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ROLE OF HIF ACTIVATION IN THE PROTECTION OF CARDIOMYOCYTES FROM DOXORUBICIN TOXICITY

MED/04

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ABSTRACT

Anthracyclines are important and effective anticancer drugs used in the treatment of many adult and children malignancies. Doxorubicin (DOX) is the anthracycline most commonly used in cancer patients but its cardiotoxicity limits its clinical use. The precise molecular basis of anthracyclines cardiotoxicity remains elusive, but a number of theories have been proposed, one of which is the formation of reactive oxygen species (ROS).

Iron aggravates the cardiotoxicity of DOX; indeed, dexrazoxane (DRZ) is the only agent able to protect the myocardium from anthracycline-induced toxicity both in experimental and clinical settings. Iron has been proposed to catalyse ROS formation in reactions primed by DOX. However, the oxidative nature of the role of iron in cardiotoxicity is challenged by results showing that antioxidants do not always protect against cardiotoxicity. Therefore, the mechanisms of DOX-mediated cardiotoxicity, and the protective role of DRZ, remain to be established.

The hypoxia inducible factors (HIF, HIF-1α and HIF-2α) are transcription factors which regulate the expression of several genes mediating adaptive responses to lack of oxygen. Iron is required for HIF degradation and therefore decreased iron availability activates HIF in normoxic cells. In consideration of the antiapoptotic and protective role of some HIF-induced genes, we tested the hypothesis that DRZ-dependent HIF activation may mediate the cardioprotective effect of DRZ.

Treatment with DRZ induced HIF protein levels and transactivation capacity in the H9c2 cardiomyocytes cell line. DRZ also prevented the induction of cell death and apoptosis caused by the exposure of H9c2 cells to clinically-relevant concentrations of DOX. Experiments involving suppression of HIF-1α activity or HIF-1α overexpression showed that the protective effect of DRZ was dependent on HIF-1 activity.

By examining the expression of HIF target genes with a possible role in cell survival in DRZ-treated H9c2 cells we found that a strong increase in protein levels of antiapoptotic genes and haem oxygenase (HO-1) plays a role in the HIF-mediated cardioprotection offered by DRZ.

We also explored two possible alternative pharmacological strategies to prevent DOX-induced toxicity.

The first one was based on a small molecular mimic of hypoxia that could be exploited in an attempt to limit anthracycline cardiotoxicity. We examined HIF-1α levels and activity, as well as protection from DOX damage, in H9c2 cardiomyocytes pre-exposed to DMOG, an antagonist of α-ketoglutarate which activates HIF under normoxic conditions. However, we did not find any kind of protection from damage induced by DOX in H9c2 cells pre-treated with DMOG.

The second one was based on the activation of the sodium-dependent glucose transporter-1 (SGLT-1), which has been shown to protect different types of cells from various injuries. We found that pre-treatment with D-glucose protected H9c2 cells from DOX-induced toxicity, but the non-metabolizable glucose analog 3-O-methylglucose, and the SLGT-1 agonist BLF50 were ineffective, thus indicating that the protection was not mediated by the activation of SGLT-1.

INTRODUCTION

1. INTRODUCTION

1.1 DOXORUBICIN

Anthracyclines are important and effective anticancer drugs used in the treatment of many adult and children malignancies. The most commonly used anthracyclines are Doxorubicin (DOX), Daunorubicin and Epirubicin.

DOX, (also called adriamycin) consists of a planar tetrahydro-anthracene ring containing quinone and hydroquinone groups located on adjacent rings, one short side chain with a carbonyl group at the C-13 and an amino-sugar linked through a bond glycoside at C-7 ring tetracyclic (Minotti G, et al., 2000).

For many years DOX has assumed an important role in cancer chemotherapy and it is commonly used in the treatment of leukemias and lymphomas, breast cancer and carcinomas. Despite its wide clinical use, the antitumor mechanism of DOX is not yet well understood but is probably related to anthracyclines biochemical properties.

The structure of DOX is responsible for many different biochemical properties: the simultaneous presence of quinone and hydroquinone groups on adjacent rings allows anthracyclines to interact with metals and undergo a series of reactions, many of which lead to the formation of free radicals, resulting in accumulation of toxic intermediates such as superoxide anion or hydroxyl radical. The planar tetrahydro-anthracene permits the anthracycline intercalation between DNA base pairs, and the complex stability is enhanced by the formation of hydrophobic bonds and hydrogen bonds formed between the amino groups and the ribophosphate of DNA helix. It is believed that these mechanisms may interfere with cell growth and survival. The antitumor activity of DOX may also be explained by the inhibition of DNA polymerase that determines a block in the synthesis of nucleic acids. A splitting of DNA strands mediated by inhibition of topoisomerase II, an enzyme that favors the positive super-coiling of DNA, may also occur. Despite its efficacy as an antineoplastic drug, the use of DOX is unfortunately limited by the onset of severe cardiomyopathy, which can be acute or chronic. The acute effects, including tachycardia and hypotension, are usually reversible, while chronic effects lead to irreversible changes that result in severe heart failure. In the case of DOX, the risk of cardiotoxicity is dose-

dependent and increases rapidly when the cumulative dose exceeds 550 mg/m^2 of body surface area. The indication to not exceed this critical threshold could imply the interruption of the chemotherapeutic regimen before completion and may thus interfere with cancer therapy (Minotti et al., 2004).

1.1.1 MECHANISMS OF DOX-INDUCED CARDIOTOXICITY

a) Role of iron

Iron seems to play an important role in anthracycline cardiotoxicity. Studies conducted some 30 years ago have shown that derivatives of EDTA, which have metal chelating properties, prevent injury and cardiac dysfunction due to the use of the drug (Herman EH, et al., 1979). As further evidence of the negative effect of iron, in the last years it has been also highlighted the cardioprotective effect of dexrazoxane (DRZ), an iron chelator that reduces the incidence of cardiotoxicity with a long-term effect, and has proved to be effective in numerous clinical trials both in adults and children (Pouillart P. 2004; Wouters KA, et al., 2005; Lipshultz SE, et al., 2010).

Conversely, the involvement of iron in DOX-induced cardiotoxicity has been highlighted by the demonstration that primary and secondary iron overloads exacerbate the drug's cardiotoxic effects (Hershko C, et al., 1993; Link G, et al., 1996; Miranda CJ, et al., 2003).

As it regards the mechanisms undergoing the toxic effect of DOX-iron interaction, the mechanism that received the major support was based on the hypothesis that iron may catalyze ROS formation in DOX-primed reactions. Indeed, the toxicity of reactive oxygen species increases significantly in the presence of iron ions in an aqueous environment because the metal can form the hydroxyl radical ($OH^{\bullet}$) through the Fenton reaction, which uses ferrous iron (Fe II) and through the Haber-Weiss reaction which uses ferric iron (Fe III). The removal of metal ions (for example by DRZ), prevents the formation of the $Fe3^{+}$ - DOX complex and the subsequent formation of reactive free radicals.

b) Metabolism of the drug and ROS formation

To better understand the mechanisms underlying the toxicity induced by DOX it is necessary to examine the metabolic fate of the drug.

Anthracyclines undergo redox cycling which begins with the monovalent reduction of the quinone residues localized on the tetracyclic ring of the drug. This reaction is catalyzed by

mitochondrial NADH-dehydrogenase or by mitochondrial and nuclear NADPH-cytochrome P450 reductase and leads to the formation of a highly unstable semiquinone radical ($DOX^{\cdot}$). $DOX^{\cdot}$ tends to regenerate the quinonic form, by transferring electrons to iron-rich structures, or by reducing molecular oxygen to superoxide anion with consequent formation of hydrogen peroxide (H_2O_2) (Gewirtz DA 1999; Minotti G, et al., 2004).

The reactive intermediates can attack cellular macromolecules such as DNA lipids and eventually lead to cell damage and death (FIG. A). The link between the formation of free radicals during DOX metabolism and the cardiotoxicity induced by DOX was highlighted by the fact that anthracyclines activate a transduction signal pathway that induces cell death by apoptosis (Minotti G, et al., 2004).

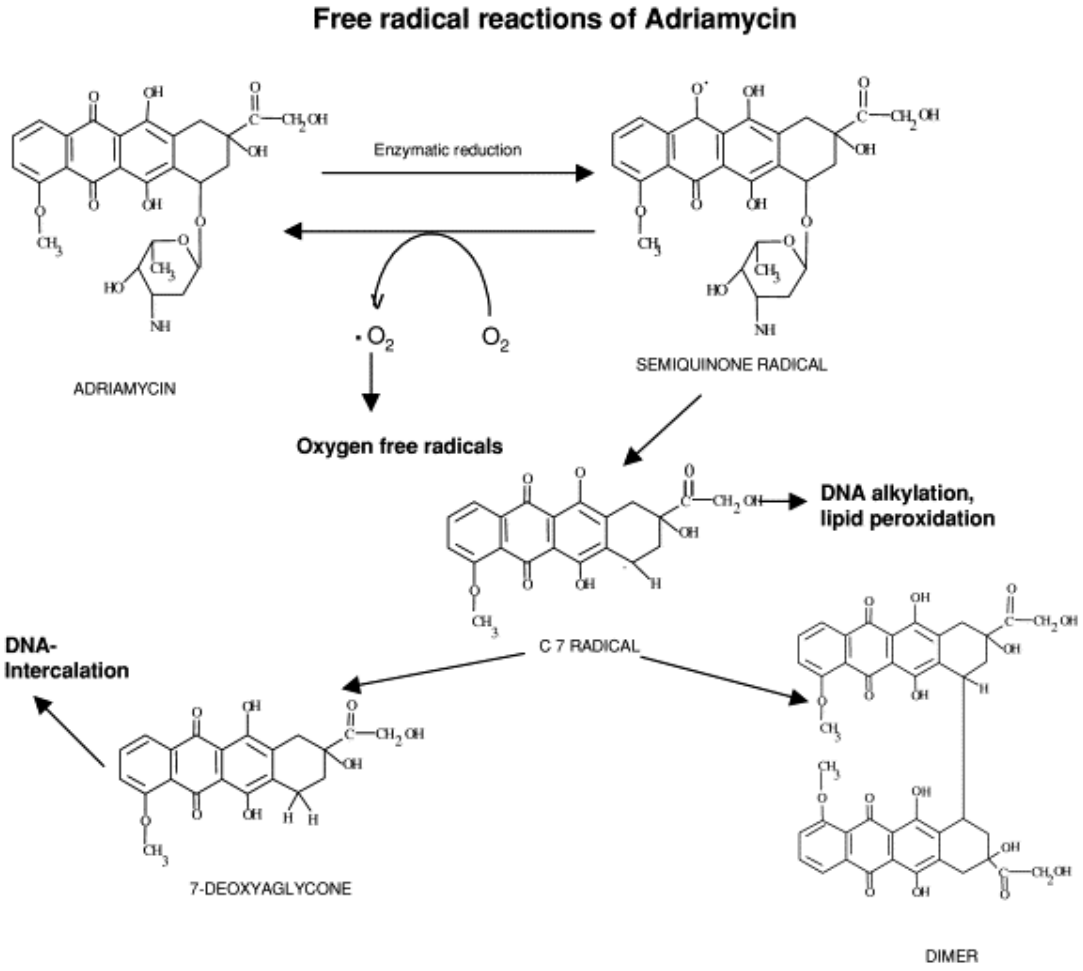

FIG. A - ***Metabolic activation of DOX***

This redox cycling is particularly harmful to cardiomyocytes that express low levels of antioxidant defenses, such as superoxide dismutase and catalase, whose function is to convert the active species into less harmful forms. This would account at least in part for the particular susceptibility of heart cells to DOX injury as well as for the role of iron in DOX cardiotoxicity. As already mentioned above, during DOX metabolism, iron increases the toxicity of the intermediates that are formed in some reactions. As a typical "redox cycling" agent that is metabolized through redox reactions, DOX releases electronegative intermediates or superoxide anions able to relocate the iron from intracellular stores.

The iron-free radicals model would imply a protective and beneficial effect of antioxidant interventions. Indeed, the use of a ‘chain breaker’ antioxidant like vitamin E was able to prevent the onset of the typical ultrastructural changes of the endoplasmic reticulum, sarcolemma and mitochondria that occurs after DOX transformation in the semiquinone radical. However, these results obtained in experimental models were not confirmed in clinical settings. Human studies have revealed that vitamin E, at doses able to prevent cardiovascular disease, protect from the onset of acute cardiomyopathy but does not offer any protection against chronic diseases (Minotti G, et al., 1999; Ladas EJ, et al., 2004; Simunek T, et al., 2009).

An alternative mechanism to explain DOX-mediated cardiotoxicity is the formation of the alcohol metabolite of DOX (Doxol) by reduction of the carbonyl group in C13 (FIG. B).

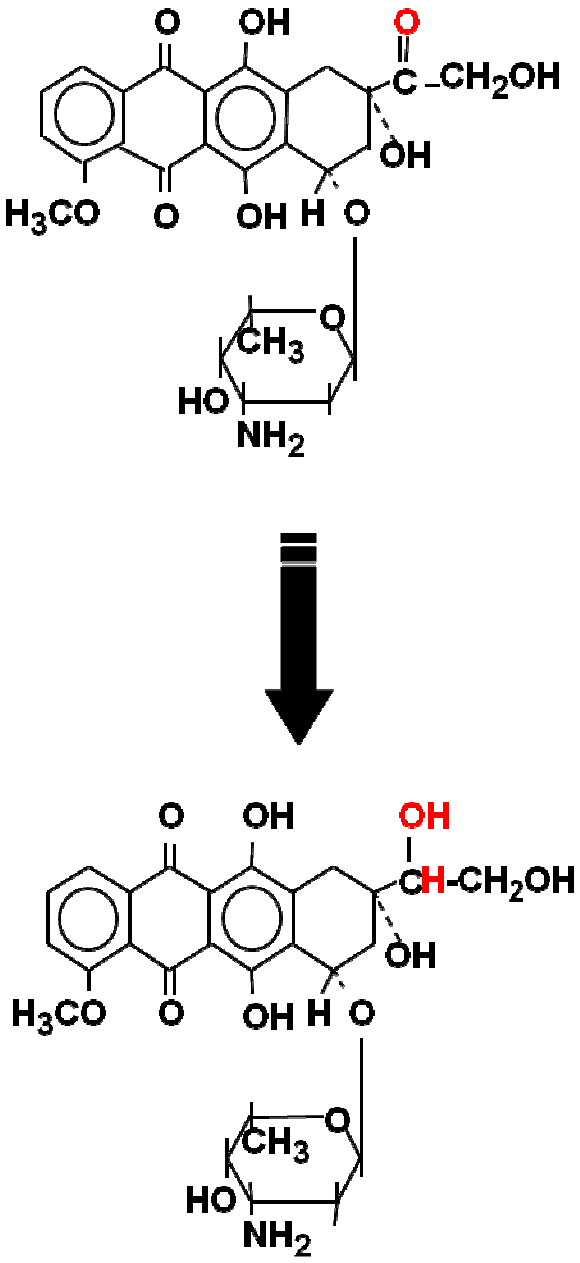

FIG. B - ***DOX is converted to its alcohol metabolite doxorubicinol***

It has been shown that Doxol accumulates in the heart of rats treated with DOX, at concentrations two/three fold higher than in the liver whereas DOX concentrations in the two organs are similar (Peters JH, et al., 1981) and this accumulation reflects the intramyocardial metabolism of the drug rather than the uptake of the metabolite from the blood (Olson RD, et al., 1990).

This suggested that the cardiotoxicity may depend on the ability of cardiomyocytes to form and accumulate Doxol. This seems to occur also in patients undergoing treatment with DOX as an antineoplastic agent, as evidenced from investigations performed on postmortem cardiac tissue (Stewart DJ, et al., 1993).

Regarding the mechanism underlying Doxol toxicity, it has been demonstrated that Doxol can inhibit Ca_2^+/Mg_2^+ ATPase of the sarcoplasmic reticulum, the mitochondrial F_0F_1 pump, the Na^+/K^+ ATPase of the sarcolemma (Boucek RJ Jr, et al. 1987; Olson, RD, 1988). Studies in which human myocardial lysates were exposed in vitro to DOX (Minotti G, et al. 1998; Brazzolotto X, et al., 2003) also showed that Doxol interferes with iron metabolism by interacting with the Iron Regulatory proteins (IRP), which are cytoplasmic proteins that modulate the expression of transferrin receptor and ferritin and play a key role in controlling iron uptake, use and intracellular accumulation use (Beinert H and Kennedy MC. 1993). IRP-1 can exist as apoprotein with the ability to bind RNA or as holoprotein after assembly of a Fe-S cluster. In the latter case IRP-1 has enzymatic activity similar to mitochondrial aconitase.

It has been shown that Doxol may cause the removal of iron (FeII) from the 4Fe-4S cluster of cytoplasmic aconitase. Iron removal proceeds through the reoxidation of Doxol to DOX and the consequent release of the DOX - Fe (II) complex as final product. Cluster disassembly abolishes aconitase activity and leads to the formation of an apoprotein that can bind to IRE sequences (Iron Responsive Elements) in the mRNAs of transferrin receptor or ferritin. (Minotti G, et al., 1998). However, DOX, thanks to the formation of a DOX-Fe complex can also oxidatively modify the Cys residues of IRP-1, probably Cys^{437}, which mediates the binding to IRE sequences and this also leads to the loss of RNA binding activity. The result of the interaction between Doxol and IRP1 is therefore the simultaneous loss of both RNA binding capacity and aconitase activity and the formation of a "null protein" with consequent impairment of iron homeostasis.

Concerning iron homeostasis, the "null protein" is not able to detect the intracellular level of the metal and to adjust the uptake and release of iron in the cell as needed. It is also important to emphasize that, in addition to modulate the level of ferritin and transferrin

receptor, IRP-1 is able to regulate the mRNA for other enzymes related to the use (erythroid aminolevulinate synthase), the uptake (DMT1/Nramp2) and the release (ferroportin-1, IREG1, MTP1) of iron. This could also lead to impaired formation of several enzymes and oxygen binding proteins, whose function depends on the metal (catalase, lipoxygenase, cytochromes, and myoglobin).

DOX may undergo not only reductive metabolism but also other types of metabolism. It has been recently discovered that DOX can also undergo oxidative degradation, releasing a compound consisting of a single ring, called 3-methossiphtalic acid. DOX oxidative degradation requires peroxidase reactions that in the heart seem to be due to the action of oxidized myoglobin and/or inducible prostaglandin H_2 synthase (iPGH2,) more commonly known as cyclooxygenase-2 (COX-2), both having peroxidase activity. Interestingly, 3-methossiphtalic acid proved to be non toxic when administrated to cardiomyocytes at doses exceeding several fold those of DOX (Minotti G, et al., 2004). This degradation pathway thus appears to decrease DOX toxicity that requires the action of the intact drug or its reductive metabolites and is probably used by cardiomyocytes as a way to counteract DOX toxicity. It should be noted that this oxidative degradation may decrease DOX cardiotoxicity but at the same time it may also impair its antitumor activity, but the effect on tumor cells have not been investigated, yet.

1.1.2 DOXORUBICIN AND APOPTOSIS

As already mentioned above, anthracyclines activate a signal transduction pathway that induces cell death by apoptosis (Minotti G, et al., 2004).

Apoptosis can be induced by many stimuli that vary from cell to cell. These stimuli can be both intracellular and extracellular and activate different mechanisms: an intrinsic pathway and an extrinsic pathway depending on the origin of death signals.

The extrinsic pathway is triggered by the binding of an extracellular "death ligand" (eg Fas) to its receptor on the cell surface. The complex then uses a molecule that acts as an adapter, called Fadd (Fas-associated death domain protein), to bind and recruit molecules that eventually determine the activation of procaspase-8.

The intrinsic pathway is activated in response to both internal damage (such as DNA damage) and extracellular signals. DNA damage activates the p53 protein, which blocks

cell cycle progression and promotes DNA repair. If the damage is too extensive and irreversible, p53 promotes cell apoptosis.

Apoptotic signals induce mitochondrial cytochrome c release, which leads to activation of caspase-9 through the formation of an apoptosome complex between 'apoptosis activating factor-1' (Apaf-1), cytochrome c and procaspase-9. The opening of the mitochondrial pores, resulting in the release of cytochrome c, is regulated by the formation of heterodimers between proapoptotic proteins (Bid, Bax, Bad etc..) (FIG. C).

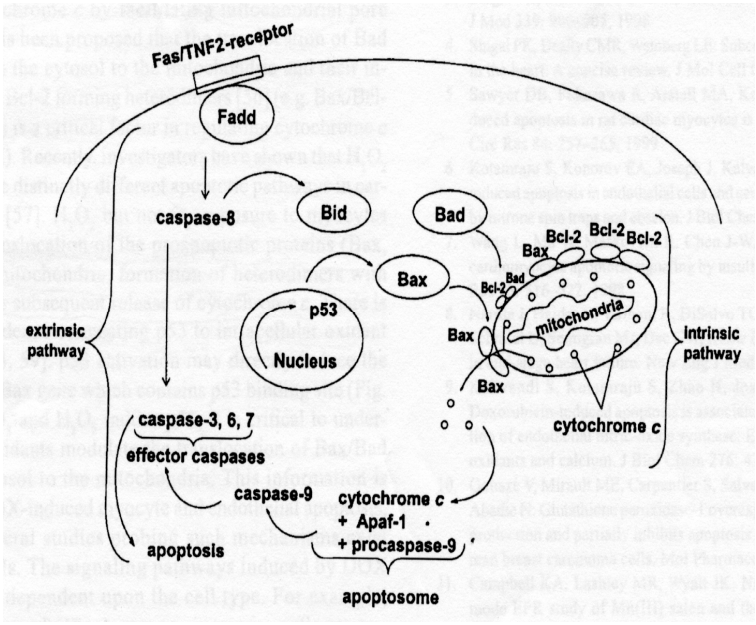

FIG. C

Apoptotic Pathways

DOX may trigger apoptosis through several mechanisms. One of these is the formation of ROS following redox cycling (see above). Moreover, as reported before, DOX can interact with DNA and enzymes involved in DNA maintenance thus leading to persistent DNA damage. Moreover, DOX can affect the mitochondrion, an organelle which is a target of DOX toxicity because it is the site of drug accumulation over time. It has been shown that, at clinically relevant plasma DOX concentrations (0.5–1 μM), the intramitochondrial

concentration is approximately about 100 times higher (50–100 μM). (Kalyanaraman B, et al. , 2002) (FIG. D).

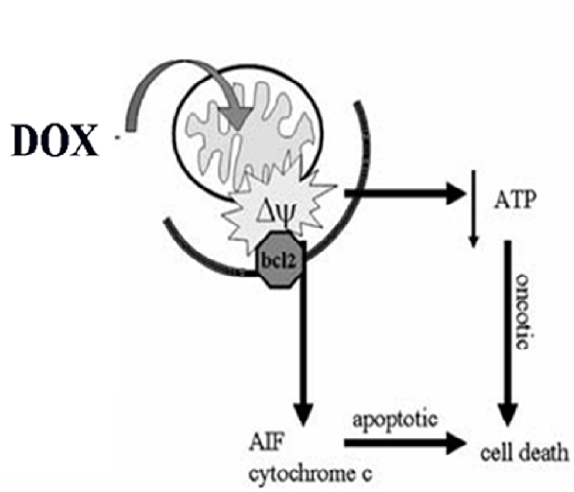

FIG. D

Mitochondrial DOX accumulation and cell death

Although there are strong evidences on the mechanisms that induce apoptosis in cardiomyocytes treated with DOX in vitro, it is not yet clear how apoptosis may contribute to the cardiotoxicity induced by DOX in vivo.

Moreover, studies in animal models have indeed highlighted the link between apoptosis and acute cardiotoxicity, but whether apoptosis is one of the mechanisms involved in chronic cardiotoxicity is still controversial (Minotti G, et al., 2004).

1.2 HYPOXIA AND HYPOXIA-INDUCIBLE FACTORS

Oxygen is both an environmental and developmental signal that governs important cellular pathways and plays a critical role in several physiological and pathophysiological conditions. Its cellular demand vary considerably among different tissues, largely due to variations in energy requirements from cell to cell.

Hypoxia is a condition that leads to inadequate availability of O_2 in the blood and tissues. It may be due to a reduction in the partial pressure of oxygen, low oxygen transport or the inability of the tissue to use oxygen, mainly caused by the disrupted microcirculation (Sitkovsky M, et al., 2005. Therefore, hypoxia (or low oxygen tension) is part of both physiological and pathological processes.

Oxygen sensing is crucial for cell survival and is a key element for the possibility of living organisms to adapt to changing environments or physiological conditions. In addition, oxygen-sensing mechanisms are involved in many pathophysiological conditions, including stroke, sleep apnea, cancer, hypertension, inflammation, heart failure and sudden infant death syndrome (Sharp FR, et al., 2004). To deal with hypoxic conditions, cells and organisms have developed efficient mechanism for adaptation and survival.

HIF-1 is a fundamental mediator of adaptation of cells to hypoxia (Semenza GL. 2001). It is a heterodimeric transcription factor consisting of an inducible HIF-1α subunit and a constitutively expressed HIF-1β subunit.

HIF-1 activation leads to increased transcription of more than 100 target genes involved in a number of different cellular functions, such as cell survival, cell proliferation, apoptosis, glucose metabolism, angiogenesis, erythropoiesis, iron homeostasis, energy metabolism. Moreover, gene inactivation experiments in mice have shown that HIF-1 is essential for embryonic development (Semenza GL. 2009) (FIG. E).

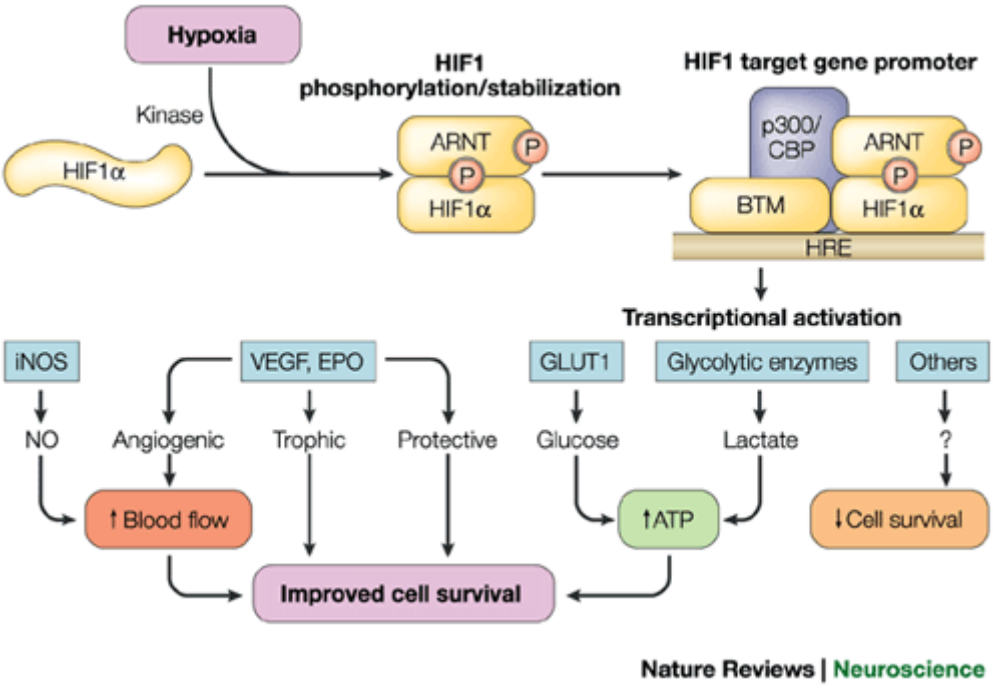

FIG. E

HIF-1 is also the main transcription factor responsible for the tissue adaptation to ischemia and plays an important role also in the oxygen sensing organ called carotid body that immediately sends to the body signals of adaptation to the low oxygen tension (Sharp FR, et al., 2004).

HIF-1 is a ubiquitously expressed transcription factor that was originally discovered in 1992 as being responsible for the expression of erythropoietin in hypoxic conditions (Semenza GL. 2001). Its subunits, HIF-1α and HIF-1β, coded by differently located genes (human chromosome 14 for HIF-1α and chromosome 1 HIF-1β, are basic helix-loop-helix (bHLH) proteins of the Per-ARNT-Sim (PAS) family.

The two proteins have some common characteristics, they both contain nuclear localization signals, a basic helix-loop-helix motif (bHLH), which is essential for DNA binding to the Hypoxia Responsive Element (HRE) in the promoter region of target genes and responsible for subunit dimerization, and the PAS domain. This last sequence identifies a protein superfamily, which was initially founded by the Drosophila proteins period (Per) and single-minded (Sim) and the vertebrate protein aryl hydrocarbon receptor nuclear translocator (ARNT), later discovered to be identical to HIF-1β. HIF-1α has some

unique characteristics: it contains the oxygen-dependent degradation domain (ODD) found between residues 401-603, this region is highly oxygen regulated and its deletion confers stability to the protein in the presence of oxygen. HIF-1α also contains two transactivation domains (TAD-N and TAD-C), which are responsible for the transcriptional regulation of HIF-1 target genes. These TADs are also involved in the binding of co-activators such as p300/CBP and Ref-1, which are essential for HIF-1's transcriptional activation. HIF-1β also contains a TAD domain, but this is not necessary for HIF-1's transcriptional activity (Dèry et al., 2005).

HIF-1α has several homologues (HIF-2α and HIF-3α) that seem to have partially different functions. Although HIF1α/HIF1β dimers and HIF2α/HIF1β dimers bind the same DNA sequence, they might be differentially expressed in different cells or tissue, resulting in the activation of different target genes with non-overlapping functions (Sharp FR, et al., 2004). HIF-1α and HIF-2α knockouts are embryonically lethal, showing that each gene has unique functions that cannot be replaced by its homologue. This has been confirmed by the evidence that HIF-1α is ubiquitously expressed, whereas HIF-2α expression is restricted to endothelial cells and perhaps other specific cell types. HIF-3α seems to act as an antagonist of the HIF system because the inhibitor of PAS domain protein (IPAS), a dominant negative regulator of HIF-1, was identified as an alternatively spliced variant of HIF-3α (Lee JW et al., 2004).

While HIF-1β is constitutively expressed, HIF-1α is an extremely labile protein with a half-life of less than 5 minutes in normoxia. The rapid and continuous degradation of HIF-1α in normoxic condition is effectively blocked if oxygen availability is reduced (Sharp FR, et al., 2004).

In the presence of O_2, iron and 2-oxoglutarate, HIF-1α is hydroxylated by three specific oxygen-dependent proline-hydroxylases (PHD1-3) in the cytoplasm and nucleus, thereby targeting it for proteosomal degradation (Kaelin et. al., 2008; Harten SK, et al., 2010). These enzymes hydroxylate proline residues 402 and 564 in the ODD domain of HIF-1α, leading to changes in the conformation of HIF-1α and allowing the von Hippel Lindau protein (VHL) to recognize and bind to it. Other factors then bind to VHL, including elongin B and elongin C, Cullin 2 and RBX1. This complex acts as an E3 ubiquitin ligase for HIF-1α poly-ubiquitylation in the ODD, and HIF-1α is eventually degraded by the proteasome (Kaelin, 2008) (FIG. F).

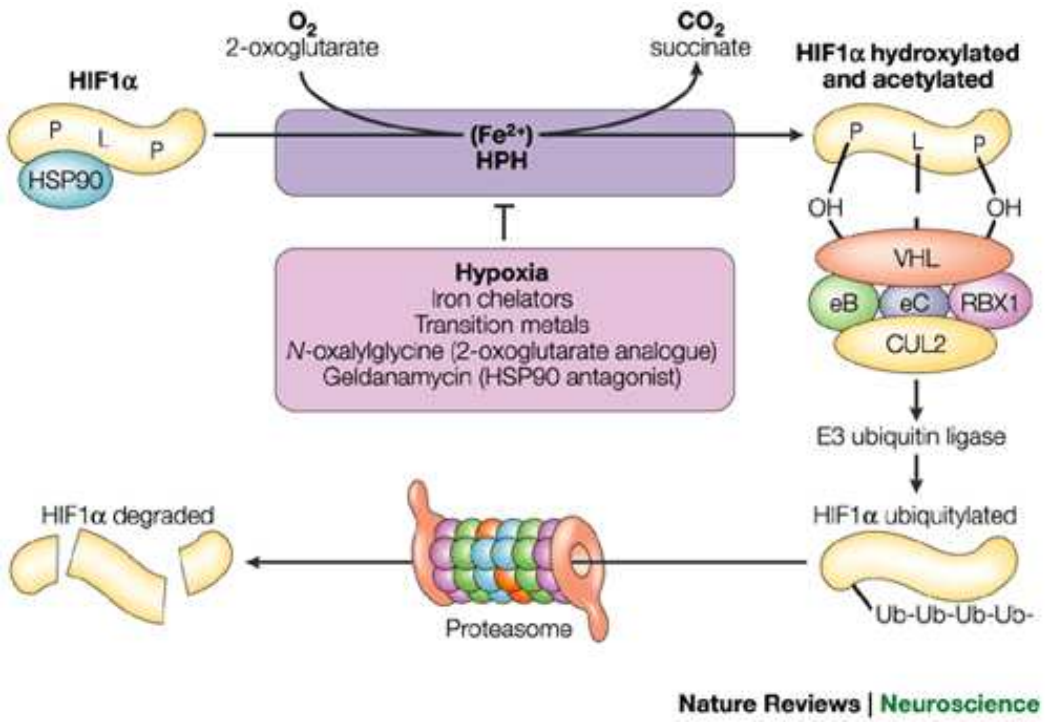

FIG. F

Degradation of HIF-1α by the proteasome

When one of the co-factors of the PHD (O_2, Fe^{2+}, 2-oxoglutarate) is not present, HIF-1α and HIF-2α bind the HIF-1β subunit forming a heterodimer able to recognize the consensus sequence HRE present in target genes.

Other mechanisms that determine the proteasomal degradation of HIF-1α in normoxia condition are: activation of the protein ARD1, a protein acetyl-transferase of the p300/CBP family that by acetylating lysine 532 enhances the interaction with VHL and promotes the proteasomal degradation of HIF-1α. The factor inhibiting HIF-1 (FIH1) abrogates the interaction between HIF-1 and the transcriptional co-activator p300/CBP. FIH1 is a unique asparaginyl-hydroxylase that contains a ferrous ion and uses O_2 and 2-oxoglutarate as co-factors to hydroxylate asparagine 803 in HIF-1α. This decreases binding of p300/CBP to HIF-1α and prevents the full transcriptional activation of HIF target genes (FIG. G).

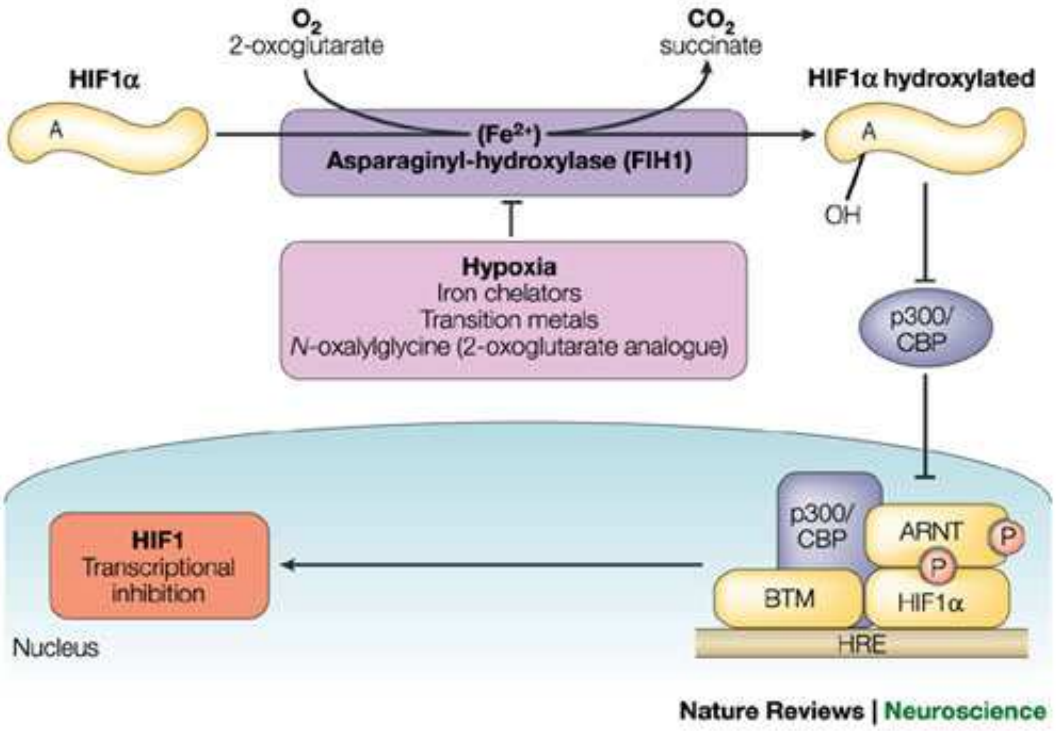

FIG. G

Lack of the cofactors thus promotes both HIF stability and transcriptional activity as prolyl- and asparaginyl-hydroxylases are inhibited, thereby preventing hydroxylation of HIF-1α and so its degradation, leading to rapid HIF-1α accumulation. In addition to lack of oxygen, deficiencies of one of the PHD cofactors can lead to HIF stabilization. This was supported by the demonstration that PHD inhibition by the competitive inhibitor dimethyl-oxalylglycine (DMOG), a cell-permeable analog of 2-oxoglutarate (Jaakkola P, et al., 2001) results in increased HIF-1 activity.

Moreover, in line with the iron requirement of PHD for HIF-1 degradation, also decreased intracellular iron availability activates HIF (Peyssonnaux C, et al., 2008; Mole DR. 2010). Accordingly, it has been found that exposure to iron chelators such as desferrioxamine (DFO) (Wang GL and Semenza GL. 1993; Bianchi L, et al., 1999) or growth under iron deficiency conditions (Jones DT, et al., 2006; Knowles HJ, et al., 2006) is associated with a increased level of HIF-1 activity in cultured cells, and HIF-1 has been induced *in vivo* by iron depletion (Dongiovanni P, et al., 2008) or starvation (Peyssonnaux C, et al., 2007).

Additionally, the activity of HIF-1 can be increased through phosphorylation and HIF-1α can be directly phosphorylated by p42/p44-mitogen-activated protein kinase (p42/44-MAPK). A study showed that the phosphorylation of threonine 796 enhances the transcriptional response in hypoxia and prevents the hydroxylation of Asn-803 by FIH. Alternatively, another study demonstrated that p42/p44–MAPK can phosphorylate the p300/CBP co-activator, leading to increased HIF-1 transcriptional activity.

Although hypoxia is the main activator of HIF-1, there is an increasing body of evidence demonstrating that a number of non-hypoxic stimuli are also highly able of turning on this transcription factor. Such stimuli include growth factor, cytokines, hormones, viral proteins (Feldser D, et al., 1999; Richard DE, et al., 2000; Tacchini L, et al., 2001; Zhong H, et al., 2000), inflammatory mediators, NO, (bacterial lipopolysaccharide) LPS (Blouin CC, et al., 2004), TNF-α (Dèry MA, et al., 2005) and adenosine (De Ponti C, et al., 2007).

Interestingly, the mechanisms that are involved in activating HIF-1 in hypoxic and normoxic conditions are strikingly different (Déry MA, et al., 2005). The main mechanism implicated in this induction is an increase of both the transcription and translation of the HIF-1α mRNA. The degradation of HIF-1α does not appear to be inhibited when HIF-1 is activated by non-hypoxic conditions. These mechanisms seem sufficient to shift the balance between synthesis and degradation towards a normoxic accumulation of HIF-1α (Déry MA, et al., 2005).

An interesting mechanism proposed suggests that the activation of phosphatidylnositol 3-kinase (PI3K) could increase the rate of HIF-1α translation. This involves the activation of the ribosomal S6 protein by the PI3K/p70S6K/mTOR pathway. P70S6K regulates the translation of a group of mRNAs possessing a 5'-terminal oligopyrimidine tract (5'-TOP), a stretch of 4-14 pyrimidines found at the extreme 5' terminus of certain mRNAs. HIF-1α's 5'-UTR contains these tracts, including a long conserved sequence in the extreme 5' terminus. Phosphorylation of the S6 protein of the 40S ribosomal unit by p70S6K increases the translation of the 5'TOP mRNAs. Some stimuli like vasoactive hormones and LPS can also increase the rate of HIF-1α mRNA transcription. Increases in HIF-1α gene transcription are possibly mediated through activation of diacilglycerol-sensitive protein kinase C (PKC), a kinase known to stimulate Sp1 gene transcription.

It is also important to note that non-hypoxic stimuli also strongly activate p42/p44 mitogen activated protein kinase (p42/p44-MAPK). In this situation, strong HIF-1

induction along robust p42/p44-MAPK activation should lead to elevated HIF-1 activity (Déry MA, et al., 2005).

1.3 THE IRON CHELATOR DEXRAZOXANE

Chelators are small molecules that bind very tightly to metal ions. Some chelators are simple molecules that are easily manufactured (e.g., ethylene diamine tetra acetic acid; EDTA). Others are complex proteins made by living organisms (e.g., transferrin). The key property shared by all chelators is that the metal ion bound to the chelator is chemically inert. Consequently, one of the important roles of chelators is to detoxify metal ions and prevent poisoning.

Iron chelators can be classified using a number of criteria such as their origin (synthetic versus biologically produced molecules), their interaction with solvents such as water (hydrophobic versus hydrophilic) or their stechiometric interaction whit the metal (bidentate versus hexadentate). Some of these properties have an important impact on the clinical utility of a chelator. Iron chelators are mainly used and developed to treat the iron overload in subjects with primary or secondary iron overload, mainly hemochromatotic and thalassemic patients, respectively. However, the use of these compounds as therapeutic agents in the treatment of cancer is envisaged. They may act by depleting iron, a necessary nutrient, thus limiting tumor growth. Alternatively or additionally, they may form redox-active metal complexes that cause oxidative stress via production of reactive oxygen species, damaging critical intracellular targets and thereby eliciting a cytotoxic response again cancer cells. Studies in vitro have evaluated the structure-activity relationships and mechanism of action of many classes of iron chelators and many animal studies have confirmed the antitumor activity of several chelators (Lipshultz SE, et al., 2010).

Dexrazoxane (DRZ) is the only iron chelator clinically approved to prevent anthracycline mediated cardiotoxicity in cancer patients since it has been shown that it can protect the myocardium from anthracycline-induced toxicity under both experimental and clinical condition (Wouters KA, et al., 2005) with long-term effect (Lipshultz SE, et al., 2010).

DRZ has two biological activities: a strong inhibitor of topoisomerase II which prevents the separation of DNA strands during meiosis and also it is a pro-drug that is enzymatically hydrolyzed inside cardiomyocytes to its metal chelating metabolite with EDTA-type structure. In particular, after hydrolysis inside the cell, DRZ initially forms two intermediate products with an open ring (B and C) and then, a metabolite with two

open rings (ADR-925) similar to EDTA and with a strong iron chelator activity (Hasinoff BB, et al., 2007) (FIG. H).

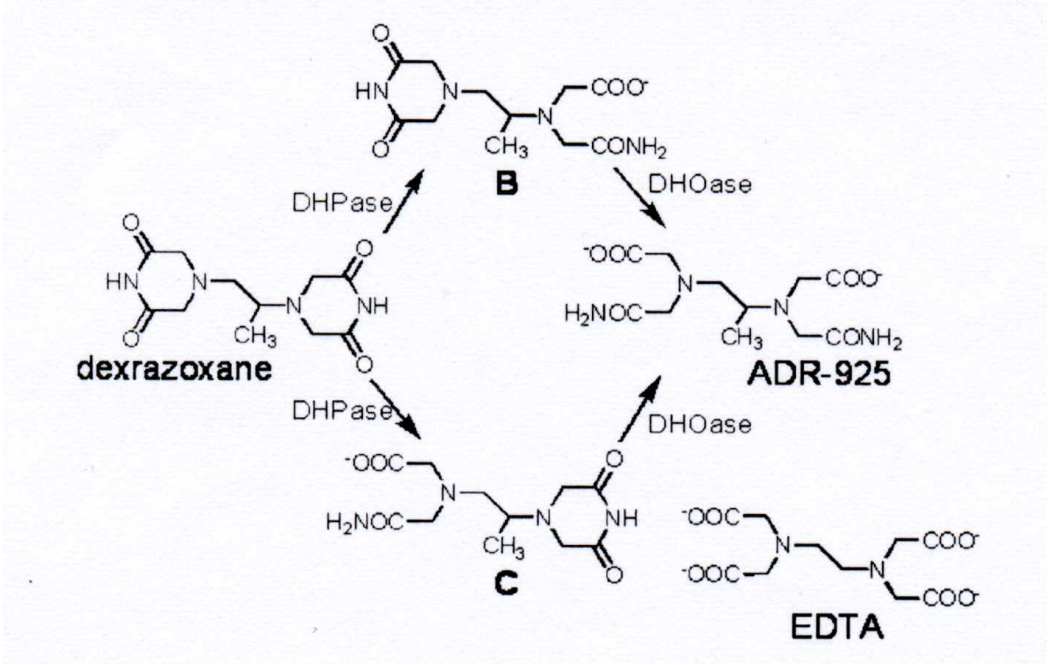

FIG. H

Intracellular metabolism of DRZ

The cardioprotective effect of DRZ has been consistently demonstrated by many studies in vitro, in animal models and in clinical trials. Its protective effect was observed in adults and in children and, importantly, does not reduce the antitumor efficacy of anthracyclines (Pouillart P. 2004).

As it regards the mechanisms underlying the cardioprotective effect of DRZ, in consideration of the chelating properties of DRZ and the role of iron in oxidative stress triggered by DOX metabolism it has been suggested that the action mechanism of DRZ cardioprotection is linked to the decreased iron dependent formation of free O_2 radical.

1.4 PROTECTIVE EFFECTS OF GLUCOSE TRANSPORTERS

The oxidation of glucose represents a major source of metabolic energy for mammalian cells. The cellular uptake of this important nutrient is accomplished by membrane-associated carrier proteins that bind and transfer it across the lipid bilayer (Bell GI, et al., 1990).

Two classes of glucose carriers have been described in mammalian cells: the Na (+)-dependent glucose cotransporter (SGLT, members of a large family of Na-dependent transporters, gene name SLC5A) and the facilitative Na (+)-independent glucose transporters (GLUT family, gene name SLC2A) (FIG. I).

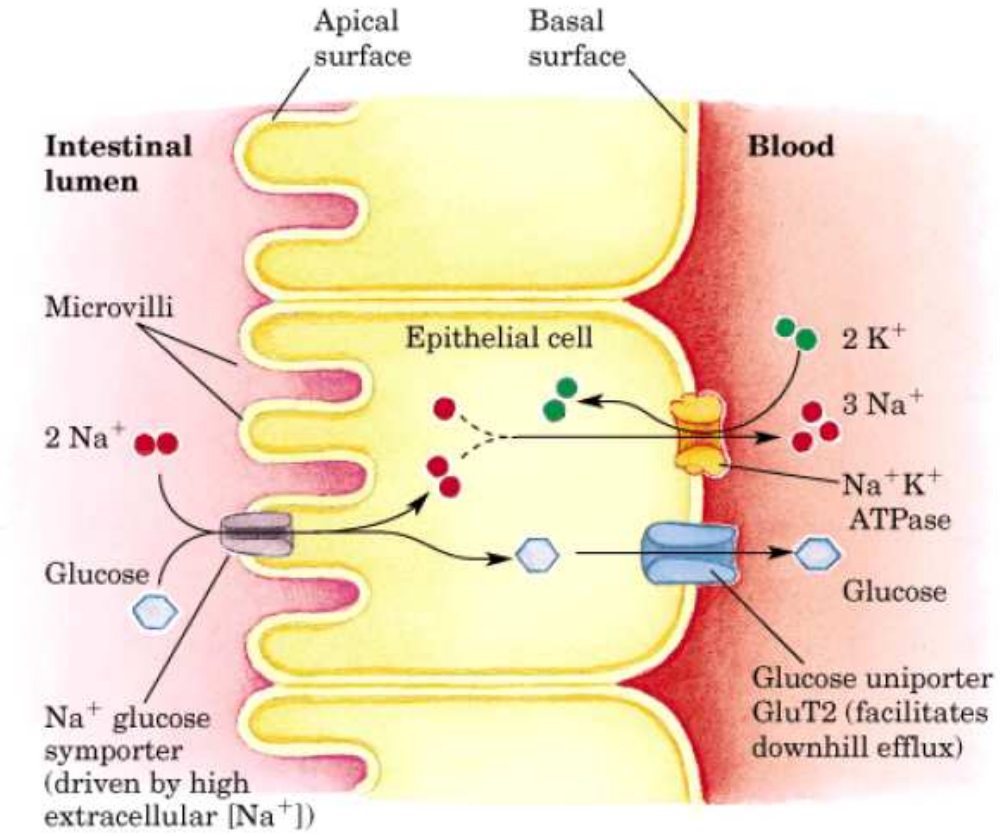

FIG. I

Classical model of intestinal sugar transport

Glucose does not represent only a key fuel and an important metabolic substrate for mammalian cells, as many studies both in vitro and vivo have shown a cytoprotective action of glucose. In particular, in vitro studies found that elevated glucose concentrations in the cell culture medium protected the intestinal epithelial cell line Caco-2 from LPS-induced apoptosis, suggesting that glucose has a cytoprotective action on enterocytes, at least in vitro (Yu LC, et al., 2005; Yu LC, et al., 2006).

The protective effect of glucose is mainly dependent on the activation of the SGLT pathways. SGLT-1 activation protected from damages induced by TLR ligands in intestinal epithelial cells and in a murine model of septic shock. In intestinal epithelial cell lines, glucose inhibited the IL-8/keratinocyte-derived chemokine production and the activation of the TLR-related transcription factor NF-κB stimulated by LPS or CpG-oligodeoxynucleotide (Palazzo M, et al., 2008).

Oral ingestion of glucose was found to protect 100% of mice from lethal endotoxic shock induced by i.p. LPS administration. This protective effects resides in activation of SGLT-1; in fact, the glucose analog 3-*O*-methyl-D-gluco-pyranose, which induces the transporter activity, but is not metabolized, exerted the same inhibitory effects as glucose both in vitro and in vivo (Palazzo M, et al., 2008).

It has been also demonstrated that oral administration of D-glucose, but not of either D-fructose or sucrose, prevents LPS-induced liver injury, as well as liver injury and death induced by an overdose of acetaminophen, also in this case the effect is the likely consequence of glucose-induced activation of the SGLT-1.

In addition, D-glucose was found to protect the liver from alpha-amanitin-induced liver injury although in this case a second signal had to be present in addition to glucose to achieve protective efficacy (Zanobbio L, et al., 2009).

Expression of SGLT1 is mainly seen in intestinal and epithelial cells, but a recent study also characterized SGLT-1 expression in cardiac myocytes (Sanjay K. Banerjee et al., 2009). High levels of SGLT-1 RNA were also found in human cardiomyocytes (Zhou L, et al., 2003) and recent studies affirms that positive inotropic effect in failing human ventricular myocardium are partially substrate-dependent and are stronger in glucose-containing solution (Von Lewinski D, et al., 2010).

In this context, it has been developed a new not metabolized glucoderivative, named BLF50, able to activate SGLT-1 and protect against damages induced by LPS. Given the cytoprotective and anti-inflammatory effects linked to SGLT-1 activation (La Ferla B, et

al., 2010), this new synthetic molecule BLF50, acting at very low dosages, might represent a new and effective drug against various cell injuries (FIG. L).

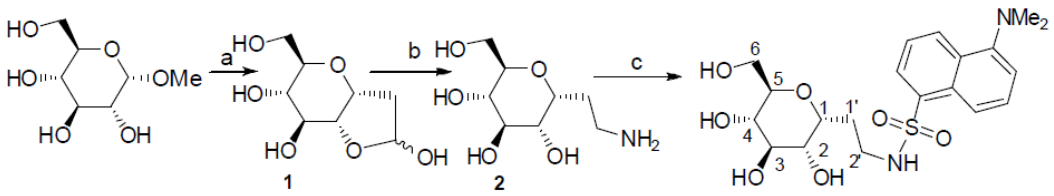

FIG. L

Synthesis and Structure of BLF50

AIM

2. AIM

DOX is effective in the treatment of a variety of malignancies, 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, one of which is the formation of reactive oxygen species (ROS).

Iron aggravates the cardiotoxicity of DOX and the iron chelator DRZ is the only agent protecting against DOX cardiotoxicity; however, the mechanisms underlying the role of iron in DOX-mediated cardiotoxicity and the protective role of DRZ 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, the major aim of my PhD project was to test the hypothesis that HIF activation by DRZ-dependent iron chelation may be involved in the protective effect against DOX-induced toxicity. To this purpose, we tested the cardioprotective effect of DRZ in cells treated with DOX.

These experiments have been performed in the H9c2 embryonic rat heart-derived cell line, that has been shown to represent a reliable model for evaluating various characteristics of cardiomyocytes, including DOX toxicity.

Another aim of my project was to investigate two other possible alternative pharmacological strategies to prevent DOX-induced toxicity were also explored.

The first one was based on the demonstration of the involvement of HIF and was aimed at evaluating whether small molecular mimics of hypoxia could be exploited in an attempt to limit anthracycline cardiotoxicity. To this purpose, HIF levels and activity, as well as protection from DOX damage, in H9c2 cardiomyocytes pre-exposed to DMOG, an antagonist of α-ketoglutarate that activates HIF under normoxic conditions, were examined.

In the second approach, the protective activity of the sodium-dependent glucose transporter-1 (SGLT-1), which has been shown to exert protective effects against different types of injuries in various cell types, was evaluated

METHODS

3. METHODS

3.1 CELL CULTURE AND TREATMENTS

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% CO2 in Dulbecco's modified minimal essential medium adjusted to contain 4 mM glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 1 mM sodium pyruvate, 100 U/mL penicillin and 0.1 ng/mL streptomycin, supplemented with 10% heat inactivated fetal calf serum. Subconfluent cells were treated for 24 hours with 0.5 µM DOX (Pharmacia, Milan, Italy) in complete growth medium. When appropriate, various concentrations of DRZ (Sigma, Milan, Italy) were added to the culture medium for 3 hours, followed or not by DOX treatment.

Cells were also exposed to 100 µM DFO (Sigma) or 1 mM dimethyloxalyl glycine (DMOG; Alexis Biochemicals, Lausen, Switzerland) for 24 hours, 1 mM buthionine sulphoximine (BSO) for 3 h and 100 µM H_2O_2 for 3 hours (all from Sigma). When appropriate, cells were treated for 3 or 24 hours with various concentrations of DMOG (Alexis Biochemicals).

Cells were also treated for 24 hours with D-Glucose 9 g/L, 13.5 g/L, 22.5 g/L, with 3-O-methylglucose at the same concentrations of D-Glucose (all from Sigma) and with 1,1 µM, 0.11 µM, 0.011 µM BLF50 (La Ferla B, et al., 2010). At the end of the various treatments, the medium was removed, and the cells were washed with phosphate-buffered saline (PBS) and used for several assays.

3.2 TRANSIENT TRASFECTION ASSAY

Subconfluent H9c2 cells maintained in complete medium were transfected with the following plasmid constructs:

- pGL3PGK6TKp vector (a kind gift of PJ Ratcliffe, Oxford, UK), which contains an HRE multimer (Tacchini L, et al., 2003);
- the expression vector pcDNA3ARNTdelta_b (ΔARNT) (obtained from M Schwarz, Tübingen, Germany), which codes for the dominant-negative mutant form of the HIF-1β ARNT subunit (Tacchini L, et al., 2004);
- the expression vector pCMV4HIF-1α, which codes for HIF-1α (kindly provided by Dr Wenger, Leipzig, Germany) using TransIT^{TM} LT1 (Mirus, Bologna, Italy).

Six hours after transfection, the cells were washed with PBS, the culture medium was replaced by fresh medium and the cells were exposed to the various treatments.

3.3 SHORT HAIRPIN RNA KNOCKDOWN

Short hairpin RNA (shRNA) constructs against Mus musculus HIF-1a (catalogue number TR517255) were purchased from Origene Technologies, Inc. (Rockville, MD, USA). The targeted sequences were:

CTGTTCACCAAAGTTGAATCAGAGGATA(#1)
CTTCTGTTATGAGGCTCACCATCAGTTA(#2)
TCAAGAAACGACCACTGCTAAGGCATCA(#3)
TTACCTTCATCGGAAACTCCAAAGCCACT(#4)
(Tacchini L, et al., 2008; Gammella E, et al., 2010).

H9c2 cells maintained in complete medium were plated onto T25 flasks (1 x 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-1α-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 DOX. 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 verify transfection efficiency, the cells were transfected with a rhodaminelabelled siRNA (Qiagen SpA, Milano, Italy), fixed and observed using fluorescence microscopy (excitation 530 nm, emission 570 nm); nuclei were counterstained with 10 µg/mL 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.

3.4 GENE REPORTER ASSAY

Subconfluent H9c2 cells maintained in complete medium were transfected in six-well multiwell plates using TransIT^{TM} 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 (ΔARNT) 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 L, et al., 2003). The empty vectors showed practically undetectable luciferase activity.

All of the transfection experiments were carried out in duplicate.

3.5 IMMUNOBLOTTING

To detect the expression of aldolase A, survivin, Mcl1, haem oxygenase (HO-1), P-glycoprotein (Pgp), BclxL and α-tubulin, the cells were homogenized in 10 mM HEPES,

pH 7.6, 3 mM $MgCl_2$, 40mM KCl, 5% glycerol, 0.2% Nonidet P40 (Sigma), 1 mM dithiothreitol (DTT) and a protease inhibitor cocktail (Sigma).

Cells lysate was centrifuged at 16000x g for 5 minutes at 4°C and the supernatant was saved for immunoblotting analysis. Nuclear extracts for the determination of HIF-1α, HIF-2 α and transcription factor II D (TFIID) were prepared as previously described (De Ponti C, et al., 2007). In order to analyse cytochrome c release, the cells were resuspended in 500 mM sucrose, 2 mM NaH_2PO_4, 16 mM Na_2HPO_4, pH 7.6, 150 mM NaCl, 1 mM DTT, a protease inhibitor cocktail and 10 µg digitonin per 10^6 cells were added with vortexing. The heavy organelles and cell debris were pelleted at 14000x g for 60 seconds at 4°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 electrophoresis in acrylamide-SDS gels and electroblotted onto Hybond ECL membranes (Amersham Co., Milan, Italy).

After assessing transfer and the correct loading protein by means of Ponceau S staining, the membranes were incubated with antibodies against HIF-1α (H1α 67, 1:1000, Novus Biologicals, Littleton, CO, USA), HIF-2 α (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), SGLT-1 (1:1000, Cell Signaling) and α -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 α-tubulin or TFIID, which is an essentially nuclear protein.

3.6 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 x 10^6 cells per sample were lysed in 50 µL lysis buffer, and the protein concentrations in the samples were estimated using the Bio-Rad protein assay.
After incubation on ice for 10 minutes, the samples were centrifuged at 16000x g for 3 minutes at 4°C. Each supernatant was mixed with 50 µL of a 2X Reaction Buffer/DTT mix and 5 µL of 1 mM caspase-3 substrate (DEVDpNA, 50 µM final concentration) and the samples were then incubated for 1 hour at 37°C in the dark. Developed colour was measured at 405 nm and caspase activity was calculated in terms of absorbance units per µg protein.

3.7 ANNEXIN V ASSAY

Externalization of phosphatidylserine to the outer side 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 minutes in the dark with Annexin V-FITC and propidium iodide. Then cells were 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 using three coverslips for each experimental group.

3.8 MTT ASSAY

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

3.9 STATISTICS

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

RESULTS

4. RESULTS

4.1 DEXRAZOXANE INDUCES HIF BINDING ACTIVITY AND TRANSACTIVATION CAPACITY IN H9c2 CELLS

Considering that the depletion of cellular iron stores leads to the induction of HIF-1 (Peyssonnaux C, et al., 2008; Mole DR. 2010; Wang GL and Semenza GL. 1993; Bianchi L, et al., 1999), we investigated whether exposure to the iron chelator DRZ activates HIF-1 in cardiomyocytes.

Immunoblot analysis of nuclear extracts of H9c2 cells showed that exposure to DRZ for 3 hours increased HIF-1α protein levels. The activation was detectable at 10 μM and there was no additional increase at 100 μM (Figure 1A). Similar activation was found in extracts of cells exposed to the iron chelator deferoxamine (DFO), a well-known inducer of HIF-1 (Wang GL and Semenza GL. 1993). Under the same experimental conditions, HIF-2α (which is also detectable in untreated cells) was also induced but to a lesser extent than HIF-1α (Figure 1A).

We then used transactivation capacity experiments to determine whether the HIF subunits induced by DRZ were transcriptionally actived. 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 C, et al., 2007; Tacchini L, et al., 2008), the expression of the reporter gene increased about threefold in response to DRZ and about fivefold in response to DFO (Figure 1B). Further indications of the involvement of HIF in the DRZ- and DFO-dependent activation of luciferase activity were obtained by experiments in which HIF transactivating capacity was almost completely abolished by the cotransfection of a plasmid expressing a dominant negative of the HIF-1β subunit (ΔARNT), which maintains the capacity of forming a heterodimer but cannot bind DNA (Tacchini L, et al., 2004, 2008) (Figure 1B).

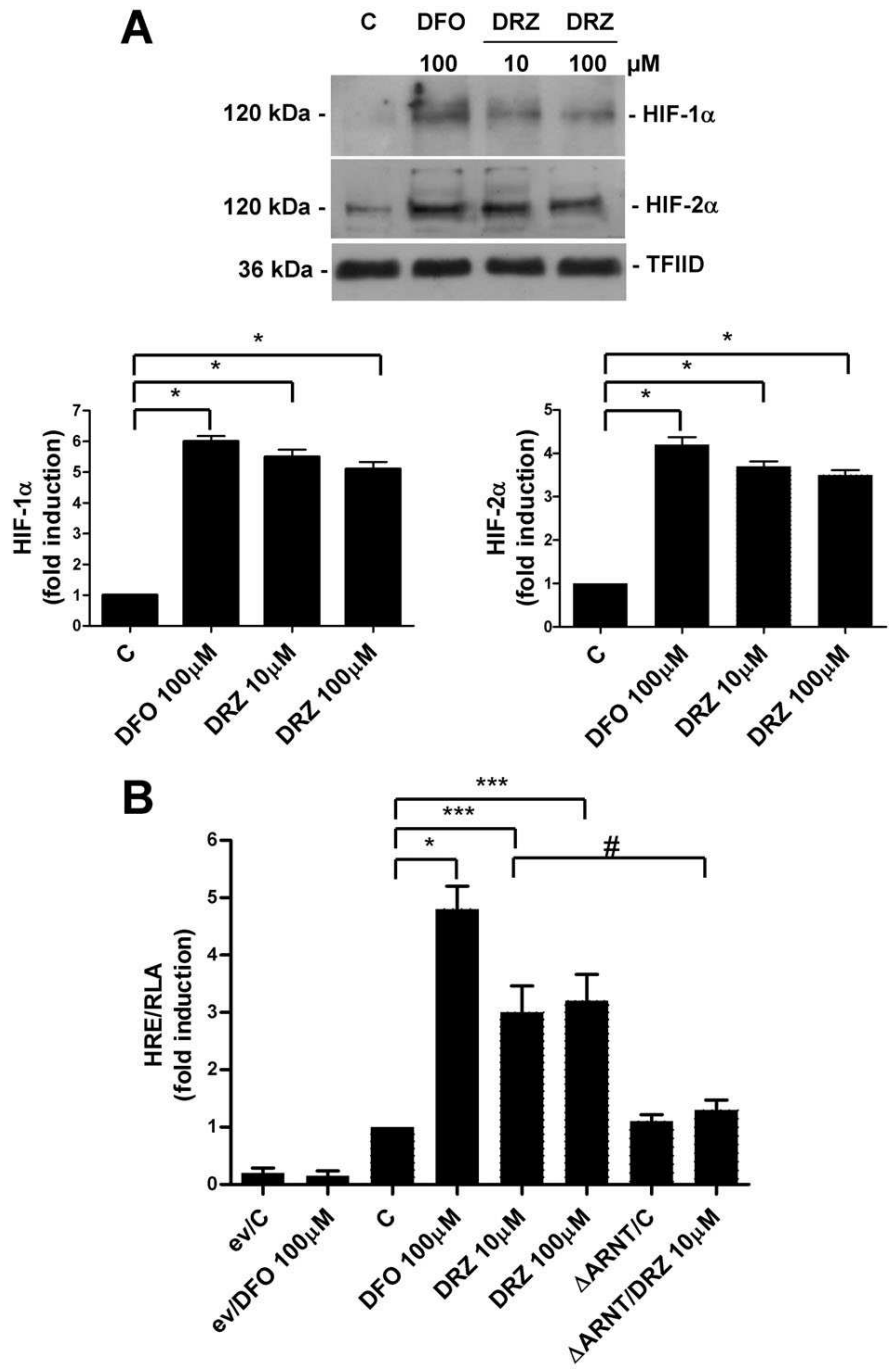

Figure 1

Dexrazoxane (DRZ) induces HIF-α 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-1α and anti-HIF-2α 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 DRZ, 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-1β subunit (ΔARNT). 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 ± 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.

4.2 PRE-EXPOSURE TO DEXRAZOXANE PREVENTS DOXORUBICIN-MEDIATED APOPTOTIC CELL DEATH

To investigate the cytoprotective activity of DRZ, H9c2 cells were exposed to 0.5 µM DOX, a concentration within the range of the plasma levels found in patients undergoing chemotherapy (Gianni L, et al., 1997). MTT assays showed that 24 hours treatment with DOX reduced cell viability by 50% (Figure 2A).

We also assessed whether exposure to the DRZ concentration that was sufficient to activate HIF prevented cell death in H9c2 cardiomyocytes treated with 0.5 µM DOX. Figure 2A shows that the cells pretreated with 10 µM DRZ were significantly protected as 77% of the cells were viable after exposure to DOX.

In line with previous reports (Bernuzzi F, et al., 2009), the cells exposed to 0.5 µM DOX did not show 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).

Since previous results indicated that apoptosis is the prevalent form of cell death in H9c2 cells exposed to low DOX doses (Sawyer DB, et al., 1999; Reeve JL, et al., 2007; Bernuzzi F, et al., 2009), we decided to check the activity of caspase-3 (a major effector protein of apoptosis) and we found that it was double that observed in untreated cells at 0.5 µM DOX and returned to control values in cells pre-incubated with DRZ (Figure 2B). We also used another assay to evaluate apoptosis and Figure 2C shows that DRZ pretreatment also counteracted the increase in DOX-induced apoptotic cell death assessed by measuring Annexin V binding to externalized phosphatidylserine (Brumatti G, et al., 2008).

As the mitochondrion-mediated apoptotic pathway is important in DOX-induced apoptosis (Minotti G, et al., 2004), we also measured cytochrome c release. Figure 2D shows that expression of cytochrome c increased about twofold in cells treated with 0.5 µM DOX, while a decreasing expression is observed in cells treated with DRZ plus DOX.

Exposure to DRZ alone did not significantly affect apoptosis (Figure 2B–D).

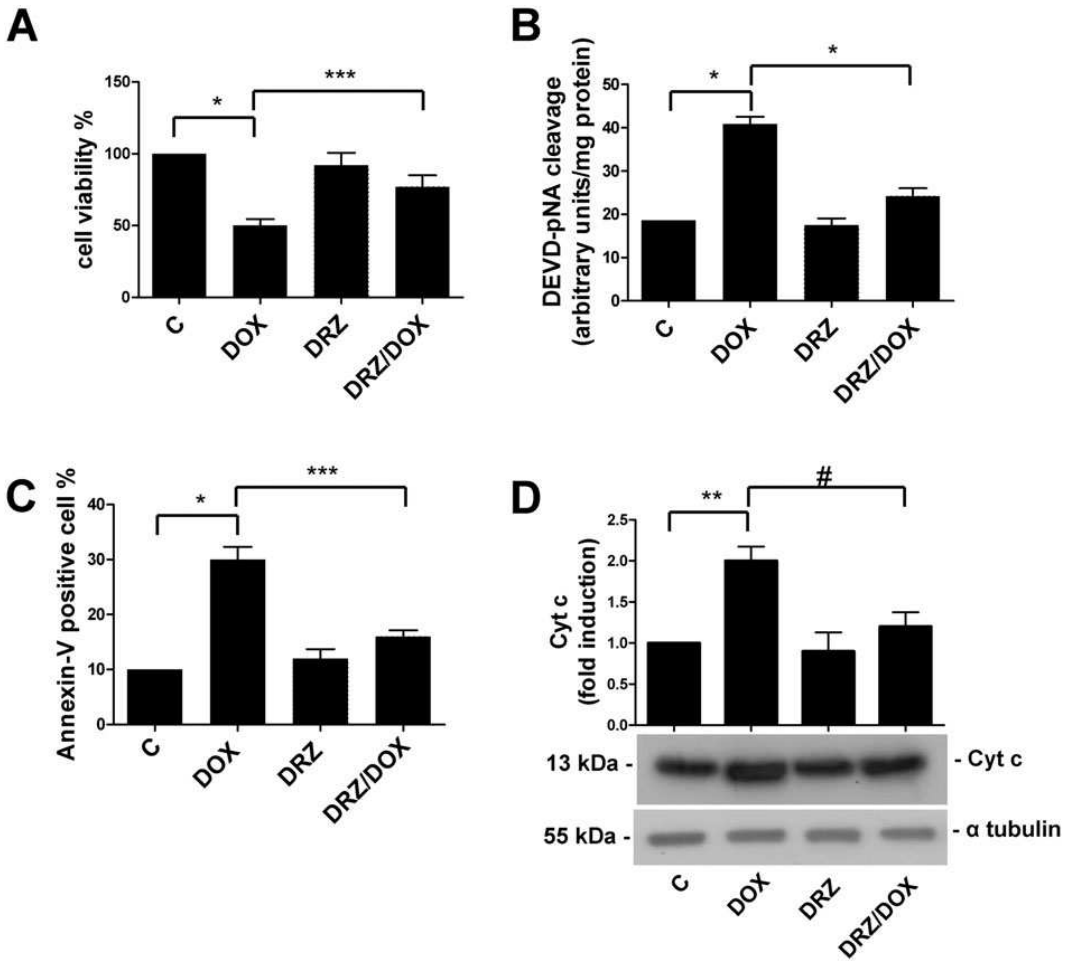

Figure 2

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 DRZ alone or pre-treated with DRZ for 3 h and then exposed to DOX. 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. α-Tubulin was used as a loading control. The figure shows one representative immunoblot and the densitometric quantification relative to *C*-values. Mean values ± 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.

4.3 THE PROTECTIVE EFFECT OF DEXRAZOXANE DEPENDS ON HIF-1 ACTIVITY

In order to investigate the role of HIF-1 in DRZ-mediated cardioprotection directly, we investigated the capacity of DRZ to prevent DOX toxicity in H9c2 cells lacking HIF-1 activity. First, we performed a control experiment in order to check whether the induction of HIF-1 transcriptional activity found in cells exposed to DRZ was maintained in cells exposed to DRZ plus DOX because DOX, which affects the expression of muscle-specific genes (Ito H, et al., 1990) and HIF-1-dependent transcriptional activity (Lee K, et al., 2009), may blunt HIF-1 activation and thus impair the protective effect of iron chelation.

However, Figure 3A shows that HIF-1α protein levels were similar in the cells exposed to DRZ and those exposed to DRZ plus DOX, as expected. Moreover, the luciferase activity driven by the multiple HRE sequences was slightly inhibited in the cells exposed to DRZ plus DOX in comparison with those treated with DRZ alone but was still significantly higher than in the untreated cells (Figure 3B).

Having demonstrated that HIF-1 is activated in H9c2 cells exposed to 0.5 μM DOX and DRZ, we evaluated cell viability in H9c2 cells transfected with the dominant negative HIF-1β subunit ΔARNT and exposed to DOX with or without DRZ pretreatment.

MTT assays revealed that the protective effect of DRZ was lost in the transfected cells as mortality was not significantly higher than in the cells exposed to DOX without DRZ pretreatment. Also in this case exposure to DRZ alone did not significantly affect cells viability (Figure 4A).

This results indicate that HIF plays a role in the DRZ-mediated protection of H9c2 cells. Similarly, a protective effect of DRZ was found when caspase 3 activity was measured to see the effect on apoptosis (Figure 4B).

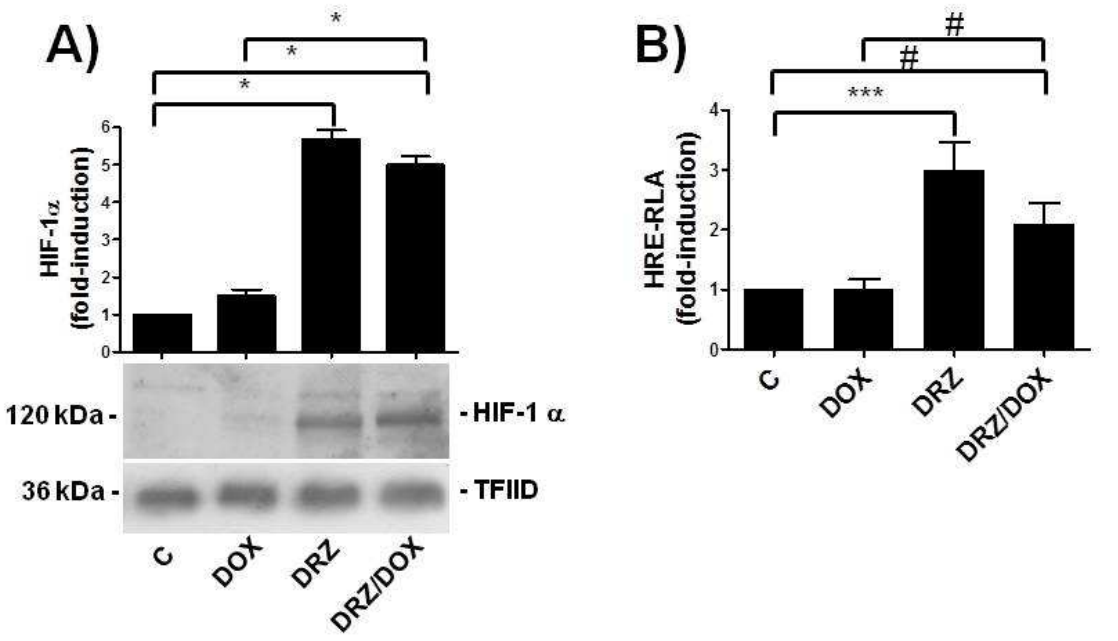

Figure 3

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 DOX, for 3 h to dexrazoxane (DRZ) alone or pre-treated with DRZ for 3 h and then exposed to DOX, using the anti-HIF-1α 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 ± SD. **P* < 0.001; ****P* < 0.01; #*P* < 0.05, *n* = 3. HIF, hypoxia-inducible factor; TFIID, transcription factor II D.

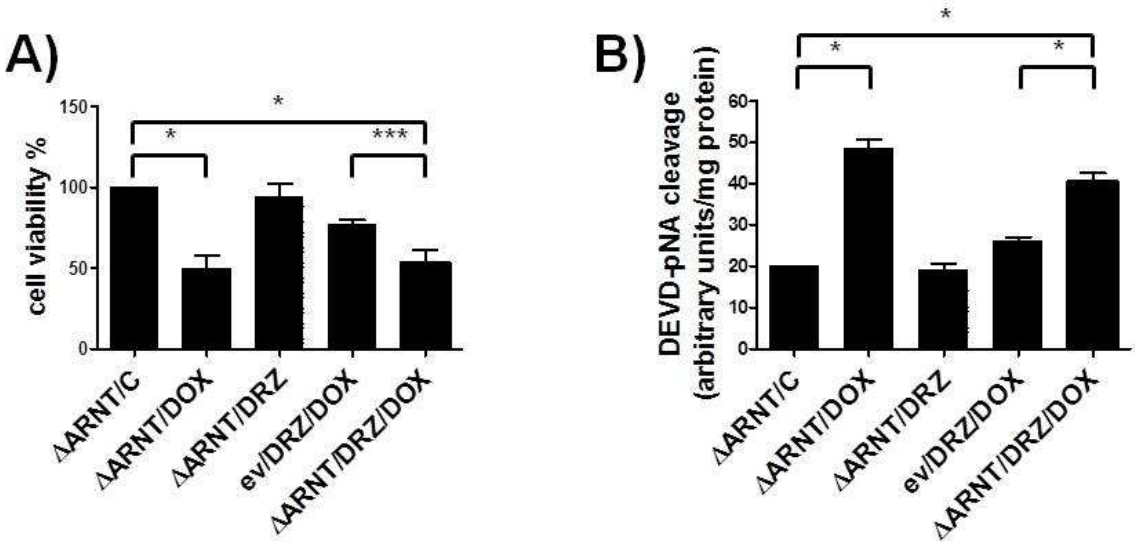

Figure 4

Suppression of HIF activity blocks dexrazoxane (DRZ)-mediated cardioprotection. **(A)** H9c2 cells were transfected with the expression vector ΔARNT 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 ± SD. **P* < 0.001; ****P* < 0.01; *n* = 5. MTT, 3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

In order to verify further the role of HIF-1 in the DOX protection of DRZ-pretreated H9c2 cells, we used shRNA technology to specifically knockdown HIF-1α.

First, we tested the transfection efficiency by using a rhodamine-labelled siRNA. Figure 5A shows a merged image with DAPI-stained nuclei (blu) and extranuclear punctuate fluorescence of rhodamine-labelled siRNA (red).

The efficient (84±5%, see Figure 5A) transfection of H9c2 cells with a set of four expression vectors coding shRNAs against HIF-1α led to a reduction in HIF-1 protein levels (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 ΔARNT dominant negative, the knockdown of HIF-1 abolished the protection offered by DRZ. Transfecting cells with a vector containing a non-effective shGFP sequence cassette, we did not find appreciable cytoprotection (Figure 5C).

In order to demonstrate further that HIF-1 is important for the protective effect of DRZ, we investigated whether HIF-1 activation also provides cardioprotection from DOX in cells not exposed to the iron chelator.

Transfection with an expression vector coding for HIF-1α resulted in greatly elevated HIF-1 protein levels and markedly stimulated HRE-dependent transcription as shown in Figure 6A.

Then we checked whether the overexpression of HIF-1 play a role in the protection of H9c2 cells treated with DOX in absence of DRZ.

Cells transfected with the expression vector coding for HIF-1 shows a significant cytoprotection from DOX-mediated cell death as revealed by MTT assay(Figure 6B).

As expected, we also observed a reduction of DOX-mediated apoptosis by caspase 3 assays (Figure 6C).

These experiments demonstrated that HIF-1α overexpression protects H9c2 cardiomyocytes from DOX-induced toxicity in the absence of DRZ.

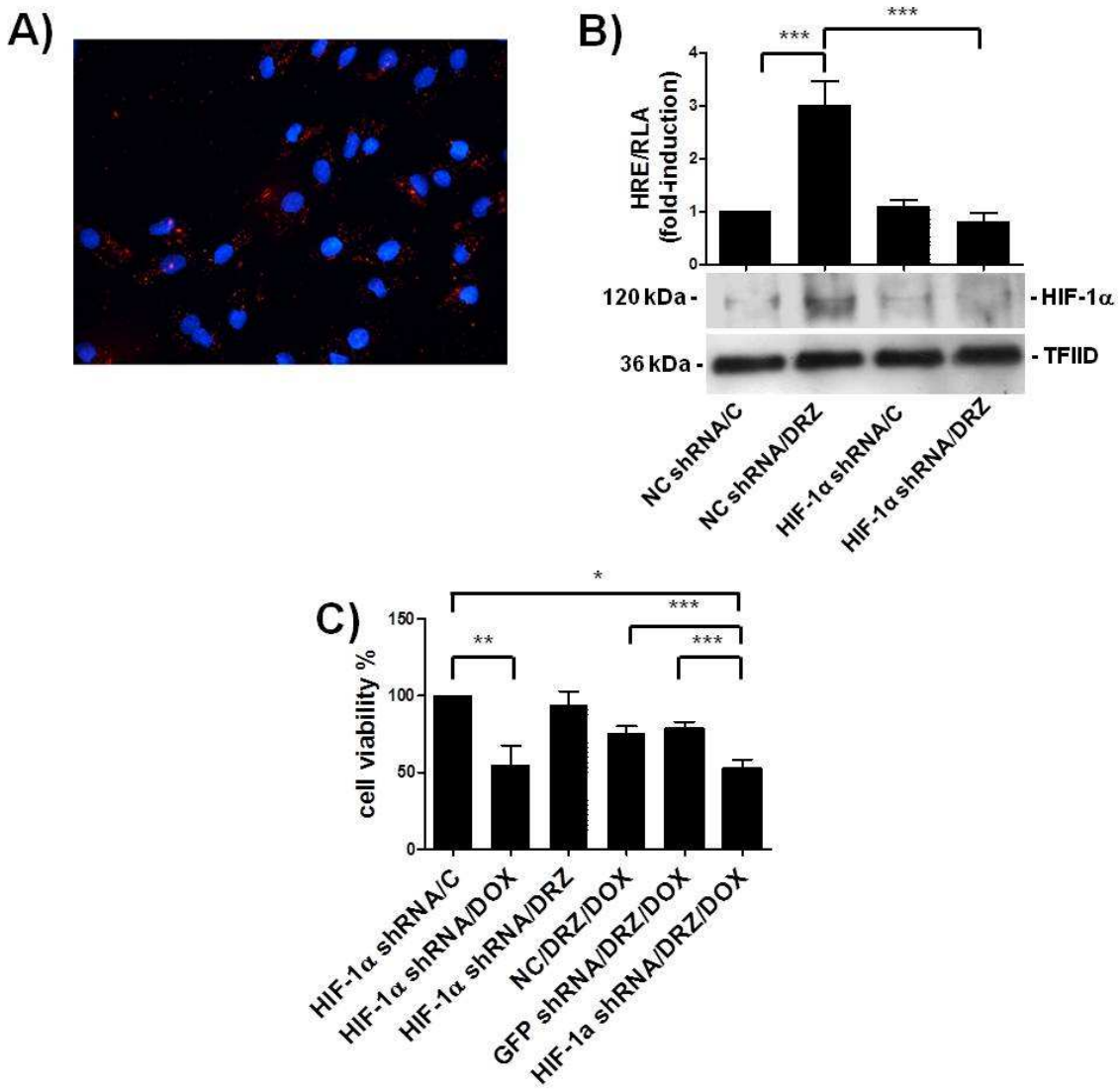

Figure 5

Knockdown of HIF-1α 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-1α protein levels in untreated H9c2 cells (C) or cells exposed to DRZ 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-1α (HIF-1α 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-1α 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-1α (HIF-1α shRNA) and treated as indicated in Figure 3A. Cell viability was evaluated by means of the MTT assay. Mean values ± 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.

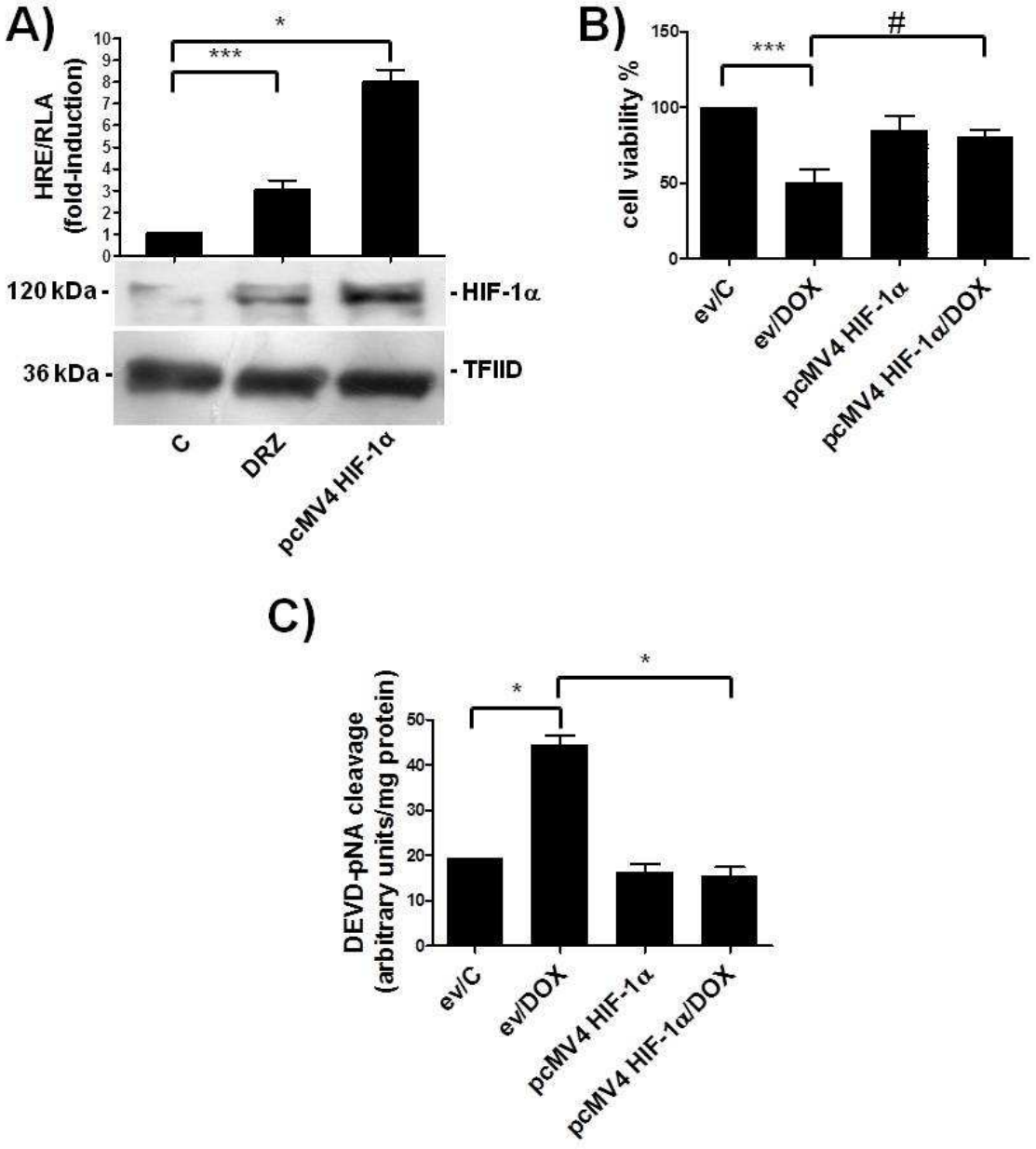

Figure 6

HIF-1α overexpression is cardioprotective in the absence of dexrazoxane (DRZ). **(A)** Relative luciferase activity (RLA) and HIF-1α protein levels in untreated H9c2 cells (C), exposed to DRZ for 3 h or transfected with a construct that induced the overexpression of HIF-1α (pcMV4 HIF-1α). 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-1α 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-1α 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 ± 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.

4.4 EFFECT OF DEXRAZOXANE ON THE EXPRESSION OF HIF TARGET GENES IN H9c2 CELLS

In order to investigate further the transcriptional function of HIF activation in DRZ-treated cells, we examined the expression of endogenous genes known to be under the transcriptional control of HIF in cells exposed to DRZ plus DOX. The immunoblots in Figure 7A show that the levels of aldolase A, a typical HIF target gene (Semenza GL, et al., 1996), increased after DRZ plus DOX treatment as expected and returned to control levels in the cells transfected with ΔARNT. Moreover, we didn't find increased levels of this protein after treatment with DOX alone.

Having demonstrated that DRZ prevents apoptosis (see Figure 2), we investigated whether HIF target genes, that may play a role in favouring cell survival after DOX-mediated damage, were induced in DRZ-treated H9c2 cells.

We therefore examined a variety of anti-apoptotic genes induced by HIF such Mcl1, survivin and haem oxygenase (HO-1) (Craig RW. 2002; Bernuzzi F, et al., 2009; Guha M and Altieri DC. 2009).

Immunoblot analysis showed that exposure to DRZ plus DOX increased the levels of all of these anti-apoptotic proteins and that this increase was significantly prevented by ΔARNT expression (Figure 7B–D).

Since it has been shown that mice overexpressing manganese superoxide dismutase (MnSOD), which is an HIF-2α target gene (Scortegagna M, et al., 2003), are protected from DOX-induced acute toxicity (Yen HC, et al., 1996), we also examined the expression of this antioxidant enzyme.

However, MnSOD levels, which increased as expected in H9c2 cells undergoing oxidative stress obtained by exposure to H_2O_2 or the glutathione-depleting compound BSO, were not significantly affected by DRZ alone or combined with DOX (Figure 7E).

As DOX is a substrate of the Pgp, a multidrug resistance (MDR)–related membrane efflux pump that transports a variety of xenobiotics (Takara K, et al., 2006) and is regulated by HIF-1 (Comerford KM et al., 2002), we assessed whether Pgp could play a role in the HIF-mediated cardioprotection offered by DRZ. This could represent and very simple and effective mechanism to avoid the toxic effects of DOX.

However, Pgp protein expression was not significantly modulated by exposure to DOX or DRZ alone. Also exposure to DRZ plus DOX did not increase the levels of this protein. Morover, 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 (Figure 7F).

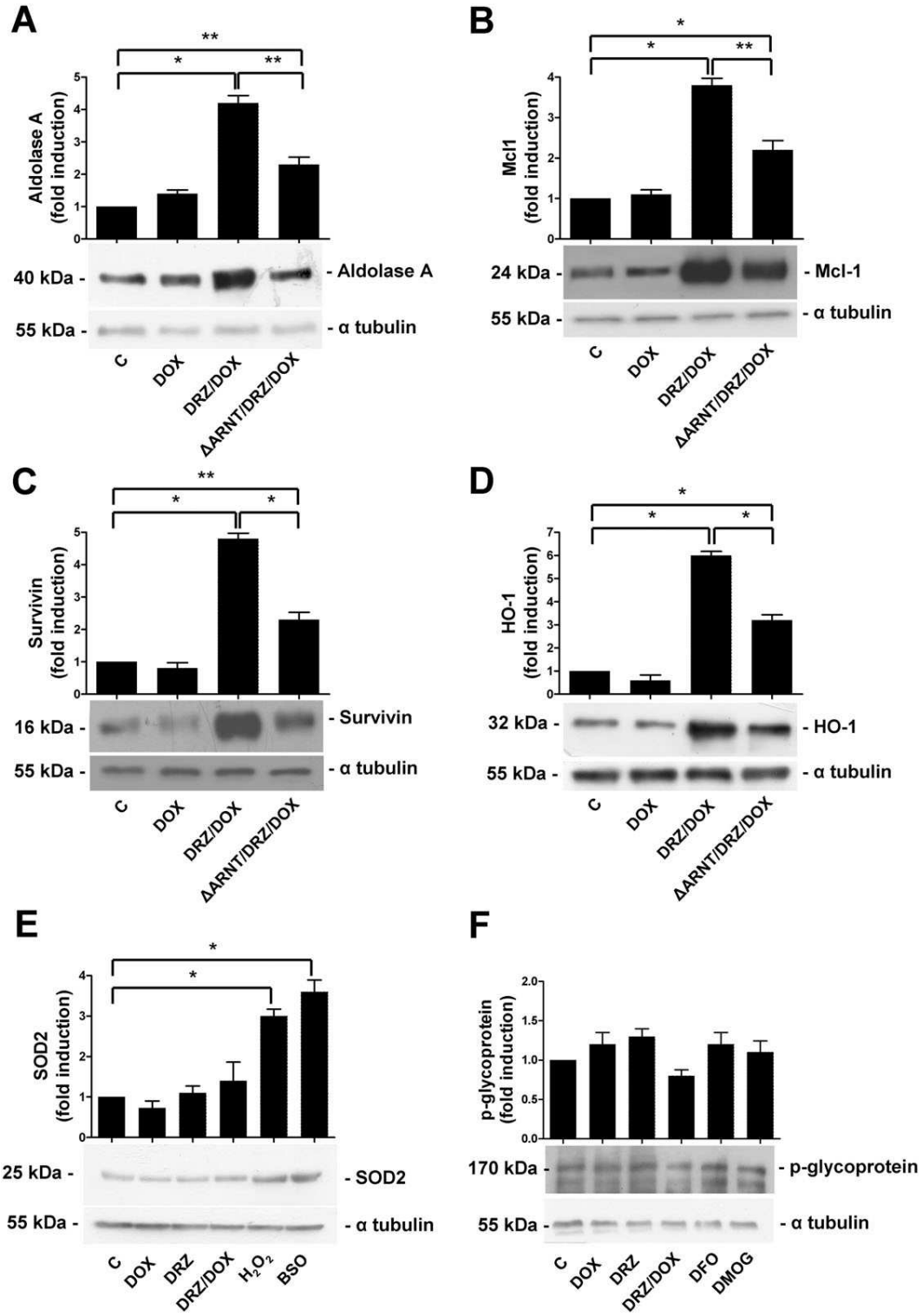

Figure 7

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 DRZ alone or pretreated with DRZ for 3 h and then exposed to DOX. When appropriate, the cells were also transfected with the expression vector ΔARNT. In some cases, the cells were exposed to DMOG for 24 h or to BSO and H_2O_2 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 α-tubulin as a loading control. The panels show one representative blot and the densitometric quantification relative to *C*-values. Mean values ± SD. **P* < 0.001; ***P* < 0.005, *n* = 3. DMOG, dimethyloxalyl glycine; BSO, buthionine sulphoximine.

4.5 PRE-EXPOSURE TO DMOG DID NOT PREVENT DOXORUBICIN-MEDIATED APOPTOTIC CELL DEATH

In order to investigate possible alternative pharmacological strategies to prevent DOX-induced toxicity, we examined a small molecular mimic of hypoxia such as dimethyloxalyl glycine (DMOG), which is a cell-permeable analog of 2-oxoglutarate able to inhibit the 2-Oxoglutarate-dependent hydroxylase enzymes, and thus prevents the hydroxylation and degradation of HIF-1α (Jaakkola P, et al., 2001).

Preliminary MTT assays showed that 3 hours treatment with 1 and 0.5 mM DMOG, which are concentrations normally used in the literature (Ockaili R, et al., 2005), reduced significantly H9c2 cells viability, while DMOG toxicity decreased when H9c2 cells were exposed to lower concentrations (0.2 and 0.1 mM) (Figure 8A).

We then assessed whether exposure to low concentrations of DMOG was sufficient to activate HIF and prevent cell death in H9c2 cardiomyocytes treated with 0.5 µM DOX.

In H9c2 cells transiently transfected with a luciferase reporter gene controlled by a DNA fragment containing multiple consensus HREs, the expression of the reporter gene increased about twofold after 3 hours exposure to 0.2 mM DMOG and about threefold after 24 hour exposure to 0.2 mM DMOG, in comparison to an about fivefold increase in response to DFO. At lower concentrations the expression of the luciferase gene was not significantly changed (Figure 8B).

Having established that 3 and 24 hour treatment with 0.2 mM DMOG was able to induce HIF-dependent transcriptional activity, we investigated the cytoprotective activity of these concentrations of DMOG in H9c2 cardiomyocytes treated with 0.5 µM DOX. Figure 8C shows that cells pretreated with 0.2 mM DMOG were not significantly protected after exposure to DOX.

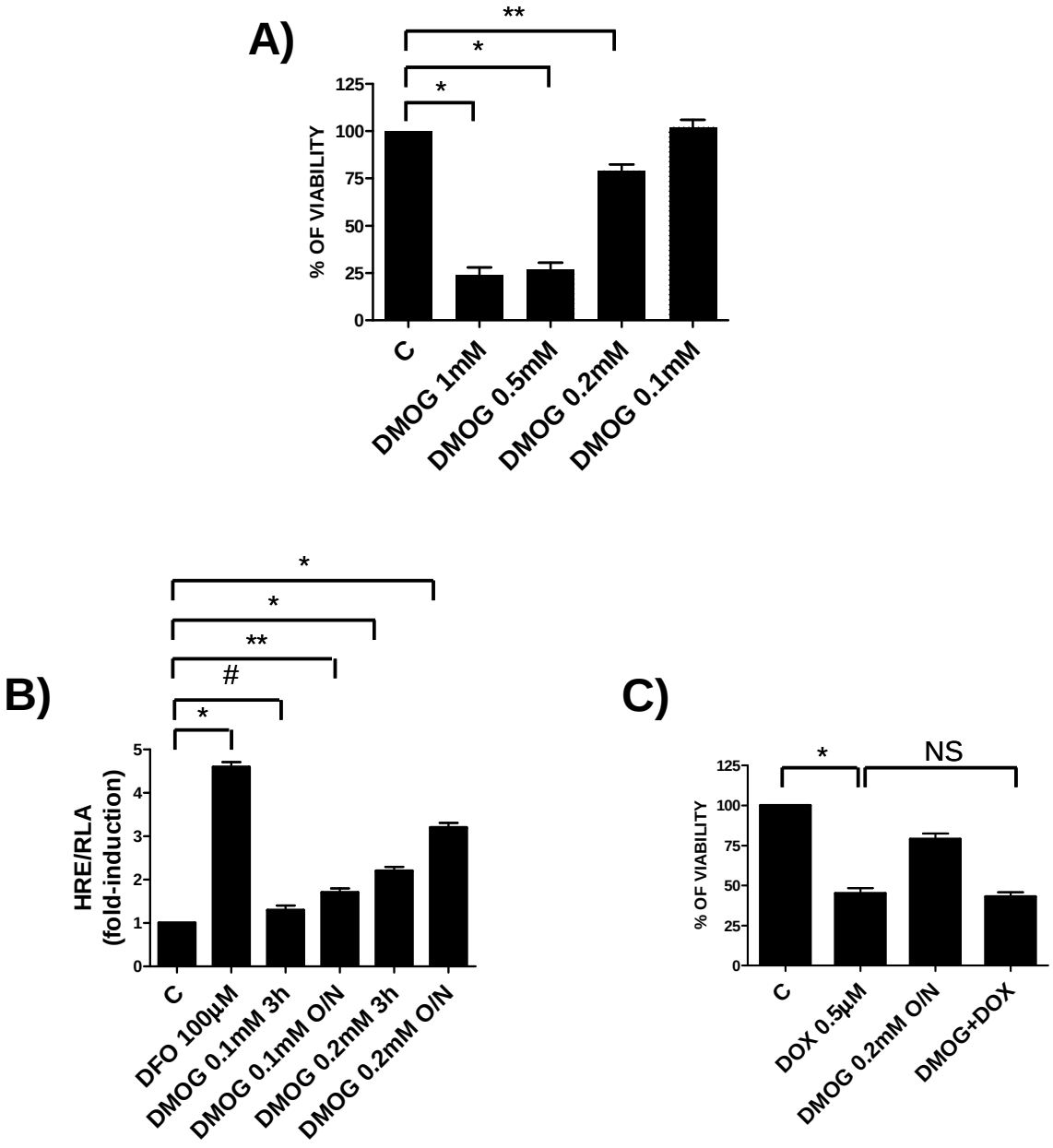

Figure 8

Dimethyloxalyl glycine (DMOG) did not prevents DOX-mediated toxicity in H9c2 cells. **(A)** H9c2 cells were left untreated (C), or exposed for 3 h to different concentration of DMOG. Viability was evaluated by the MTT assay and DMOG toxicity was calculated as the percentage of viable cells after drug exposure. **(B)** Relative luciferase activity (RLA) in H9c2 cells transiently transfected with a construct in which luciferase was controlled by an HRE multimer and treated with desferrioxamine (DFO) for 24 h or different concentrations of DMOG for 3 or 24 h. 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). **(C)** H9c2 cells were left untreated (C) ore exposed for 24 h DOX, for 24 h to DMOG alone or pretreated with DMOG for 24 h and then exposed to DOX. Viability was evaluated by means of the MTT assay. Mean values ± SD. $*P < 0.001$; $**P < 0.005$; $^{NS}P > 0.05$, $n = 3$. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; HRE, hypoxia response element.

4.6 ROLE OF SGLT-1 IN PROTECTING CARDIOMYOCYTES FROM DOX TOXICITY

In order to explore possible alternative ways to prevent DOX cardiotoxicity, we investigated the cytoprotective activity of several molecules able to activate the sodium-dependent glucose transporter-1 (SGLT-1) which is a member of a large family of Na-dependent transporters (gene name SLC5A) and has been shown to exert a protective effects from different types of injuries in various cell types (Yu LC, et al., 2005; Yu LC et al., 2006; Zanobbio L, et al., 2009; Palazzo M, et al.,2008).

Expression of SGLT1 is mainly seen in intestinal and epithelial cells, although a recent study also characterized SGLT-1 expression in cardiac myocytes (Sanjay K. Banerjee et al., 2009). In this context, we first verified whether SGLT-1 was expressed in H9c2 cells. Figure 9A shows that SGLT-1 is detectable in H9c2 cells lysates, although its expression is lower than in the rat intestinal epithelial cell line IEC-6 which was used as a positive control.

In order to understand whether SGLT1 activation could have a protective role also in our experimental system, H9c2 cells were pretreated with D-Glucose (D-GLU), the non-metabolizable glucose analog 3-O-methylglucose (3-OMG), and the SLGT-1 agonist BLF50 and then exposed to 0.5 μM DOX.

We initially exposed H9c2 cells to concentrations of 9, 13.5 and 22.5 g/L D-GLU that are 2, 3 and 5 times greater than the concentration of glucose in the standard culture medium for H9c2 cells (4.5 g/L).

MTT assays revealed that 24 hours pretreatment with 22.5 g/L D-GLU prevented DOX-mediated cell death whereas pretreatment with 13.5 g/L and 9 g/L D-GLU, did not significantly protect H9c2 cardiomyocytes from DOX toxicity (Figure 9B).

In order to understand whether the protective effect of D-GLU was due to increased cellular availability of glucose or to activation of SGLT-1, we successively tested whether 22.5 g/L 3-OMG was able to protect H9c2 cells after exposure to DOX. Figure 9C shows that 22.5 g/L 3-OMG, which reduced cell viability by about 10% when present alone, did not significantly protect H9c2 cardiomyocytes from DOX-mediated cell death.

Given the small but reproducible cytotoxic effect of 22.5 g/L 3-OMG we then tried to see whether lower concentrations of 3-OMG were able to decrease the damage induced by DOX; however, also 9 g/L and 13.5 g/L 3-OMG seem to exhibit a form of slight toxicity

in cardiomyocytes and thus also these lower concentrations failed to show any significant cytoprotective effect (Figure 9D).

BLF50 is a new not-metabolized synthetic glucoderivative that act as a potent activator of SGLT-1 at very low dosages. Given the cytoprotective and anti-inflammatory effects linked to SGLT-1 activation (La Ferla B, et al., 2010), we investigated the cytoprotective activity of various concentrations of this SGLT-1 agonist.

MTT assays in figure 9E show that 24 hours pretreatment with BLF50 0.011µM protected H9c2 cells from DOX toxicity, possibly because treatment with 0.011µM alone increased significantly cell viability. On the other hand, higher concentrations of BLF50 did not significantly prevent DOX-mediated cell death although also the treatment with BLF50 0.11µM alone significantly increased cell viability.

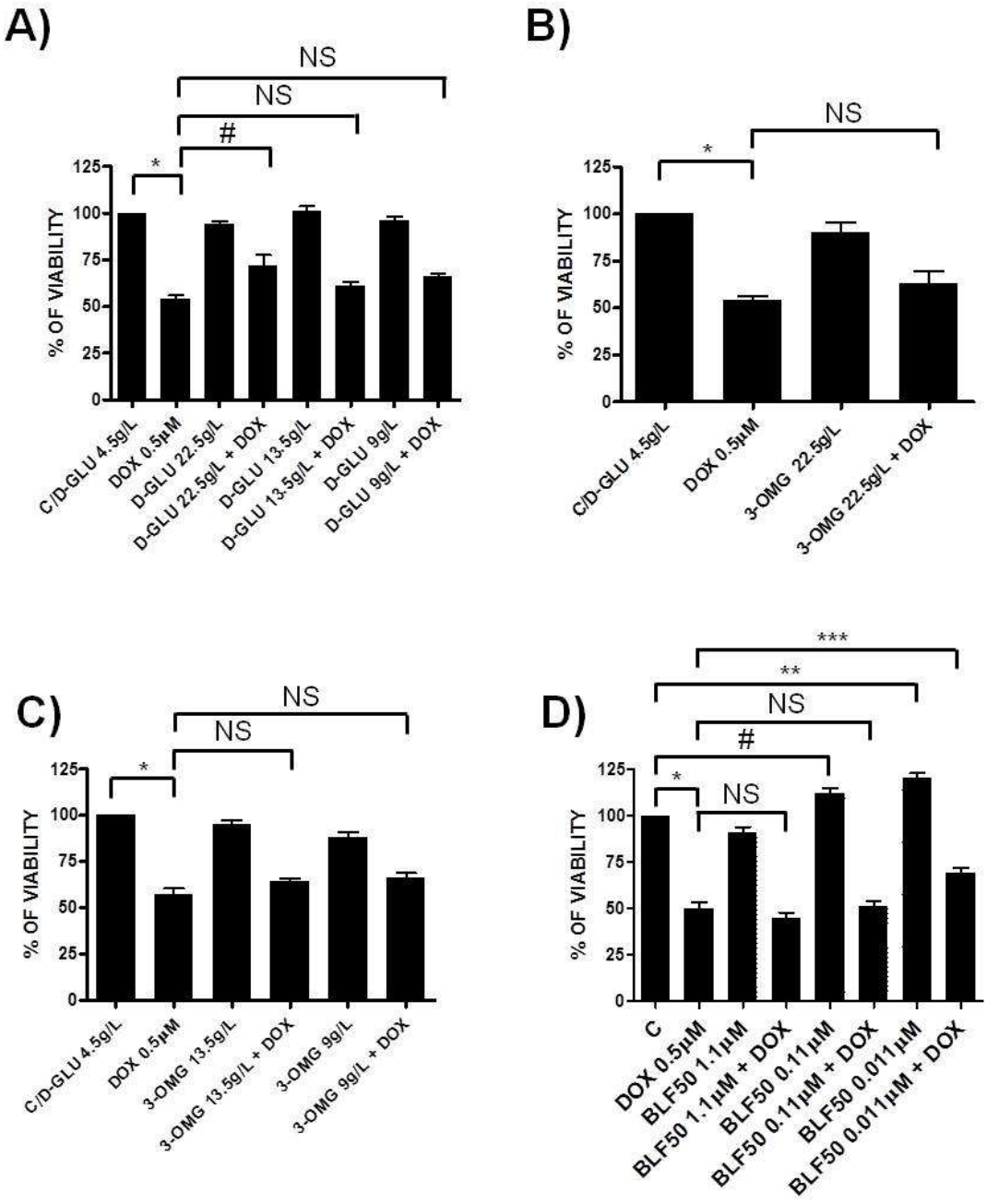

Figure 9

D-Glucose (D-GLU) prevents DOX-mediated toxicity in H9c2 cells. **(A)** Immunoblot analysis of SGLT-1 in untreated H9c2 and IEC-6 cells. **(B)** H9c2 cells were left untreated (C/D-GLU 4.5 g/L), or exposed for 24 h DOX, for 24 h to different concentrations of D-GLU alone, or treated with different concentrations of D-GLU and then exposed to DOX. Viability was evaluated by means of the MTT assay. **(C)** H9c2 cells were exposed to DOX as described above and were pretreated for 24 h with 22.5 g/L 3-OMG. Viability was measured as described for panel A. **(D)** H9c2 cardiomyocytes were left untreated (C/D-GLU 4.5 g/L), or exposed to DOX as describe above, or pretreated for 24 h with decreasing concentrations of 3-OMG alone or pretreated with 3-OMG and then exposed to DOX. Viability was estimated by MTT assay. **(E)** H9c2 cardiomyocytes were left untreated (C/D-GLU 4.5 g/L), or exposed to BLF50 alone or pretreated with decreasing concentrations of BLF50 for 24 h and then exposed to DOX. Viability was evaluated as described above. Mean values ± SD. $*P < 0.001$; $**P < 0.005$; $***P < 0.01$; $\#P < 0.05$, $^{NS}P > 0.05$, $n = 3$. MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium.

DISCUSSION

5. DISCUSSION

Anthracyclines are important and effective anticancer drugs used in the treatment of many adult and children malignancies. The most commonly used anthracyclines are Doxorubicin (DOX), Daunorubicin and Epirubicin.

The clinical use of anthracyclines to treat many human tumors is limited by its severe dose-related cardiotoxicity: clinical studies have established that in order to prevent the chronic cardiotoxicity there is a threshold dose of anthracyclines that should not be passed. However, it should be noted that compliance with this threshold dose can lead to suspension of the use of the drug in cancer chemotherapy even though a therapeutic effect has not been obtained.

The mechanisms that lead to DOX-mediated chronic cardiotoxicity are still controversial but have been mainly attributed to oxidative stress (Minotti G, et al., 2004; Chen B, 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 an important role in this toxicity (Minotti G, et al., 1999, 2004). This view is supported by the efficient cardioprotection induced by DRZ, 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 $O_2^{\bullet-}$ and H_2O_2 to more damaging oxidants (Hasinoff BB and Herman EH. 2007).

In particular, since iron can form the hydroxyl radical ($OH^{\bullet}$) through the Fenton reaction, which uses ferrous iron (Fe II) and through the Haber-Weiss reaction which uses ferric iron (Fe III), DRZ, through the removal of metal ions, may prevent the formation of the Fe 3^+ - DOX complex and the subsequent formation of reactive free radicals whose toxicity increases significantly in the presence of “free” iron ions in an aqueous environment.

On the basis of these premises, iron chelators and antioxidants should both prevent the cardiotoxicity induced by anthracyclines, but this is not the case. A number of studies have shown that antioxidants offer protection in animal models but not in patients (Ladas EJ, et al., 2004; Minotti G et al., 2004; Simunek T, et al., 2009). Therefore, despite the clinical usefulness of DRZ, the mechanisms underlying its cardioprotective effects are still not fully understood.

To understand whether there are protective mechanisms mediated by DRZ that are independent of oxidative stress, we hypothesized that HIF-1, a transcription factor that is activated not only by the lack of oxygen, but also by low levels of intracellular iron (Peyssonnaux C,et al., 2008; Mole DR. 2010), may play an important role in DRZ-dependent protection of cardiomyocytes. This compound, through its ability to sequester intracellular iron, could induce HIF-1 in cardiomyocytes thereby activating an HIF-1-dependent cytoprotection.

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 CJ and Ratcliffe PJ. 2004; Higgins DF, et al., 2008; Semenza GL. 2009), is central to cardioprotection in models of ischaemic preconditioning (Eckle T, et al., 2008) and infarction (Zhou L, et al., 2010).

Moreover HIF-1 is involved in several other cellular processes, including the regulation of energy metabolism; when mitochondrial activity is compromised (for example following treatment with DOX, which accumulates in the mitochondria; Kalyanaraman B, et al., 2002), this transcription factor promotes glycolytic metabolism by favoring an increased expression of the glucose transporter GLUT-1 or the glycolytic enzyme aldolase A (Kilic M, et al., 2007). This increase in energy production could therefore promote cell survival.

Our results show that the iron chelation obtained by exposure to DRZ 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 DOX toxicity (Corna G, et al., 2004; L'Ecuyer T, et al., 2004; Spallarossa P, et al., 2004; Li K, et al., 2006; Mukhopadhyay P, et al., 2007; Reeve JL, et al., 2007; Turakhia S, et al., 2007; Konorev EA, et al., 2008; Xu X and Richardson DR. 2008; Bernuzzi F, et al., 2009) and DRZ cardioprotection (Lyu YL, et al., 2007).

The fact that (in agreement with previous evidence; Weiss G, et al., 1997) DRZ administration up-regulated iron regulatory proteins (results not shown), whose activity is known to depend on intracellular iron availability (Recalcati S, et al., 2010), indicates that the effects of DRZ are mediated by decreased iron levels rather than by any other unforeseen effects. HIF was induced by DRZ concentrations as low as 10 µM, which allowed us to use doses that were well within pharmacological levels achieved in patients (Hasinoff BB, et al., 2003) and to respect the recommended DOX: DRZ ratio (Thompson KL, et al., 2010).

In line with the findings of previous studies showing the protective effect of DRZ *in vitro* (Simunek T, et al., 2009) and *in vivo* (Popelova O, et al., 2009), we showed that pre-exposure to DRZ prevents DOX-mediated cell death, particularly apoptosis (the prevailing mechanism for low-dose DOX cardiotoxicity) (Sawyer DB, et al., 1999; Bernuzzi F, et al., 2009), although it has been reported that DOX-dependent depletion of GATA4 triggers cardiomyocyte autophagic death (Kobayashi S, 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 DRZ-mediated protection against DOX-induced damage in H9c2 cardiomyocytes.

The involvement of HIF in the survival of DOX-treated H9c2 cells was shown by the fact that the protection was abolished by shRNA-mediated HIF-1α knockdown or the ΔARNT-mediated inhibition of the DNA binding activity of both HIF-1α and HIF-1β isoforms, whereas the overexpression of HIF-1α a was sufficient to provide a level of protection against DOX-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 DOX in breast carcinoma cells (Fang D, et al., 2007) and suggest that similar mechanisms may be operating in tumour cells and cardiomyocytes.

HIF-1α and HIF-2α 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 GL. 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 GL. 2009), were induced by DRZ, 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-1α-specific knockdown and the resistance to DOX toxicity in cells overexpressing HIF-1α suggest that this isoform plays an important role.

DOX severely inhibits HIF-1 transcriptional activity in tumour cells (Lee K, et al., 2009), although another study did not find any inhibitory effect (Yamazaki Y, et al., 2006).

We observed only partial inhibition of HIF transactivating capacity in DOX-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 DOX and was also up-regulated in H9c2 cells exposed to DRZ plus DOX (see Figure 7). The different experimental conditions (3 hours pretreatment with DRZ before DOX

administration in our study vs. hypoxic exposure in the presence of DOX in the study of Lee et al., 2009) and the different cell types may explain this discrepancy.

We also evaluated the effects of DRZ 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 GL, et al., 1996).

In line with the antiapoptotic role of some HIF-regulated genes (Higgins DF, et al., 2008) and the prevention of apoptosis induced by DRZ pretreatment (see Figure 2), we observed the strong up-regulation of surviving 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 M and Altieri DC. 2009), as well as with the recent demonstration that the overexpression of survivin in cardiomyocytes inhibits DOX-induced apoptosis (Levkau B, 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 RW. 2002).

Our previous results suggested that DOX may facilitate the apoptosis of cardiomyocytes by inhibiting the antiapoptotic HO-1 (Bernuzzi et al., 2009), which is an HIF-1 target gene (Kim HP, et al., 2006). In line with these findings, exposure to DRZ was able to counteract the inhibition of HO-1 expression exerted by DOX 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 ΔARNT prevented DOX-induced cell death (see Figure 4) and suppressed the induction of these antiapoptotic genes (see Figure 7), thus suggesting their role in DRZ-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 DOX-induced apoptosis (Reeve JL, et al., 2007). Our results are in line with previous findings indicating that Bcl-xL is probably not regulated by HIF-1; NF-*k*B (but not HIF-1) is important in hypoxia-induced apoptosis (Glasgow JN, et al., 2001), and the pathway underlying anoxia- and hypoxia-induced cell death is initiated by the loss of function of Bcl-xL (Shroff EH, et al., 2007).

Taken together, these results indicate that the cardioprotective action of DRZ involves the HIF-mediated activation of antiapoptotic genes and is in line with the recent

demonstration that DRZ prevents apoptosis and heart damage in a rat model of cardiac infarction (Zhou L, et al., 2010).

The role of DRZ 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 DOX concentrations (Bernuzzi F, et al., 2009; Shi R, et al., 2009). Accordingly, we did not detect any significant alteration in MnSOD expression, an HIF-2α target gene (Scortegagna M, et al., 2003) whose overexpression in mice protects against DOX-induced acute toxicity (Yen HC, 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 ironcatalysed ROS production.

Recent evidence showing that Pgp, a membrane efflux pump involved in the development of the MDR phenotype (Takara K, et al., 2006), is induced in tumour cells exposed to 100 µM DRZ (Riganti C, et al., 2008) suggests that, by actively extruding DOX 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 DRZ 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 downregulation in leukaemic K562 cells exposed to an iron chelator (Fang D, et al., 2010).

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

The protective effect of DRZ may depend on additional and possibly unique mechanisms, such as interference with topoisomerase II b-mediated DNA double-strand breaks (Lyu YL, 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 DRZ to prevent cardiotoxicity *in vivo*. However, our findings demonstrate a novel ROS-independent mechanism based on the HIF mediated activation of protective genes, which seems to account for the antiapoptotic effect of DRZ against low-dose DOX toxicity in the H9c2 model. This indicates that HIF plays a role in DRZ cardioprotection.

We also explored two possible alternative pharmacological strategies to prevent DOX-induced toxicity.

The first one was based on dimethyloxalyl glycine (DMOG), a small molecular mimic of hypoxia that can inhibit 2-oxoglutarate-dependent hydroxylase enzymes, thus preventing hydroxylation of HIF-1α and its proteasomal degradation (Jaakkola P, et al., 2001).

DMOG is a well known activator of hypoxia-inducible factor (HIF) and some studies have reported that DMOG is able to inhibit apoptosis in neurons deprived of nerve growth factor (NGF) by inhibiting cytochrome c release and caspase activation (Lomb DJ, et al., 2007). Other studies have shown that DMOG prevents the decrease in glucose uptake and the accumulation of ROS that occur in primary cultures of sympathetic neurons after NGF withdrawal. These data implicate HIF-2α in the neuroprotective mechanisms activated by prolyl hydroxylase inhibitors and as an obligatory player in a survival pathway activated by NGF in developing neurons (Lomb DJ, et al., 2009).

In line with these and others studies, we therefore tried to demonstrate a HIF-1-mediated protection of cardiomyocytes treated with DMOG. However, we did not find any kind of protection from damage induced by DOX in cells pretreated with DMOG. We found that at concentrations normally used in the literature (0.5 – 1 mM; Ockaili R, et al., 2005), DMOG is substantially toxic to cardiomyocytes . The cytotoxic effect of DMOG in H9c2 cells was rather unexpected although studies in human mesencephalic neural progenitor cells have already highlighted the toxicity of this molecule (Milosevic J, et al., 2009).

Given the intrinsic toxicity of this compound, H9c2 cells were exposed to lower doses of DMOG. Indeed, treating H9c2 cells with these low concentrations of DMOG 0.1-0.2 mM did not have any toxic effect and DMOG was still able to induce HIF-1, but to a very lesser extent than that achieved with an established iron chelator like DFO. Importantly, this treatment was not cytoprotective, thus suggesting that the lower extent of HIF-1 activation might not be sufficient to protect cardiomyocytes from DOX-induced toxicity and indicating that the action of HIF may be dose-dependent.

The second approach was based on the activation of the sodium-dependent glucose transporter-1 (SGLT-1) which has been shown to protect cells from various injuries (Yu LC, et. al., 2005; Yu LC, et al., 2006; Zanobbio L, et al., 2009; Palazzo M, et al., 2008; La Ferla B, et al., 2010; Zhou L, et al., 2003).

The rationale for these experiments was provided by a large body of evidence suggesting that the protective effects of D-Glucose is due to the activation of SGLT-1, as the glucose

analog 3-OMG, which induces the transporter activity but is not metabolized, and the SGLT-1 agonist BLF50 exerted the same effects as glucose both in vitro and in vivo (Palazzo M, et al., 2008).

Previous studies have shown that human cardiomyocytes express high levels of SGLT-1 RNA (Zhou L, et al., 2003) and indeed we verified that SGLT-1 is also expressed in the H9c2 cell line (see Fig. 9A). Thus, we decided to assess the protective role SGLT-1-mediated in the H9c2 model of DOX toxicity.

We found that D-glucose protects H9c2 from DOX-induced toxicity, but this protection does not seem to be mediated by the activation of glucose transporter SGLT-1. This conclusion is based on the fact that by exposing cells to 3-OMG and BLF50, that have been shown to induce the transporter activity (Yu LC, et. al., 2005; Yu LC, et al., 2006; Zanobbio L, et al., 2009; Palazzo M, et al., 2008; La Ferla B, et al., 2010; Zhou L, et al., 2003), we did not find any protection from DOX-toxicity in H9c2 cardiomyocytes.

These data contrast with those reported in a recent study showing that another glucose analog, 2-deoxy-D-glucose (2-DG), is able to prevent DOX-induced damage (Chen K, et al., 2011). This glucose analog can enter the cells and be phosphorylated but not further metabolized; therefore, it competitively blocks glucose utilization, presumably by mimicking caloric restriction effects at the cellular level. The mechanisms by which 2-DG protects against DOX toxicity appear to be complicate and are mediated through multiple factors, including the preservation of ATP content, the activation of AMP-activated protein kinase and the inhibition of autophagy (Chen K, et al., 2011). 3-OMG and 2-DG are very similar and they both compete with glucose for uptake into cells and do not support glycolysis, but 2-DG is not phosphorylated by hexokinase (Xu YZ, et al., 2001). The discrepancy of the results obtained with 2-DG and 3-OMG does not appear to be due to the different structure of the molecules but may result from the fact that Chen K, et al., used isolated neonatal rat cardiomyocytes and a greater concentration of DOX (1µM).

Preserved or enhanced glucose uptake has been shown to protect also hematopoietic cells (Rathmell JC, et al., 2003; Vander Heiden MG, et al., 2001), neurons (Ravikumar B, et al., 2003), and cardiomyocytes (Morissette MR, et al., 2003). Furthermore, pretreatment of cells with high extra-cellular glucose concentrations or hyperglycemia has been shown to enhance de novo synthesis of diacylglycerol and promote protein kinase C (PKC) activation to protect cardiomyocytes and neurons from ischemic injury. The mechanism of this protection, however, is not clear (Malliopoulou V, et al., 2006; Peter-Riesch B, et al., 1988; Raval AP, et al., 2003; Whiteside CI and Dlugosz JA. 2002).

An interesting study conducted in primary lymphocytes suggested that increased glucose metabolism protected cells against the proapoptotic Bcl-2 family protein Bim and attenuated degradation of the antiapoptotic Bcl-2 family protein Mcl-1 through the phosphorylation of GSK-3, most likely via PKC activity (Zhao Y, et al., 2007).

These results suggest that the H9c2 protection induced by glucose could be mediated by signalling pathway initiated by glucose catabolism which may inhibit apoptotic cells death. Since it has been shown that p53 is activated in response to glucose deprivation (Assaily W, et al., 2011), one may speculate that in DOX-treated H9c2 cells increased glucose availability may blunt DOX-dependent p53 activation and thus prevent apoptosis. However, further studies are necessary to understand the mechanisms underlying D-glucose-mediated cardioprotection in H9c2 cardiomyocytes exposed to DOX. Alternatively or concomitantly, glucose may exert its protective effect by simply acting as a substrate for glycolysis-dependent energy production in cells in which the mitochondria have been damaged by DOX, which specifically affects these organelles.

In conclusion, our results showing that the activation of the HIF-dependent pathway is one of the molecular mechanisms at the basis of the well established and successful cardioprotective effect of iron chelation in patients treated with high cumulative doses of DOX indicate the HIF pathway as a druggable target to limit anthracycline cardiotoxicity in cancer patients.

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