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Biochemical Pharmacology 65 (2003) 1701–1707
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Deregulation of gene expression in fetal oocytes exposed to doxorubicin

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Received 15 October 2002; accepted 3 February 2003

Abstract

Doxorubicin is an effective anticancer drug but its use is limited due to its adverse side effects such as infertility and cardiomyopathy. Some possible mechanisms of the action of doxorubicin have been postulated, but the initial gene deregulation response has not been investigated. Fetal life stages are critical periods in mammalian oogenesis. This study analyzes gene expression alterations in mouse fetal oocytes exposed *in vitro* to this anticancer agent. cDNA libraries were generated from isolated fetal oocytes and differential screenings performed with cDNAs from *in vitro* doxorubicin-treated and -untreated oocytes. Differentially expressed genes were assessed by real-time RT-PCR to quantify the extent of their transcriptional control in doxorubicin-exposed oocytes. The results show that doxorubicin alters the expression of genes involved in the mitochondrial respiratory chain, intracellular transport and cell differentiation. Finally, the up-regulation of a differentially expressed gene (metaxin) mediated by its promoter was evaluated in a functional assay. When treated with doxorubicin, somatic cells transfected with a genetic construct including the promoter of metaxin and a reporter gene showed increases in expression similar to those observed in fetal oocytes. This demonstrates the direct effect of agent on the regulation of a specific gene.

Keywords: Doxorubicin; Fetal oocyte; Gene regulation; Metaxin; Mitochondria

1. Introduction

Doxorubicin (DOX) (trade name ADRIAMYCIN) is a cytotoxic anthracycline antibiotic that has been widely used for more than 30 years to treat many forms of cancer. Despite this extensive clinical use, the mechanisms of its antiproliferative and cytotoxic effects have been the subject of controversy. DNA is considered its main cellular target, on which it is thought to act by incurring DNA damage through the inhibition of topoisomerase II, DNA alkylation, DNA intercalation and binding, the inhibition of DNA biosynthesis, and interference with DNA strand separation and helicase activity. Free radical formation, the induction of lipid peroxidation and direct membrane effects have also been documented (for a review, see [1]). The cytotoxic effects of DOX and its derivatives may also be responsible for its adverse side effects, e.g. cardiotoxicity and infertility.

The recognized clinical effects of DOX are preceded by changes at the cellular and molecular levels. Alterations in the expression of genes involved in the structural integrity and enzymatic functions of cardiac muscle have been reported by Ito *et al.* [2]. Changes in the expression of genes in different cell types treated with DOX have also been reported. These include: c-*myc* in breast tumor cells [3], *Bcl-xl* in cardiac myocytes [4], and CD95 in endothelial cells [5]. Recently, the use of microarrays has shown changes in the expression of genes involved in different pathways in cells treated with DOX and in multidrug-resistant cell lines [6–8]. The analysis of DOX-induced gene expression changes might help us understand the effect of this drug, its side effects, its antineoplasic action [7] and drug resistance.

Infertility is a common side effect of chemotherapy which has a substantial effect on the quality of life for young survivors of cancer. Chemotherapy-induced inferti-

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Abbreviations: AdoMet, adenosylmethionine; MAT, methionine adenosyltransferase; COX II, cytochrome *c* oxidase subunit II; COX III, cytochrome *c* oxidase subunit III; 16CE, mouse sixteen-cell-embryo cDNA; DMEM, Dulbecco-modified Eagle medium; DOX, Doxorubicin; GV, germinal vesicle; MRC, mitochondrial respiratory chain; MTX, metaxin; ND2, NADH-ubiquinone oxidoreductase chain 2; PBS, Phosphate buffered saline; SYNT, syntaxin 5; S16, ribosomal protein S16.

0006-2952/03/$ – see front matter
doi:10.1016/S0006-2952(03)00119-9

lity may be temporary or permanent, and may occur with low as well as high dose treatments [8]. Chemotherapy, especially combination chemotherapy, can damage or even destroy germ cells. After DOX treatment, apoptosis has been reported as the main cause of cell destruction, including that of germ cells. *In vivo* treatment with anticancer agents has been shown to cause inhibition of stage-specific DNA synthesis in the rat testis and to induce DNA damage and apoptosis in spermatogonia [9]. Unfertilized mouse oocytes exposed to therapeutic levels of DOX undergo apoptosis, as assessed by caspase activity [10].

The number of oocytes produced in mammalian females is limited and production occurs during fetal life. Although the pharmacological treatment of cancer during pregnancy is not clinically routine, some protocols of combination chemotherapy treatment, including DOX treatment, are used [11]. The mechanisms by which DOX operates and affects cells appear to be different depending on the cell type and their developmental stage. However, the effects of most potential reprotoxicants have not been investigated in fetal oocytes—a critical period in female germ cell development.

This study analyzes gene expression alterations in mouse fetal oocytes exposed *in vitro* to DOX. cDNA libraries of isolated fetal oocytes were created and differential screenings performed to identify target genes. The levels of expression of differentially expressed genes in DOX-exposed oocytes were also assessed by real-time RT-PCR to quantify the extent of transcriptional control. Finally, the promoter of metaxin, a DOX-induced up-regulated gene in fetal oocytes, was evaluated in a functional assay to determine the mechanism of the drug's action at transcription level. Genes related to mitochondrial metabolism appear to be the main target of DOX's deregulation effect.

2. Materials and methods

2.1. In vitro culture of fetal oocytes and DOX treatment

Ovaries from 17 days postcoitum mouse CD-1 embryos were dissected in HEPES buffered MEM (Gibco BRL) to which had been added 1 mg/mL BSA (FR V; Sigma Chemical Co.) and 100 μg/mL penicillin–streptomycin. *In vitro* cultures of fetal oocytes was performed as previously described [12]. Briefly, each ovary was cut into small fragments (0.5–1 mm) with fine needles (G 5/8) under a stereomicroscope and transferred to FlexiPERM tissue culture vessels (Heraeus Instruments) attached to tissue culture dishes. Each well was prefilled with 250 μL of Waymouth culture medium (Gibco) supplemented with 5% horse serum, 2.5% fetal calf serum (both heat-inactivated) and 100 μg/mL penicillin–streptomycin. Oocytes were cultured at 37° in a 5% CO_2 atmosphere. After 2–3 days, when ovary fragments were attached to the dish, the FlexiPERM wells were removed and 10 mL of culture medium were added. After 6 days of culture, the oocytes had reached the germinal vesicle (GV) stage and had a diameter of 40–70 μm. These were isolated from the ovary fragments by pipetting up and down several times until they were released. They were then washed in PBS-DEPC and stored in PCR tubes at −70° (10 oocytes per tube in 0.5 μL PBS) until use.

On day 6 of culture, when most oocytes had reached the GV stage, the cultures were exposed to different doses of DOX (10, 100 and 1000 nM) for 24 hr. They were then washed with fresh medium and oocyte survival was evaluated on days 7, 11, 16 and 21 after culture initiation.

2.2. Library construction

Reverse transcription (RT) reactions were performed using whole-oocyte lysates to avoid RNA loss during isolation. Immediately before the assay, 10 oocytes were lysed by two rapid thawing and freezing steps. The RT reagents [1× RT buffer, 10 mM DTT, 0.5% NP40, 1.5 U/μL RNase inhibitor (Promega), 0.5 mM each nucleotide, and 3.75 μM oligo $(dT)_{17}$] were mixed on ice, and 4 μL of this added to cell lysates. Samples were heated for 1 min at 65°, then cooled at room temperature for 2 min to allow annealing of the (dT) primer. They were then placed on ice. Reverse transcriptase Superscript (160 U, Gibco) (omitted in negative controls) was added to the oocyte lysates and RT was performed at 37° for 15 min. The reverse transcriptase was inactivated at 65° for 10 min, and the cDNA formed was stored at −20°.

Elongation of the 3′ end of first-strand cDNA with deoxyadenosine residues [poly(dA) tailing] was carried out at 37° for 15 min using 12 U terminal deoxynucleotidyl transferase (Roche Diagnostics) in a total volume of 10 μL which included 0.67 mM dATP.

The oligo(dA)-tailed first-strand cDNA was PCR amplified using the dT-EcoR1 primer for 25 cycles. Ninety μL of PCR mix [10 μL of 10× PCR buffer, 10 μL of 25 mM $MgCl_2$, 1 μL of each 100 mM deoxynucleotide triphosphate, 4 μL of 100 pmol/μL (dT)-Eco R1 primer, 60 μL H_2O, 2 μL of Taq polymerase (Promega)] were added to each tube. The reaction cycle consisted of an initial denaturation step at 94° for 5 min, 25 cycles of denaturation at 94° for 1 min, annealing at 42° for 2 min, and elongation at 72° for 6 min, with a 10 s extension time at each cycle. A final elongation step was performed at 72° for 10 min. When these 25 first cycles were complete, 2 μL of Taq polymerase were added to each tube and 25 more cycles were performed under the following PCR conditions: denaturation at 94° for 1 min, annealing at 42° for 2 min, elongation at 72° for 6 min, and a final elongation step at 72° for 30 min.

Upon completion of amplification, the cDNA was purified using the High Pure PCR Product Purification kit (Roche). To enable ligation into the vector, the cDNAs

were digested for 1 hr at 37° with EcoR1, and purified as before.

Approximately, 100 ng of cDNA were ligated into the Lambda Zap III vector (Stratagene), packaged *in vitro* and used to transform XL1-Blue MRF *Escherichia coli* cells. The libraries were amplified and titered according to standard protocols.

2.3. Selection of cDNA clones by differential screening

Approximately, 10,000 plaques were screened at a density of 2000 pfu/plate. Plaques were transferred onto nitrocellulose membranes in duplicate. One of these membranes was incubated with a radioactive cDNA probe obtained from untreated control oocyte mRNAs; the other was incubated with a radioactive cDNA probe from DOX-treated oocytes. Differentially expressed genes were detected by autoradiography, and the respective clones PCR amplified, resolved by electrophoresis, and transferred onto membranes in duplicate to perform a second differential analysis under the same conditions. Only clear differential signals appearing in the second screenings were considered genuinely positive.

2.4. Nucleotide sequencing and analyses of sequence homologies

PCR products were sequenced using a Perkin-Elmer (Wellesley) sequencer. Analysis of sequence homologies involved the use of the Basic Local Alignment Search Tool (BLAST) algorithm [13] to search the GenBank, EMBL and dbEST databases.

2.5. Quantification of gene expression by real-time RT-PCR

Genes showing differential expression in differential screenings were analyzed by real-time RT-PCR using a high-speed thermal cycler (LightCycler™; Roche Diagnostics) to quantify the extent of transcriptional control of these genes in DOX-exposed oocytes [14].

RT reactions were performed using whole oocytes. Amplification of genomic DNA was prevented by digesting it with DNase I free of RNase, before the RT step. Immediately before the assay, 10 oocytes were lysed by two rapid thawing and freezing steps. The RT reagents (1× RT buffer, 10 mM DTT, 0.5% NP40, 1.5 U/μL RNase inhibitor, 0.5 mM each nucleotide, and 3.75 μM oligo (dT_{17}) were mixed on ice and 7.5 μL were added to cell lysates. Samples were then heated for 1 min at 65° before being cooled on ice. Two units of DNase free of RNase were then added and DNA digestion performed by incubation at 37° for 5 min. DNase was then inactivated by incubating at 65° for 5 min. Tubes were cooled at room temperature for 2 min to allow annealing of the (dT) primer, and then placed on ice. Reverse transcriptase (160 U) (omitted in negative controls) was added to the oocyte lysates and RT performed at 37° for 15 min. The enzyme was inactivated at 65° for 10 min and the cDNA formed was stored at −20° until use.

The expressions of methionine adenosyltransferase (*MAT*), cytochrome *c* oxidase subunit II (*COX II*) and III (*COX III*), metaxin (*MTX*), syntaxin 5 (*SYNT*), ribosomal protein S16 (*S16*), NADH-ubiquinone oxidoreductase chain 2 (*ND2*) and a mouse sixteen-cell-embryo cDNA (*16CE*) were quantified. The primers used for PCR were, respectively: MAT-U (5′-GTTGGGGACTGTAAGTTGGG-3′), MAT-L (5′-CATTACAAATTCATGCTGAC-3′), COX II-U (5′-AGGGCACCAATGATACTGAA-3′), COX II-L (5′-TCTAGGACAATGGGCATAAA-3′), COX III-U (5′-CGAGACGTAATTCGTGAAGG-3′), COX III-L (5′-GGGCTTGATTTATGTGGTTT-3′), MTX-U (5′-ATCAGAGAACGAGGAGGAAC-3′), MTX-L (5′-GATAGAAACGATGCCACTGA-3′), SYNT-U (5′-CGCCCATTCAGAGATCCTCA-3′), SYNT-L (5′-GGCCATTCTTAGCAGTTCCA-3′), S16-U (5′-AGGAGCGATTTGCTGGTGTGGA-3′), S16-L (5′-GTCACCAGGCCTTTGAGATGGA-3′), ND2-U (5′-TCCTTACAACCCATCCCTCA-3′), ND2-L (5′-TCCTGTTAGTGGTGGAAGGC-3′), 16CE-U (5′-GGGGTAGGGTTATTGTGCTT-3′), 16CE-L (5′-TACTCGCCTAATTTATTCCA-3′).

PCR and melting curve determinations were performed in 20-μL volumes in glass capillaries. The PCR mix was prepared as follows: 10.8 μL H_2O, 3.2 μL $MgCl_2$ 25 mM, 1 μL upper primer and 1 μL lower primer at 5 pmol/μL each, 2 μL DNA-Master SYBR Green I (Roche Diagnostics) containing *Taq* polymerase, reaction buffer and dNTP mixture. Two μL of oocyte retrotranscriptions (equivalent to 2 oocytes) were used for amplification. A standard concentration curve was produced by amplifying S16 from serial dilutions (1:100, 1:1000 and 1:5000) of mouse testis cDNA. Cycling conditions were: initial denaturation at 94° for 2 min, followed by 40 cycles with denaturation at 94° for 1 s, annealing at 61° for 10 s and extension at 72° for 16 s with a ramping time of 20°/s. To assess the specificity of the amplified PCR products, melting curves were generated after amplification by holding the samples at 50° for 5 s and then slowly heating them to 95° at a ramp rate of 0.2°/s while monitoring fluorescence decline. Melting curves were converted to melting peaks by plotting the negative derivative of the fluorescence with respect to temperature ($-dF/dT$) against temperature.

2.6. Functional assays of the metaxin promoter

The metaxin promoter was previously cloned in the pGL2-Basic reporter vector, which carries luciferase as reporter [15]. The metaxin promoter-luciferase plasmid construct (kindly provided by Dr. Bornstein) includes 1389 nucleotides upstream of the metaxin translation start site.

NIH-3T3 cells were cultured as monolayers in Dulbecco-modified Eagle medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum, 100 U/mL

penicillin and 100 μg/mL streptomycin. Sub-confluent NIH-3T3 cells were transiently transfected with 1.25 μg of the metaxin promoter-luciferase plasmid construct using fuGENE 6 transfection reagent (Roche) following the manufacturer's instructions. After 24 hr the cells were exposed to different doses of DOX (0, 1, 5 and 10 μM) for 24 hr. Transfected cells not exposed to DOX were used as a controls. Cell lysates were prepared from the transfected cells using the cell culture lysis reagent (Promega). The luciferase activity in the extracts was measured with the Luciferase Reporter Assay System (Promega), according to the manufacturer's instructions, using a luminometer (TD-20/20) (Promega).

2.7. Statistics

Data were subjected to the Mann–Whitney *U*-test using SAS statistical programs (SAS Institute). Statistical significance was set at $P < 0.05$.

3. Results

3.1. Effect of DOX on the survival of fetal oocytes

After 6 days of culture, approximately 100 GV oocytes had grown from each fetal ovary cultured. The effect of DOX on the survival of fetal oocytes cultured *in vitro* is shown in Fig. 1. A value of 100% was assigned to the number of oocytes present in ovary explants on day 6 of culture. Cultures were exposed on this day to different doses of DOX, and oocyte survival evaluated on days 7, 11, 16 and 21 postculture initiation. DOX had no effect on oocyte survival at a concentration of 10 nM. However, oocyte survival decreased significantly to 83 and 48% after 24 hr exposure to 100 and 1000 nM DOX, respectively. Oocytes exposed to 100 nM DOX were selected for gene expression analysis since, although the number oocytes decreased, cell morphology was normal at this concentration.

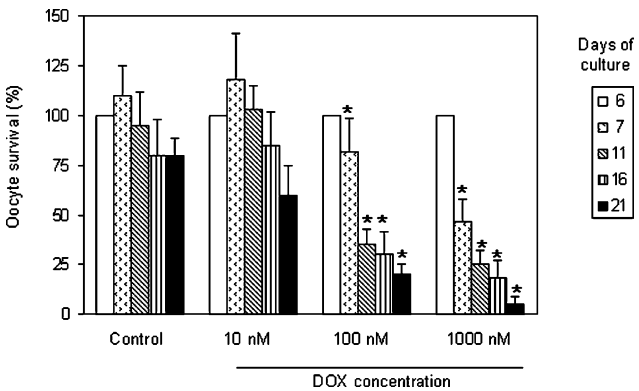

Fig. 1. Effect of DOX on the survival of fetal oocytes cultured *in vitro*. Cultures were exposed for 24 hr to different doses of DOX on day 6 after explant. Oocyte survival was evaluated on days 7, 11, 16 and 21 postinitiation of culture. Values represent means ± SEM (bar) of three independent experiments. (∗) As compared with the paired controls, $P < 0.05$.

3.2. Genes differentially expressed

Using only 20 oocytes representative cDNA libraries were generated. Analysis of the cDNA libraries obtained showed a titer of 10^6 pfu/mL with 98% of recombinants, which was amplified to 10^9 pfu/mL. Electrophoretic analysis and the sequence of randomly selected clones revealed no redundancy.

To identify differentially expressed genes in oocytes exposed to DOX, differential screenings were performed using cDNAs of untreated vs. treated oocytes (exposed during 24 hr to 100 nM DOX). Clones corresponding to deregulated genes, detected by differential autoradiographical signals in the first screening, were subjected to a second differential screening using Southern blots with inserted PCR-amplified cDNAs (Fig. 2). Of over 10,000 clones screened, only 7 were found to be clearly differentially expressed in oocytes exposed to DOX. This may be due to the differential screenings used, that allowed us only the identification of genes expressed at moderate to abundance levels and which are highly up- or down-regulated. These genes were sequenced and compared to known genes in data banks. Four nuclear-encoded genes showed altered expression in oocytes exposed to the drug: non-hepatic-type methionine adenosyltransferase (*MAT*),

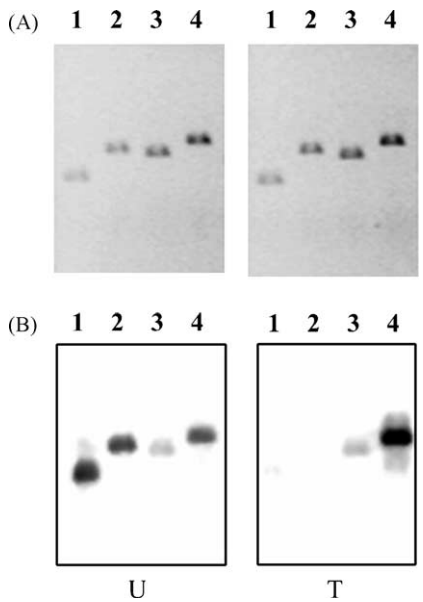

Fig. 2. Differential screening of gene expression. Cloned cDNAs corresponding to genes differentially expressed in oocytes treated with DOX in the first plate screening were PCR amplified, electrophoresed (A) and transferred onto membranes (B). A second confirmatory screening was performed by hybridization of membranes (B) using labeled cDNAs from untreated (U) vs. DOX-treated oocytes (T). Samples 1 and 2 correspond to the genes for cytochrome *c* oxidase subunits III and II, respectively. Down-expression is seen in treated oocytes. Sample 4 corresponding to the metaxin gene, demonstrates overexpression in treated oocytes. Sample 3 corresponds to a gene that does not modify its expression.

metaxin (*MTX*), syntaxin 5 (*SYNT*), and a mouse sixteen-cell-embryo cDNA (*16CE*). These were down-regulated, with the exception of metaxin which was up-regulated. Three mitochondrial-encoded genes were also identified which were down-regulated in DOX-exposed oocytes: cytochrome *c* oxidase subunit II (*COX II*), cytochrome *c* oxidase subunit III (*COX III*), and NADH-ubiquinone oxidoreductase chain 2 (*ND2*).

The seven genes showing differential expression in differential screenings were analyzed by real-time RT-PCR to quantify the extent of their transcriptional control in oocytes exposed to DOX. The specificity of RT-PCR and the absence of primer–dimer amplification was assessed by the analysis of amplicon melting curves. The type of deregulation (up- or down-regulation) correlated with that obtained from the differential screenings. Compared to control oocytes, the percentages of expression of *MAT*, *COX II*, *COX III*, *ND2*, *SYNT* and *16CE* decreased significantly to 7 ± 6, 29 ± 21, 3 ± 2, 7 ± 4, 12 ± 8, and 19 ± 13, respectively, while the percentage of expression of *MTX* increased significantly to 355 ± 61 (values represent means $\pm$ SEM of three independent experiments). These are prominent changes in relative transcript abundance, since relative expression levels are above 3-fold for all genes analyzed [16].

3.3. Functional assay of metaxin promoter

The promoter of metaxin, the only gene overexpressed in oocytes exposed to DOX, was evaluated in a functional assay to determine if the drug exerted transcriptional control at the promoter level. NIH-3T3 cells were transiently transfected with a metaxin promoter-luciferase plasmid construct and then exposed to different doses. Compared to untreated cells, luciferase activity increased significantly when transfected cells were exposed to 1 and 5 μM DOX (Fig. 3). This indicates deregulation of the metaxin gene by DOX not only in oocytes but also in somatic cells such as NIH-3T3. Activation of the metaxin promoter occurred only in the presence of DOX; cells treated with classic reprotoxicants such as ethyl-nitrosurea showed no increase in promoter activity (manuscript in preparation). There was no significant difference between cells exposed to 10 μM DOX and control cells, probably due to high levels of cell death after exposure to this dosage, and therefore the loss of the luciferase signal.

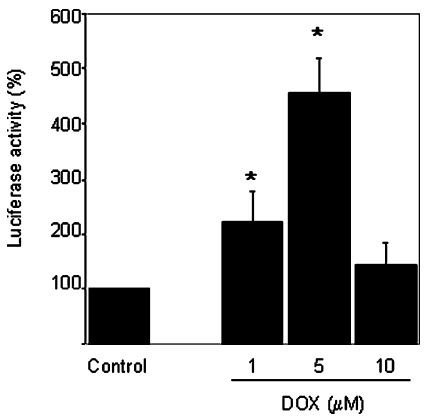

Fig. 3. Functional assay of the metaxin promoter. NIH-3T3 cells were exposed to different doses of DOX and then transiently transfected with a metaxin promoter-luciferase plasmid construct. Cells were lysed and luciferase activity in the extracts measured using the luciferase assay system. Cells transfected but not treated with DOX were used as controls. The value of control samples was designed to have a reference value of 100. Values represent means $\pm$ SEM (bar) of three independent experiments. (*) As compared with control, $P < 0.05$.

4. Discussion

The results show that in cultured oocytes the cytotoxic response to DOX involves the deregulation of expression of genes coding for the products of different biochemical pathways. This work analyzed the effects of DOX on the critical stage of fetal oocyte development, but it might be inferred that similar changes in gene expression regulation occur during other stages of oocyte maturation.

In clinical treatments the plasma DOX concentrations after bolus injections ranges between 50 and 200 nM—a concentration sustained for many days. The half life is 4–5 days. Nevertheless, DOX can be accumulate up to 30–100 times the plasma concentration in some cell types, such as leukemic cells [17]. This work analyzed gene expression response below 100 nM to understand the initial target response at the level of gene deregulation.

The differential hybridization cloning techniques we have used allowed us the identification of only seven differentially expressed genes in oocytes exposed to DOX because these techniques are effective at identifying genes that are expressed at moderate to high abundance levels and which are highly up- or down-regulated.

DOX exposure caused down-regulation of three genes involved in the mitochondrial respiratory chain (MRC): NADH ubiquinone oxidoreductase chain 2, cytochrome *c* oxidase subunit II and cytochrome *c* oxidase subunit III. Mitochondrial damage induced by DOX has been observed *in vivo* in man, rat, mouse, rabbit and dog heart. After several weeks of treatment, heart mitochondria have been seen to undergo swelling, fusion, dissolution, and/or the disruption of their cristae [18]. To analyze the mitochondrial injury induced by DOX treatment, the inhibition of respiratory chain activities has been measured in mitochondria from rat liver, rat heart and bovine heart [19]. It was found that the major drug-sensitive sites lie in complexes III and IV, their activities being reduced to 50% of control values with 175 μM DOX. Papadopoulou *et al.* [20] found that *COX II* expression was drastically reduced by DOX treatment, mainly after 8 weeks in both heart and liver mitochondria from mice treated with DOX (8 mg/kg b.wt.) for several weeks. This decrease in mitochondrial COX II gene expression was rather specific, since other

genes of COX enzyme examined (COX III and COX IV) were not affected by DOX treatment. In the present study, lower expressions of NADH ubiquinone oxidoreductase II, COX II and COX III were observed in oocytes exposed 24 hr to 100 nM DOX. Taken together, these results show that the molecular mechanisms of DOX injury in different cell types, such as cardiomyocytes, hepatocytes and oocytes, are strongly associated with the deregulation of several genes involved in the MRC.

DOX also altered the expression of genes involved in intracellular transport, such as metaxin and syntaxin 5. Metaxin is a component of a preprotein import complex in the outer mitochondrial membrane [21]. The present study demonstrates that the metaxin gene is inducible by DOX. This induction is basically regulated by the promoter region of the gene, as observed in the transfection experiments with NIH-3T3 cells. The results also demonstrate that the induction of gene expression is independent of cell type, although the final effect of metaxin overexpression could be modulated for other cell-specific factors. The presence in the metaxin promoter region of both NF-κB and AP-2 elements for the corresponding inducible transcription factors, suggests that metaxin gene expression could be regulated in response to external stimuli [15]. It is unknown whether DOX exerts a direct effect on the regulation of metaxin gene expression or whether some intermediate(s) promote the up-regulation of the ubiquitously expressed metaxin gene. The elements of this putative deregulation cascade merit further studies to clarify the therapeutic and side effects of DOX in different cells.

Syntaxin 5, a vesicular receptor involved in vesicle transport from the endoplasmic reticulum to the Golgi stack [22], was also affected in oocytes exposed to DOX. Syntaxin 5 expression was reduced to 12% of control values in oocytes exposed to 1 μM DOX. A consequence of syntaxin 5 down-regulation may be the blockade of cortical granule formation that normally occurs during oocyte growth. Cortical granules are required in mammals to produce the zona pellucida which is involved in the blockage of polyspermy [23]. If cortical granules formation is altered in DOX-exposed oocytes, this would be an additional infertility factor: even if oocytes survive DOX treatment, the possibility of successful fertilization would be limited by polyspermy.

DOX also caused down-regulation of methionine adenosyltransferase (MAT), the enzyme responsible for the synthesis of adenosylmethionine (AdoMet) from L-methionine and ATP. AdoMet is the major donor of methyl groups for transmethylation reactions in eukaryotic systems, and it is essential for normal cellular metabolism [24,25]. AdoMet is used for DNA and histone methylation, biochemical mechanisms associated with the functional and transcriptional inactivation of a number of genes during the processes of cellular differentiation and drug resistance (probably by altering chromatin structure and/or by preventing the binding of transcription factors) [24,26,27]. Further, AdoMet is a precursor of the polyamines spermidine and spermine, which are essential for cell growth and regulation of the cell cycle [23], and of the antioxidant agent glutathione [25]. It has been reported that a decrease in AdoMet content generates oxidative stress [26]. It might therefore be expected that the observed down-regulation of MAT in oocytes would affect: (1) DNA and histone methylation—and therefore the transcriptional regulation of several genes essential in cell differentiation, (2) the synthesis of spermidine and spermine—which would affect oocyte growth and differentiation, and (3) the synthesis of glutathione-causing oxidative stress. This hypothesis is still to be tested.

It has been proposed that DOX exerts an antitumor activity by interaction with DNA and its metabolism. However, some of the toxicity of this drug has been related to the interference of mitochondrial functions. Enzyme inhibitions, lipid peroxidations, membrane disorders and oxidative stress, are now being associated with the toxic side effects of DOX, with the mitochondria as subcellular targets [27]. The deregulation of mitochondrial gene expression seen in the present work supports these findings. It is clear that DOX and other anthracyclines interact with the structure and function of mitochondria, and we here demonstrate that this drug also affects genes specific to the mitochondrial genome. The potential synergistic effect of interaction with different biochemical pathways and deregulation of gene expression should be considered when trying to understand cell damage observed in DOX treatment. DOX-mediated changes in gene expression could be due to the drug itself, one of its metabolites, or a consequence of initial mitochondrial injury. Although nuclear-coded, metaxin is a protein involved in mitochondrial function; the up-regulation of the metaxin gene could thus represent a cellular response to mitochondrial damage.

Since DOX shows a wide range of effects on mammalian oocytes, additional analysis is needed for a global view of oocyte response to this agent at the genome level. Such analysis would yield insight into the molecular mechanisms of DOX injury in oocytes and the rationale design of more effective treatment strategies to circumvent the unwanted side effects of this drug on fertility.

Acknowledgments

The authors wish to thank Dr. Luis A. López-Fernández and Dr. Mario Párraga for their advice and suggestions. We thank Fernando Escolar for excellent technical assistance. We are indebted to Dr. M. De Felici for his assistance in the culture of fetal oocytes. This work was supported by grants awarded to J.M. by the DGICYT (PB95-0119), EU (PL 960183) and CAM (07B/0022/1997). E.B. was granted a fellowship from the CONACyT (Mexico) and AECI-Mutis (Spain).

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