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ORIGINAL ARTICLE

Uncoupling protein downregulation in doxorubicin-induced heart failure improves mitochondrial coupling but increases reactive oxygen species generation

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Abstract
Purpose Doxorubicin-based chemotherapy is limited by the development of dose-dependent left ventricular dysfunction and congestive heart failure caused by reactive oxygen species (ROS). Uncoupling proteins (UCP) can inhibit mitochondrial ROS production as well as decrease myocyte damage from exogenous ROS. Prior studies have shown that cardiac UCP2 and UCP3 mRNA expression is decreased with acute doxorubicin treatment. However, the expression of UCP protein in hearts with doxorubicin cardiotoxicity and the resultant changes in mitochondrial function and oxidant stress have not been determined.
Methods Heart failure was induced in Sprague–Dawley rats with intraperitoneal injections of doxorubicin (2 mg/kg t.i.w., total dose: 18 mg/kg). Mitochondria were isolated from mice receiving doxorubicin or saline injections for determination of UCP2 and UCP3 expression. In addition, mitochondrial respiration, ATP synthesis and ROS production were determined.
Results Doxorubicin-induced heart failure was associated with significant decreases in UCP2 and UCP3 protein expression compared with nonfailing hearts ($P < 0.05$). While the rates of state 3 and state 4 respiration and ATP synthesis were lower in mitochondria isolated from failing hearts, the respiratory control ratio was 15% higher ($P < 0.05$), and the ratio of ATP production to oxygen consumption was 25% higher ($P < 0.05$) in mitochondria from failing hearts, indicating greater coupling between citric acid cycle flux and mitochondrial ATP synthesis. However, the decrease in UCP expression was associated with 50% greater mitochondrial ROS generation ($P < 0.05$).
Conclusions Downregulation of myocardial UCP2 and UCP3 in the setting of doxorubicin-induced heart failure is associated with improved efficiency of ATP synthesis, which might compensate for abnormal energy metabolism. However, this beneficial effect is counterbalanced by greater oxidant stress.

Keywords Cardiotoxicity · Mitochondria · Reactive oxygen species · Energetics

Introduction

One of the major limitations of chemotherapy with anthracycline agents, such as doxorubicin, is their dose-dependent cardiotoxicity. This cardiotoxicity is due, at least in part, to reactive oxygen species (ROS) generated by the metabolism of the anthracycline agents [9, 24, 50]. In support of this mechanism of cardiotoxicity, several studies have demonstrated that antioxidant treatment can ameliorate the left ventricular dysfunction caused by doxorubicin [23, 50]. However, the innate cellular responses to the oxidant stress induced by doxorubicin treatment have not been completely characterized.

Uncoupling proteins (UCPs) are members of the superfamily of mitochondrial transport proteins that regulate the mitochondrial membrane potential created by the proton gradient across the inner mitochondrial membrane. The initial

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characterization of the first described member of this family, UCP1, demonstrated that UCPs uncouple flux through the electron transport chain from mitochondrial ATP synthesis, thereby decreasing the efficiency of the conversion of metabolic substrates to high-energy phosphates. In addition to this function, UCPs are also thought to play a role in the detoxification of ROS produced by the mitochondria because mitochondrial production of ROS is dependent on the mitochondrial membrane potential [31]. Thus, UCPs may attenuate free radical damage [35, 48].

In the mammalian heart, UCP2 and UCP3 are the predominant isoforms that are expressed [6, 7, 39]. Recent studies have demonstrated that a single injection of doxorubicin can decrease the expression of UCP2 and UCP3 on the mRNA level in the heart [46]. However, no studies to date have determined the effect of heart failure induced by doxorubicin on UCP2 and UCP3 expression and the resultant effects on mitochondrial function. Given the role of UCPs in regulating ROS production, decreased expression of myocardial UCPs may be an important mechanism that exacerbates the toxic effects of doxorubicin on the heart.

The first purpose of this study was to determine whether changes in UCP2 and UCP3 expression occur at the protein level in the setting of doxorubicin-induced cardiotoxicity. The second goal of this study was to determine whether changes in UCP expression were associated with changes in ATP production and ROS generation in mitochondria from hearts with doxorubicin-induced cardiotoxicity.

Materials and methods

Doxorubicin-induced heart failure

To determine the effects of doxorubicin-induced heart failure on mitochondrial function, heart failure was induced in male Sprague–Dawley rats (initial weight: 175–200 g) by intraperitoneal injections of doxorubicin (2 mg/kg three times a week) for 3 weeks (cumulative dose: 18 mg/kg). Left ventricular function was determined echocardiographically using a 15-MHz ultrasound probe (Sonos 5500, Phillips Medical Systems, Bothell, WA) in rats anesthetized with 1% isoflurane. Left ventricular cavity end-systolic and end-diastolic dimensions were used to determine fractional shortening. All studies were approved by the Yale Animal Care and Use Committee.

Tissue collection and mitochondrial isolation

Hearts were harvested from anesthetized rats as described earlier and used for the isolation of mitochondria as previously described [42]. Mitochondrial respiration was measured using a Clark-type oxygen electrode (YSI, Yellow Springs OH) in respiration media containing 200 mM mannitol, 70 mM sucrose, 10 mM Tris HCl and 5 mM KH_2PO_4 [11]. Mitochondrial membrane integrity was determined by measuring latent and total citrate synthase activity in fresh mitochondria and mitochondria incubated with 2.5% Triton X-100, respectively [13]. The mitochondrial protein concentration was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA). Immunoblot analysis of UCP2 and UCP3 was performed using specific antibodies (AB3226 [UCP2] and AB3044 [UCP3], Chemicon International, Temecula, CA) and enhanced chemiluminescence (Western Lightning, PerkinElmer Life Sciences, Boston, MA). Autoradiographic bands were quantified by densitometry.

Determination of mitochondrial ATP production, reactive oxygen species production and oxidant stress

Mitochondrial adenosine 5′-triphosphate (ATP) production was determined for rat heart mitochondria incubated with pyruvate (1 mM), malate (1 mM), palmitoyl-L-carnitine (0.05 mM), α-ketoglutarate (10 mM) and ADP (40 mM) as substrates [51]. Previous studies have demonstrated that the rate of muscle mitochondrial ATP production is greatest with this combination of substrates [44, 51, 52]. Mitochondrial ATP release into the media was measured luminometrically (ATP Bioluminescent Assay, Sigma–Aldrich, St. Louis, MO). Mitochondrial ROS production was determined using a lucigenin-derived chemiluminescence (LDCL) assay [28, 41]. Mitochondrial superoxide dismutase (SOD) activity was determined using a commercial assay (Cayman Chemical Company, Ann Arbor, MI).

Statistical analysis

Values are reported as the mean ± standard error of the mean (SEM). Differences between the control and doxorubicin-treated groups were analyzed by unpaired Student's *t* test. Temporal changes in left ventricular function were assessed by analysis of variance with post hoc comparisons performed using Dunnett's test. A value of $P < 0.05$ was considered statistically significant.

Results

Treatment of Sprague–Dawley rats with doxorubicin resulted in time-dependent changes in left ventricular fractional shortening, with a significant decrease in fractional shortening noted after 3 weeks of doxorubicin treatment (Fig. 1). The depression of left ventricular function persisted after completion of the doxorubicin treatment protocol. In addition, there was evidence of left ventricular

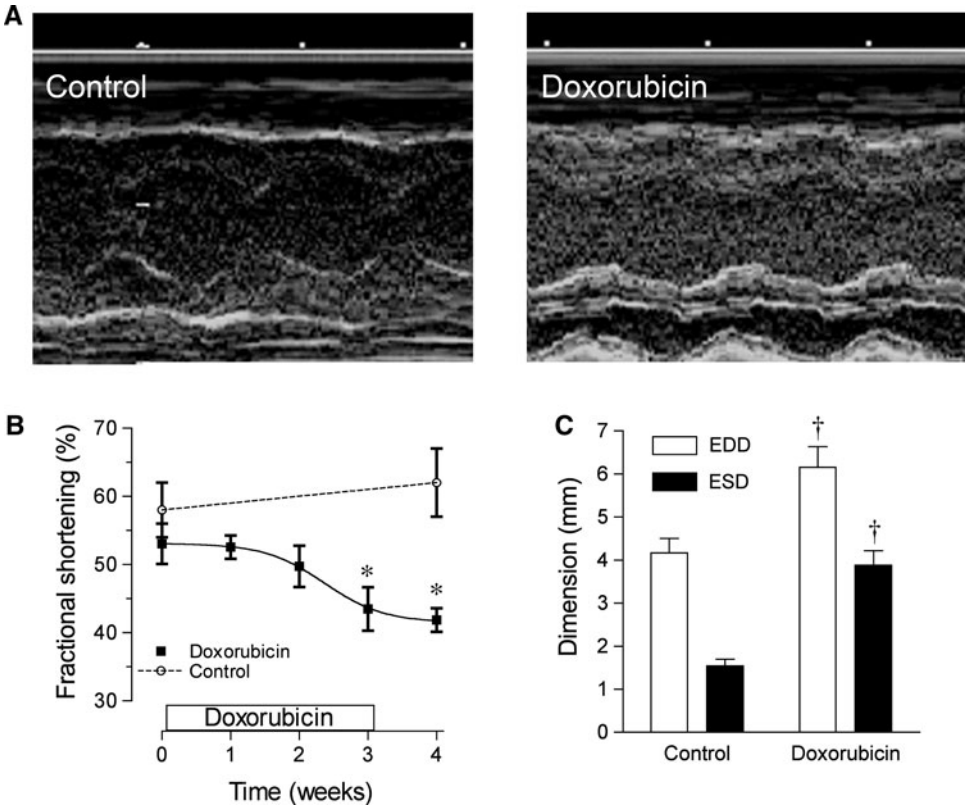

Fig. 1 Echocardiographic assessment of left ventricular function and dimensions. Representative M-mode echocardiograms of saline-treated (Control) and doxorubicin-treated hearts (**a**). Changes in left ventricular fractional shortening (**b**) and left ventricular end-systolic and end-diastolic dimensions assessed at the 4-week time point (**c**) evaluated echocardiographically in rats treated with doxorubicin. * $P < 0.05$ and † $P < 0.01$ compared with control measurements. Values are reported as the mean ± SEM, $n = 10$ for each measurement

dilation following treatment with doxorubicin consistent with adverse remodeling of the left ventricle (Fig. 1). Therefore, studies of mitochondrial function were performed comparing vehicle-treated (control) animals and animals that received 3 weeks of doxorubicin treatment followed by 1 week of no doxorubicin therapy. In keeping with previous studies demonstrating the downregulation of UCP2 and UCP3 mRNA with doxorubicin treatment and in the setting of heart failure [40, 46], contractile dysfunction induced by doxorubicin was associated with decreased expression of both UCP2 and UCP3 proteins (Fig. 2).

In order to determine the energetic changes associated with the downregulation of the UCPs, respiration and ATP synthesis were determined for mitochondria isolated from control and failing hearts. The mitochondria isolated from control and failing hearts had similar degrees of structural integrity based on the ratio between the latent and free citrate synthase activity (control: 25.8 ± 6.3, doxorubicin-treated: 21.5 ± 1.6, n.s.). However, state 3 (ADP-dependent) respiration in the mitochondria from doxorubicin-treated failing hearts was only 47% of the value for control mitochondria (Fig. 3). There was a similar decrease in state 4 (ADP-independent) respiration in the mitochondria from failing hearts. These changes in mitochondrial respiration most likely reflect the well-documented decreases in the activity of proteins involved in both the citric acid cycle and the electron transport chain [32, 37, 38, 47]. In keeping with the decreased rate of state 3 respiration, the mitochondrial production of ATP from failing hearts from doxorubicin-treated animals was ~25% lower than in mitochondria from nonfailing hearts (Fig. 4). Despite the lower rate of ATP production, the P/O ratio was ~25% higher in mitochondria from doxorubicin-treated failing hearts than control hearts and the respiratory control ratio (RCR) was also increased in mitochondria from failing hearts (Fig. 3). These findings indicate greater coupling between ATP synthesis and oxygen consumption in the mitochondria from failing hearts from animals treated with doxorubicin and are consistent with the effects of decreased expression of UCPs [10].

To determine whether the downregulation of UCP2 and UCP3 was associated with an increased risk of damage by ROS, the mitochondrial generation of the superoxide radical was determined based on lucigenin-derived chemiluminescence during state 3 and state 4 respiration. Under state 3 conditions, in which the mitochondrial membrane potential is dissipated by the phosphorylation of ADP to ATP, there were similar rates of superoxide production in mitochondria from normal and doxorubicin-treated hearts (Fig. 5a). Under state 4 conditions, in which the mitochondrial membrane potential is not dissipated by ATP synthesis but is determined by the degree of uncoupling, there was a significant increase in the generation of ROS for both groups. However, there was significantly more superoxide

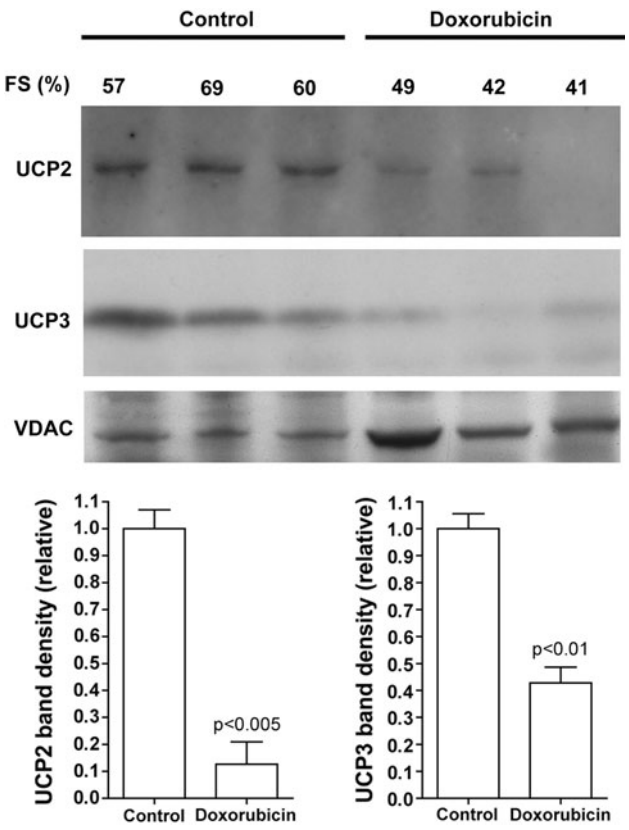

Fig. 2 UCP2 and UCP3 expression at the 4-week time point assessed by immunoblot analysis in heart mitochondria from vehicle-treated rats and those treated with doxorubicin. The voltage-dependent anion channel (VDAC) was used to control for loading of mitochondrial protein. FS: Echocardiographic fractional shortening measured at 4-week time point. * $P < 0.01$ versus control. Values are reported as the mean ± SEM ($n = 5$ for both groups)

radicals produced by mitochondria from doxorubicin-treated failing hearts ($P < 0.05$). The greater ROS production in mitochondria from failing hearts could not be explained by a decrease in Mn-SOD activity (Fig. 5b), which is in keeping with previous studies [27].

Discussion

In this study, we demonstrated that left ventricular contractile failure induced by doxorubicin is associated with downregulation of UCP2 and UCP3 in the heart. This is the first demonstration of a decrease in UCP expression on the protein level in association with doxorubicin cardiotoxicity. As might be expected, this decrease in UCP expression was associated with greater coupling of citric acid cycle flux and mitochondrial ATP production, as shown by an increase in the P/O ratio during state 3 respiration and an increase in the RCR. This greater coupling helps to compensate for the decreased rate of mitochondrial respiration by improving the efficiency of ATP production. However, the downregulation of UCPs was also associated with an increased generation of ROS and may predispose the failing heart to a greater susceptibility to damage from ROS.

Effects of doxorubicin on myocardial energetics and mitochondrial function

Doxorubicin has multiple effects on mitochondrial proteins involved in energy production. Doxorubicin is known to bind to cardiolipin, a phospholipid component of the inner mitochondrial membrane that disrupts the proper association of respiratory chain proteins [16, 37]. In addition, the carnitine palmitoyltransferases, which are responsible for fatty acid transport into the mitochondria, are inhibited by doxorubicin [22]. Doxorubicin also causes downregulation of a variety of genes involved in mitochondrial function and energy metabolism, including UCP2 and UCP3 [46]. As a result, the enzymatic activity of the respiratory chain complexes is decreased by ~15–50% by doxorubicin [32, 37, 38, 47], which in turn inhibits mitochondrial respiration [4, 34]. The direct effects of doxorubicin on mitochondrial function are likely compounded by the effects that heart failure can have on cardiac mitochondrial function [15, 26].

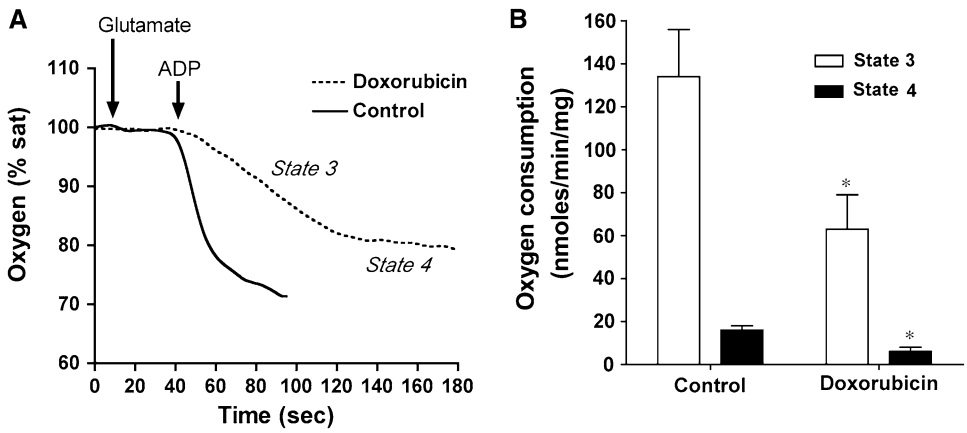

Fig. 3 Alterations in mitochondrial oxygen consumption in hearts from rats with doxorubicin-induced left ventricular dysfunction. Representative tracings of oxygen consumption for mitochondria from hearts of saline-treated animals (control) and doxorubicin-treated animals (**a**). State 3 (ADP-dependent) and state 4 (ADP-independent) mitochondrial respiration (**b**). * $P < 0.05$ versus control. Values are reported as the mean ± SEM ($n = 6$ for both)

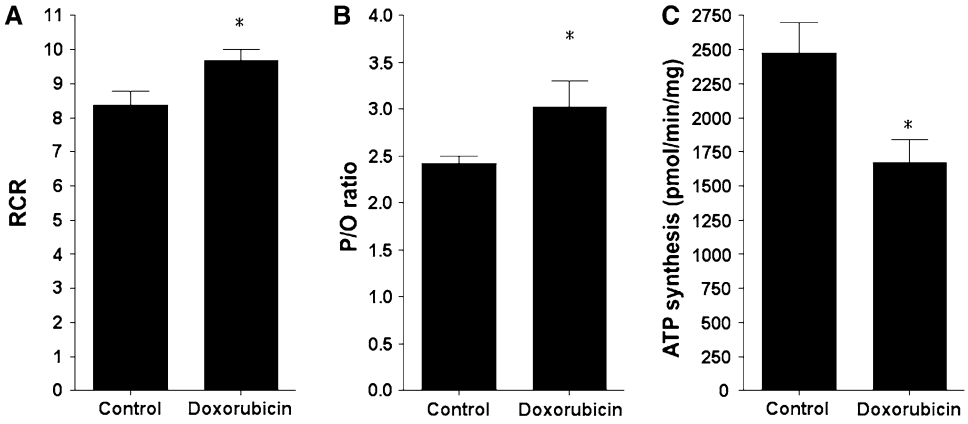

Fig. 4 Changes in mitochondrial coupling and ATP synthesis in hearts from rats with doxorubicin-induced left ventricular dysfunction. Changes in the respiratory control ratio (RCR, **a**) and the ratio of ATP generated to oxygen consumed (P/O ratio, **b**). ATP synthesis in mitochondria isolated from saline- and doxorubicin-treated hearts (**c**). * $P < 0.05$ versus control. Values are reported as the mean ± SEM ($n = 5$ for both)

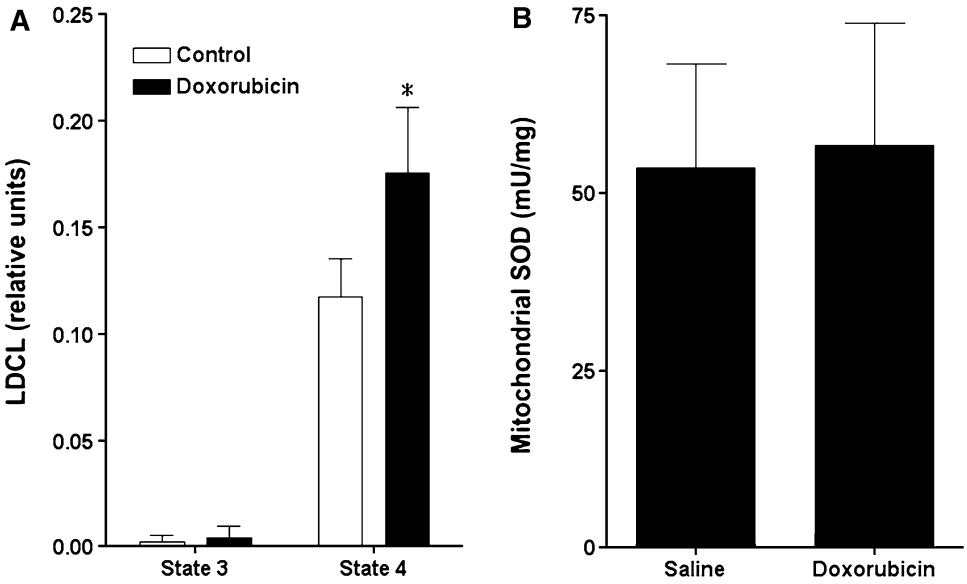

Fig. 5 Mitochondrial superoxide production based on lucigenin-derived chemiluminescence (LDCL) in saline- and doxorubicin-treated hearts (**a**). Mitochondrial superoxide dismutase (SOD) activity (**b**). * $P < 0.05$ versus control. Values are reported as the mean ± SEM ($n = 6$ for both groups)

Activities of UCPs

The proposed functions of UCPs include regulation of ATP production and control of mitochondrial ROS production, both of which rely on the regulation of the mitochondrial membrane potential ($\Delta\psi$) by UCPs. Interestingly, studies of loss-of-function and gain-of-function mutants of UCP3 in skeletal muscle have demonstrated variable effects on $\Delta\psi$. Specifically, knockout of functional UCP3 from skeletal muscle can either increase [3] or have no effect [8] on $\Delta\psi$. In contrast, overexpression of UCP3 has been shown to decrease $\Delta\psi$ [14]. As discussed below, a decrease in UCP expression, potentially increasing $\Delta\psi$ could have both beneficial and detrimental effects.

The coupling between citric acid cycle flux and ATP synthesis has been measured in vivo in skeletal muscle using $^{31}P/^{13}C$-NMR spectroscopy [10]. The previous study demonstrated that there is a greater rate of ATP production for a given rate of citric acid cycle flux in the skeletal muscle of UCP3 knockout mice compared with wild-type littermates. Furthermore, treatment of rats with thyroid hormone increased skeletal muscle UCP3 expression and decreased the coupling between ATP production and citric acid cycle flux [21]. In the setting of heart failure, in which a decrease in F_0F_1-ATPase activity impairs ATP synthesis [29], greater coupling of citric acid cycle flux and ATP production would be important to maintain cellular energetics and contractile function. Hemodynamic alterations, including those caused by cardiac hypertrophy, heart failure or mechanical unloading, have been shown to decrease UCP2 and UCP3 mRNA expression [40, 53], and doxorubicin treatment has also been shown to decrease UCP expression [46].

The changes in UCP2 and UCP3 mRNA in these prior studies were presumed to be secondary to the development of heart failure. In vivo echocardiographic measurements and in vitro hemodynamic measurements of hearts of UCP2 and UCP3 null mice demonstrate no abnormalities in left ventricular function compared with wild-type mice, indicating that changes in UCP expression do not cause heart

failure and suggesting that heart failure causes the decrease in UCP2 and UCP3 expression (unpublished observations).

In the current study, we found that the decrease in cardiac UCP2 and UCP3 expression was associated with increases in the P/O ratio and the RCR, indicating that there was greater coupling in failing hearts following doxorubicin treatment compared with nonfailing hearts. Prior studies have suggested that doxorubicin increases uncoupling in heart mitochondria [1, 12]. However, those studies utilized a single high dose of doxorubicin (≥20 mg/kg). In contrast, the present study utilized fractionated dosing of doxorubicin and assessed mitochondrial function 1 week after the last dose of doxorubicin, which may explain the differences in the findings. Furthermore, we assessed coupling not only by evaluating the respiratory control ratio, which is the ratio between state 3 and state 4 respiration, but also by evaluating the P/O ratio, while the prior studies did not evaluate the P/O ratio [18]. Our current findings are in keeping with other studies that have demonstrated an increased P/O ratio following subacute doxorubicin treatment [9].

Counteracting the beneficial energetic effects of the downregulation of UCP2 and UCP3 expression in the failing heart is the greater degree of oxidant stress caused by either increased production of ROS or decreased ability to remove ROS. Previous studies have revealed an inverse relationship between UCP3 expression and the production of ROS [35, 48]. Increases in $\Delta\psi$ result in an exponential increase in the production of ROS [30, 31], most likely through transfer of an unpaired electron from complex I or complex III of the respiratory chain to molecular oxygen [25]. Overexpression of UCP1 in H9C2 cardiac myoblast cells improves survival following hypoxia/reoxygenation [5]. Furthermore, there is greater functional recovery and less oxidant damage in the setting of ischemia and reperfusion in hearts overexpressing UCP1 [19]. In addition to the ability of UCPs to regulate the mitochondrial production of ROS, there is evidence that UCPs may also protect against exogenous oxidant stress. Specifically, there is diminished apoptosis in cardiac myocytes overexpressing UCP2 that are exposed to hydrogen peroxide, and of relevance to the current study, doxorubicin [45].

Given this importance of uncoupling proteins in protecting against exogenous oxidant stress, the oxidant stress caused by doxorubicin may be amplified with the onset of doxorubicin cardiotoxicity because of decreased UCP expression. In the setting of doxorubicin-induced heart failure, in which the levels of ROS would be expected to be increased because of exogenous oxidant stress both from the chemotherapeutic agent and from ROS generated in the setting of left ventricular dysfunction [2, 20, 33], a decrease in the expression of UCP2 and UCP3 would be expected to augment the oxidant stress caused by doxorubicin. In support of the idea that decreasing ROS production may decrease doxorubicin cardiotoxicity, a recent study demonstrated that genetic deletion of nitric oxide synthase 3 attenuates the left ventricular dysfunction and improves survival associated with doxorubicin treatment [36]. This may be due to the fact that NOS3 acts as a flavoprotein reductase that catalyzes the reduction of a quinine moiety of doxorubicin to a semiquinolone and thereby generates ROS,

Conclusions

In the present study, we demonstrated a decrease in the expression of UCP2 and UCP3 in the setting of doxorubicin-induced heart failure. While this alteration in expression is associated with a potentially beneficial metabolic effect, namely greater coupling between citric acid cycle flux and mitochondrial ATP synthesis, it also predisposes the failing heart to greater oxidant stress. Given the fact that polymorphisms in the human UCP genes have been shown to affect the expression or function of the protein [17, 43, 49], genetic variations in human UCP2 and/or UCP3 may affect the susceptibility of patients to doxorubicin-induced cardiotoxicity. In addition, polymorphisms in the genes that regulate UCP expression directly or indirectly in the heart, such as peroxisome proliferator-activated receptor (PPAR)-α, may impact the susceptibility to doxorubicin-induced cardiotoxicity.

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