusion in liver. Moreover, Ozkan et al. (2009) demonstrated that RESV significantly ameliorated the intestinal injury decreased MDA, NO and myeloperoxidase (MPO) levels.

Resveratrol was found to have pro-apoptotic effects on cancer cells but displayed minimal toxicity to human peripheral blood lymphocytes, indicating its lack of toxicity for normal cells (Clement et al., 1988). Resveratrol has also been shown to exhibit anticancer properties in leukemia (Gao et al., 2002). Resveratrol demonstrated activity to inhibit the overexpression of the heat shock proteins or stress protein HSP-70 and HSP-90, been identified as contributors to oncogenesis (Caroline and Morimoto, 2000), as well as induced apoptosis (Chakraborty et al., 2008) in K562 cells.

Our results showed that RESV was not able to mobilize calcium at any of the used concentrations but significantly decreased MDA and NO production increasing SOD activity both in CML as well as in controls cells. RESV, was able to inhibit ATP, IONO, and $InsP_3$ activity both of intracellular calcium concentration as well as on the NO and MDA production and on the SOD activity.

Our data demonstrate in CML cells a lower activity of $InsP_3$-dependent release of from stores as well as a decreased function of purinergic dependent calcium channels associated with a high oxidative stress status. CML cells also showed a significant lower SOD activity and it is conceivable that the higher oxidative stress observed can be justified by the altered intracellular calcium homeostasis and SOD activity. It is known that an abnormal accumulation of superoxide anions may lead to abnormal interactions between superoxide anions and DNA, which in turn may lead to erroneous signals resulting in transcription errors responsible to induce neoplastic diseases.

Such dysregulations observed in leukocytes from CML patients associated to the capacity of RESV to down-regulate them, might suggest novel therapeutic strategies for the treatment of CML.

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