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Prevention of Oxidation and Apoptosis in Human Peripheral Blood Mononuclear Cells Exposed to Calcium Dobesilate

Gabriele A. Losa,[1] Riccardo Graber,[1] Jean-Claude Farine[2]

[1]*Laboratorio di Patologia Cellulare, Instituto Cantonale di Patologia 6601 CH-Locarno/Switzerland and [2]OM Pharma, CH-1217 Meyrin/Switzerland

Abstract. The antioxidant effects of calcium dobesilate (CD) (Doxium®) were investigated in relation to the oxidative status, apoptosis, and *in vitro* proliferation of human peripheral blood mononuclear cells (PBMC) isolated from healthy donors. Calcium dobesilate alone did not modify cell growth *in vitro* until it reached 10 μM. This molecule counteracted oxidative damage generated by the highly reducing sugar 2-deoxy-D-ribose (dR) and was shown to reduce apoptosis by delaying both membrane permeability changes and DNA fragmentation. Calcium dobesilate (10 μM) was effective in a time-dependent manner on several parameters, representative of the cellular oxidative status. In particular, CD significantly increased the activity of glutathione S-transferase (GST) after 3 days of treatment and also the activity of γ-glutamyltransferase (γ-GT). Both of these enzymes are known to be involved in the glutathione (GSH) metabolic cycle. This enzymatic behavior was reversed after 7 days of treatment, with a significant GST decrease and a γ-GT activation. After 7 days of CD exposure, the intracellular GSH content was enhanced and this resulted in a dramatic decrease of lipid peroxidation, underlining the powerful antioxidant properties of CD in human PBMC.

Introduction

Among the biochemical mechanisms responsible for apoptotic cell death of human T lymphocytes, particular attention has been devoted to those related to oxidative stress generated by oxygen-free radicals and peroxides [10,11]. The main feature of oxidative stress is the loss of protective action of biological scavengers, which results in an increased level of peroxides, hydroperoxides, aldehydes, and free radicals [1]. Among molecules acting as antioxidants or scavengers of free radicals, there are the intracellular GSH, glutathione disulfide (GSSG), as well as antioxidant enzymes involved in the glutathione cycle, such as γ-glutamyltransferase (γ-GT) and glutathione-S-transferase (GST) [13,14]. GST exerts its protective functions using intracellular GSH though two main mechanisms. First, it catalyzes the transfer of sulfydryl groups to alkylating molecules by forming GSH conjugated metabolites that are less toxic and easily excretable. Second, it catalyzes a peroxidase reaction with formation of oxidized glutathione GSSG [8,15,18]. Therefore, GST acts as an efficient detoxifier and repairer of oxidative damage to membrane lipids and DNA. Among exogenous agents with pharmacological effect, calcium dobesilate (Doxium) has been recently observed to possess antioxidant properties *in vitro*. It appears to act as a scavenger for superoxide anions and as an inhibitor of membrane lipid peroxidation generated by oxygen-free radicals in human erythrocytes, polymorphonuclear cells and bovine endothelial cells [3]. Calcium Dobesilate was also shown to exert an angioprotective action by strongly reducing the microvascular permeability induced by reactive oxygen species [4]. The precise cellular and biochemical mechanisms of action of calcium dobesilate *in vivo* and *in vitro* remain poorly understood. In particular, its effects on immunologic functions, metabolic features, and proliferative properties of human lymphocytes remain to be investigated. The aims of this study were to examine the effects of Doxium® on *in vitro* proliferation, apoptosis and biochemical pathways involved in the control of oxidative status of human peripheral blood mononuclear cells isolated from healthy donors.

Material and Methods

Calcium dobesilate was supplied by OM PHARMA, CH-Geneva/Meyrin. Monoclonal antibodies (anti-CD3, anti-CD4 and anti-CD8) were purchased from Becton-Dickinson, CH-Basel. Glutathione assay kit was from Calbiochem AG, CH-Luzern. Ficoll-Hypaque was from Fakola AG, CH-Basel. All other reagents used for the various assays were purchased from SIGMA AG, CH-Buchs.

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**Correspondence to:* Gabriele A. Losa, PhD, Laboratorio di Patologia Cellulare, Istituto Cantonale di Patologia, 6601 CH-Locarno, Switzerland

Lymphocyte Isolation and Treatment

Peripheral blood mononuclear cells (PBMC) were isolated from blood samples of healthy normal individuals by the Ficoll-Hypaque gradient, according to the conventional method [6]. Cells were washed twice with NaCl 0.9% pH 7.4, counted, and characterized by staining with monoclonal antibodies anti-CD3, anti-CD4, and anti-CD8. The PBMC population accounted for 84% of CD3 T lymphocytes, among which CD4 and CD8 lymphocytes amounted to 52% and 32%, respectively; 12% were B lymphocytes. Cell viability, determined by trypan blue exclusion, always exceeded 95%. Cells at the initial concentration of 10^6 cells/ml were cultured in RPMI 1640 supplemented by 5% fetal calf serum (FCS). dR and antioxidants were dissolved in RPMI 1640 and added to the cultures as indicated in Results.

Enzyme Activities

The enzyme assays were optimized for time, pH, protein, and substrate concentration after homogenization of cell suspensions in Tris-HCl buffer (10 strokes 4000 rpm up and down). γ-glutamyltransferase (γ-GT; EC 2.3.2.2) was assayed by incubating homogenates of PBMC (50 μg protein) in 1 ml reaction mixture containing 20 mM Tris-HCl buffer pH 8.0, 60 mM glycylglycine, 300 mM NaCl, and 2.5 mM γ-glutamyl-p-nitroanilide. The reaction was terminated by adding 2 ml 1.5 N acetic acid. The mixture was cleared by centrifugation (800 × *g*, 20 minutes at 4°C) and the absorbance of the p-nitroaniline in the supernatant was measured at 410 nm with a Beckman DU-65 spectrophotometer [12].

The glutathione S-transferase (GST; EC 2.5.1.18) activity was determined by the method of Anderson et al. [3] with slight modifications. The GST activity was assayed in 1 ml of a reaction mixture containing 1 mM GSH, 1 mM CDNB (1-chloro-2,4-dinitrobenzene) dissolved in ethanol at a final concentration of 2.5% (v/v), 100 mM potassium phospahte pH 6.5, and 0.1% Triton X-100. The reaction was started by adding 50 μg of cell homogenate and incubating for 30 minutes at 25°C. The amount of dinitrobenzene (DNB) liberated from CDNB was measured with a Beckman DU-65 spectrophotometer at 340 nm.

Analysis of Lipid Peroxidation (MDA Assay)

The binding of thiobarbituric acid to malondialdehyde formed during lipid peroxidation results in a chromogenic complex. Briefly, the homogenate obtained by lysis of 1–5.10^6 cells with 10% ice-cold TCA was centrifuged at 800 *g* for 10 minutes. Aliquots of 1 ml of the supernatant were added to 1 ml of 0.67% thiobarbituric acid and heated in a boiling water bath for 10 minutes. After cooling, samples were read at 535 nm and the concentration of malondialdehyde (MDA) was determined by comparison with a standard reference curve [17].

Measurement of Intracellular GSH Content

Intracellular GSH content was measured on total cell homogenate by using the Glutathione Assay Kit. The assay is based on a two-step chemical reaction: the formation of thioethers with a reagent and the mercaptans present in the sample and the successive alkaline β-elimination of the thioethers with the formation of a chromophoric thione detectable at 400 nm.

Characterization of Apoptosis

Membrane Permeability Assay. The change in the permeability of the cell plasma membrane, measured by flow cytometry, was used to differentiate living from apoptotic and dead cells [7]. Cells that have an intact plasma membrane exclude dyes such as trypan blue and propidium iodide (PI), whereas dead cells with damage plasma membrane do not. Apoptotic cells have intermediate patterns of membrane permeability, which results in a variable dye uptake. Untreated or treated cells (1×10^5–10^6) were collected by centrifugation, resuspended in 0.5 ml NaCl 0.9%, pH 7.4 containing 5 μg/ml propidium iodide (PI), and incubated for 7 minutes at room temperature and washed with NaCl. Histograms of cell subpopulations are obtained with a FACscan equipped with a Lysis II program, which distinguishes three main fluorescence peaks, corresponding to normal, apoptotic, and dead cell populations.

Analysis of Cell Cycle and Apoptosis. The cells (3×10^6) were washed in NaCl 0.9% solution, pH 7.4, and nuclei were obtained with hypotonic choc by resuspending PBMC in 1 ml of 0.1% sodium citrate solution containing 50 μg/ml of PI, 0.1% Triton X-100, and RNase-A (5 U/ml) for 1 hour in the dark at 4°C. Thereafter, nuclei were analyzed for cell cycle ($G0/G_1$, S. and G_2+M) with a flow cytometry equipped with Lysis II software. Fragmented DNA generated by apoptosis was visualized as a fluorescence subdiploid fluorescence peak occurring at the left of the G0/G1 diploid DNA peak [7,16]

Statistics

Data were analyzed with Student's paired *t*-test (two tailed).

Results

Effects of CD Alone or in Combination with dR on PBMC Growth

In order to investigate the effect of CD on human PBMC growth *in vitro,* cells (10^6 cells/ml) were incubated with various concentrations of CD (range 1 μM–10 mM) and the viability was measured after 5 days of culture. Figure 1 shows that the percentage of living cells in the presence of CD at the concentration from 1 μM to 10 μM was found to be similar to that of untreated PBMC or even slightly higher. In the presence of higher concentrations of CD (100 μM–1 mM) there was a rapid decrease of living cells accompanied by an enhancement of the percentage of apoptotic cells (about 30% at 1 mM CD). Finally, at 10 mM CD we observed an important increase in the number of dead cells (34%), probably due to the emergence of necrotic features at this very high concentration (Fig. 1). On the basis of these results we decided to use 10 μM CD for further experiments. The maximal effect of 2-deoxy-D-ribose (dR), known to be a potent inducer of apoptosis, was reached at 10 mM with a 40% of PBMC growth inhibition. Since a preincubation of cells with 10 μM of the antioxidant N-acetyl-L-cysteine (NAC) completely abolished the cytotoxic effects of 10 mM dR, we could conclude that apoptosis induced by dR is probably due to the oxidative stress. In order to investigate the role of CD as antioxidant agent, changes of cell membrane permeability toward propidium iodide (PI) and the amplitude of the subdiploid DNA peak reflecting DNA fragmentation were investigated in PBMC treated with both 10 μM CD and 10 mM dR. Both methods showed that 10 μM CD exerted an antioxidative effect when it was added 24 hours before dR (Fig. 2), column CD/dR). In fact, the pretreatment with CD resulted in a reduction of DNA fragmentation (restricted subdiploid peak) and permeability when compared with values measured in PBMC with dR alone. However, neither the addition of CD after the dR treatment (dR/CD) nor the contem-

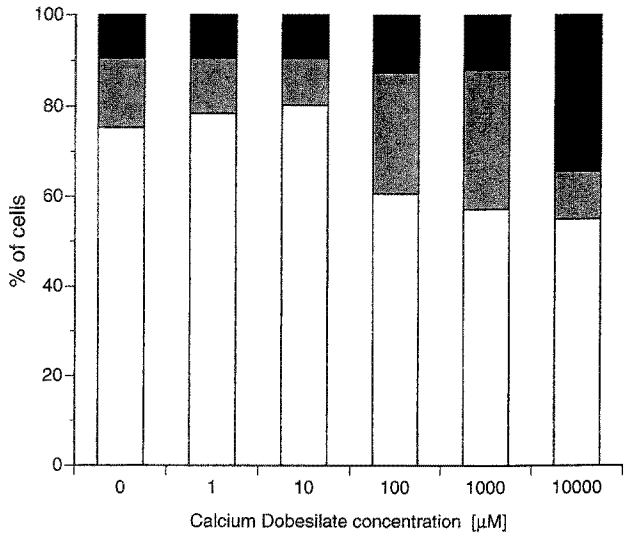

Fig. 1. Effect of calcium dobesilate (CD) concentration on viability (membrane permeability to propidium iodide) of human PBMC at 5 days of culture. (□) living, (▨) apoptotic, and (■) dead cells. Results are representative of four separate experiments.

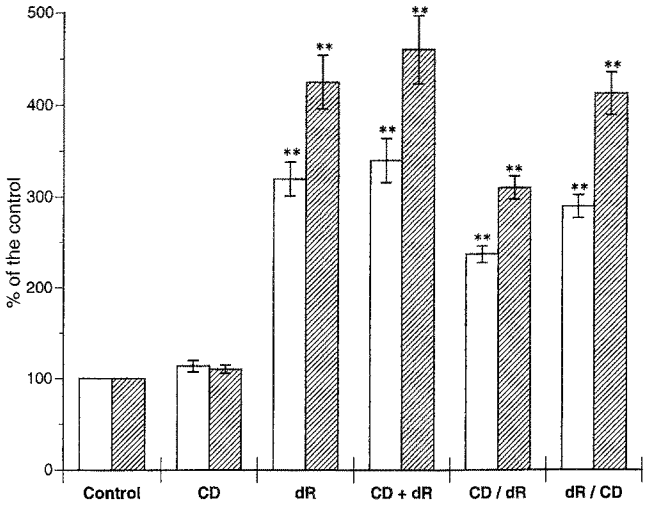

Fig. 2. Effect of CD and dR on apoptosis in PBMC, expressed as % of controls. Cells have been incubated for 5 days with CD (10 μM), dR (10 mM), or with combinations of both. CD + dR: treatment with both CD and dR; CD/dR: preincubation with CD 24 hours before dR addition; dR/CD: preincubation with dR 24 hours before Cd addition. Apoptosis was determined by the permeability test (□) and by occurrence of the subdiploid DNA peak (▨). In control cells, the mean values for the permeability and the subdiploid DNA peak were 13.8 ± 4.8% and 2.5 ± 1.2%, respectively. In treated cells, relative values are means ± SD of three separate experiments. **Significantly different ($2p < 0.01$) from the respective controls.

poraneous administration of both CD and dR (CD + dR) could protect cells from dR-induced apoptosis (Fig. 2).

Effects of CD, NAC, and dR on γ-GT and GST Activities

The behavior of both antioxidant enzymes γ-GT and GST was investigated in PBMC treated with the antioxidants NAC and CD and the reducing sugar dR in the culture conditions mentioned above. Two periods of culture have been considered: day 3 (early phase) and day 7 (late phase). After 3 days of culture, 10 μM of both antioxidant NAC or CD (a concentration that did not affect cell growth) induced a small activation of γ-GT (about 20%) whereas GST activity was highly enhanced (about 150%) when compared with the activity of control cells (Fig. 3). At day 7 of culture, γ-GT was highly stimulated in the presence of 10 μM antioxidants (more than 150%), whereas GST underwent a significant reduction of activity with respect to that measured at day 3 (about 150%) (Fig. 3). Interestingly, PBMC treated with 10 mM of the reducing sugar dR exhibited an inverse enzyme behavior, namely, the activation of γ-GT at day 3 (more than 100%) was followed by a strong inhibition at day 7 but accompanied by a strong stimulation of GST (about 200%).

Effects of CD, NAC, and dR on GSH Content and Lipid Perioxidation

We measured a progressive accumulation of intracellular GSH at day 7 in the presence of 10 μM of both antioxidant agents (CD, NAC), caused by γ-GT activation whereas GST remained unmodified (Figure 5). PBMC exposed to dR exhibited at day 7 an opposing enzyme behavior leading to a dramatic reduction in GSH content accompanied by a significant increase of MDA, an indicator of lipid peroxidation (Fig. 5). In antioxidant-treated cells, the GSH level was increased slightly at day 3 but markedly at day 7, in contrast to MDA level which strongly decreased below control values (Fig. 4).

Discussion

The results of the present study indicate that calcium dobesilate at micromolar concentration has antioxidant properties and prevents human PBMC from the oxidative damages induced by high reducing deoxyribose [19]. In analogy to the antioxidant N-acetylcysteine (NAC), CD exerted its effects by increasing the intracellular concentration of the reduced glutathione GSH. It is known that the cytoplasmic GSH is the result of two enzyme activities, namely, the γ-GT responsible for its synthesis [9] and the GST which intervenes in its catabolism. Our findings revealed that in human PBMC, the behavior of both enzymes was found to be dependent on the duration and nature of the treatment. At 3 days of culture in the presence of the antioxidant NAC and/or CD alone, a significant increment of GST was accompanied by a slight change of γ-GT, whereas in the presence of the reducing aldose dR there was a significant increase of the γ-GT but a low activation of GST. At 7 days of treatment with these antioxidants there was a drastic change in both enzyme activities. In fact, human PBMC in the presence of NAC and/or CD alone showed an activation of the γ-GT synthetic pathway and a concomitant reduction of GST activity. On the other hand, in the presence of dR, the GSH-consumptive way mediated by GST was highly activated whereas the γ-GT was strongly inhibited, provoking an increased permeability of the plasma membrane, a reinforced lipid peroxidation, and the occurrence of a subdiploid DNA peak, which finally led to apoptosis.

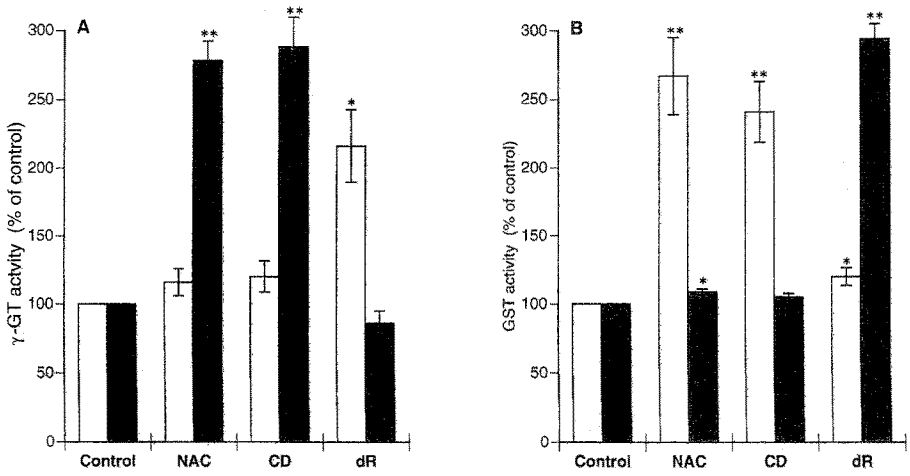

Fig. 3. Effect of antioxidants (NAC and CD at 10 μM) and oxidant (dR at 10 mM) on γ-GT **(A)** and GST **(B)** activities at 3 (□) and 7 days (▨) of culture. Enzyme activities in control cells represent 100%. Mean control values for γ-GT and GST activities at both times are similar and amounted to 8.6 ± 2.6 and 23.2 ± 2.8 nmol/min/mg, respectively. Values given are means ± SD of three separate experiments. Significantly different (*$2p < 0.05$ and **$2p < 0.01$) from the respective controls.

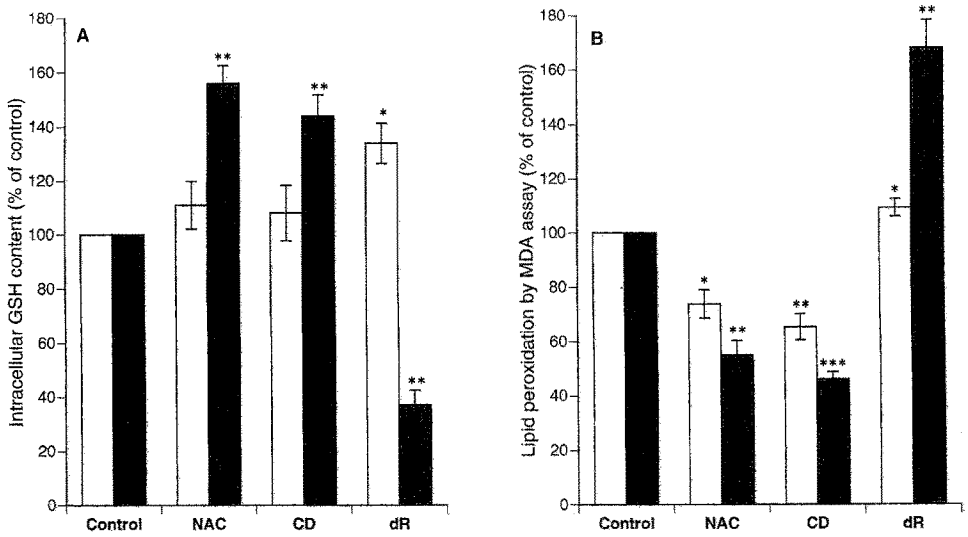

Fig. 4. Effect of antioxidants (NAC and CD at 10 μM) and oxidant (dR at 10 mM) on intracellular GSH **(A)** and on lipid peroxidation **(B)** at days 3 (□) and 7 (▨). Results are percentages of control values (100%). Mean control values at both times are similar and amounted to 12.2 ±1.8 μM GSH and 1.2 ± 0.5 nmol MDA/mg protein. MDA: malondialdehyde. Results are means ± SD of three distinct experiments. Significantly different (*$2p < 0.05$; **$2p < 0.01$; ***$2p < 0.001$) from the respective controls.

Such an enzyme activation was accompanied by a significant increase in the intracellular GSH content and a stable level of the lipid peroxidation, which might suggest that PBMC could counteract injuries in the early phases of 10 mM dR treatment. In reality, these cells were in a preapoptotic stage, the rise of γ-GT-mediated GSH synthesis being insufficient to overcome the persistent effect of dR. In fact, this aldose provoked irreversible apoptotic features such as membrane damages, dramatic fall in GSH, and rise in lipid peroxidation, findings that confirmed those previously reported [2,9]. In addition, the apoptotic effect of dR could not be blocked nor reversed by culturing dR-treated PBMC with mitogens or growth factors.

It is noteworthy that both types of molecules, either the antioxidants NAC/CD or acivicin, ensured a high level of intracellular GSH which partially prevented the apoptotic process in PBMC induced by dR. In the light of these findings one may conclude that calcium dobesilate exerted an antioxidant effect in human PBMC *in vitro* by alternative modulating γ-GT and GST enzymes, which are antagonist to GSH metabolism. This maximal effect of CD implying a maximal content of the intracellular GSH culminated at day 7 with the maximal reduction of GST and the concomitant maximal increase of γ-GT activity.

The conclusion is that CD-modulated events, reduced lipid peroxidation and hence contributed to the prevention of apoptosis. The antioxidant effect of CD observed in PBMC was consistent with that observed in other cell systems such as human polymorphonuclear cells and erythrocytes and in bovine endothelial cells. In these cells, CD was shown to decrease the concentration of superoxide anions, PAF-induced chemiluminescence, and free radicals [3,5].

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