

## RESEARCH PAPER

# Role of hypoxia-inducible factors in the dexrazoxane-mediated protection of cardiomyocytes from doxorubicin-induced toxicity

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## BACKGROUND AND PURPOSE

Iron aggravates the cardiotoxicity of doxorubicin, a widely used anticancer anthracycline, and the iron chelator dexrazoxane is the only agent protecting against doxorubicin cardiotoxicity; however, the mechanisms underlying the role of iron in doxorubicin-mediated cardiotoxicity and the protective role of dexrazoxane remain to be established. As iron is required for the degradation of hypoxia-inducible factors (HIF), which control the expression of antiapoptotic and protective genes, we tested the hypothesis that dexrazoxane-dependent HIF activation may mediate the cardioprotective effect of dexrazoxane.

## EXPERIMENTAL APPROACH

Cell death, protein levels (by immunoblotting) and HIF-mediated transcription (using reporter constructs) were evaluated in the rat H9c2 cardiomyocyte cell line exposed to low doses of doxorubicin with or without dexrazoxane pretreatment. HIF levels were genetically manipulated by transfecting dominant-negative mutants or short hairpin RNA.

## KEY RESULTS

Treatment with dexrazoxane induced HIF-1 $\alpha$  and HIF-2 $\alpha$  protein levels and transactivation capacity in H9c2 cells. It also prevented the induction of cell death and apoptosis by exposure of H9c2 cells to clinically relevant concentrations of doxorubicin. Suppression of HIF activity strongly reduced the protective effect of dexrazoxane. Conversely, HIF-1 $\alpha$  overexpression protected against doxorubicin-mediated cell death and apoptosis also in cells not exposed to the chelator. Exposure to dexrazoxane increased the expression of the HIF-regulated, antiapoptotic proteins survivin, Mcl1 and haem oxygenase.

## CONCLUSIONS AND IMPLICATIONS

Our results showing HIF-dependent prevention of doxorubicin toxicity in dexrazoxane-treated H9c2 cardiomyocytes suggest that HIF activation may be a mechanism contributing to the protective effect of dexrazoxane against anthracycline cardiotoxicity.

## Abbreviations

DAPI, 4'-6-diamidino-2-phenylindole; DFO, desferrioxamine; DMOG, dimethylxalyl glycine; HIF, hypoxia-inducible factors; HRE, hypoxia responsive elements; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; ROS, reactive oxygen species

## Introduction

Doxorubicin is effective in the treatment of a variety of malignancies (Singal and Iliskovic, 1998; Minotti *et al.*, 2004a), but

its cardiotoxicity limits its clinical use in cancer patients. The precise molecular basis of anthracycline cardiotoxicity remains elusive, but a number of theories have been proposed (reviewed by Minotti *et al.*, 2004a; Chen *et al.*, 2007), one

of which is the formation of reactive oxygen species (ROS).

Doxorubicin undergoes a one-electron reduction catalysed by NAD(P)H reductases to yield a semiquinone free-radical intermediate that regenerates the parent quinone reacting with  $O_2$  and thus generates  $O_2^{\cdot-}$  and its dismutation product  $H_2O_2$  (Gewirtz, 1999; Minotti *et al.*, 2004a,b). These ROS can then be converted into the more potent hydroxyl radical  $OH^{\cdot}$ , which is capable of damaging DNA and proteins, and initiating lipid peroxidation.

It has been suggested that iron may catalyse ROS formation in doxorubicin-primed reactions, and the involvement of iron in doxorubicin-induced cardiotoxicity has been highlighted by the demonstration that primary and secondary iron overloads exacerbate the drug's cardiotoxic effects (Hershko *et al.*, 1993; Link *et al.*, 1996; Miranda *et al.*, 2003). In line with this, dexrazoxane, a pro-drug that is enzymatically hydrolysed inside cardiomyocytes to its active metal-chelating metabolite, can protect the myocardium from anthracycline-induced toxicity under both experimental and clinical conditions (Wouters *et al.*, 2005) with a long-term effect (Lipshultz *et al.*, 2010). The concentration of intracellular iron therefore seems to be a key factor in doxorubicin cardiotoxicity. However, the concept of the oxidative nature of the role of iron in doxorubicin cardiotoxicity is challenged by results showing that antioxidants do not always protect laboratory animals against cardiotoxicity, or mitigate or delay cardiotoxicity in patients (Minotti *et al.*, 1999; Ladas *et al.*, 2004; Simunek *et al.*, 2009). Therefore, the precise mechanism of cardiotoxicity remains to be established and how it may be prevented or mitigated by the use of iron chelators such as dexrazoxane.

Hypoxia-inducible factors (HIF) are transcription factors that are activated in response to reduced oxygen levels and regulate the expression of a wide variety of genes mediating adaptive responses to a lack of oxygen (Semenza, 2009). Active HIF is a heterodimer consisting of an inducible HIF-1 $\alpha$  or HIF-2 $\alpha$  subunit and a constitutively expressed HIF-1 $\beta$  subunit. The HIF- $\alpha$  subunits are extremely labile proteins in normoxia, and hypoxia increases their intracellular levels by markedly inhibiting their rapid degradation. Under normoxic conditions, they are modified by a family of prolyl-4-hydroxylase domain enzymes, which have an absolute requirement for oxygen, iron and 2-oxoglutarate (Kaelin and Ratcliffe, 2008; Harten *et al.*, 2010). Hydroxylated HIF- $\alpha$  are bound by the von Hippel-Lindau tumour suppressor protein and targeted for proteasomal destruction (Kaelin, 2008). In the absence of one of the three cofactors, hydroxylation is blocked, and the stabilized subunits are free to bind HIF-1 $\beta$  in order to form an active heterodimer, which binds to hypoxia response elements (HREs) located in the promoter regions of a variety of genes encoding proteins that play key roles in cell functions such as angiogenesis, erythropoiesis, glucose transport, glycolysis, iron transport and cell proliferation/survival (Semenza, 2009).

In line with the iron requirement of prolyl hydroxylases for HIF-1 degradation, decreased intracellular iron availability activates HIF (Peyssonnaud *et al.*, 2008; Mole, 2010). Accordingly, it has been found that exposure to an iron chelator such as desferrioxamine (DFO) (Wang and Semenza, 1993; Bianchi *et al.*, 1999), or growth under iron deficiency condi-

tions (Jones *et al.*, 2006; Knowles *et al.*, 2006), is associated with a high level of HIF-1 activity in cultured cells, and HIF-1 has been induced *in vivo* by iron depletion (Dongiovanni *et al.*, 2008) or starvation (Peyssonnaud *et al.*, 2007).

Given the generally protective role of HIF activation against a number of different insults (Schofield and Ratcliffe, 2004; Higgins *et al.*, 2008; Semenza, 2009), the aim of this study was to test the hypothesis that iron chelation by dexrazoxane leads to HIF activation, and that this may explain its protective effect against doxorubicin-induced toxicity in the H9c2 cardiomyocyte model.

## Methods

### Cell cultures

The H9c2 embryonic rat heart-derived cell line was obtained from The American Type Culture Collection (Manassas, VA, USA) (CRL 1446) and grown at 37°C in 5%  $CO_2$  in Dulbecco's modified minimal essential medium adjusted to contain 4 mM glutamine, 1.5 g·L<sup>-1</sup> sodium bicarbonate, 4.5 g·L<sup>-1</sup> glucose, 1 mM sodium pyruvate, 100 U·mL<sup>-1</sup> penicillin and 0.1 ng·mL<sup>-1</sup> streptomycin, supplemented with 10% heat-inactivated fetal calf serum. Subconfluent cells were treated for 24 h with 0.5  $\mu$ M doxorubicin (Pharmacia, Milan, Italy) in complete growth medium. When appropriate, various concentrations of dexrazoxane (Sigma, Milan, Italy) were added to the culture medium for 3 h, followed or not by doxorubicin treatment. Cells were also exposed to 100  $\mu$ M DFO (Sigma) or 1 mM dimethyloxalyl glycine (DMOG; Alexis Biochemicals, Lausen, Switzerland) for 24 h, 1 mM buthionine sulfoximine (BSO) for 3 h and 100  $\mu$ M  $H_2O_2$  for 3 h (all from Sigma). At the end of the various treatments, the medium was removed, and the cells were washed with phosphate-buffered saline (PBS), collected and lysed as described below.

### Transient transfection

Subconfluent H9c2 cells maintained in complete medium were transfected with the following plasmid constructs: the pGL3PGK6TKp vector (a kind gift of PJ Ratcliffe, Oxford, UK), which contains an HRE multimer (Tacchini *et al.*, 2003); the expression vector pcDNA3ARNTdelta\_b ( $\Delta$ ARNT) (obtained from M Schwarz, Tübingen, Germany), which codes for the dominant-negative mutant form of the HIF-1 $\beta$  ARNT subunit (Tacchini *et al.*, 2004); and the expression vector pCMV4HIF-1 $\alpha$ , which codes for HIF-1 $\alpha$  (kindly provided by Dr Wenger, Leipzig, Germany) using TransIT<sup>TM</sup> LT1 (Mirus, Bologna, Italy). Six hours after transfection, the culture medium was replaced by fresh medium, and the cells were exposed to the various treatments.

### Short hairpin RNA knockdown

Short hairpin RNA (shRNA) constructs against *Mus musculus* HIF-1 $\alpha$  (catalogue number TR517255) were purchased from Origene Technologies, Inc. (Rockville, MD, USA). The targeted sequences were CTGTTCACCAAAGTTGAATCAGAG GATA(#1), CTCTGTTATGAGGCTCACCATCAGTTA(#2), TCAAGAAACGACCACTGCTAAGGCATCA(#3) and TTACCTTCATCGGAAACTCCAAAGCCACT (#4) (Tacchini *et al.*, 2008; Gammella *et al.*, 2010).

H9c2 cells maintained in complete medium were plated onto T25 flasks ( $1 \times 10^6$  cells per flask). After 24 h, the medium was changed, and the cells were transfected with a mixture of the four plasmids (600 ng each) containing the HIF-1 $\alpha$ -specific shRNA, or with plasmids containing a non-effective shGFP sequence cassette (Origene Technologies, Inc.) or the empty pRS vector, in the presence or absence of the pGL3PGK6TKp multimer using the transfection method described above. The medium was changed 48 h later, and the cells were treated with doxorubicin. The cytosolic extracts were then prepared, and the *Renilla* luciferase activities or 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay was performed as described below. To monitor transfection efficiency, the cells were transfected with a rhodamine-labelled siRNA (Qiagen SpA, Milano, Italy), fixed and observed using fluorescence microscopy (excitation 530 nm, emission 570 nm); nuclei were counterstained with 10  $\mu\text{g}\cdot\text{mL}^{-1}$  4'-6-diamidino-2-phenylindole (DAPI, Sigma), and fluorescence was observed (excitation 364 nm, emission 454 nm). More than 150 cells were counted, and the percentage of transfected cells was determined.

### Gene reporter assay

Subconfluent H9c2 cells maintained in complete medium were transfected in six-well multiwell plates using *TransIT* LT1 (Mirus) with a 50:1 mixture of the pGL3PGK6TKp construct and pRL-TK reporter vector containing *Renilla* luciferase, which was used to normalize transfection efficiency. When appropriate, the cells were cotransfected with the dominant-negative expression vector ( $\Delta$ ARNT) or the shRNA constructs. Six hours after transfection, the culture medium was replaced by fresh medium, and the cells were exposed to the various treatments. After 24 h, the cells were collected, washed and lysed using the reporter lysis buffer (Promega, Milan, Italy), and luciferase activities were measured in a Promega luminometer using the Dual-Luciferase Reporter Assay System (Promega) (Tacchini *et al.*, 2003). The empty vectors showed practically undetectable luciferase activity. All of the transfection experiments were carried out in duplicate.

### Immunoblotting

To detect aldolase A, survivin, Mcl1, haem oxygenase (HO-1), P-glycoprotein (Pgp), BclxL and  $\alpha$ -tubulin, the cells were homogenized in 10 mM HEPES, pH 7.6, 3 mM  $\text{MgCl}_2$ , 40 mM KCl, 5% glycerol, 0.2% Nonidet P40 (Sigma), 1 mM dithiothreitol (DTT) and a protease inhibitor cocktail (Sigma). The lysate was centrifuged at  $16\,000\times g$  for 5 min at  $4^\circ\text{C}$ , and the supernatant was saved for analysis. Nuclear extracts for the determination of HIF-1 $\alpha$ , HIF-2 $\alpha$  and transcription factor II D (TFIID) were prepared as previously described (De Ponti *et al.*, 2007). In order to analyse cytochrome c release, the cells were resuspended in 500 mM sucrose, 2 mM  $\text{NaH}_2\text{PO}_4$ , 16 mM  $\text{Na}_2\text{HPO}_4$ , pH 7.6, 150 mM NaCl, 1 mM DTT and a protease inhibitor cocktail, and 10  $\mu\text{g}$  digitonin per  $10^6$  cells were added with vortexing. The heavy organelles and cell debris were pelleted at  $14\,000\times g$  for 60 s at  $4^\circ\text{C}$ , and the supernatant was collected for analysis.

Aliquots of cytosolic or nuclear extracts containing equal amounts of proteins as assessed using the Bio-Rad protein assay kit (Bio-Rad, Segrate, Italy) were separated by electro-

phoresis in acrylamide-SDS gels and electroblotted onto Hybond ECL membranes (Amersham Co., Milan, Italy). After assessing transfer by means of Ponceau S staining, the membranes were incubated with antibodies against HIF-1 $\alpha$  (H1 $\alpha$ 67, 1:1000, Novus Biologicals, Littleton, CO, USA), HIF-2 $\alpha$  (1:500 Novus Biologicals); TFIID (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), survivin (1:500 Santa Cruz Biotechnology), Mcl1 (1:200, Santa Cruz Biotechnology), cytochrome c (1:5000, BD Biosciences, Buccinasco, Italy), Pgp (1:500, Sigma), BclxL (1:1000, Cell Signaling Technology, EuroClone, Pero, Italy) and  $\alpha$ -tubulin (1:8000, Sigma). After incubation with appropriate secondary antibodies and extensive washing, the antigens were detected by means of chemiluminescence using an ECL Plus immunodetection kit (Amersham Co.). The proteins were quantified densitometrically, making sure that the signals were in the linear range. All of the data were calculated by comparing the intensity of the bands using the same film exposure. The values were calculated after normalization to the amount of  $\alpha$ -tubulin or TFIID, which is an essentially nuclear protein.

### Caspase activity assay

Caspase activity was determined using the ApoAlert Caspase Colorimetric Assay kit (Clontech, EuroClone, Pero, Italy) in accordance with the manufacturer's protocol. In brief, at least  $2 \times 10^6$  cells per sample were lysed in 50  $\mu\text{L}$  lysis buffer, and the protein concentrations in the samples were estimated using the Bio-Rad protein assay. After incubation on ice for 10 min, the samples were centrifuged at  $16\,000\times g$  for 3 min at  $4^\circ\text{C}$ . Each supernatant was mixed with 50  $\mu\text{L}$  of a 2X Reaction Buffer/DTT mix and 5  $\mu\text{L}$  of 1 mM caspase-3 substrate (DEVD-pNA, 50  $\mu\text{M}$  final concentration), and the samples were then incubated for 1 h at  $37^\circ\text{C}$  in the dark. Developed colour was measured at 405 nm, and caspase activity was calculated in terms of absorbance units per  $\mu\text{g}$  protein.

### Annexin V assay

Externalization of phosphatidylserine to the outer leaflet of the plasma membrane of apoptotic cells was assessed with Annexin V-fluorescein isothiocyanate (FITC). After the various treatments, H9c2 cells grown on a coverslip were washed with PBS and incubated at room temperature for 5 min in the dark with Annexin V-FITC and propidium iodide and then observed under a fluorescence microscope according to the instructions of the kit (PromoCell, Heidelberg, Germany). The number of positive cells was determined on at least four randomly selected areas from three coverslips for each experimental group.

### MTT assay

H9c2 cells were seeded in quadruplicate in 24-well plates and then left untreated or treated with doxorubicin for 24 h in the presence or absence of shRNA or  $\Delta$ ARNT. At the end of the treatments, cell viability was measured as previously described (Corna *et al.*, 2004; Bernuzzi *et al.*, 2009) using thiazolyl blue (MTT, Sigma) as an indicator of mitochondrial function. Briefly, 50  $\mu\text{L}$  of MTT solution (5  $\text{mg}\cdot\text{mL}^{-1}$ ) was added to each well with 450  $\mu\text{L}$  of medium. After incubation at  $37^\circ\text{C}$  for 2 h, formazan crystals were dissolved by adding 500  $\mu\text{L}$  of the MTT solubilization solution and thorough

up-and-down pipetting. Absorbance was read at 570 nm, and the background absorbance at 690 nm was subtracted.

### Statistics

The data are expressed as mean values  $\pm$  SD and were statistically analysed using InStat-3 statistical software (GraphPad Software Inc, San Diego, CA, USA) and one-way ANOVA.

## Results

### *Dexrazoxane induces HIF binding activity and transactivation capacity in H9c2 cells*

As it has been shown that the depletion of cellular iron stores leads to the induction of HIF-1 (Peyssonnaud *et al.*, 2008; Mole, 2010), we investigated whether dexrazoxane activates HIF-1 in cardiomyocytes. Immunoblot analysis of nuclear extracts of H9c2 cells showed that exposure to dexrazoxane for 3 h increased HIF-1 $\alpha$  protein levels; activation was detectable at 10  $\mu$ M, and there was no additional increase at 100  $\mu$ M (Figure 1A). Similar activation was found in extracts of cells exposed to the iron chelator DFO, a well-known inducer of HIF-1 (Wang and Semenza, 1993). Under the same experimental conditions, HIF-2 $\alpha$  (which is also detectable in untreated cells) was also induced but to a lesser extent than HIF-1 $\alpha$  (Figure 1A).

We then used transactivation capacity experiments to verify whether the HIF subunits induced by dexrazoxane were transcriptionally active. In H9c2 cells transiently transfected with a luciferase reporter gene controlled by a DNA fragment containing multiple consensus HREs, which has previously been shown to drive HIF-1-dependent transcription in response to hypoxia and hypoxia-mimics (De Ponti *et al.*, 2007; Tacchini *et al.*, 2008), the expression of the reporter gene increased about threefold in response to dexrazoxane and about fivefold in response to DFO (Figure 1B). Further indications of the involvement of HIF in the dexrazoxane- and DFO-dependent activation of luciferase activity were obtained by means of experiments in which HIF transactivating capacity was almost completely abolished by the cotransfection of a plasmid expressing a dominant negative of the HIF-1 $\beta$  subunit ( $\Delta$ ARNT), which maintains the capacity of forming a heterodimer but cannot bind DNA (Tacchini *et al.*, 2004, 2008) (Figure 1B).

### *Pre-exposure to dexrazoxane prevents doxorubicin-mediated apoptotic cell death*

To investigate the cytoprotective activity of dexrazoxane, H9c2 cells were exposed to 0.5  $\mu$ M doxorubicin, a concentration within the range of the plasma levels found in patients undergoing chemotherapy (Gianni *et al.*, 1997). MTT assays showed that 24 h treatment with doxorubicin reduced cell viability by 50% (Figure 2A). In line with previous reports (Bernuzzi *et al.*, 2009), the cells exposed to 0.5  $\mu$ M doxorubicin showed no release of the cytosolic enzyme lactic dehydrogenase, which is commonly used as a measure of drug-induced damage and is indicative of necrotic cell death (results not shown).

We then assessed whether exposure to the dexrazoxane concentration that was sufficient to activate HIF prevented

cell death in H9c2 cardiomyocytes treated with 0.5  $\mu$ M doxorubicin. Figure 2A shows that the cells pretreated with 10  $\mu$ M dexrazoxane were significantly protected, as 77% were still alive after exposure to doxorubicin. In line with previous results indicating that apoptosis is the prevalent form of cell death in H9c2 cells exposed to low doxorubicin doses (Sawyer *et al.*, 1999; Reeve *et al.*, 2007; Bernuzzi *et al.*, 2009), the activity of caspase-3 (a major effector protein of apoptosis) was double that observed in untreated cells at 0.5  $\mu$ M doxorubicin and returned to control values in cells preincubated with dexrazoxane (Figure 2B). Dexrazoxane pretreatment also counteracted the increase in doxorubicin-induced apoptotic cell death assessed by measuring Annexin V binding to externalized phosphatidylserine (Figure 2C). As the mitochondrion-mediated apoptotic pathway is important in doxorubicin-induced apoptosis (Minotti *et al.*, 2004a), we also measured cytochrome c release and found that it was enhanced at 0.5  $\mu$ M doxorubicin and was prevented by dexrazoxane pretreatment (Figure 2D). Exposure to dexrazoxane alone did not significantly affect apoptosis (Figure 2B–D).

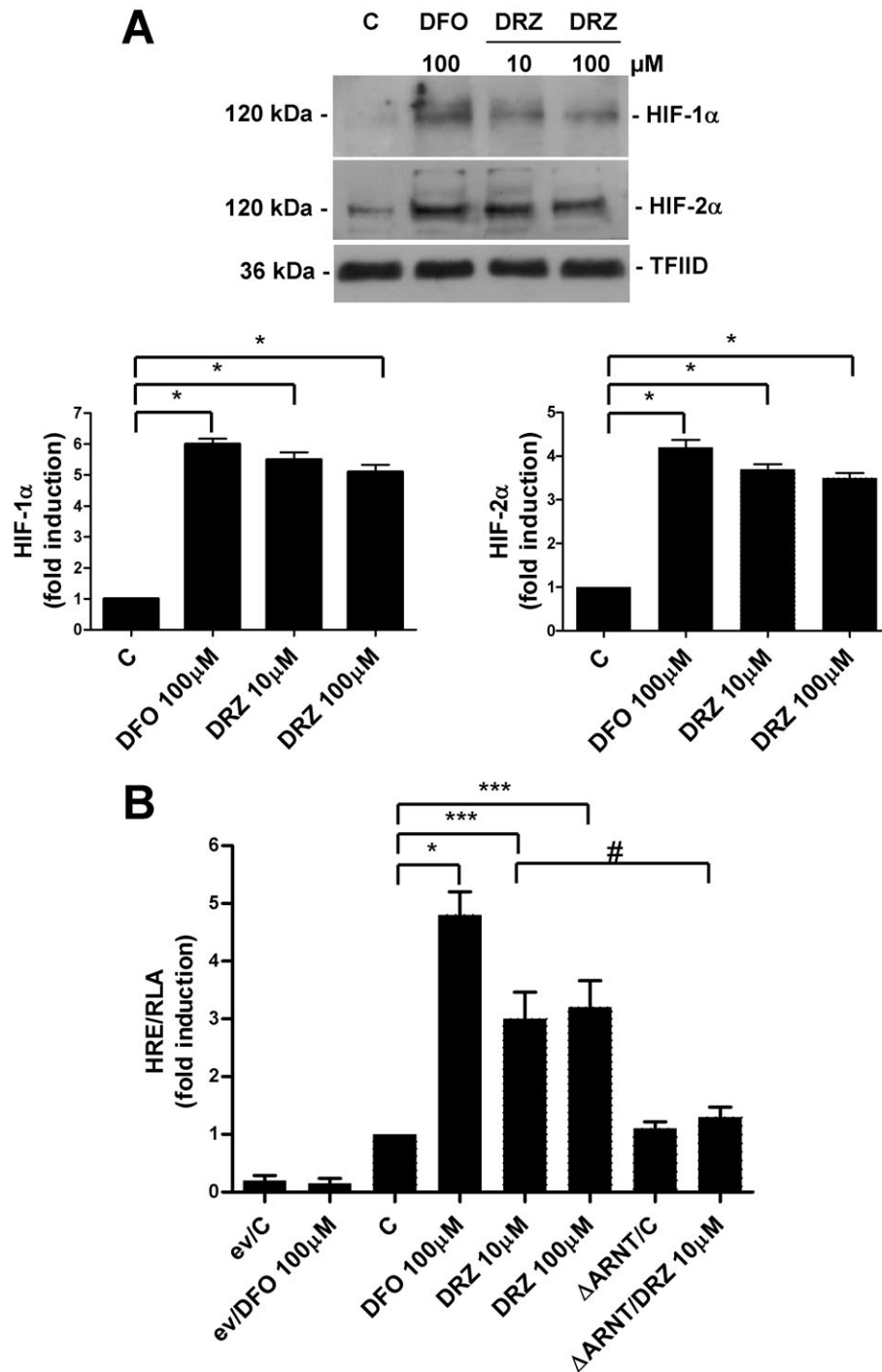
### *The protective effect of dexrazoxane depends on HIF-1 activity*

In order to investigate the role of HIF-1 in dexrazoxane-mediated cardioprotection directly, we investigated the capacity of dexrazoxane to prevent doxorubicin toxicity in H9c2 cells lacking HIF-1 activity. First of all, we performed a control experiment in order to check whether the induction of HIF-1 transcriptional activity found in cells exposed to dexrazoxane was maintained in cells exposed to dexrazoxane plus doxorubicin because doxorubicin, which affects the expression of muscle-specific genes (Ito *et al.*, 1990) and HIF-1-dependent transcriptional activity (Lee *et al.*, 2009), may blunt HIF-1 activation and thus impair the protective effect of iron chelation. However, Figure 3A shows that HIF-1 $\alpha$  protein levels were similar in the cells exposed to dexrazoxane and those exposed to dexrazoxane plus doxorubicin, as expected. Moreover, the luciferase activity driven by the multiple HRE sequences was slightly inhibited in the cells exposed to dexrazoxane plus doxorubicin in comparison with those treated with dexrazoxane alone but was still significantly higher than in the untreated cells (Figure 3B).

Having demonstrated that HIF-1 is active in H9c2 cells exposed to 0.5  $\mu$ M doxorubicin, we evaluated cell viability in H9c2 cells transfected with the dominant negative HIF-1 $\beta$  subunit  $\Delta$ ARNT and exposed to doxorubicin with or without dexrazoxane pretreatment. MTT assays revealed that the protective effect of dexrazoxane was lost in the transfected cells as mortality was not significantly higher than in the cells exposed to doxorubicin without dexrazoxane pretreatment, thus indicating that HIF plays a role in the dexrazoxane-mediated protection of H9c2 cells (Figure 4A). Similarly, a protective effect of dexrazoxane was found when caspase 3 activity was measured to see the effect on apoptosis (Figure 4B).

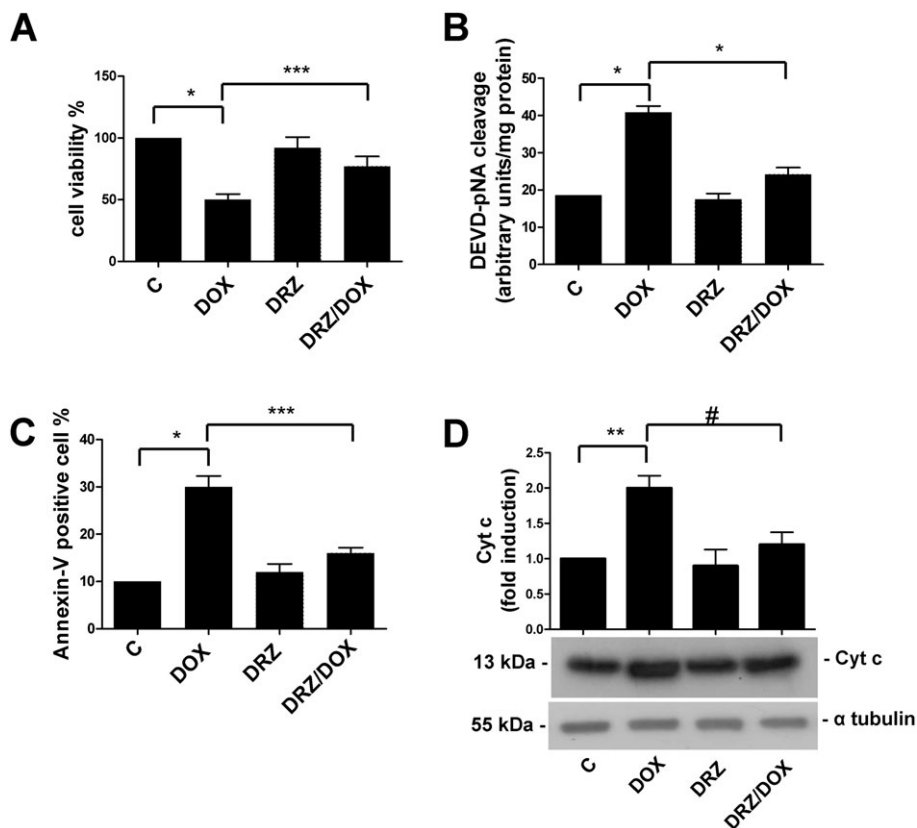
In order to verify further the role of HIF-1 in the doxorubicin protection of dexrazoxane-pretreated H9c2 cells, we used shRNA technology to specifically knockdown HIF-1 $\alpha$ . The efficient ( $84 \pm 5\%$ , see Figure 5A) transfection of H9c2 cells with a set of four expression vectors coding shRNAs against HIF-1 $\alpha$  led to a reduction in HIF-1 protein levels





**Figure 1**

Dexrazoxane (DRZ) induces HIF- $\alpha$  expression and transactivation capacity. (A) Immunoblot analysis of the nuclear extracts of untreated H9c2 cells (C) and cells treated with desferrioxamine (DFO) for 24 h, or different concentrations of DRZ for 3 h, using anti-HIF-1 $\alpha$  and anti-HIF-2 $\alpha$  antibodies. The blots were reprobed using the antibody against TFIID as a loading control. The panel shows one representative blot and the densitometric quantification relative to C-values. (B) Relative luciferase activity (RLA) in untreated H9c2 cells (C) and cells exposed to DFO or dexrazoxane, as described above. The cells were transiently transfected with the empty pGL3 vector (ev) or a construct in which luciferase was controlled by an HRE multimer and cotransfected using a control vector containing the *Renilla* luciferase gene. When appropriate, the cells were also cotransfected with an expression vector coding for a dominant-negative mutant of the constitutive HIF-1 $\beta$  subunit ( $\Delta$ ARNT). Luciferase activity was determined after 24 h, corrected for transfection efficiency on the basis of *Renilla* luciferase activity and normalized to the activity recorded in untreated cells (arbitrarily set to 1). Mean values  $\pm$  SD. \* $P$  < 0.001; \*\*\* $P$  < 0.01; # $P$  < 0.05,  $n$  = 3. HIF, hypoxia-inducible factor; HRE, hypoxia response element; TFIID, transcription factor II D.



**Figure 2**

Dexrazoxane (DRZ) protects H9c2 cells from apoptotic cell death. (A) H9c2 cells were left untreated (C), or exposed for 24 h to doxorubicin (DOX), for 3 h to dexrazoxane alone or pretreated with DRZ for 3 h and then exposed to doxorubicin. Viability was evaluated by the MTT assay and DOX toxicity was calculated as the percentage of viable cells after drug exposure. (B) H9c2 cells were treated as described for panel A, and apoptosis was determined by measuring caspase-3 activity. (C) H9c2 cells were treated as described for panel A, and apoptosis was determined by measuring Annexin V-FITC as described in Methods. (D) H9c2 cells were treated as described for panel A, and apoptosis was determined by measuring cytochrome c (Cyt c) release.  $\alpha$ -Tubulin was used as a loading control. The figure shows one representative immunoblot and the densitometric quantification relative to C-values. Mean values  $\pm$  SD. \* $P < 0.001$ ; \*\* $P < 0.005$  \*\*\* $P < 0.01$ ; # $P < 0.05$ ,  $n = 5$  for experiments reported in panels A and B, and 3 for experiments reported in panel C and D. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

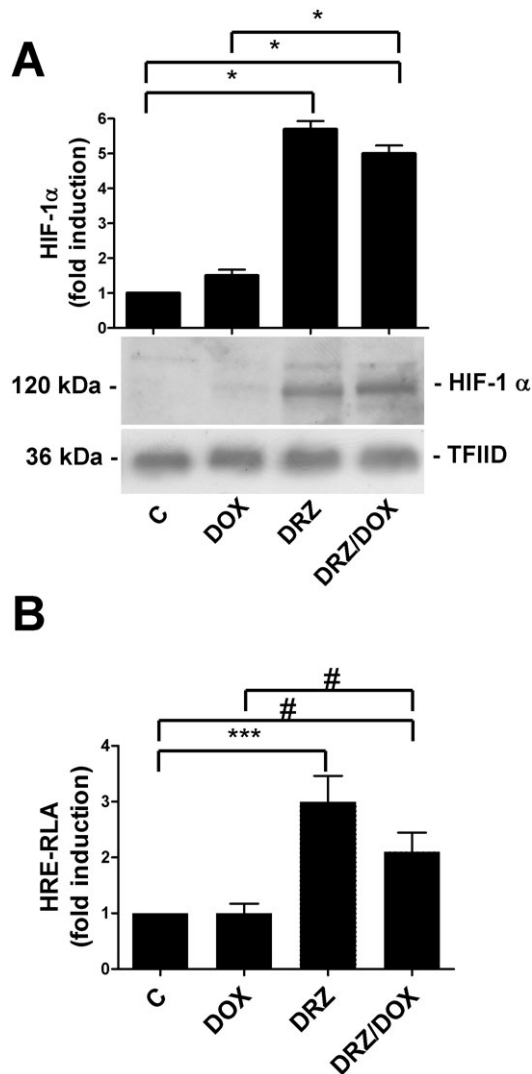
(Figure 5B) and transactivation activity, as demonstrated by the complete inhibition of the activation of the luciferase reporter gene under the control of the consensus HREs, unlike the cells transfected with an empty control plasmid (Figure 5B). In line with the results obtained with the  $\Delta$ ARNT dominant negative, the knockdown of HIF-1 abolished the protection offered by dexrazoxane, whereas the transfection of a vector containing a non-effective shGFP sequence cassette had no appreciable effects (Figure 5C).

In order to demonstrate further that HIF-1 is important for the protective effect of dexrazoxane, we investigated whether HIF-1 activation also provides cardioprotection from doxorubicin in cells not exposed to the iron chelator. Transfection with an expression vector coding for HIF-1 $\alpha$  resulted in greatly elevated HIF-1 protein levels and markedly stimulated HRE-dependent transcription (Figure 6A), and provided significant cytoprotection from doxorubicin-mediated cell death and apoptosis as revealed by MTT and caspase 3 assays (Figure 6B,C). These experiments demonstrated that HIF-1 $\alpha$  overexpression protects H9c2

cardiomyocytes from doxorubicin-induced toxicity in the absence of dexrazoxane.

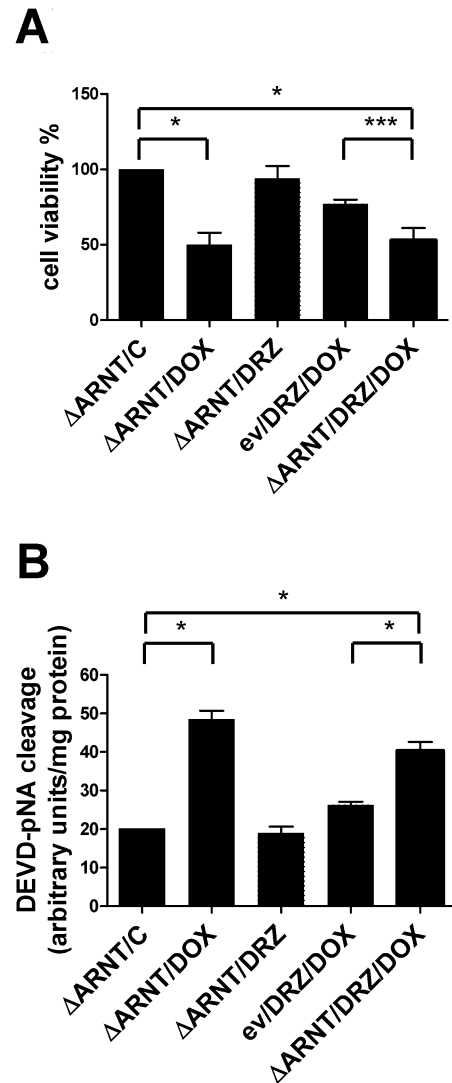
### *Effect of dexrazoxane on the expression of HIF target genes in H9c2 cells*

In order to investigate further the transcriptional function of activated HIF in dexrazoxane-treated cells, we examined the expression of endogenous genes known to be under the transcriptional control of HIF in cells exposed to dexrazoxane plus doxorubicin. The immunoblots in Figure 7A show that the levels of aldolase A, a typical HIF target gene (Semenza *et al.*, 1996), increased after dexrazoxane plus doxorubicin treatment as expected and returned to control levels in the cells transfected with  $\Delta$ ARNT. Having demonstrated that dexrazoxane prevents apoptosis (see Figure 2), we investigated whether HIF target genes that may play a role in favouring cell survival after doxorubicin-mediated damage were induced in dexrazoxane-treated H9c2 cells. Immunoblot analysis showed that exposure to dexrazoxane plus doxorubicin increased the levels of the antiapoptotic proteins Mcl1,

**Figure 3**

Doxorubicin (DOX) does not inhibit HIF expression and transactivation capacity. (A) Immunoblot analysis of nuclear extracts of untreated H9c2 cells (C) and cells exposed for 24 h to doxorubicin, for 3 h to dexrazoxane (DRZ) alone or pretreated with DRZ for 3 h and then exposed to DOX, using the anti-HIF-1 $\alpha$  antibody. The blots were reprobed using the antibody against TFIID as a loading control. The panel shows one representative blot and the densitometric quantification relative to C-values. (B) Relative luciferase activity (RLA) in H9c2 cells transiently transfected with a construct in which luciferase was controlled by an HRE multimer and treated as described for panel A. The cells were cotransfected using a control vector containing the *Renilla* luciferase gene. Luciferase activity was determined after 24 h, corrected for transfection efficiency on the basis of *Renilla* luciferase activity and normalized to the activity recorded in untreated cells (arbitrarily set to 1). Mean values  $\pm$  SD. \* $P$  < 0.001; \*\*\* $P$  < 0.01; # $P$  < 0.05,  $n$  = 3. HIF, hypoxia-inducible factor; TFIID, transcription factor II D.

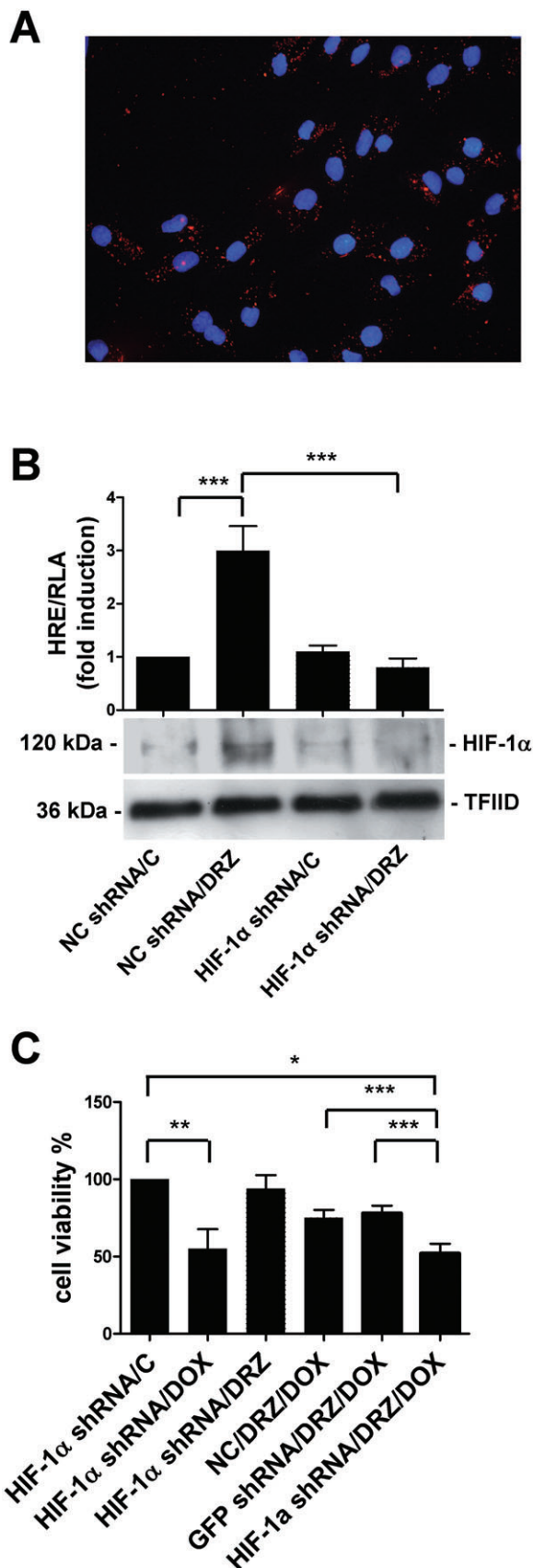
survivin and haem oxygenase (HO-1) (Craig, 2002; Bernuzzi *et al.*, 2009; Guha and Altieri, 2009), and that this increase was significantly prevented by  $\Delta$ ARNT expression (Figure 7B–D).

**Figure 4**

Suppression of HIF activity blocks dexrazoxane (DRZ)-mediated cardioprotection. (A) H9c2 cells were transfected with the expression vector  $\Delta$ ARNT or an empty vector (ev) and treated as indicated in Figure 3A. Viability was evaluated by means of the MTT assay. (B) H9c2 cells were transfected and treated as described for panel A, and apoptosis was evaluated by measuring caspase-3 activity. Mean values  $\pm$  SD. \* $P$  < 0.001; \*\*\* $P$  < 0.01;  $n$  = 5. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

We also examined the expression of manganese superoxide dismutase (MnSOD), an HIF-2 $\alpha$  target gene (Scortegagna *et al.*, 2003) that protects against doxorubicin-induced acute toxicity when overexpressed in mice (Yen *et al.*, 1996). However, MnSOD levels, which increased as expected in H9c2 cells undergoing oxidative stress obtained by exposure to H<sub>2</sub>O<sub>2</sub> or the glutathione-depleting compound BSO, were not significantly affected by dexrazoxane alone or combined with doxorubicin (Figure 7E).

As doxorubicin is a substrate of the Pgp, a multidrug resistance (MDR)-related membrane efflux pump that transports a variety of xenobiotics (Takara *et al.*, 2006) and is



**Figure 5**

Knockdown of HIF-1 $\alpha$  blocks dexrazoxane (DRZ)-mediated cardioprotection. (A) Transfection efficiency in H9c2 cells. The figure shows a merged image with DAPI-stained nuclei (blue) and punctuate fluorescence of rhodamine-labelled siRNA (red). (B) Relative luciferase activity (RLA) and HIF-1 $\alpha$  protein levels in untreated H9c2 cells (C) or cells exposed to dexrazoxane for 3 h. The cells were transiently transfected with a HRE multimer and also cotransfected with vectors containing the negative control shRNA (NC) or shRNA targeting HIF-1 $\alpha$  (HIF-1 $\alpha$  shRNA). Luciferase activity was determined after 48 h, corrected for transfection efficiency on the basis of *Renilla* luciferase activity and normalized to the activity recorded in untreated cells (arbitrarily set to 1). HIF-1 $\alpha$  protein levels were determined by immunoblot analysis of the nuclear extracts, as described in the legend to Figure 1. (C) H9c2 cells were transfected with vectors containing the negative control shRNA (NC), shRNA targeting GFP (GFP shRNA) or HIF-1 $\alpha$  (HIF-1 $\alpha$  shRNA) and treated as indicated in Figure 3A. Cell viability was evaluated by means of the MTT assay. Mean values  $\pm$  SD. \* $P < 0.001$ ; \*\* $P < 0.005$  \*\*\* $P < 0.01$ ;  $n = 3$ . siRNA, small interfering RNA; shRNA, short hairpin RNA; HIF, hypoxia-inducible factor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

regulated by HIF-1 (Comerford *et al.*, 2002), we assessed whether Pgp may play a role in the HIF-mediated cardioprotection offered by dexrazoxane. However, Pgp protein expression was not significantly modulated by exposure to dexrazoxane (Figure 7F). The lack of induction in cells treated with DFO or DMOG (which inhibits HIF degradation) further indicated that HIF is not involved in Pgp activation in H9c2 cardiomyocytes.

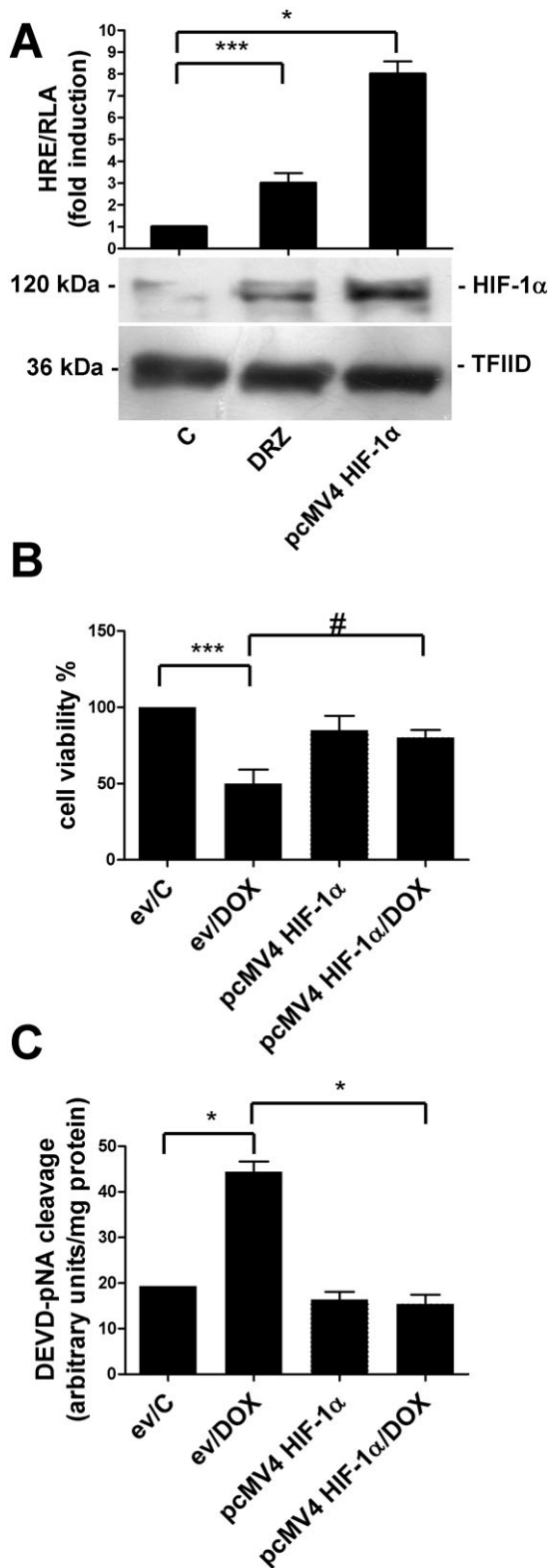
## Discussion

The clinical use of anthracyclines to treat many human tumours is limited by its life-threatening dose-related cardiotoxicity, which has been attributed to oxidative stress (Minotti *et al.*, 2004a; Chen *et al.*, 2007) and the fact that anthracyclines generate ROS and impair iron homeostasis is in line with a large body of evidence suggesting that iron also plays a role in this toxicity (Minotti *et al.*, 1999, 2004a). This view is supported by the efficient cardioprotection afforded by dexrazoxane, a clinically approved *bis*-ketopiperazine that diffuses into cells, hydrolyses to an EDTA-like diacid-diamide, and thus meets the structural requirements necessary to chelate iron before it catalyses the conversion of  $O_2^{\cdot-}$  and  $H_2O_2$  to more damaging oxidants (Hasinoff and Herman, 2007).

On the basis of these premises, iron chelators and antioxidants should both prevent the cardiotoxicity induced by anthracyclines, but this is not the case. Antioxidants offer protection in animal models but not in patients (Ladas *et al.*, 2004; Minotti *et al.*, 2004a; Simunek *et al.*, 2009) and so, despite the clinical usefulness of dexrazoxane, the mechanisms underlying its cardioprotective effects are not fully understood.

As decreased iron levels activate HIF (Peyssonnaud *et al.*, 2008; Mole, 2010), we hypothesized that HIF activation triggered by iron chelation may be an alternative mechanism



**Figure 6**

HIF-1 $\alpha$  overexpression is cardioprotective in the absence of dexrazoxane (DRZ). (A) Relative luciferase activity (RLA) and HIF-1 $\alpha$  protein levels in untreated H9c2 cells (C), exposed to dexrazoxane for 3 h or transfected with a construct that induced the overexpression of HIF-1 $\alpha$  (pcMV4 HIF-1 $\alpha$ ). The cells were transiently transfected with a construct in which luciferase was controlled by an HRE multimer. Luciferase activity was determined after 24 h, corrected for transfection efficiency on the basis of *Renilla* luciferase activity and normalized to the activity recorded in untreated cells (arbitrarily set to 1). HIF-1 $\alpha$  protein levels were determined by immunoblot analysis of the nuclear extracts, as described in the legend to Figure 1. (B) Untreated H9c2 cells (C) or cells exposed to doxorubicin (DOX) for 24 h were transiently transfected with the empty pGL3 vector (ev) or the pcMV4 HIF-1 $\alpha$  vector. Cell viability was evaluated by means of the MTT assay. (C) H9c2 cells were treated and transfected as described for panel B and apoptosis was determined as described in Figure 4B. Mean values  $\pm$  SD. \* $P$  < 0.001; \*\*\* $P$  < 0.01; # $P$  < 0.05,  $n$  = 3. HIF, hypoxia-inducible factor; HRE, hypoxia response element; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

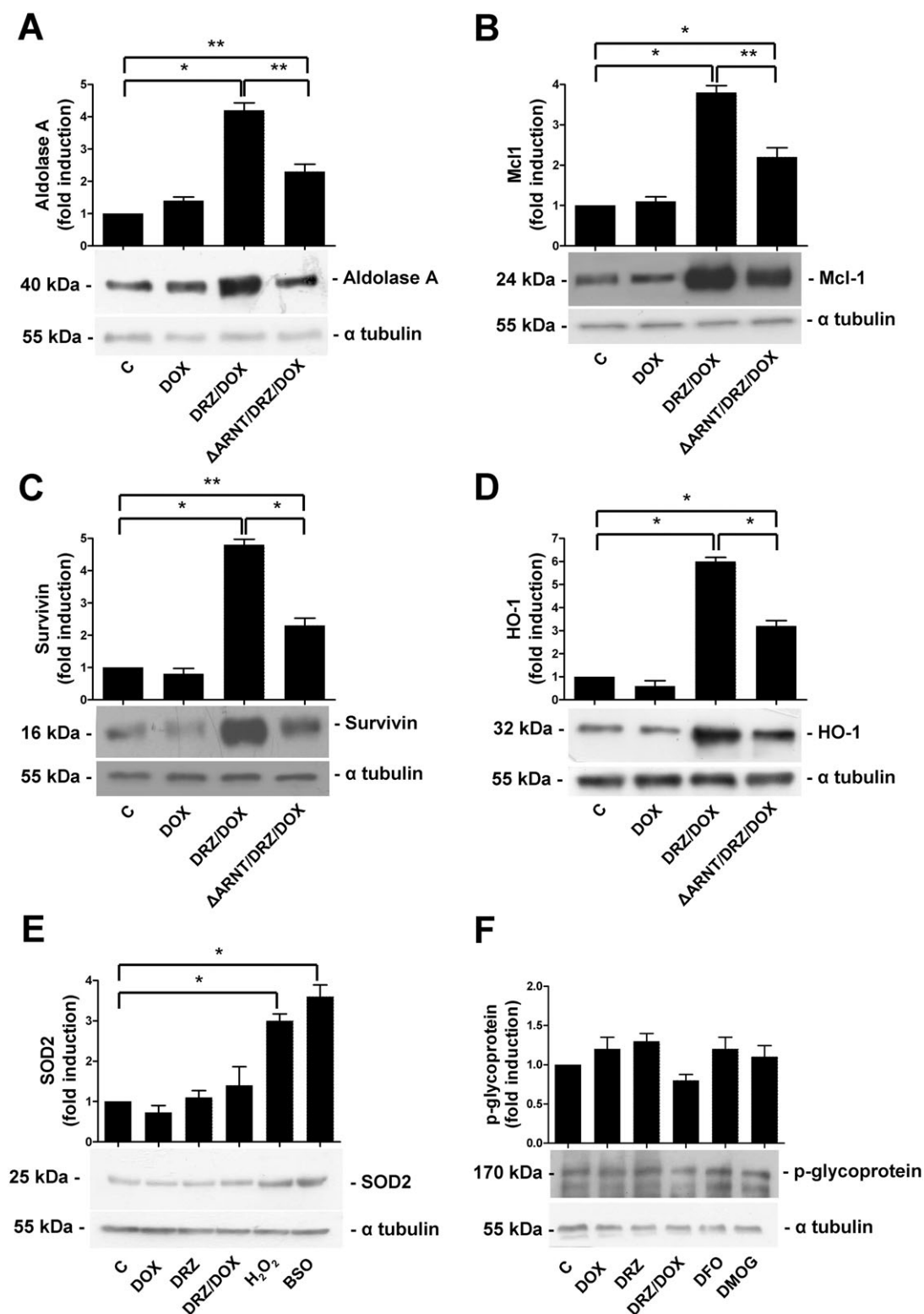
2009), is central to cardioprotection in models of ischaemic preconditioning (Eckle *et al.*, 2008) and infarction (Zhou *et al.*, 2010).

Our results show that the iron chelation obtained by exposure to dexrazoxane induces HIF binding activity and transactivation capacity in H9c2 cells, which we and others have shown represent a reliable model for evaluating various characteristics of cardiomyocytes, including doxorubicin toxicity (Corna *et al.*, 2004; L'Ecuyer *et al.*, 2004; Spallarossa *et al.*, 2004; Li *et al.*, 2006; Mukhopadhyay *et al.*, 2007; Reeve *et al.*, 2007; Turakhia *et al.*, 2007; Konorev *et al.*, 2008; Xu *et al.*, 2008; Bernuzzi *et al.*, 2009) and dexrazoxane cardioprotection (Lyu *et al.*, 2007). The fact that (in agreement with previous evidence; Weiss *et al.*, 1997) dexrazoxane administration up-regulated iron regulatory proteins (results not shown), whose activity is known to depend on intracellular iron availability (Recalcati *et al.*, 2010), indicates that the effects of dexrazoxane are mediated by decreased iron levels rather than by any other unforeseen effects. HIF was induced by dexrazoxane concentrations as low as 10  $\mu$ M, which allowed us to use doses that were well within pharmacological levels achieved in patients (Hasinoff *et al.*, 2003) and to respect the recommended doxorubicin : dexrazoxane ratio (Thompson *et al.*, 2010).

In line with the findings of previous studies showing the protective effect of dexrazoxane *in vitro* (Simunek *et al.*, 2009) and *in vivo* (Popelova *et al.*, 2009), we showed that pre-exposure to dexrazoxane prevents doxorubicin-mediated cell death, particularly apoptosis (the prevailing mechanism for low-dose doxorubicin cardiotoxicity) (Sawyer *et al.*, 1999; Bernuzzi *et al.*, 2009), although it has been reported that doxorubicin-dependent depletion of GATA4 triggers cardiomyocyte autophagic death (Kobayashi *et al.*, 2010).

Importantly, using genetic manipulations involving the loss and gain of function of HIF-1 levels and activity, we demonstrated the contribution of HIF to dexrazoxane-mediated protection against doxorubicin-induced damage in H9c2 cardiomyocytes. The involvement of HIF in the survival of doxorubicin-treated H9c2 cells was shown by the fact that the protection was abolished by shRNA-mediated HIF-1 $\alpha$

underlying dexrazoxane-mediated cardioprotection. It has been previously shown that this transcription factor, which plays a key role in regulating alterations in the expression of genes that promote cell survival and maintain homeostasis (Schofield and Ratcliffe, 2004; Higgins *et al.*, 2008; Semenza,



**Figure 7**

Dexrazoxane (DRZ) induces the expression of antiapoptotic genes. Immunoblot analysis of cytosolic extracts of untreated H9c2 cells (C), and cells exposed for 24 h to doxorubicin (DOX), for 3 h to dexrazoxane alone or pretreated with DRZ for 3 h and then exposed to DOX. When appropriate, the cells were also transfected with the expression vector  $\Delta$ ARNT. In some cases, the cells were exposed to DMOG for 24 h or to BSO and H<sub>2</sub>O<sub>2</sub> for 3 h, and then washed and re-incubated for 2 h. Antibodies against the indicated proteins were used, and the blots were reprobed using the antibody against  $\alpha$ -tubulin as a loading control. The panels show one representative blot and the densitometric quantification relative to C-values. Mean values  $\pm$  SD. \* $P$  < 0.001; \*\* $P$  < 0.005,  $n$  = 3. DMOG, dimethylxalyl glycine; BSO, buthionine sulfoximine.

knockdown or the  $\Delta$ ARNT-mediated inhibition of the DNA binding activity of both HIF-1 $\alpha$  and HIF-1 $\beta$  isoforms, whereas the overexpression of HIF-1 $\alpha$  was sufficient to provide a level of protection against doxorubicin-induced damage that is similar to that obtained with the iron chelator. These findings are in line with the demonstration that HIF-1 is required for confluence-dependent resistance to doxorubicin in breast carcinoma cells (Fang *et al.*, 2007) and suggest that similar mechanisms may be operating in tumour cells and cardiomyocytes.

HIF-1 $\alpha$  and HIF-2 $\alpha$  have different tissue distributions but are activated by common stimuli and share a large number of target genes and functions, which hinders any clear determination of their specific roles (Semenza, 2009). We showed that both HIF isoforms, which are susceptible to similar degradation mechanisms through the von Hippel-Lindau-mediated ubiquitin-dependent proteasome pathway (Semenza, 2009), were induced by dexrazoxane, but we cannot define the contribution of either to the protection of H9c2 cells. However, the loss of cardioprotection in cells with shRNA-mediated HIF-1 $\alpha$ -specific knockdown and the resistance to doxorubicin toxicity in cells overexpressing HIF-1 $\alpha$  suggest that this isoform plays an important role.

Doxorubicin severely inhibits HIF-1 transcriptional activity in tumour cells (Lee *et al.*, 2009), although another study did not find any inhibitory effect (Yamazaki *et al.*, 2006). We observed only partial inhibition of HIF transactivating capacity in doxorubicin-treated H9c2 cells (Figure 3B), and not enough to prevent its protective function (Figure 4). Importantly, we showed that the expression of HIF target genes was not affected by doxorubicin and was also up-regulated in H9c2 cells exposed to dexrazoxane plus doxorubicin (see Figure 7). The different experimental conditions (3 h pretreatment with dexrazoxane before doxorubicin administration in our study vs. hypoxic exposure in the presence of doxorubicin in the study of Lee *et al.*, 2009) and the different cell types may explain this discrepancy.

We also evaluated the effects of dexrazoxane on the expression of a number of HIF target genes in H9c2 cells and found that it triggered the expression of the typical HIF target gene aldolase A, a glycolytic enzyme which has a positive effect on cell function and adaptation to hypoxia (Semenza *et al.*, 1996). In line with the antiapoptotic role of some HIF-regulated genes (Higgins *et al.*, 2008) and the prevention of apoptosis induced by dexrazoxane pretreatment (see Figure 2), we observed the strong up-regulation of survivin and Mcl1, both of which are members of the apoptosis protein inhibitor family. Our findings are in line with the known essential function of survivin in cell division and the inhibition of apoptosis (Guha and Altieri, 2009), as well as with the recent demonstration that the overexpression of survivin in cardiomyocytes inhibits doxorubicin-induced apoptosis (Levkau *et al.*, 2008). Moreover, it has been shown that Mcl1 (a pro-survival protein belonging to the Bcl2 gene family) is associated with cardiac myocyte viability (Craig, 2002). Our previous results suggested that doxorubicin may facilitate the apoptosis of cardiomyocytes by inhibiting the antiapoptotic HO-1 (Bernuzzi *et al.*, 2009), which is an HIF-1 target gene (Kim *et al.*, 2006). In line with these findings, exposure to dexrazoxane was able to counteract the inhibition of HO-1 expression exerted by doxorubicin and resulted

in a strong increase in HO-1 levels. We point out that in our study, the inhibition of HIF activity by the expression of  $\Delta$ ARNT prevented doxorubicin-induced cell death (see Figure 4) and suppressed the induction of these antiapoptotic genes (see Figure 7), thus suggesting their role in dexrazoxane-mediated cardioprotection. The incomplete effect exerted by the inhibition of HIF-1 activity may be due to the fact that not all the cells were transfected (see Figure 5A), but the involvement of other transcription factors cannot be ruled out.

On the other hand, we did not detect any significant modulation of Bcl-xL (results not shown), an antiapoptotic protein that protected H9c2 cardiomyocytes against doxorubicin-induced apoptosis (Reeve *et al.*, 2007). Our results are in line with previous findings indicating that Bcl-xL is probably not regulated by HIF-1; NF- $\kappa$ B (but not HIF-1) is important in hypoxia-induced apoptosis (Glasgow *et al.*, 2001), and the pathway underlying anoxia- and hypoxia-induced cell death is initiated by the loss of function of Bcl-xL (Shroff *et al.*, 2007).

Taken together, these results indicate that the cardioprotective action of dexrazoxane involves the HIF-mediated activation of antiapoptotic genes and is in line with the recent demonstration that dexrazoxane prevents apoptosis and heart damage in a rat model of cardiac infarction (Zhou *et al.*, 2010).

The role of dexrazoxane in preventing anthracycline-dependent cardiotoxicity does not seem to involve the prevention of ROS formation, as we and others have recently obtained evidence indicating that oxidative stress does not play a role in the apoptotic cell death of H9c2 cardiomyocytes exposed to low doxorubicin concentrations (Bernuzzi *et al.*, 2009; Shi *et al.*, 2009). Accordingly, we did not detect any significant alteration in MnSOD expression, an HIF-2 $\alpha$  target gene (Scortegagna *et al.*, 2003) whose overexpression in mice protects against doxorubicin-induced acute toxicity (Yen *et al.*, 1996). Our findings are therefore in line with the idea that the toxic role of iron in anthracycline cardiotoxicity is a result of reactions that extend beyond canonical oxidative damage and involve other mechanisms unrelated to iron-catalysed ROS production.

Recent evidence showing that Pgp, a membrane efflux pump involved in the development of the MDR phenotype (Takara *et al.*, 2006), is induced in tumour cells exposed to 100  $\mu$ M dexrazoxane (Riganti *et al.*, 2008) suggests that, by actively extruding doxorubicin and thus lowering its intracellular concentration, Pgp may be a potential mediator of HIF-dependent cardioprotection. However, we found that Pgp expression was not affected by dexrazoxane in H9c2 cells despite concomitant HIF activation; this discrepancy may be explained by the cell-specific response of Pgp to iron deprivation, as suggested by a recent study showing Pgp down-regulation in leukaemic K562 cells exposed to an iron chelator (Fang *et al.*, 2010).

The fact that dexrazoxane offers unquestionable protection *in vivo*, whereas other iron chelators whose bioavailability is similar to that of dexrazoxane have either not been protective (Popelova *et al.*, 2008; Hasinoff and Patel, 2009) or have only been effective at low-intermediate but not at higher doses (Sterba *et al.*, 2006), has still not been explained. The protective effect of dexrazoxane may depend on

additional and possibly unique mechanisms, such as interference with topoisomerase II  $\beta$ -mediated DNA double-strand breaks (Lyu *et al.*, 2007). As we found that DFO-mediated iron chelation also activates HIF in H9c2 cells, our results do not explain the unique capacity of dexrazoxane to prevent cardiotoxicity *in vivo*. However, our findings do demonstrate a novel ROS-independent mechanism based on the HIF-mediated activation of protective genes, which seems to account for the antiapoptotic effect of dexrazoxane against low-dose doxorubicin toxicity in the H9c2 model. This indicates that HIF plays a role in dexrazoxane cardioprotection and suggests that novel pharmacological strategies based on small molecular mimics of hypoxia (Nagel *et al.*, 2010) could be explored in an attempt to limit anthracycline cardiotoxicity.

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## Conflict of interest

The authors declare no conflict of interest.

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