Chronic Anthracycline Cardiotoxicity: Molecular and Functional Analysis with Focus on Nuclear Factor Erythroid 2-Related Factor 2 and Mitochondrial Biogenesis Pathways

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ABSTRACT

Anthracycline anticancer drugs (e.g., doxorubicin or daunorubicin) can induce chronic cardiotoxicity and heart failure (HF), both of which are believed to be based on oxidative injury and mitochondrial damage. In this study, molecular and functional changes induced by chronic anthracycline treatment with progression into HF in post-treatment follow-up were analyzed with special emphasis on nuclear factor erythroid 2-related factor 2 (Nrf2) and peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α) pathways. Chronic cardiotoxicity was induced in rabbits with daunorubicin (3 mg/kg, weekly for 10 weeks), and the animals were followed for another 10 weeks. Echocardiography revealed a significant drop in left ventricular (LV) systolic function during the treatment with marked progression to LV dilation and congestive HF in the follow-up. Although daunorubicin-induced LV lipoperoxidation was found, it was only loosely associated with cardiac performance. Furthermore, although LV oxidized glutathione content was increased, the oxidized-to-reduced glutathione ratio itself remained unchanged. Neither Nrf2, the master regulator of antioxidant response, nor the majority of its target genes showed up-regulation in the study. Instead, down-regulation of manganese superoxide dismutase and NAD(P)H dehydrogenase [quinone] 1 were observed together with heme oxygenase 1 up-regulation. Although marked perturbations in mitochondrial functions were found, no induction of PGC1α-controlled mitochondrial biogenesis pathway was revealed. Instead, especially in the post-treatment period, an impaired regulation of this pathway was observed along with down-regulation of the expression of mitochondrial genes. These results imply that global oxidative stress need not be a factor responsible for the development of anthracycline-induced HF, whereas suppression of mitochondrial biogenesis might be involved.

Introduction

Introduction of anthracycline (ANT) antibiotics to clinical practice in the 1960s represented a significant milestone in cancer treatment. High antitumor efficacy and a broad clinical spectrum made ANTs one of the most useful chemotherapeutics ever developed (Minotti et al., 2004). Unfortunately, the benefits from ANTs have been found to be notably counterbalanced by the risk of cardiac toxicity. Major clinical concerns are related to the chronic type of ANT cardiotoxicity associated with dilated cardiomyopathy and heart failure with either early- or late-onset forms (Lipszultz et al., 2008; Ewer and Suter, 2010). Unlike other complications of cancer chemotherapy, chronic ANT cardiotoxicity usually stays si-
lent during cancer treatment, whereas it can strike back weeks, months, or even years later to significantly affect the morbidity and prognosis of cancer survivors (Ewer and Suter, 2010). Chronic cardiotoxicity is known to be a class effect typical of all ANT derivatives introduced to clinical practice so far (Minotti et al., 2004). Although many modifiable and nonmodifiable risk factors have been described, the most important one is the lifetime cumulative dose of the drug (Lipschultz et al., 2008; Ewer and Suter, 2010).

Despite many experimental and clinical studies performed throughout the last 40 years, the unequivocal explanation of molecular basis for ANT cardiotoxicity development remains elusive and is still a matter of debate and controversy (Gianni et al., 2008; Šimůnek et al., 2009; Menna et al., 2012). The prevailing mechanistic concept highlights ANT-induced and iron-catalyzed formation of reactive oxygen species (ROS), resulting in direct oxidative damage to the myocardium (Keizer et al., 1990; Šimůnek et al., 2009). In addition, anthracyclines have been shown to interfere with the normal regulation of cellular iron homeostasis in cardiomyocytes (Kwok and Richardson, 2003, 2004). Other hypotheses point to ANT-induced impairment in mitochondrial bioenergetics (Wallace, 2003; Tokarska-Schlattner et al., 2006), damage to mitochondrial DNA (Berthiaume and Wallace, 2007; Lebrecht and Walker, 2007) with subsequent perturbations in expression of mitochondria-encoded genes (Lebrecht et al., 2010), disruption in mitochondrial and cellular Ca2+ homeostasis (Wallace, 2007; Lebrecht et al., 2010), and alterations in expression and stability of cardiac myofilaments (Lím et al., 2004).

Indeed, in the clinical setting it is very difficult to directly assess ANT-induced molecular changes in the myocardium and their further development in the post-treatment period. Furthermore, it is also challenging to appropriately mimic the nature of chronic ANT cardiotoxicity in vitro or by using single high doses in vivo models (Gianni et al., 2008). This can partially explain our poor insight into molecular events determining the development of chronic ANT cardiotoxicity and its transition into heart failure in the post-treatment follow-up (FU). In this regard, it might be particularly important to understand what happens with ANT-induced myocardial oxidative stress, mitochondrial damage, perturbations in bioenergetics, and cell death. Furthermore, because oxidative stress is the most frequently cited mechanism of chronic ANT cardiotoxicity development, it is rather surprising that there is no information on the response of a key antioxidant and cytoprotective pathway regulated by nuclear factor erythroid 2-related factor 2 (Nrf2). Cellular oxidative stress is known to stabilize Nrf2 and induce its translocation in to the nucleus where it markedly up-regulates expression of a battery of antioxidant response element (ARE)-associated target genes (Baird and Dinkova-Kostova, 2011). In addition to its own expression, Nrf2 up-regulates the expression of superoxide dismutase (both copper zinc superoxide dismutase (CuZnSOD) and manganese superoxide dismutase (MnSOD)), inducible heme oxygenase 1 (HO1), NAD(P)H dehydrogenase [quinone] 1 (NQO1), numerous enzymes involved in the glutathione system, and many others (Baird and Dinkova-Kostova, 2011).

Furthermore, mitochondria have been repeatedly suggested as the main target for chronic ANT cardiotoxicity (Wallace, 2003; Tokarska-Schlattner et al., 2006). However, involvement of the mitochondrial biogenesis pathway remains uncertain in the chronic ANT cardiotoxicity setting. The master regulation of mitochondrial biogenesis is performed by peroxisome proliferator-activated receptor-γ coactivator-1α (PGC1α), which is induced by numerous conditions including energy starvation and mitochondrial oxidative stress (Ventura-Clapier et al., 2008; Rimbaud et al., 2009). Once activated, PGC1α acts as a key coactivator up-regulating the expression of numerous important mitochondrial proteins. With respect to the respiratory chain subunits, there is a requirement for coordination of expression of components encoded by both nuclear and mitochondrial genome, and this is mediated by downstream pathway members, nuclear respiratory factors (NRFs) and mitochondrial transcription factor A (TFAM), respectively (Rimbaud et al., 2009). Despite the potential importance of this pathway, its role in the development of chronic anthracycline cardiotoxicity and transition into heart failure remains to be determined.

Therefore, in this study we analyzed left ventricular (LV) molecular and functional changes induced by repeated administration of ANT and their further progress in the post-treatment follow-up with a focus on the involvement of oxidative stress and mitochondrial damage. Moreover, we investigated the response of two logically related endogenous protective machineries, the Nrf2-regulated antioxidant response pathway and the mitochondrial biogenesis pathway, to assess their role in chronic ANT cardiotoxicity.

**Materials and Methods**

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated.

**Animals and Experimental Design.** The study was performed by using the previously well established animal model of chronic ANT cardiotoxicity (Šimůnek et al., 2004; Popelová et al., 2009; Štěrba et al., 2011). Adult male Chinchilla rabbits (~4 months old; ~3.5 kg; n = 46; Velaz, Košelé u Kladna, Czech Republic) were housed under a 12-h light cycle with constant temperature and humidity and free access to tap water and a standard laboratory pellet diet. All procedures were performed according to the Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) as approved and supervised by the Animal Experimentation Committee of the Faculty of Medicine, Charles University in Prague, Hradec Králové. Cardiotoxicity was induced in rabbits with daunorubicin (DAU; 3 mg/kg i.v., once weekly for 10 weeks; n = 27; Daunoblastina, Pfizer, Rome, Italy), whereas control (CTR) animals received saline (1 ml/kg i.v.; n = 19). A week after the last drug administration, the animals were randomized into two groups. The first group was sacrificed, whereas the second was followed for the next 10 weeks and designated as the FU group (DAU FU, n = 11; CTR FU, n = 10). Mortality was determined in the treatment period. During the FU animals were sacrificed whenever weekly echocardiography examination showed LV fractional shortening (FS) to be lower than 20% (indicating decompensated heart failure) to avoid loss of myocardial samples because of sudden deaths.

All experimental procedures were performed under light anesthesia consisting of ketamine (30 mg/kg i.m.; Narketan, Vétoquinol AG, Ittigen, Switzerland) and midazolam (2.5 mg/kg i.m.; Midazolam Torrex, Torrex Chiesi Pharma, Vienna, Austria), and freshly prepared pentobarbital solution [4% (w/w), i.v.] was used for animal overdose.

During autopsy hearts were rapidly excised and briefly retrogradely perfused with ice-cold saline. A piece of the LV free wall was removed for analysis of myocardial bioenergetics, and the rest was shock-frozen, homogenized under liquid nitrogen, and stored at ~80°C.
**Examination of Cardiac Functions.** Echocardiographic measurements were performed by using Vivid 4 equipped with a 10-MHz probe (GE Medical Systems Ultrasound; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) during the treatment period and weekly in the FU period. Guided M-mode measurements were performed at the tips of the mitral valve to obtain the LV end-systolic and end-diastolic diameters (LVESD and LVEDD, respectively, in the equation). LV FS as an index of the systolic function was determined as follows:

\[
\text{LVFS(\%)} = \left( \frac{\text{LVEDD} - \text{LVESD}}{\text{LVEDD}} \right) \times 100
\]

**Plasma Troponin T.** Cardiac troponin T was determined in plasma samples by using Elecsys Troponin T high sensitivity assay (Roche Diagnostics, Basel, Switzerland) with a limit of detection of 0.003 µg/l. Blood samples were collected before the first, fifth, seventh, eighth, and 10th drug administration, a week after the last administration of DAU and weekly in the FU period. Area under the curve of plasma troponin T was determined by using Prism 5.0 (GraphPad Software, Inc., San Diego, CA).

**Oxidative Damage to the Myocardium.** Markers of oxidative damage were analyzed in LV samples homogenized in ice-cold buffer [25 mM Tris and 0.1% (v/v) Triton X-100, pH 7.6] on ice, and supernatants were collected and stored in −80°C. For measurement of LV glutathione content, the low-spin supernatant was treated with 10% (w/v) metaphosphoric acid on ice and further centrifuged at 15,000 g to yield final supernatants.

LV malondialdehyde (MDA) content was measured as a marker of lipoperoxidation in supernatants prepared as described above using a HPLC method according to Pilz et al. (2000) with minor modifications (Popelová et al., 2009). This TBARS-independent technique is based on the derivation of MDA with 2,4-dinitrophenylhydrazine yielding a fluorescent compound, which is then selectively detected by using a HPLC system.

For the measurement of individual oxidized and reduced glutathione forms (GSSG and GSH, respectively) in the LV myocardium a selective HPLC method was used (Kandár et al., 2007). The method is based on the derivation of GSH with o-phthalaldehyde yielding a HPLC-detectable fluorescent derivative, whereas for selective GSSG measurement the samples were incubated with N-ethylmaleimide to remove GSH from the sample before the o-phthalaldehyde derivation.

**Activity of Glutathione System Enzymes.** Activity of glutathione system enzymes were analyzed in the LV samples homogenized in ice-cold buffer [5 mM HEPES, 1 mM EDTA, 1 mM DTT, and 0.1% (w/v) Triton X-100, pH 8.7]. Enzyme-coupled spectrophotometric assays for detection of activity of glutathione peroxidase (GPx) and glutathione reductase (GR) were performed as described previously (Vávrová et al., 2011). Glutathione transferase (GST) activity was determined by using the SensoLyte GST Activity Assay Fluorimetric Kit (Anaspec, Inc., San Jose, CA) according to the manufacturer’s instructions.

**Mitochondrial Function Measurement.** Fresh LV myocardium was homogenized in ice-cold buffer (25 mM sucrose, 75 mM sorbitol, 100 mM KCl, 10 mM H$_2$PO$_4$, 5 mM MgCl$_2$, 10 mM Tris, 20 mM EDTA, and 1 mg/ml bovine serum albumin) by using a glass/Teflon Potter-Elvejem grinder (P-LAB, Prague, Czech Republic), and the homogenate was passed through a nylon filter. Mitochondrial respiration was measured by using a high-resolution Oxygraph 2K (Oroboros, Innsbruck, Austria) in a MR05 respiration buffer (Oroboros) at 30°C. Oxygen consumption was induced by glutamate (10 mM), malate (2.5 mM), and ADP (1 mM). The status of the mitochondrial outer membrane was evaluated by the addition of cytochrome c (10 µM), and an increase of oxygen consumption by approximately 69% suggested partial damage to the membrane, which occurred during the preparation of the tissue homogenate. No significant differences were found between the treatment groups. Rotenone (1 µM) was used for the inhibition of the complex I, whereas succinate (10 mM) was used for the activation of complex II. The activity of cytochrome c oxidase (COX) was evaluated separately by using cytochrome c (10 µM) as a substrate, N,N,N,N'-tetramethyl-p-phenylenediamine (0.5 mM) as an artificial electron donor, and ascorbate (2 mM) as a regenerating system for reduced N,N,N,N'-tetramethyl-p-phenylenediamine. Oroboros software (DatLab 4.1) was used for evaluating oxygen consumption. The data were corrected for by the rate of chemicals autoxidation and are expressed as relative fold over the control group.

Additional rotenone-sensitive complex I activity assay was performed according to Long et al. (2009) with minor modification in the mitochondria-enriched fraction as described previously (Štěrba et al., 2011). Citrate synthase activity in the LV samples was analyzed in the LV homogenates as prepared as described under Activity of Glutathione System Enzymes by using an enzyme-coupled spectrophotometric method as described previously (De Sousa et al., 1999).

**Active Form of Nrf2 in the Nuclear Fraction.** The amount of transcriptionally active form of Nrf2 in the LV nuclear extract was analyzed by using a commercial kit (TransAM Nrf2; Active Motif, Inc., Carlsbad, CA) according to the manufacturer’s recommendations. The assay contains 96-well plates coated with immobilized oligonucleotides containing the ARE consensus binding site, which specifically binds with the active form of Nrf2 contained in the nuclear extract. Ten micrograms of nuclear extract were loaded into each well, and samples were incubated for 1 h. Nrf2 bound to the imbedded ARE oligonucleotides on the 96-well plates was detected colorimetrically at 450 nm by incubation with a primary antibody against Nrf2 and secondary antibody conjugated to horseradish peroxidase. Nuclear extracts from COS-7 cells transfected with Nrf2 were used as a positive control for Nrf2 binding activity. The fold increase in Nrf2 binding activity was determined by comparison of these results with the levels determined in the samples from control animals. Nuclear extract were isolated from LV myocardium by using Dignam’s protocol (Dignam et al., 1983). In brief, approximately 50 mg of the tissue was homogenized in ice-cold buffer A [10 mM HEPES, 1.5 mM MgCl$_2$, 10 mM KCl, 0.5 mM DTT, 0.005% nonidet P-40 (v/v), and protease inhibitor cocktail, pH 7.9]. After centrifugation (3000 g, 10 min), the pellet was collected and dissolved in a mixture of ice-cold buffer B [5 mM HEPES, 1.5 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM DTT, and 26% (v/v) glycerol, pH 7.9] and 4.6 M NaCl.

**Quantitative Real-Time PCR.** Total RNA from LV myocardium was isolated by using TRIzol reagent. For analysis of the expression of ND1, ND4, NDUF52, COX1, and COX41, the isolated total RNA was further treated with rDNase and cleaned with a Nucleospin RNA XS kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocol. Isolated RNA was converted into cDNA via a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Commercially available qPCR assays based on the combination of two primers and sequence-specific hydrolysis probe (Supplemental Table 1) were obtained from Generi Biotech (Hradec Králové, Czech Republic). The amplification was performed in triplicate with TaqMan Fast Universal PCR Master Mix (Applied Biosystems) by using a 7500HT Fast Real-Time PCR System (Applied Biosystems). The assay was performed in fast mode of 50 cycles with the following time-temperature profile: 95°C for 3 min, 95°C for 5 s, and 60°C for 25 s. All results were normalized by geometric mean of hypoxanthine phosphoribosyltransferase 1 expression.

For analysis of the change of mitochondrial DNA (mtDNA)/nuclear DNA (nDNA), total DNA was extracted from the LV myocardium by using a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA). Commercial qPCR assays (Generi Biotech) for three mtDNA-encoded genes (ND1, ND4, and COX1) were used to analyze extracted DNA to assess the relative abundance of mtDNA. Averaged results were normalized over the results of qPCR analysis of nDNA-encoded leptin. The assay was performed in fast mode of 50 cycles with following time-temperature profile: 95°C for 3 min, 95°C for 10 s, and 60°C for 30 s.

**Determination of Protein Concentration.** Protein concentration was determined by using a BCA Protein Assay Kit (Sigma-Aldrich).
Statistical Analyses. The results are shown as individual values along with their means/medians according to data character unless otherwise stated. Statistical significance was determined by using one-way ANOVA/ANOVA on Ranks or paired t test/Wilcoxon Signed Rank Test according to the data character by using Sigmastat 3.5 (SPSS, Chicago, IL). Correlation analyses were performed by using Pearson’s or Spearman’s methods.

Results

DAU-Induced Cardiotoxicity and Its Progression into Dilated Cardiomyopathy and Heart Failure in the FU. The repeated administration of DAU induced a moderate mortality in the treatment period (5 of 27 animals), and 9 of 11 DAU-treated rabbits were gradually sacrificed according to the protocol in the post-treatment FU because of the progression of cardiac dysfunction (Fig. 1A). No premature deaths occurred in the control groups. There was no significant body weight gain in the DAU group, neither at the end of the treatment nor in the FU, which contrasted with significant body weight gain in both intervals of the control group (Fig. 1B). A heart weight-to-body weight ratio was significantly higher in the DAU-treated animals compared with the control group, both at the end of the treatment (2.72 ± 0.21 versus 2.06 ± 0.06 g/kg, respectively) and in the FU (2.69 ± 0.08 versus 1.80 ± 0.07 g/kg; p ≤ 0.001).

Echocardiographic examination revealed a significant decrease of the LV FS at the end of the DAU treatment, which was followed by marked and significant progression in the
Fig. 2. Daunorubicin-induced myocardial lipoperoxidation. A, HPLC determination of total MDA (a marker of lipoperoxidation) in the left ventricular myocardium (TBARS-independent method). Data are shown as individual values along with their medians. Statistical analysis was performed by using ANOVA on Ranks. Significance is shown as: ***p < 0.001. FU, 10-week post-treatment follow-up. B, correlation of left ventricular performance with lipoperoxidation (malondialdehyde content) in individual animals as determined by Spearman correlation and regression analysis. Correlation coefficient and statistical significance are shown.

FU (to ~48% of the initial value; p ≤ 0.001; Fig. 1C). This corresponded with a higher incidence of pleural effusions (>1.0 ml) in the FU than at the end of the DAU treatment (55 versus 13%, respectively). The LVEDD was not changed at the end of the DAU treatment, whereas it rose significantly in the FU compared with the control group in initial as well as end-of-treatment values (Fig. 1D). These data describe moderate LV dysfunction with no change in the LV dimension by the end of the treatment, whereas there was a marked progression into dilated cardiomyopathy and congestive heart failure in the FU period.

Plasma concentrations of cardiac troponin T were significantly elevated in the DAU group by the fifth week, and they continued to rise further until the end of the treatment in the 11th week (Fig. 1E). It is noteworthy that the concentrations remained similarly elevated in the FU for at least another 3 weeks with slow decreases thereafter. In addition, the area under the curve of troponin T plasma concentrations was found to be markedly higher in the DAU group than in the control group during treatment (0.64 ± 0.14 versus 0.09 ± 0.02 µg/l; p ≤ 0.001, respectively) and increased further in the FU (1.12 ± 0.02 versus 0.16 ± 0.02 µg/l; p ≤ 0.001, respectively). Because cardiac troponin T has a short plasma half-life, these data indicate that cardiomyocyte degeneration and cell death take place during the time course of ANT treatment, but also in the post-treatment period.

DAU-Induced Myocardial Oxidative Stress and Response of Nrf2 Pathway. Analyses of oxidative stress markers in the LV myocardium revealed significantly increased MDA content at the end of the DAU treatment (Fig. 2A) and further significant elevation in the post-treatment period. Although a significant association (p < 0.05) between individual values of MDA and LV FS was identified (Fig. 2B), the relationship was relatively weak (R = −0.417). Furthermore, there was a significant increase in the content of oxidized glutathione caused by the treatment persisting in the FU period (Fig. 3A). Nevertheless, the GSSG/GSH ratio was not altered (Fig. 3C), because the reduced glutathione content also increased in the DAU-treated animals (Fig. 3B). Correlation analysis found no association between the GSSG/GSH ratio and cardiac function (Fig. 3D). Activities of Nrf2-regulated enzymes involved in glutathione system maintenance, such as glutathione transferase, glutathione reductase, and glutathione peroxidase, showed no changes caused by the DAU treatment (Fig. 3, E–G) with the exception of a slight decrease of the latter enzyme in the FU period.

We were surprised that no significant changes caused by the DAU treatment were found in the expression of the master regulator of the oxidative stress response, Nrf2 (Fig. 4A). The relative gene expression of Nrf2 was without significant changes both during the treatment and in subsequent FU. In addition, determination of the transcriptionally active form of Nrf2 in the myocardial nuclear fraction revealed no significant changes caused by the treatment in either interval (Fig. 4B). Gene expression of key Nrf2 target genes showed no coordinated induction caused by DAU treatment. Although CuZnSOD and glutamate-cysteine ligase catalytic subunit (GCLC) gene expression showed no significant change caused by DAU treatment (Fig. 4, C and D), MnSOD and NQO1 were markedly and significantly down-regulated at the end of the DAU treatment, and this change did not progress in the FU period (Fig. 4, E and F). In contrast, HO1 (Fig. 4G) was markedly up-regulated (up to 2.3- and 2.6-fold, respectively) in the same periods. Correlation analyses of Nrf2 pathway components with the LV systolic function (LV FS) showed only insignificant or weak association in individual animals, with the exception of MnSOD, HO1, and NQO1 (Supplemental Fig. 1, A-C). The relationship was particularly strong for the former two genes (r = 0.75 and R = −0.77, respectively), although they showed opposite regulation caused by the treatment.

DAU-Induced Mitochondrial Damage and Response of Mitochondrial Biogenesis Pathway. High-resolution respirometric measurements revealed significantly decreased activity of complex I and II in the post-treatment FU, whereas the values at the end of the treatment showed no change in either case (Fig. 5, A and B). Furthermore, complex IV function was significantly decreased by DAU treatment in both periods (Fig. 5C). Rotenone-sensitive enzymatic function of complex I determined in mitochondria-enriched fractions of LV myocardium was significantly decreased both by the end of the DAU treatment and in the FU (by 34 and 26%, respectively, Fig. 5D). Moreover, citrate synthase activity (general marker of mitochondria content) was decreased sig-
significantly at the end of the treatment by 19.7 and 20.9% in the FU period (Fig. 5E) compared with the corresponding controls.

Subsequent analyses of mitochondrial biogenesis pathway showed either no changes or significant down-regulation of key pathway components caused by the chronic DAU treatment (Fig. 6). Gene expression of PGC1α, a master regulator of mitochondrial biogenesis, did not show any change caused by DAU administration by the end of the treatment (Fig. 6A). The levels of PGC1α transcripts in the control group showed a slight, but significant, relative increase in the FU period. However, PGC1α mRNA levels did not follow the same trend in the DAU FU group; hence, a significant difference was found between the groups. A similar pattern was found in the gene expression of nuclear respiratory factor 1 (NRF1), although with somewhat lower values in the DAU groups and insignificant rise in the controls in the FU (Fig. 6B). On the other hand, TFAM (Fig. 6C), exhibited a profound drop in gene expression (by more than 50%) caused by DAU treatment (p < 0.001) with no further progression in the FU period. Expression of the essential mtDNA-encoded subunit of complex I (ND1) showed an insignificant tendency toward down-regulation by the end of the treatment, whereas expression of another mtDNA-encoded subunit (ND4) was significantly suppressed by nearly 40%. The expression of both complex I subunits was evidently severely depressed in the FU with significant difference between both DAU-treated groups in the case of ND4 (Fig. 6, D and E). A nuclear-encoded complex I subunit (NDUFS2) exhibited a marked down-regulation at the end of the treatment to a similar extent to ND4 (Fig. 6F). In addition, the apparent tendency to decrease was found in the gene expression of the complex IV subunits encoded by both mtDNA and nDNA (Fig. 6, G and H, respectively), although it did not reach statistical significance in the latter case at the end of the treatment. Furthermore, analysis of gene expression of another nuclear-encoded mitochondrial protein outside the oxidative phosphorylation machinery, sarcomeric mitochondrial creatine kinase (smtCK), also revealed profound down-regulation (Fig. 6I) in both study periods (by nearly 50 and 75%, respectively). No change in mtDNA/nDNA copy ratio was found at the end of treatment, whereas a significant drop in this ratio was observed in the FU period.
was found in the FU period (Fig. 6J). It is noteworthy that the majority of changes in the expression of mitochondrial proteins remained significant when the data were normalized over mtDNA abundance (Supplemental Fig. 2, A–G).

Discussion

In the present study, we administered 10 clinically relevant doses of daunorubicin (~50 mg/m²) to rabbits to mimic individual chemotherapeutic cycles without their overlap. As expected, significant cumulative dose induced chronic ANT cardiotoxicity, which progressed into dilated cardiomyopathy and heart failure in the post-treatment FU. It is noteworthy that we also have demonstrated that ANT-induced myocardial lipoperoxidation continued in the drug-free post-treatment period. Nevertheless, its loose association with toxicity parameters argues against its direct executive role in ANT cardiotoxicity. This might correspond with our previous data (Popelová et al., 2009) where the clinically available cardioprotective agent dexrazoxane showed nearly complete protection from ANT-induced cardiotoxicity and had only a minor and insignificant effect on myocardial lipoperoxidation. Furthermore, although the presence of ANT-induced oxidative stimuli was also obvious from increased GSSG content, no changes in the key GSSG/GSH ratio suggest that the glutathione antioxidant system does not lose its overall competence upon chronic ANT treatment. Using a completely different technique, we had previously obtained similar results by the end of chronic ANT treatment (Vávrová et al., 2011). All these data might be consistent with the outcomes of clinical trials showing conclusively a lack of cardioprotective effects of classic antioxidants and ROS scavengers including N-acetylcysteine and vitamin E in ANT cardiotoxicity (Gianni et al., 2008; Šimuš et al., 2009; Menna et al., 2012). In addition, others (Berthiaume et al., 2005) have demonstrated that although vitamin E can decrease myocardial oxidative stress it has a little or no effect on key parameters of chronic ANT cardiotoxicity. Hence, although global oxidative stimuli are evidently taking place during ANT carousel.
diotoxicity development, they need not be the direct perpetrators of chronic ANT cardiotoxicity and resulting heart failure. Nevertheless, one cannot exclude a more sophisticated (e.g., signaling) or considerably compartmentalized role of ROS in ANT-induced heart failure. Several authors have implicated the role of p53 in the triggering of mitochondrial oxidative stress and apoptotic signaling (Sardão et al., 2009; Velez et al., 2011). Nevertheless, the potential involvement of this pathway in chronic ANT cardiotoxicity setting remains to be determined.

Despite calls for revisiting these questions (Gianni et al., 2008; Šimušek et al., 2009), oxidative injury remains the most frequently cited mechanism of ANT cardiotoxicity development. Therefore, in the present study we have focused on the involvement of the Nrf2 pathway. It is noteworthy that we show no changes in either abundance of active Nrf2 in the nuclear fraction or Nrf2 expression after chronic ANT treatment. One can speculate that the level of oxidative stress in the cytosolic compartment is perhaps not sufficient to activate Nrf2 or its activation might be counteracted by other signaling cascades. We also convincingly demonstrated no coordinated activation of Nrf2 target genes upon chronic ANT treatment. There was no change in the expression of CuZnSOD, which corresponds with previously published data (Li and Singal, 2000), and we detected no orchestrated activation of glutathione system enzymes, which also rank among important Nrf2 targets.

In contrast, we found severe down-regulation of gene expression of MnSOD and NQO1 persisting a long time after treatment. The former finding is in line with our proteomic data (Štěrba et al., 2011) and protein abundance and activity reported by others (Li and Singal, 2000). MnSOD down-regulation can be seen even when the data are normalized over mtDNA abundance, which suggests that the change cannot be simply explained by a drop in the mitochondrial content. However, it should be noted that, in addition to transcriptional control, the activity of SOD isoforms is markedly affected by post-translational modifications; this requires further study in anthracycline cardiotoxicity settings (Dhar and St Clair, 2012). In addition, we, for the first time, demonstrate that chronic ANT cardiotoxicity is associated with the marked and persistent increase of expression of HO1. HO1 is an essential cardioprotective molecule induced in response to cardiac damage and stress (Peers and Steele, 2012). So far, very little and rather variable data are available regarding HO1 and ANT cardiotoxicity, and virtually nothing has been reported regarding the clinically relevant chronic forms resulting in heart failure. Using proliferating H9c2 cells, a suppression of HO1 expression has been reported after in vitro ANT treatment (Bernuzzi et al., 2009), whereas others have suggested the opposite in a microarray study (Choi et al., 2008). Little to no change in HO1 expression has been seen at protein and mRNA levels 2 weeks after a single supratherapeutic ANT dose (Suliman et al., 2007) and 70 days after injection of 1 mg/kg for 10 days (Richard et al., 2011), respectively. Piantadosi et al. (2008) have also reported HO1 as an important checkpoint involved in the cross-talk between the Nrf2 pathway and mitochondrial biogenesis with the possibility of providing powerful cardioprotection against acute ANT cardiotoxicity induced by a single high dose of doxorubicin. However, translatable to clinically more relevant chronic ANT cardiotoxicity settings needs to be verified in further studies. It is noteworthy that carbon monoxide produced by HO1 enzymatic activity is known to interfere with the electron transport chain to release electrons and produce ROS (Peers and Steele, 2012). Bearing in mind the mitochondrial damage and down-regulation of MnSOD, one cannot exclude that an adaptive role of HO1 becomes maladaptive upon chronic exposure. In either
In accordance with previous reports we have detected important perturbations in mitochondrial function. Mitochondria have been repeatedly suggested as the most important subcellular target for ANT toxicity (Wallace, 2003; Tokarska-Schlattner et al., 2006), and this has also been supported by recent unbiased proteomic investigations (Šteřba et al., 2011). Furthermore, complex I, which can also catalyze the redox cycling of ANT aglycone (Davies and Doroshow, 1986), has been suggested as a key and early target for cardiotoxicity of ANT chemotherapeutics (Ohkura et al., 2003). In our study, high-resolution respirometry detected a significant change in complex I function in the post-treatment FU, whereas markedly decreased enzymatic function of complex I was found in the mitochondria-enriched fraction in both study periods. The latter results seem to fit better our proteomic data obtained on the same model as well as results reported by others (Ohkura et al., 2003), and it might be related to the measurement of respiration in the tissue ho-
mogenate. It is noteworthy that complex IV function was found compromised by the end of the treatment, and it persisted further in the post-treatment period, which may correspond with decreased enzymatic function of this complex in the late-onset form of chronic ANT cardiotoxicity (Lebrecht et al., 2003). Our data also suggested a decline in the LV mitochondria content with no recovery in the drug-free period. Hence, our findings confirm that chronic ANT cardiotoxicity is associated with marked mitochondrial alterations, which persist or deteriorate further in the post-treatment period.

Therefore, we addressed the function of mitochondrial biogenesis pathway, which might provide an endogenous protective response against such impairment (Ventura-Clapier et al., 2008). Earlier reports provided only limited insights into the role of mitochondrial biogenesis in ANT cardiotoxicity. Although two studies (Suliman et al., 2007; Miyagawa et al., 2010) reported down-regulation of this pathway caused by the ANT treatments, opposing findings have been recently published (Marechal et al., 2011). It is noteworthy that all of these investigations have been performed on acute ANT cardiotoxicity models using single rather supratherapeutic ANT doses. Thus, the connection to chronic ANT cardiotoxicity and heart failure remains uncertain.

In the present study, we demonstrate for the first time that PGC1α as well as its downstream transcription factor NRF1 show no change by the end of chronic ANT treatment, although evident mitochondrial alterations were in place. The fact that TFAM was markedly depressed in the same period may imply more complex regulation of some parts of mitochondrial biogenesis. Gene expression of PGC1α showed a slight, but significant, tendency toward increase in the control group comparing FU and end-of-treatment values and a similar, but insignificant, trend was observed in several downstream pathway members. Although the reasons for these observations are unknown, considering a significant length of the FU period, different factors such as animal aging or challenge by the scheduled experimental procedures could be involved. In either case DAU-treated animals were unable to follow this trend, which might imply different regulation than in the controls. On the other hand, TFAM showed marked down-regulation in both study periods, which was evidently caused by the treatment. Most importantly, a severe decrease in gene expression was found in all investigated subunits of complex I and IV without marked down-regulation of gene expression of numerous mitochondrial proteins encoded by both mtDNA or nDNA-encoded ones. Because this trend has been observed even after the normalization of the data on mtDNA abundance, this change may be interpreted as down-regulation caused by the treatment.

We have not found any change in mtDNA/nDNA ratio at the end of the chronic treatment, whereas the significant drop that was identified in the FU period suggests continuing mitochondrial damage in the post-treatment period. Previously reported data seem to depend on this model, because Lebrecht et al. (2003) have also shown a decrease in mtDNA/nDNA ratio with a late-onset chronic ANT cardiotoxicity model, whereas others have reported either decreases (Miyagawa et al., 2010) or even increases (Marechal et al., 2011) after acute ANT dosing.

It should be noted that decreased mitochondrial content caused by the treatment evidenced by significant decrease of the citrate synthase activity (by ~20%) may have an impact on some mitochondria-related parameters. However, the mean relative changes of all significantly affected mitochondria-related parameters seem to be too high (average change 47.4%) to be solely determined by this event. Furthermore, normalization of the data over mtDNA argues against the loss of mitochondria in surviving cardiomyocytes as the main driving force for these observations. This is in line with markedly decreased complex I activity, which was found even in the mitochondria-enriched tissue fractions. Future studies should work out the present results to encounter the protein level and follow the changes identified in this study throughout the whole time course of cardiotoxicity development. Furthermore, to draw firm mechanistic conclusions it will be necessary to use a genetic manipulation approach. In addition, although the induction of mitochondrial biogenesis through the administration of CO, CO-releasing molecules, or metformin treatment seemed to be helpful in acute and subacute anthracycline cardiotoxicity settings (Piantadosi and Suliman, 2006; Asensio-López et al., 2011; Soni et al., 2011; Ashour et al., 2012), it will be important to reveal whether this intervention may have a therapeutic value against chronic anthracycline cardiotoxicity.

In conclusion, in the present study we describe molecular and functional changes associated with the development of chronic ANT cardiotoxicity and its progression to heart failure in the post-treatment FU. Our data strongly suggest that ANT-induced global oxidative stress is not a key factor directly responsible for heart failure development. Furthermore, we demonstrate that the Nrf2 pathway coordinating antioxidant response is not induced by chronic ANT treatment. It is noteworthy that we have observed Nrf2-independent regulation of expression of MnSOD, NQO1, and HO1, which merits further study. Despite evident damage to mitochondria that persists or further develops in the FU, chronic ANT cardiotoxicity was not associated with the induction of the mitochondrial biogenesis pathway controlled by PGC1α. Instead, this pathway seemed impaired, which was associated with the severe down-regulation of gene expression of numerous mitochondrial proteins encoded by both nuclear and mitochondrial genomes. This is particularly evident in the FU, and it may be connected with ongoing myocardial damage and transition to heart failure.

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Authorship Contributions

Participated in research design: Jirkovský, Popelová, Vávrová, Mičuda, Šimůnek, Červínková, Geršl, and Štěrba.

Conducted experiments: Jirkovský, Popelová, Kriváková-Staňková, Vávrová, Hroch, Hašková, Brčákova-Doleželová, Adamcová, and Štěrba.

Performed data analysis: Jirkovský, Popelová, Kriváková-Staňková, Vávrová, Hroch, Hašková, Brčákova-Doleželová, Mičuda, Adamcová, Šimůnek, Červínková, Geršl, and Štěrba.

Wrote or contributed to the writing of the manuscript: Jirkovský, Popelová, Kriváková-Staňková, Vávrová, Mičuda, Adamcová, Šimůnek, Červínková, Geršl, and Štěrba.

References


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Supplementary Figure 2

A ND1/mtDNA

B ND4/mtDNA

C NDUF52/mtDNA

D COX1/mtDNA

E COX4I1/mtDNA

F MnSOD/mtDNA

G smtCK/mtDNA
Supplementary Figure 2. Relative gene expression of several mitochondrial proteins normalized over mtDNA abundance – (A) mitochondrial DNA-encoded subunit of complex I (ND1), (B) mitochondrial DNA-encoded subunit of complex I (ND4), (C) nuclear DNA-encoded subunit of complex I (NDUFS2), (D) mitochondrial DNA-encoded subunit of complex IV (COX1), (E) nuclear DNA-encoded subunit of complex IV (COX4I1), (F) manganese superoxide dismutase (MnSOD) and (G) sarcomeric mitochondrial creatine kinase (smtCK). Data are shown as individual values with their means/medians. Statistical significances (One Way ANOVA/ANOVA on Ranks) are displayed as follows: “*” p<0.05, “**” p<0.01, “***” p<0.001. CTR – control group, DAU – daunorubicin group, FU – 10-week post-treatment follow up. LV FS – left ventricular fractional shortening.
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Supplementary Table 1. Quantitative PCR assays for reference and target genes

<table>
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**Supplementary Figure 1**

![Graphs A, B, and C showing correlation of left ventricular performance with relative gene expression of MnSOD, NQO1, and HO1](image)

**Supplementary Figure 1.** Correlation of left ventricular performance with relative gene expression of (A) MnSOD - manganese superoxide dismutase, (B) NQO1 - NAD(P)H dehydrogenase [quinone] 1, (C) HO1 - heme oxygenase 1 in individual animals as determined by Spearman correlation and regression analysis. LV FS – left ventricular fractional shortening. Correlation coefficient and statistical significance are shown in each graph.