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Unexpected Anthracycline-Mediated Alterations in Iron-Regulatory Protein-RNA-Binding Activity: The Iron and Copper Complexes of Anthracyclines Decrease RNA-Binding Activity

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ABSTRACT

Anthracyclines are effective antineoplastic agents. However, the interaction of these drugs with iron (Fe) is an important cause of myocardial toxicity, limiting their therapeutic use (*J Lab Clin Med* **122:**245–251, 1993). To overcome this limitation, it is crucial to understand how anthracyclines interact with the Fe metabolism of myocardial and neoplastic cells. Iron-regulatory proteins (IRPs) play vital roles in regulating cellular Fe metabolism via their mRNA-binding activity. We showed that doxorubicin (DOX) and its analogs interfere with tumor and myocardial cell Fe metabolism by affecting the RNA-binding activity of IRPs. Unexpectedly, experiments with the free radical scavengers, catalase, superoxide dismutase, ebselen, and Mn(III) tetrakis (4-benzoic acid) porphyrin complex, suggested that the effects of DOX on IRP-RNA-binding activity were not due to anthracycline-mediated free radical production. In contrast to previous studies, we showed that the DOX metabolite, doxorubicinol, had no effect on IRP-RNA-binding activity. Rather, the anthracycline-Fe and -copper (Cu) complexes decreased IRP-RNA-binding activity, indicating that formation of anthracycline-metal complexes may affect cellular Fe metabolism. In addition, anthracyclines prevented the response of IRPs to the depletion of intracellular Fe by chelators. This information may be useful in designing novel therapeutic strategies against tumor cells by combining chelators and anthracyclines. Interestingly, the effect of DOX on primary cultures of cardiomyocytes was similar to that observed using neoplastic cells, and particularly notable was the decrease in IRP2-RNA-binding activity. Our results add significant new information regarding the effects of anthracyclines on Fe metabolism that may lead to the design of more effective treatments.

Anthracyclines [e.g., doxorubicin (DOX); Fig. 1] are highly effective agents for the treatment of a wide variety of tumors. However, the major limitation of anthracyclines is cardiotoxicity (Gianni and Myers, 1992; Gerwirtz, 1999), which is thought to be associated with its marked ability to bind Fe (Garnier-Suillerot, 1988; Gianni and Myers, 1992).

The fact that DOX can avidly bind Fe suggests that it may act as a chelator to perturb intracellular Fe pools. Certainly, DOX binds Fe from ferritin, transferrin, and microsomal membranes (Gianni and Myers, 1992). Moreover, Fe loading potentiates anthracycline toxicity, and the clinically used chelator desferrioxamine (DFO) reduces the toxic effects of DOX in vitro (Hershko et al., 1993; Link et al., 1996) and in vivo (Herman et al., 1994; Saad et al., 2001). Another Fe chelator known as ICRF-187 (also called dexrazoxane) reduces anthracycline-induced cardiotoxicity in many experimental models and clinical trials (Gerwirtz, 1999). These studies indicate that DOX cardiotoxicity is due, in part, to its interaction with Fe. However, there have been few studies examining the effects of anthracyclines on Fe metabolism of myocardial and neoplastic cells.

Cellular Fe homeostasis is largely regulated post-transcriptionally by iron-regulatory proteins (IRPs) (Hentze and Kühn, 1996). The mRNAs of certain molecules involved in Fe metabolism, including ferritin and transferrin receptor (TfR), contain hairpin loop structures in their 5′- or 3′-untranslated regions (UTRs), called iron-responsive elements (IREs) (Hentze and Kühn, 1996). The IRPs bind to IREs and either stabilize the mRNA against degradation or inhibit translation (Hentze and Kühn, 1996). In ferritin mRNA, the IRE is in the 5′-UTR, and IRP binding inhibits translation, thereby decreasing Fe storage. However, in TfR mRNA the IREs are in the 3′-UTR, and IRP binding confers mRNA stability,

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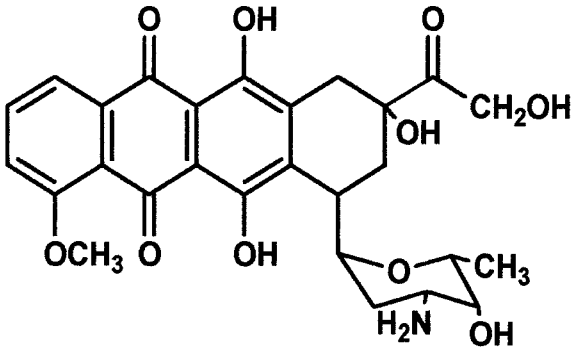
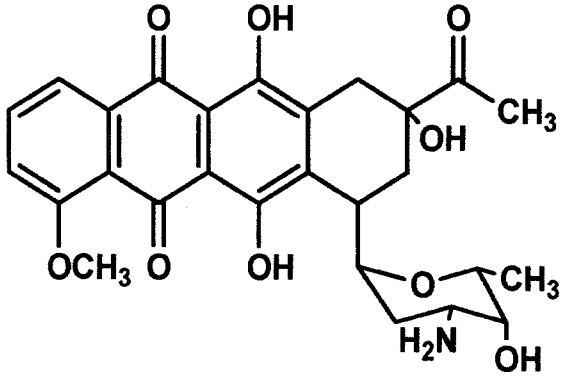
ABBREVIATIONS: DOX, doxorubicin; DFO, desferrioxamine; ICRF-187, dexrazoxane; IRP, iron-regulatory protein; TfR, transferrin receptor; UTR, untranslated region; IRE, iron-responsive element; c-acon, cytoplasmic aconitase; *cis*-acon, *cis*-aconitate; DOXol, doxorubicinol; FAC, ferric ammonium citrate; NEM, *N*-ethylmaleimide; SOD, superoxide dismutase; MnTBAP, Mn(III) tetrakis (4-benzoic acid) porphyrin complex; DAU, daunorubicin; EPI, epirubicin; FeCN, ferricyanide; β-ME, β-mercaptoethanol; TBS, Tris-buffered saline; Apo-IRP1, iron regulatory protein 1 without iron-sulfur cluster; Holo-IRP1, iron regulatory protein 1 with iron-sulfur cluster.

Doxorubicin (DOX)



Daunorubicin (DAU)



Epirubicin (EPI)

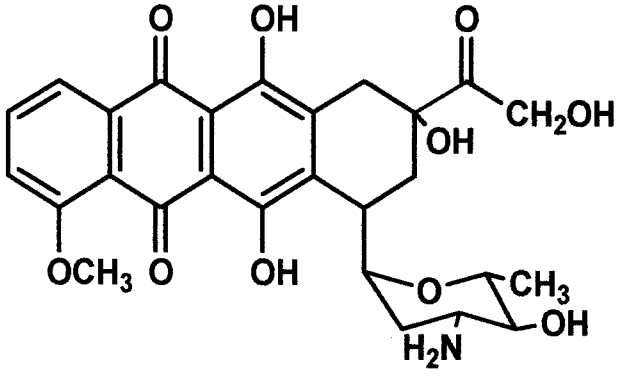


Fig. 1. Chemical structure of the anthracycline, doxorubicin (DOX), and its analogs daunorubicin (DAU) and epirubicin (EPI).

enhancing translation and Fe uptake via the TfR (Hentze and Kühn, 1996). Thus, the IRP-RNA-binding mechanism is crucial in regulating Fe homeostasis, and it is therefore the focus of this investigation.

The mRNA-binding activity of IRP1 is regulated by the presence of an [4Fe-4S] cluster within the protein (Hentze and Kühn, 1996; Theil and Eisenstein, 2000). When cells are Fe-depleted, the [4Fe-4S] cluster is absent (apo-IRP1), allowing for increased IRP1-IRE binding affinity. Conversely, when Fe levels are high, an [4Fe-4S] cluster becomes incorporated into the protein (holo-IRP1), preventing IRP1-IRE binding (Hentze and Kühn, 1996). Interestingly, the [4Fe-4S] cluster of IRP1 confers the molecule with aconitase activity. In fact, IRP1 is the cytoplasmic aconitase (c-acon), which is capable of converting citrate to isocitrate, with *cis*-aconitate (*cis*-acon) being an intermediate (Beinert and Kennedy, 1993). Apart from IRP1, a related RNA-binding molecule called IRP2 has been described that does not contain an [4Fe-4S] cluster. In Fe-replete cells, IRP2 is degraded by a proteasome-dependent mechanism (Guo et al., 1995).

The effect of DOX on IRP1 has been studied using heart tissue lysates in which the [4Fe-4S] cluster had been artificially reconstituted in vitro using Fe salts and cysteine (Minotti et al., 1995, 1998). The DOX metabolite, doxorubicinol (DOXol), in the presence of *cis*-acon, was suggested to interact with the [4Fe-4S] cluster of IRP1 to decrease IRP1-RNA-binding activity (Minotti et al., 1995, 1998). In contrast, in a more recent investigation using a rat heart-derived cell line, DOXol increased IRP1-RNA binding activity (Minotti et al., 2001). However, these latter experiments were performed in a cell line that has lost many differentiated features of myocardial cells (Hescheler et al., 1991). Hence, the relevance of these studies to normal heart cells was uncertain. Our current study is the first to examine the effect of anthracyclines on IRP-RNA-binding activity using intact and differentiated myocardial cells. Furthermore, the effects of anthracyclines on the IRP-RNA-binding activity of tumor cells were examined because this has not been assessed previously.

Our experiments using intact tumor cells show that anthracyclines induce a significant decrease in active IRP-RNA-binding activity after 6 h and a decrease in total IRP-RNA-binding activity after 24 h. Unexpectedly, these effects were not due to DOXol or the generation of free radicals by anthracyclines. Rather, the Fe or Cu complexes with DOX decreased active IRP-RNA-binding activity, suggesting that the formation of these compounds may result in the initial decrease in active IRP-RNA-binding. In primary cultures of cardiomyocytes, DOX had an effect on IRP1 similar to that described for neoplastic cells, and caused a decrease in IRP2-RNA-binding activity. Our results may be vital for understanding the effects of anthracyclines on Fe metabolism and the generation of novel treatment strategies.

Materials and Methods

Cell Treatments and Reagents. Desferrioxamine was obtained from Novartis (Basel, Switzerland). Catalase, *cis*-acon, ebselen, ferric ammonium citrate (FAC), *N*-ethylmaleimide (NEM), and superoxide dismutase (SOD) were obtained from Sigma-Aldrich (St. Louis, MO). The Mn(III) tetrakis (4-benzoic acid) porphyrin complex (MnTBAP) was obtained from ICN Pharmaceuticals Biochemicals Division (Aurora, OH). ICRF-187 was purchased from Chiron B.V. (Paasheuvelweg, Amsterdam, The Netherlands). DOX, daunorubicin

(DAU), and epirubicin (EPI) were obtained from Pharmacia (Sydney, Australia). DOXol was kindly provided by Dr. G. Minotti, Department of Drug Sciences, G. D'Annunzio University School of Medicine, Chieti, Italy. The anthracycline-metal complexes were prepared as described previously (Garnier-Suillerot, 1988; Gianni and Myers, 1992).

Cell Culture. The human SK-Mel-28 melanoma, human SK-N-MC neuroepithelioma, and rat L8 skeletal muscle cell lines were obtained from the American Type Culture Collection (Manassas, VA). The M15 mouse kidney cell line was obtained from Dr. S. Wardrop, Institute of Molecular Biosciences, The University of Queensland, Brisbane, Australia. The human BE-2 neuroblastoma cell line was a gift from Dr. G. Anderson, Queensland Institute of Medical Research, Brisbane, Australia. Cells were grown and subcultured as described previously (Richardson and Baker, 1992a,b).

Primary cultures of beating neonatal myocardial cells were isolated from 2- to 3-day-old rats using well established methods (Link et al., 1985, 1996; Hershko et al., 1993; Terman and Brunk, 1998). Briefly, ventricles were minced and incubated in the presence of 0.05% collagenase type II (Worthington Biochemicals, Freehold NJ) at 37°C, with stirring. The cell suspension was centrifuged in a Percoll gradient (1.05 g/ml) to purify cardiac myocytes from other cell types including fibroblasts and red blood cells (Terman and Brunk, 1998). Cells were plated on collagen-coated plates and cultured at 37°C in an atmosphere of 8% O_2 and 5% CO_2 (Terman and Brunk, 1998). Experiments were performed on day 4 of culture. Purity of cardiomyocyte cultures was confirmed by immunofluorescent staining of the cells using an α-actinin antibody (Goncharova et al., 1992) (Sigma). These cells were used because they demonstrate most of the functional characteristics of the intact heart, including contractility, rhythmicity, and automaticity (Link et al., 1985). Furthermore, the effects of anthracyclines, Fe loading, and Fe chelators on these cells have been well characterized, and this model has been shown to closely mimic the in vivo situation (Link et al., 1985, 1996; Shiloh et al., 1992; Hershko et al., 1993).

Preparation and Treatment of Cytosolic Extracts. Cellular extracts were prepared by incubating cells in culture with medium alone (control) or medium containing DFO (100 μM), FAC (100 μg/ml), or the anthracyclines (0.5–20 μM). The concentrations of anthracyclines examined were within the range used in previous studies (Minotti et al., 1995, 1998; DeAtley et al., 1999; Konorev et al., 1999; Sawyer et al., 1999). In experiments where Fe-depleted or Fe-loaded lysates were required, cells were incubated for 24 h at 37°C with the above concentrations of DFO or FAC, respectively. This incubation procedure has been shown, respectively, to deplete and load cells with Fe, as indicated by TfR mRNA levels, transferrin-bound ^{59}Fe uptake, IRP-IRE binding, c-acon activity, and the susceptibility of IRP-RNA binding to the sulfhydryl alkylating agent, NEM (Wardrop et al., 2000). Using the protocol with DFO and FAC, we showed in this study and previous investigations (Wardrop et al., 2000) that IRP1 was predominantly present without or with the [4Fe-4S] cluster, respectively. Indeed, after a 24-h incubation with control media, DFO (100 μM), or FAC (100 μg/ml), the c-acon activities were 12.3 $\pm$ 1.9 U/mg, 3.4 $\pm$ 0.3 U/mg, and 11.6 $\pm$ 1.0 U/mg (three experiments), respectively. These studies demonstrate that the control cells used in our experiments were Fe-replete. Approximately 2 to 5 $\times$ 10^6 cells were lysed at 4°C in ice-cold Munro extraction buffer and processed (Leibold and Munro, 1988). In some experiments, the cytosolic extracts (2 μg) were directly incubated with the anthracyclines and other agents for 3 h at 4°C or 1 h at 37°C in 25 mM Tris/40 mM KCl (pH 8) (Wardrop et al., 2000).

RNA-Protein Gel Retardation Assays. The gel retardation assay was used to measure the interaction between IRPs and IREs via established techniques (Leibold and Munro, 1988; Wardrop et al., 2000). A ^{32}P-labeled pGL66 RNA transcript containing 118 base pairs of the ferritin H-chain (pGL66 was kindly provided by Dr. E. Leibold, University of Utah, Salt Lake City, UT) was transcribed in vitro using the Promega Riboprobe In Vitro Transcription Kit (Promega, Madison, WI). The riboprobe was then purified on a 6% urea/polyacrylamide gel.

Aliquots of cell lysate containing 2 μg of protein were incubated with 0.1 ng (approximately 1 $\times$ 10^5 cpm) of ^{32}P-labeled riboprobe for 30 min at room temperature. Using this protocol, the level of RNA added was saturating. Unprotected probe was degraded by incubation with 1 U of RNase T1 for 10 min at room temperature. Heparin (5 mg/ml) was then added for another 10 min to exclude nonspecific binding. The IRE-protein complexes were analyzed on 6% nondenaturing polyacrylamide gels.

In parallel experiments, samples were treated with ferricyanide (FeCN, 5 mM) and 0.5% β-mercaptoethanol (β-ME), before addition of the IRE probe (Hirling et al., 1994; Abboud and Haile, 2000). This procedure results in the conversion of IRP1, whether spontaneously active or not, into an active IRE-binding form (Hentze and Kühn, 1996; Abboud and Haile, 2000). This treatment measures the total amount of IRP present in the cell that can be activated to bind IRE, whether initially active or not (Hentze and Kühn, 1996; Abboud and Haile, 2000). The combination of ferricyanide together with β-ME is highly efficient in removing the [4Fe-4S] cluster of IRP1, and this was used in preference to β-ME alone in terms of determining total IRP1-RNA-binding activity. Unlike IRP1, IRP2 is not regulated by an [4Fe-4S] cluster and is not responsive to FeCN and β-ME (Guo et al., 1995). Hence, total IRP2 levels cannot be estimated by treatment with these latter agents.

To optimally demonstrate the complex changes in IRP-RNA-binding observed (e.g., see Fig. 2), gels for the active and total IRP-RNA-binding activity needed to be exposed for different lengths of time. Hence, one cannot quantitatively compare the active and total IRP-RNA-binding activity at a particular time point. Rather, all results must be interpreted with respect to the relevant control for that particular gel.

Supershift experiments were performed by standard procedures (Guo et al., 1995) using a rabbit anti-human IRP2 polyclonal antibody kindly provided by Dr. E. Leibold (University of Utah).

Cytoplasmic Aconitase Activity Determination. Cytoplasmic aconitase activity was measured using well established procedures (Drapier and Hibbs, 1996). Briefly, cells were treated with the detergent digitonin, which selectively permeabilizes the plasma membrane and leaves the inner mitochondrial membrane intact (Drapier and Hibbs, 1996). Samples were spun at 1,800*g* for 8 min at 4°C. The supernatant was then ultracentrifuged at 230,000*g* for 20 min at 4°C to remove mitochondria. The cytosolic supernatant was subsequently desalted and concentrated using an Amicon concentrator (M_r cutoff = 30,000; Millipore Corporation, Bedford, MA) so that the protein concentration was 3 to 4 mg/ml. The c-acon activity of extracts was determined spectrophotometrically by measuring the disappearance of *cis*-acon at 240 nm as described previously (Drapier and Hibbs, 1996; Wardrop et al., 2000). Units represent nanomoles of substrate consumed per minute at 37°C ($\epsilon_{240\ nm}$ = 3.6 mM^{-1} cm^{-1}) (Drapier and Hibbs, 1996; Wardrop et al., 2000).

Western Blot Analysis. Western blot analysis was performed essentially as described previously (Gao and Richardson, 2001). Briefly, for cytoplasmic extracts, cells were collected and incubated on ice for 20 min with the lysis buffer [20 mM HEPES, pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.1% Triton X-100, 1 mM dithiothreitol, 10 mM NaCl, and Complete protease inhibitors (Roche Diagnostics, Mannheim, Germany)] and centrifuged at 14,000 rpm/45 min at 4°C. The protein concentrations of lysates were assessed by the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA).

The lysates were then mixed with loading buffer containing 20% β-ME and loaded at 100 μg per sample onto a SDS-polyacrylamide gel consisting of 4% stacking and 8% resolving gels. After electrophoresis, the proteins were transferred onto polyvinylidene difluoride membranes (Amersham Biosciences Inc., Piscataway, NJ) overnight at 4°C. These membranes were then soaked in methanol and immediately blocked with 3% skim milk and 2% bovine serum albu-

min in Tris-buffered saline (TBS) for 2 h. After blocking, the membranes were incubated with the anti-IRP1 polyclonal antibody (kindly supplied by Professor K. Pantopoulos, Lady Davis Institute for Medical Research, Montréal, QC, Canada) diluted to 1/800 in 3% skim milk and 2% bovine serum albumin in TBS for 3 h at room temperature. To ensure even loading of proteins, membranes were probed using an anti-β-actin monoclonal antibody (clone AC-15; Sigma) diluted to 1/10,000. Membranes were then washed four times in TBS containing 0.1% Tween 20 (Sigma) for 10 min each. After washing, anti-rabbit (1/10,000) (Zymed Laboratories, South San Francisco, CA) or anti-mouse (1/10,000) (Sigma) antibodies conjugated with horseradish peroxidase were incubated with the membranes for 1 h at room temperature. After washing, the membranes were developed using the ECL Plus (Amersham Biosciences) Western blot detection reagents by using a 1-min incubation and exposure to X-ray film for 10 s to 15 min. All densitometric data were normalized to β-actin.

Statistical Analysis. Experimental data were compared using Student's t test. Results were considered statistically significant when $p < 0.05$.

Results

Effect of Anthracyclines on IRP-RNA-Binding Activity of Neoplastic Cells as a Function of Incubation Time and Concentration. The cardiotoxic effects of anthracyclines have been linked to their ability to perturb cellular Fe metabolism (Garnier-Suillerot, 1988; Gianni and Myers, 1992). Considering the ability of anthracyclines to bind Fe (Garnier-Suillerot, 1988; Gianni and Myers, 1992), a potential target for these cytotoxic agents may be the IRPs that are crucial in controlling cellular Fe metabolism (Hentze and Kühn, 1996). Therefore, the effect of DOX on IRP-RNA-binding activity in human SK-Mel-28 melanoma cells was examined after incubation times of 0.5 to 30 h (Fig. 2). This cell type was assessed initially because its Fe metabolism has been well characterized (Richardson and Baker, 1992a,b), and the proliferation of these cells was sensitive to anthracyclines (data not shown). In the current investigation, the gel retardation assay was used to examine IRP-RNA binding. The active IRP-RNA-binding activity represents the proportion of IRP in the high affinity form that can spontaneously bind mRNA. Total IRP-RNA binding represents all cellular IRP present (both high and low affinity forms) that can be activated to bind mRNA after incubation with β-ME and FeCN treatment (Hentze and Kühn, 1996) (see *Materials and Methods*).

The effect of DOX on IRP-RNA binding was complex and could be divided into two components, an initial and a delayed response (Fig. 2). In the initial response, treatment with DOX (20 μM) after 30 min caused an insignificant ($p > 0.05$) increase in active IRP-RNA-binding activity. More notably, a 6-h incubation with DOX significantly ($p < 0.01$) decreased active IRP-RNA-binding activity to 47% of the relevant control level (Fig. 2). In addition, total IRP-RNA-binding activity was significantly ($p < 0.01$) decreased to 76% of the relevant control after a 3-h incubation with DOX and remained at relatively the same level up to 18 h (Fig. 2). As part of the delayed response, active IRP-RNA-binding activity increased after 6 h and was shown to slightly exceed ($p > 0.05$) relevant control levels after a 24-h incubation with DOX (Fig. 2). Indeed, it should be noted that in some experiments (e.g., see Figs. 2C and 5B), there was no marked difference in active IRP-RNA-binding activity between DOX and the control after a 24-h incubation. In contrast, total IRP-RNA-binding activity decreased further after 18 h, reaching 54% of the relevant control level ($p < 0.05$) after a 30-h incubation with DOX (Fig. 2). Experiments were performed using Western blotting to determine whether the reduction in total IRP-RNA-binding activity could be correlated to a decrease in IRP1 protein levels. After a 24-h incubation with DOX (20 μM), the IRP1 protein levels had decreased to 52% of the relevant control. This result was in agreement with the decrease in total IRP1-RNA-binding ac-

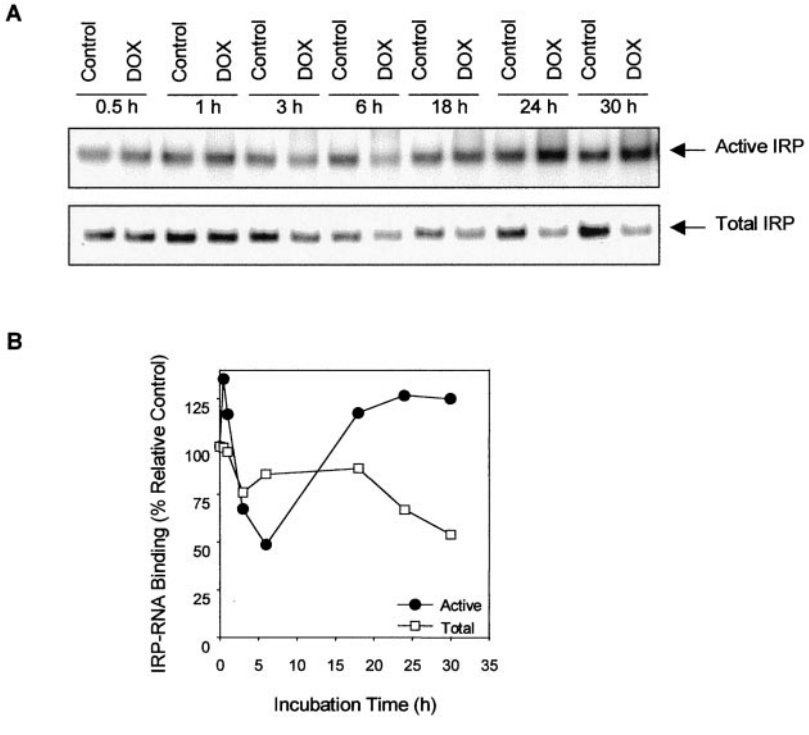


Fig. 2. Effect of DOX on cellular IRP-RNA-binding activity as a function of incubation time with SK-Mel-28 melanoma cells. A, SK-Mel-28 cells were treated with or without 20 μM DOX for 0.5 to 30 h at 37°C, and IRP-RNA-binding activity was assessed by the gel retardation assay. B, densitometric analysis of A. Results are a representative experiment of four performed.

tivity reported in Fig. 2. Collectively, these experiments indicated that in this tumor cell type DOX interferes with Fe metabolism by affecting both the active and total IRP-RNA-binding activity.

Considering the results in Fig. 2, it seems that the initial cellular reaction to DOX involved a decrease in active and total IRP-RNA-binding activity. In contrast, the delayed response after 18 h involved a restoration of active IRP-RNA-binding activity to untreated levels, but the total IRP-RNA binding further decreased (Fig. 2). This latter observation indicates that as a proportion of the total IRP-RNA-binding pool, the active RNA-binding form has increased.

Gel retardation assays using native polyacrylamide gel electrophoresis with human cells results in comigration of IRP1 and IRP2 (Chitambar and Wereley, 1995). To examine whether the effects of DOX observed in the present study were targeted to IRP1, IRP2, or both, supershift experiments were performed. These studies demonstrated that the major IRP type in SK-Mel-28 cells was IRP1, with undetectable amounts of IRP2 (data not shown).

To assess whether the time-dependent effects described in Fig. 2 were unique to DOX, we examined two other structurally similar anthracyclines, namely, DAU and EPI (Fig. 1). The SK-Mel-28 cells were treated at 37°C with the anthracyclines for 6 or 24 h (Fig. 3) to determine the presence of the initial and delayed response seen in Fig. 2. Similar to DOX, incubation of cells for 6 h with either DAU or EPI caused a significant ($p < 0.005$) decrease in active IRP-RNA-binding activity without significantly affecting total IRP-RNA-binding activity (Fig. 3, A and B). In addition, all three anthracyclines caused a concentration-dependent decrease in total IRP-RNA-binding activity after incubation with cells for 24 h, while maintaining active IRP-RNA-binding activity at near-control levels (Fig. 3, C and D). At an anthracycline concentration of 20 μM, total IRP-RNA-binding activity had significantly ($p < 0.001$) decreased to 51 to 64% of control values (Fig. 3D). The Fe chelator, DFO, was used as a positive control (Hentze and Kühn, 1996; Wardrop et al., 2000) and caused a marked increase in active IRP-RNA-binding activity after 24 h (Fig. 3C) as expected. The Fe donor, FAC, should decrease IRP-RNA-binding activity (Wardrop et al., 2000). However, it seemed that the untreated cells were already Fe-replete, as incubation with FAC had little effect on IRP-RNA-binding activity (Fig. 3C). Collectively, the results above demonstrated that all three anthracyclines affect IRP-RNA-binding activity in a similar way, suggesting that this is a general effect of these compounds.

To investigate whether the changes in IRP-RNA-binding activity induced by DOX were specific for SK-Mel-28 melanoma cells, other DOX-sensitive neoplastic cell types were examined, including BE-2 neuroblastoma and SK-N-MC neuroepithelioma cells. The effects of DOX on IRP-RNA-binding activity were similar in all three cell lines (data not shown), suggesting that the responses observed were not unique to one tumor cell type.

The effect of anthracyclines on IRP could not be explained by the cytotoxic effects of these agents, because high concentrations of cisplatin (1–20 μM) or bleomycin (10–500 U/ml) did not affect IRP-RNA-binding activity despite marked cell death (data not shown). Moreover, after a 6-h incubation with the anthracyclines, no cytotoxic effects were observed, despite the decrease in active IRP-RNA-binding activity (Figs. 2 and 3, A and B).

Effect of Anthracyclines on IRP-RNA-Binding Activity of Cardiomyocytes as a Function of Concentration and Incubation Time. Rodent IRP1 and IRP2 migrate at different rates in native polyacrylamide gels (Chitambar and Wereley, 1995), and in rat cardiomyocytes, the mRNA-binding activity of both proteins was observed (Fig. 4). Considering the results above in tumor cells (Figs. 2 and 3), the effects of DOX (1–20 μM) were assessed in cardiomyocytes after a 6-h (Fig. 4, A and B) and 24-h (Fig. 4, C and D) incubation in comparison with DFO (100 μM).

After 6 h, DFO had little effect on IRP-RNA-binding activity (Fig. 4A), whereas it markedly increased active IRP1- and IRP2-RNA-binding after 24 h (Fig. 4C). The effects of DOX on IRP1-RNA-binding activity in myocardial cells (Fig. 4) were somewhat similar to those reported in neoplastic cells (Figs. 2 and 3). Indeed, after an incubation period of 6 h with increasing DOX concentrations, active IRP1-RNA-binding activity decreased, whereas total IRP1 binding remained relatively constant (Fig. 4, A and B). The IRP2-RNA-binding activity decreased to less than 50% of the control at a DOX concentration of 20 μM, being more markedly affected than IRP1 (Fig. 4, A and B).

After a 24-h incubation of cardiomyocytes with DOX, there was a slight decrease in active IRP1-RNA-binding activity at 1 μM DOX that was not consistently observed in repeat experiments (Fig. 4, C and D). At higher DOX concentrations there was no significant effect on active IRP1-RNA-binding activity in cardiomyocytes (Fig. 4, C and D), as found for neoplastic cells (Fig. 3, C and D). Total IRP1-RNA-binding activity decreased to 74% of the control at 20 μM DOX after 24 h (Fig. 4, C and D). Concentrations of DOX from 1 to 10 μM were slightly less effective at reducing total IRP1-RNA-binding activity in cardiomyocytes (Fig. 4, C and D) than that found in neoplastic cells (Fig. 3, C and D). As found after 6 h (Fig. 4, A and B), a 24-h incubation with DOX decreased IRP2-RNA-binding activity, although to a much lesser extent (Fig. 4, C and D). A similar effect of DOX on IRP2 was also observed in several other rodent cell types, including the mouse M15 kidney cell line and the rat L8 skeletal muscle cell type (data not shown).

Role of Free Radicals in Anthracycline-Mediated Alterations in IRP-RNA-Binding Activity. Numerous studies have implicated a role for free radical production in anthracycline-mediated cardiotoxicity (Minotti et al., 1998; Konorev et al., 1999; Kotamraju et al., 2002). To determine whether free radicals mediate anthracycline-induced changes in IRP-RNA-binding activity in tumor cells, SK-Mel-28 cells were treated with or without DOX in the presence or absence of a range of free radical scavengers for 6 or 24 h (Fig. 5, A and B). Free radical scavengers examined included the cell-impermeable agents, SOD (1000 U/ml) and catalase (1000 U/ml), the cell-permeable SOD mimetic, MnT-BAP (200 μM), and the cell-permeable glutathione peroxidase mimetic, ebselen (15 μM) (Konorev et al., 1999). The concentrations of free radical scavengers examined were within the effective range used in the literature (Minotti et al., 1998; Konorev et al., 1999; Kotamraju et al., 2002). None of the radical scavengers had any significant effect on IRP-RNA-binding activity in SK-Mel-28 cells after a 6-h (Fig. 5A) or 24-h incubation (Fig. 5B) in the presence or absence of

DOX. Furthermore, whereas MnTBAP markedly protected tumor cells against DOX-induced cytotoxicity after 24 h (data not shown), total IRP-RNA-binding activity remained depressed (Fig. 5B). These results suggested that the effect of DOX on IRP-RNA-binding activity after 6 or 24 h was not mediated by free radical production.

The Effect of Doxorubicinol and *Cis*-Aconitate on IRP-RNA-Binding and Cytoplasmic Aconitase Activity. Anthracyclines are metabolized in vivo to form a number of intermediates that have been shown to possess important biological effects (Takanashi and Bachur, 1976; Minotti et al., 1995, 1998). It was therefore important to further examine which intracellular metabolite was directly affecting IRP-RNA-binding activity. To investigate this, cell lysates were prepared and treated directly with the metabolites of interest, and the IRP-RNA-binding activity was assessed (see *Materials and Methods*).

Previous studies using Fe-loaded lysates from myocardial tissue have suggested the importance of the combination of the DOX metabolite, doxorubicinol (DOXol), and *cis*-acon on

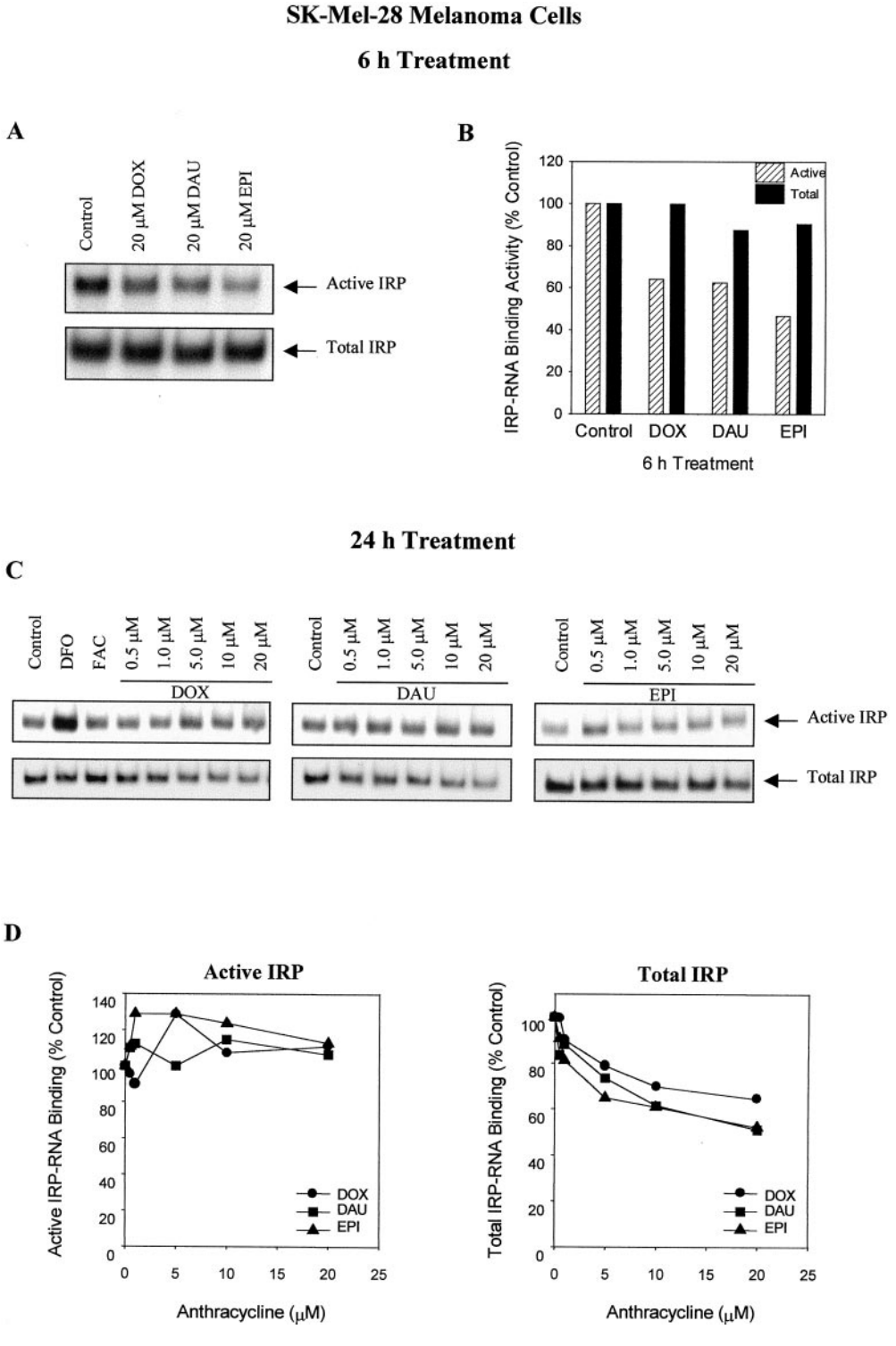


Fig. 3. The anthracyclines, DOX, DAU, and EPI, all affect IRP-RNA-binding in a similar manner in neoplastic cells after a 6- or 24-h incubation. A, SK-Mel-28 cells were incubated with control media or the anthracyclines (20 μM) for 6 h at 37°C, and IRP-RNA-binding activity was assessed. B, densitometric analysis of the results in A. C, SK-Mel-28 cells were incubated with either control media, DFO (100 μM), FAC (100 μg/ml), or anthracycline (0.5–20 μM) for 24 h at 37°C, and the IRP-RNA-binding activity was assessed. D, densitometric analyses of the results in C. Results are a representative experiment of three performed.

IRP-RNA-binding activity (Minotti et al., 1998). Lysates from Fe-loaded cells were used because previous studies by Minotti et al. (1998) have suggested that DOXol together with *cis*-acon interacts with the [4Fe-4S] cluster of IRP1 to decrease IRP1-RNA-binding activity. Indeed, the cellular Fe-loading procedure used results in IRP1 predominately with the [4Fe-4S] cluster (see *Materials and Methods* for details). To examine the effect of DOXol, lysates from Fe-loaded SK-Mel-28 cells were incubated with this metabolite (1.5, 5, and 20 μM) or *cis*-acon (20 and 40 μM) alone or in combination for 1 h at 37°C (Fig. 6) or 3 h at 4°C (data not shown). Concentrations examined were the same and higher than that used in myocardial tissue lysates (Minotti et al., 1998). Interestingly, no significant change in IRP-RNA-binding activity was detected after 1 h at 37°C (Fig. 6) or 3 h at 4°C (data not shown). NEM (1 mM) was used as a positive control (Philpott et al., 1993; Hirling et al., 1994; Wardrop et al., 2000) and caused a marked decrease in active and total IRP-RNA-binding activity (Fig. 6). Similar results were obtained using cardiomyocyte cultures (data not shown).

Minotti et al. (1995, 1998) have previously suggested that DOXol, in the presence of *cis*-acon, may affect IRP1 by partially dissociating the [4Fe-4S] cluster. This partial dissociation has been suggested to inhibit c-acon activity but was insufficient to activate IRP1-RNA-binding activity (Minotti et al., 1995, 1998). The ability of DOXol to affect the [4Fe-4S] cluster of IRP1 was assessed by examining the influence of this compound on c-acon activity in lysates derived from control and Fe-loaded SK-Mel-28 cells. In lysates derived from Fe-loaded cells after a 1-h incubation at 37°C with either control medium, DOX (20 μM), or DOXol (20 μM) and *cis*-acon (40 μM), c-acon activity was equal to 11.3 $\pm$ 0.5, 11.0 $\pm$ 0.2, and 11.1 $\pm$ 0.9 U/mg of protein, respectively. Similar results were observed using lysates derived from control SK-Mel-28 cells. As a positive control in each experiment, lysates derived from cells pretreated with DFO (100 μM) for 24 h were used which gave c-acon activity of 3.4 $\pm$ 0.3 U/mg of protein.

The Effect of Anthracyclines and Their Metal Ion Complexes on IRP-RNA-Binding Activity and Cytoplasmic Aconitase Activity. Because anthracyclines are capable of readily forming Fe and Cu complexes (Garnier-

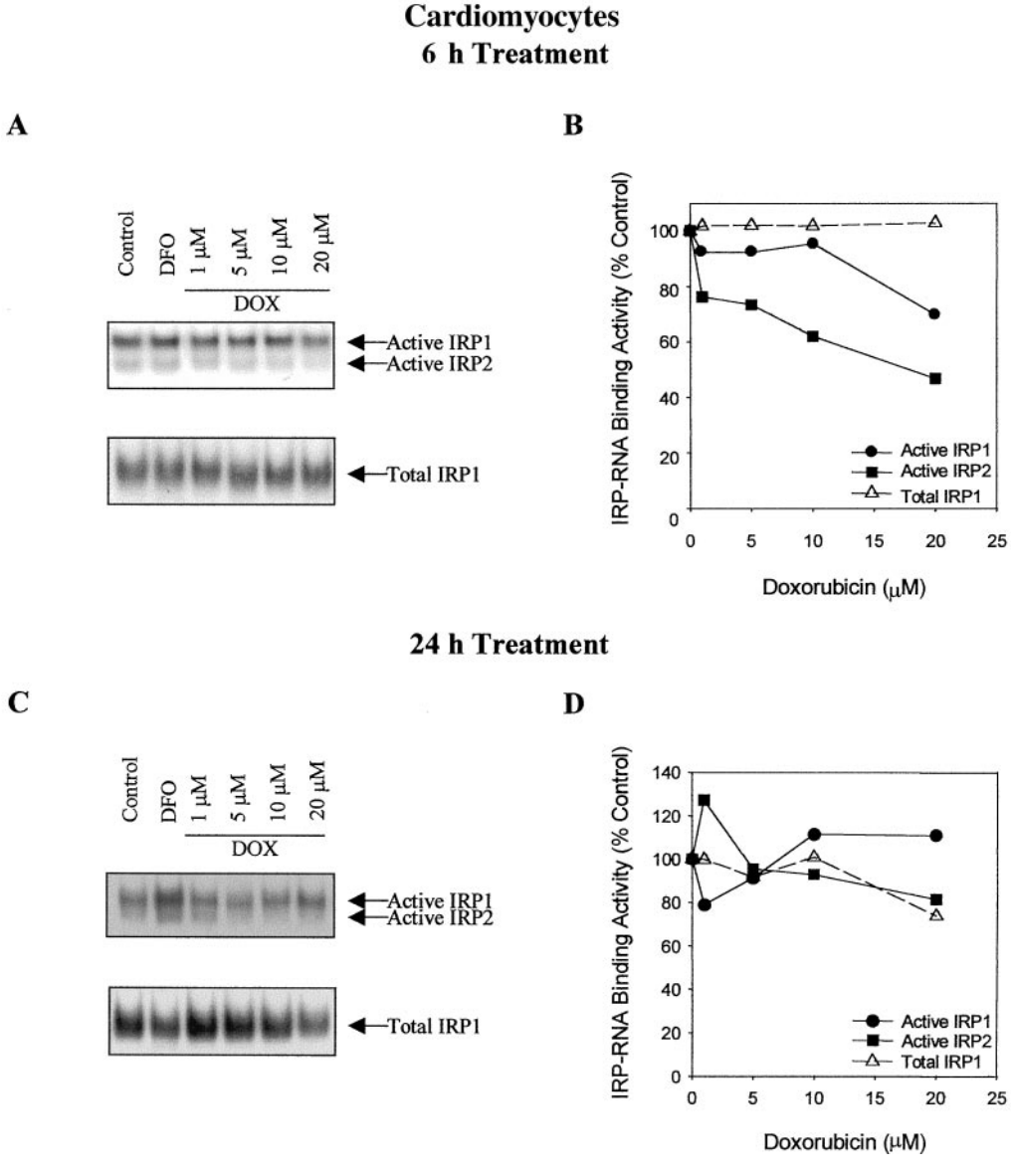


Fig. 4. Effect of DOX on cellular IRP-RNA-binding activity after a 6- or 24-h incubation with cardiomyocytes. Primary cultures of cardiomyocytes were treated with or without 1 to 20 μM DOX or DFO (100 μM) for 6 h (A) or 24 h (C) at 37°C, and the IRP-RNA-binding activity was assessed by the gel retardation assay. B and D are densitometric analyses of A and C, respectively. Results are a representative experiment of four performed.

Suillerot, 1988; Gianni and Myers, 1992), and because both metal ions are found at relatively high levels in cells, it was important to determine the effects of anthracycline-Fe or -Cu complexes directly on IRP-RNA-binding activity (Fig. 7). Indeed, DOX-metal ion complexes can catalyze a range of redox reactions (Gianni and Myers, 1992), and their effects were important to assess. In all studies, the complexation of anthracyclines with metal ions was ensured by examination using UV-visible spectrophotometry (Garnier-Suillerot, 1988; Gianni and Myers, 1992).

Cell lysates from control SK-Mel-28 cells were incubated with or without DOX (20 μM), the DOX-Fe (3:1) complex, or the DOX-Cu (2:1) complex (all complexes at 20 μM DOX equivalent) for 3 h at 4°C (Fig. 7A) or 1 h at 37°C (data not shown), and the effect on IRP-RNA-binding activity was examined. The effects of these agents on apo-IRP1 or holo-IRP1 were also examined by treating lysates derived from cells either depleted of or loaded with Fe using incubations of 24 h with DFO (100 μM) or FAC (100 μg/ml), respectively (see *Materials and Methods*).

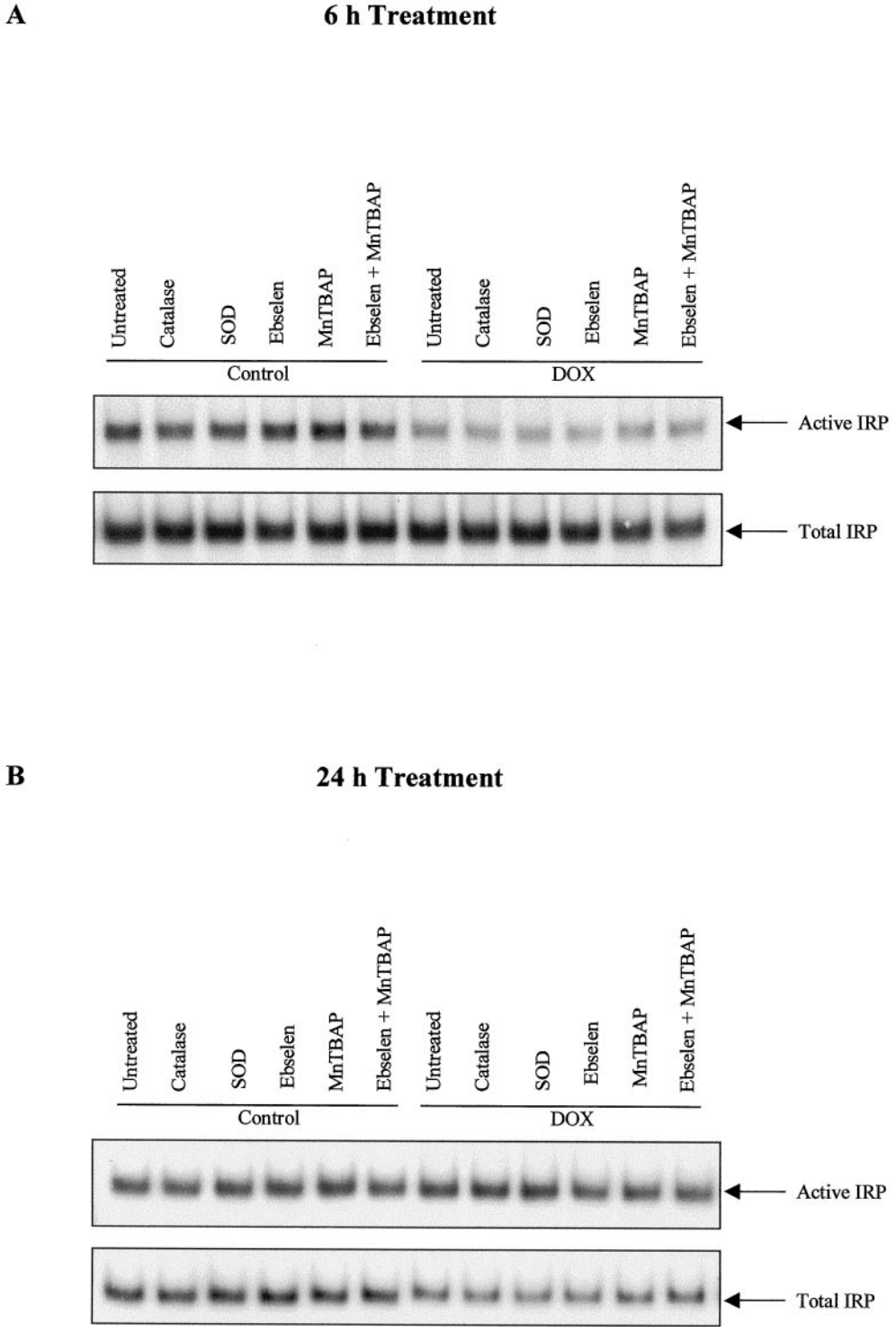


Fig. 5. Free radical scavengers do not protect cells against the DOX-induced changes in IRP-RNA-binding activity. SK-Mel-28 cells were treated simultaneously with control media or 20 μM DOX and the free radical scavengers, catalase (1000 U/ml), SOD (1000 U/ml), ebselen (15 μM), MnTBAP (200 μM), or the combination of ebselen and MnTBAP, for 6 h (A) or 24 h (B) at 37°C; and IRP-RNA-binding activity was assessed. These results are a representative experiment of three performed.

Interestingly, DOX alone had no significant effect on either active or total IRP-RNA-binding activity compared with untreated lysates from SK-Mel-28 cells (Fig. 7A). In contrast, compared with the relevant control, the DOX-Fe complex caused a significant ($p < 0.01$) decrease in active IRP-RNA-binding activity in lysates derived from control cells, or cells preincubated with DFO or FAC (Fig. 7A). These data suggested that the effect of the DOX-Fe complex on IRP1 was independent of the presence or absence of the [4Fe-4S] cluster. The effect of the DOX-Fe complex was at least partially reversible upon treatment with β-ME and FeCN (Fig. 7A). These experiments demonstrated that DOX alone had no effect on IRP-RNA binding but, rather, the formation of the DOX-Fe complex within cells may inhibit this activity. Similar results were obtained when cell lysates were incubated with the agents of interest for 1 h at 37°C (data not shown). In contrast to the DOX-Fe complex, the DOX-Cu complex significantly ($p < 0.01$) decreased IRP-RNA-binding activity only in lysates derived from cells pretreated with DFO (Fig. 7A). These results suggested that the DOX-Cu complex more readily affects apo-IRP1. The same concentration of Fe (as $FeCl_3$) or Cu (as $CuSO_4$) added to the lysates had no effect on IRP-RNA-binding activity (data not shown).

The effect of the Fe and Cu complexes on IRP-RNA-binding activity in myocardial cultures was also examined (data not shown). Similar to melanoma cells, DOX alone had no effect on IRP-RNA-binding activity in myocardial cell lysates. However, both the DOX-Fe and DOX-Cu complexes decreased active IRP1- and IRP2-RNA-binding activity in cardiomyocyte lysates.

The ability of the DOX-Fe or DOX-Cu complexes to dissociate the [4Fe-4S] cluster of IRP1 was assessed by examining c-acon activity in lysates derived from control and Fe-loaded melanoma cells. In all experiments, DOX-Fe or DOX-Cu complexes had no effect on c-acon activity. After a 1-h incubation at 37°C with either control medium, the DOX-Fe complex (20 μM), or the DOX-Cu complex (20 μM), c-acon activity in lysates from Fe-loaded cells was equal to 11.3 ± 0.5, 10.9 ± 0.7, and 11.1 ± 0.2 U/mg of protein, respectively. These experiments examining the effect of the DOX-Fe complexes on c-acon activity were in agreement with the RNA-binding studies above (Fig. 7A), which suggested that its effect was independent of the presence or absence of an [4Fe-4S] cluster.

It is well known that the DOX-Fe complex is unstable and can engage in spontaneous redox cycling, resulting in the reduction of Fe(III) to Fe(II) and the oxidation of DOX to an oxygen-centered radical (Gianni and Myers, 1992). This reduction of Fe(III) requires the presence of the hydroxyl group on the alkyl side chain of DOX (Gianni et al., 1988). However, DAU lacks this hydroxyl group (Fig. 1), and the Fe complex of this molecule does not engage in spontaneous redox cycling (Gianni et al., 1988; Gianni and Myers, 1992). Therefore, to determine whether the ability of the DOX-Fe complex to decrease active IRP-RNA-binding activity was due to this latter process, the DAU-Fe complex was examined. However, like DOX, the Fe complex of DAU also significantly ($p < 0.01$) decreased active IRP-RNA-binding (Fig. 7, B and C), suggesting that this effect was not solely due to spontaneous redox cycling of the anthracycline-Fe complex. The EPI-Fe complex also exerted a similar effect (Fig. 7, B and C).

Doxorubicin Prevents the Chelator-Mediated Increase in IRP-RNA-Binding Activity. Cellular Fe sequestration by DFO and ICRF-187 results in activation of IRP-RNA-binding activity (Weiss et al., 1997). Having demonstrated that anthracyclines decrease total IRP-RNA-binding activity in SK-Mel-28 cells after 24 h (Figs. 2; 3, C and D; and 5B), we examined the ability of melanoma cells to respond to changes in Fe status at the same time as DOX treatment (Fig. 8). The SK-Mel-28 cells were simultaneously incubated with DOX (5 or 10 μM) and either DFO (6.25–25 μM) or ICRF-187 (300 or 1000 μM) for 24 h, and the IRP-RNA-binding activity assessed (Fig. 8).

As shown in Fig. 8, ICRF-187 at 1000 μM and all concentrations of DFO caused a significant ($p < 0.01$) increase in active IRP-RNA-binding activity compared with control cells, whereas 300 μM ICRF-187 only caused a slight increase. However, simultaneous treatment with DOX significantly ($p < 0.001$) diminished the IRP-RNA-binding response to both DFO and ICRF-187 (Fig. 8). In addition, neither DFO nor ICRF-187 could protect cells against the DOX-mediated decrease in total IRP-RNA-binding activity compared with control cells (Fig. 8). This suggested that the ability of cells to respond to changes in Fe levels via IRP-RNA-binding activity was inhibited by DOX.

Discussion

The current investigation is the first to assess the effects of anthracyclines on IRP-RNA-binding activity in both tumor cells and differentiated cardiomyocytes. We show using these cells that anthracyclines interfere with Fe metabolism by affecting both the active mRNA-binding activity of IRP and the total cellular IRP pool (Fig. 2). Short-term incubation

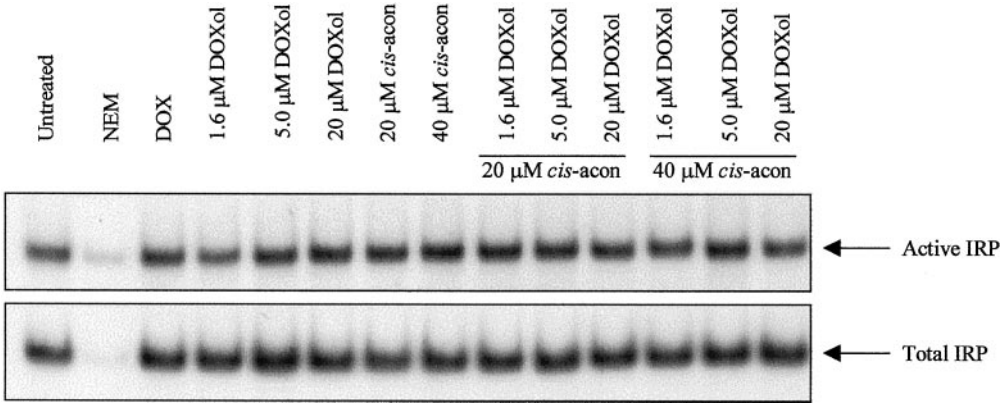


Fig. 6. Doxorubicinol (DOXol) and *cis*-aconitate have little effect on IRP-RNA-binding activity in Fe-loaded lysates from SK-Mel-28 melanoma cells. Lysates from Fe-loaded cells were prepared as described under *Materials and Methods* and then incubated for 1 h at 37°C with DOXol (1.6, 5, and 20 μM), *cis*-acon (20 or 40 μM), or combinations of both, and IRP-RNA-binding activity was assessed. These results are a representative experiment of three performed.

with DOX (3–6 h) resulted in a dramatic decrease in IRP-RNA-binding activity, with a less pronounced effect on total IRP pools (Fig. 2). This may be due to the intracellular formation of anthracycline-Fe and/or -Cu complexes. Interestingly, DOX had a similar effect on IRP-RNA-binding activity in cardiomyocytes and neoplastic cells. A notable effect of DOX in cardiomyocytes was the decrease in IRP2-RNA-binding activity, particularly after a 6-h incubation (Fig. 4).

Because DOX forms a number of metabolites that have been shown to be important in its biological effects (Takanashi and Bachur, 1976; Minotti et al., 1998), these were assessed to understand their roles in IRP-RNA-binding activity. Our experiments in cell lysates demonstrated that DOX alone or DOXol in the presence or absence of *cis*-acon had no influence on IRP-RNA-binding (Fig. 6) or c-acon activity. These results are important because they demonstrate that DOX and one of its critical metabolites are not directly affecting IRP-RNA-binding activity and cellular Fe metabolism. In contrast, the Fe and Cu complexes of DOX reversibly reduced active IRP-RNA-binding activity in cell lysates (Fig. 7), suggesting that these metal complexes may be responsible for the short-term cellular effects observed after a 6-h incubation (Fig. 2). The effect of the DOX-Fe complex on IRP-RNA-binding activity rather than DOX itself is interesting (Fig. 7). This is because previous studies have shown that loading cells with Fe potentiates the cytotoxic effects of DOX (Hershko et al., 1993; Link et al., 1996).

It is important to consider the mechanism of how the

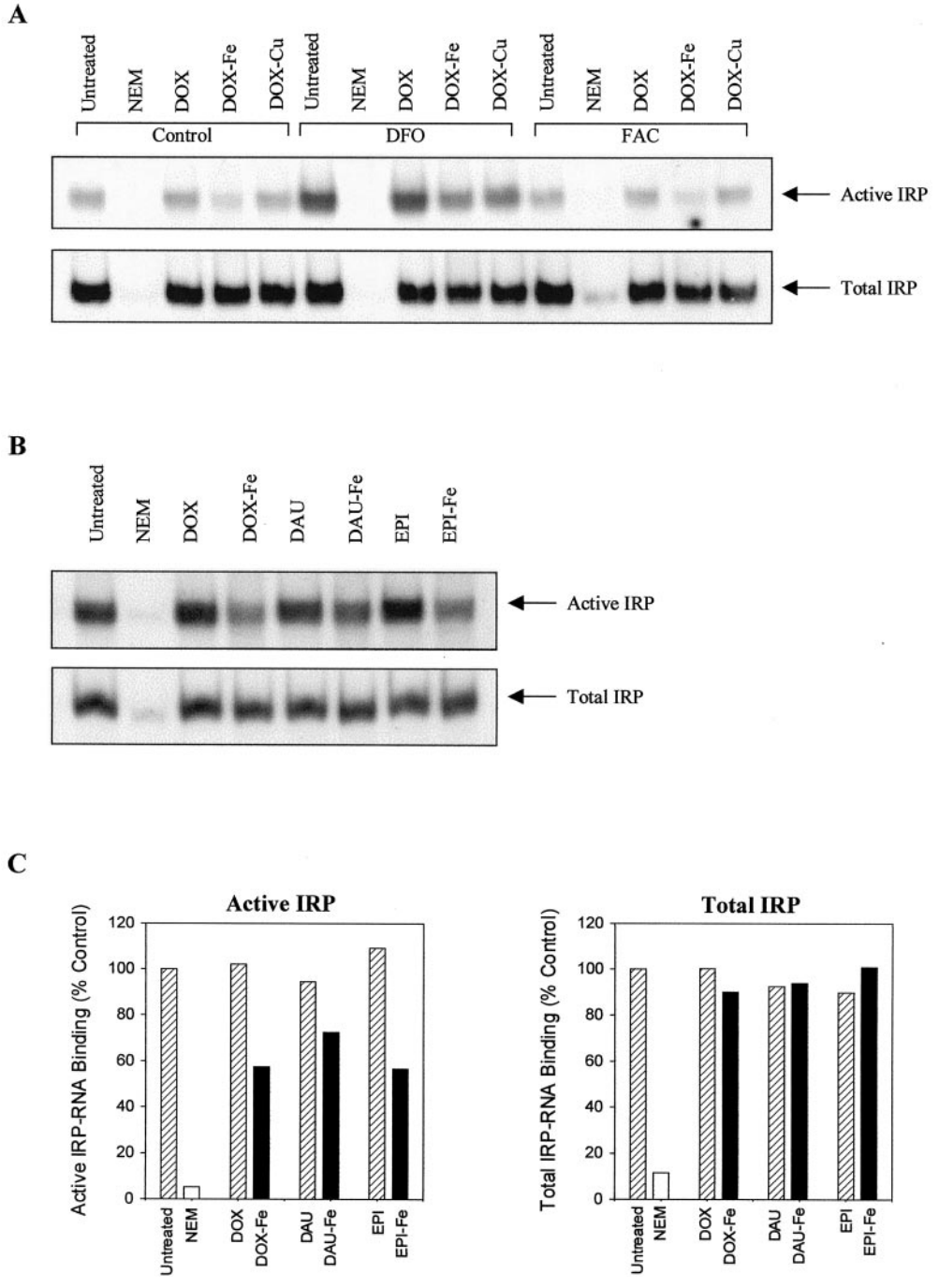


Fig. 7. The Fe and Cu complexes of anthracyclines cause a reversible decrease in active IRP-RNA-binding activity. A, SK-Mel-28 cells were treated with control media, DFO (100 μM), or FAC (100 μg/ml) for 24 h at 37°C, and cell lysates were prepared (see *Materials and Methods*). Lysates were then treated with NEM (1 mM), anthracycline (20 μM), anthracycline-Fe (3:1) complex, or anthracycline-Cu (2:1) complex (metal complexes at 20 μM anthracycline equivalent) for 3 h at 4°C, and IRP-RNA-binding activity was assessed. B, lysates prepared from control SK-Mel-28 cells were treated with NEM (1 mM), the anthracyclines, DOX (20 μM), DAU (20 μM), and EPI (20 μM), and the respective anthracycline-Fe complexes (at 20 μM anthracycline equivalent as in A) for 3 h at 4°C; and IRP-RNA-binding activity was assessed. Results are a representative experiment of three performed. C, densitometric analysis of B.

DOX-Fe complex reduces IRP-RNA-binding activity (Fig. 7). It is clear from our IRP-RNA-binding studies (Fig. 7A) and the lack of effect of the DOX-Fe complex on c-acon activity, that the effect of this compound was independent of the [4Fe-4S] cluster. The DOX-Fe complex can catalyze a range of redox reactions (Gianni and Myers, 1992). For instance, it reacts with a range of reductants including glutathione to yield oxidized thiols (Muindi et al., 1984; Gianni and Myers, 1992). Therefore, the DOX-Fe complex could oxidize critical sulfhydryl groups involved in the IRP-mRNA-binding mechanism (Hentze and Kühn, 1996). In fact, DOX may reversibly inhibit IRP-RNA-binding activity via a similar mechanism to the sulfhydryl-oxidizing agent, diamide (Philpott et al., 1993; Hirling et al., 1994). It has been shown that diamide catalyzes disulfide bridging between crucial thiol groups in IRP1, namely, the cysteine-437 residue with either cysteine-503 or -506, inhibiting mRNA-binding activity (Hirling et al., 1994). Similar to the DOX-Fe complex (Fig. 7), inhibition by diamide could be recovered by treatment with β-ME that reduces disulfide bonds (Philpott et al., 1993; Hirling et al., 1994). Considering that DOX can bind cellular Fe pools to form the DOX-Fe complex (Gianni and Myers, 1992), this molecule may then oxidize crucial IRP thiol groups and thereby inhibit mRNA binding. Hence, this reaction could explain the decrease in active IRP-RNA-binding activity observed within the first 6 h of incubation with DOX (Fig. 2).

Apart from the ability of the DOX-Fe complex to decrease IRP-RNA-binding activity, similar activity was also found for the DOX-Cu complex (Fig. 7). The mechanism of this effect could be similar to that of the DOX-Fe complex discussed above. Alternatively, it has been suggested that non-Fe metals can be incorporated into the Fe-S cluster to decrease IRP1-RNA-binding and increase c-acon activity (Oshiro et al., 2002). Hence, it is possible to speculate that the DOX-Cu complex and DOX-Fe complex may be able to directly donate their metal ions to IRP1 for cluster assembly and thus de-

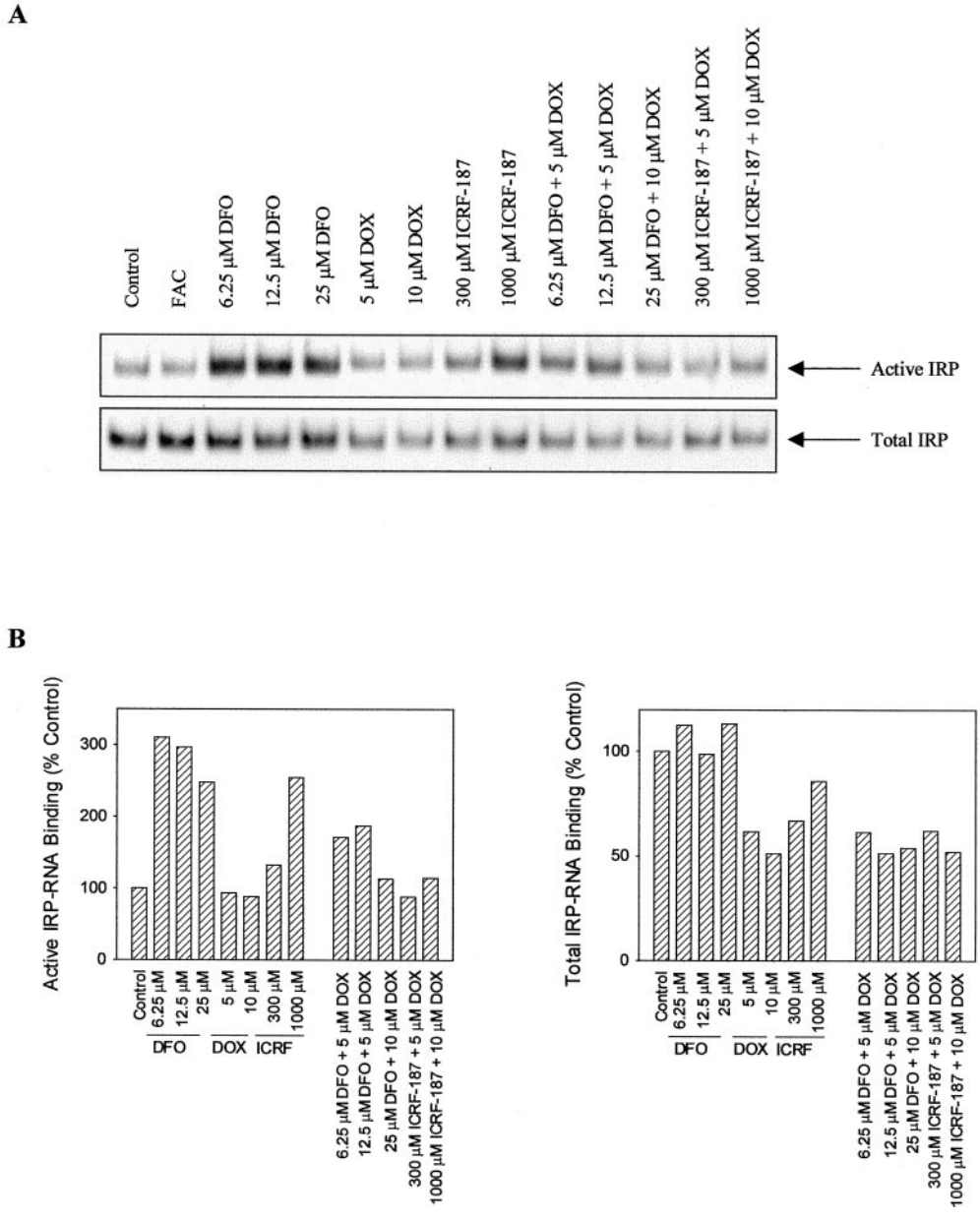


Fig. 8. Cells can no longer respond to cellular Fe deficiency by increasing active IRP-RNA-binding activity after treatment with DOX. A, SK-Mel-28 cells were treated with control media, DFO (6.25–25 μM), DOX (5 and 10 μM), ICRF-187 (300 and 1000 μM), and DFO or ICRF-187 plus DOX for 24 h at 37°C; and IRP-RNA-binding activity was assessed. **(B)** Densitometric analyses of A. Results are a representative experiment of three performed.

crease RNA-binding affinity. However, we found no change in c-acon activity after incubation with DOX-Fe or DOX-Cu, which argues against this hypothesis.

Another reaction of the DOX-Fe complex is the generation of free radicals that could affect IRP-RNA-binding activity (Gianni and Myers, 1992). However, a range of radical scavengers could not protect against the DOX-mediated decrease in active IRP-RNA-binding activity (Fig. 5A). Furthermore, the SOD mimetic, MnTBAP, provided protection against toxicity in melanoma cells without preventing the DOX-mediated decrease in the total IRP pool after 24 h (Fig. 5B). These results again demonstrate that free radical production by DOX or its Fe complex was not the mechanism responsible for the changes in IRP mRNA-binding activity. These observations in neoplastic cells are again different from that observed in a heart cell line, where the effect of DOXol and free radicals irreversibly inactivated both IRP1 and 2, but only at a DOX concentration of 10 μM (Minotti et al., 2001).

The effect of the anthracyclines on active and total IRP-RNA-binding activity as a function of time was complex (Fig. 2). We have suggested above that the initial decrease observed within 6 h (Fig. 2) may be due to the effect of the Fe or Cu complex of DOX (Fig. 7). However, after this initial response, active IRP-RNA-binding activity then increased back to control levels. The mechanism by which this restoration occurs is not clear, although it may be due to a compensatory response that involves conversion of holo-IRP1 to its active RNA-binding form. The reason for the decrease in the total IRP pool after longer incubations (18–24 h) with anthracyclines (Fig. 2) is also not understood at present, although we showed that it was not a free radical-mediated effect (Fig. 5).

Simultaneous treatment of cells with DOX and the Fe chelators, DFO and ICRF-187, did not prevent the decrease in total IRP-RNA-binding activity induced by DOX after 24 h (Fig. 8). In addition, the presence of DOX inhibited the DFO- and ICRF-187-mediated increase in IRP-RNA-binding activity (Fig. 8). This result indicates that in the presence of DOX, cells can no longer respond to changes in Fe status via the IRP-regulatory system.

Our current data are clearly different from those reported by others using heart homogenates (Minotti et al., 1995, 1998) and a heart cell line (Minotti et al., 2001). These studies suggested a role for DOXol in removing the [4Fe-4S] cluster from IRP1, thereby affecting cellular Fe metabolism (Minotti et al., 1995, 1998; 2001). These observations are not in agreement with our results in which the formation of Fe or Cu complexes with DOX, rather than DOXol itself, affects IRP1-RNA-binding activity. Furthermore, the effect of DOX in our primary cultures of cardiomyocytes (Fig. 4) was somewhat similar to the changes observed in tumor cells (Figs. 2 and 3). The different response of cardiomyocytes to DOX shown in this investigation compared with studies using a rat heart-derived cell line (Minotti et al., 2001) could be due to the dedifferentiated state of this latter cell type (Hescheler et al., 1991). Also, it is difficult to compare our studies using intact cells or cell lysates to previous work using heart tissue homogenates in which IRP1 was loaded with Fe by chemical means (Minotti et al., 1995, 1998).

The effects of DOX on IRP-RNA-binding activity observed in this study using neoplastic cells and myocardial cells may facilitate the design of anthracyclines that exhibit high antitumor activity. For example, in tumor cells, the ability of DOX to inhibit the response of the IRPs to cellular Fe chelation may be important (Fig. 8). Indeed, Fe chelators have been assessed for their potential to treat cancer (Richardson et al., 1995; Richardson and Milnes, 1997), and DFO can markedly enhance the antitumor effect of DOX (Blatt and Huntley, 1989). Therefore, the combination of anthracyclines with Fe chelators that possess potent antiproliferative activity in neoplastic cells (Richardson et al., 1995; Richardson and Milnes, 1997) could be beneficial. Using this regimen, the ability of anthracyclines to effectively prevent the response to Fe chelation via the IRP system would inhibit the subsequent compensatory increase in TfR levels and Fe uptake. Hence, this would potentiate the antitumor activity observed and may partly explain the greater activity of combining chelators and DOX than either agent alone (Blatt and Huntley, 1989). Furthermore, by screening a variety of anthracycline analogs in combination with Fe chelators and assessing their effects on antiproliferative activity and indices of Fe metabolism (e.g., IRP-RNA-binding activity, etc.), effective treatment regimens may be developed.

In summary, our experiments demonstrated for the first time that anthracyclines interfere with the Fe metabolism of tumor cells and myocardial cells by affecting both the active IRP-RNA-binding activity and the total cellular IRP pool. Furthermore, both the Fe and Cu complexes of anthracyclines could reversibly decrease active IRP-RNA-binding activity, and the intracellular formation of these complexes could be responsible for the initial response to these drugs. The fact that DOX inhibited the response of the IRP system to Fe chelation may be important in terms of designing novel antitumor therapies combining anthracyclines with potent Fe chelators. Our results add significant new information regarding the effects of anthracyclines on the regulation of cellular Fe metabolism by IRPs.

Acknowledgments

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