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SIRT1 activation attenuates diastolic dysfunction by reducing cardiac fibrosis in a model of anthracycline cardiomyopathy

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ABSTRACT

Background: Doxorubicin (DOXO) is an effective anti-neoplastic drug but its clinical benefits are hampered by cardiotoxicity. Oxidative stress, apoptosis and myocardial fibrosis mediate the anthracycline cardiomyopathy. ROS trigger TGF-β pathway that activates cardiac fibroblasts promoting fibrosis. Myocardial stiffness contributes to diastolic dysfunction, less studied aspect of anthracycline cardiomyopathy. Considering the role of SIRT1 in the inhibition of the TGF-β/SMAD3 pathway, resveratrol (RES), a SIRT1 activator, might improve cardiac function by interfering with the development of cardiac fibrosis in a model of DOXO-induced cardiomyopathy.
Methods: F344 rats received a cumulative dose of 15 mg/kg of DOXO in 2 weeks or DOXO + RES (DOXO and RES, 2.5 mg/kg/day, concomitantly for 2 weeks and then RES alone for 1 more week). The effects of RES on cardiac fibroblasts were also tested *in vitro*.
Results: Along with systolic dysfunction, DOXO was also responsible of diastolic abnormalities. Myocardial stiffness correlated with fibroblast activation and collagen deposition. DOXO + RES co-treatment significantly improved ± dP/dt and, more interestingly, ameliorated end-diastolic pressure/volume relationship. Treatment with RES resulted in reduced fibrosis and fibroblast activation and, most importantly, the mortality rate was significantly reduced in DOXO + RES group. Fibroblasts isolated from DOXO + RES-treated rats, in which SIRT1 was upregulated, showed decreased levels of TGF-β and pSMAD3/SMAD3 when compared to cells isolated from DOXO-exposed hearts.
Conclusions: Our findings reveal a key role of SIRT1 in supporting animal survival and functional parameters of the heart. SIRT1 activation by interfering with fibrogenesis can improve relaxation properties of myocardium and attenuate myocardial remodeling related to chemotherapy.

1. Introduction

Doxorubicin (DOXO) is one of the most effective antitumor agents but its clinical application is limited due to major side effect, the cumulative dose-dependent cardiotoxicity. Despite decades of research, the complexity and the plethora of pathophysiologic mechanisms underlying DOXO-cardiotoxicity make difficult the identification of a defined strategy to prevent or treat such complication. Traditionally, progressive cardiac dilation, contractile dysfunction and ultimately congestive heart failure are attributed to DOXO cardiotoxicity with a consequent standard pharmacologic approach with ACEIs, ARBs, β-blockers, diuretics, ionotropic agents and Ca^{2+} antagonists that, unfortunately, give rather disappointing results. However, it is becoming clearer that to adjudicate cardiotoxicity only when a patient develops symptoms of congestive heart failure or presents with the asymptomatic decrease of left ventricular ejection fraction, can be reductive. A decline in systolic function can have different evolution, depending on baseline myocardial conditions. Left ventricular ejection fraction can return to normal value not only when chemotherapy is stopped but even when treatment is ongoing [1]. In this regard, it has recently been observed that diastolic dysfunction can be present in cancer survivors early during the course of treatment or after ending chemotherapy [2–7]. Additionally, in various conditions and/or in presence of comorbidities (hypertension, diabetes, aging and metabolic dysfunction), diastolic abnormalities can precede systolic dysfunction that can evolve into congestive heart failure with reduced (HFrEF) or preserved ejection fraction (HFpEF). Therefore, the presence of a diastolic dysfunction, in absence or concomitantly with a systolic dysfunction, may have a superior predictive value, helping to identify patients with a higher risk to develop heart failure.

Abnormal diastolic loads and increased diastolic wall stress are the parts of the pathophysiologic continuum in all forms of cardiac failure.

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Because mechanical stimulus activates programmed cell death in ventricular myocytes [8], wall stress can sum with the effect of DOXO toxicity on the cardiomyocytes. Additionally, diastolic dysfunction is associated with an adverse myocardial remodeling that also involves changes in the major structural proteins of the extracellular matrix [9]. Accumulation of fibrous tissue has a harmful impact on myocardial mechanics, and its excessive amount explains the onset of several features of a ventricular dysfunction that first appear during diastole and subsequently involve also systole [10,11]. Therefore, the interference with the mechanisms that increase myocardial stiffness and wall stress, independently from the presence of systolic abnormalities, may represent a valid option to oppose the development of DOXO-cardiomyopathy.

The current knowledge on DOXO-induced cardiotoxicity reckons the generation of reactive oxygen species (ROS) as the most upstream event in the cascade of intracellular modifications [12]. Several processes underlying anthracycline cardiotoxicity such as myocardial remodeling, cardiomyocyte death and alteration of cardiac contractile properties are modulated by oxidative stress [13–15]. Moreover, ROS have important role in the progression of fibrosis and can influence the expression as well as the activation of transforming growth factor-β (TGF-β) [16], a cytokine that has been associated to the evolution of tissue fibro-inflammatory signaling. TGF-β is able to modulate fibroblast proliferation and extracellular matrix production, particularly of type I collagen and fibronectin, while reducing degradation of those components. TGF-β also regulates the differentiation of fibroblasts into myofibroblasts that have a role in several diseases associated with fibrosis and tissue remodeling by producing extracellular matrix components as well as pro-fibrotic mediators, including TGF-β [11].

Numerous compounds with antioxidant activity have been tested in anthracycline cardiotoxicity models with contradictory findings, and similar image emerges from the clinical experience [14,17–21]. Nonetheless, with all the limitations, dexrazoxane is the only effective cardioprotective agent approved by FDA for anthracycline cardiotoxicity. Dexrazoxane was initially thought to diminish ROS generation in the heart by binding to iron and acting as a free radical scavenger. Given the critical role of topoisomerase IIβ, its inhibition and consequent reduction of ROS and DNA double-strand breaks is considered to be the key mechanism of dexrazoxane-mediated cardioprotection from DOXO [22–24]. Furthermore, inhibition of topoisomerase IIβ also reduces p53 and apoptotic pathway activation in response to DOXO-induced damage [24]. Altogether, it is clear that the search for compounds able to interfere with different but converging cellular and molecular features of DOXO cardiotoxicity is still of value.

Sirtuin family members have gathered a significant interest due to their cardiovascular protective role. SIRT1 is implicated in the regulation of cardiomyocyte growth and survival under stress conditions and, when upregulated, reduces the progression of heart failure and aging in mice [25]. Transgenic SIRT1 overexpression in a mouse heart protects from paraquat-induced oxidative stress [26], while a depletion of SIRT1 aggravates ischemia/reperfusion-induced oxidative injury [27]. Interestingly, in a model of hypertrophic cardiomyopathy, SIRT1 ablation in endothelial cells associates not only with impaired angiogenesis but also with diastolic dysfunction [28]. Our previous studies have demonstrated that *in vitro* activation of SIRT1 by resveratrol (RES) stimulated IGF-1/Akt pro-survival signaling, decreased p53 acetylation, enhanced oxidative stress defense and prevented senescence of human cardiac progenitor cells exposed to DOXO [29]. Similar results were reported for H9c2 cells, were RES prevented DOXO-induced toxicity through the inhibition of endoplasmic reticulum stress and the activation of the SIRT1 pathway [30]. Finally, RES ameliorated DOXO-induced cardiotoxicity in the aged heart through the restoration of SIRT1 activity [31] and reduced the pro-fibrotic effects of DOXO in adult male rats [32]. Although these results suggest that SIRT1 can be a downstream effector in the protection against oxidative stress and apoptosis, very little information is available regarding the role of SIRT1 in the development of myocardial fibrosis. The possible involvement of SIRT1 in a development of diastolic abnormalities remains an untested hypothesis. Therefore, the present study aimed to test whether SIRT1 activation, by interfering with pro-fibrotic mechanisms, can represent a preventive or an early interventional strategy to mitigate diastolic dysfunction in a model of anthracycline cardiomyopathy.

2. Materials and methods

2.1. In vivo treatment with RES in an animal model of DOXO cardiomyopathy

The present study conforms to the National Ethical Guidelines (Italian Ministry of Health; D.L.vo 26, March 4, 2014) and has been performed upon approval of local ethics committee in accordance with the ethical standards of the 1964 Declaration of Helsinki and its later amendments. Three months-old female Fischer 344 rats (Harlan Laboratories, Udine, Italy) were randomly divided into two groups: 1) DOXO group (n = 20), 6 i.p. injections of 2.5 mg/kg of DOXO over a period of 2 weeks to reach a cumulative dose of 15 mg/kg [29,33]; 2) DOXO + RES group (n = 18), received DOXO and RES (2.5 mg/kg/die, by gavage) concomitantly and then they were maintained on the intervention with RES for one more week. Saline-injected rats (n = 10; CTRL) were used as control. The regimen of six injection of low-average dosage made every other day over a period of two weeks was design to minimize the risk of drug-induced peritonitis.

2.2. Echocardiographic and hemodynamic measurements

Rats were anesthetized with an intramuscular injection of ketamine (100 mg/kg) and medetomidine (0.25 mg/kg) and echocardiography was performed with Vevo 770 (VisualSonics, Toronto, Ontario, Canada). Serial M-mode images were recorded along the minor axis at the level of the papillary muscles to measure LV diameter and wall thickness in diastole and systole and to calculate ejection fraction. Hemodynamic parameters were evaluated at sacrifice; animals were anesthetized and the right carotid artery was cannulated with a microtip pressure transducer (Millar Instruments, Houston, TX, USA). The catheter was advanced into LV cavity for the measurements of pressures and volumes in the closed-chest preparation.

2.3. Fixation of the heart

After hemodynamic measurements, the abdominal aorta was cannulated, the heart was arrested in diastole by injection of $CdCl_2$ (100 mM; (Sigma-Aldrich, St. Louis, MO, USA), the thorax was opened and perfusion with phosphate-buffered formalin was started, as previously described [29,33]. After fixation, the heart was dissected and weighted. Tissue specimens were embedded in paraffin and histological sections of 5 μm were cut.

2.4. Immunohistochemistry

Histological sections were deparaffinized with xylene and rehydrated with aqueous solutions of decreasing ethanol concentrations and stained with Masson's trichrome (Sigma-Aldrich) or used for immunolabeling and confocal microscopy. Cardiac fibroblasts were labeled with vimentin (Abcam, Cambridge, UK) and α-SMA (Sigma-Aldrich), myocytes were identified by α-sarcomeric actin (Sigma-Aldrich); SIRT1 (Abcam) was also used. Apoptosis was detected by TdT assay (Clontech Laboratories, Milan, Italy). Oxidative stress was detected by using an antibody against 8-OH-dG (Abcam). Nuclei were stained with DAPI (Sigma-Aldrich). Samples were analyzed with a Leica DM5000B microscope and a Zeiss LSM700 confocal microscope.