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Dicoumarol relieves serum withdrawal-induced $G_{0/1}$ blockade in HL-60 cells through a superoxide-dependent mechanism

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Abstract

This work was set to study how dicoumarol affects the cell cycle in human myeloid leukemia HL-60 cells. Cells were accumulated in $G_{0/1}$ after serum deprivation. However, when cells were treated with 5 μM dicoumarol in serum-free medium, a significant increment in the number of cells in S-phase was observed. Inhibition of $G_{0/1}$ blockade was confirmed by the increase of thymidine incorporation, the phosphorylation of retinoblastoma protein, and the promotion of cell growth in long-term treatments in the absence of serum. Dicoumarol treatment increased superoxide levels, but did not affect peroxide. Increase of cellular superoxide was essential for inhibition of $G_{0/1}$ blockade, since scavenging this reactive species with a cell-permeable form of SOD and the SOD mimetics 2-amino-3,5-dibromo-*N*-[*trans*-4-hydroxycyclohexyl]benzylamine (ambroxol, 100 μM) and copper[II]diisopropyl salicylate (CuDIPS, 10 μM) completely abolished the effect of dicoumarol. However, *N*-acetyl-cysteine, overexpression of Bcl-2 or a cell-permeable form of catalase were not effective. 5-Methoxy-1,2-dimethyl-3-[(4-nitrophenol)methyl]-indole-4,7-dione (ES936), a mechanism-based irreversible inhibitor of NAD(P)H:quinone oxidoreductase 1 (NQO1), did not promote S phase entry, and dicoumarol still inhibited $G_{0/1}$ blockade in the presence of ES936. We demonstrate that dicoumarol inhibits the normal blockade in $G_{0/1}$ in HL-60 cells through a mechanism involving superoxide, but this effect is not dependent solely on the inhibition of the NQO1 catalytic activity. Our results send a precautionary message about use of dicoumarol to elucidate cellular processes involving oxidoreductases.

Keywords: Cell cycle; Dicoumarol; HL-60 cells; NAD(P)H:quinone oxidoreductase 1; Superoxide

1. Introduction

Dicoumarol is a coumarin-derived natural anticoagulant. By far, the best characterized action of dicoumarol on cells is the competitive inhibition of NAD(P)H:(quinone acceptor) oxidoreductase 1 (NQO1, EC 1.6.99.2), a cytosolic flavoenzyme that catalyzes the obligatory two-electron reduction of a variety of quinone substrates to give their corresponding hydroquinones using both NADH and NADPH as electron donors. NQO1 avoids the formation of reactive oxygen species generated by redox cycling [1,2] and is an effective superoxide scavenger [3]. By competing with NAD(P)H at the pyridine nucleotide binding site, dicoumarol inhibits NQO1 very effectively in the low micromolar range [4]. Thus, dicoumarol is used for routine analysis of NQO1 activity in vitro [2] and has been also widely employed to determine the involvement of NQO1 in biochemical reactions and cellular processes [5–9].

Several reports have shown that dicoumarol has profound effects on cell growth and death. Treatment of HeLa cells with dicoumarol causes the blockade of stress-activated protein kinase/c-Jun NH_2-terminal kinase and NFκB pathways, and potentiate apoptosis induced by tumor necrosis factor-α (TNF-α) [5]. Also,

Abbreviations: ES936, 5-methoxy-1,2-dimethyl-3-[(4-nitrophenol)-methyl]-indole-4,7-dione; FCS, fetal calf serum; NAC, *N*-acetyl-cysteine; NQO1, isoform 1 of the cytosolic NAD(P)H:(quinone acceptor) oxidoreductase; DT, diaphorase; ROS, reactive oxygen species; TNF-α, tumor necrosis factor-α; pRb, retinoblastoma tumor suppressor gene product (hypophosphorylated); pRb-P, retinoblastoma tumor suppressor gene product (hyperphosphorylated)

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dicoumarol suppress the malignant phenotype of pancreatic cancer cells, most likely due to an increase of superoxide levels [8,10]. Dicoumarol also binds to bovine brain tubulin and strongly stabilizes the growing and shortening dynamics of microtubules in vitro, and inhibits the first cleavage of sea urchin embryos [11]. On the other hand, dicoumarol treatment results in the degradation of p53 and blocks wild-type p53-mediated apoptosis in γ-irradiated normal thymocytes and in M1 myeloid leukemic cells overexpressing wild-type p53. These latter studies have enabled to demonstrate the pivotal role of NQO1 in the regulation of p53 stability and p53-dependent apoptosis by increasing p53 stabilization, particularly under oxidative stress conditions [7,12]. However, this function does not require NQO1 activity, but relies on a direct interaction between NQO1 and p53 proteins, which is impaired by dicoumarol [13].

Recent results obtained in our group demonstrated that treatment of human myeloid leukemia HL-60 cells with dicoumarol at micromolar concentrations apparently inhibits growth of cells in the absence of serum [14]. Later we showed that this inhibition was mainly accounted by a significant activation of apoptosis, but this effect could be avoided by preculturing HL-60 cells in serum-free medium before treatment with the inhibitor [15]. Since growth of a cell population is the sum of cell division and survival, the present work was set to analyze how dicoumarol affects the cell cycle in this myeloid leukemia cell line, which lacks p53 [16]. This is particularly interesting because p53 is often mutated in various tumors [17]. In addition, since HL-60 cells have been previously reported to have a defect in NQO1 expression [18], we were interested in elucidating whether or not inhibition of NQO1 catalytic activity was actually related with the observed effects of dicoumarol on cells.

We demonstrate here that dicoumarol relieves growth arrest in $G_{0/1}$ induced by removal of growth factors, favoring entry into the S phase. Once its apoptosis-promoting effect was overcome, dicoumarol stimulated the growth of HL-60 cells in serum-free medium. Treatment with dicoumarol increased superoxide levels, and this elevation was essential for dicoumarol-induced S phase entry. However, specific inhibition of NQO1 catalytic activity was not sufficient to impair $G_{0/1}$ blockade, since no effect was obtained with 5-methoxy-1,2-dimethyl-3-[(4-nitrophenol)methyl]-indole-4,7-dione (ES936), a novel and highly selective mechanism-based irreversible inhibitor of NQO1 [19]. Furthermore, dicoumarol still promoted entry into the S phase in cells treated with ES936. Our results indicate that dicoumarol alters the redox balance of HL-60 cells towards a more prooxidant condition, resulting in the inhibition of the normal blockade in $G_{0/1}$ in the absence of growth factors through a superoxide-mediated mechanism. However, this effect is not dependent solely on the inhibition of NQO1 catalytic activity.

2. Materials and methods

2.1. Cell cultures and treatments

Human myeloid leukemia HL-60 cells were cultured in RPMI-1640 medium (BioWhittaker) supplemented with 10% fetal calf serum (FCS, Linus-Cultek), 100 units/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B and 2 mM of L-glutamine (Sigma) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Cells were concentrated from stock cultures by centrifugation at 1000 × *g* for 5 min, washed twice in serum-free RPMI-1640 medium and then cultured in the same medium without FCS in the presence of quinone reductase inhibitors. For some experiments, cells were precultured for 24 h in serum-free medium before they were treated with the inhibitors. Dicoumarol (3,3′-methylenebis[4-hydroxycoumarin]) was obtained from Sigma. Stock solutions of dicoumarol were prepared at 2 mM in 6 mM NaOH, and added to cells to a final concentration of 1–5 μM. 5-Methoxy-1,2-dimethyl-3-[(4-nitrophenol)-methyl]-indole-4,7-dione was kindly provided by Prof. C.J. Moody (University of Exeter, Exeter, UK). Stock solutions of ES936 were prepared at 10 mM in DMSO, and added to cells to a final concentration of 30–1000 nM. The same amount of the corresponding vehicle was added to controls in all experiments. To target different checkpoints of the oxidative cascade potentially triggered by dicoumarol, cells were treated with the quinone reductase inhibitor in the presence of the following antioxidants: *N*-acetyl-cysteine (NAC, final concentration 5 mM), an antioxidant and glutathione precursor; and 2-amino-3,5-dibromo-*N*-[*trans*-4-hydroxycyclohexyl]benzylamine (ambroxol, 100 μM) and copper[II]diisopropyl salicylate (CuDIPS, 10 μM), two SOD mimetics [20]. We also performed the specific scavenging of superoxide and peroxide by culturing dicoumarol-treated cells in the presence of the cell-permeable forms of SOD (polyethylene glycol-SOD, 300 U/ml) and catalase (polyethylene glycol-catalase, 1000 U/ml) respectively. These compounds were obtained from Sigma. The effect of dicoumarol was also tested in a HL-60-derived cell line overexpressing Bcl-2, that was obtained as described in the next section. Cell viability was determined by the Trypan blue exclusion method.

2.2. Plasmid construction and stable transfection of HL-60 cells

Plasmid pSFFV-Neo-Bcl-2 was obtained from expression plasmid pSFFV-Neo [21] by cloning the cDNA for human Bcl-2 (850 bp EcoR1–EcoR1 fragment) into the EcoR1 site adjacent to the Friend spleen focus-forming virus 5′ long terminal repeat (SFFV-LTR) promoter element. For transfection, HL-60 cells were grown

(2×10^6 cells/ml) and recovered by centrifugation at $500 \times g$ for 5 min. Cells were washed with serum-free RPMI-1640 medium, centrifuged again under the same conditions. Cells were then resuspended in serum-free RPMI-1640 medium at a final density of 2×10^7/400 μl and mixed with 0.5 μg plasmid/10^6 cells. After a preincubation for 10 min on ice, cells were transferred to electroporation chambers (BTX) (2×10^7 cells/chamber). Electroporation (13 Ω, 960 μF and 300 V) was carried out using a BTX electroporator. After electroporation cells were incubated again for 10 min on ice, and cultured at a density of 5×10^5 cells/ml in RPMI-1640 medium supplemented with 10% FCS. After 48 h, transfected cells were selected by progressively increasing the concentration of antibiotic G418 sulphate (Calbiochem) from 200 μg/ml to 1 mg/ml. Stably transfected HL-60 cells were cultured in the presence of G418 sulphate.

2.3. Cell cycle and apoptosis analysis

The number of cells in different phases of the cell cycle and apoptotic cells were measured by flow cytometry. Briefly, 10^6 cells were recovered by centrifugation at $500 \times g$ for 5 min, fixed with 70% ethanol for at least 24 h at 4 °C and stained with propidium iodide. Cells were then assayed for DNA content using a Beckman/Coulter EPICS XL cytometer equipped with a 488 nm Argon laser. Fluorescence was determined at 620 nm (FL3). Population in each of cell cycle phases, G_1, S and G_2/M, was determined with the software Cylchred (Cardiff University), based on the algorithm described by Watson et al. [22]. The number of apoptotic cells was determined as the percentage of population showing $subG_{0/1}$ DNA content. Cell death (apoptosis versus necrosis) was also studied by staining the cells with annexin V-FITC/propidium iodide following the protocol recommended by the manufacturer (Immunotech-Beckman/Coulter). Fluorescence was measured by flow cytometry at 525 nm (FL1) for fluorescein, and 620 nm (FL3) for propidium. The number of necrotic cells was calculated from the propidium iodide-positive, annexin V-negative population, whereas annexin V-positive cells were considered as apoptotic.

2.4. Measurement of intracellular reactive oxygen species

Levels of peroxide and superoxide in cells were quantified using the probes 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) and hydroethidine (Het) (Sigma, Spain) respectively [23,24]. Cells were incubated in the dark with the corresponding probe (final concentrations: 10 μg/ml DCFH-DA and 4 μM Het) for 30 min at 37 °C. After washing, fluorescence was determined by flow cytometry at 525 nm (FL1) for fluorescein, and 620 nm (FL3) for ethidium.

2.5. Thymidine incorporation experiments

Proliferation was measured from the ability of cells to incorporate thymidine. Briefly, cells were incubated with 0.25 μCi/ml [methyl-^{3}H]thymidine for 30 min. After incubation, cells were recovered by centrifugation (5 min, $1500 \times g$) and washed with cold 0.9% NaCl. Cold trichloroacetic acid was added to a final concentration of 5%. After incubating for 30 min at 4 °C, samples were centrifuged again and the supernantants were discarded. Precipitates were then lysed with 1N NaOH for 1 h at room temperature. Lysates were used for measuring incorporation of radioactivity using a liquid scintillation counter (Beckman). Incorporation was referred to protein contained in the samples to obtain specific values.

2.6. Cell lysis and polyacrylamide gel electrophoresis

For preparation of cytosolic fractions, cells were recovered by centrifugation at $1000 \times g$ for 5 min and washed with cold 130 mM Tris–HCl pH 7.6, containing 1 mM EDTA, 0.1 mM DTT and 1 mM PMSF. Cells were centrifuged again and resuspended in 1 ml of hypotonic lysis buffer (10 mM Tris–HCl pH 7.6, containing 1 mM EDTA, 0.1 mM DTT, 1 mM PMSF and 20 μg/ml each of the following protease inhibitors: chymostatin, leupeptin, antipain and pepstatin A (CLAP). Homogenization of cells was carried out for 5 min with the aid of a glass–glass potter and then for 5 s with a mechanical cell homogenizer. After disruption of cells, the concentration of the lysis buffer was raised to 100 mM Tris by adding enough volume of 250 mM Tris buffer, pH 7.6 containing 1 mM EDTA, 0.1 mM DTT, 1 mM PMSF and CLAP). Unbroken cells and debris were separated by centrifugation at $800 \times g$ for 5 min and the supernatant was saved. Cytosolic fractions were separated from membranous material by ultracentrifugation at $100{,}000 \times g$ for 30 min. These fractions were used both for determination of NQO1 activity and for immunodetection of NQO1 polypeptide (see below). For analysis of phosphorylated (pRB-P) and hypophosporylated (pRB) retinoblastoma, about 10^7 cells were centrifuged at $500 \times g$ for 5 min, washed with PBS, and centrifuged again under the same conditions. Cell pellets were resuspended in 250 μl of extraction buffer (50 mM Tris–HCl, pH 7.5 containing 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 1 mM sodium ortovanadate, 1 mM PMSF and (CLAP) and, after gentle agitation, extracts were centrifuged at $10{,}000 \times g$ for 15 min at 4 °C to remove undisrupted cells and debris. Supernatants were saved for determinations of total protein and polyacrylamide gel electrophoresis.

For detection of NQO1 polypeptide, about 100 μg of cytosolic protein were denatured by boiling in SDS-dithiothreitol loading buffer [10% sucrose, 2 mM EDTA, 1.5% (w/v) SDS, 20 mM dithiothreitol, 0.01% (w/v) bromophenol blue and 60 mM Tris–HCl, pH 6.8], separated by SDS-

PAGE (12% acrylamide) and then blotted onto nitrocellulose sheets. For detection of pRB, cellular extracts (50 μg) were suspended in SDS-dithiothreitol loading buffer. After boiling for 5 min, samples were separated by SDS-PAGE (5% acrylamide) and blotted onto nitrocellulose sheets. Blots were stained with Ponceau S for visualization of protein lanes. Protein determinations were carried out by the dye-binding method described by Stoscheck [25].

2.7. Determination of NQO1 and pRB polypeptides

Determination of NQO1 and pRB polypeptides was carried out by immunostaining of Western blots with the corresponding specific antibodies. Primary antibodies were a goat polyclonal antiserum raised against NQO1 (Santa Cruz Biotech) diluted at 1:100, and a monoclonal antibody, which recognizes both hypophosphorylated and phosphorylated forms of pRB (Santa Cruz Biotech) diluted at 1:500. Anti-goat and anti-mouse IgG secondary antibodies coupled to horseradish peroxidase (Sigma) were used, respectively, to reveal binding sites by enhanced chemiluminescence. pRB and pRB-P were distinguished on the basis of their different electrophoretic mobilities. To control protein loading, nitrocellulose sheets were stripped from bound antibodies by incubation with 62.5 mM Tris–HCl, pH 6.7 containing 2% SDS and 100 mM mercaptoethanol at 70 °C for 30 min with agitation. After stripping, membranes were reprobed again with either a goat anti-actin polyclonal antibody (Sigma) diluted at 1:100 or a mouse anti-β-tubulin monoclonal antibody (Boehringer) at a 1:50 dilution. Anti-goat or anti-mouse IgG antibodies coupled to horseradish peroxidase (Sigma) were used as secondary, and developing of images was also performed by enhanced chemiluminescence. Photographic films were scanned to obtain digital images for quantification of intensity reaction using Quantity One software (Bio-Rad). Data obtained from quantification of stained bands (in arbitrary units) were normalized to that of the corresponding lane stained with anti-actin or anti-β-tubulin antibodies to correct for possible differences in protein loading between samples.

2.8. NQO1 activity assays

NQO1 activity in cytosolic fractions was measured from the NADH and menadione-dependent dicoumarol-inhibitable reduction of cytochrome *c* [2]. Assays were carried at 37 °C with constant gentle stirring in a final volume of 1 ml in 50 mM Tris–HCl (pH 7.5) containing 70 μg of cytosolic proteins, 0.08% Triton X-100, 0.5 mM NADH, 10 μM menadione and 77 μM cytochrome *c*. Assays were carried out either in the absence or in the presence of 10 μM dicoumarol and absorbance was recorded at 550 nm in a Beckman DU-640 UV–vis spectrophotometer. NQO1 activity was calculated from the difference in reaction rates obtained with and without dicoumarol. An extinction coefficient of 18.5 mM^{-1} cm^{-1} was used in calculations of specific activities.

3. Results

3.1. Dicoumarol stimulates $G_{0/1}$/S transition in HL-60 cells cultured in the absence of serum

We recently demonstrated [14] that simultaneous removal of serum and treatment with micromolar concentrations of dicoumarol apparently inhibited growth of HL-60 cells in the absence of serum in short-term experiments. Dicoumarol at a concentration of 5 μM also increased significantly the number of apoptotic cells in the absence, but not in the presence of 10% serum [15]. Since growth of a cell population is the sum of cell division and survival, the aim of this study was to evaluate how dicoumarol affected the cell cycle in HL-60 cells grown in serum-free medium. To accomplish this task we stained ethanol-fixed cells for DNA with propidium iodide, and analyzed their distribution among the phases of the cell cycle by flow cytometry. Analysis of HL-60 cells populations demonstrated a strong effect of dicoumarol treatment on the distribution of HL-60 cells among the different phases of the cell cycle. As expected, serum removal itself delayed progression through the cell cycle with a concomitant increase in the number of cells in $G_{0/1}$ period after 24 h (66.6 ± 7.3% of cells versus 39.7 ± 4.6% of cells under control conditions; see also Figs. 1A and C and 2). However, in the presence of dicoumarol cells were not arrested in G_1. Instead, the number of cells in G_1 was significantly lower (20.7 ± 9.7% of cells), whereas a population of cells with a DNA content intermediate between G_1 and G_2/M was significantly higher at 24 h (69.6 ± 9.8% of cells, see also Figs. 1D and 2). These results could indicate that dicoumarol promoted $G_{0/1}$/S transition of HL-60 cells. After 48 h in the presence of the inhibitor, cells in S phase were then decreased (42.2 ± 5.3% of cells), with a concomitant increase of the number of sub$G_{0/1}$ and G_2/M cells (see Figs. 1F and 2). Necrosis, measured from the propidium iodide-positive and annexin V-negative population, only accounted for less that 0.5% of cells, whereas the vast majority of nonviable cells were apoptotic (annexin V-positive) (not shown). In the presence of 10% FCS, treatment with dicoumarol had no effect on cell cycle distribution of HL-60 cells (compare Fig. 1A and B).

To prove that the increase in this population with an intermediate DNA content actually corresponded to the promotion of $G_{0/1}$/S transition, we analyzed this phenomenon by independent methodological approaches. Phosphorylation of the RB tumor suppressor gene product by cyclin-dependent kinases is one of the hallmarks of $G_{0/1}$/S transition [26]. We have measured levels of the phosphorylated (pRB-P) and the hypophosphorylated (pRB) forms of this protein on the basis of their different electrophoretic

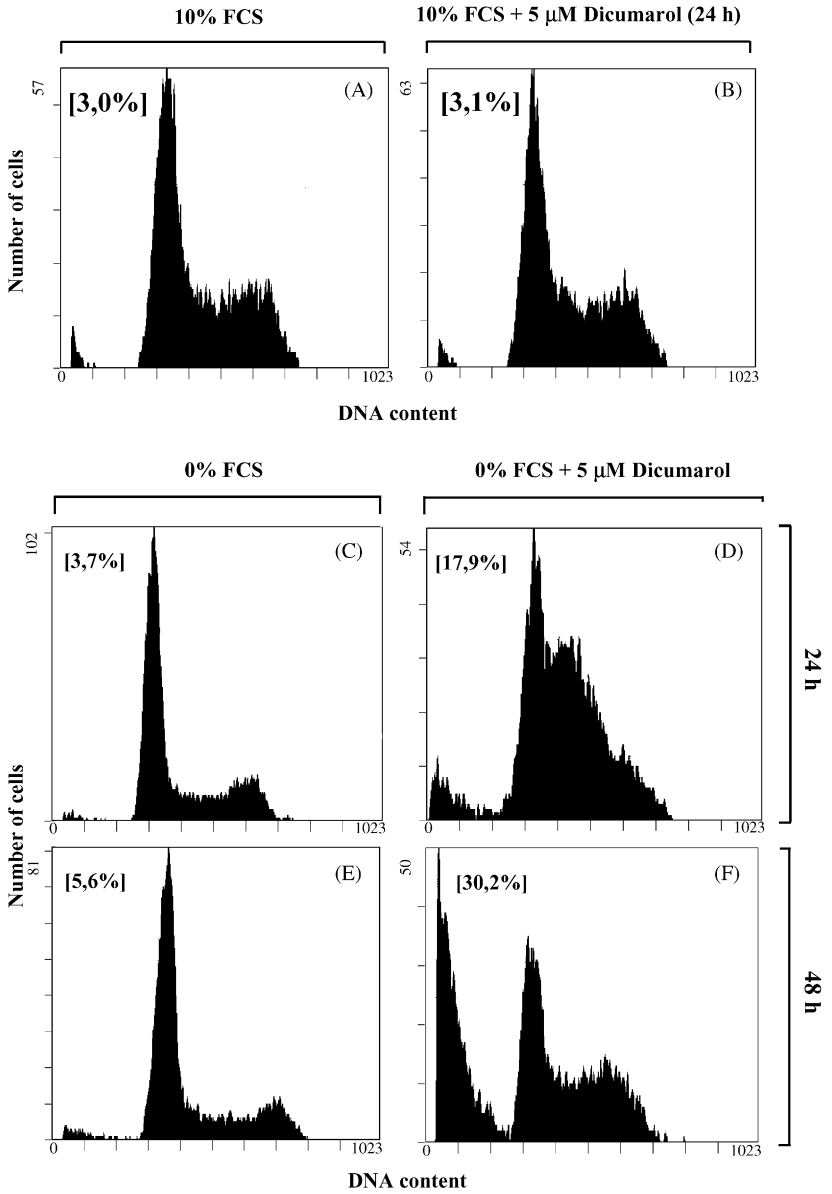

Fig. 1. Effect of dicoumarol on cell cycle and apoptosis in HL-60 cells. Culture medium of cells growing in the presence of 10% serum was replaced by serum-free RPMI-1640 containing or not 5 μM dicoumarol. The inhibitor was also added to cells cultured in the presence of 10% serum. After incubation for 24 or 48 h, cells were fixed with cold 70% ethanol, stained with propidium iodide and analyzed for DNA content by flow cytometry. The amount of apoptotic cells (in %) for each experimental condition is depicted in brackets on each panel. Results shown are representative of five separate experiments.

mobility, by using immunodetection in Western blots with a specific monoclonal antibody, which recognizes both forms (Fig. 3A). Quantification of pRB and pRB-P expression levels was accomplished by densitometry of stained bands. Staining intensity was referred to that of the corresponding β-tubulin band to obtain specific values (Fig. 3B). Levels of this tumor suppressor were very low in HL-60 cells cultured in 10% serum, which agrees with their proliferating phenotype. Upon removal of serum, pRB levels were considerably increased at 24 and 48 h. In the absence of serum and the presence of 5 μM dicoumarol, levels of the protein at 24 h were higher than in cells only subjected to serum removal. Dicoumarol treatment did not increased further pRB expression levels at 48 h. Interestingly, phosphorylation status of pRB resembled that of proliferating HL-60 cells at 24 and 48 h of treatment. Induction of pRB phosphorylation was already observed at 24 h of treatment, which fully agrees with a promotion of S phase entry. Thereafter, pRB remained in a phosphorylated state in cells treated with dicoumarol for 48 h.

We next studied thymidine incorporation, as a direct estimation of DNA synthesis. For these experiments, we precultured HL-60 cells in serum-free medium for 24 h to increase the number of cells in G_1 before treatment with dicoumarol. Since we had previously demonstrated that this pretreatment protects HL-60 cells against dicoumarol-induced apoptosis [15], we first tested if dicoumarol still

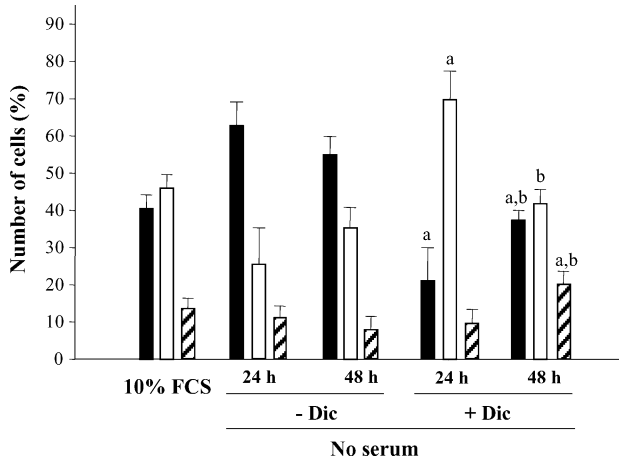

Fig. 2. Distribution of HL-60 cells in the different phases of the cell cycle. Cells were treated and analyzed as described in Fig. 1. Quantification of the number of cells in each phase of the cell cycle was performed using the algorithm described by Watson (20). G_1 (■), S (□), G_2/M (▨). Experiments were repeated five times. Results are mean ± S.D. Differences were statistically significant vs. the corresponding control without dicoumarol ($^a p < 0.05$). Differences were statistically significant vs. 24 h of treatment with dicoumarol ($^b p < 0.05$).

had the same effect on cell cycle distribution, i.e. increasing the population with a DNA content intermediate between G_1 and G_2. As shown in Fig. 4, after a 24 h preincubation without serum, dicoumarol treatment also resulted in the apparent promotion of $G_{0/1}$/S transition and, as previously reported [15], apoptosis was significantly inhibited. At 24 h, about 75% of dicoumarol-treated cells were found in S phase, whereas only 36% of cells were in S phase in controls (see Fig. 4 and Table 1). At 48 h, the number of dicoumarol-treated cells in S phase was still very high (about 61%), and they apparently had progressed through the cell cycle compared to cells at 24 h of treatment. The high number of cells apparently in S phase at 48 h of treatment is most likely due to the inhibition of apoptosis provided by preculturing cells without serum, thus increasing the number of viable cells. However, the above-described results indicate that the action of dicoumarol on the cell cycle is not necessarily linked to the activation of the apoptotic program, but both effects can be separated. DNA synthesis was then studied by giving 30 min pulses of [methyl-^{3}H]thymidine (0.25 μCi/ml) at different times after addition of dicoumarol to serum-free medium. The inhibitor was replaced by the same volume of 6 mM NaOH in controls. After the pulse with [methyl-^{3}H]thymidine, cells were harvested and processed as described in Section 2 for measurement of radioactivity incorporation. As depicted in Fig. 5, thymidine incorporation of cells treated with dicoumarol was not different from that of control cells until 15 h of treatment. However, a significant increase in the incorporation of thymidine was observed after 16 h in cells treated with 5 μM dicoumarol, whereas no significant changes in thymidine incorporation were observed in control cells cultured in serum-free medium without dicoumarol addition. Thus, dicoumarol actually stimulates DNA synthesis in HL-60 cells in the absence of serum. Taken together, our results clearly demonstrate that dicoumarol treatment impairs $G_{0/1}$ blockade and stimulates entry of HL-60 cells into the S phase in the absence of growth factors.

3.2. Dicoumarol can promote growth of HL-60 cells in serum-free medium

Since dicoumarol inhibited growth arrest of HL-60 cells in $G_{0/1}$, we tested if this treatment resulted in growth stimulation of this tumor cell line in the absence of serum in long-term experiments. For these experiments, cells were precultured for 24 h with serum-free medium to protect against dicoumarol-induced apoptosis, and then stimulated with dicoumarol as described in the previous section. Control cells were cultured without dicoumarol. The number of viable cells was scored over a 15 days period, the culture medium and dicoumarol being refreshed every 2–3 days. As shown in Fig. 6, HL-60 cells were able to grow for 5 days in medium without serum but then, the number of viable cell sharply declined. However, in the presence of 1 μM dicoumarol, cells were still able to maintain growth for 9 days, when almost all control cells were already dead. These results clearly demonstrate that, once the initial effect of dicoumarol potentiating apoptosis is overcome, treatment with this compound provides HL-60 cells with an advantage to grow and survive in the absence of serum.

3.3. Dicoumarol increases superoxide levels in HL-60 cells in the absence of serum, and this increase is required for the stimulation of $G_{0/1}$/S transition

It has been previously shown that dicoumarol treatment of pancreatic cancer cells increases superoxide levels, resulting in the inhibition of their malignant phenotype [8,10]. A redox-sensitive pathway operates in early G_1 to control the progression of cells into the S phase, and this transition requires mild prooxidant conditions [27]. Thus, it is plausible that a putative increase of cellular reactive oxygen species (ROS) could be involved in the stimulation of S phase entry we show here. We have used the probes Het and DCFH-DA to monitor intracellular levels of superoxide and peroxide, respectively, by flow cytometry. The use of flow cytometry with Het as probe demonstrated that viable HL-60 cells were distributed amongst two populations according to intracellular levels of superoxide (not shown). Treatment with dicoumarol resulted in a significant increase in the population of high superoxide levels (Fig. 7A), which is consistent with the increase of superoxide in dicoumarol-treated pancreatic cancer cells, as demonstrated with the same technique [8,10]. On the other hand, levels of peroxide, as measured by monitoring the fluorescence of DCFH oxidized into the cells, were

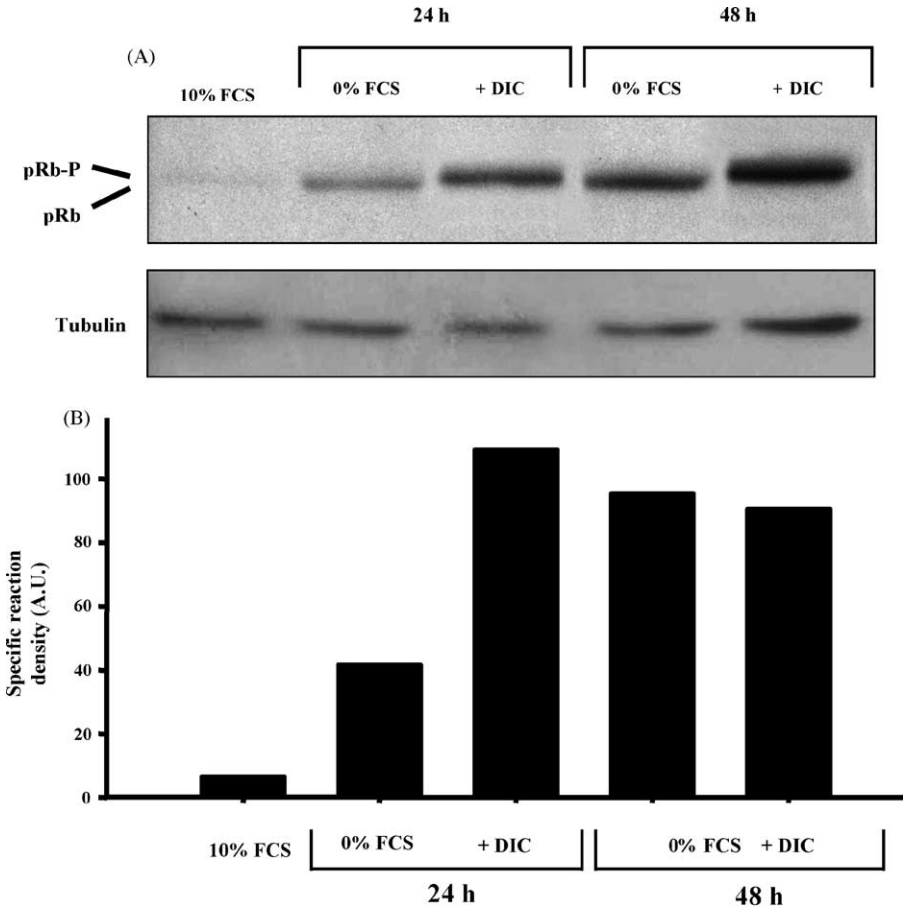

Fig. 3. Dicoumarol increases phosphorylation of RB in HL-60 cells in the absence of serum. Cells were treated as described in Fig. 1. At the indicated times, cells were recovered and protein extracts were obtained for SDS-PAGE, Western blotting and immunostaining with anti-RB antibody. After staining and quantification, blots were stripped and then reprobed again with anti-β-tubulin monoclonal antibody for control of protein loading (A). Quantification of specific reaction intensity is depicted in (B). Results shown are representative of three independent experiments.

apparently unaffected by dicoumarol treatment (Fig. 7B). To verify that the two fluorescent probes actually detected superoxide and peroxide, respectively, we tested the effect of ambroxol and CuDIPS, two SOD mimetics [20]. As expected, in the presence of the SOD mimetics, the population with high superoxide levels was significantly decreased (Fig. 7A), and this effect was accompanied by a substantial increase in peroxide (Fig. 7B).

To test whether or not prooxidant conditions provoked by dicoumarol were important determinants for inhibition of G_1 blockade we carried out treatments with this quinone reductase inhibitor in the presence of several antioxidants that block the oxidative cascade at different levels. Since we had found a significant increase in the population with higher superoxide levels, we first tested the effects of ambroxol, CuDIPS and a cell-permeable form of SOD. As depicted in Fig. 8, dicumarol-induced entry into the S phase was largely abolished by SOD and both SOD mimetics. However, NAC, an antioxidant and glutathione precursor that has been widely used to block oxidative cascades in cells, and a cell-permeable form of catalase had no effect. Since it has been very recently reported that the antiapoptotic protein Bcl-2 retards G_1/S transition through an antioxidant pathway by decreasing intracellular ROS levels [28], we also generated a HL-60-derived cell line stably transfected with a plasmid bearing the *BCL-2* gene, to test the effect of Bcl-2 overexpression in dicoumarol-induced entry into the S phase. As expected due to their higher Bcl-2 levels, HL-60 cells that were stably transfected with plasmid pSFFV-Neo-Bcl-2 displayed enhanced resistance against dicoumarol-induced apoptosis and showed a higher growth rate that parental cells (not shown). However, transfected cells were promoted to enter the S phase to the same extent than parental cells upon treatment with dicoumarol (Fig. 8). Taken together, our results support that an increase of superoxide by dicoumarol treatment is critical for inhibition of $G_{0/1}$ blockade, but a change in cellular sulphydryls, peroxide, or a pathway involving Bcl-2 may not play a major role in this response.

3.4. Dicoumarol-induced S phase entry is not due solely to inhibition of NQO1 catalytic activity

Physiological effects of dicoumarol on cells have been widely interpreted on the basis of the involvement of

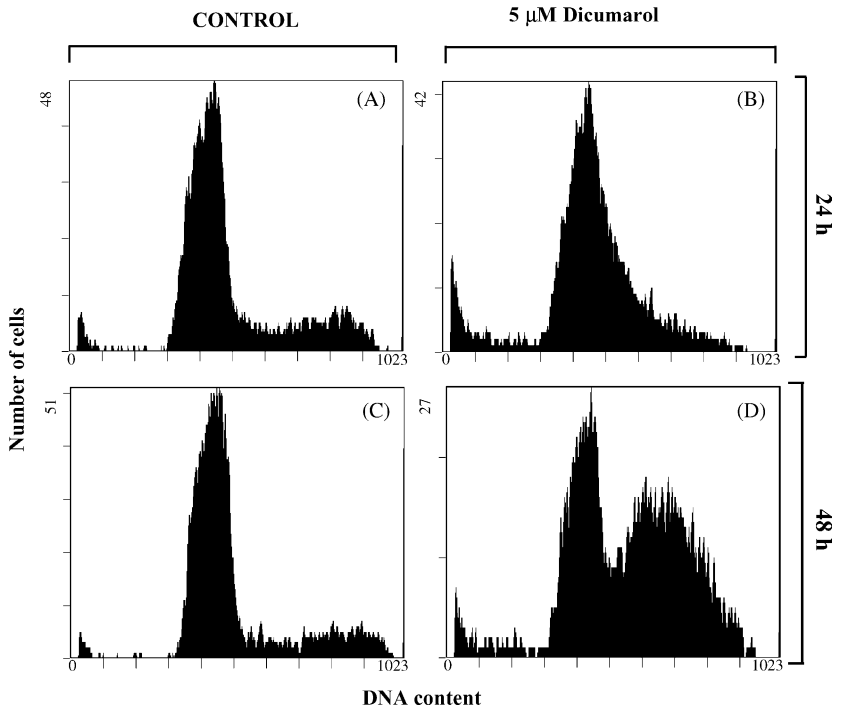

Fig. 4. Stimulation of $G_{0/1}$/S transition in HL-60 cells treated with 5 μM dicoumarol after a 24 h preculture in the absence of serum. After treatments, cells were stained with propidium iodide and analyzed for DNA content by flow cytometry. Pretreatment without serum greatly reduced dicoumarol-induced apoptosis, but inhibition of $G_{0/1}$ blockade was still observed. Results shown are representative of nine separate experiments.

NQO1 activity. However, dicoumarol can also interfere with NQO1 functions independently of its catalytic activity [13]. Moreover, dicoumarol may also exerts its effects through mechanisms unrelated to NQO1, either by inhibiting other enzymes, including UDPglucuronosyltransferase [29], glutathione transferases and glutathione peroxidase II [30], or affecting interactions between other proteins such as tubulin [11]. In addition, dicoumarol is a powerful mitochondrial uncoupler [31]. The possibility that dicoumarol could target other cellular systems different to NQO1 is an important issue in this study, inasmuch as HL-60 cells have been described as defective in NQO1 expression [18].

Thus, we first measured NQO1 activity in cytosolic fractions obtained from HL-60 cells cultured under control conditions, and compared this value with the activity of a cell line with high NQO1 expression, such as confluent human carcinoma HeLa cells [32]. As depicted in Fig. 9A, NQO1 activity could be measured in cytosols from HL-60 cells, but at levels about two orders of magnitude lower than in HeLa cells. Differences in specific activities between both cell lines corresponded with very different levels of NQO1 expression. When equal amounts of cytosolic protein from HeLa or HL-60 cells were loaded in electrophoresis gels and stained bands were developed simultaneously, NQO1 could be detected in HeLa, but almost no reaction was found in HL-60 (Fig. 9B). However, a very faint band corresponded to the molecular mass of NQO1 could be distinguished in these Western blots that prompted us to study the possible expression of NQO1 in HL-60 cells with more detail. Actually, when a higher amount of protein (100 μg) was loaded in SDS-gels, and

Table 1
Effect of dicoumarol treatment on cell cycle parameters and apoptosis of HL-60 cells precultured for 24 h in serum-free medium

Treatment	G_1	S	G_2/M	Apoptosis	*n*
Control, 24 h	53.86 ± 7.47	35.96 ± 6.08	10.66 ± 3.28	3.98 ± 1.96	16
Control, 48 h	54.48 ± 9.54	36.1 ± 8.35	9.39 ± 4.27	7.39 ± 6.69	10
Dicoumarol, 24 h	17.36 ± 10.4[a]	76.55 ± 12.0[a]	6.09 ± 3.95[a]	7.46 ± 4.85	10
Dicoumarol, 48 h	26.50 ± 6.49[a,b]	60.78 ± 13.54[a,b]	12.71 ± 7.74[b]	11.36 ± 5.67	9

HL-60 cells were precultured for 24 h in serum-free RPMI-1640 medium before they were treated with 5 μM dicoumarol for 24 or 48 h. Dicoumarol was omitted in controls. Cells were then fixed with cold 70% ethanol and stained with propidium iodide for analysis of cell populations by flow cytometry. Number of apoptotic cells and cells in each phase of the cycle were determined using the algorithm described by Watson et al. [22]. Apoptosis values (expressed in %) are referred to total cell population (apoptotic plus nonapoptotic). Values corresponding to cells in G_1, S or G_2/M (expressed in %) are referred to the nonapoptotic cell population. Results are mean ± S.D. *n* = number of determinations.

[a] Differences were statistically significant vs. the corresponding control without dicoumarol ($p < 0.05$).

[b] Differences were statistically significant vs. 24 h of treatment with dicoumarol ($p < 0.05$).

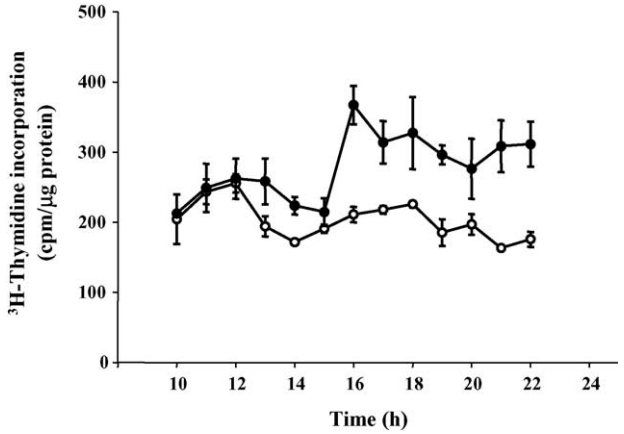

Fig. 5. Dicoumarol increases incorporation of ^{3}H-thymidine in HL-60 cells in the absence of serum. Cells were precultured for 24 h in serum-free medium, and then stimulated with 5 μM dicoumarol (closed symbols). Control cells (open symbols) received an equal volume of 6 mM NaOH. At the indicated times, 30 min pulses of [methyl-^{3}H]thymidine (0.25 μCi/ml) were given. Cells were then washed by centrifugation and precipitated with trichloroacetic. Radioactivity was measured in precipitates, resuspended with 1N NaOH and referred to total protein. Experiments were carried out in triplicate. Results are mean ± S.D.

films were exposed for a longer time, NQO1 band could be easily detected. For experiments depicted in Fig. 9C, we precultured HL-60 cells for 24 h without serum and treatments with dicoumarol were then carried out for additional 24 or 48 h. Levels of NQO1 protein did not change significantly at 24 h (with or without 5 μM dicoumarol) and at 48 h without serum. However, expression of NQO1 was considerably increased after 48 h in the presence of

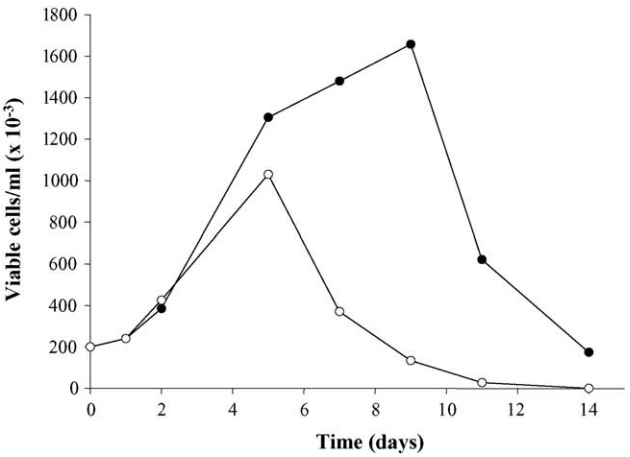

Fig. 6. Dicoumarol stimulates the growth of HL-60 cells in the absence of serum. Cells were precultured for 24 h in serum-free medium, and then stimulated with 1 μM dicoumarol (at zero time; closed symbols). Control cells (open symbols) received an equal volume of 6 mM NaOH. Culture medium was changed each 2–3 days with addition of fresh dicoumarol or vehicle. At the times of medium changing, total viable cells were counted and then seeded again at about 500,000 viable cells/ml. Values depicted in the figure were calculated according to the ability of the culture to growth at each point and the initial number of cells at the beginning of the experiment. Results shown are a representative growth curve from three different experiments.

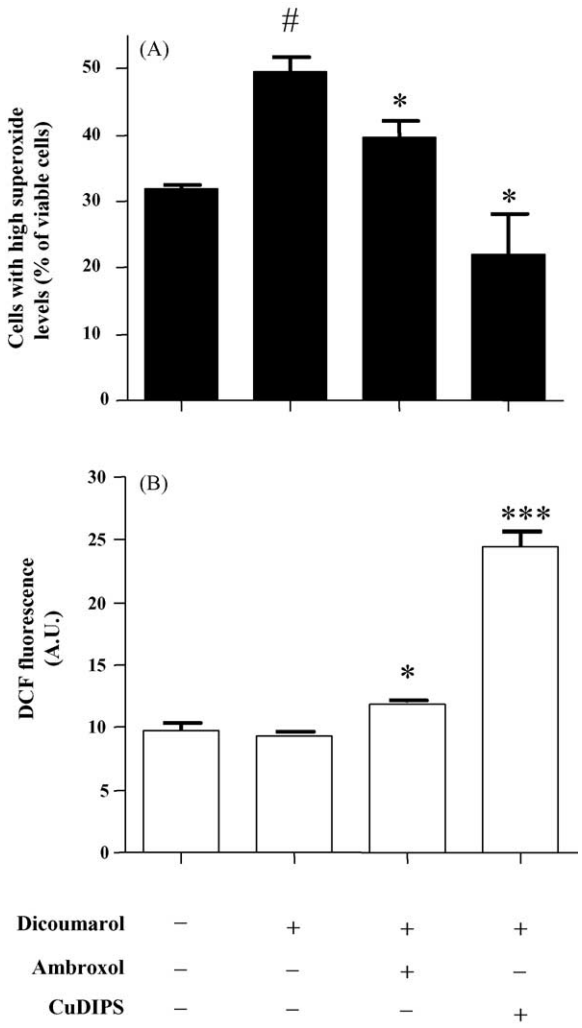

Fig. 7. Effect of dicoumarol on the regulation of ROS levels in HL-60 cells in the absence of serum. Cells were preincubated for 24 h in serum-free medium, and then treated with 5 μM dicoumarol for another 24 h. Treatments were also carried out in the presence of two SOD mimetics: ambroxol (100 μM) and CuDIPS (10 μM). After treatments cells were stained with DCFH-DA and Het for detection of peroxide and superoxide, respectively, by flow cytometry. Dicoumarol produced a significant increase of superoxide ($^{\#}p < 0.05$), but did not affect peroxide. Both ambroxol and CuDIPS decreased superoxide ($^{*}p < 0.05$), and produced a significant increase of peroxide ($^{*}p < 0.05$, $^{***}p < 0.001$). Data are mean ± S.E.M. of two independent experiments.

5 μM dicoumarol in serum-free medium. Thus, although expressed at low levels in HL-60 cells compared to other cell lines such as HeLa, these cells do not completely lack NQO1, and its expression may be enhanced by dicoumarol treatment.

To test whether or not dicoumarol-induced promotion of S phase entry involved inhibition of NQO1 activity, we tested the effect of ES936, a novel mechanism-based inhibitor of NQO1 that inhibits irreversibly this enzyme at nanomolar concentrations without affecting other cellular reductases [19]. HL-60 cells were precultured in serum-free medium, treated with ES936 (at 30, 100, 500 or 1000 nM) for 24 or 48 h and then processed for flow

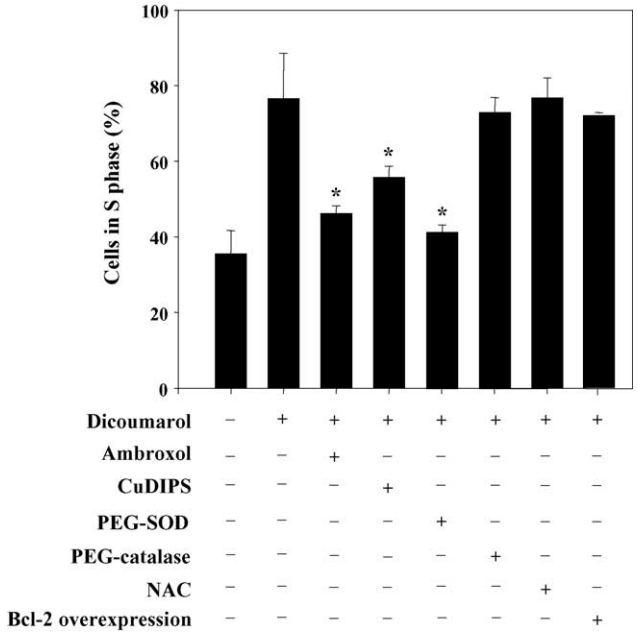

Fig. 8. Effect of antioxidants on dicoumarol-induced S phase entry. HL-60 cells were treated with 5 μM dicoumarol either alone, or in the presence of the following antioxidants: ambroxol (100 μM), CuDIPS (10 μM), NAC (5 mM), polyethylene glycol-SOD (300 U/ml) and polyethylene glycol-catalase (1000 U/ml). The effect of dicoumarol was also tested in a HL-60-derived cell line overexpressing Bcl-2. After treatments (24 h), cells were fixed with cold 70% ethanol, stained with propidium iodide and analyzed for DNA content by flow cytometry. Experiments were carried out in triplicate. Results are mean ± S.D.

cytometry analysis. As shown in Fig. 10A and C (which depict results obtained with 100 nM ES936), this NQO1 inhibitor was completely unable to promote entry of HL-60 cells into the S phase. Similar results were obtained with all the concentrations of ES936 tested, despite the fact that almost a 100% inhibition in NQO1 activity was achieved in all cases (data not shown). Interestingly, 5 μM dicoumarol was still able to promote G_1/S transition even in the presence of ES936 at all the concentrations tested (Fig. 10B and D depicts results obtained with 100 nM ES936 and 5 μM dicoumarol). These results clearly demonstrate that dicoumarol-induced impairment of $G_{0/1}$ blockade cannot be explained solely on the basis of the inhibition of NQO1 catalytic activity.

4. Discussion

Recent reports have documented that dicoumarol, a quinone reductase inhibitor, exerts profound effects on cell growth and death in various cellular systems by altering the function of several signaling pathways. Of particular importance was the demonstration that dicoumarol treatment results in the degradation of p53, thus blocking wild-type p53-mediated apoptosis in γ-irradiated normal thymocytes and in M1 myeloid leukemic cells overexpressing wild-type *p53* [7,12]. We recently reported that simultaneous withdrawal of serum and treatment with dicoumarol apparently potentiated growth arrest and apoptosis in HL-60 cells [14,15]. Since these cells completely lack p53, our previous results clearly indicate that important processes of cell growth regulation that are affected by dicoumarol do not involve this tumor suppressor. We were then interested in studying how dicoumarol affects the cell cycle in this leukemia myeloid cell line. This is very important because p53 is often mutated in various tumors [17].

Serum withdrawal delayed the growth of HL-60 cells with a concomitant increase of the number of cells in G_1 phase. Interestingly, dicoumarol impaired G_1 blockade in the absence of growth factors and favored transition of viable cells into the S phase. This conclusion is supported by several experimental independent observations: (i) the decrease of cells in G_1 and the concomitant increase in a population with DNA content intermediate between G_1 and G_2, as detected by flow cytometry, (ii) the phosphorylation of pRB, one of the hallmarks of G_1/S transition, (iii) the increase of DNA synthesis, measured as thymidine incorporation, observed after 16 h in cells treated with 5 μM dicoumarol in the absence of serum, but not in control cells; and (iv) the promotion of cell growth observed by

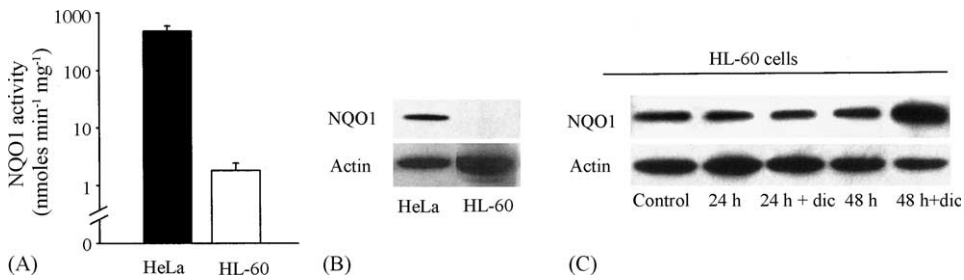

Fig. 9. Measurement of NQO1 activity and protein in HL-60 cells. (A) NQO1 specific activity measured in cytosolic fractions isolated from HL-60 and confluent HeLa cells. Enzymatic activity was assayed with 70 μg of cytosolic protein. Data are means ± S.D. of two independent determinations ($p < 0.001$). (B) Immunodetection of NQO1 polypeptide in HeLa and HL-60 cells. About 70 μg of cytosolic protein were loaded in SDS-gels. Bands corresponding to HeLa and HL-60 cells were developed simultaneously. (C) Immunodetection of NQO1 polypeptide in HL-60 cells preincubated for a 24 h without serum (control), and then treated for 24 or 48 h with or without 5 μM dicoumarol. About 100 μg of cytosolic protein were loaded in SDS-gels. Films were exposed for a longer time than in (B) until bands were clearly recognized. In all cases, after immunolabeling, blots were stripped and reprobed with anti-actin antibody as a marker for protein loading. Results depicted in (B) and C) are representative of three independent determinations.

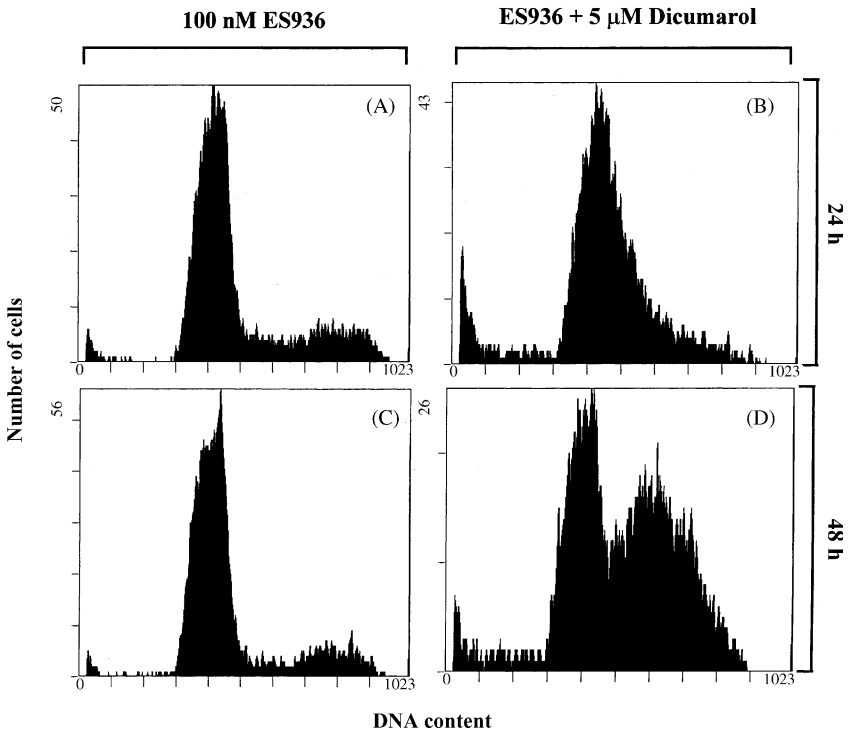

Fig. 10. Inhibition of NQO1 enzymatic activity with ES936 does not stimulate $G_{0/1}$/S transition of HL-60 cells in the absence of serum. Cells were precultured for 24 h in serum-free medium and then treated with either ES936 (at 30, 100, 500 or 1000 nM) or ES936 plus 5 μM dicoumarol. The figure represents results obtained with 100 nM ES936, but similar results were obtained at all the concentrations of ES936 tested. After 24 or 48 h treatment, cells were fixed with cold 70% ethanol, stained with propidium iodide and analyzed for DNA content by flow cytometry.

long-term culturing of HL-60 cells without serum but in the presence of dicoumarol. Dicoumarol at 5 μM had no significant effect on cells in the presence of 10% serum. This effect is likely related to the complexation of dicoumarol by serum albumin, which avoids its uptake into the cells [9,32].

This is the first demonstration that dicoumarol can stimulate the growth in a human tumor cell line. Despite the stimulatory effect we show here, most effects of dicoumarol on cells reported to date are inhibition of cell growth and potentiation of cell death. These effects include the blockade of stress-activated protein kinase/c-Jun NH_2-terminal kinase and NFκB pathways and the potentiation of apoptosis induced by TNF-α [5] and serum withdrawal in HeLa cells [32], the suppression of the malignant phenotype of pancreatic cancer cells [8,10], and the inhibition of the first cleavage of sea urchin embryos [11]. In accordance, a variety of coumarin compounds display antiproliferative and antitumor activities inhibiting the proliferation of a number of human malignant cells lines in vitro [11]. Cell growth stimulation by dicoumarol in HL-60 cells could reflect a cell type-specific effect. In accordance, 5 μM dicoumarol did not increase thymidine incorporation in HeLa and 3T3 cells cultured in the absence of serum (F.J. Alcaín, D. González-Aragón and J.M. Villalba, unpublished observations; see also ref. [32]). On the other hand, it should be also taken into account that concentrations of dicoumarol that have been used in some of the cited studies are much higher (100–300 μM) than that used in our study (1–5 μM), and toxic effects caused by dicoumarol at these high concentrations could unmask a putative stimulatory action on some cell types. For instance, dicoumarol at 75 μM increases cell viability of M1-t-p53 myeloid leukemic cells overexpressing wild-type *p53* gene, but concentrations above 125 μM are toxic to these cells [7].

Reactive oxygen species are important mediators of cell signaling cascades controlling cell growth. Depending on the intensity and duration of the stimulus, the site of ROS generation, and the cell type, responses elicited in cells by ROS may be growth stimulation, transient or permanent growth arrest, senescence, and apoptosis or necrosis [33,34]. It has been reported very recently that a redox-sensitive pathway operates in early G_1 to control the progression of cells into the S phase, and this transition requires mild prooxidant conditions [27]. In this way, *N*-acetyl-cysteine induces cell cycle arrest at G_1 through its reducing activity [35]. Furthermore, ROS that are generated during the metabolism of anticancer quinones cause inappropriate S/G_2 entry [36]. It has been previously shown that dicoumarol increases superoxide levels in pancreatic cancer cells [8]. However, in that study dicoumarol was used at a very high concentration (50–250 μM). Thus, we tested whether or not ROS levels were also increased in HL-60 cells by treatment with low concentrations of dicoumarol (5 μM). Our results showed that dicoumarol at 5 μM also increased superoxide, but did not affect peroxide. This increase of superoxide is an important factor that determines the inhibition of $G_{0/1}$ blockade,

because S phase entry was significantly inhibited by a cell-permeable form of SOD and by ambroxol and CuDIPS, two SOD mimetics. Our results are in agreement with the recent demonstration that overexpression of MnSOD inhibits the growth of androgen-independent prostate cancer cells by retarding G_1 to S transition [37]. NAC is an antioxidant and glutathione precursor that has been widely employed to inhibit oxidative cascades in cells. NAC is a powerful scavenger of hypochlorous acid, it reacts very easily with hydroxyl radical, and it also reacts slowly with H_2O_2, but it does not react with superoxide [38]. In accordance, dicoumarol also induced S phase entry in the presence of NAC. Peroxide was also unlikely to be involved in the mitogenic response of cells to dicumarol, because both SOD mimetics, which inhibited dicoumarol-stimulated G_1 to S transition, increased peroxide and furthermore, the effect of dicoumarol was not inhibited by a cell-permeable form of catalase. Furthermore, whereas it has been reported that the antiapoptotic protein Bcl-2 retards G_1/S transition by decreasing intracellular ROS levels [28], overexpression of the *BCL-2* gene in HL-60 cells did not inhibit the effect of dicoumarol. Taken together, our results support that an increase of superoxide by dicoumarol treatment is very important for inhibition of $G_{0/1}$ blockade, but a change in cellular sulphydryls, peroxide or a pathway involving Bcl-2 may not play a major role in this response.

Dicoumarol has been widely employed to determine the involvement of NQO1 in biochemical reactions and cellular processes, but physiological consequences of dicoumarol on cells do not necessarily mean involvement of NQO1 activity. In fact, dicoumarol can also inhibit other enzyme activities, including UDPglucuronosyltransferase [29], glutathione transferases and glutathione peroxidase II [30]. A cytosolic NADPH-ubiquinone reductase that is separate from NQO1 can be also inhibited by dicoumarol, although concentrations required to inhibit this enzyme are 3–4 orders of magnitude higher than those required to inhibit NQO1 [39]. In addition, dicoumarol is a powerful mitochondrial uncoupler [31]. Thus, an important question in our study was to elucidate whether or not dicoumarol-induced potentiation of S phase entry was indeed related to the inhibition of NQO1 activity. Since HL-60 cells are defective in NQO1 expression [18], we first studied levels of NQO1 under our experimental conditions. By using both an enzymatic assay, and immunodetection in Western blots, we can conclude that NQO1 is actually expressed in HL-60 cells, although at levels much lower than in HeLa cells, a cell line with a high NQO1 content [32]. Furthermore, we show here that levels of NQO1 protein are significantly increased after 48 h treatment with 5 μM dicoumarol. To investigate whether or not inhibition of NQO1 was responsible for inhibition of $G_{0/1}$ blockade, we tested the effect of ES936, a novel mechanism-based inhibitor of NQO1 that inhibits irreversibly this enzyme at nanomolar concentrations without affecting other cellular reductases [19]. Specific abolishment of NQO1 activity with ES936 did not impaired G_1 blockade of HL-60 cells in the absence of serum at any of the concentrations tested. Furthermore, dicoumarol was still able to promote G_1/S transition even in the presence of ES936, demonstrating clearly that the sole inhibition of NQO1 catalytic activity is not sufficient for inhibiting $G_{0/1}$ blockade in HL-60 cells.

Given that NQO1 inhibition per se was not the stimulus responsible for dicoumarol-induced and superoxide-dependent S phase entry in HL-60 cells, the actual site of ROS production in dicoumarol-treated cells remains to be elucidated. It has been proposed that ROS generated during mitochondrial oxidative phosphorylation could act as a signaling mechanism early in G_1 in preparation for entry of mouse embryo fibroblasts into S phase [27]. Interestingly, recent studies using HL-60 cells and gene knockout mitocondria-deficient HL-60ρ^0 cells demonstrate that dicoumarol inhibits nonmitochondrial oxygen consumption, but increases mitochondrial oxygen consumption, most likely due to its effect as uncoupler [31]. A possible role for mitochondria-derived ROS in the mitogenic response of HL-60 cells to dicoumarol is currently under investigation.

In summary, our results are the first evidence supporting the existence of a dicoumarol-inhibitable $G_{0/1}$ checkpoint in human myeloid leukemia HL-60 cells. Treatment of cells with dicoumarol at micromolar concentrations in the absence of serum increases superoxide and impairs $G_{0/1}$ blockade through a mechanism involving this ROS, thus favoring transition towards the S phase. This effect does not rely solely on the inhibition of NQO1 catalytic activity. Our results send a precautionary message about use of dicoumarol to elucidate cellular processes involving oxidoreductases.

Acknowledgements

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