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Biochemical Pharmacology 63 (2002) 823–831

Biochemical Pharmacology

Alterations in the expression of cytochrome *c* oxidase subunits in doxorubicin-resistant leukemia K562 cells

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Received 28 November 2000; accepted 23 May 2001

Abstract

Doxorubicin (DOX), a widely used antitumoral drug, induces numerous modifications in sensitive cells, interacting with nuclear and mitochondrial DNA. In previous studies achieved in two K562 DOX-resistant sublines (K562/0.2R and K562/0.5R), we have shown stable mitochondrial damage comparatively with sensitive parental cells, such as decrease of cytochrome *c* oxidase activity (COX; EC 1.9.3.1) and cytochrome *aa3* content. In order to explain these data, we have studied several *COX* genes and their expression, in relationship with altered COX activity and multidrug resistance (MDR) phenotype. We have observed a lower expression of the catalytic subunits COX I and II in MDR sublines, which was neither related to mutations in the corresponding mitochondrial genes, nor to a reduced transcription rate. In contrast, we have noticed an increase in both MDR K562 variants, in the mRNA expression of the catalytic subunit COX III, related to an increase in the half-life of these transcripts. Moreover, the doxorubicin resistance phenotype in K562 cells was accompanied by modifications of the expression and steady-state mRNA levels of several nuclear-encoded regulatory COX subunits. Thus, doxorubicin-resistant K562 cells represent an interesting model to study stable modifications concomitant to MDR phenotype. Our results seem to indicate compensatory mechanisms which highlight the complexity of regulatory systems of COX enzyme, involving coordinate regulation of both nuclear and mitochondrial subunit expression.

Keywords: Doxorubicin (adriamycin); Cytochrome *c* oxidase; Mitochondria; Multidrug resistance

1. Introduction

DOX is an anticancer drug of the anthracycline family, which is able to induce DNA double strand breaks through interaction with the DNA–topoisomerase II complex [1]. This interaction is currently believed to trigger cell death through apoptosis induction [2]. In addition to its direct activity on the genome, doxorubicin is able to interact with multiple other targets in the cytoplasm: it is able to bind to the plasma membrane bilayer [3], to stimulate the formation of reactive oxygen species [4] and, consequently, to promote lipid peroxidation [5]. At the level of mitochondria, doxorubicin impairs functional integrity, desegregates the organelle structure [6] and interacts tightly with cardiolipin [7–9], which could be responsible to the cardiac toxicity [7,10,11].

Several mechanisms are involved in doxorubicin resistance. *In vitro*, resistance to this antineoplasic has been obtained in many cell lines by prolonged contact of the cell cultures to infratoxic, progressively increasing concentrations of the drug. The resistance phenotype is generally characterized by a cross-resistance pattern to many unrelated drugs (MDR), mediated by the overexpression of a membrane ATP-driven pump of the ABC protein family, P-glycoprotein [12]. Several mitochondrial modifications have been observed in doxorubicin-resistant leukemia cell lines [13]. Whether the alterations encountered at the mitochondrial level in MDR cells are involved in the mechanism of resistance to doxorubicin or are simple side effects related to drug contact is presently not known. We have shown in a previous study [13] that several modifications of mitochondrial oxido–reductase activities were encountered in human K562 leukemia cells rendered

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Abbreviations: BSA, bovine serum albumin; COX, cytochrome *c* oxidase; DOX, doxorubicin; MDR, multidrug resistance.

0006-2952/02/$ – see front matter
PII: S0006-2952(01)00865-6

resistant to doxorubicin. They are characterized by a significant increase in the activity of complex II in contrast with a decrease in COX activity and cytochrome *aa3* content during stationary growth phase.

Cytochrome *c* oxidase or complex IV is the terminal enzyme of the mitochondrial respiratory chain and catalyzes the transfer of electrons from cytochrome *c* to molecular oxygen. This complex of respiratory chain in mammalian mitochondria is composed of 13 different subunits [14–16]. Three of which (subunits I–III) are encoded by the mitochondrial genome [17] and are responsible for the catalytic functions of the enzyme [15]. The 10 smaller subunits are encoded by nuclear genes and their function is not yet available, although regulatory roles have been inferred from their binding of allosteric effectors.

In this study, we analyzed the mitochondrial alterations occurring in K562 doxorubicin-resistant cells at the level of complex IV in comparison with the parental cells, which should explain the alteration of COX activity. We performed quantitative evaluations of the expression of its subunits, at the mRNA and protein levels, and determination of the sequence of their mitochondrial genes. The various alterations we report in this paper could be related to the known modifications of apoptosis induction in K562-resistant cells [18].

2. Materials and methods

2.1. Cell culture

DOX-sensitive K562 cells [19] and their doxorubicin-resistant counterparts [20] were grown in suspension in RPMI-1640 medium (Rosewell Park Memorial Institute) containing 10% (v/v) heat-inactivated fetal calf serum (Seromed) and 2 mM L-glutamine (Life Technologies). Cells were incubated at 37° in a humidified atmosphere containing 5% CO_2. The resistant cell lines were obtained by progressive adaptation of the parental sensitive cells (K562/S) to 0.2 and 0.5 μg/mL of DOX for K562/0.2R and K562/0.5R cells, respectively.[1] Pharmacological characterization of sensitive and DOX-resistant cells was performed in a previous study [21]. These two resistant cell lines were maintained without drug for at least 1 month in order to get the cells free of remaining drug [13].

2.2. Preparation of mitochondria from K562 cells

Mitochondria were prepared according to Bourgeron *et al*. [22] modified by Denis-Gay *et al*. [13]. Cells (5×10^6) were collected in exponential phase of growth by centrifugation (600 *g* for 10 min and at 4°). Cells were then washed twice in 100 mM sucrose, 1 mM ethylene glycol-bis(β-aminoethyl ether)-tetraacetic acid (EGTA), 20 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), pH 7.4 and 1 mg/mL BSA. The pellet was resuspended in the same buffer solution supplemented with 10 mM triethanolamine, 5% (v/v) Percoll, 0.1 mg/mL digitonin for 3 min at 4°. Finally, the mixture was homogenized in a Potter homogenizer (10 strokes, 1000 rpm) before being diluted at the ratio of 1/5 in 300 mM sucrose, 1 mM EGTA, 20 mM MOPS, pH 7.4 and 1 mg/mL BSA, and centrifuged at 2500 *g* for 5 min at 4°. The supernatant containing mitochondria was collected and the pellet was resuspended in this last buffer and centrifuged again. This supernatant was added to the first one and the resulting mixture was centrifuged at 10,000 *g* for 10 min at 4°, to collect mitochondria as a pellet. Isolated mitochondria were washed twice in the same conditions before being resuspended in this last buffer solution supplemented with 1 mM phenylmethanesulfonyl fluoride (PMSF). Mitochondrial protein content was measured accordingly to Bradford [23].

2.3. Immunoblotting of human COX subunits

Monoclonal antibodies directed against human COX I and II subunits were purchased from Molecular Probes. MAbs specific for human nuclear subunits (COX IV, VIa+c and VIIa+b) were purified by chromatography on protein G-Sepharose (Pharmacia Biotech), from hybridoma cells kindly provided by Kadenbach (Marburg, Germany). Detection was performed with rabbit anti-mouse POD-secondary antibody purchased from Dako. Detection and analyses of the two mitochondrial subunits (COX I and II) in the mitochondrial fraction isolated from parental cells and MDR variants were performed by immunoblotting. Equivalent amounts of isolated mitochondria were incubated at 96° for 3 min in a denaturing buffer (Tris–HCl 10 mM pH 8 containing 5% (v/v) glycerol, 2.5% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 0.005% (w/v) bromophenol blue). Mitochondrial proteins were resolved by SDS-PAGE according to Laemmli [24], and electroblotted onto nitrocellulose (Amersham Pharmacia Biotech). After blocking overnight at 4° with 1% blocking reagent, the membranes were then incubated for 1 hr at room temperature with the relevant primary antibody. Detection was performed by chemiluminescence using a POD-conjugated secondary antibody and the ''BM chemiluminescence blotting substrate (POD)'' kit (Roche) according to the manufacturer's instructions. Chemiluminescence was detected for the indicated time with a Fuji X-ray film.

2.4. Semi-quantitation of COX subunits expression

Semi-quantitative analysis were performed using ELISA in order to study the steady-state level of COX subunits expression. Mitochondrial proteins ranging from 2.5 to 20 μg were coated overnight at 4° in 100 mM

[1] Robert *et al*., unpublished.

Na_2CO_3, pH 9.6, in a 96-wells plate. Then, the plate was washed for 30 s with PBS 0.1% Tween 20 (v/v) four times. After blocking for 1 hr at 37° with PBS 5% (w/v) BSA, the plate was incubated for 2 hr at 37° with the relevant primary antibody, at a concentration ranging from 0.25 to 1 μg/mL. After four washes as indicated above, detection was performed using a POD-conjugated secondary antibody (1.3 μg/mL, Dako) incubated for 1 hr at 37° with the plate, which was revealed by addition of OPD (200 μL per well). The colored reaction was stopped by 100 μL of 4N H_2SO_4, and its intensity, proportional to the antigen amount, was evaluated spectrophotometrically at 490 nm, comparatively with appropriate controls.

2.5. Extraction of nucleic acids

Total DNA was prepared from 10^6 cells from both K562 parental and variant sublines. The pellet was resuspended in a lysis buffer (10 mM Tris–HCl, pH 8, 10 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.5% w/v SDS containing 50 μg proteinase K, at 50° for 2 hr). After extraction of proteins with phenol/chloroform, DNA was recovered by ethanol precipitation and incubated at 65° for 10 min with RNase A (Roche).

Total cellular RNA was extracted from the sensitive and resistant K562 cells by the ''High Pure RNA Isolation'' kit (Roche). All aqueous solutions used in the manipulations were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC) and autoclaved. RNA concentration was estimated spectrophotometrically at 260 nm.

2.6. Cloning of PCR products from COX I–III genes

Mitochondrial *COX I–III* genes were amplified by PCR from total DNA with specific primers (Table 1) and with 5 U of *Taq* DNA polymerase. PCR products were gel-purified with the Jetquick kit (Genomed) and then ligated into the plasmid pGEM-T (Promega). The ligated product was used to transform *Escherichia coli* JM109 competent cells. Ampicillin-resistant colonies were screened by PCR using primers specific for *COX* genes (Table 1). The bacteria were grown overnight at 37° in LB liquid medium supplemented with 100 μg/mL of ampicillin. Plasmid purification was carried out by standard procedures [25].

2.7. DNA sequencing and analysis

Plasmidic DNA sequencing was performed by the dideoxy chain termination method [26] using an automated sequencer (ABI PRISM 310, Perkin-Elmer). All mitochondrial genes encoding *COX I–III*, were sequenced twice on both strands in the three cell lines. Sequence comparisons were performed by searching the Genbank Database at the Internet Web site.

2.8. Sybr®Green real-time reverse transcriptase (RT)-PCR assay

The PCR primers for all *COX* genes studied were designed using a Software program (Primer Express) from Applied Biosciences according to the published cDNA sequences (summarized in Table 2). For reverse transcription, 3 μg of total RNA solution were heated at 70° for 10 min with random hexadeoxyribonucleotides {pd(*N*)6} primers (Applied Biosciences), ice-cooled and subjected to first-strand DNA synthesis using 200 U of Superscript II, Rnase H^- (Life Technologies) for 1 hr at 42°.

A thermal stable Ampli*Taq* Gold DNA polymerase was used for the second strand cDNA synthesis and DNA amplification. Real-time PCR was performed with 5 μL of RT products (ranging from 10 ng to 0.1 pg) of total RNA in the case of *COX* mitochondrial encoded genes, and ranging from 1 μg to 1 ng for nuclearly-encoded *COX* genes, 1X Taqman buffer, 5 mM $MgCl_2$, 200 μM dATP, dGTP and dCTP, 400 μM dUTP, 200 nM primers (forward and reverse), 0.01 U/μL AmpErase and 0.025 U/μL Ampli*Taq* Gold DNA polymerase in a total volume of 50 μL. PCR was performed, after incubation at 50° for 2 min (for AmpErase UNG incubation to remove any uracil incorporated into the cDNA), and at 95° for 1 min (for Ampli*Taq* Gold activation). In all, 40 cycles were run at 95° for 15 s, 60° for 1 min on the ABI Prism 5700 Detection System. Each sample was run in duplicate and threshold cycle (C_t) values, defined as the cycle at which PCR amplification reaches a significant value (i.e. usually 10-fold the standard deviation of the baseline), were averaged from each reaction. The C_t values showed linear correlation with relative DNA copy numbers [27]. However,

Table 1
Sense and antisense primers used for *COX I, II* and *III* genes sequencing

Studied genes	Names of primers	Sequence of primers	Localisation on mtDNA
COX I	Ext-5′-COX I	5′-CCAATGCTTCACTCAGCCATTTTACCTCACCCCCAC-3′	5865–5901
COX I	Ext-3′-COX I	5′-CTAATACCTTTTTGAAAAAGTCATGGAGGCCATGGGGTTG-3′	7488–7527
COX I	Int-5′-COX I	5′-CATAAGCTTCTGACTCTTACCTCCCTC-3′	6200–6226
COX I	Int-3′-COX I	5′-GGCGTAGTTTGGTCTAGGTGTAGC-3′	7107–7133
COX II	5′-COX II	5′-GTCAAAGTTAAATTATAGGCTAAATCC-3′	7548–7574
COX II	3′-COX II	5′-AGTTAGCTTTACAGTGGGCTCTAGA-3′	8286–8310
COX III	5′-COX III	5′-AGCCTCTACCTGCACGACAACACATA-3′	9181–9206
COX III	3′-COX III	5′-GTCAAAACTAGTTAATTGGAAGTTAACGGTACT-3′	10008–10040

Table 2
Sense and antisense primers used for quantitative RT-PCR analysis

Studied cDNA	Sequences of primers
COX I	5′-TCCGCTACCATAATCATCGCT-3′ 5′-CCGTGGAGTGTGGCGAGT-3′
COX II	5′-CGATCCCTCCCTTACCATCA-3′ 5′-TCCGCCGTAGTCGGTGTACT-3′
COX III	5′-CCATTTCCGACGGCATCTAC-3′ 5′-CGGCATCTACGGCTCAACAT-3′
COX IV	5′-CCCAGCTTATATGGATCGGC-3′ 5′-AGGTGCTTGACATGGGCC-3′
COX Va	5′-CAAAGCAGGACCTCATAAGGAAA-3′ 5′-GGAGtGGAGATTCCCAGTTCAT-3′
COX Vb	5′-GGACAATACCAGCGTCGTCTG-3′ 5′-CAGCTTGTAATGGGCTCCACA-3′
COX VIaL	5′-GCACGAGAGACCCGAGTTCA-3′ 5′-AGGGAAACGGCTTGGTCCT-3′
COX VIb	5′-GGTCACAGACTGGGATGAGCA-3′ 5′-TGCAGCCAGTTCAGATCTTCC-3′
COX VIc	5′-TCCAGTAATCTGAAAAATAAACTCTAATGG-3′ 5′-AAATCTGCGTATGCCTTCTTTCTT-3′
COX VIIaL	5′-GCGGAATCTGCTGGCTCTT-3′ 5′-TTTAAAATGCCTGCGGGAAG-3′
COX VIIb	5′-GCACCAAAGCAGCAGCTGT-3′ 5′-CGCTTTTGACCAAGGGAAAC-3′
COX VIIc	5′-CAGTGGAAAACAAGTGGTCGTTAC-3′ 5′-TGGTGTCTTACTACAAGGAAGGGT-3′
COX VIII	5′-CTCGCGCGCCAAGATCC-3′ 5′-CCAATTCCATGATCCCAAGC-3′

since we are unable to determine the efficiency of the RT, which depends in part on the length of 3′ untranslated sequence of the mRNA molecule for each gene, it is not possible to extrapolate this value to absolute copy number of mRNA. Nevertheless, relative changes in mRNA copy number between sensitive K562/S cells considered as the reference, and MDR sublines for a given gene, can be determined in this way. Data were analyzed using a sequence Detector V1.6 program (Applied Biosciences).

2.9. Analysis of COX I–III mRNAs stability

Sensitive and resistant cells were incubated in the presence of 4 μM actinomycin D (Sigma–Aldrich) at time 0, and total cellular RNA was isolated by the ''High Pure RNA Isolation'' kit (Roche) every 3 or 4 hr during 15 hr. Total RNA (10 μg) was resolved on 1% (w/v) denaturing agarose gel and transferred onto Hybond-N^+ filters by capillarity. Filters were hybridized overnight at 42° in the presence of 50% formamide and ^{32}P-labeled DNA probes of the COX I–III and human β-actin cDNAs. Autoradiograms were scanned by laser densitometry using Imagequant Software (Molecular Dynamics), and were normalized in comparison of β-actin signal.

2.10. Statistics

An unpaired Student's *t*-test of the Statview™ software was used for all experiments.

3. Results

3.1. Analysis of catalytic COX subunits expression

In order to explain the decrease of cytochrome *c* oxidase activity in doxorubicin-resistant cells [13], we have analyzed by immunoaffinity the expression of the COX subunits encoded by mitochondrial DNA and involved in catalytic functions of the enzyme. This was done using both Western blotting for qualitative comparison and ELISA for semi-quantitative estimation. For these mitochondria encoded subunits, only MAbs against COX I and II were available. Both proteins were detected by Western blot in sensitive and MDR cell lines (Fig. 1), and no appreciable difference was observed concerning the molecular mass of these subunits between the three sublines. Nevertheless, differences in signal intensity between MDR and sensitive cells seemed to indicate eventual quantitative modifications of expression, particularly for COX I subunit. These results were confirmed by semi-quantitative analysis of their expression by ELISA method. This study revealed that COX I and II expression significantly decreased ($P < 0.05$) in both MDR sublines comparatively with sensitive cells (Fig. 2). The expression level of COX I decreased by about 40 and 34% in K562/0.2R and K562/0.5R cells, respectively, and that of COX II by 36.5 and 40% in K562/0.2R and K562/0.5R cells, respectively. No significant difference was observed for

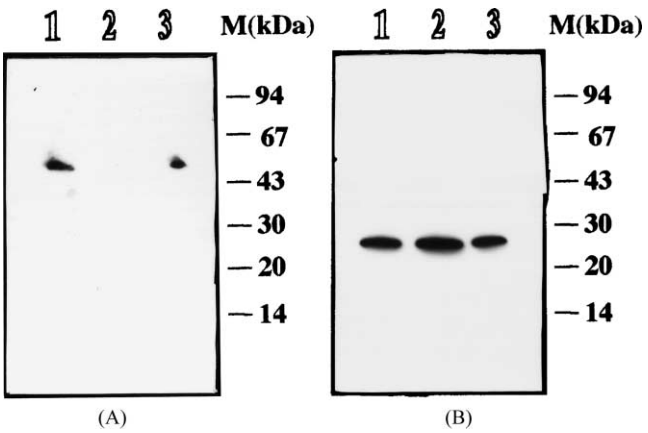

Fig. 1. Western blot analysis of COX I (A) and COX II (B) subunits expression in mitochondrial proteins (50 μg) extracted from K562/S cells (lane 1) and resistant K562/0.2R (lane 2) and K562/0.5R (lane 3) cells.

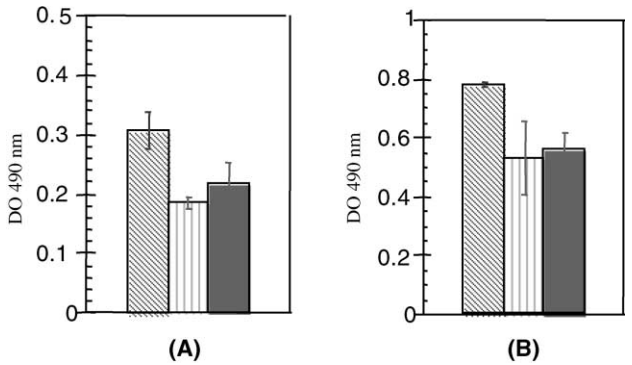

Fig. 2. Semi-quantitative analysis by ELISA method of the expression of COX I (A) and COX II (B) subunits of 25 μg mitochondrial proteins extracted from the three sublines: K562/S: (▨); K562/0.2R: (▥); K562/0.5R: (■). OD was measured spectrophotometrically at 490 nm.

COX I and II expression between K562/0.2R and K562/0.5R cells.

3.2. Sequencing of COX I–III genes

The decrease of COX I and II expression in MDR sublines could be due to mtDNA modifications. Therefore, we have cloned and sequenced their corresponding genes, as well as the *COX III* gene. No mutations or deletions have been detected for these three genes in MDR sublines as compared with parental cells, in reference to Genbank Database (access number: J01415 [17]).

3.3. Quantification of COX I–III subunit mRNAs

We have investigated the steady state levels of COX I–III expression at the mRNA level by quantitative RT-PCR. Concerning COX I and II, no significant difference in their transcript levels could be detected between sensitive and both MDR variants. The C_t values, which represent the threshold cycle, were the same in each case for the same quantity of total RNA (data not shown). We have also studied the COX III mRNA level in both resistant sublines compared with parental cells. In this case, a significant increase ($P < 0.05$) of the mRNA level was related with the resistance degree of cells. COX III mRNA copy number increased by about 30-fold in K562/0.2R cells as compared to K562/S cells, and by about 170-fold in K562/0.5R cells as compared to sensitive cells.

3.4. Analysis of stability of COX I–III subunit mRNAs

The reduced expression of COX I and II in MDR sublines could result from difference in mRNA stability. Therefore, we have determined the half-life of these transcripts and we have also studied the stability of COX III transcripts (Fig. 3). The half-life of COX I and II mRNAs were similar ($P > 0.05$): it was estimated to about 12 hr in each cell line. In contrast, the half-life of COX III mRNA increased with the degree of resistance of the cells. In sensitive cells, it was of about 3 hr, and increased in K562/0.2R and K562/0.5R cells to 15 and 18 hr, respectively ($P < 0.05$).

3.5. Study of different nuclear-encoded COX subunits

We have completed the investigations in studying the expression of several nuclear-encoded COX subunits in view of their putative regulatory role in COX activity. Western blot analysis (Fig. 4) suggested that COX IV expression tended to increase with the degree of resistance of the cells. On the other hand, the level of COX VIac and VIIab expression diminished in MDR sublines in comparison with parental K562/S cells. All these results

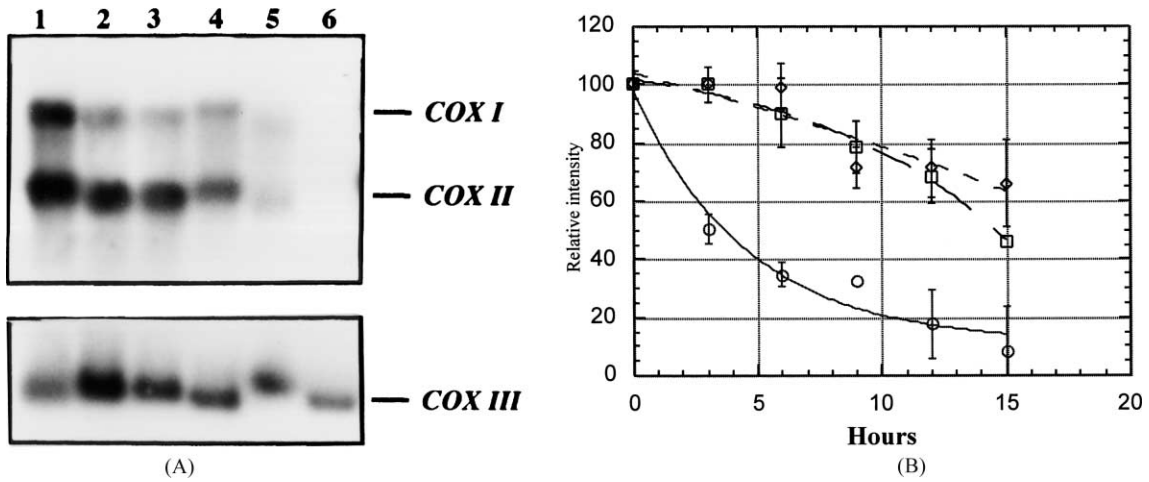

Fig. 3. Determination of COX I–III mRNAs half-life. (A) Northern blot for COX I–III mRNAs in K562/S cells. Lane 1: control mRNAs extracted from non-treated cells by actinomycin D. Lanes 2–6: mRNAs extracted after 4, 6, 9, 12 and 15 hr of treatment by actinomycin D, respectively. (B) Quantitations of Northern blot for COX III mRNA in K562/S cells (○), K562/0.2R cells (□) and K562/0.5R cells (◇). Quantitations were obtained from at least two independent experiments.

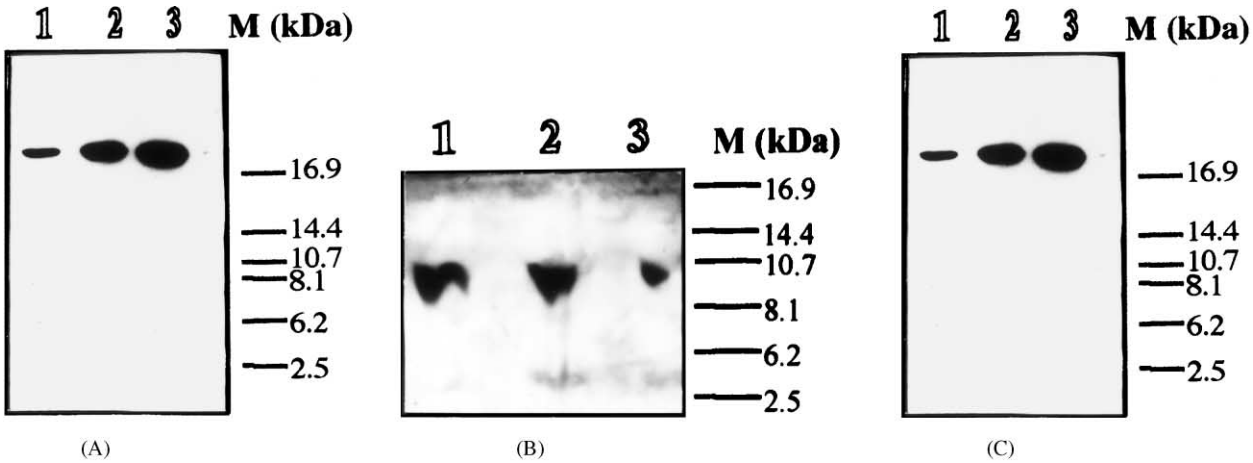

Fig. 4. Western blot analysis of nuclear COX subunits of mitochondrial proteins (50 μg) extracted from sensitive cells (lane 1), and both MDR variants: K562/0.2R (lane 2) and K562/0.5R (lane 3). (A) COX IV subunit; (B) COX VIac subunits; (C) COX VIIab subunits.

Table 3
Study of mRNAs levels for the nuclear COX subunits in MDR K562 cells (K562/0.2R and K562/0.5R cells) versus sensitive cells (K562/S). The mRNA level in K562/S cells is considered as reference, so had a value of 1, and mRNA levels in MDR variants were relative to this value. Statistical analysis using T-test was performed in order to compare mRNA steady-state levels in both MDR variants with sensitive cells

COX subunits	K562/0.2R	K562/0.5R	Remarks	Statistical analysis
COX IV	1.3 ± 0.4	1.4 ± 0.5	NS	$P > 0.05$
COX Va	1.1 ± 0.1	1.9 ± 0.5	NS	$P > 0.05$
COX Vb	1.2 ± 0.3	1.7 ± 0.5	NS	$P > 0.05$
COX VIaL	0.2 ± 0.06	0.6 ± 0.1	S	$P < 0.05$
COX VIb	0.6 ± 0.3	0.3 ± 0.1	S	$P < 0.05$
COX VIc	1.1 ± 0.3	1.1 ± 0.4	NS	$P > 0.05$
COX VIIaL	0.8 ± 0.08	0.7 ± 0.2	S	$P < 0.05$
COX VIIb	0.2 ± 0.03	0.3 ± 0.15	S	$P < 0.05$
COX VIIc	1.5 ± 0.4	0.2 ± 0.1	S only in K562/0.5R cells	$P < 0.05$ only in K562/0.5R cells
COX VIII	1.9 ± 0.8	1.3 ± 0.3	NS	$P > 0.05$

NS: Non significant difference; S: Significant difference.

should be taken with care because Western blot does not constitute an accurate quantification method. Therefore, we have also quantified mRNA levels in these three sublines by real-time RT-PCR assay, as described for mitochondrial-encoded COX subunits (Table 3). In the case of COX VIaL, VIb, VIIaL and VIIb, a lesser expression of these mRNAs in MDR sublines appeared when compared with sensitive cells ($P < 0.05$), and varied in the same way as their respective proteins. For the other nuclear subunits (COX IV, Va+b, VIc, VIIc and VIII), no difference was noticed between sensitive and drug-resistant cell lines.

4. Discussion

In the past decade, studies have been carried out on cellular changes associated with doxorubicin resistance, and more particularly on mitochondrial functionality. In this way, studies of cytochrome *c* oxidase revealed a significant decrease of its activity [13], and of V_{max} values associated with the MDR phenotype (data not shown).

The modifications of COX activity that we have observed were not due to the direct effect of doxorubicin in cells, but were rather associated to the acquired resistance phenotype to this drug. We have previously monitored COX activity as a function of passage number in a doxorubicin-free culture medium, and observed that COX activity remained unchanged in both resistant K562 sublines after 18 passages in drug-free medium [13]. The stable phenotype, due to doxorubicin treatment or selection (Fig. 5), resulted from its binding with genomic [28], and/or mitochondrial DNA [29]. The acquisition of the MDR phenotype is concomitant to the stable alterations in oxido–reductase activities in MDR K562 variants. Drug treatment could affect DNA and more particularly COX subunits: the three catalytic subunits (COX I–III) encoded by mtDNA, and the ten regulatory subunits (IV–VIII) encoded by nuclear DNA.

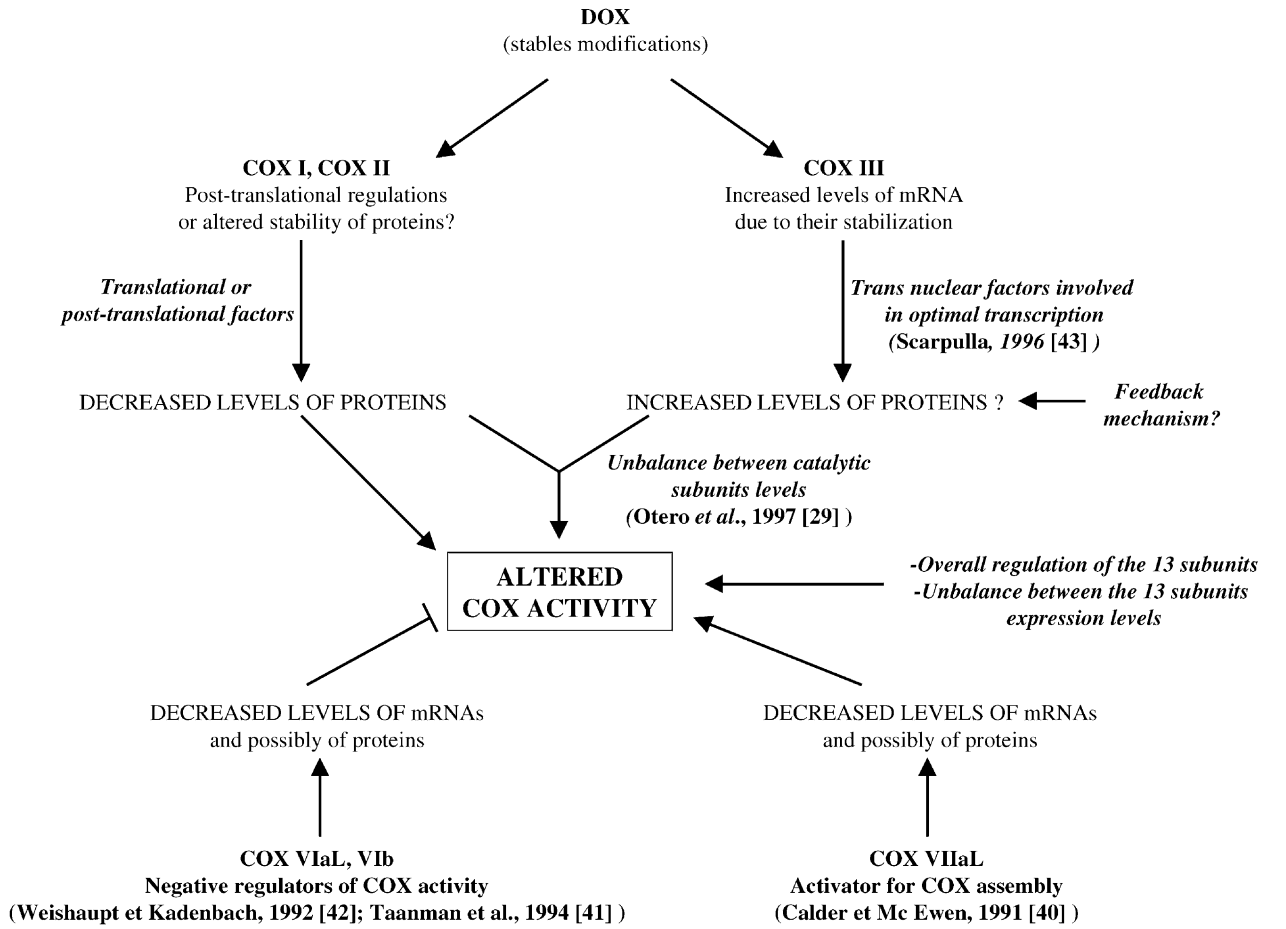

Fig. 5. Altered expression of COX subunits paralleled with reduced COX activity in MDR K562 sublines comparatively to sensitive cells. Different hypothesis are typed in italic.

In a first approach, we have studied the catalytic COX subunits encoded by mtDNA, and possibly involved in the alterations of enzyme activity. The expression of COX I and II subunits decreased in both MDR sublines, but without noticeable modification of their molecular mass. A recent study [30] suggested that altered expression of mitochondrial subunits was sufficient to impair the biosynthesis and final assembly of the COX holoenzyme, leading to a lesser activity. Moreover, the lower COX activity and protein expression could be explained by alterations in mtDNA, as observed in mitochondrial myopathy (reviewed in [31]). However, in MDR K562 sublines, we did not observe any modifications in mitochondrial genes encoding COX subunits.

In doxorubicin-resistant K562 cells, quantitative RT-PCR and stability studies of COX I and II mRNAs have revealed no difference between sensitive and MDR cells. Thus, no alteration at the transcriptional level can be responsible for the reduced expression. We hypothesize that post-transcriptional modifications or different stability of these proteins could be implicated. For example, this discrepancy may be attributable to a reduced translation rate of mitochondrial mRNA of the complex, in which case the quantity of available, synthesized, catalytic subunits would constitute a limiting factor for functional complex IV assembly. In yeast, specific nuclear factors are involved in the control of translation of several mitochondrial mRNAs, notably COX III and cytochrome *b* [32,33]. No factor of this type is currently known in mammals. Several authors have nevertheless postulated the role of such factors, apparently operative at the level of interactions between mRNA and mitoribosomes [34,35], or at the level of protein synthesis initiation [36]. Whatever its mechanism, the lower expression of COX I and II subunits could lead to the reduced value of COX activity and V_{max}.

Concerning COX III subunit, its protein expression could not be studied because no monoclonal antibody directed against this subunit was available. The quantitative analysis of the COX III transcripts showed a significant increase at the mRNA level, correlated with the degree of resistance of the cells. This enhanced mRNA levels paralleled the greater stability of these transcripts in MDR K562 sublines, but this result could not be explained by any mutation or deletion of the corresponding genes. The unbalance transcription in the three mitochondrial sequences encoding catalytic COX subunits is puzzling. Indeed, COX

I–III are encoded by the same polycistronic mRNA, and the transcription level is identical for all of them [37,38]. A similar unbalance into expression of different mitochondrial COX subunits has already been observed, causally associated with a decrease in COX activity [30], without real satisfactory explanation.

We can, therefore, hypothesize that the overexpression of COX III, due to the stabilization of its transcripts in MDR cells, could result from a feedback regulation, in order to compensate for the decreased steady-state levels of COX I and II subunits. The enhanced expression of COX III may represent a mechanism to improve the efficiency of their assembly and stability in a functional COX. A recent study suggests the involvement of COX III subunit in assembly and stability of the holoenzyme [39]. These stable modifications observed in DOX-resistant K562 cells could concern the efficiency of factors involved in translational or post-translational mechanisms. Fig. 5 summarizes all our results in relationship with MDR phenotype and altered COX activity.

Several authors attempted to assess a functional role of nuclear subunits in cytochrome *c* oxidase activity, especially a regulatory function or a role in the assembly or stability of the holoenzyme [15]. This is why we have completed our investigations on catalytic mitochondrial subunits, by a study of several regulatory COX subunits. We have observed a decrease in COX VIaL+b and COX VIIab mRNA level. Western blot analysis paralleled this result in both MDR K562 variants. The function of the COX VIIb polypeptide is completely unknown. On the other hand, COX VIIa subunit could contribute in assembly of the holocomplex, possibly involving the proper folding and/or heme incorporation of subunit I [40,41]. Moreover, an inhibitory function has been established for subunits COX VIaL and VIb [42,43]. In view of their negative properties, their reduced expression could explain the decreased COX activity and V_{max} value. Conversely, the fact that the mRNA and possibly the expression of COX VIIaL, estimated by Western blot analysis, was reduced in MDR cells, could be responsible for the decrease in COX activity and V_{max} value of the complex (Fig. 5).

Deletion analysis of nuclear genes encoding for mitochondrial proteins have indeed revealed a mosaic of *cis*-acting upstream and intron sequence elements required for maximal transcription (reviewed in [44]). Consequently, not only mutations in the coding regions of the *COX* nuclear genes may affect enzymatic activity, but mutations in the *trans*-acting factors may also result in decreased subunit synthesis and eventually lead to an inadequate assembly of complex.

Finally, it is likely that the decrease of these parameters is the result of the overall regulation of the 13 subunits, instead of that of the individual polypeptides, or of an alteration of the proportion between negative and positive regulatory subunits, as supposed by Aksenov *et al*. [45] (Fig. 5). The unbalance between different nuclear and mitochondrial COX subunits expression of COX subunits, could be compensated by the formation of a COX complex dimer (super-COX-complex), as suggested by [46].

We have shown in this paper that a series of coordinate alterations of COX subunit expression occurred in multidrug resistant K562 cells. Therefore, the K562-resistant cells may represent an interesting model for the analysis of the complex regulation pathways of cytochrome *c* oxidase activity at the molecular level. The function of nuclear subunits are indeed poorly understood and even more the regulation of expression by two genomes. In addition, it has been shown that the parental cell line is unable to undergo apoptosis at doxorubicin concentrations leading to cell death, whereas the multidrug-resistant variant K562/0.2R could undergo apoptosis when exposed to similarly cytotoxic concentrations, able to overcome the P-gp mediated efflux [2,18]. The mechanism of these changes in cell death pathway is yet unknown, but could involve alterations in cytochrome *c* release from mitochondria in response to drug action. A role for cytochrome *c* oxidase can be hypothesized at this level, whose alterations in resistant cells would lead to a facilitated release of its substrate in the cytosol. This is presently under investigation in our laboratory.

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