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Review article

New insights into doxorubicin-induced cardiotoxicity: The critical role of cellular energetics

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Abstract

Cardiotoxic side-effects represent a serious complication of anticancer therapy with anthracyclines, in particular with doxorubicin (DXR) being the leading drug of the group. Different hypotheses, accentuating various mechanisms and/or targets, have been proposed to explain DXR-induced cardiotoxicity. This review focuses on the myocardial energetic network as a target of DXR toxic action in heart and highlights the recent advances in understanding its role in development of the DXR related cardiac dysfunction. We present a survey of DXR-induced defects in different steps of cardiac energy metabolism, including reduction of oxidative capacity of mitochondria, changes in the profile of energy substrate utilization, disturbance of energy transfer between sites of energy production and consumption, as well as defects in energy signaling. Considering the wide spectrum and diversity of the changes reported, we attempt to integrate these facts into a common framework and to discuss important functional and temporal relationships between DXR-induced events and the possible underlying molecular mechanisms.

Keywords: Doxorubicin; Anthracyclines; Energy metabolism; Cardiotoxicity; Creatine kinase; AMP-activated protein kinase

Contents

Abbreviations: AMPK, AMP-activated protein kinase; ANT, adenylate nucleotide translocator; ATP, ADP, AMP, adenosine tri-, di-, monophosphate, respectively; CK, creatine kinase; BCK, MCK, sMtCK, uMtCK, cytosolic brain-type, cytosolic muscle-type, sarcomeric mitochondrial, and ubiquitous mitochondrial CK isoforms, respectively; DXR, doxorubicin; Cr, creatine; PCr, phosphocreatine; PFK, phosphofructokinase; ROS, RNS, reactive oxygen and nitrogen species, respectively; VDAC, voltage dependent anion channel.

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Since the late 1960s, the anthracycline antibiotic doxorubicin (Adriamycin; DXR) has been one of the most largely prescribed chemotherapeutic drugs for the treatment of a variety of human cancers [1]. Unfortunately, in addition to its potent antitumor effect, the use of DXR is associated with a number of unwanted side effects, especially with serious cardiac toxicity. This complication represents a major obstacle to prolonged use of the drug and/or cumulative dose exceeding 550 mg/m^2 body surface area [2–5].

Over the years, different hypotheses, accentuating various molecular mechanisms and/or intracellular targets, have been proposed to explain DXR-induced cardiotoxicity (see [3–5]), but no single one of these is considered fully satisfying. Rather, DXR cardiotoxicity seems to be a multifactorial process that leads to cardiomyocyte death as the terminal downstream event [3,6,7]. It has long been considered that DXR exerts its anticancer and cardiotoxic action by distinct mechanisms: while the anticancer response was associated with DNA intercalation, topoisomerase-II inhibition and apoptosis, the cardiotoxicity was mainly ascribed to oxidative stress. At present it appears that such separation is not fully justified [3]. It seems that beneficial (anticancer/therapeutic) and detrimental (cardiotoxic) responses to DXR are to some extent overlapping: they share common effectors, such as oxidative stress, and both involve apoptosis.

This review focuses on the myocardial energetic network as a target of DXR-induced toxicity in the heart. There are several reasons to recapitulate the energetic aspect of DXR-cardiotoxicity. First, in spite of a vast literature on this topic, a comprehensive review is not available. Second, there is an increasingly apparent relationship between cellular metabolic status and apoptosis [8,9], emerging as an important factor of DXR-induced cardiac cell loss [3]. Finally, cellular energy deficit has been recognized as an important common factor in the development of cardiac myopathies of different origin [10–12]. In case of DXR, impairment of cardiac energetics has been primarily associated with compromised mitochondrial energy production [13]. Recently, evidence has accumulated indicating regulatory and compensatory components of myocardial energy metabolism among the most sensitive and early targets of DXR-induced damage. We shall review these defects in the energetic network of the myocardium induced by DXR and discuss their temporal and functional relationships and consequences. First, we briefly describe the clinical aspects and ultrastructural features of DXR cardiomyopathy in patients, review different experimental models used to study effects of DXR on cardiac energetics and their relevance to the clinical situation, including DXR-induced cell death. This will be followed by a characterization of DXR-induced changes in high-energy phosphate levels (adenosine triphosphate, ATP, and phosphocreatine, PCr) and in mitochondrial oxidative capacity, as well as accompanying changes in the profile of cardiac substrate utilization. Further, we will emphasize DXR-induced defects in the creatine kinase (CK) energy storage and transport system, as well as a potential involvement of energy sensing and signaling by the AMP-activated protein kinase (AMPK) pathway. In a last section, molecular mechanisms by which DXR may exert its toxic action on cardiac energetics will be highlighted. Although we will mainly focus on data concerning DXR effects in heart, most of the considerations will be valid also for other anthracyclines as well as other non-cancer tissues. For further information on DXR, the reader is referred to previous reviews that have dealt with different aspects of the drug, including detailed description of therapeutic and toxic action of DXR [3], DXR chemical structure and related oxidative chemistry [1,3,5], interaction with iron [14], intracellular production of DXR-induced free radicals [15] or generation and action of DXR secondary metabolites [3,4,16].

1. Clinical aspects and morphological characteristics of DXR-induced cardiomyopathy

Chronic cardiomyopathy usually occurs within the first year after therapy (early onset), but occasionally much later [17]. Such late onset cardiac toxicity was documented especially in children. The probability of developing cardiomyopathy is clearly dose-dependent [18]. At a dose of 550 mg/m^2 the incidence is up to 7%. However, subclinical cardiac damage can be observed at doses much lower than this threshold using echocardiography or radionuclide ventriculography. The clinical course and manifestation of DXR-induced cardiomyopathy is highly variable ranging from acute cardiogenic shock to gradually progressive deterioration and congestive heart failure [19]. Acute cardiotoxicity with anthracyclines occurring during or immediately after the treatment was reported in patients as relatively rare and usually transient. It is typically characterized by electrocardiographic abnormalities, such as arrhythmias, non-specific ST-T alterations, QT-prolongation, and associated with reversible hypotension and pericarditis [4,20,21]. Of note, the electrical manifestations are not predictive of subsequent cardiomyopathy. Diastolic functional impairment arises first after DXR therapy. Later on, diastolic dysfunction becomes more pronounced and is accompanied with alterations in systolic function [22]. Tissue Doppler imaging isovolumic

relaxation time (an index for diastolic function), measured at the mitral annulus during early assessment was found to be shorter (<80 ms) in patients who developed subsequently an ejection fraction below 50% of original values and cardiomyopathy (positive and negative predictive value of 100% and 91%, respectively). Certain factors were identified to predispose to cardiomyopathy: age over 65 years (but also very young children), diabetes, pre-existing heart disease and hypertension, liver disease or mediastinal radiotherapy. Although DXR-induced cardiomyopathy was found to have a poor outcome (up to 61% mortality rate), recent studies show a clear survival benefit associated with proper medical management. There is no specific treatment for cancer therapy-related cardiomyopathy. Symptomatic patients receive standard treatments for congestive heart failure, such as angiotensin-converting enzyme inhibitors, beta-blockers, diuretic, digoxin and spironolactone [23,24]. Recent experimental evidence supports the preventive effects of erythropoietin on cardiac dysfunction in DXR-induced cardiomyopathy [25]. Unfortunately, effective means to detect cardiotoxicity sufficient early as to avoid subsequent irreversible cardiac damage remains a problem. Attempts to reduce DXR-toxicity were directed towards schedule modifications, developing less cardiotoxic analogs, or concurrent administration of cardioprotective agents like dexrazoxane, an iron chelator [26,27]. However, these strategies were of limited effect [28].

DXR cardiotoxicity has been termed type I chemotherapy-related cardiac dysfunction [29], which in contrast to type II is characterized by ultrastructural changes and has a greater tendency to become irreversible. The major morphological changes in myocardium of patients treated with DXR were described as myofibrillar loss, dilatation of sarcoplasmic reticulum and swollen mitochondria. The severity of these changes can be assessed semiquantitatively according to the score by Billingham and colleagues [30], but these histopathological features are not specific for DXR toxicity.

2. Experimental models

Effects of DXR on the global cardiac energetic network or its individual components have been studied in different model systems, including animal models, as well as in vitro models of different complexity, such as perfused heart, cultured cardiomyocytes, isolated mitochondria, submitochodrial particles, or purified proteins. Very few data are available from studies in humans.

A comparison between models, as well as a critical evaluation of the demonstrated effects of DXR, must consider the fact that the choice of a correct, clinically relevant DXR concentration strongly depends on the model system used. This issue requires particular care when analyzing in vitro models. It must be kept in mind that different tissue [31,32] and intracellular structures [33] accumulate the drug to various degrees. For example, for mitochondria, which selectively accumulate DXR to concentrations much higher than plasma levels (see [16]), the relevant concentrations were evaluated to be in the range of 5-30 μM [16], for isolated cells they should not exceed 1 μM [34]. Both, in isolated mitochondria and in cultured cardiomyocytes, any comparison of data should also consider the effective DXR doses, i.e. the drug concentration per mg protein in the experimental system. For perfused heart, a good indication for a clinically relevant DXR concentration can be the peak plasma concentration of the drug measured in patients, which reaches up to 2-6 μM after bolus injection, with a typical value of 1-2 μM [34]. For in situ models with a short life time and thus short time of drug persistence, including the perfused heart or isolated mitochondria, very often supraclinical DXR concentrations have been applied, that is up to several hundred μM. However, such an approach, which is intended to simulate the effects of lower DXR doses over longer time, must be always evaluated with caution. According to Herman and Ferrans [35], the use of smaller repeated doses (individual dose <20% of the median lethal dose) in animals adequately mimics chronic myocardial alterations in patients. Typical cumulated cardiotoxic DXR doses cited by these authors are 36.4 mg/kg for mice, and 15 mg/kg for rats; these cumulated doses are usually applied over a couple of weeks.

3. DXR-induced cell death

A common endpoint of anthracycline-therapy in cardiomyocytes and non-myocytes is cell death, although this is likely to be only the final response to a variety of upstream events and longer-lasting stress conditions. Adult cardiomyocytes are terminally differentiated muscle cells which do no longer proliferate. Therefore, repetitive induction of cardiomyocyte cell death in the absence of sufficient regenerative capacity is a plausible mechanism for DXR-induced, irreversible cardiotoxicity. However, the use of elevated, supraclinical drug concentrations, which may kill cells through non-physiological mechanisms, can be misleading in the discussion about the role of cell death in anthracycline-induced cardiotoxicity [34]. More recent evidence suggests that terminally differentiated cells can exhibit one or more characteristics of cell death independently, and that the results of the widely used TUNEL-assay could lead to an overestimation of apoptosis [36]. This may explain the high variability in the apoptotic rates reported for different cardiomyopathies. In general, cellular damage and cell loss in human heart failure has been recently described in detail and tentatively grouped in apoptosis, oncosis, and autophagy [37,38]. According to this more detailed classification, apoptosis is characterized by DNA degradation, oncosis by the rapid loss of plasma membrane integrity and autophagy by the disturbance of protein degradation pathways. The term necrosis describes the end stage of cellular destruction by unspecified mechanisms. The difference in morphology between oncosis and apoptosis is that oncosis is defined as cell injury with swelling, and apoptosis as cell injury with shrinkage. There is evidence from in vitro and animal studies that all three types of cell injury can occur in parallel and play a role in DXR-induced cardiotoxicity. However, the reported rate and the observed types of cell death vary in different experimental models. Classical apoptosis as defined by DNA fragmentation, caspase activation and changed morphology of

nuclei has been observed in isolated cardiomyocytes treated with DXR and was shown to be related to oxidative cellular damage [5,6]. Studies on cardiomyocytes in culture suggest that low doses of anthracyclines do induce several forms of cellular damage, but not necessarily rapid apoptotic cell death. After one hour of exposure to clinically relevant doses of DXR (1 μM), a significant degradation of the sarcomeric protein titin by a calpain-dependent mechanism has been observed in cultured adult rat cardiomyocytes [39]. In contrast to cultured neonatal cardiomyocytes, no DNA degradation was observed in the adult cells despite increased caspase-3 activity during a 48 hours period. Since titin is thought to play an important role in the complex process of myofibrillogenesis, titin degradation in the working myocardium could lead to destabilization of the myofilaments, as well as disrupt incorporation of newly synthesized proteins into the myofilament lattice and ultimately lead to sarcomere disarray [39]. Concerning the autophagy pathway of cell death, it has been observed that DXR induces the formation of polyubiquitin-positive inclusions and down-regulation of proteasome activity in cultured cardiomyocytes (C. Zuppinger, unpublished observations). Accumulation of oxidatively damaged macromolecules and organelles due to imperfect autophagic degradation is considered an important contributor to aging and final cell death of cardiomyocytes [40]. Such a mechanism may enhance the susceptibility of the aging heart to DXR and contribute to the long-term effects of DXR-cardiotoxicity.

4. Myocardial high-energy phosphate levels

The heart requires large amounts of energy to sustain its contractile performance. Adenosine triphosphate (ATP) serves as primary, immediate source of energy, however intracellular ATP pools are rather small (ca. 5 mmol/kg heart wet weight). Upon increasing demand, ATP can be efficiently replenished from the larger intracellular pools of phosphocreatine (PCr; ca. 10 mmol/kg heart wet weight [41,42]). DXR has been reported to diminish cardiac energy reserves, by reducing both ATP and PCr levels as well as the PCr/ATP ratio. This effect has been consistently observed in different models of cardiotoxicity, as well as in patients (Table 1).

In animal models, DXR-induced depletion of the high energy phosphates ATP and PCr has been demonstrated by different independent techniques—both by biochemical determination in hearts isolated from treated animals, as well as by in vivo ^{31}P-NMR spectroscopy. As shown in rats with ^{31}P-NMR, the longer-term effects of repetitive lower doses of DXR on high energy phosphates were more pronounced than acute effects of an equivalent single dose [43]. In another study using in vivo ^{31}P-NMR, Eidenschink and colleagues [44] demonstrated a decrease in the PCr/ATP ratio in children as late as 4 years after completion of the treatment. While in DXR-treated cardiomyocytes or perfused hearts deterioration of the mechanical function has been reported to coincide with reduced high energy phosphates content [45,46], in patients the PCr/ATP ratio was diminished even in the absence of clinical manifestations of cardiomyopathy [44].

5. Mitochondrial function

More than 90% of the ATP utilized by cardiomyocytes is produced by mitochondrial respiration [12]. Therefore, any alterations of mitochondrial structure and function will likely manifest themselves in terms of cardiomyocyte function. Progression of DXR cardiotoxicity is associated already at an early stage with morphological abnormalities of mitochondria, including mitochondrial swelling that is typical for apoptotic cells. Such ultrastructural changes have been observed in different cardiotoxicity models, as well as in patients [4,47,48]. In parallel, DXR has been reported to interfere with mitochondrial respiratory function (Table 2), to decrease mitochondrial membrane potential, as well as induce mitochondrial permeability transition [13].

Numerous reports based on studies either with isolated mitochondria exposed to DXR or with mitochondria isolated from treated animals evidence that DXR affects mitochondrial respiration (Table 2), both through complex I and II substrates

Table 1
DXR-induced changes in high-energy phosphate levels

Effect	Experimental model	Species	Extent of change*–applied DXR dose*–time of treatment	Refs
Decreased ATP	Cultured cardiomyocytes	Rat	By 30%-1.7 μM-3 h (effect delayed 24-48 h)	[45]
			By 50%-1 μM-24 h	[64]
			By 90%-800 μM-1 h; significant already after several min	[176]
			By respectively 40, 70, 80%-0.5, 2, 5 μM-18 h (effect delayed 24 h)	[177]
	Heart slices	Rat	By 30%-24 μM-60 min	[178]
	Perfused heart	Rat	By 20%-10 μM-70 min	[179]
			To 77%-16 μM-60 min, 27 %-50 μM-60 min, 55 %-80 μM-30 min	[46]
			To 68%-1 mM-15 min	[71]
	Animal model	Rat	By 50%-3 mg/week-3 weeks	[180]
			By 30%-2 mg/week-6 weeks	[158]
Decreased PCr, PCr/ATP	Cultured cardiomyocytes	Rat	By 30%-1.7 μM-3 h (effect delayed 24 -48 h)	[45]
	Perfused heart	Rat	To 73%-16 μM- 60 min, 21 %-50 μM-60 min, 36 %-80 μM-30 min	[46]
	Animal model	Rat	By 20%-25 mg/kg-30-60 min	[43]
	Patients	Human	By 20%-270±137 mg/m^2- 4 years after treatment	[44]

* If necessary, the original data were recalculated to give percent changes and DXR concentration in μM.

Table 2
Effects of DXR on mitochondrial respiration

Effect	Experimental model	Species	Applied DXR dose/IC_{50} *–time of treatment	Refs
Stimulation of state 4	Mitochondria isolated from hearts of treated animals	Rat	8 mg/kg in 2 injections-24 h and 1 h before analysis	[52]
		Rabbit	Several injections resulting in total cumulative dose up to 22 mg/kg	[181]
Inhibition of state 3	Isolated heart mitochondria treated with DXR	Rat	IC_{50} ~ 750 μM per 0.75 mg protein/ml (FCCP stimulated)	[50]
		Rat	IC_{50} ~ 766 μM (glutamate + malate) and 121 μM (succinate) per 1 mg protein (ADP stimulated)	[51]
		Pigeon	IC_{50} ~ 200 μM per 0.4 mg protein/ml (ADP stimulated)	[49]
	Mitochondria isolated from heart of treated animals	Mice	By ca. 20-30%-20 mg/kg per day-5 days	[123]
		Rat	By ca. 20% (glutamate + malate) and 15 % (succinate)-2 mg/kg per week-7 weeks (ADP stimulated)	[182]

* IC_{50}, drug concentration inducing decrease by 50%.

[49–52] by inhibiting the respiratory state 3 (maximal oxygen uptake in presence of ADP or uncoupler) [49–51] and sporadically stimulating state 4 (basal oxygen uptake; [51–53]). The response of mitochondrial respiration to DXR has been characterized as relatively non-specific (see [49]). DXR can interfere with mitochondrial function at multiple levels, by inhibiting different components of the respiratory chain, by inhibiting phosphorylation steps or by exerting partial uncoupling.

Respiratory complexes, embedded in the inner mitochondrial membrane representing an important site of DXR accumulation, have to be considered as obvious potential targets for DXR toxic action. Several studies aimed to determine the sensitivity of respiratory complexes to DXR (Table 3). Numerous enzymatic activities of the respiratory chain have been found affected, some of them, however, at relatively high micromolar IC_{50}. DXR-sensitive sites were mainly located in complex I, III and IV, with a particular vulnerability of NADH dehydrogenase and cytochrome c oxidase [50,54–56]. The variability of the reported IC_{50} values can have numerous reasons in addition to obvious differences in the experimental systems. Apparent activities of respiratory enzymes depend on the composition of assay media, e.g. the presence of detergent, which can affect accessibility of sites of interest as well as on condition of the actual experiments (see [55]).

It is also of note that different processes, including drug distribution, binding specificity, and drug metabolism, e.g. via iron, peroxidase systems or other metabolic pathways are not always considered in the studies with isolated organelles. For example, DXR activated by horseradish peroxidase (HRP) and hydrogen peroxide (H_2O_2) seems to be a more potent inhibitor of enzymatic activities than DXR alone. Studies of Muraoka and Miura [57], using pig heart submitochondrial particles, have shown that mitochondrial succinate dehydrogenase is inactivated by 50% within 30 min already at 0.8 μM DXR if the drug is activated by HRP/H_2O_2, while the non-activated drug becomes a potent inhibitor of this enzyme only at 500 μM [54].

Reduced activity of respiratory complexes has been also observed in animal models of DXR-cardiotoxicity (Table 3). It should be stressed, that in view of the complexity of the respiratory control, the relation between reduction in maximal capacity of the respiratory enzymes and a decrease in mitochondrial ATP production is not straightforward, in particular in vivo (see [58,59]).

A particular sensitivity of the mitochondrial phosphorylation step to DXR has been suggested by the study of Muhammed and coworkers with isolated mitochondria [51]. The IC_{50} for inhibiting phosphorylation was lower than for the inhibition of substrate oxidation in mitochondria from different tissues, including heart. Although an inhibitory effect of DXR on mitochondrial ATPase activity has been reported for heart mitochondria [50], the more important targets of DXR seem to be peripheral proteolipid complexes involved in channeling of ATP and phosphocreatine from the mitochondria into the cytosol. These multienzyme transmembrane complexes, together with the mitochondrial respiratory chain and the ATP synthase, are necessary for the efficient mitochondrial ATP synthesis and export. They contain adenine nucleotide translocator (ANT) of the inner mitochondrial membrane and voltage dependent anion channel (VDAC) of the outer membrane. Another important component of this complex, especially in tissues with high and fluctuating energy requirements, such as cardiac and skeletal muscle, is mitochondrial creatine kinase (MtCK), which is situated in the intermembrane space and functionally and/or structurally associated with ANT and VDAC [60–63]. As suggested by several lines of evidence, DXR compromises the functioning of these "channeling complexes". DXR affects ANT1, the ANT isoform predominantly expressed in heart [64,65], as well as the inorganic phosphate carrier, an ANT homologue [66,67]. In addition, it inhibits the functions of MtCK and the functional association between MtCK and ANT [68]. The interference of DXR with MtCK is described in more detail in one of the following sections.

The uncoupling activity of DXR is corroborated by the stimulation of respiratory state 4 observed in isolated mitochondria that were DXR treated [51,53] or obtained from hearts of DXR treated animals [52]. Uncoupling explains a number of other findings observed particularly in DXR-treated isolated mitochondria, like a decrease in respiratory state 3, a decrease in respiratory control ratio, a decrease in the P/O ratio, and increased activity of ATP synthase [51]. DXR-induced uncoupling has been ascribed to increased proton leak of the inner membrane due to the membrane interaction of the drug [53,69] and/or to its action as a so-called redox cycling alternate

Table 3
Effects of DXR on respiratory enzymes

Effect	Experimental model	Species	Extent of change–applied DXR dose/IC_{50}–time of treatment	Refs
Inactivation of complex I *NADH dehydrogenase*	Isolated mitochondria	Rat, bovine	IC_{50} ~ 400-425 μM/100-200 μg protein/ml-immediate effect	[55]
	Submitochondrial preparations	Bovine	By 70%-25-50 μM DXR/100 μg protein/ml-15 min	[54]
	Animal model	Mice	By ca. 15%-20 mg/kg DXR per day-5 days	[123]
	Patient autopsy	Human	To 79%-chemotherapy	[76]
Inactivation of complex II *succinate dehydrogenase*	Isolated mitochondria	Rat, bovine	Unaffected up to 1 mM/100-200 μg protein/ml-immediate effect	[55]
	Submitochondrial preparations	Bovine	$IC_{50} \geq$ 500 μM DXR/100 μg protein/ml-15 min	[54]
	Animal model	Pig	IC_{50} ~ 0.8 μM DXR activated by HRP/H_2O_2/100 μg protein/ml	[57]
		Mice	By ca. 13%-20 mg/kg DXR per day-5 days	[123]
Inactivation of complex III *ubiquinol-cytochrome c oxidoreductase*	Isolated mitochondria	Rat, bovine	IC_{50} ~ 150-200 μM/100-200 μg protein/ml-immediate effect	[55]
Inactivation of complex IV *cytochrome c oxidase*	In vitro-purified enzyme		By 10-50%-40-200 μM/10 μM protein-60 min	[165]
	Isolated mitochondria	Rat, bovine	IC_{50} ~ 150-200 μM/100-200 μg protein/ml-immediate effect	[55]
		Rat	IC_{50} ~ 750 μM per 0.75 mg protein/ml-2 min	[50]
		Bovine	IC_{50} ~ low micromolar/0.015 mg protein/ml-20 min	[140]
	Submitochondrial preparations	Bovine	By 30%-1 mM/100 μg protein/ml-15 min	[54]
		Pig	To 70%-10-100 μM/100 μg protein/ml with excess of NADH-2h	[139]
	Cultured cardiomyocytes	Rat	To resp. 64 and 27%-0.5, 2 μM-18 h (effect delayed 24 h)	[177]
	Animal model	Mice	By ca. 50%-single dose of 40 mg/kg-48 h later (similar chronic effect after cumulative dose of 12 or 20 mg/kg)	[47]
		Rat	To 24%-1 mg/kg per week-7 weeks (delayed effect observed after several weeks)	[74]
	Patient autopsy	Human	To 59%-chemotherapy	[76]
Inactivation of complex V *ATP-synthase*	Isolated mitochondria	Rat	IC_{50} ~ 750 μM per 0.75 mg protein/ml-2 min	[50]
Inactivation of phosphate carrier	Carrier reconstituted in lipid vesicles		IC_{50} ~ 30 μM per 250 μl lipids	[66]
Expression of repiratory enzymes	Cultured cardiomyocytes	Rat	~40-50% decrease in mRNA for Rieske iron-sulfur protein and ANT1- 1 μM-2-3 h	[64]
	Animal model	Mice	Diminished COXI protein-1 mg/kg per week-7 weeks (delayed effect observed after several weeks)	[74]
			Diminished mRNA for COXII-1 mg/kg per week-8 weeks	[48]
		Rat	Diminished ANT protein-2 mg/kg per week-7 weeks	[182]

electron acceptor. The latter term denotes compounds which can be both reduced and further oxidized by the respiratory chain, thus allowing electrons to bypass the proton-pumping complexes and finally decreasing terminal phosphorylation ([70] see also [56]).

It is of interest to which extent impaired mitochondrial respiration contributes to decreased cardiac function in situ and how this process evolves over years into chronic DXR toxicity. Instantaneous effects on isolated mitochondria were reported with a rather high DXR IC_{50} of several hundred μM ([51,53,71] see also [72]). An exception is succinate oxidation under phosphorylating conditions in the presence of hexokinase, which has been found inhibited with an IC_{50} of ~121 μM DXR [51]. However, the potentiating effect of hexokinase was not observed in other experimental setups [50]. It seems that in cardiac models like the perfused heart, DXR can acutely affect cardiac performance without any marked change in mitochondrial respiration and oxidative phosphorylation [46,71,73]. In DXR treated animals, however, mitochondrial dysfunction has been observed together or even before an apparent decrease in cardiac performance. In rats treated weekly with DXR at 1 mg/kg for 7 weeks, a significant score of cardiomyopathy (according to clinical, macroscopic, and histological parameters) was accompanied by a dramatically depressed cytochrome c oxidase activity to 24% of control. However, this effect was rather associated with decreased protein level of the subunit COXI [74]. In rats treated with a total DXR dose of 15 mg/kg over 2 weeks, activity of complex I was found to be decreased before left ventricular ejection fraction and fatty acid metabolism became significantly impaired [75]. Decreased activities of NADH dehydrogenase and cytochrome c oxidase, related at least partially to lower expression of these enzymes, were found in DXR-treated patients showing no clinical symptoms of cardiomyopathy (Table 3; [76]). Positron emission tomography (PET) was used to study DXR effects on heart oxygen metabolism in patients [77]. In this study, no significant effect of DXR on blood flow or metabolic flux through the citric

acid cycle was observed, neither acutely, 24 h after administration of 50 mg/m^2, nor 3 weeks after cumulative administration of 300 mg/m^2, despite a small but significant decrease in left ventricular ejection fraction. The authors interpreted the lack of DXR effects on blood flow and citric acid cycle flux as indicative for the absence of changes in the mitochondrial oxidative phosphorylation. Although it has been shown in the normal heart that myocardial blood flow and oxygen consumption highly match each other [78], the interpretation of the above data and the conclusion made are nonetheless fairly indirect. They are based on a relatively limited study performed in an early phase after DXR treatment. Nevertheless, the applied method is a valuable approach to investigate mitochondrial function in vivo.

6. Alteration in myocardial substrate utilization

6.1. Fatty acid oxidation

The heart metabolizes multiple substrates to synthesize ATP. Fatty acids are known to be a major cardiac energy source under aerobic conditions. During the development of cardiac pathologies, at least in their early stages, a decrease in fatty acid oxidation is accompanied by up-regulation of glucose utilization as a compensatory response. DXR-induced cardiomyopathy is associated with a decreased utilization of both substrates (Table 4). Wakasugi and colleagues [79] have demonstrated in DXR-treated rats that utilization of fatty acids and glucose is markedly reduced before the occurrence of functional changes, with fatty acid utilization being less affected than utilization of glucose. In cultured cardiomyocytes, however, higher DXR concentrations were required for inhibiting glucose oxidation as compared to fatty acid oxidation [80].

Perturbed fatty acid metabolism with increased serum lipids, in particular free fatty acid levels, has been found in cell culture and animal models of DXR cardiotoxicity following DXR treatment [79–83], as well as in DXR-treated patients [84]. Decreased oxidation of palmitate, a long chain fatty acid, was shown to occur acutely in isolated cardiomyocytes treated with DXR, or in the chronic situation in cardiomyocytes isolated from DXR-treated rats. As underlying mechanism, impairment of carnitine palmitoyl transferase I (CPT I) and/or depletion of its substrate L-carnitine was proposed [80]. External L-carnitine and probucol, a drug with hypolipidemic and antioxidant action, have been shown to diminish the cardiotoxic side effects in treated animals [32,85–90] as well as in patients [91]. DXR-induced down-regulation of fatty acid oxidation is another manifestation of disturbed mitochondrial function that is characteristic for heart pathology. It is to note that reduced oxidation and excessive accumulation of fatty acids not only leads to perturbation at the energy substrate level, but can also modify the energy-coupling properties of mitochondria [92].

6.2. Glucose transport and glycolysis

The inability of the DXR-challenged heart to sustain cellular energy production by increased glycolysis may be due to DXR effects on glucose supply and/or the ability of cells to stimulate glucose uptake. In cardiomyocytes, treatment with DXR led to a transient increase in glucose uptake, followed by a marked decrease [93]. This transient stimulation of glucose uptake was associated with a recruitment of the glucose transporter GLUT1 into the plasma membrane while there was no change in the level of the membrane-associated GLUT4 [93]. Another cause limiting the glycolytic flux in DXR-challenged heart can be impaired activity of phosphofructokinase (PFK), a key regulator of glycolysis. As shown by

Table 4
DXR-induced changes in energy-substrate utilization

Affected parameter/process	Experimental model	Species	Extent of change–applied DXR dose/ IC_{50}–time of treatment	Refs
Serum free fatty acid/lipids	Animal model	Rat	Increased level of long-chain fatty acids as palmitic, linoleic, oleic and stearic acids-4 injections of 5 mg/kg over 2 weeks (changes measurable already 1d after single dose 5 mg/kg)	[83]
			Increase in plasma triglycerides, total cholesterol, high- and low-density lipoproteins	[85]
Substrate metabolism	Animal model	Rat	Decrease by 35% and 80% in glucose utilization-2 mg/kg/week-at 6th and 10th week respectively; less pronounced effect on fatty acids utilization	[79]
	Patients	Human	Change in kinetics of I-123 BMIPP-total dose of 38-640 mg/m2-7-28 days after therapy	[183] also [84]
Fatty acids oxidation	Isolated cardiomyocytes	Rat	Oxidation of palmitate decreased by 40% and of octanoate by 30%-0.5 mM-20 min; (2 mM abolished oxidation of palmitate)	[80] also [178]
	Cardiomyocytes isolated from DXR treated animals	Rat	Oxidation of palmitate decreased to 40%-18 mg/kg in 6 doses over 2 weeks	[89]
	Cardiomyocytes isolated from DXR perfused heart	Rat	Oxidation of palmitate decreased by 70%-0.5 mM DXR-10 min	[89]
Glucose oxidation	Isolated cardiomyocytes	Rat	Significantly decreased-1 mM-20 min (50% decrease-2 mM)	[80]
Glucose uptake	Cultured cardiomyocytes	Rat	Increased by ~100%-1 μM-1 h, followed by decrease	[93]
Expression of glycolytic enzymes	Cultured cardiomyocytes	Rat	~50% decrease in mRNA of PFK-1 μM-2-3 h	[64]

Jeyaseelan and colleagues for cardiomyocytes [64], exposure to DXR rapidly decreases the mRNA level of PFK. These findings demonstrate the limitations of the DXR-treated heart to compensate for a low cellular energy state by increasing glycolytic ATP generation.

7. Energy transfer and regulatory pathways

7.1. High energy phosphate storage and transfer

DXR not only diminishes general energy production, but already very early leads to a rather specific impairment of creatine kinase (CK) isoenzymes (Table 5). Cytosolic and mitochondrial CK isoenzymes, together with easily diffusible creatine (Cr) and phosphocreatine (PCr), provide an important cellular energy buffer and energy transport system, bridging sites of energy production and consumption (for reviews see [60,94,95]) that is particularly important in heart [96]. CK isoenzymes are expressed in vertebrates in a tissue-specific as well as compartment-specific manner. Two cytosolic isoenzymes, muscle-type MCK and brain-type BCK, as well as two mitochondrial isoenzymes (MtCK), sarcomeric sMtCK (restricted to heart and skeletal muscles) and ubiquitous uMtCK (found in other organs and tissues, as brain, spermatozoa, skin) are encoded by separate nuclear genes. In heart tissue, both cytosolic CK isoenzymes are expressed, with a prevalence of MCK. They form two homodimers (MM- and BBCK), as well as a heterodimer (MBCK) that is specific for heart. Heart mitochondria contain also considerable amounts of sMtCK, which forms mainly octamers. The mitochondrial isoenzyme shows high affinity to the outer surface of the inner mitochondrial membrane and its major negatively charged phospholipid, cardiolipin. It is located in the peripheral intermembrane space and the cristae of mitochondria and functionally and/or structurally associated with the membrane proteins ANT and VDAC in the inner and outer mitochondrial membrane, respectively [61]. The enzyme fulfills a dual function by intramitochondrial regeneration of ADP, and generation of easily diffusible PCr from ATP and Cr [62]. Generated ADP is immediately re-imported by ANT into the matrix space, thus functionally coupling the MtCK reaction to oxidative phosphorylation. PCr leaves mitochondria via VDAC and is used by cytosolic CK isoenzymes, in particular a subfraction that is associated with various ATP consuming sites to maintain high and stable local ATP/ADP ratios. These include main ATP utilizing sites such as the myofibrillar M-band close to actomyosin ATPases [97], the Ca^{2+}-ATPase of sarcoplasmic reticulum [98] and the Na^{+}, K^{+}-ATPase in the sarcolemma [99].

Interference of DXR with CK has been found at different levels of complexity, from the purified enzyme in solution to animals, and the involved molecular mechanisms have been elucidated in detail. Our group has shown that DXR impairs structure and function of purified CK isoforms in

Table 5
Effects of DXR on energy- transfer and signaling

Affected parameter	Experimental model	Species	Effect–extent of change–applied DXR dose–time of treatment	References
CK isoenzymes *Enzymatic activity*	Purified enzyme		Up to ~100% decrease in human and chicken recombinant mitochondrial MtCK activity-5-750 μM/50 μg/ml protein-hours to days	[100]
			60% decrease in MMCK (0.5 mg/ml)-10 μM DXR activated by 1.2 μM HRP/100 μM H_2O_2-10 min	[108] also [106]
	Isolated heart mitochondria	Rat	60% decrease in sMtCK activity-30 μM DXR/Fe^{3+}/0.1 mg protein-2 h	[107]
	Cultured cardiomyocytes	Rat	67% decrease in total CK activity of detached cells-50 μM-24 h; 40% increase in attached cells	[122]
	Heart homogenate	Rat	80% decrease in total CK activity-10 μM DXR activated by 1.2 μM HRP/200 μM H_2O_2 per 0.5 mg protein/ml-30 min	[106]
	Perfused heart	Rat	20% decrease in total CK activity-20 μM-1 h; shift in isoenzyme activity pattern MM-, BB-CK activity respectively de- and increased 2 μM-1 h	[116]
			50% decrease in total CK activity-3 doses of 10 mg/kg within 6 days	[122]
	Animal model	Rat	~20% decrease in total CK activity to-2 mg/kg per week during 13 weeks	[184]
			30% decrease in MMCKactivity-20 mg/kg-5 days after injection	[124]
		Mice	15% decrease in mitochondrial sMtCK activity-20 mg/kg for 5 days	[123]
Inhibited membrane binding of MtCK	Purified protein		Decrease in mobility restriction induced by MtCK in cardiolipin dispersion	[101]
			90% decrease in adsorbtion and increase of the solubilisation of MtCK from liposomes	[185]
			By ~40% with 100 μM per 50 μg/ml protein-20 min (SPR)	[100]
	Isolated heart mitochondria	Rat	IC_{50} ~140 μM/1 mg protein-15 min at 10 °C	[102]
	Perfused heart	Rat	20 μM-1 h	[116]
Dimerization of MtCK	Purified protein		100 μM per 50 μg/ml protein-7 days	[100]
	Perfused heart	Rat	20 μM-1 h	[116]
AMPK	Perfused heart	Rat	Decreased basal phosphorylation-2 and 20 μM-1 h	[116]
Expression of energy regulating enzymes	Cultured cardiomyocytes	Rat	Marked reduction mRNA for MCK-0.5 μM-24 h	[119] also [166]
	Perfused heart	Rat	M-, and BCK protein level respectively de- and increased-2 and 20 μM-1 h	[116]
	Animal model	Mice	5-fold upregulation of SNF-1 gene; ~15% downregulation of AK3 gene-3 mg/kg per week-12 weeks	[167]

vitro, including MtCK [100] and cytosolic CK isoforms (M. Tokarska-Schlattner, unpublished data). DXR-induced damage leads not only to inactivation, which was observed with all CK isoforms, but also to further specific injury of the MtCK isoform, namely dissociation of octamers into dimers and inhibition of MtCK binding to mitochondrial membranes, in particular to cardiolipin [100–102]. Most importantly, the cardiac isoform sMtCK occurred to be more sensitive to DXR than the ubiquitous uMtCK, which could be explained by the slightly different molecular properties and structures of these isoforms [103–105]. Such differential sensitivity of s- and uMtCK to DXR may contribute to the selective toxicity of the drug in heart tissue. As shown for purified enzyme, the DXR effect on MtCK membrane binding is immediate, probably due to a competitive binding of the drug to the main MtCK receptor, cardiolipin. In contrast, enzymatic inactivation and dissociation of MtCK octamers into dimers by 5-750 μM DXR occur in solution only after hours to days. At DXR concentrations below 100 μM inactivation of MtCK is due to direct oxidative modification of the active site cysteine, while generalized damage of different residues, partially mediated by superoxide anion, becomes significant at higher DXR doses. It must be stressed that for in vitro experiments with purified MtCK, the clinically relevant concentrations should be higher than for isolated mitochondria, i.e. of the order of a few tens of μM, since MtCK and DXR share cardiolipin as a common binding partner in the inner mitochondrial membrane, leading to high local concentrations of DXR in the microenvironment of MtCK. In addition, activation of the drug by peroxidase/H_2O_2 or its complexation with iron has been shown to significantly accelerate inactivation of purified CK or CK in heart homogenate [106,107]. In such systems, 10–30 μM DXR induce CK inactivation within a few tens of minutes. Recently, Muraoka and Miura [108] have suggested that oxidative activation of the p-hydroquinone B ring by DXR-HRP/H_2O_2 is essential for oxidation of sulfhydryl (SH) groups of CK and other SH proteins, and that this effect is prevented by thiol compounds, e.g. reduced glutathione. This mechanism, as well as the more intensive production of reactive oxygen and nitrogen species (ROS, RNS, respectively) in situ [5,109,110] can enhance inactivation of CK, which is known to be very sensitive to oxidation and nitration [111–115], in particular in case of MtCK situated at the very origin of free radical formation.

In situ, acute effects of DXR on the CK system have been studied in the perfused heart model [116]. In this condition, dimerization of MtCK and inhibition of cardiolipin binding were detectable already after 1 h perfusion at 2 μM DXR, and became significant at 20 μM. At the latter concentration, also a mild decrease in total CK activity by about 20% was observed. In spite of these defects, which occurred relatively early, the CK system probably maintained its functionality under such acute conditions. Interestingly, the acute response of the CK system to DXR is accompanied by a shift in the expression of cytosolic CK isoforms, with decreased MCK and increased BCK protein, characteristic for an early differentiation state. This isoform shift was already apparent after 1 h of perfusion of the heart at the 2 μM DXR. The mechanisms responsible for the shift in CK isoforms remain largely speculative at this time and can involve changes in transcription, translation, degradation or leakage. For example, it could be due to rapid changes in gene expression, either because of a general induction of a fetal expression profile in response to stress [117,118] or because of specific susceptibility of CK genes to DXR (see Table 5). Fast changes in gene expression after DXR exposure were already observed earlier [119]. Re-expression of a fetal-like CK isoenzyme pattern, with BCK accumulation, is known for chronic heart pathologies as cardiac hypertrophy or failure [12,117,120] and has been hypothesised to have a compensatory character. Myocardial contractile efficiency may increase in proportion to the fetal shift from MCK to BCK in chronically infarcted hearts [121]. A functional advantage would be related to higher affinity of BCK for ADP and PCr as compared to MCK, possibly allowing more efficient ATP regeneration [117].

Gradual accumulation of substantial direct and radical-mediated molecular damage of CK has been shown to occur with prolonged DXR exposure. In cultured cardiomyocytes treated with 50 μM DXR, a pronounced impairment of the CK system was observed after 24 h [122]. Interestingly, total CK activity decreased significantly in detached cells by about 67%, while it increased in attached cells by about 40% during 48 h of treatment. The latter may also be indicative of a compensatory mechanism within the CK system. Inactivation of CK under chronic DXR exposure has been reported for several animal models (see Table 5). In studies with DXR treated mice, sarcomeric sMtCK occurred to be more sensitive to inhibition as compared to the respiratory chain complexes, which would make this kinase a prime target of DXR-induced damage in vivo [123]. Significant nitration of the myofibrillar MCK isoform together with a marked reduction in CK activity was observed in DXR-treated mice by Mihm and colleagues [124].

Long-term CK damage could be at the origin of numerous deleterious processes, which promote chronic DXR-induced cardiac dysfunction [3,12]. Compromised MtCK functions, including inactivation, dimerization and inhibition of its binding to cardiolipin, would not only impair the energy channeling and signaling between mitochondria and cytosol [62,96,125], but would additionally affect mitochondrial respiration. This molecular damage would also contribute to destabilization of the so-called mitochondrial contact sites involving ANT, MtCK and VDAC [126,127]. In support of this, disappearance of contact sites between the two membranes has been observed in DXR-treated liver mitochondria [53]. In view of a postulated role of MtCK in preventing opening of the mitochondrial permeability pore [128], MtCK defects would sensitize cardiac cells to mitochondrial permeability transition, which has been observed as a result of DXR treatment (for review see [13]). In the cytosol, where MCK is functionally coupled to the Ca^{2+}-pump of sarcoplasmic reticulum [98], inhibition of this isoenzyme would result in Ca^{2+} imbalance, which in turn would interfere with muscle contraction and relaxation [95] and could lead via chronic Ca^{2+}-overload to apoptotic and/or necrotic cell death.

7.2. Energy state signaling

In a normal physiological setting, perturbations in intracellular nucleotide ratios activate signaling cascades, which would trigger a coordinated response to protect the cell from a dangerous fall in energy state. In the heart, AMP-activated protein kinase (AMPK) plays a crucial role in such emergency signaling. AMPK, a key energy sensor, signaling system and regulator of cellular energy substrate utilization [129,130], is activated by a fall in cellular energy state, in particular an increased AMP/ATP ratio, as well as by oxidative stress and some other extracellular stimuli. Activated AMPK triggers catabolic pathways that generate ATP, e.g. fatty acid oxidation, and glycolysis, and down-regulates anabolic ATP-consuming processes that are not essential for short-term cell survival, such as the synthesis of lipids, carbohydrates and proteins.

Recent work of our laboratory suggests AMPK as a new and highly sensitive target of DXR-induced damage in heart ([116], see Table 5). The toxicity limit in term of DXR dose and time of onset is very low. A dose of 2 μM DXR, which corresponds to peak plasma concentration of DXR in patients, downregulated AMPK protein expression and phosphorylation as well as phosphorylation of its downstream target acetyl-CoA carboxylase. Decreased phosphorylation within the AMPK pathway was observed earlier than changes in myocardial function. The mechanisms of inhibition are not yet known, but they may include direct effects on AMPK, compromised upstream signaling or altered phosphatase activity.

DXR induces both, energetic imbalance and generation of reactive oxygen and nitrogen species [4,110]. Under these conditions, down-regulation of the fuel-sensing AMPK pathway, which would be expected to initiate responses preventing such energy depletion, seems paradoxical. On the other hand, inhibition of AMPK is consistent with several known DXR effects. AMPK inhibition could explain the change in energy substrate utilization after DXR treatment, first of all the decreased fatty acid oxidation as observed in different studies (see Table 4). Inactivated AMPK would be unable to down-regulate acetyl-CoA carboxylase activity, possibly leading to higher malonyl-CoA levels that inhibit carnitine palmitoyl transferase (CPT1), and thus mitochondrial import and oxidation of fatty acids [131]. Similarly, impaired AMPK signaling would reduce the capacity of the cell to initiate a compensatory increase in glycolytic rate and to stimulate glucose uptake.

Disturbance of cell regulatory systems, such as CK and AMPK, can be especially harmful at elevated work-loads or other situations of energetic emergency. In the perfused heart model, hypoxia combined with DXR, has been shown to significantly potentiate harmful effect of the drug [132]. As shown in exercised rats, DXR hampers the exercise-stimulated activation of respiration in response to ADP [52]. Perturbed regulation could be at the origin of reduced exercise tolerance, affecting up to 70 % of cancer patients during and after therapy [133]. In long-term cancer survivors maximal oxygen consumption (VO_{2max}) averages only 50-70% of normal.

8. Molecular mechanisms

Existing evidence points to a complex situation with a multitude of molecular mechanisms involved in DXR-induced impairment of cardiac energetics and other cellular targets, finally leading to cardiac dysfunction (Fig. 1).

An important factor, which can mediate the toxic action of DXR, especially in mitochondria, is high affinity binding of DXR to cardiolipin, an anionic phospholipid specific for the inner mitochondrial membrane, which has been recognized as an essential phospholipid in eukaryotic energy metabolism [134–137]. Cardiolipin with its particular ability to interact more or less specifically with many proteins, is very important not only for mitochondrial structure and function, but also for overall cardiac energy metabolism as well as for cell survival [137]. Binding of DXR would modify membrane properties and thus change phospholipid environment and function of numerous crucial mitochondrial integral membrane proteins, which depend on cardiolipin in their function, as e.g. cytochrome c oxidase [138–141], inorganic phosphate carrier [66], and possibly ANT, which has firmly bound cardiolipin [142]. Cardiolipin-bound DXR would also induce dissociation of cardiolipin-associated peripheral proteins from the inner mitochondrial membrane, like e.g. cytochrome c and MtCK (see [116]). This could affect electron transport chain and energy channeling, as well as favor initiation of programmed cell death.

Toxicity of mitochondrial, mostly cardiolipin-bound DXR is mediated by oxidative stress [3], which represents a particular threat to cellular energetics in the myocardium and is considered as the main mediator of DXR cardiotoxic action. Heart tissue is rich in mitochondria, since it heavily relies on oxidative metabolism, and thus produces significant amounts of free radicals. Accumulation of redox active DXR in these organelles [16,33,34] would enhance mitochondrial production of reactive oxygen (ROS), and–as more recently evidenced–also of nitrogen species (RNS) [4,5,14,70,143]. DXR generates free radicals and other related reactive oxygen and nitrogen species both through an enzymatic mechanism utilizing cellular oxidoreductases (NADH dehydrogenase of complex I, cytochrome P-450 reductase, xanthine oxidase) and through nonenzymatic pathways involving complexation with iron (Fe^{3+}) [4,144–148]. The redox cycling of DXR catalysed by oxidoreductive enzymes is shown in Fig. 2. DXR, a quinone-containing drug, can be converted to the semiquinone form by one electron reduction. The DXR semiquinone can subsequently transfer an electron to the oxygen molecule (O_2) to form superoxide anion radical ($O_2^{\bullet -}$) [4,5,14,70,143]. The latter can dismutate to form hydrogen peroxide (H_2O_2) and further hydroxyl radical ($HO^{\bullet}$) or may react with nitric oxide to form peroxynitrite ($ONOO^-$). The noxious action of these reactive compounds includes the peroxidation of lipids and oxidative damage to proteins and DNA. Peroxidation of membrane phospholipids associated with a decrease in membrane fluidity, as well as oxidation or nitration of proteins have been reported after DXR treatment [124,149,150]. Oxidative and nitrosative stress interfere with many aspects of cardiac function, inducing

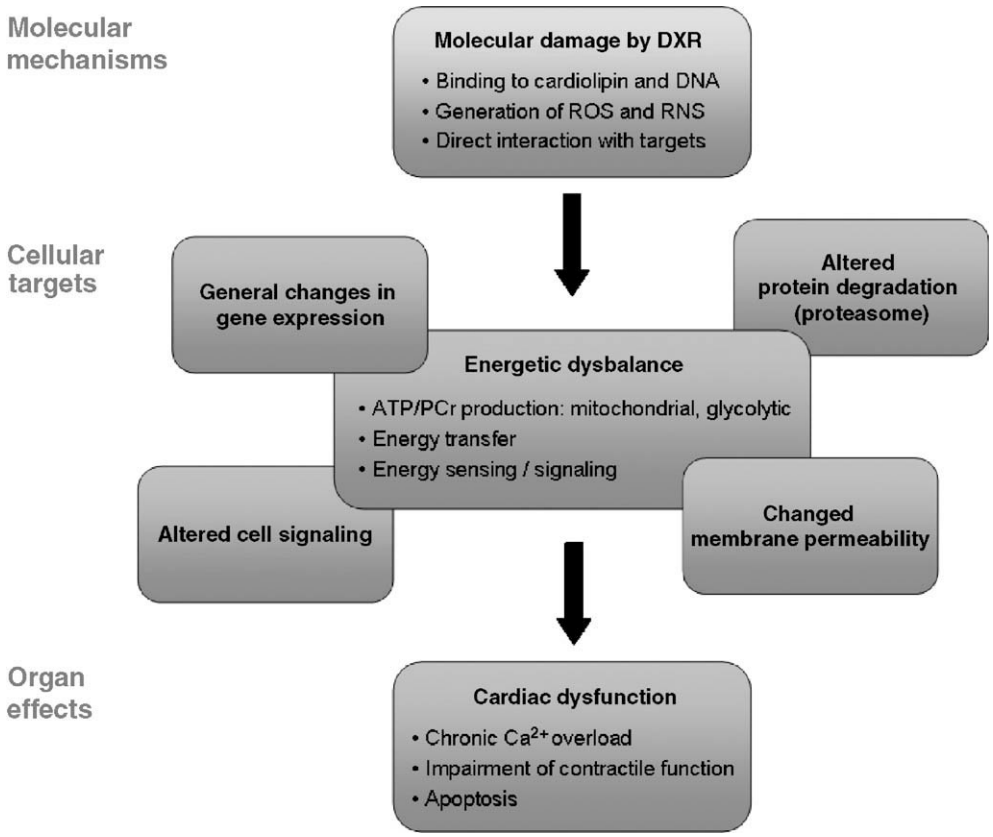

Fig. 1. Mechanisms and targets of doxorubicin cardiotoxic action. The schematic diagram summarizes multiple molecular mechanisms and intracellular targets, including cellular energetic network, implicated in development of doxorubicin-induced cardiac dysfunction. The depicted processes represent a simplified view of the most pertinent mechanisms, but are not exhaustive.

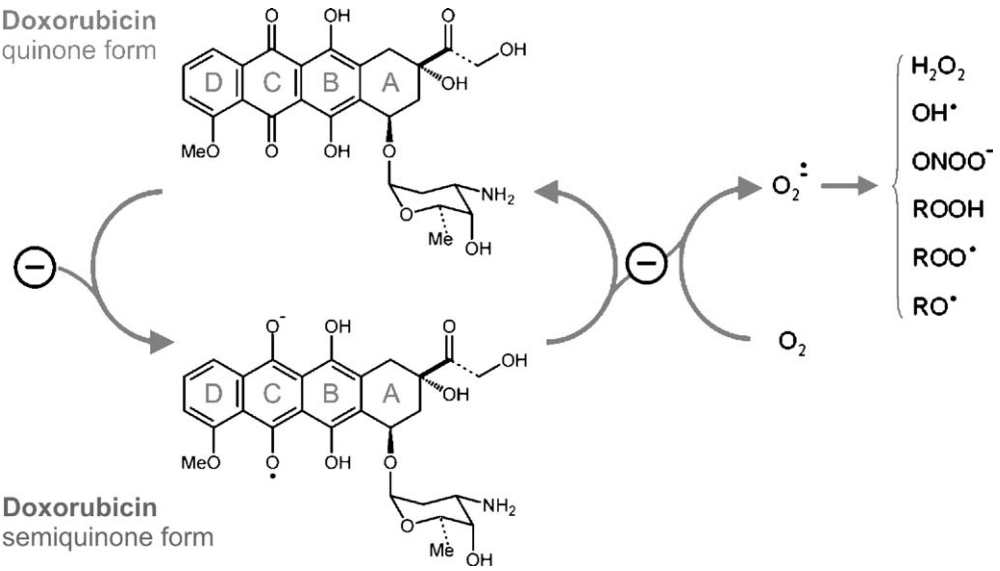

Fig. 2. Chemical structure of doxorubicin and related redox cycling by enzymatic mechanism. Doxorubicin consists of a tetracyclic ring containing adjacent quinone-hydroquinone groups in rings C–B, coupled with the sugar daunosamine attached by a glycosidic linkage to the ring A. One-electron reduction of the quinone moiety mediated by the cellular oxidoreductases results in formation of a semiquinone radical that regenerates the parent quinone by reducing oxygen molecule (O_2) to superoxide radical ($O_2^{\bullet -}$). This initiates a reaction cascade leading to the formation of other reactive oxygen and nitrogen species (ROS, RNS): superoxide dismutases can convert superoxide radical ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2); $O_2^{\bullet -}$ and H_2O_2 can interact with iron or other transition metal ions to generate hydroxyl radical ($OH^{\bullet}$); $O_2^{\bullet -}$ can also initiate lipid peroxidation forming lipid peroxides and derived alkoxyl and peroxyl radicals (ROOH, $RO^{\bullet}$, $ROO^{\bullet}$) or react with nitric oxide ($NO^{\bullet}$) to form peroxynitrite ($ONOO^{-}$). Besides the depicted enzymatic mechanism, doxorubicin can generate free radicals and other related reactive oxygen and nitrogen species through a nonenzymatic pathway involving complexation with iron (Fe^{3+}).

among others energetic imbalance, mitochondrial permeability transition and apoptosis, as well as activation of various related signaling pathways [151–153]. Signaling via NO [143], MAPK [154], NF-kB [155], NFAT, FAS ligand [156], ceramide [90], Ca^{2+} [4,13] or PARP activation [157], may serve as examples of such pathways associated with DXR cardiotoxic action. In particular the response of cardiac Ca^{2+} to DXR can be closely related with the perturbation of heart energy homeostasis. DXR-induced alterations in Ca^{2+} handling and their possible consequences are, however, beyond the scope of this review, but are described in several original papers and review articles [4,13,14,65,158–163]. It is to note, that the actual extent and impact of oxidative and nitrosative stress produced by DXR in different toxicity models, as well as in patients, are a matter of discussion (see [34,148]). Interestingly, as suggested by Feng and coworkers [164], quinone-containing molecules can exert their effect independently of free radicals by directly affecting some sensible protein residues, as e.g. reactive sulfhydryl groups. Such a direct mechanism may play a role in in vitro inactivation of MtCK [100]. In line with this concept, DXR has been shown to interact directly with the prosthetic group of heme in hemoproteins, as e.g. cytochrome c oxidase [165].

Finally, DXR can interfere with cardiac gene expression [64], in particular by down-regulating genes of several enzymes implicated in energy metabolism. Among these are genes of enzymes involved in mitochondrial oxidative phosphorylation (cytochrome c oxidase [48,74], Rieske iron-sulfur protein, and ANT1 [64,65]), in glycolysis (phosphofructokinase [64]), and enzymes in energy transfer (MCK [119,166], adenylate kinase AK3 [167]). The mechanisms by which DXR inhibits cardiac gene expression are not yet clear. DXR was found to inhibit DNA replication in cultured rat cardiomyocytes [168] and to alter cardiac DNA in animals [74,169] and in humans [76], inducing cardiac-specific mtDNA lesions. Although such interference with DNA is considered important for the anticancer action of the drug, it may also affect macromolecular biosynthesis in the heart [170]. In addition, oxidative stress has been implicated in DXR-impaired gene expression [171]. Reduced protein levels seen after DXR exposure may be also explained by elevated degradation of proteins that were oxidized by DXR. Fragmentation and proteolysis of DXR-modified proteins were shown to occur in DXR-treated mitochondrial preparations [172]. In addition to lower biosynthetic rate and enhanced degradation rate, reduced cellular levels of soluble proteins and metabolites in DXR-challenged cells can occur through leakage. This is well known for cytosolic MBCK, the plasma level of which serves as clinical marker of different cardiac pathologies, including DXR toxicity [15]. However, it should be noted that certain genes encoding proteins implicated in energy metabolism have been found up-regulated in a mice model of chronic DXR-cardiotoxicity [167]. This may reflect induction of a protective or compensatory response.

Further mechanisms contributing to the toxic effect of DXR in cardiac tissue can be mediated by iron or DXR metabolites [148], as well as by an important damage of endothelial origin [155,173]. Collectively, the response of cardiac energetics to DXR involves a complex cross-talk between different pathways and mechanisms.

9. Concluding remarks

Alterations in myocardial energy metabolism have been recognized as a hallmark for different cardiac pathologies ([10,11], Nahrendorf, 2006 #390, [12,174]). This review summarized the amazing spectrum of changes in cardiac energy metabolism caused by DXR. The drug induces damage at multiple sites of cardiac energy metabolism, including a fall of basal high energy phosphate levels, PCr and ATP, a reduction of oxidative capacity of mitochondria, changes in the profile of energy substrate utilization with marked reduction of fatty acid oxidation, a disturbance of energy transfer between sites of energy production and consumption by CK isoenzymes, as well as defects in the AMPK signaling pathway.

The clinical relevance of all these changes, as well as their onset in DXR-treated patients, remains to be established. Further studies will be necessary to elucidate the relative impact of DXR on the different components of the cellular energy network and on cardiac function in general, and to clarify the onset of molecular damage in treated patients. Especially valuable will be novel non-invasive techniques, which allow insight into structural and functional changes including evaluation of energy metabolism in the heart in vivo [175]. Identification of the most sensitive components of the cellular energy network may help to design a strategy for cardioprotection. Vice versa, testing protective strategies addressing specific energetic defects can be helpful to identify the critical steps that are affected by DXR.

Finally, it is of particular interest that in DXR-treated patients, changes in heart ultrastructure appear much earlier than any clinical manifestation of heart failure [20]. This implies that compensatory mechanisms are operative that can preserve cardiac function in spite of increasing myocardial damage. We support the view that the accumulation of various deficiencies in high-energy phosphate metabolism, encompassing the failure of regulatory and compensatory mechanisms, is a very critical event in the deterioration of cardiac function and in the onset and progression of chronic DXR-induced cardiotoxicity in patients.

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