Original Research

Daunorubicin therapy is associated with upregulation of E3 ubiquitin ligases in the heart

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Abstract

Daunorubicin (DNR) and doxorubicin (DOX) are two of the most effective anthracycline drugs known for the treatment of systemic neoplasms and solid tumors. However, their clinical use is hampered due to profound cardiotoxicity. The mechanism by which DNR injures the heart remains to be fully elucidated. Recent reports have indicated that DOX activates ubiquitin proteasome-mediated degradation of specific transcription factors; however, no reports exist on the effect of DNR on the E3 ubiquitin ligases, MURF-1 (muscle ring finger 1) and MAFbx (muscle atrophy F-box). The aim of this study was to investigate the effect of DNR treatment on the protein and organelle degradation systems in the heart and to elucidate some of the signalling mechanisms involved. Adult rats were divided into two groups where one group received six intraperitoneal injections of 2 mg/kg DNR on alternate days and the other group received saline injections as control. Hearts were excised and perfused on a working heart system the day after the last injection and freeze-clamped for biochemical analysis. DNR treatment significantly attenuated cardiac function and increased apoptosis in the heart. DNR-induced cardiac cytotoxicity was associated with upregulation of the E3 ligases, MURF-1 and MAFbx and also caused significant increases in two markers of autophagy, beclin-1 and LC3. These changes observed in the heart were also associated with attenuation of the phosphoinositide 3-kinase/Akt signalling pathway.

Keywords: cardiotoxicity, antracyclines, daunorubicin, ubiquitin ligases, autophagy

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Introduction

Anthracyclines such as daunorubicin (DNR), doxorubicin (DOX), epirubicin and idarubicin are widely used for treatment of various hematological and solid tumor malignancies, including breast cancer, leukemia and sarcomas.[1] Although these anthracyclines are very effective, its clinical use is limited due to cardiotoxicity which leads to congestive heart failure, reduced quality of life or death.[2,3] On a molecular level, anthracyclines induce apoptosis, alterations in iron homeostasis, deregulation of calcium homeostasis and mitochondrial dysfunction.[4]

Antracycline cardiotoxicity can be classified as acute or chronic. Acute cardiotoxicity is independent of the anthracycline dose and is characterized by hypotension, tachycardia, arrhythmias and depression of left ventricular function.[5] Chronic or delayed cardiotoxicity is dose-related, typically irreversible and usually presents within one year after the end of treatment.

Since the early detection and treatment of cardiotoxicity can reduce its clinical outcome, it is particularly important to understand the molecular events leading to these adverse effects in order to develop new treatment strategies to manage the side-effects appropriately. Several mechanisms have been proposed for anthracycline-mediated cardiac toxicity such as the formation of reactive oxygen species[6] and the formation of secondary alcohol metabolites within cardiac tissue.[7] Furthermore, it was also shown that the ubiquitin–proteasome system (UPS) is deregulated by DOX in the heart.[8,9] The UPS is one of two major pathways which are responsible for the clearance of proteins and organelles in the eukaryotic cell. The UPS predominantly degrades short-lived normal protein molecules after they have fulfilled their duty in the cell, such as proteins involved in regulation of cell division, gene transcription, signal transduction and endocytosis.[10] The UPS also degrades abnormal proteins such as misfolded, oxidized and mutant proteins, thereby serving as a critical step of post-translational protein quality control in the cell.[11] Targeted proteolysis by the UPS includes two main steps: (1) the attachment of a series of ubiquitin molecules to the target

protein molecule via a process known as ubiquitination and (2) degradation of the ubiquitinated proteins by the proteasome.[12,13] A series of energy-consuming reactions, involving ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3), are required to tag targeted proteins. Two E3 ligases, muscle ring finger 1 (MURF-1) and muscle atrophy F-box (MAFbx), are expressed specifically in striated (cardiac and skeletal) muscle and are central players in the UPS-regulated turnover of sarcomeric proteins.[14,15]

The other major pathway responsible for degradation of cytoplasmic proteins, organelles and long-lived proteins is the autophagy–lysosomal pathway.[16] Although there is a constant low level of autophagic activity under normal conditions in the heart,[17] autophagy is upregulated in response to stressors such as ischemia/reperfusion injury, cardiac hypertrophy, heart failure and nutrient deprivation.[18] Autophagy requires a cascade of evolutionarily conserved proteins (Atg proteins) that comprise two conjugation pathways: (1) the Atg12–Atg5 pathway and (2) the light chain 3-phosphatidylethanolamine (LC3 or Atg8-PE) pathway.[19] Beclin 1 (Atg6) is part of a phosphoinositide 3-kinase (PI3-K) complex and seems to play an important role during the initial steps of autophagosome formation by mediating the localization of other Atg proteins to the isolation membrane.[20]

The distinction of substrate preference between these two proteolytic systems is relative as recent studies indicate that the UPS can participate in the degradation of long-lived proteins while autophagy can also be involved in the degradation of short-lived proteins.[21,22] Together, these systems play an essential role in the maintenance of sarcomeric function in the face of physiological and pathophysiological stimuli. Therefore, the aim of this study was to characterize these proteolytic systems after DNR-induced cardiotoxicity in a rat model.

Materials and methods

Animals

Male Wistar rats (180–200 g, $n = 14$) were fed a standard rat chow diet (SRC) with free access to water. All experiments were approved by the ethics committee at the Faculty of Health and Wellness Sciences, Cape Peninsula University of Technology, South Africa (Ref: CPUT/HW-REC 2008/009) and conform with the European Communities Council Directive of 1986 (86/609/EEC) and the United States National Institute of Health guidelines.

Experimental protocol

Adult rats were divided into two groups where one group received DNR treatment and the other saline injections as control (Figure 1). Animals of the DNR group received six intraperitoneal injections of 2 mg/kg on alternate days: 12 mg/kg cumulative dose. The evaluation of heart function was performed the day after the last injection.

Working heart perfusions

After the experimental protocol, rats were injected with sodium pentobarbitone solution (Euthenase, 50 mg/kg), and their hearts were rapidly excised and placed in cooled Krebs Henseleit buffer (KHB), before being mounted on a working heart perfusion apparatus by canulating the aorta and pulmonary vein. Retrograde aortic perfusion was initiated and sustained for 10 min (stabilization period) at a constant perfusion pressure of 100 cm KHB. After the stabilization period, the hearts were switched to the working heart mode for 35 min, during which aortic output, coronary flow, heart rate and aortic pressure were measured every five minutes. At the end of the perfusion protocol, hearts were freeze-clamped for biochemical analysis.

Western blot analysis

Tissue proteins were extracted with a lysis buffer containing (in mmol/L): Tris 20, *p*-nitrophenylphosphate 20, EGTA 1, sodium fluoride 50, sodium orthovanadate 0.1, phenylmethyl sulfonyl fluoride 1, dithiothreitol 1, aprotinin 10 μg/mL and leupeptin 10 μg/mL. The tissue lysates were diluted in Laemmli sample buffer, boiled for five minutes and 10 μg (for kinases, E3 ligases and LC3 and beclin) or 50 μg protein (for caspase-3 and PARP [poly ADB ribose polymerase]) were subjected to electrophoresis. The lysate protein content was determined using the Bradford technique.[23] The separated proteins were transferred onto a polyvinylidene fluoride membrane (Immobilon™ P; Millipore, Billerica, MA, USA). These membranes were routinely stained with Ponceau Red for visualization of proteins and stripped and re-probed with antiactin antibody to ensure equal loading. Non-specific binding sites on the membranes were blocked with 5% fat-free milk powder dissolved in Tris-buffered saline-0.1% Tween 20 (TBST) and then incubated with the primary antibodies that recognize phospho-specific and total PKB Ser^{473} and FoxO1, caspase-3 (p17 fragment pAb), PARP (p89 fragment pAb), Bcl-2, Bax, LC3, beclin-1 (all from Cell Signalling Technology, Boston, MA, USA), MAFbx, MURF-1 and ubiquitin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were subsequently washed with large volumes of TBST (5 × 5 min) and the immobilized antibody conjugated with a diluted horseradish peroxidase-labelled secondary antibody (GE Healthcare, Buckinghamshire, UK). After thorough washing with TBST, membranes were covered with ECL™ detection reagents and quickly exposed to an autoradiography film (Hyperfilm ECL, RPN 2103, Amersham, Buckinghamshire, UK) to detect light emission through a non-radioactive method (ECL™ Western blotting, Amersham). Films were densitometrically analyzed (UN-SCAN-IT; Silk Scientific Ltd, Orem, UT, USA) and phosphorylated protein values were corrected for minor differences in protein loading, if required. All blots were scanned at a resolution of 150 dpi. The exact outline of each band was demarcated in the UN-SCAN-IT programme, which takes all aspects of density and distribution into account. The full experimental range was analyzed on a particular blot. These analyses were performed under

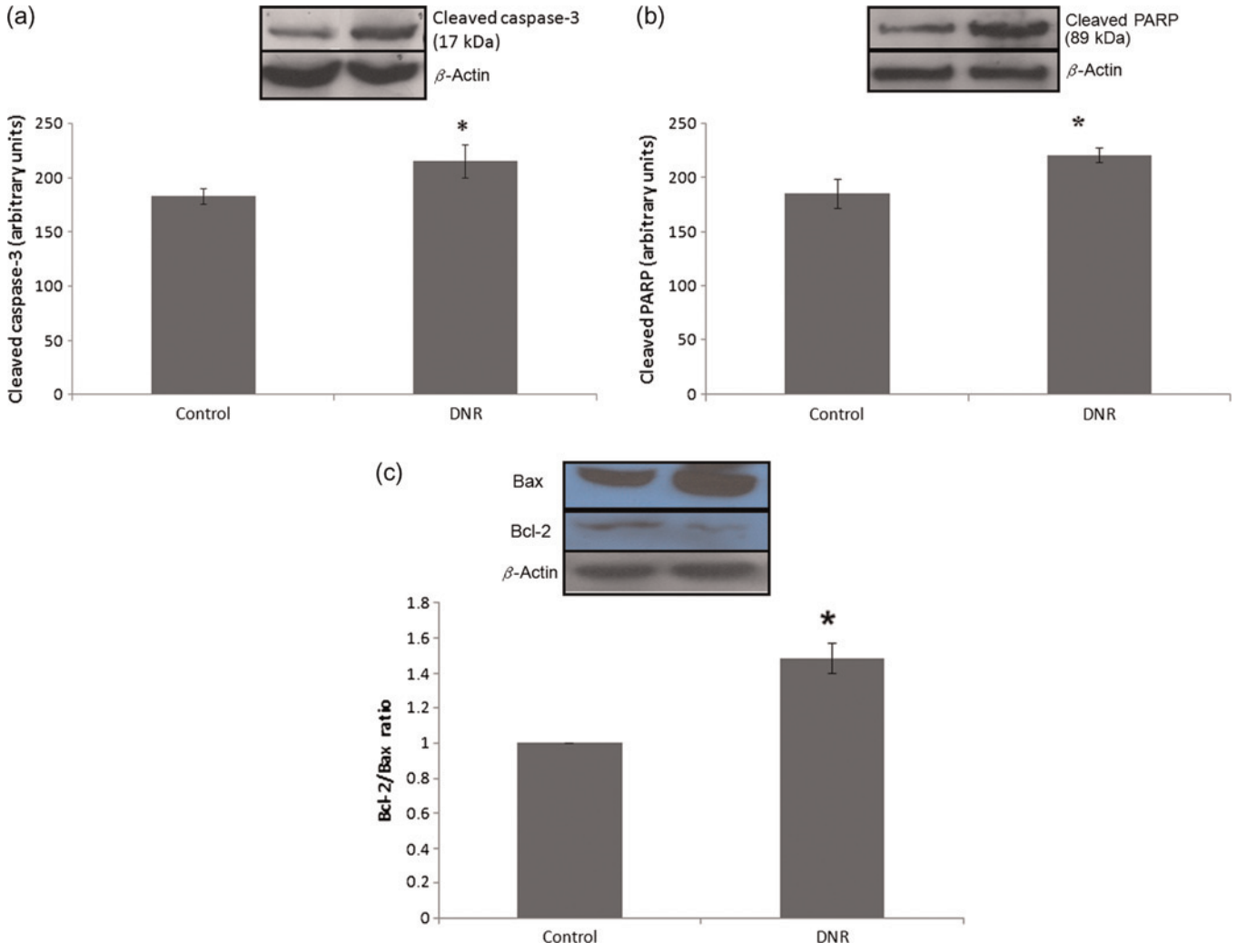

Figure 1 Effect of daunorubicin treatment on apoptosis in the heart. After the perfusion protocol, the heart tissue was freeze-clamped and analyzed for apoptosis. (a) Cleaved caspase-3, $^{*}P = 0.029$, $n = 7$; (b) cleaved PARP (poly ADB ribose polymerase), $^{*}P = 0.014$, $n = 7$; and (c) Bcl-2/Bax ratio, $^{*}P = 0.009$, $n = 7$. (A color version of this figure is available in the online journal)

conditions where autoradiographic detection was in the linear response range.

Statistics

Values are presented as mean ± standard error of the mean (SEM). Western blot data are presented as means ± SEM of triplicate analysis of the protein samples from seven rats per group. The Student's unpaired *t*-test was used to determine statistical significance. A value of $P \leq 0.05$ was considered statistically significant.

Results

DNR suppresses cardiac function

We have previously demonstrated that the anticancer treatment regimen with the anthracycline, DNR, significantly attenuated cardiac function in the isolated rat heart.[24] DNR significantly decreased aortic pressure (93.98 ± 8.53 mmHg versus 117.80 ± 2.99 mmHg, $P < 0.05$), aortic output (31.73 ± 2.20 mL/min versus 38.25 ± 1.25 mL/min, $P < 0.05$) and coronary flow rate (17.07 ± 1.41 mL/min versus 21.88 ± 1.21 mL/min, $P < 0.05$) compared with their respective control groups (Table 1).

DNR induces apoptosis in the rat heart

DNR significantly increased caspase-3 (215.00 ± 8.88 versus 182.70 ± 4.05 avg pixels, $P = 0.029$) as well as PARP cleavage (221.00 ± 3.78 versus 185.30 ± 7.79 avg pixels, $P = 0.014$) in the rat heart. In animals treated with DNR, Bcl-2 protein expression in the hearts decreased, but Bax protein expression increased compared with control. More importantly, the ratio of Bcl-2/Bax increased during DNR treatment, indicating that DNR down-regulated Bcl-2

Table 1 Functional characteristics of the hearts

	Control	DNR
Aortic pressure (mmHg)	117.80 ± 2.99	93.98 ± 8.53*
Aortic output (mL/min)	38.25 ± 1.25	31.73 ± 2.20*
Coronary flow rate (mL/min)	21.88 ± 1.21	17.07 ± 1.14*

*$P < 0.05$ versus control

expression and upregulated Bax expression (1.482 ± 0.086 versus control, $P = 0.009$) (Figures 1a–c).

DNR activates the ubiquitin ligases, MURF-1 and MAFbx

DNR caused a significant increase (44%) in the induction of MURF-1 (137.90 ± 10.15 versus 77.22 ± 9.45 avg pixels, $P = 0.001$), as well as a 40% increase in MAFbx (172.90 ± 4.82 versus 103.90 ± 10.94 avg pixels, $P < 0.0001$). DNR treatment caused a significant increase in the accumulation of ubiquitin proteins (153.2 ± 4.09 versus 141.4 ± 4.66 avg pixels, $P = 0.049$) (Figures 2a–c).

DNR induces autophagy in the rat heart

DNR caused significant increases in two markers of autophagy. A 24% increase in beclin-1 (206.10 ± 2.51 versus 156.60 ± 6.40 avg pixels, $P = 0.0002$) as well as a 46% increase in LC3 lipidation (210.60 ± 2.58 versus 114.10 ± 4.85 avg pixels, $P = 0.0002$) was observed after DNR treatment. DNR also caused a significant attenuation of p62 (174.5 ± 2.446 versus 152.7 ± 5.813 avg pixels, $P = 0.008$) (Figures 3a–c).

DNR attenuates the PI3-kinase/Akt signalling pathway

Akt (Ser^{473}) phosphorylation was significantly inhibited after DNR treatment ($90.00 \pm 0.58\%$, $P = 0.001$). One substrate of Akt, FoxO1, was also significantly dephosphorylated after DNR treatment ($95.60 \pm 1.2\%$, $P = 0056$) (Figures 4a and b).

Discussion

The data presented in this study support a model where acute DNR-induced cardiac dysfunction is associated with an upregulation of the ubiquitin–proteasome pathway and autophagy. Duanorubicin-induced cardiac dysfunction is reflected in significant decreases in aortic pressure, aortic output and coronary flow rate.[24] The attenuation of heart

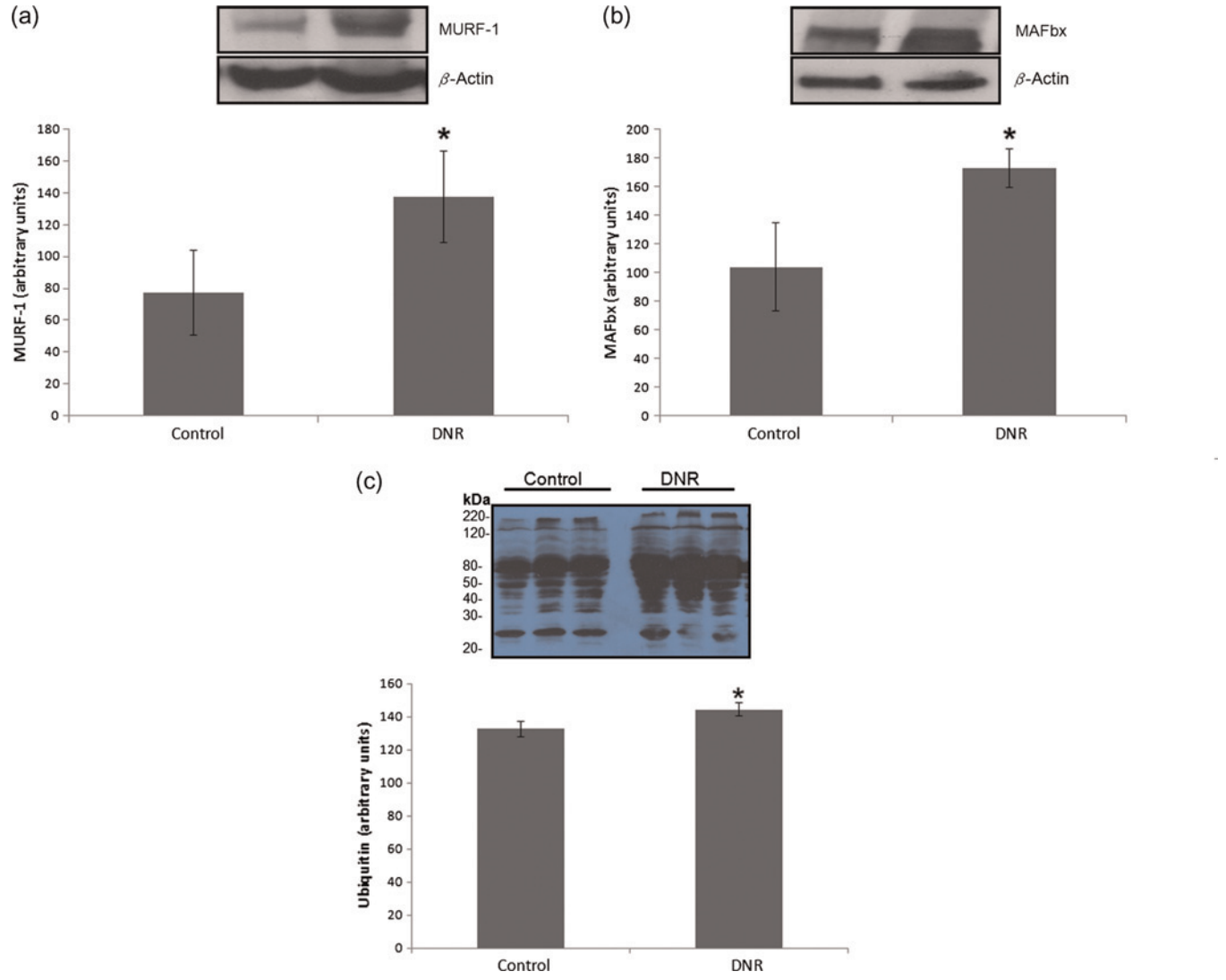

Figure 2 Effect of daunorubicin (DNR) treatment on the ubiquitin–proteasome system in the heart. After the perfusion protocol, the heart tissue was freeze-clamped and analyzed for the E3 ligases, MURF-1 (muscle ring finger 1) and MAFbx (muscle atrophy F-box) as well as for ubiquitin conjugates. (a) MURF-1, $^{*}P = 0.0011$, $n = 7$; (b) MAFbx, $^{*}P = 0.0001$, $n = 7$; and (c) ubiquitin, $^{*}P = 0.049$. (A color version of this figure is available in the online journal)

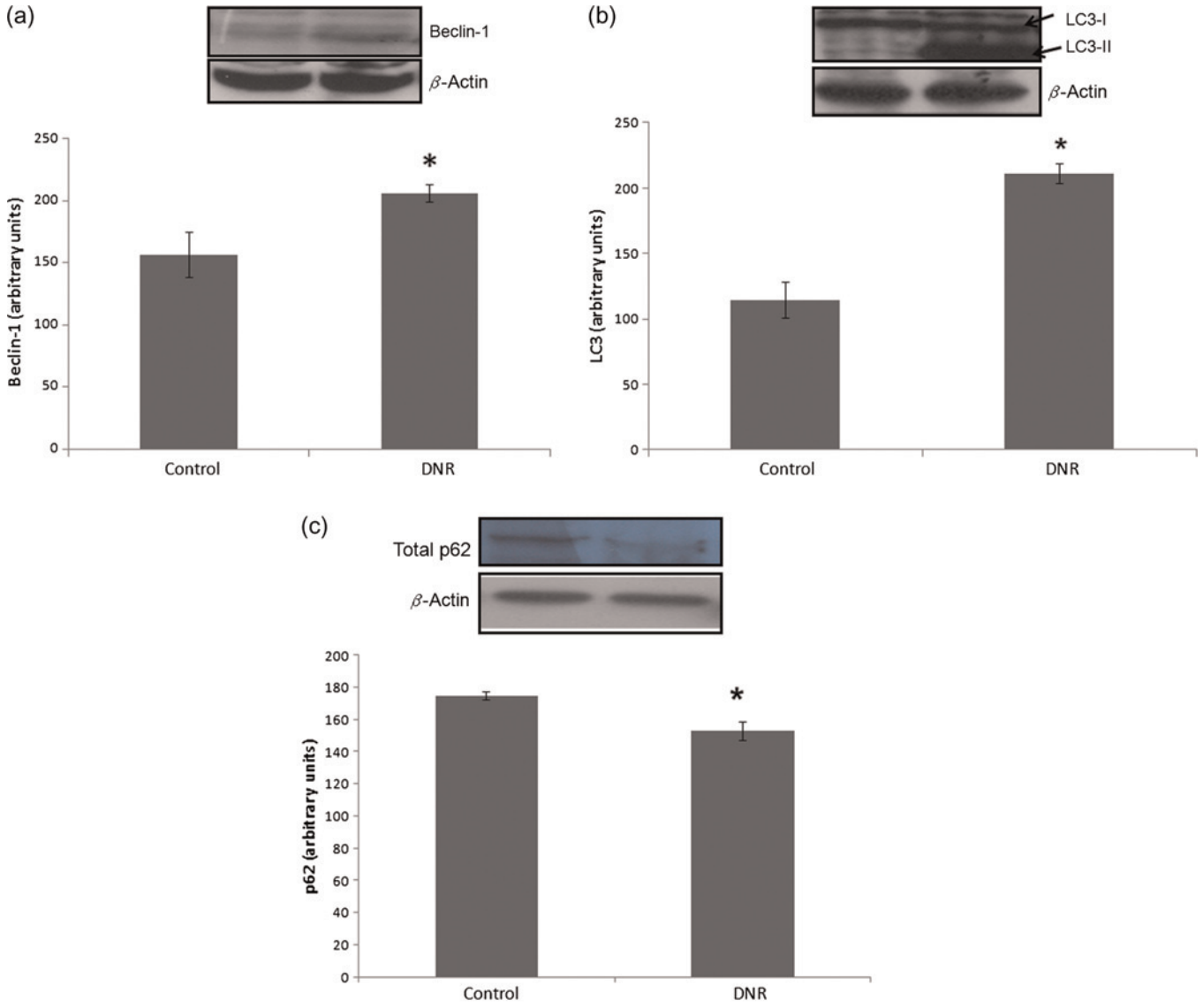

Figure 3 Effect of daunorubicin (DNR) treatment on autophagy in the heart. After the perfusion protocol, the heart tissue was freeze-clamped and analyzed for autophagy. (a) Beclin-1, $^{*}P = 0.0002$, $n = 7$; (b) LC3, $^{*}P = 0.0002$, $n = 7$; and (c) p62, $^{*}P = 0.008$, $n = 7$. (A color version of this figure is available in the online journal)

function induced by DNR was also associated with increased apoptosis in our model (Figures 1a–c). It is well accepted that apoptosis of cardiomyocytes could be one of the fundamental mechanisms that initiates or aggravates heart failure after acute anthracycline therapy.[25] It was also previously demonstrated that very low levels of myocyte apoptosis are sufficient to cause lethal, dilated cardiomyopathy.[26]

Although the involvement of the UPS and the E3 ligases, MURF-1 and MAFbx, in the turnover of skeletal muscle proteins is clearly established,[27,28] no evidence exists for a role for these two E3 ligases after acute DNR therapy. We have demonstrated for the first time that acute DNR therapy is associated with upregulation of MURF-1 and MAFbx (Figures 2a and b) and concomitant accumulation of ubiquitin proteins (Figure 2c). Although it was previously shown that a therapeutic dose of DOX activates the UPS by acting directly on both the ubiquitination apparatus and the proteasome, these researchers explore the mechanisms involved rather than investigating the role of these two ligases.[29] Although the pathophysiological role of MURF-1 and MAFbx has been largely confined to diseases that involve peripheral skeletal muscle wasting,[30,31] only recent evidence exists for a possible role for these ligases in ventricular remodelling. Several investigators have demonstrated that the UPS is activated during cardiac hypertrophy.[32,33] Upregulation of MURF-1 and MAFbx are also associated with increased protein degradation via the ubiquitin–proteasome pathway during myocardial remodelling in chronic heart failure.[34] Molecular targets ubiquitinylated by MAFbx and MURF include myofibrillar proteins like troponin-1, titin, nebulin and myosin light chain 2, as well as metabolic enzymes involved in energy production.[35,36] MURF-mediated degradation of troponin-1 seems to be very specific since structural proteins such as myosin, actin or MyBP-C are not downregulated.[37]

Although autophagy has long been depicted as a survival pathway which allow cells to maintain energy production

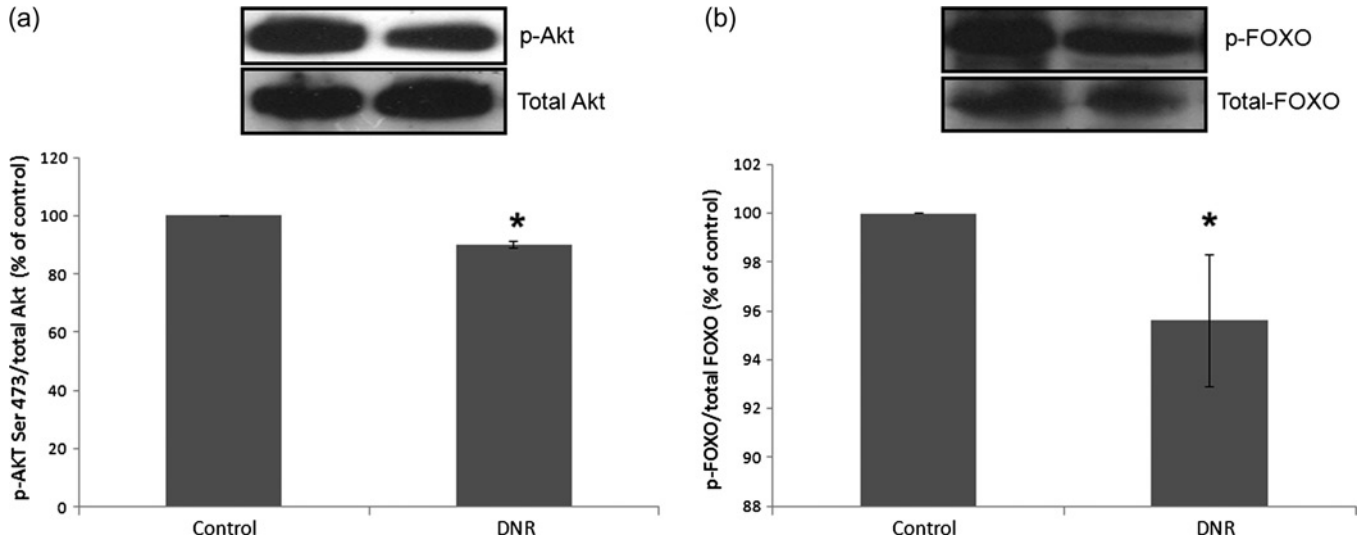

Figure 4 Effect of daunorubicin (DNR) treatment on the phosphoinositide 3-kinase (PI3-kinase)/Akt signalling pathway. After the perfusion protocol, the heart tissue was freeze-clamped and analyzed for phosphorylated and total Akt and FOXO. (a) Akt(Ser^{473}), $^{*}P = 0.001$, $n = 7$; (b) FoxO1, $^{*}P = 0.0056$, $n = 7$

under various stress and starvation conditions,[38] it has also been shown to contribute to cell death in other contexts, suggesting autophagy could either be protective or be detrimental, depending on the cellular environment.[39,40] Therefore, the functional significance of autophagy induction has to be determined individually within the specific context of each study. In our model, DNR-induced cardiotoxicity is associated with an upregulation of the autophagy markers, beclin-1 and LC3 and downregulation of p62 (Figures 3a–c). Beclin-1 is part of the PI(3) kinase class III lipid kinase complex which plays a central role in the induction of autophagy.[41] When autophagy is induced, microtubule-associated protein light-chain 3 (LC3), encoded by autophagy-related gene ATG8, is processed from LC3-1 (18 kDa) to LC3-11 (16 kDa) and incorporated into autophagic vacuoles.[42] p62 (also known as SQSTM1/sequestome 1) is an adaptor protein which targets protein aggregates and damaged organelles for autophagic degradation; in so functioning, p62 is selectively incorporated into autophagosomes through binding to LC3-II, degraded by autophagy and a good marker for efficient autophagic activity.[43] Autophagy is often also associated with apoptosis, which makes it even more difficult to determine the role of autophagy in cell death and cell survival. The interaction between autophagy and apoptosis has been characterized as follows: (1) autophagy and apoptosis can act as partners to coordinately induce cell death; (2) autophagy can act as an agonist to block cell death and; (3) autophagy can act as an enabler of apoptosis.[40] In accordance with our results, Kobayashi *et al*. (2010)[44] also demonstrated that DOX dramatically increased autophagy flux in cardiomyocytes which was associated with elevated apoptosis. These researchers also demonstrated that inhibition of autophagy resulted in the significant attenuation of cell death while the activation of autophagy with rapamycin exacerbated DOX-induced cardiomyocyte death, suggesting that autophagy is linked to apoptosis and act as partners to promote cell death.

The induction of the UPS and autophagy is tightly controlled by many positive and negative regulators.[40,45] The PI3-kinase/Akt signalling pathway, which is activated by insulin, growth factors and metabolic signals, are well known to inhibit autophagy and the UPS. The activation of Akt results in the phosphorylation of both cytoplasmic and nuclear target proteins which include FOXO proteins, a subgroup of the Forkhead transcription factors and mTOR. Phosphorylation of FOXO proteins by Akt promotes FOXO sequestration by 14-3-3 proteins in the cytoplasm, leading to inhibition of their transcriptional functions. On the other hand, dephosphorylation of FOXO leads to nuclear entry and transcription of ubiquitin ligases.[46] In the present study, we observed a significant attenuation in Akt and Foxo1 activity (Figures 4a and b), which might be responsible for the increased induction of MURF-1 and MAFbx in the DNR group. Furthermore, the attenuation of Akt by DNR might also be responsible for increased autophagy observed in our model through inhibition of mTOR, another downstream target of Akt. Our findings are indirectly supported by Zhu *et al*. (2009)[25] who observed that DOX treatment decreased mTOR activity in non-transgenic mice.

In summary, the results reported here suggests that acute DNR-induced cardiotoxicity, which is reflected in attenuation of cardiac function and increased apoptosis, is associated with an increased induction of the ubiquitin proteasome and autophagy as well as blunted Akt/FOXO signalling. Although a molecular link between the UPS-activating effects of DNR and its cardiotoxicity remains to be established, the present study is the first to demonstrate that DNR activates the E3 ubiquitin ligases, MURF-1 and MAFbx. This might implicate that the modulation of MURF-1/MAFbx might represent a novel strategy to attenuate cardiotoxicity after DNR treatment.

Author contributions: All authors participated in the design, interpretation of the studies and analysis of the

data and review of the manuscript. BJNS, DJB and AW conducted the experiments; BJNS and A-ME wrote the manuscript; BL analyzed the results; and JvR and AKJ initiated and planned the project.

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