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Ranolazine protects from doxorubicin-induced oxidative stress and cardiac dysfunction

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Aims Doxorubicin is widely used against cancer; however, it can produce heart failure (HF). Among other hallmarks, oxidative stress is a major contributor to HF pathophysiology. The late I_{Na} inhibitor ranolazine has proven effective in treating experimental HF. Since elevated $[Na^+]_i$ is present in failing myocytes, and has been recently linked with reactive oxygen species (ROS) production, our aim was to assess whether ranolazine prevents doxorubicin-induced cardiotoxicity, and whether blunted oxidative stress is a mechanism accounting for such protection.

Methods and result In C57BL6 mice, doxorubicin treatment for 7 days produced LV dilation and decreased echo-measured fractional shortening (FS). Ranolazine (305 mg/kg/day) prevented LV dilation and dysfunction when co-administered with doxorubicin. Doxorubicin-induced cardiotoxicity was accompanied instead by elevations in atrial natriuretic peptide (ANP), BNP, connective tissue growth factor (CTGF), and matrix metalloproteinase 2 (MMP2) mRNAs, which were not elevated on co-treatment with ranolazine. Alterations in extracellular matrix remodelling were confirmed by an increase in interstitial collagen, which did not rise in ranolazine-co-treated hearts. Levels of poly(ADP-ribose) polymerase (PARP) and pro-caspase-3 measured by western blotting were lowered with doxorubicin, with increased cleavage of caspase-3, indicating activation of the proapoptotic machinery. Again, ranolazine prevented this activation. Furthermore, in HL-1 cardiomyocytes transfected with HyPer to monitor H_2O_2 emission, besides reducing the extent of cell death, ranolazine prevented the occurrence of oxidative stress caused by doxorubicin. Interestingly, similar protective results were obtained with the Na^+/Ca^{2+} exchanger (NCX) inhibitor KB-R7943.

Conclusions Ranolazine protects against experimental doxorubicin cardiotoxicity. Such protection is accompanied by a reduction in oxidative stress, suggesting that I_{Na} modulates cardiac redox balance, resulting in functional and morphological derangements.

Keywords Doxorubicin cardiotoxicity • Ranolazine • Heart failure • Oxidative stress • Na^+

Introduction

Anthracyclines are well established and effective antineoplastic agents in cancer treatment. Unfortunately, their use is limited by cardiotoxicity, characterized by progressive cardiac dilatation, contractile dysfunction, and ultimately heart failure (HF).[1] Indeed, with improvement in cancer survival, cardiovascular side effects of anti-cancer treatments have emerged as a relevant clinical problem,[2,3] and are also acknowledged in the most recent guidelines of the Heart Failure Association of the European Society of Cardiology.[4]

The chronic response to doxorubicin-induced myocardial injury involves complex reorganization of the extracellular matrix and

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substantial alterations in cardiomyocyte biology, such as apoptosis and changes in cell growth, excitation–contraction coupling, and cytoskeleton organization. Multiple mechanisms are involved in doxorubicin-induced HF (reviewed in Octavia *et al.*[5]). Increased oxidative stress certainly plays a role in such cardiomyopathy, as witnessed by reactive oxygen species- (ROS) induced damage, such as lipid peroxidation, along with reduced levels of endogenous antioxidant defences. Myofibrillar deterioration and intracellular calcium dysregulation are also important mechanisms commonly associated with doxorubicin-induced cardiac dysfunction. Furthermore, doxorubicin cardiotoxicity is mediated, at least in part, by changes in the high-energy phosphate pool, endothelin-1 levels, and disturbances of myocardial adrenergic signalling.

Many of the mechanisms underlying doxorubicin-induced LV dysfunction are shared by other forms of HF. This is the case of the above-mentioned alterations in cardiac redox balance and for the dysregulation of intracellular Ca^{2+} homeostasis due to reduced SERCA2a (sarcoplasmic reticulum Ca^{2+}-ATPase 2a) Ca^{2+} reuptake and increased Ca^{2+} extrusion from the ryanodine receptor (RyR2). In HF, late sodium current (I_{Na}) develops in ventricular myocytes.[6,7] Elevated $[Na^+]_i$ aggravates Ca^{2+} overload because of the decreased efflux of Ca^{2+} through the forward mode of the sodium–calcium exchanger (NCX), with increased Ca^{2+} influx through its reverse mode,[8] and increases H_2O_2 generation in myocytes.[9] The late I_{Na} inhibitor ranolazine has proven to reduce cardiac dysfunction in experimental HF studies.[10–13] Also, ranolazine reduced the composite primary endpoint (cardiovascular death, myocardial infarction, and recurrent ischaemia) in patients with elevated BNP in a subgroup analysis of the MERLIN trial.[14] Here, we hypothesize that ranolazine is also able to prevent cardiac dysfunction induced by doxorubicin in animal and cellular models, and we investigate whether oxidative stress is a mechanism involved in such protection.

Methods

Doxorubicin treatment protocol *in vivo*

C57Bl/6 mice (2–4 months old, Harlan Italy, San Piero al Natisone, Udine, Italy) were injected with a cumulative dose of 15 mg/kg doxorubicin via seven daily intraperitoneal injections (2.17 mg/kg i.p., DOXO group), as for our well-established protocol.[15] No mortality was associated with this dosing regimen. Another group of mice was treated orally with ranolazine (Ranexa, Menarini, 150 mg/kg/day or 305 mg/kg/day, doses comparable[16] with those used clinically in humans of 375 mg twice daily and 750 mg twice daily, below the human maximal dosing of 1 g twice daily) for 10 days (RAN group), and another group, after 3 days of ranolazine, started receiving doxorubicin concomitantly with ranolazine for 7 days (RAN + DOXO group). Sham animals were used as controls. For *ex vivo* analyses, animals were sacrificed by cervical dislocation after anaesthesia with tilotamine (0.09 mg/g), zolazepam (0.09 mg/g), and 0.01% atropine (0.04 ml/g); hearts were then excised and processed for further studies. Eight to ten animals per group were studied for all protocols.

Transthoracic echocardiography

In vivo cardiac function was assessed by transthoracic echocardiography in sedated 2- to 4-month-old wild-type C57Bl/6 mice using a Vevo 2100 high-resolution imaging system (VisualSonics, Toronto, ON, Canada, 40 MHz transducer). Mice were anaesthetized with tilotamine (0.09 mg/g), zolazepam (0.09 mg/g), and 0.01% atropine (0.04 ml/g). Cardiac function was evaluated by non-invasive echocardiography in basal conditions, after 7 days of doxorubicin, after 3 and 10 days of ranolazine, and after 3 days of ranolazine followed by co-administration of ranolazine and doxorubicin for 7 days. More details are available in the Supplementary material online, *Methods*.

Studies and analysis were performed blinded to heart condition. Data are presented as mean ± standard error of the mean (SEM) unless otherwise noted. Between-group differences were assessed by Student's *t*-test or one-way analysis if variance (ANOVA) as appropriate. Statistical significance was defined as $P < 0.05$.

The animal experiments described herein were conducted in accordance with the Italian regulations for experimentation on animals. All *in vivo* experiments were carried out with ethical committee approval and met the standards required by Directive 2010/63/EU of the European Parliament.

Real-time reverse transcription–polymerase chain reaction

Total RNAs were extracted using Trizol (Invitrogen, Milan, Italy) according to the manufacturer's protocol. Reverse transcription of total miRNA was performed starting from equal amounts of total RNA per sample (1 μg) using a SuperScript® III Reverse Transcriptase Kit (Invitrogen). Quantitative analysis of atrial natriuretic peptide (ANP), BNP, connective tissue growth factor (CTGF), matrix metalloproteinase 2 (MMP2), and glyceraldehyde phosphate dehydrogenase (GAPDH; as an internal reference) were performed by real-time PCR using specific primers and iQ™ SYBR Green Supermix (Biorad, Milan, Italy), respectively. The reaction for detection of mRNAs was performed as follows: 95 °C for 15 min, 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s. The threshold cycle (CT) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. For relative quantification the $2^{(-\Delta CT)}$ method was used as previously described.[17] Experiments were carried out in triplicate for each data point, and data analysis was performed by using software from Bio-Rad. Primer sequences are shown in the Supplementary material online, *Table S1*.

Cardiac fibrosis analysis

Interstitial fibrosis was evaluated by staining 5 μm thick tissue sections with 1% Sirius red in picric acid (Carlo Erba Laboratories, Milan, Italy) as previously described.[15] Further details are available in the Supplementary material online, *Methods*.

Western blot analysis of the apoptotic pathway

Mouse specimens were mechanically lysed in JS buffer (50 mM HEPES pH 7.5 containing 150 mM NaCl, 1% glycerol, 1% Triton X-100, 1.5 mM $MgCl_2$, 5 mM EGTA, 1 mM Na_3VO_4, and 1× protease inhibitor cocktail). Protein concentration was determined by the Bradford assay (BioRad) using bovine serum albumin as the standard, and equal

amounts of proteins were analysed by SDS–PAGE (12.5% acrylamide). Gels were electroblotted onto nitrocellulose membranes (Millipore, Bedford, MA, USA). For immunoblot experiments, membranes were blocked for 1 h with 5% non-fat dry milk in Tris-buffered saline (TBS) containing 0.1% Tween-20, and incubated at 4 °C overnight with primary antibody. Detection was performed by peroxidase-conjugated secondary antibodies using the enhanced chemiluminescence system (GE Healthcare, Milan, Italy). Primary antibodies used were: anti-GAPDH, anti-PARP [poly(ADP-ribose) polymerase], and anti-caspase-3 (Santa Cruz Biotechnologies, Santa Cruz, CA, USA).

HL-1 cardiomyocyte culture and transfection

HL-1 cardiomyocytes, an immortalized cell line derived from mouse atrial cardiomyocytes which maintains a differentiated adult cardiac phenotype, were obtained from Dr Claycomb (New Orleans, LA, USA) and were grown as previously described.[18] Further details are available in the Supplementary material online, *Methods*. HL-1 cardiomyocytes were transfected using a standard transfection method of adherent eukaryotic cell lines. Cells were plated in order to reach a confluence level of ~80% after 48 h. They were maintained in antibiotic- and serum-free media during the transfection procedure. Ranolazine (10 μM) or the NCX inhibitor KB-R7943 (5 μM, Sigma) were present when required. Transfection with Lipofectamine 2000 reagent (Invitrogen) was performed according to the manufacturer's instructions.

In vitro assessment of cardioprotective effects of ranolazine

HL-1 cardiomyocytes were seeded in 24-well plates at a density of 5×10^4/well and ranolazine (10 μM) or KB-R7943 (5 μM)[19] were added. After 48 h, cells were treated either with 0.1 μM doxorubicin or with 0.5 mM 2-mercaptopropionyl glycine (MPG) for 24 h. HL-1 viability was assessed by measuring the lactate dehydrogenase (LDH) released in the culture supernatant. LDH activity was evaluated by a well established procedure.[20] Cell-free culture supernatant was collected and cells were lysed in 1% (v/v) Triton X-100 to estimate the remaining LDH cellular content. The LDH activity was expressed as a percentage of the total LDH activity.

Measurement of formation of reactive oxygen species

Intracellular ROS generation was monitored using the cytoplasmic HyPer (Evrogen), a genetically encoded fluorescent sensor capable of detecting the intracellular formation of hydrogen peroxide (H_2O_2), one of the main ROS generated by cells.[21] Further details are available in the Supplementary material online, *Methods*. HL-1 cells were transfected and cultured in the presence of 10 μM ranolazine or 5 μM KB-R7943. At 48 h after transfection, cells were incubated either with 0.1 μM doxorubicin or the antioxidant MPG or their combination for 24 h. The analysis was then performed using the fluorescence microscope Zeiss Axiovert 100 M.

Statistical analysis

For most studies, between-group differences were assessed by Student's *t*-test or one-way ANOVA as appropriate. Differences among the groups in parameters assessed by reverse transcription–PCR (RT–PCR) or western blotting were evaluated using the non-parametric Kruskal–Wallis test and adjusted for multiple comparisons

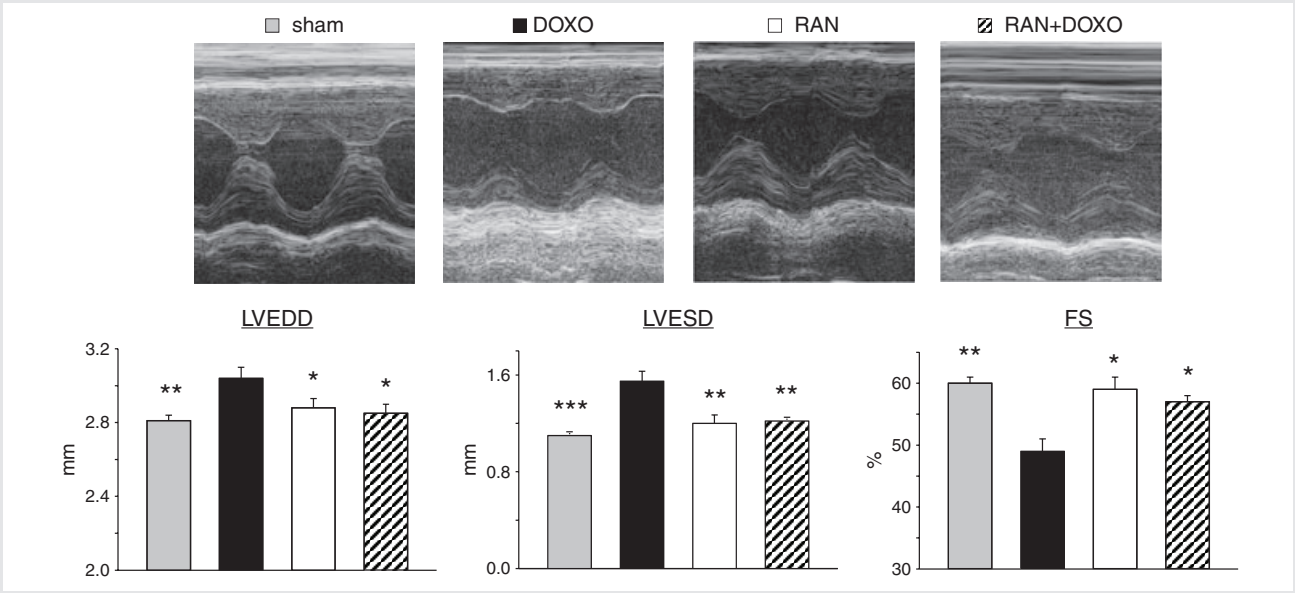

Figure 1 Ranolazine prevents left ventricular dilation and reduction in fractional shortening (FS) produced by doxorubicin (DOXO). Top: sample M-mode short axis echocardiographic images showing LV dilation and reduction of FS induced by DOXO, and the protective effects of ranolazine (RAN) in the RAN + DOXO group. Bottom: in mice treated with RAN + DOXO, LV diameters are significantly smaller, with milder reduction in FS. *$P < 0.05$ vs. DOXO; **$P < 0.005$ vs. DOXO; ***$P < 0.0005$ vs. DOXO. LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter.

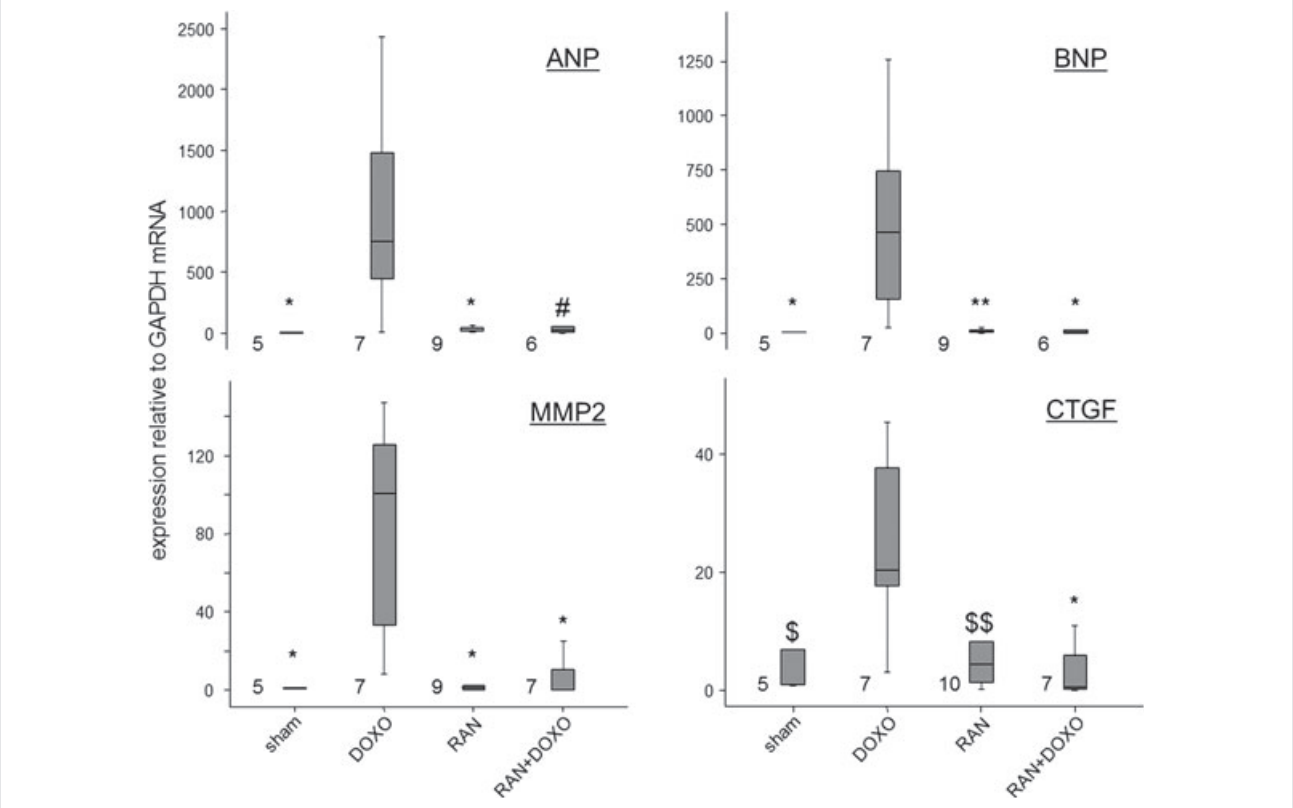

Figure 2 Ranolazine (RAN) blunts the elevation of the mRNA levels of atrial natriuetic peptide (ANP), BNP, connective tissue growth factor (CTGF), and matrix metalloproteinase 2 (MMP2) induced by doxorubicin (DOXO). Box-plots show that mRNA levels increase with DOXO, while they are lower in the RAN + DOXO group vs. DOXO. ANP, Kruskal–Wallis *P* for global model = 0.004; *Bonferroni $P < 0.05$ vs. DOXO; #Bonferroni $P = 0.07$ vs, DOXO. Bonferroni *P* = NS (non-significant) between RAN vs. sham and between RAN vs. RAN + DOXO. BNP, Kruskal–Wallis *P* for global model = 0.002; *Bonferroni $P < 0.05$ vs. DOXO; **Bonferroni $P < 0.01$ vs. DOXO. The remaining contrasts were not statistically significant. MMP2, Kruskal–Wallis *P* for global model = 0.008; *Bonferroni $P < 0.05$ vs. DOXO. The remaining contrasts were not statistically significant. CTGF, Kruskal–Wallis *P* for global model = 0.01; *Bonferroni $P < 0.05$ vs. DOXO; $Bonferroni $P = 0.09$ vs DOXO; $$Bonferroni $P = 0.216$ vs. DOXO. Bonferroni *P* = NS between RAN vs. sham and between RAN vs. RAN + DOXO. GAPDH, glyceraldehyde phosphate dehydrogenase.

with the Bonferroni method Statistical analysis were performed with SPSS statistical package (14.0 version).

Statistical significance was defined as $P < 0.05$.

Results

Ranolazine blunts doxorubicin-induced left ventricular dysfunction in mice

First, we tested the protective role of ranolazine on doxorubicin-induced cardiotoxicity *in vivo*. To this end, groups of 8–10 mice were injected with doxorubicin for 7 days, or, after 3 days of oral pre-treatment with ranolazine alone, doxorubicin and ranolazine were co-administered for 7 days. Another group of animals was treated with ranolazine alone for 10 days. Echocardiography was performed before and after the treatments.

After 7 days, doxorubicin produced LV dilation: the LV end-diastolic dimension (LVEDD) increased from 2.81 ± 0.03 nm (sham) to 3.04 ± 0.06 nm (DOXO), $P = 0.002$, with the LV end-systolic dimension (LVESD) increasing from 1.1 ± 0.03 nm (sham) to 1.55 ± 0.08 nm (DOXO), $P = 0.0001$. Fractional shortening (FS) decreased to $49 \pm 2\%$, $P = 0.002$ vs. $60 \pm 1\%$ (sham). Interestingly, in mice treated with RAN + DOXO, LV diameters were significantly smaller (LVEDD 2.85 ± 0.05 nm, LVESD 1.22 ± 0.03 nm, both $P < 0.05$ vs. DOXO), and the reduction in FS was milder: $57 \pm 1\%$, $P = 0.01$ vs. DOXO alone (*Figure 1*). These beneficial effects of ranolazine were not present when the drug was administered at a lower dose (150 mg/kg/day) with DOXO, since FS remained at the same level observed with DOXO alone: $50 \pm 2\%$, *P* = NS (non-siginificant) vs. DOXO.

Ranolazine prevents cardiac foetal gene reprogramming in doxorubicin-treated hearts

At the end of the protocol, mice were euthanized, and hearts were removed and processed for mRNA expression, histological

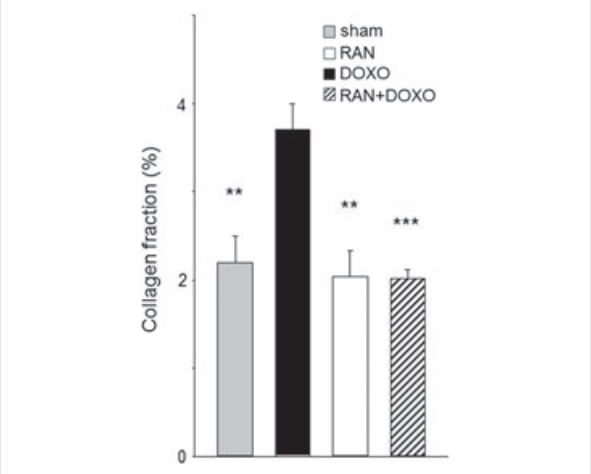

Figure 3 Effect of doxorubicin (DOXO) and ranolazine (RAN) + DOXO on collagen deposition. $^{**}P < 0.005$ vs. DOXO; $^{***}P < 0.0005$ vs. DOXO. The remaining contrasts were not statistically significant.

examination, and detection of myocardial stress and apoptosis. All analyses were carried out in parallel experiments on control, untreated mice. In accordance with the deterioration in function, significant elevations in ANP and BNP mRNAs were found in DOXO compared with sham. In RAN + DOXO hearts, these levels were lower compared with DOXO (*Figure 2*).

Ranolazine blunts doxorubicin-induced extracellular matrix remodelling and cell death in doxorubicin-treated murine hearts

We then evaluated the impact of ranolazine on doxorubicin-induced extracellular matrix remodelling and cell death. In the DOXO group, CTGF and MMP2 mRNAs were increased by 5-fold and 80-fold, respectively, compared with sham animals. Co-treatment with ranolazine prevented the increase in these mRNAs (*Figure 2*). The alterations in extracellular matrix remodelling were confirmed by an increase in interstitial collagen with DOXO (3.66%), $P = 0.004$ vs. 2.19% (sham), which did not increase in hearts co-treated with RAN (2.02%, $P = 0.0002$ vs. DOXO) (*Figure 3*).

Finally, the levels of uncleaved PARP and pro-caspase-3 were significantly decreased in the DOXO group. Activation of apoptosis was also indicated by increased cleavage of caspase-3. Caspase-3 fragmentation did not occur when hearts were co-treated with RAN + DOXO (*Figure 4*).

Ranolazine protects HL-1 cells from doxorubicin toxicity

To test whether ranolazine protection is achieved by blunting oxidative stress caused by treatment with doxorubicin, ROS formation and cell death were monitored in HL-1 cardiomyocytes. HL-1 cells were pre-treated with ranolazine (10 μM) for 48 h and then treated with doxorubicin (0.1 μM), MPG (0.5 mM), or their combination for an additional 24 h. Cell viability was evaluated as the percentage of LDH release in the culture medium. Doxorubicin increased total LDH release by 20%, which was reduced by 15% by co-treatment with ranolazine ($P < 0.05$ vs. doxorubicin alone, see *Figure 5A*). Interestingly, similar results were obtained with doxorubicin and co-treatment with the NCX inhibitor KB-R7943 (5 μM). For comparison, a conventional antioxidant treatment with MPG[22] reduced LDH release to 10% ($P < 0.005$ vs. doxorubicin alone, *Figure 5A*).

Ranolazine reduces forrmation of reactive oxygen species induced by doxorubicin in HL-1 cardiomyocytes

HL-1 cells, transfected with HyPer and treated for 24 h with doxorubicin, showed a significant increase of probe fluorescence, compared with untreated cells, reflecting induction of oxidative stress (*Figure 5B*). Based on the evidence that ranolazine protects against doxorubicin-induced cell death, we investigated whether ranolazine affects ROS formation consequent to doxorubicin treatment. To this aim, the degree of HyPer oxidation observed in cells incubated with doxorubicin was compared with that afforded by cell co-incubation with ranolazine or MPG. *Figure 5B* shows that ROS formation induced by doxorubicin was significantly decreased by ranolazine treatment, analogously to what happened with MPG antioxidant intervention. Since ranolazine does not interact directly with ROS, these findings suggest that I_{Na} contributes to doxorubicin-induced ROS formation, explaining how ranolazine can counteract doxorubicin toxicity. Similar findings obtained with the NCX inhibitor KB-R7943 confirmed our suggested link between Na^+ levels and ROS generation.

Discussion

Anthracyclines are the most widely used anticancer therapies, but unfortunately can bring on LV dysfunction and HF. To the best of our knowledge, our data show for the first time that the late I_{Na} inhibitor ranolazine is able to blunt doxorubicin cardiotoxicity *in vivo* and *in vitro*; such beneficial effects are accompanied by a reduction in oxidative stress.

Beside anthracycline-induced cardiotoxicity, intracellular ROS formation is involved in the development or evolution of many pathological conditions.[23] In particular, low levels of ROS have a role in normal cardiac signalling, growth adaptations, and matrix changes. Higher levels play a role in pathophysiological remodelling, cell death, and chamber dysfunction. The molecular signalling pathways linking ROS to cardiac hypertrophy, remodelling, and failure include alpha- and beta-adrenergic and angiotensin II (AT1) receptor agonism, as well as modifications of a wide array of proteins including stress kinases, nuclear transcription factors, collagen and metalloproteinases, calcium channels, and

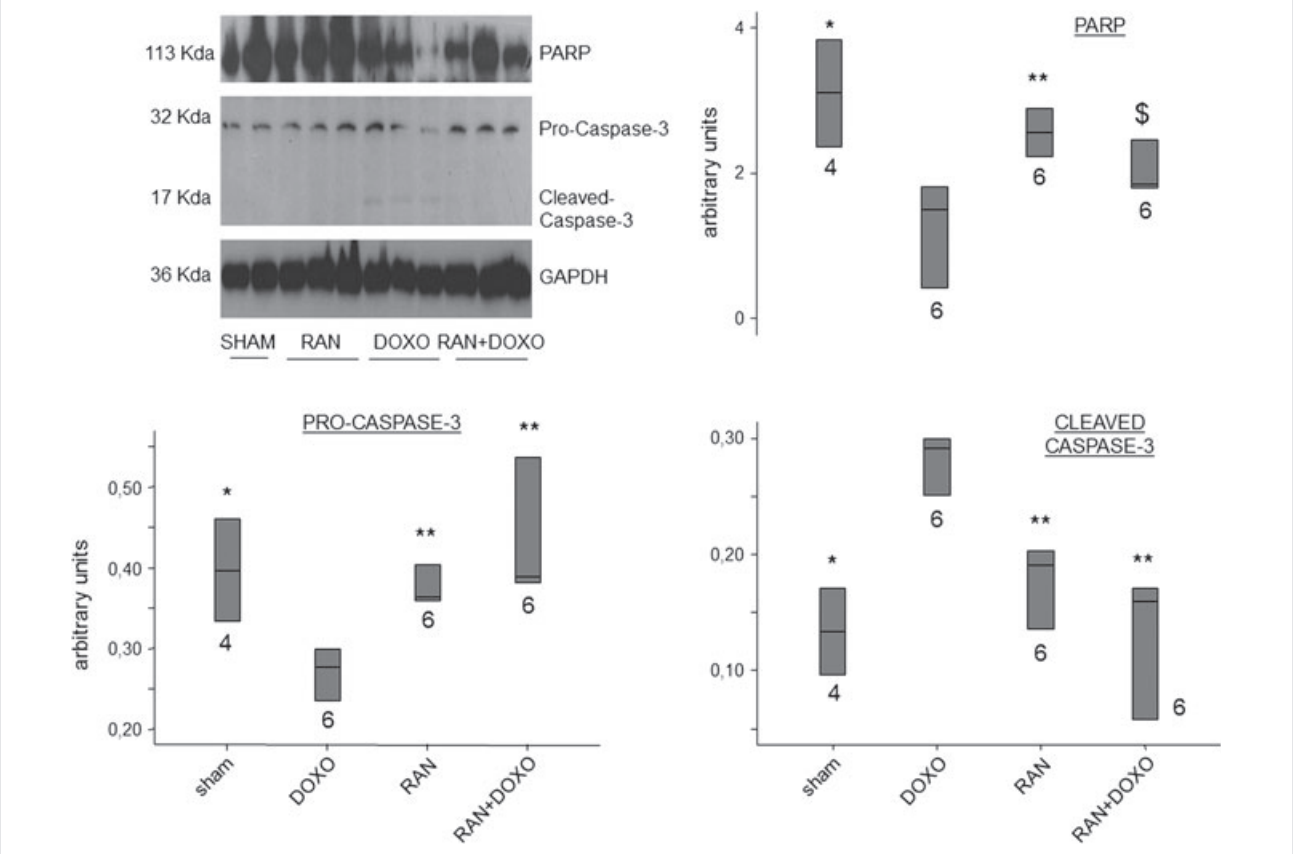

Figure 4 Western blot analysis of poly(ADP-ribose) polymerase (PARP) and caspase-3 activation. Upper left: example of western blot analysis of protein lysates from heart tissues of four different mice groups. Cleavage of PARP and pro-caspase-3 in mice treated with doxorubicin (DOXO; lanes 6–8) is prevented by pre-treatment with ranolazine (RAN; lanes 9–11). PARP, Kruskal–Wallis P for global model = 0.01; * Bonferroni $P < 0.05$ vs. DOXO; **Bonferroni $P < 0.01$ vs. DOXO; $Bonferroni $P = 0.13$ vs. DOXO. Bonferroni P = NS (non-significant) between RAN vs. sham and between RAN vs. RAN + DOXO. Pro-caspase-3, Kruskal–Wallis P for global model = 0.006; * Bonferroni $P < 0.05$ vs. DOXO; ** Bonferroni $P < 0.01$ vs. DOXO. The remaining contrasts were not statistically significant. Cleaved caspase-3, Kruskal–Wallis P for global model = 0.001; *Bonferroni $P < 0.05$ vs. DOXO; ** Bonferroni $P < 0.01$ vs. DOXO. The remaining contrasts were not statistically significant.

sarcomeric and excitation–contraction coupling proteins. A relevant outcome is the increase in cytosolic Ca^{2+} levels resulting in expression changes of several genes involved in pathophysiological hypertrophy and remodelling of the heart.[24]

Anthracycline-related cardiotoxicity is a multifactorial process sustained by energy collapse, disruption of ion homeostasis and sarcomeric proteins, suppression of cardiac-specific gene programmes, necrotic or apoptotic loss of myocytes, followed by remodelling and dilative cardiomyopathy.[25] Moreover, the toxic effects on cell membranes and related enzymes could cause metabolic changes. Anthracyclines also produce cardiac dysfunction by inducing myocardial Ca^{2+} overload and stiffness by inhibiting or reducing the expression levels of SERCA2a, by inhibition of the energy build-up that assists Ca^{2+} loading in mitochondria, and by inappropriate opening of the RyR2,[25] generating further ROS. Interestingly, reducing intracellular Ca^{2+} levels has proven to inhibit doxorubicin-induced ROS generation and cell death.[26]

A recent study[27] identified the molecular basis of doxorubicin-induced cardiotoxicity, showing that cardiomyocyte-specifc deletion of Top2b (encoding topoisomerase-IIβ) protects from doxorubicin-induced DNA double-strand breaks and alteration in the transcriptome that affects oxidative phosphorylation, and leads to altered mitochondrial biogenesis and ROS formation. Mice with cardiomyocyte-specific deletion of Top2b were protected from doxorubicin-induced LV dysfunction. Anthracycline metabolites or redox activation products such as secondary alcohol metabolites and ROS or reactive nitrogen species (RNS) are likely to represent important players in cardiotoxicity.[28,29] Interestingly, experimental doxorubicin-induced cardiac contractile dysfunction and adverse morphological changes are inhibited by exogenous antioxidant treatment or overexpression of endogenous antioxidant enzymes.[28,30] Furthermore, besides lowering the cumulative anthracycline doses and encapsulating anthracycline preparations into liposomes, strategies aimed at lowering oxidative stress (i.e. dexrazoxane) can be used in oncology patients, in spite of some

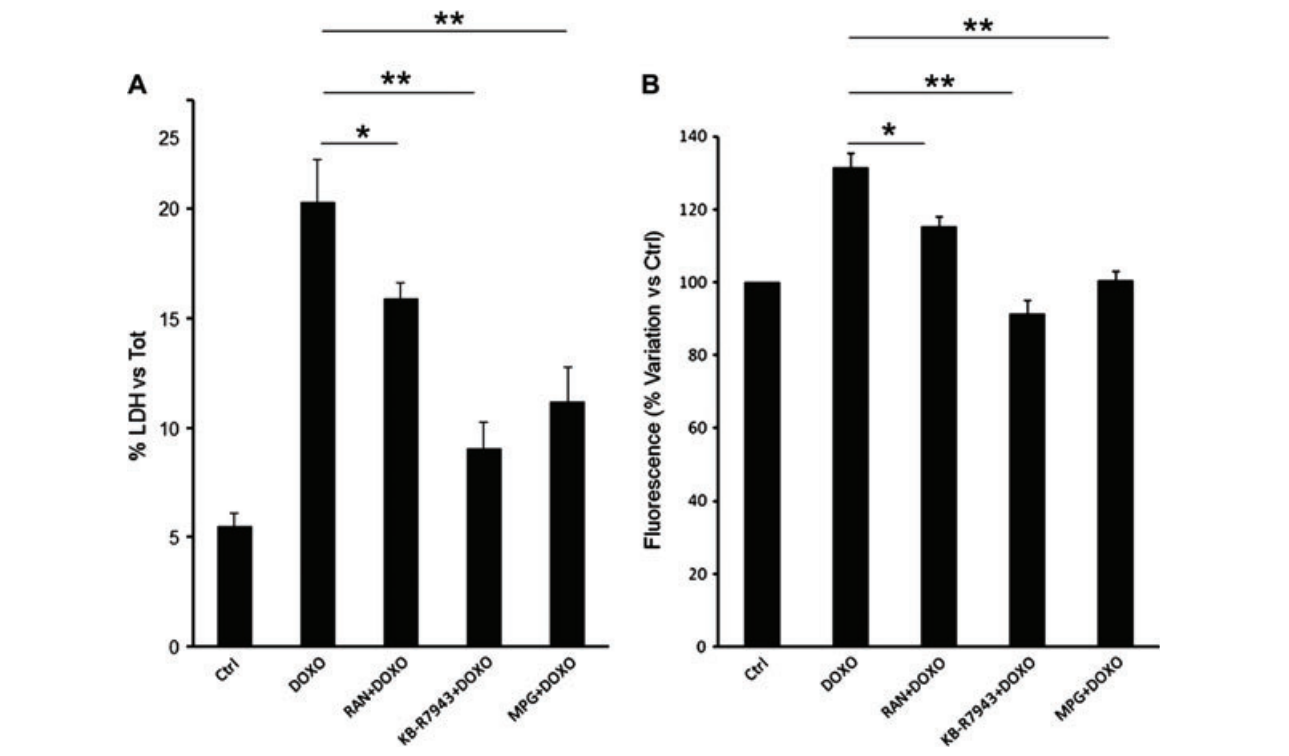

Figure 5 (*A*) Ranolazine (RAN) reduces cell death induced by doxorubicin (DOXO). HL-1 cells untreated and treated for 48 h with 10 μM RAN or with 5 μM KB-R7943 were incubated for 24 h with 0.1 μM DOXO. The degree of cell death was evaluated as a percenatge of lactate dehydrogenase (LDH) released in the culture medium. To evaluate the effect of oxidative stress on cell viability, cells were treated with DOXO and 0.5 mM of the antioxidant 2-mercaptopropionyl glycine (MPG) (MPG + DOXO). $n = 6$; *$P < 0.05$; **$P < 0.005$. (*B*) RAN reduces reactive oxygen species (ROS) formation induced by DOXO. HL-1 cells untreated and treated for 48 h with 10 μM RAN or with 5 μM KB-R7943 were incubated for 24 h with 0.1 μM DOXO. To evaluate the degree of intracellular oxidative stress, cells were treated with DOXO and 0.5 mM of the antioxidant MPG (MPG + DOXO). Microscopy analysis of cells expressing the cytoplasmic ratiometric fluorescent probe HyPer was performed to detect the degree of intracellular oxidative stress. $n = 30$; *$P < 0.05$; **$P < 0.005$.

controversy.[5,31] Our results in HL-1 cells show that attenuation of doxorubicin toxicity with ranolazine is indeed achieved with reduction in oxidative stress, and our *in vivo* data show better heart function with ranolazine + doxorubicin compared with doxorubicin alone.

Previous studies have supported late I_{Na} inhibition with ranolazine as a therapeutic strategy in different experimental models of systolic dysfunction. Acute infusion of ranolazine has been proven to increase EF and stroke volume in dogs with tachypacing-induced HF,[12] but not in control animals. In a more recent paper by the same group, ranolazine showed long-term effects, reducing myocardial remodelling in failing dogs. Chronic treatment with ranolazine prevented LV dysfunction; when combined with metoprolol or enalapril, it improved LV function even further.[13] Indeed, the failing myocyte is characterized by imbalances not only of Ca^{2+}, but also of Na^+ homeostasis:[6,7] late I_{Na} is markedly increased, persisting throughout the duration of the action potential plateau.[8] Elegant studies from Maack and O'Rourke have shown how elevated $[Na^+]_i$ would then be responsible for ROS formation by reducing mitochondrial Ca^{2+} uptake.[9] Accordingly, an inhibitor of the mitochondrial Na^+/Ca^{2+} exchanger, by blocking Na^+-induced Ca^{2+} exportation,[32] prevented increased ROS formation. The ROS could then activate Ca^{2+}/calmodulin kinase II[33] that would then interact with the Na^+ channel, increasing late I_{Na} and $[Na^+]_i$,[34,35] establishing a vicious cycle of elevated $[Na^+]_i$ and oxidative stress.[9] Such an increase of $[Na^+]_i$, would lead to increased exchange of intracellular Na^+ for extracellular Ca^{2+} through the NCX that, together with ROS inhibition of SERCA2a and activation of RyR2, would lead to Ca^{2+} overload, with electrical and mechanical dysfunction.[6,36] Hence, elevated $[Na^+]_i$ would constitute an attractive therapeutic target for LV dysfunction.

This mechanism would also be relevant in the ischaemia/reperfusion setting, characterized by Na^+ overload[37] and oxidative stress and cell death. In our *in vivo* model, we pretreated mice with ranolazine, and we continued such treatment during doxorubicin administration, similarly to what others have done with ranolazine protection against ischaemic damage.[10,38] Indeed, myocyte death (responsible, at least in part, for doxorubicin cardiotoxicity[28]) would stimulate reparative extracellular matrix remodelling, with increases in interstitial fibrosis and expression of profibrotic genes, with doxorubicin activating MMPs

in a redox-sensitive manner.[39] Derangements of the extracellular matrix and adverse remodelling are likely also to be worsened by oxidative stress.[40] Accordingly, our data show that ranolazine is able to blunt the effects induced by doxorubicin on fundamental components of myocardial remodelling such as myocyte death and fibrosis, preventing the onset of LV dysfunction measured by echocardiography.

Limitations of the study

Anthracyclines in human pathology are administered to cancer patients, while here we studied experimental doxorubicin toxicity in C57BL6 mice without cancer. Of course, further studies in mice with cancer are to be performed. Nevertheless, C57BL6 mice have a compromised immune system that in part may mimic cancer. Furthermore, these mice are commonly used in models of experimental heart failure.

The main focuses of our lab are redox modulation of myocardial function and cardiac dysfunction induced by anticancer drugs, and in our paper we did not aim at detecting precocious subtle changes of cardiac dysfunction, as specific assessment of diastolic dysfunction is sometimes difficult in mice. Our study aimed at showing that ranolazine is able to prevent the occurrence of doxorubicin-induced systolic dysfunction, while the INTERACT study[41] was specifically designed to assess the effects of ranolazine on diastolic dysfunction as an early sign of doxorubicin cardiotoxicity.

Conclusions

Our data support previous findings[10–13] on the efficacy of ranolazine in experimental HF. Importantly, in doxorubicin-induced cardiac damage, we show for the first time that such beneficial effects are accompanied by a reduction in oxidative stress, and we obtained similar results with the NCX inhibitor KB-R7493 in HL-1 cardiomyocytes. The antioxidant efficacy of ranolazine offers an advantage over conventional antioxidant treatments. In fact, this latter approach aims at quenching ROS after their production, while ranolazine, by reducing ROS formation, prevents the occurrence of oxidative damage. Further studies (both experimental and clinical) will be important to assess whether ranolazine might be introduced in clinical practice in the therapeutic armamentarium against anthracycline-induced cardiotoxicity; of note, the INTERACT study,[41] recently designed to assess whether ranolazine is able to relieve diastolic dysfunction induced by anthracyclines, indicates ranolazine as a promising cardio-oncological drug.

Supplementary Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Primer sequences.

Appendix S1. Supplementary Methods.

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Conflict of interest: none declared.

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