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Dicoumarol impairs mitochondrial electron transport and pyrimidine biosynthesis in human myeloid leukemia HL-60 cells

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ABSTRACT

Dicoumarol, a competitive inhibitor of NAD(P)H:quinone oxidoreductase 1 (NQO1), increases intracellular superoxide and affects cell growth of tumor cells. This work was set to establish a mechanistic link between dicoumarol, superoxide and cell cycle alterations in HL-60 cells. Using ES936, a mechanism-based irreversible inhibitor of NQO1, we demonstrate that NQO1 inhibition is not a major factor involved in superoxide boost. Mitochondrial Complexes II, III and IV were directly inhibited by dicoumarol. Succinate, which inhibits superoxide generation by reversed electron flow in Complex II, significantly decreased superoxide boost in dicoumarol-treated cells and in isolated mitochondria incubated with dicoumarol and decylubiquinol. Superoxide generation in cells was strongly potentiated by blocking the quinone site of Complex II with thenoyltrifluoroacetone, supporting the involvement of cytochrome b_{560} to drive electrons for increasing superoxide. Simultaneous inhibition of the mitochondrial chain upstream ubiquinone and displacement of succinate from the Complex II active site is proposed as a major mechanism to explain how dicoumarol increases superoxide in HL-60 cells. Dicoumarol-treated cells accumulated in S phase due to the impairment of pyrimidine biosynthesis at dihydroorotate dehydrogenase step because blockade was overcome by addition of exogenous uridine or orotate, but not by dihydroorotate. We demonstrate for the first time that dicoumarol inhibits mitochondrial electron transport, induces superoxide release by reversed electron flow in Complex II, and inhibits pyrimidines biosynthesis. These actions must be taken into account when considering dicoumarol effects on cells.

1. Introduction

Dicoumarol is a coumarin-derived natural compound used clinically by its anticoagulant properties. Its best defined action on mammalian cells is the inhibition of NAD(P)H:(quinone acceptor) oxidoreductase 1 (NQO1) by competing with NAD(P)H at the pyridine nucleotide binding site [1]. However, dicoumarol can also interfere with NQO1 functions independently of

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Abbreviations: CLAP, chymostatin, leupeptin, antipain and pepstatin; ES936, 5-methoxy-1,2-dimethyl-3-[(4-nitrophenol)methyl]-indole-4,7-dione; DB, decylubiquinone; DBH_2, decylubiquinol; DCPIP, 2,6-dichlorophenolindophenol; FCS, fetal calf serum; HEt, hydroethidine; NQO1, isoform 1 of the cytosolic NAD(P)H:(quinone acceptor) oxidoreductase, DT-diaphorase; ROS, reactive oxygen species; TTFA, thenoyltrifluoroacetone

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inhibiting its catalytic activity by preventing NQO1–p53/p73 interactions [2], and it can also exerts its effects through mechanisms unrelated to NQO1, by inhibiting UDPglucuronosyltransferase [3], glutathione transferases and glutathione peroxidase II [4], or affecting the stability of microtubules [5]. In addition, dicoumarol has been described as a mitochondrial uncoupler [6,7].

Recent works have documented that dicoumarol produces a significant increase in steady-state levels of intracellular superoxide in different cellular models such as pancreatic cancer cells [8,9] and human myeloid leukemia HL-60 cells [10]. Interestingly, dicoumarol-induced augmentation of superoxide inhibits the malignant phenotype of pancreatic cancer cells [8,9]. In HL-60 cells, dicoumarol increases significantly S phase population of cultures in the absence of serum [10]. Elucidating the mechanism by which dicoumarol increases superoxide in cells is clearly of great importance because superoxide affects the growth rate and malignancy of tumor cells, and dicoumarol has been proposed as a putative antitumor agent *in vivo* mostly due to its ability to increase superoxide [8,9]. Inhibition of the malignant phenotype of pancreatic cancer cells by dicoumarol *via* a superoxide-mediated mechanism was previously interpreted on the basis of NQO1 inhibition [8,9]. While this interpretation would be in accordance with the proposed role played by NQO1 as a superoxide scavenger [11], the participation of NQO1 inhibition in dicoumarol-stimulated generation of superoxide was not unambiguously demonstrated. In fact, it has been shown recently that ES936 (5-methoxy-1,2-dimethyl-3-[(4-nitrophenol)methyl]-indole-4,7-dione), a mechanism-based irreversible inhibitor of NQO1, does not increase superoxide in pancreatic cancer cells [12], which agrees with our previous data showing that S phase accumulation of HL-60 cells treated with dicoumarol was also independent on NQO1 inhibition [10]. The relative unspecificity of dicumarol towards different cellular targets are indicative that separate mechanisms different to the inhibition of NQO1 might be involved in superoxide increase.

Mitochondria largely contribute to the production of reactive oxygen species (ROS) into the cells *via* the respiratory chain. The one-electron reduction of oxygen generates superoxide, that can be transformed into hydrogen peroxide by the various cellular superoxide dismutases [13,14]. The participation of Complex I (NADH-ubiquinone oxidoreductase) and Complex III (ubiquinol-cytochrome *c* oxidoreductase) in ROS production by mitochondria is well established [13,14]. In addition, Complex II can be also a significant source of superoxide under particular conditions that lead to reversed electron transport from cytochrome b_{560} to FAD [15]. Studies using HL-60 cells and gene knockout mitochondria-deficient HL-60ρ^0 cells demonstrate that dicoumarol increases mitochondrial oxygen consumption [16]. Dicoumarol also increases oxygen consumption in isolated mitochondria obtained from EMT6 and CHO cells as well as total levels of ROS measured in these cells [7], indicating that the impairment of mitochondrial function might be related to superoxide increase.

This work was set as a first attempt to establish a mechanistic link between dicoumarol, superoxide generation and cell cycle alterations in HL-60 cells. ES936 had only a minor effect on superoxide, and dicoumarol still increased superoxide under conditions of complete depletion of NQO1 activity, which indicates that inhibition of NQO1 is not the major cause of superoxide increase. We demonstrate by the first time that dicoumarol inhibits mitochondrial Complexes II, III and IV, stimulates superoxide release by reversed electron flow at Complex II and inhibits the biosynthesis of pyrimidines at the dihydroorotate dehydrogenase step. The action of dicoumarol on the mitochondrial physiology must be taken into account when explaining its effects on cells.

2. Materials and methods

2.1. Cell cultures

Human myeloid leukemia HL-60 cells were cultured in RPMI-1640 medium (BioWhittaker, Verviers, Belgium) supplemented with 10% fetal calf serum (PAA Laboratories, Les Mureaux, France), 100 units/ml penicillin, 100 μg/ml streptomycin and 250 ng/ml amphotericin B (Sigma, Madrid, Spain) and 2 mM of L-glutamine (Sigma) at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. Before we carried out the treatments described in the next section, cells were concentrated from stock cultures by centrifugation at 500 × *g* for 5 min, washed twice in serum-free RPMI-1640 medium and then cultured in the same medium without FCS for additional 24 h. Cell viability of cultures was determined by the Trypan blue exclusion method.

2.2. Treatments

Unless otherwise stated, the compounds used in cell treatments were obtained from Sigma. Dicoumarol (3,3′-methylenebis[4-hydroxycoumarin]) was prepared at 2 mM in 6 mM NaOH and added to cells to a final concentration of 5–50 μM. Since dicoumarol can strongly bind serum albumin, which precludes its uptake into the cells [17], all experiments with this inhibitor were carried out in serum-free medium. ES936 was kindly provided by Prof. C.J. Moody (University of Exeter, Exeter, U.K.). Stock solutions of ES936 were prepared at 10 mM in DMSO, and added to cells to a final concentration of 100 nM. Thenoyltrifluoroacetone (TTFA) was prepared at 100 mM in ethanol. The inhibitor was added to cells to reach a final concentration of 25–50 μM. Uridine and ambroxol were dissolved in water at 10 mM and 20 mM, respectively; dihydroorotate and orotate were prepared at 30 mM in NaOH 6 mM. Optimal concentrations of all compounds used in our study were chosen from dose response curves in order to find those concentrations producing the maximal effect without decreasing significantly cell viability (data not shown). In all experiments, the same amount of the corresponding vehicle was added to controls.

2.3. Superoxide measurements

Steady-state levels of intracellular superoxide in cells were quantified using the probe hydroethidine (HEt) (Sigma, Spain). After its uptake into the cells, this compound can be oxidized by superoxide to yield a product (2-hydroxyethidium) that is

strongly fluorescent upon binding to nuclear DNA [18]. For superoxide analysis cells were incubated in the dark with 4 μM HEt for 30 min at 37 °C. After washing, fluorescence was measured by flow cytometry using a Coulter EPICS XL cytometer equipped with a 488 nm Argon laser. Fluorescence was determined at 620 nm (FL3). At least 20,000 cell events were recorded in the flow cytometer using the logarithmic mode. Since HEt can be oxidized by other molecules to yield different oxidation products with excitation and emission spectra overlapping with those of 2-hydroxyethidium, the specific reaction product of superoxide-mediated oxidation of HEt [18], it is important to verify that fluorescence changes were actually due to superoxide-mediated oxidation of the probe. To accomplish this task, controls were carried out by the simultaneous incubation of the cells with dicoumarol and a SOD mimetic. We used 2-amino-3,5-dibromo-*N*-[trans-4-hydroxycyclohexyl]benzylamine (ambroxol, 100 μM) or copper[II] diisopropyl salicylate (CuDIPS, 10 μM) [10]. Histogram data obtained by flow cytometry were analyzed using EXPO 32 ADC Analysis software (Beckman-Coulter, USA). The median was used for fluorescence quantification because this value is more appropriate when dealing with distributions on logarithmic scales [19]. In another series of experiments carried out *in vitro* with isolated mitochondria, the SOD-sensitive reduction of cytochrome *c* was also used as another estimation of superoxide radicals generation [20].

2.4. Preparation of cytosols and mitochondria from HL-60 cells

All procedures were carried out at 4 °C. For preparation of cytosolic fractions, cells were recovered by centrifugation at 500 × *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. After disruption of cells, the concentration of the lysis buffer was rinsed to 130 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 500 × *g* for 5 min and the supernatant was saved. Cytosolic fractions were separated from membranous material by ultracentrifugation at 100,000 × *g* for 30 min.

For mitochondria isolation, about 75–100 × 10^6 cells were recovered by centrifugation at 500 × *g* for 5 min and washed with cold PBS. Cells were then resuspended in hypotonic lysis buffer (10 mM Tris–acetate pH 6.7 containing 10 mM KCl, 1 mM $MgCl_2$, 1 mM PMSF and CLAP) and left to swell for 5 min at 4 °C. Homogenization of cells was carried out for 2 min using a Teflon pestle rotating at about 1500 rpm. After disruption of cells, the osmolarity of the lysis buffer was raised by adding sucrose to 0.25 M. Unbroken cells, debris and nuclei were separated by sedimentation at 500 × *g* for 3 min. Supernatants were recovered and centrifuged again at 5000 × *g* for 15 min. The pellet, consisting in a membrane fraction enriched in mitochondria, was resuspended in 10 mM Tris–acetate pH 7.0 containing 10 mM KCl, 1 mM $MgCl_2$, 1 mM PMSF, CLAP and 0.25 M sucrose, and washed by centrifugation at 500 × *g* for 3 min. The final mitochondria-enriched fraction was recovered by spinning the supernatant at 5000 × *g*, for 15 min, resuspended in the same buffer and used for enzymatic assays of mitochondrial complexes. Protein determinations were carried out with the Bradford method.

2.5. NQO1 activity

NQO1 activity in cytosolic fractions was measured from the NADH and menadione-dependent dicoumarol-inhibitable reduction of cytochrome *c*. Assays were carried out 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.

2.6. Enzymatic assay of mitochondrial complexes

The activity of mitochondrial complexes was assayed in a final volume of 1 ml, essentially as described by Trounce et al. [21] excepting for Complex IV, that was assayed basically as described by Storrie and Madden [22]. Assays were carried out by continuous recording of absorbance changes in a thermostatized spectrophotometer (Beckman, DU-640) at 30 °C (Complexes I, II and III) or 37 °C (Complex IV) with gentle stirring. All assays were carried out in the absence and in the presence of dicoumarol (50 μM).

Activity of complex I (NADH-ubiquinone oxidoreductase) was measured from the NADH-dependent and rotenone (2 μg/ml)-sensitive reduction of decylubiquinone (DB) in a medium containing 50 mM Tris–HCl, pH 7.4, 250 mM sucrose, 1 mM EDTA, 10 μM DB, 2 mM KCN and 45 μg cell mitochondrial protein. The reaction was initiated by adding 50 μM NADH. Reduction of DB was monitored at 272 nm (extinction coefficient of 8 mM^{-1} cm^{-1}).

The activity of complex II (succinate-ubiquinone oxidoreductase) was measured from the succinate and DB-dependent reduction of 2,6-dichlorophenolindophenol (DCPIP) in a reaction medium consisting of 50 mM potassium phosphate, pH 7.4, 20 mM succinate, 2 μg/ml antimycin A, 2 μg/ml rotenone, 2 mM KCN, 50 μM DCPIP and 30 μg cell mitochondrial protein. Complex II was activated by preincubating samples for 10 min at 30 °C, in order to remove inhibitor oxalacetate before running the assays of enzymatic activity [21]. The reaction was started with 50 μM DB and monitored at 600 nm (extinction coefficient of 19.1 mM^{-1} cm^{-1}).

The activity of Complex III (ubiquinol-ferricytochrome *c* oxidoreductase) was measured from the decylubiquinol (DBH_2)-dependent and stigmatellin (5 μM)-sensitive reduction of cytochrome *c*. The reaction mixture contained 250 mM sucrose, 1 mM EDTA, 50 mM Tris–HCl, pH 7.4, 50 μM cytochrome *c*, 2 mM KCN and 30 μg cell mitochondrial protein.

After initiating the reaction with 50 μM DBH_2, the increase of absorbance at 550 nm (due to reduction of cytochrome *c*) was monitored (extinction coefficient of 19 mM^{-1} cm^{-1}). DBH_2 was obtained by reducing DB with borohydride [21].

The activity of Complex IV (ferrocytochrome *c* oxidase) was assayed basically according to Storrie and Madden [22] with only one minor modification: 0.025% Triton X-100 instead Lubrol-PX was used in the assay. The reaction medium contained 25 μM reduced cytochrome *c*, 0.025% Triton X-100 and 50 μg of purified mitochondrial protein, in 40 mM phosphate buffer, pH 6.2. Oxidation of cytochrome *c* was monitored at 550 nm (extinction coefficient of 19 mM^{-1} cm^{-1}).

2.7. Cell cycle analysis

The number of cells in different phases of the cell cycle was measured by flow cytometry. Briefly, 10^6 cells were recovered by centrifugation at 500 × *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 by flow cytometry at 620 nm (FL3). At least 20,000 cell events were recorded in the flow cytometer. Population in each of cell cycle phases, G_1, S and G_2/M, was determined with the software Cylchred (Cardiff University). Cells with a sub$G_{0/1}$ DNA content were considered apoptotic, and this parameter was used as a marker for cytotoxicity of the compounds used in cell treatments.

2.8. Statistical analysis

Data shown in this article are medians (flow cytometry data acquired in the logarithmic mode) or means ± standard deviations from at least three different determinations. Significant differences were assessed using Student's *t*-test. The effect of dicoumarol concentration was analyzed by

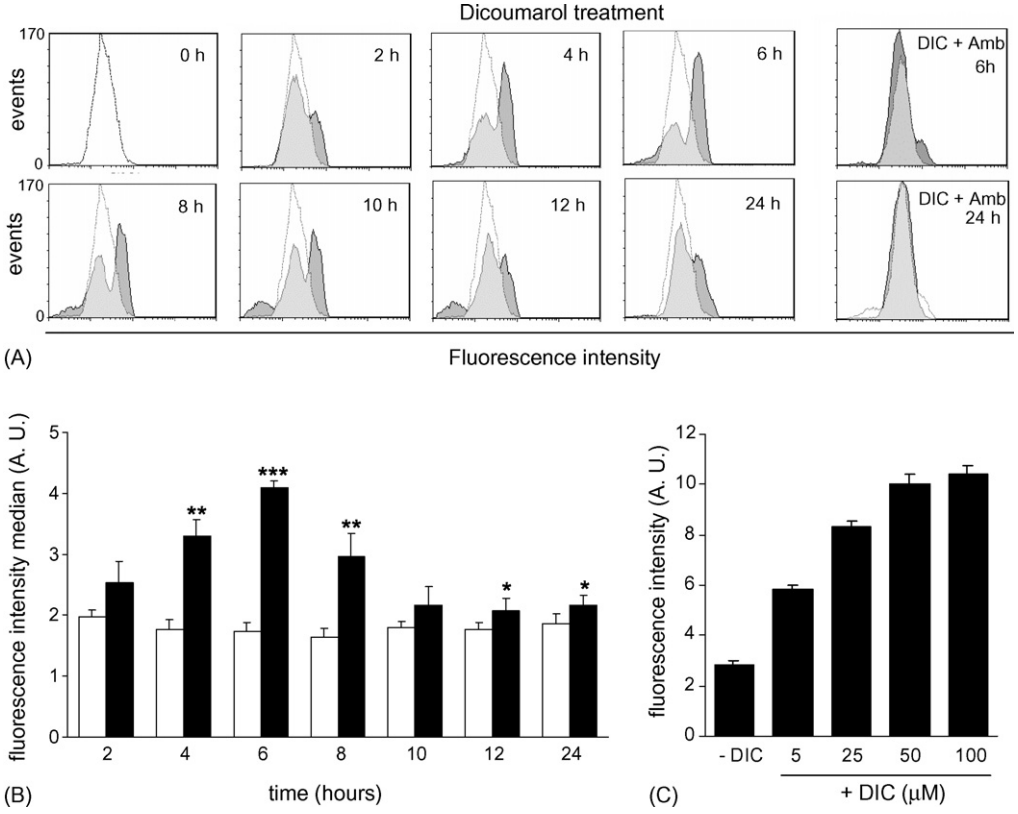

Fig. 1 – Kinetics of superoxide production in HL-60 cells treated with dicoumarol. (A) Fluorescence signal of oxidized HEt as detected by flow cytometry. Cells were preincubated for 24 h in serum-free medium, and then treated with 5 μM dicoumarol. At the indicated times, cells were labeled with 4 μM HEt, harvested by centrifugation and then analyzed by flow cytometry at 620 nm (FL3). Control cells were co-incubated with 5 μM dicoumarol and 100 μM ambroxol to verify that fluorescence signal was specific for superoxide-mediated oxidation of the probe. Open histogram depicts control values of superoxide in the absence of dicoumarol. Closed histograms represent superoxide signal in cells treated with dicoumarol for the indicated times. (B) Quantification of median values of fluorescence in time-course experiments. Values obtained for cells cultured for indicated times in serum-free medium but the absence of dicoumarol are presented in open bars, whereas closed bars represent values obtained for cells cultured in serum-free medium and in the presence of 5 μM dicoumarol. Depicted values are medians ± S.D. ($n = 3$). Significant differences with respective controls in the absence of dicoumarol for a given incubation time are represented by asterisks ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$). (C) Effect of dicoumarol concentration on superoxide levels. Cells were treated with the given concentrations of dicoumarol for 4 h. Cells were then labeled with HEt, and superoxide levels measured by flow cytometry. Median fluorescence values are shown ($n = 3$). The effect of concentration was statistically significant with $p < 0.0001$ (one-way analysis of variance).

one-way analysis of variance. Differences were considered significant with $p < 0.05$. Data were analyzed using Graphpad Prism 4.00 (Graphpad Software Inc., San Diego, CA, USA).

3. Results

3.1. Kinetics of superoxide generation in HL-60 cells treated with dicoumarol

We showed in a previous report that fluorescence due to oxidation of HEt (as an estimation for superoxide) was elevated after a 24 h treatment of HL-60 cells with 5 μM dicoumarol in the absence of serum [10]. Before we studied those factors that are mechanistically linked to dicoumarol-stimulated generation of superoxide in HL-60 cells, we first studied the kinetics of superoxide generation as a function of incubation time and of dicoumarol concentration. In addition, we wanted to confirm that fluorescence changes induced by dicoumarol treatment were actually mediated by superoxide, and not by other oxidizing species acting on the probe [18].

As depicted in Fig. 1A and B, addition of 5 μM dicoumarol to cells that had been precultured for 24 h in serum-free medium induced a rapid increase of fluorescence due to oxidized HEt, that was already detected at 2 h of treatment. Maximal accumulation was observed at 4–8 h and thereafter, the signal decayed progressively and was finally stabilized at 12 and 24 h of treatment (Fig. 1A and B). No significant changes in fluorescence signal were observed in cells that were cultured for the indicated times in serum-free medium but without addition of dicoumarol (see control bars in Fig. 1B). To verify that fluorescence increase after treatment of cells with dicoumarol was actually due to superoxide-mediated oxidation of the probe, control experiments were carried out by co-incubating cells with dicoumarol and SOD mimetics. The fluorescence signal of oxidized HEt was significantly reduced when HL-60 cells were co-incubated with dicoumarol plus 100 μM ambroxol both in 6 and 24 h treatments (Fig. 1A). The same result was obtained with CuDIPS, another SOD mimetic, as we reported is a previous publication [10]. Furthermore, as expected from the action of a SOD mimetic accelerating dismutation of superoxide to hydrogen peroxide, the decrease in fluorescence of oxidized HEt was accompanied by an increase of peroxide, measured by flow cytometry using the probe 2′,7′-dichlorodihydrofluorescein diacetate [10]. Taken together, these results clearly demonstrate that, in our model, the increase of fluorescence signal of oxidized HEt we observe upon addition of dicoumarol is specific for superoxide throughout the 24 h incubation period.

We next studied how the concentration of dicoumarol affected the superoxide signal in HL-60 cells. In experiments carried out at 4 h, the fluorescence of oxidized HEt was increased from 5 to 25 μM dicoumarol. The signal was then stabilized at a maximal value with 50 and 100 μM dicoumarol (Fig. 1C). Since a 5 μM concentration was found optimal because it did not decrease significantly the viability of cells in serum-free medium (about 85%), we chose this concentration for further experiments carried out with cells. Furthermore, this concentration would give enough sensitivity to detect both inhibitions and activations of superoxide generation due to the different treatments we carried out with the cells (see next sections). On the other hand, in experiments carried out with isolated mitochondria, a 50 μM concentration was chosen because this was the minimal concentration that gave a maximal response.

3.2. NQO1 inhibition plays a minor role in superoxide boost induced by dicoumarol in HL-60 cells

A role for NQO1 as a superoxide scavenger has been proposed recently [11]. Thus, we wanted to test whether or not superoxide increase induced by dicoumarol in HL-60 cells was caused by the inhibition of NQO1. To accomplish this task we studied the effect of ES936, a novel and highly selective mechanism-based irreversible inhibitor of NQO1 [23]. Treatment of HL-60 cells with 100 nM ES936 was sufficient to produce a complete depletion of NQO1 activity in cytosol extracts (data not shown). However, compared with dicoumarol, ES936 only produced a minor modification of superoxide levels. More interestingly, dicoumarol was still able to stimulate dramatically superoxide generation in the presence of ES936 (Fig. 2). These results clearly indicate that inhibition of NQO1 is not a major factor involved in superoxide generation in HL-60 cells, but dicoumarol targets other cellular components.

3.3. Dicoumarol inhibits mitochondrial Complexes II, III and IV

Since mitochondria are considered as the main generators of reactive oxygen species in mammalian cells [24] and it is known that dicoumarol can increase mitochondrial oxygen

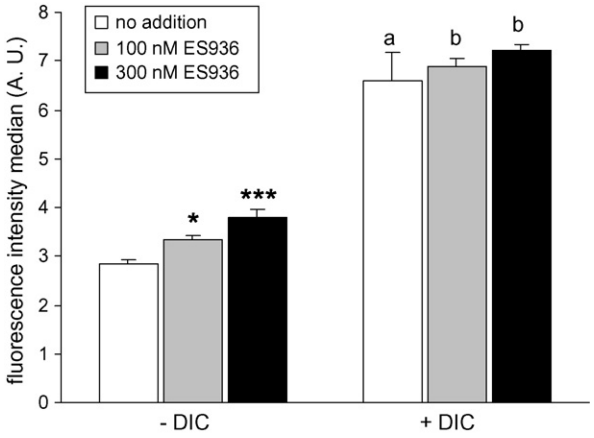

Fig. 2 – NQO1 plays a minor role in the regulation of superoxide levels in HL-60 cells. Cells were treated for 4 h with ES936 (100 or 300 nM) or with 5 μM dicoumarol plus ES936. A concentration of 100 nM ES936 was sufficient to produce the complete depletion of NQO1 activity (not shown). After incubation with the inhibitors, cells were stained with HEt and steady-state levels of intracellular superoxide analyzed by flow cytometry as described in the legend of Fig. 1. Depicted values are medians ± S.D. ($n = 3$). Significant differences with respect to their controls without ES936 are represented by asterisks ($^{*}p < 0.05$; $^{***}p < 0.001$). Significant differences with respect to controls with the same concentration of ES936 but without dicoumarol are represented with letters (a, $p < 0.01$; b, $p < 0.001$).

consumption [7], we considered the possibility that dicoumarol could increase superoxide levels by affecting the mitochondrial electron transport chain. Inhibition of the mitochondrial complexes by different compounds is well known to increase univalent reduction of molecular oxygen generating superoxide [25]. Thus, we studied how dicoumarol affected the activity of the mitochondrial complexes in their *in vitro* assays.

Complex I activity, measured from the NADH-dependent and rotenone-sensitive reduction of DB, was not affected significantly by dicoumarol (Fig. 3A), but Complex II activity, measured from the succinate and DB-dependent reduction of DCPIP, was inhibited by dicoumarol. Significant decrease of Complex II activity in the presence of dicoumarol was observed at low concentrations of succinate, whereas increasing substrate concentration in the reaction mixture resulted in a less degree of inhibition. This is in accordance with a competitive inhibition of Complex II activity by dicoumarol, as shown in an early report [6]. Complex III activity, measured from the DBH_2-dependent and stigmatellin-sensitive reduction of cytochrome *c* was slightly inhibited by dicoumarol (Fig. 3C), and the same result was obtained when specificity of Complex III activity was tested by employing antimycin (data not shown). Finally, Complex IV activity, measured from the oxidation rates of reduced cytochrome *c*, was significantly inhibited (about 50%) by dicoumarol (Fig. 3D).

3.4. *Dicoumarol-stimulated superoxide generation involves reversed electron flow at Complex II*

Inhibition of the mitochondrial chain upstream ubiquinone (i.e. at Complexes III and IV) might have an effect similar to hypoxia, and thus it could result in increased generation of superoxide in Complex III [26] and/or in Complex II by reversed electron transport [27]. Complex III inhibition per se is also known to increase superoxide [14].

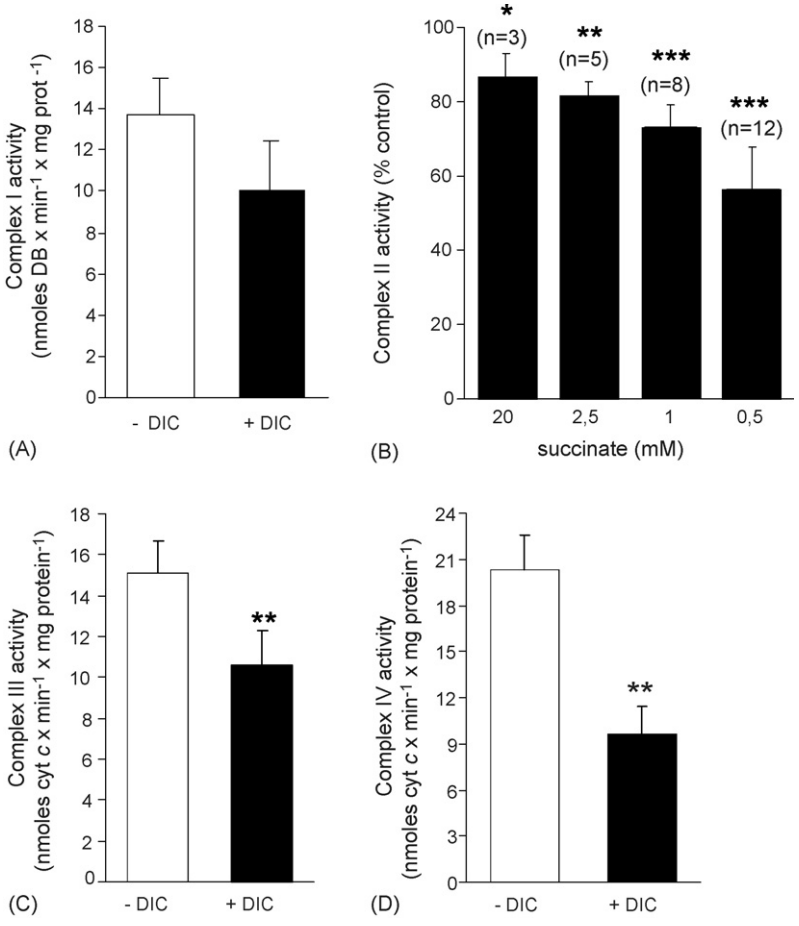

Fig. 3 – Effect of dicoumarol on the activity of mitochondrial complexes. Assays were carried out with fractions of purified mitochondria freshly isolated from HL-60 cells, either in the absence or in the presence of 50 μM dicoumarol. (A) Complex I. Activity was not affected significantly by dicoumarol ($n = 4$). (B) Complex II. Results depict the activity obtained with dicoumarol with respect to that obtained at the same concentration of succinate but without dicoumarol, the latter being normalized to 100% activity in each case. Succinate dehydrogenase was significantly inhibited by dicoumarol at low concentrations of substrate, but increasing succinate concentrations in the reaction medium resulted in a loss of inhibition (see number of repetitions in the figure). (C) Complex III. Activity was inhibited significantly by dicoumarol ($n = 4$). (D) Complex IV. Cytochrome c oxidase activity was significantly inhibited by dicoumarol ($n = 5$). Depicted values are means ± S.D. Significant differences in relation to respective controls without dicoumarol are represented with asterisks ($^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$).

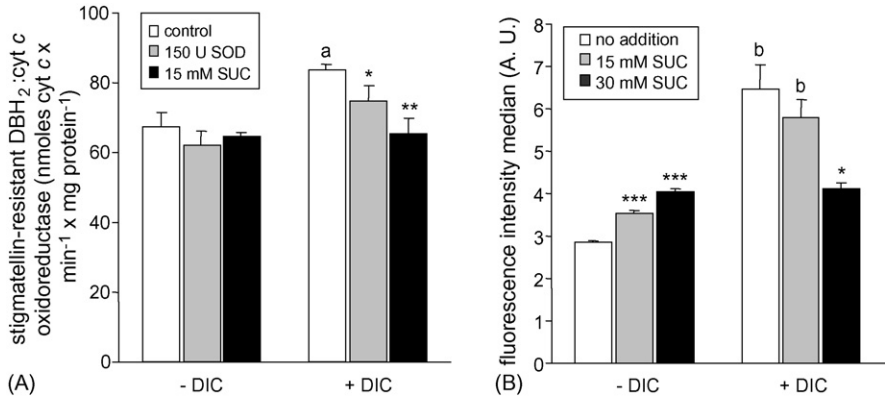

Fig. 4 – Reversed electron transport in Complex II is involved in dicoumarol-stimulated generation of superoxide. (A) Stigmatellin-resistant and DBH_2-dependent reduction of cytochrome c in mitochondria isolated from HL-60 cells. Dicoumarol produced a significant stimulation of DBH_2-dependent cytochrome c reduction that was inhibited by both SOD and succinate, supporting the involvement of superoxide anions and reversed electron transport in Complex II. Depicted values are means ± S.D. ($n = 3$; $^{**}p < 0.01$). Significant differences with respect to their controls without addition (succinate or SOD) are represented by asterisks ($^{*}p < 0.05$). Significant differences with respect to controls with succinate or SOD but without dicoumarol are represented with letters (a, $p < 0.05$). (B) Succinate inhibits superoxide production in dicoumarol-treated cells *in vivo*. Cells were treated for 4 h with 5 μM dicoumarol either in the absence or in the presence of 15 or 30 mM succinate. Cells were then harvested and steady-state levels of intracellular superoxide measured by HEt labelling and flow cytometry. Depicted values are medians ± S.D. ($n = 3$). Significant differences with respect to their controls without succinate are represented by asterisks ($^{*}p < 0.05$; $^{***}p < 0.001$). Significant differences with respect to controls with the same concentration of succinate but without dicoumarol are represented with letters (b, $p < 0.01$).

To investigate the site of superoxide generation in cells treated with dicoumarol, we tested the effect of succinate, because the reversed enzymatic reaction of Complex II (i.e. fumarate reductase), and hence superoxide generation due to this reaction, can be specifically inhibited by application of succinate [20,27], whereas superoxide production at Complex III is significantly increased when electrons are delivered into the mitochondrial chain through Complex II [13]. Interestingly, the portion of DBH_2-dependent reduction of cytochrome c that was resistant to the Complex III inhibitor stigmatellin was also significantly stimulated by dicoumarol. Addition of SOD decreased this reduction of cytochrome c, which supports the involvement of superoxide radicals. Consistent with a role for reversed electron transport at Complex II in the generation of these radicals, succinate inhibited completely the DBH_2-dependent and dicoumarol-stimulated reduction of cytochrome c in the presence of stigmatellin (Fig. 4A). The same results were obtained using antimycin A as Complex III inhibitor (data not shown). Since succinate competes with dicoumarol ([6] see also this work), these results support that competitive interaction of dicoumarol with Complex II (thus displacing succinate from the active site) might stimulate superoxide generation by reversed electron flow, particularly under conditions of Complexes III and IV inhibition.

We next tested how succinate affected superoxide levels measured by flow cytometry in whole cells. Succinate alone produced a slightly increase of superoxide in intact HL-60 cells, which is consistent with enhanced entry of electrons into the redox chain, thus increasing the reduction state of electron transporters. However, succinate also produced a dose-dependent reduction in steady-state levels of superoxide in cells treated with dicoumarol. When cells were cultured in the presence of 30 mM succinate, dicoumarol was completely unable to increase superoxide levels (Fig. 4B). These results also agree with the involvement of reversed electron transport in Complex II for generation of superoxide radicals in cells treated with dicoumarol *in vivo*.

Then, we were interested in studying the effect of TTFA, a frequently employed Complex II inhibitor that interferes with ubiquinone interaction [25,27,28]. This inhibitor was tested at concentrations of 25 and 50 μM because two different binding sites in Complex II have been reported that can be blocked by TTFA at these two concentrations, respectively [29]. These experiments were carried out both at 4 h, when dicoumarol provoked a boost of intracellular superoxide, and at 24 h, when a stabilization of superoxide levels was observed (see above, Fig. 1). TTFA alone did not affect significantly superoxide levels at any of the concentrations tested, both in 4 and 24 h treatments (Fig. 5A and B). At a concentration of 25 μM, TTFA did not affect significantly superoxide signal in cells treated with dicoumarol, either at 4 h (Fig. 5A) or at 24 h (Fig. 5B). However, a significant increase of superoxide was observed when cells were treated simultaneously with 5 μM dicoumarol and 50 μM TTFA. This stimulation by TTFA was particularly evident at 24 h of treatment (Fig. 5B). Fig. 5C depicts representative histograms of fluorescence signal due to oxidation of HEt in cells treated with dicoumarol alone or with dicoumarol plus TTFA.

3.5. *Superoxide is not involved in partial S phase blockade induced by dicoumarol*

We have previously shown that, in addition to causing a significant superoxide increase, dicoumarol also provoked cell

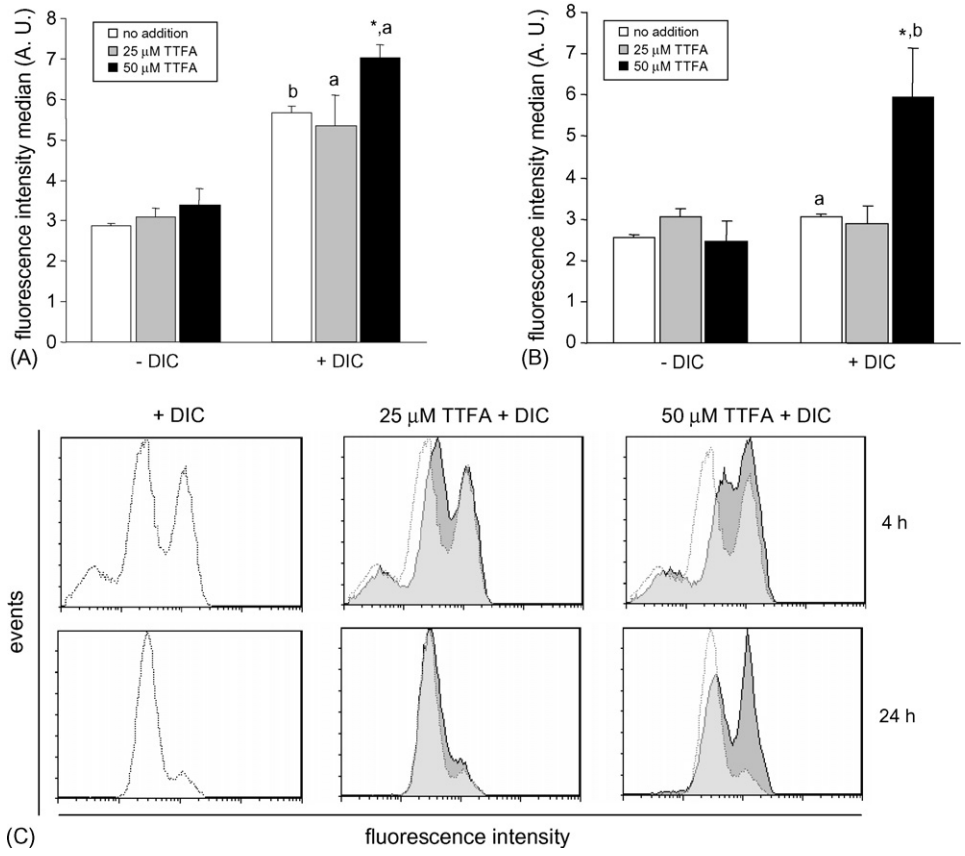

Fig. 5 – Effect of the Complex II inhibitor thenoyltrifluoroacetone (TTFA) in dicoumarol-stimulated generation of superoxide. Cells were treated for 4 or 24 h with 5 μM dicoumarol either in the absence or in the presence of TTFA at 25 or 50 μM. Cells were then harvested and steady-state levels of intracellular superoxide measured by HEt labelling and flow cytometry. (A) 4 h treatments and (B) 24 h treatments. Depicted values are medians ± S.D. ($n = 3$). Significant differences with respect to their controls without TTFA are represented by asterisks ($^*p < 0.05$). Significant differences with respect to controls with the same concentration of TTFA but without dicoumarol are represented with letters (a, $p < 0.05$; b, $p < 0.01$). (C) Representative histograms of fluorescence signal due to oxidation of HEt in cells treated with dicoumarol alone or with dicoumarol plus TTFA. Open histogram depicts values of superoxide signal in cells treated with dicoumarol alone. Closed histograms represent superoxide signal in cells treated with dicoumarol plus TTFA (25 or 50 μM) for the indicated times.

cycle alterations in HL-60 cells, inducing a significant increase in the number of S phase cells in the absence of serum [10]. Thus, we were also interested in elucidating whether or not superoxide boost induced by dicoumarol was causally linked to cell cycle alterations.

To answer this question HL-60 cells were treated with 5 μM dicoumarol for 24 h in serum-free medium to induce a significant accumulation of cells in S phase and then, three different treatments were carried out: (i) further incubation with dicoumarol for another 24 h period, (ii) culture in its absence for the same time, and (iii) culture in the presence of both 5 μM dicoumarol and 100 μM ambroxol. Consistent with our previous report [10], a significant accumulation in early S phase was observed after a 24 h treatment with dicoumarol (Fig. 6A). In the presence of dicoumarol, cells progressed slowly from early to late S phase during a 24 h period, which indicates the existence of a partial blockade in S phase. However, cells fully retained their ability to grow because they progressed rapidly through S phase after removal of the inhibitor. After 24 h most cells had already undergone mitosis and were found in G_1 phase of the next cycle (see Fig. 6A). To test the putative involvement of superoxide in partial S phase blockade, cells were incubated with dicoumarol plus ambroxol during the second 24 h period of treatment. As depicted in Fig. 6A, the rate of progression through S phase of cells cultured with dicoumarol plus ambroxol was not altered in comparison with that obtained with dicoumarol alone. However, incorporation of ambroxol clearly decreased the number of cells in early S phase compared with cells treated with dicoumarol alone (see arrows in Fig. 6A). This findings are indicative that increased steady-state levels of superoxide that are observed in cells treated with dicoumarol are not related to S phase blockade, and scavenging of this reactive species inhibits G_1 to S transition.

3.6. *Partial S phase blockade induced by dicoumarol is due to the inhibition of pyrimidines biosynthesis*

Mitochondrial inhibitors acting upstream ubiquinone affect indirectly the activity of dihydroorotate dehydrogenase, a

mitochondrial enzyme required for biosynthesis of pyrimidines that uses ubiquinone as natural electron acceptor [30,31]. To test the hypothesis that partial S phase blockade induced by dicoumarol could be due to the inhibition of pyrimidines biosynthesis, we studied the effect of adding exogenous uridine. As shown in Fig. 6B, uridine completely released S phase blockade induced by dicoumarol and, after 9.5 h of uridine addition in the presence of dicumarol, cells were found in G_2/M. This clearly demonstrates that dicoumarol provokes S phase blockade in HL-60 cells due to a lack of pyrimidine nucleotides.

To elucidate the step of pyrimidines biosynthesis that was affected by dicoumarol, we investigated the effects of dihydroorotate or orotate (the substrate and the product of the reaction catalyzed by dihydroorotate dehydrogenase, respectively) on cells that had been blocked in S phase with dicoumarol. For these experiments, we used dihydroorotate and orotate at a concentration of 1 mM because these substances are not efficiently incorporated into the cells [32]. The effect of orotate was similar to that of uridine, releasing cells from S phase blockade induced by dicoumarol. However, dihydroorotate was completely unable to release this blockade (Fig. 6B), demonstrating that dicoumarol inhibits the biosynthesis of pyrimidines at the dihydroorotate dehydrogenase step.

4. Discussion

Dicoumarol produces a boost of intracellular superoxide in pancreatic cancer cells [8,9] and myeloid leukemia HL-60 cells [10]. It is of great importance to understand how dicoumarol increases superoxide because this compound has been proposed as a putative antitumor agent, mainly due to its ability to increase superoxide [8,9]. An obvious explanation could be NQO1 inhibition [8], which is in accordance with a role for NQO1 as a superoxide scavenger [11]. However, although dicoumarol effects have been widely interpreted on the basis of NQO1 involvement, this compound has other cellular targets as well [2–5]. This work represents the first attempt to establish a mechanistic link between dicoumarol, superoxide generation and cell cycle alterations in a tumor cell line.

Maximal levels of superoxide were achieved at 4–6 h of dicoumarol treatment, and a progressive decline was then observed with longer incubations. Since dicoumarol was given after a 24 h preculture in serum-free medium to improve cell survival [10], this decrease may be due to an antioxidant response that is elicited in HL-60 cells after 32 h culture without serum [33].

Given the relative low specificity of dicoumarol, it was important to elucidate whether or not NQO1 inhibition is a major factor involved in superoxide boost. Previous works have indicated that superoxide could be formed in the mitochondria of dicoumarol-treated cells. Dicoumarol increases mitochondrial oxygen consumption in HL-60 cells [16] and in isolated mitochondria obtained from EMT6 and CHO cells, as well as total levels of reactive oxygen species measured in these cells [7]. Because subcellular fractionation studies showed that a minor portion of NQO1 is detected in mitochondria-enriched fractions, it was argued that superoxide increase could be due to the inhibition of a mitochondrial NQO1 [9]. However, as suggested by immunolocalization studies, localization of NQO1 in mitochondria could be an artifact of subcellular fractionation [34]. The use of ES936, a novel and highly selective mechanism-based irreversible inhibitor of NQO1 [23], has allowed us to demonstrate that, although superoxide levels are slightly increased in HL-60 cells when NQO1 is fully inhibited with ES936, dicoumarol produces a significant increase of superoxide that can not be explained on the basis of NQO1 inhibition, as recently found for pancreatic cancer cells [12]. The very limited effect ES936 exerts on superoxide levels, together with the fact that mitochondrial superoxide is increased in cells treated with dicoumarol, support that dicoumarol could target the mitochondrial electron transport chain.

We have shown that dicoumarol produced a substantial inhibition of Complexes II and IV. A minor effect on Complex III was also observed. Consequences of inhibiting the mitochondrial chain upstream ubiquinone might be similar to those of hypoxia, that results in increased generation of superoxide in Complexes II and/or III [26,27]. Succinate is able to suppress superoxide formation through reversed electron transport in Complex II, possibly by avoiding flavin autooxidation [15,20]. On the other hand, superoxide production at Complex III is significantly increased when electrons are delivered into the mitochondrial chain through Complex II [13]. Since succinate inhibited dicoumarol-induced generation of superoxide, both in whole cells and in isolated mitochondria, dicoumarol might activate reversed electron transport in Complex II. Interestingly, dicoumarol interaction with Complex II is competitive with respect to succinate, and significant inhibition of Complex II activity was observed at succinate concentrations that fall within the physiological range found in the mitochondrial matrix (1–3 mM) [35]. Succinate could be displaced by dicoumarol from the Complex II active site *in vivo*, thus allowing for a more pronounced generation of superoxide under conditions of Complexes III and IV inhibition. The fact that dicoumarol stimulates superoxide generation at Complex II is of great importance because several mutations in Complex II also result in increased oxidative stress with overproduction of superoxide, nuclear DNA damage, mutagenesis, apoptosis, tumorigenesis or premature aging [36]. Spurious utilization of oxygen at Complex II yielding superoxide could also contribute partially to the increase of mitochondrial oxygen consumption in dicoumarol-treated cells.

What is the source of electrons for dicoumarol-stimulated generation of superoxide in Complex II? Since DBH_2-dependent production of superoxide by isolated mitochondria in the presence of Complex III inhibitors was stimulated by dicoumarol, and the reaction was completely inhibited by succinate, the ubiquinol pool could be a source of electrons to drive this reversed electron transport. TTFA is a well-known inhibitor, which interferes with ubiquinone binding to Complex II. The recent elucidation of the crystal structure of porcine Complex II has allowed the identification of two ubiquinone binding sites. These sites (proximal, Qp and distal, Qd), coincide with the respective sites where TTFA binds to Complex II with high (TTFA1) or low (TTFA2) affinities [28]. This also agrees with the previous demonstration of two inhibition sites by TTFA, one of them being occupied at a

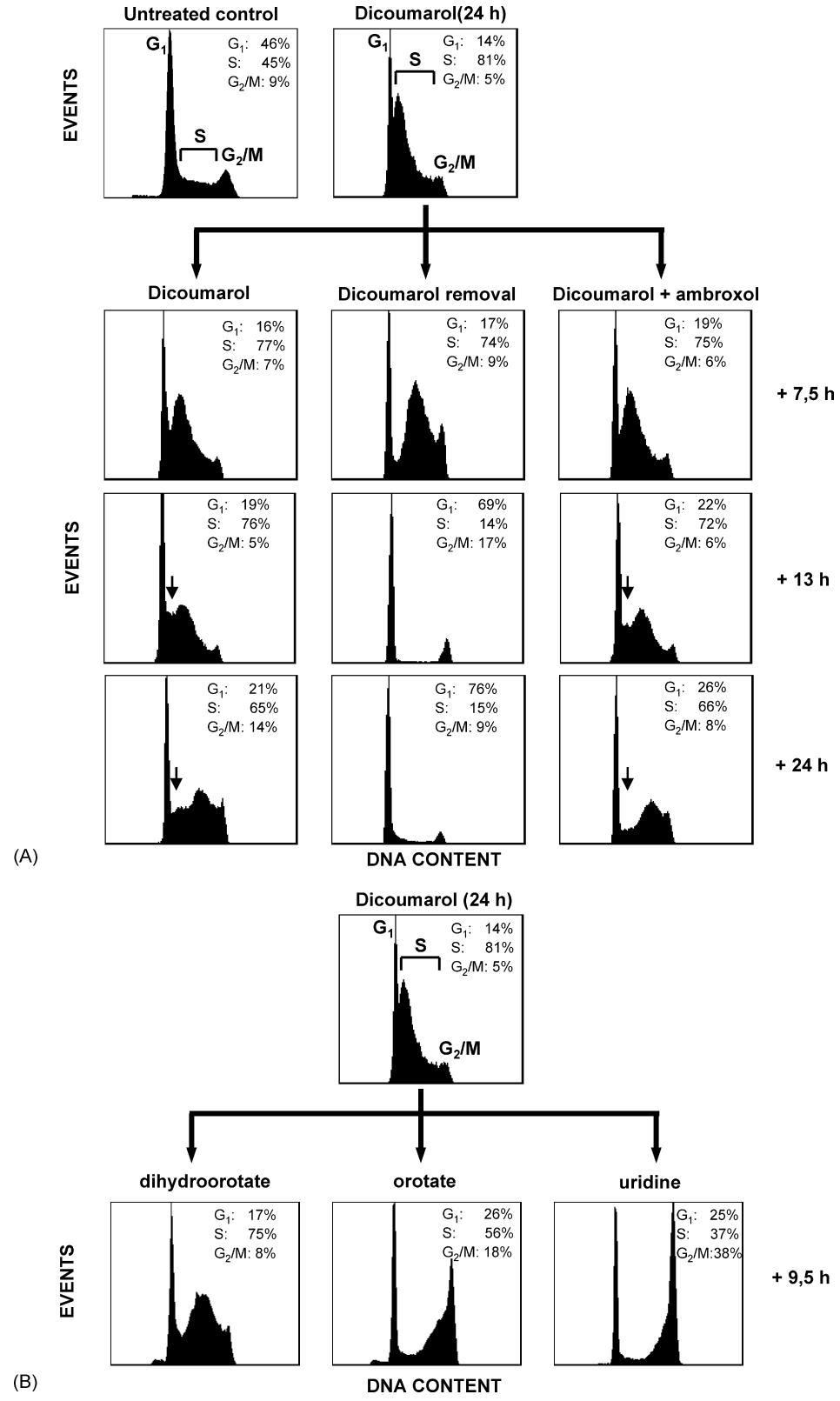

Fig. 6 – Partial S phase blockade induced by dicoumarol does not involve superoxide but the impairment of pyrimidine biosynthesis. Cells were precultured for 24 h in serum-free medium, and then for another 24 h in the same medium containing 5 μM dicoumarol to accumulate cells in S phase (dicoumarol, 24 h in (A) and (B)). (A) Culture of cells was continued under the following conditions: serum-free medium containing 5 μM dicoumarol, serum-free medium without dicoumarol, and 5 μM dicoumarol plus 100 μM ambroxol to scavenge superoxide. (B) Culture of cells was continued under the following conditions: 5 μM dicoumarol plus 25 μM uridine to replenish the pool of pyrimidine nucleotides and 5 μM dicoumarol plus 1 mM dihydroorotate or orotate to investigate the involvement of dihydroorotate dehydrogenase

concentration of about 20 μM, and the other requiring a concentration higher than 20 μM for binding [29]. In our experiments, we tested TTFA at 25 or 50 μM. At any of the concentrations tested, TTFA alone did not affect superoxide levels. TTFA at 25 μM did not alter significantly superoxide levels in cells treated with dicoumarol, supporting that, although excess DBH_2 can drive electrons for dicoumarol-stimulated generation of superoxide *in vitro*, the ubiquinol pool might not play a major role *in vivo*. In contrast, 10 μM TTFA reduces slightly ROS production in pulmonary vasculature cells under hypoxia [27].

Despite the lack of effects at 25 μM, TTFA at 50 μM dramatically potentiated superoxide boost. This stimulatory effect cannot be explained on the basis of the inhibition of reversed electron transfer form the ubiquinol pool because, if this was the case, a decrease would be expected. Qd is apparently the site where ubiquinone can be reduced by the cytochrome b_{560} of Complex II [28]. The function of this low-potential (−185 mV) cytochrome in mammalian mitochondria is unclear because it cannot be reduced by succinate, and thus, it is not supposed to become part of the electron pathway from succinate to ubiquinone. Instead, there is a general agreement that cytochrome b_{560} serves as the electron entrance for the reversed reaction [28,37]. NADH can reduce cytochrome b_{560} in the presence of type II NADH-dehydrogenase [38] and, once reduced, the cytochrome can efficiently donate electrons to ubiquinone. It is thus believed that this heme is in redox equilibrium with the quinone pool in the membrane [37]. Thus, a plausible interpretation of our results is that blocking both quinone sites with 50 μM TTFA avoids the exit of electrons from the reduced cytochrome b_{560} to the ubiquinone pool, which might force electrons to follow the reversed pathway [3Fe–4S] → [4Fe–4S] → [2Fe–2S] → FAD, resulting in significant production of ROS. Stimulation of superoxide production by TTFA would be only observed in the presence of dicoumarol because in its absence, endogenous succinate could inhibit the reversed electron flow and, hence, superoxide production.

Although we have explained our results on the basis of the existence of two quinone/TTFA binding sites in the porcine structure of Complex II at 3.5 Å resolution, a second TTFA binding site (Qd) has not been recognized in the chicken X-ray structure of Complex II at 2.1 Å resolution [39]. The porcine structure would be phylogenetically closer to the human enzyme than the chicken structure and anyway, a second TTFA site is not strictly needed to interpret our results because the same conclusion would be reached considering a single quinone site receiving electrons from both [3Fe–4S] and cytochrome b_{560}, that is blocked at 50 μM TTFA.

Cell damage induced by ionizing radiation inhibits cell progression through G_1 and G_2 and induces a transient delay in the progression through S phase [40]. Because some DNA damage could be also provoked by superoxide, we wanted to study whether or not this reactive species was involved in dicoumarol-induced accumulation of S phase cells. Our results clearly demonstrate that accumulation in S phase induced by dicoumarol is mainly due to a partial blockade in this phase that is independent on superoxide generation, but this ROS is required for G_1–S transition as we suggested previously [10]. Here, the novelty of our results is that dicoumarol not only increases superoxide, but it also impairs pyrimidines biosynthesis at the dihydroorotate dehydrogenase step because S phase blockade can be overcome by exogenous uridine or orotate, but not by dihydroorotate. Mitochondrial inhibitors acting upstream ubiquinone affect indirectly the activity of dihydroorotate dehydrogenase, a mitochondrial enzyme that uses ubiquinone as natural electron acceptor [31]. Well characterized inhibitors of Complex III, as antimycin A, myxothiazol and stigmatellin also increased number of S phase cells (as dicoumarol did) but this effect was not observed with rotenone (Complex I inhibitor) or TTFA (Complex II inhibitor) (D. González-Aragón and J.M. Villalba, unpublished). Pyrimidines biosynthesis is also inhibited under hypoxia [41]. Our findings can also give additional insights to better understand how dicoumarol suppress proliferation in several urogenital cancer cell lines irrespective of their NQO1 levels [42].

Finally, we have to consider that our experiments were carried out in the absence of serum because serum albumin strongly binds dicoumarol, which precludes its uptake into the cells [10,17]. Very high concentrations of dicoumarol (>100 μM) are required to affect cells when the inhibitor is given in the presence of serum, which makes results so obtained much more difficult to interpret [43]. Although clearly an unphysiological situation for cells, it is very likely that performing our experiments in serum-free medium was a factor that allowed us to demonstrate unambiguously the role of dicoumarol as an inhibitor of pyrimidine biosynthesis because serum contains uridine at concentrations (1–10 μM) that are sufficient to support cell growth. Moreover, de novo biosynthesis of pyrimidines is almost completely inhibited in L1210 cells when grown in the presence of uridine at concentrations above 12 μM and, under these conditions, L1210 cells decrease their dependency on de novo biosynthesis and utilize their salvage pathway [44]. Anyway, our results are clinically relevant for anticoagulation therapy in humans because effects of dicoumarol were observed in a concentration range similar to that found in plasma of patients subjected to this therapy [45].

In summary, we demonstrate here that dicoumarol severely affects the mitochondrial physiology, enhancing superoxide production and inhibiting the endogenous synth-

inhibition. At the times indicated in (A) and (B), cells were harvested, fixed and stained for DNA with propidium iodide for quantification of DNA by flow cytometry. Experiments were carried out in triplicate, and a representative result is shown. Removal of dicoumarol or addition of uridine or orotate released cells from S phase blockade, which was not reproduced by ambroxol or dihydroorotate treatments. Position of cells in G_1, S or G_2/M is indicated in the flow cytometry histogram corresponding to cells cultured for 24 h with dicoumarol before the treatments described here were carried out. Position of cells in early S phase is also indicated (arrows in (A)). Quantification of the percentage of cells in each phase of the cell cycle is also indicated for each histogram.

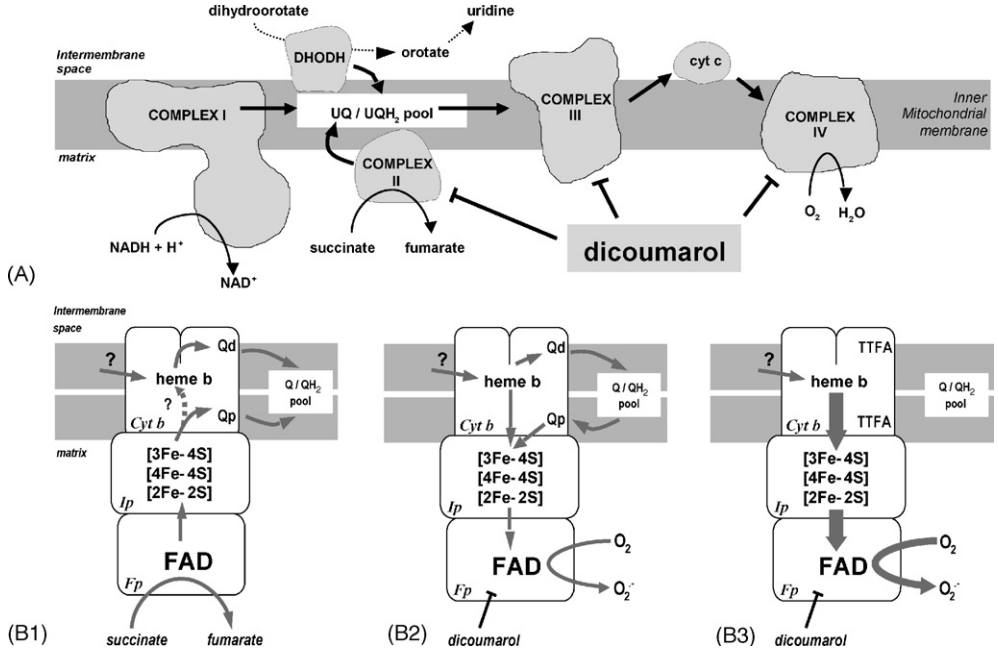

Fig. 7 – A model summarizing our findings about the action of dicoumarol on the mitochondrial physiology. (A) According to our results, dicoumarol inhibits Complexes II, III and IV. Inhibition of dihydroorotate dehydrogenase (DHODH) causes partial S phase blockade. This effect is overcome by addition of exogenous uridine or orotate, but not by dihydroorotate. (B) The interaction of dicoumarol with Complex II promotes superoxide generation. The structure of Complex II with two TTFA/quinone binding sites as reported by Sun et al. [28] is considered. (B1) Normal electron transfer in Complex II. Electrons derived from the oxidation of succinate are transferred to iron sulfur centers and then to ubiquinone at the Qp site. The electron donor that reduces cytochrome b_{560} has not been identified (see "?" symbol in the models). However, once reduced, cytochrome b_{560} is efficiently oxidized by ubiquinone bound at the Qd site. (B2) In the presence of dicoumarol, inhibition of Complexes III and IV promotes reversed electron transport in Complex II. This effect is potentiated due to displacement of succinate by dicoumarol from the flavoprotein subunit, resulting in easier auto-oxidation of reduced FAD and hence in superoxide generation. (B3) Blocking Qd and Qp sites with TTFA avoids exit of electrons from Complex II to the ubiquinone pool. TTFA inhibits reversed electron transfer from ubiquinol, but it also potentiates reversed electron transfer from cytochrome b_{560}. Under these conditions, cytochrome b_{560} is not in equilibrium with ubiquinone and electrons are forced to follow the reversed pathway resulting in enhanced generation of superoxide. Fp, flavoprotein subunit of Complex II; Ip, iron protein of Complex II; Cyt b, cytochrome b_{560}.

esis of pyrimidines (see Fig. 7 for our model). These actions should be taken into account when explaining dicoumarol effects on cells.

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