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Doxorubicin induces protein ubiquitination and inhibits proteasome activity during cardiotoxicity

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ABSTRACT

Anthracycline-induced cardiotoxicity is a clinically complex syndrome that leads to substantial morbidity and mortality for cancer survivors. Despite several years of research, the underlying molecular mechanisms remain largely undefined and thus effective therapies to manage this condition are currently non-existent. This study therefore aimed to determine the contribution of the ubiquitin–proteasome pathway (UPP) and endoplasmic reticulum (ER)-stress within this context. Cardiotoxicity was induced with the use of doxorubicin (DXR) in H9C2 rat cardiomyoblasts (3 μM) for 24 h, whereas the tumour-bearing GFP-LC3 mouse model was treated with a cumulative dose of 20 mg/kg. Markers for proteasome-specific protein degradation were significantly upregulated in both models following DXR treatment, however proteasome activity was lost. Moreover, ER-stress as assessed by increased ER load was considerably augmented (*in vitro*) with modest binding of DXR with ER. These results suggest that DXR induces intrinsic activation of the UPP and ER stress which ultimately contributes to dysfunction of the myocardium during this phenomenon.

1. Introduction

Cardiotoxicity, defined as a multifactorial process that ultimately triggers cardiomyocyte death as the terminal downstream event (Minotti et al., 2004), is a well-known side effect of cytotoxic drugs particularly anthracyclines (ACs) such as doxorubicin (DXR). This debilitating condition poses a major threat to cancer survivors as it can lead to irreversible heart failure (HF), a reduced quality of life or even death (Lefrak et al., 1973; Swain et al., 2003; O'Shaughnessy et al., 2002). AC-induced cardiotoxicity is understood to be dose-related, with the incidence of complications increasing with each subsequent dose administered (Lefrak et al., 1973; Von Huff et al., 1977). The mechanism by which ACs induce cardiotoxicity appears to involve the production of oxygen-derived free radicals leading to oxidative stress (Schimmel et al., 2004), which initiates cell death of cardiomyocytes (Zhang et al., 1993). Although the 'oxidative stress hypothesis' is widely accepted, alternative mechanisms have been suggested to play significant roles during this phenomenon.

Studies by Timolati et al. (2009) and Lim et al. (2004) have demonstrated that contractile dysfunction, as a result of protease damage to myofibrillar proteins, is induced by oxidative stress and elevated levels of intracellular calcium in different models of AC-induced cardiotoxicity. The endoplasmic reticulum (ER) is highly receptive to alterations in calcium homeostasis and exposure to free radicals (Glembotski, 2007). Under these conditions, changes in the protein folding capacity, as well as the accumulation of misfolded proteins within the ER lumen elicits a phenomenon known as ER stress (Kaufman, 1999). The removal of these oxidatively damaged proteins is conducted by the ubiquitin–proteasome pathway (UPP), one of the major proteolytic pathways responsible for intracellular protein degradation (Gruene et al., 2004). It has previously been suggested that dysfunction of this pathway plays a significant role in various cardiac pathologies including myocardial ischaemia (Bulteau et al., 2001), atherosclerosis (Herrmann et al., 2004) and numerous cardiomyopathies. Moreover, in hearts challenged by ACs, excessive activation of the UPP has been implicated as a potential underlying mechanism for acute cardiotoxicity (Kumarapeli et al., 2005), although it remains to be determined how the UPP is activated in response to ACs.

The process of ubiquitination refers to the conjugation of free ubiquitin (Ub) with a substrate protein. Ub is a small, highly conserved protein that is ubiquitously expressed in all eukaryotes. It covalently binds to proteins in linear chains *via* three enzymatic reactions: (i) The Ub-activating enzyme (E1) covalently attaches to Ub in an ATP-dependent fashion, thus resulting in its activation. (ii) The Ub-conjugating enzyme (E2) consequently transports the activated Ub molecule from E1 to itself. (iii) The Ub-ligase (E3) then recognizes a specific substrate and thus transfers the activated

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Ub molecule from E2 to a particular lysine residue (Willis and Patterson, 2006; Patterson et al., 2007; Powell, 2006). Substrates marked with multiple Ub molecules (poly-ubiquitination) at lysine 48, results in the targeted substrate being degraded by the 26S proteasome (Spence et al., 1995; Pickart, 2001). In catabolic states where protein degradation is elevated, specific E3 ligases, MuRF-1 (Muscle Ring Finger-1) and atrogin1/MAFbx (Muscle Atrophy F-box), are upregulated in a FoxO (forkhead box)-dependent manner (Bodine et al., 2001), and thus mediate the ubiquitination of myofibrillar proteins for subsequent degradation during muscle atrophy (de Palma et al., 2008). Whilst the turnover of skeletal muscle proteins involving the ubiquitin ligases MuRF-1 and MAFbx is well established (Glass, 2003) the contribution of these E3 ubiquitin ligases in cardiac remodelling during DXR-induced cardiotoxicity remains to be fully elucidated. The present study thus sought to determine the impact of DXR therapy on the activity of the UPP and on ER stress in different models of acute DXR-induced cardiotoxicity.

2. Materials and methods

2.1. Cell culture preparation

H9C2 rat cardiomyoblasts (European Collection of Cell Cultures, Salisbury, UK) were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM) containing glutamine (Life Technologies, 32430, Johannesburg, South Africa) and supplemented with 10% heat-inactivated foetal bovine serum (FBS, Life Technologies, 10270, Johannesburg, South Africa) and 1% penicillin/streptomycin (Sigma, P4333, Johannesburg, South Africa). Incubation conditions were 100% humidified atmosphere at 37 °C in the presence of 5% CO_2. Seeding density was as follows: 1×10^6 myoblasts per 75 cm^2 tissue culture flask, 3×10^5 myoblasts per 25 cm^2 tissue culture flask (Flow cytometry), 1×10^5 myoblasts per 6-well plate (9.6 cm^2, Western blotting), 3×10^4 myoblasts per 96-well culture plates (0.3 cm^2, Proteasome assay) and 2×10^4 myoblasts per 8-well chamber slide (0.8 cm^2, Live cell imaging).

2.2. Treatment of H9C2 cells DXR

At 70–80% confluency, cells were treated with 3 μM DXR (Sigma, D1515, Johannesburg, South Africa) for a period of 24 h.

2.3. Preparation of cell extracts

Cell extracts were prepared as follows: 100 μl lysis buffer (pH 7.4) containing (in mM): TRIS–HCl 50, NP-40 1%, Na-deoxycholate 0.25%, EDTA 1, NaF 1, SBTI 4 μg/ml, PMSF 1, benzamidine 1, leupeptin 1 μg/ml and Triton X-100 1000 μl was added to the cells and the cell solution transferred to new tubes. Cells were sonicated, centrifuged [8000 rpm (4301 × *g*) at 4 °C for 10 min], and the supernatant was transferred to new tubes. Protein concentrations were determined with the Bradford method (Bradford, 1976), whilst others were diluted in Laemmli buffer and stored for Western blot analysis.

2.4. Western blot analysis

Proteins extracted from H9C2 myoblasts were subjected to 10% gel electrophoresis to separate proteins. These proteins were transferred to nitrocellulose membranes (Immobilon™ P, Millipore, IPVH00010, Billerica, MA) which were subsequently blocked for 2 h in Tris-buffered saline and Tween-20 (TBST, pH 7.6) containing 5% non-fat dry milk and incubated overnight at 4 °C with antibodies against anti-MuRF-1 (Imgenex: IMX-3924, 1:1000), MAFbx (Santa Cruz: Sc-33782, 1:1000), anti-ubiquitin (Cell Signalling: 3936, 1:1000), K48 ubiquitin (Cell Signalling: 8081, 1:1000) phosphorylated FoxO1/FoxO3a (Cell Signalling: 9464, 1:1000) and FoxO3a (Cell Signalling: 9467, 1:1000). β-Actin (Cell Signalling: 4967, 1:1000) was used for standardization of protein loading. All membranes (except the anti-ubiquitin and MuRF-1) were probed with ECL™ anti-rabbit IgG, HRP-linked whole antibody from donkey (Amersham Life Science: NA934V) in a 1:1000 dilution. The anti-Ubiquitin and MuRF-1 membranes were probed with the ECL™ peroxidase labelled anti-mouse (Amersham Life Science: NA931V, 1:1000) and the peroxidase conjugated affinity purified anti-goat IgG (Rockland: 605-703-125, 1:2000) antibodies respectively, at room temperature for 1 h. This was then followed by washes with TBST. The protein bands were detected by chemiluminescence (ECL™, Amersham Life Science, RPN 2108, Johannesburg, South Africa) and quantified using densitometry (UN-SCAN-IT, Silkscience version 5.1). All blots were scanned at a resolution of 150 dpi. The exact outline of each band (MuRF-1, MAFbx and FoxO) or lane (anti-ubiquitin and K48 Ubiquitin) 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 conditions where autoradiographic detection was in the linear response range.

2.5. Proteasome activity analysis

Proteasome activity was measured using the Proteasome-Glo chymotrypsin-like cell-based assay (Promega, G8661) following the manufacturer's instructions. This luminescent cell based assay measures the chymotrypsin-like protease activity associated with the proteasome complex in cultured cells. H9C2 cells were cultured and treated as previously described in white-walled 96-well luminometer plates where after 100 μl of the Proteasome-Glo cell-based reagent was added to each well. The contents of the wells were mixed at 700 rpm (33 × *g*) using a plate shaker for 2 min and incubated for 10 min at room temperature. This was followed by measuring the luminescent signal, which is directly proportional to the activity of this catalytic site, using a luminometer (GLOMAX 96 microplate Luminometer, Promega).

2.6. Assessment of ER load

In order to determine ER load as an indicator of ER-stress, the cell-permeant ER-tracker (Molecular Probes, E12353) was utilized. This fluorescent probe is highly selective for ER labelling. H9C2 cells were grown and treated as previously described in 25 cm^2 tissue culture flasks. Growth medium was discarded and cells rinsed with warm (37 °C) sterile PBS. Warm 0.25% trypsin-EDTA (3 ml) was added and cells were incubated until cells detached from the surface (2–3 min). Culture medium (double the volume of trypsin used; 6 ml) was added to the cell suspension, which was then transferred to a 15 ml falcon tube and centrifuged for 3 min at 6000 × *g*. Medium was decanted and cells resuspended in 500 μl warm PBS. ER-tracker was directly added onto the unfixed cells, using a final concentration of 100 nM, incubated for 10 min, and analyzed on the flow cytometer (BD FACSAria I) immediately thereafter. A minimum of 10 000 events (cells) were collected. Utilizing the 407 nm laser and the 430–640 nm emission filters, fluorescence intensity signal was measured using the geometric mean on the intensity histogram.

2.7. Live cell imaging

In order to establish a dynamic approach of monitoring various changes within the cell and its organelles after treatment, live cell imaging was performed. For this purpose, H9C2 cells were maintained at 37 °C in growth medium and seeded in eight-chamber dishes with a density of 2×10^4 cells. After reaching confluency, cells were treated with DXR as previously described and stained with ER tracker to visualize DXR localization and its association (colocalization) with the ER. Image acquisition was performed on an Olympus CellR̂ system attached to an IX 81 inverted fluorescence microscope equipped with an F-view-II cooled CCD camera (Soft Imaging Systems). Using a Xenon-Arc burner (Olympus Biosystems GmbH) as light source, images were acquired using a 360 nm and 572 nm excitation filter and a UBG triple-bandpass emission filter cube (Chroma). Images were acquired through z-stacks where appropriate, using an Olympus Plan Apo N60x/1.4 oil objective. The top and bottom focus position parameter were selected, indicating the upper and lower dimensions of the sample to be acquired with a step width of 0.26 μm between the single image frames. Images were processed and background-subtracted using the CellR̂ software, and presented in a maximum intensity projection. In setting up a defined experiment in the Experiment Manager facet of the CellR̂ software, image acquisition parameters such as exposure time, illumination settings and emission filter cube selection were kept constant for all groups and ensured appropriate selection of parameters. The DAPI 360 nm excitation wave length was used for setting the focal plane, avoiding unnecessary photo-bleaching.

2.8. Animal model

Protocols utilized were ethically approved and carried out according to the guidelines for the care and use of laboratory animals implemented at Stellenbosch University (Reference number: 2009B02004) and conforms to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication no. 85-23, revised 1985). GFP-LC3 transgenic mice were obtained through the RIKEN Bio-Resource Centre (RBRC00806, Japan kindly donated by Prof Noburu Mizushima). Mice were kept in sterilized filter top cages with controlled humidity, 12-h day/night cycles at 22 °C. Standard rat chow (SRC) and tap water was provided *ad libitum*. At 8 weeks of age, female mice were inoculated in the lower abdomen, in or near the left pad of the fourth mammary gland with 200 μl of 2.5×10^5 E0771 cells (kindly donated by Fengzhi Li, Roswell Park Cancer Institute, Buffalo, New York, USA) suspended in Hanks Balanced Salt Solution (Sigma, H9394, Johannesburg, South Africa) (protocol adapted from Ewans et al., 2006). The day of tumour cell inoculation was designated day 0 and small tumours were evident by day's 12–14 post-injection. Tumour size was monitored every second day by using digital callipers and body weight was monitored twice weekly.