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ORIGINAL RESEARCH

The role of cell death and myofibrillar damage in contractile dysfunction of long-term cultured adult cardiomyocytes exposed to doxorubicin

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Abstract In failing hearts cardiomyocytes undergo alterations in cytoskeleton structure, contractility and viability. It is not known presently, how stress-induced changes of myofibrils correlate with markers for cell death and contractile function in cardiomyocytes. Therefore, we have studied the progression of contractile dysfunction, myofibrillar damage and cell death in cultured adult cardiomyocytes exposed to the cancer therapy doxorubicin. We demonstrate, that long-term cultured adult cardiomyocytes, a well-established model for the study of myofibrillar structure and effects of growth factors, can also be used to assess contractility and calcium handling. Adult rat ventricular myocytes (ARVM) were isolated and cultured for a total of 14 days in serum containing medium. The organization of calcium-handling proteins and myofibrillar structure in freshly isolated and in long-term cultured adult cardiomyocytes was studied by immunofluorescence and electron microscopy. Excitation contraction-coupling was analyzed by fura 2 and video edge detection in electrically paced cardiomyocytes forming a monolayer, and cell death and viability was measured by TUNEL assay, LDH release, MTT assay, and Western blot for LC3. Adult cardiomyocytes treated with Doxo showed apoptosis and necrosis only at supraclinical concentrations. Treated cells displayed merely alterations in cytoskeleton organization and integrity concomitant with contractile dysfunction and up-regulation of autophagosome formation, but no change in total sarcomeric protein content. We propose, that myofibrillar damage contributes to contractile dysfunction prior to cell death in adult cardiomyocytes exposed to clinically relevant concentrations of anthracyclines.

Keywords Cardiomyocytes · Doxorubicin · Contractility · Apoptosis · Necrosis · Autophagy

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Introduction

Contractile dysfunction in the failing heart is thought to be due to irreversible cardiomyocyte loss, which can be caused by cardiotoxic agents. A well-studied

example of cardiotoxicity is cancer therapy with anthracyclines, for instance with doxorubicin (Doxo), which can be associated with early cardiomyopathy as well as late-onset ventricular dysfunction (Lipshultz et al. 1991; Bristow et al. 1978). Possible mechanisms of anthracycline-induced cardiotoxicity are alterations in cellular structure (Lim et al. 2004), induction of apoptosis (Sawyer et al. 1999), disruption of energy production (Tokarska-Schlattner et al. 2005) and formation of reactive oxygen species (ROS) (Doroshow and Davies 1986). Anthracyclines have been reported to induce structural changes and cell death in the myocardium of treated patients, and the extent of structural damage has been correlated with early cardiac dysfunction (Billingham et al. 1978). However, it is not well known presently, how anthracycline-induced morphological changes of cardiac muscle cells correlate with markers for cell death and contractile dysfunction.

The study of potentially cardiotoxic agents in individual cardiac muscle cells is to some extent limited by the currently available in vitro model systems: cardiomyocytes from neonatal animals or commercially available cell lines may respond differently to cytotoxic drugs compared to adult primary cardiomyocytes due to differences in development-specific gene expression, cell cycle activity and apoptotic signaling (Konorev et al. 2008). Isolated ventricular cardiomyocytes or muscle strips from adult animals in serum-free conditions allow investigations on electric properties, contractility and other functions, but only for relatively short time. On the other hand, adult rat ventricular cardiomyocytes (ARVM) forming a monolayer of redifferentiated, contracting cells in serum-containing media can be maintained for several weeks and allow studies on cytoskeleton remodeling, metabolic changes and long-term effects of toxic agents and signaling modulators (Lim et al. 2004; Pentassuglia et al. 2009; Eppenberger-Eberhardt et al. 1997; Suzuki et al. 2001).

We have established a measurement procedure for contractile activity and Ca^{2+} transients in long-term cultured ARVM in order to simultaneously evaluate the progression of anthracycline-induced cardiotoxicity and effects on EC-coupling. To correlate changes in contractility and Ca^{2+}-handling with cell death, we have assessed a broad range of markers of cell viability and damage such as mitochondrial enzyme activity, plasma membrane integrity, DNA-degradation, a marker of autophagosomes and selected sarcomeric proteins. We also looked into the previously described detrimental effect of Doxo on the myofibrillar apparatus in cardiomyocytes, and its recovery potential after washout of the drug in this model.

Materials and methods

Isolation and culture of adult rat ventricular cardiomyocytes

Adult (250–300 g) male Wistar rats from an in-house breeding facility were killed by pentobarbital (Nembutal, Abbott Laboratories) injection. Isolation of calcium-tolerant cardiomyocytes was achieved according to previously published methods (Kondo et al. 1998). The cardiomyocytes were plated onto laminin-coated glass coverslips (10 μg/mL mouse laminin, Invitrogen) or culture dishes (Nunc). Cardiomyocytes were cultured for the long-term model in medium containing: 20% fetal calf serum (FCS) (PAA laboratories), cytosine 1-β-D-arabinofuranoside 10 μM, creatine monohydrate (Sigma) 20 mM, penicillin 100 IU/mL and streptomycin 100 μg/mL (Invitrogen) in MEM199 (Amimed). For the serum-free culture model cardiomyocytes were cultured in medium containing: fatty acid-free bovine serum albumin 2 mg/mL, L-carnitine 2 mM, creatine 5 mM, taurine 5 mM, triiodothyronine 10 nM (all from Sigma), penicillin 100 IU/mL and streptomycin 100 μg/mL (Invitrogen) in MEM199 (Amimed). All experiments were carried out according to the Swiss animal protection law and with the permission of the state veterinary office of Bern. This investigation conforms with the "Guide for the Care and Use of Laboratory Animals" published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Immunofluorescence microscopy

Cultures were washed 3 times with PBS, fixed with 3% *para*-formaldehyde for 15 min, permeabilized with 0.2% triton-X100 (Sigma) in PBS for 10 min, incubated for 30 min with bovine serum albumin (Sigma) 1 mg/mL in PBS at room temperature, incubated over night with primary antibodies at 4 °C, washed 3 times with PBS and incubated 1 h with the secondary antibodies. Anti mouse monoclonal antibodies (mAb)

for alpha-actinin (clone EA-53) and for desmin (clone DE-U-10) were from Sigma, or from Abcam (SERCA 2ATPase, clone 2A7-A1; ryanodine receptor, clone C3-33), and anti-myomesin (clone B-4) as a kind gift from J.C. Perriard, ETH-Zurich, Switzerland. Anti-mouse secondary antibodies were coupled with Alexa Fluor-488 (Invitrogen) and phalloidin coupled to Alexa Fluor-532 (Invitrogen) was used to visualize actin filaments. The morphology of the cytoskeleton was then analyzed using an inverted fluorescence microscope LEITZ DM IL (Leica) equipped with a 40× oil objective (Olympus) and with a confocal microscope (Zeiss LSM 410) using 40× and 64× objectives. Confocal images in Fig. 6 were taken using a Leica TCS NT and an inverted microscope (Leica DMIRB-E). Confocal microscopy images are shown as single optical sections.

Electron microscopy

Cardiomyocytes cultured on laminin-coated glass coverslips were fixed for 6 h at 4 °C with 2% glutaraldehyde (Fluka Chemika) in 0.1 M sodium cacodylate buffer. Samples were then postfixed for 1 h in 1% osmium tetroxide, dehydrated stepwise to 70% ethanol and stained en bloc for 1 h in 2% uranyl acetate in 70% ethanol. Dehydration was carried out by incubation in 90–100% ethanol and several mixtures of ethanol and propylenoxide (Sigma). After dehydration was completed in 100% propylenoxide, the cells were infiltrated within 24 h with an epoxy resin based on Epon812 (Epon 20 g, DDSA 16 g, NMA 7 g and DMP-30 1 g, all from Fluka Chemika) in a series of ascending mixtures of epoxy resin/propylenoxide. Gelatin capsules filled with 100% resin were placed on the coverslips and the samples were heat polymerized for 48 h at 60 °C. Ultrathin sections were cut on a Reichert Diatom ultramicrotome, placed on carbon and formvar coated copper grids (Provac) and were poststained with uranyl acetate and lead citrate according to standard procedures. Preparations were examined in a JEOL JEM100C transmission electron microscope at 80 kV.

Cell death assays

TUNEL (terminal deoxyribonucleotidyltransferase-mediated d-uridine 5′-triphosphate nick end labeling) assay was performed according to the manufacturers instructions ("in situ cell death detection kit AP", Roche Applied Science), with the exception of prolonged permeabilization and incubation steps. Incubation with DNAse-I (Sigma) was used as positive control for DNA nick labeling. DNAse incubation in fixed cardiomyocytes led to positive nuclei in 100% of the cells (not shown). LDH release test was performed according to the manufacturer ("cytotoxicity detection kit", Roche Applied Science). Results were normalized assuming cultures treated with 0.2% triton-X100 (Sigma) for 10 min as 100%. Absorption was measured using a Safire microplate reader (Tecan). The reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is thought to mainly occur in the mitochondria through the action of succinate dehydrogenase, therefore providing a measure of mitochondrial function. After incubation in MTT for 2 h cells were washed two times with PBS and lysed to release formazan from active cardiomyocytes (lysis solution: 0.6% glacial acetic acid and 10% SDS in DMSO) and lysates were analyzed in a Safire microplate reader (Tecan).

Contractility and calcium transients

Isolated ARVM were cultured for 14 days in media containing 20% FCS onto glass coverslips coated with laminin. Coverslips were taken out of the culture dishes after the indicated pharmacological treatment and assembled in a closed field-stimulation chamber including a temperature-controlled stage (Warner Instruments Corp.) on an Olympus IX-50 inverted microscope equipped with an UAPO-objective 20×/1.35 N.A. in Tyrode's buffer containing 0.5 μM of membrane-permeant fura-2-AM (Invitrogen) for 15 min and were rinsed with Tyrode's buffer containing probenecid 500 μM (Sigma) to prevent leakage of fura-2. Temperature stability of the chamber assembly and superfused buffer was continuously monitored and automatically maintained by a dual-channel temperature controller (Warner Instruments Corp.). The "SarcLen" sarcomere length recording method was used to measure contractility by fast-Fourier analysis of video images in real time with the IonWizard program (IonOptix). The region of interest was set to places where an unidirectional striation of sarcomeres was discernible in the phase contrast images. Field stimulation was performed using a

MyoPacer (IonOptix) delivering 2-ms square-wave bipolar pulses. Calcium and contraction transient plots were obtained by averaging the measurements of 10 (Fig. 3) or 27 (Fig. 5) cells for each condition. The calcium-length loops in those figures were drawn with the purpose to obtain a index of relative myofibril Ca^{2+} sensitivity based on the following assumptions: Within each contraction, the phase-plane diagram of sarcomere length and Ca^{2+} forms a counter-clockwise loop, as an analogy to the pressure–volume relationship diagrams used to assess contractile performance of the heart. During the relaxation phase, the sarcomere length and Ca^{2+} transients (which slightly vary in magnitude during each contraction) converge to a common trajectory. This common trajectory occurs during the Ca^{2+} decline at a time point shortly after sarcomere re-lengthening begins. Therefore, the trajectory of the relaxing phase can be used as an index of relative myofibril Ca^{2+} sensitivity (Spurgeon et al. 1992).

Western blot analysis

Cardiomyocytes were cultured for 10 days in culture dishes (Nunc) and treated as indicated in the "Results" section. Cells were quickly rinsed twice with ice-cold phosphate buffered saline and lysed in cold lysis buffer containing 3.7 M urea, 134.6 mM Tris, 5.4% SDS, 2.3% NP-40, 4.45% beta-mercaptoethanol, 4% glycerol, 6 mg/100 mL bromophenol blue, and boiled for 5 min. After SDS-PAGE using 12% Novex gels (Invitrogen), proteins were wet-transferred onto nitrocellulose membrane (BA85, Whatman). Immunodetection was carried out after blocking in 5% milk in TTBS (20 mM Tris base, pH 7.5, 150 mM NaCl, 0.03% Tween-20) and incubation with diluted specific antibodies. Polyclonal antibodies for LC3A/B were from Abcam (ab58610), monoclonal antibodies for α-cardiac actin (sarcomeric actin) (clone 5C5), for desmin (clone DE-U-10), and for sarcomeric alpha-actinin (clone EA-53) were from Sigma. Secondary antibodies anti-mouse IgG/IgM or anti-rabbit IgG coupled to horse radish peroxidase were from Pierce (Thermo Scientific), as was the chemiluminescence detection kit "SuperSignal Dura". Densitometry was performed using a ChemiDoc documentation system (BioRad).

Statistical analysis

All data are expressed as mean ± SEM. Statistical analysis of differences between groups was performed by Student's unpaired *t*-tests or by one-way ANOVA, followed by the Bonferroni post-test to compare single treatments when appropriate using the program Prism 5 (Graphpad).

Results

Structure and function of ARVM after isolation and in long-term culture

In cell culture medium containing serum ARVM undergo a process of de and redifferentiation, reestablish cell–cell contacts, regenerate myofibrils and resume spontaneous contractions (Eppenberger and Zuppinger 1999; Eppenberger et al. 1988) (Fig. 1a–d). The rather dramatic remodeling process from rod-shaped cells in tissue to a monolayer in culture might hinder regular EC-coupling because of a changed spatial alignment of calcium-handling proteins and Ca^{2+} stores. We therefore compared the ultrastructure of long-term cultured cells with freshly isolated cardiomyocytes or tissue sections and investigated the localization of Ca^{2+}-handling proteins before further exploring pharmacologic interventions (Fig. 1). The ultrastructural features of the sarcoplasmic reticulum and myofibrils were present in the polymorphic cultured cells, although at a lesser degree of overall organization compared to adult rat ventricular tissue. M-lines were occasionally discernible in sarcomeres (Fig. 1c, d). While freshly isolated cells presented a regular striated pattern of ryanodine-receptor (RyR) and sarco-endoplasmic reticulum ATPase (SERCA) proteins along the cell axis in immunofluorescence microscopy (Fig. 1e, g), ARVM cultured for 12 days showed a robust expression of the same proteins, with regional differences in striation presumably corresponding to myofibrils (Fig. 1f, h). Adult cardiomyocytes usually started spontaneous contractions around day 5 in culture.

We then tested the stability of paced contractions in cultured cells with field pacing at increasing frequencies in the range of 0.5–5 Hz (Fig. 2) and using known modulators of cardiac EC-coupling (Fig. 3). Despite the morphological differences between

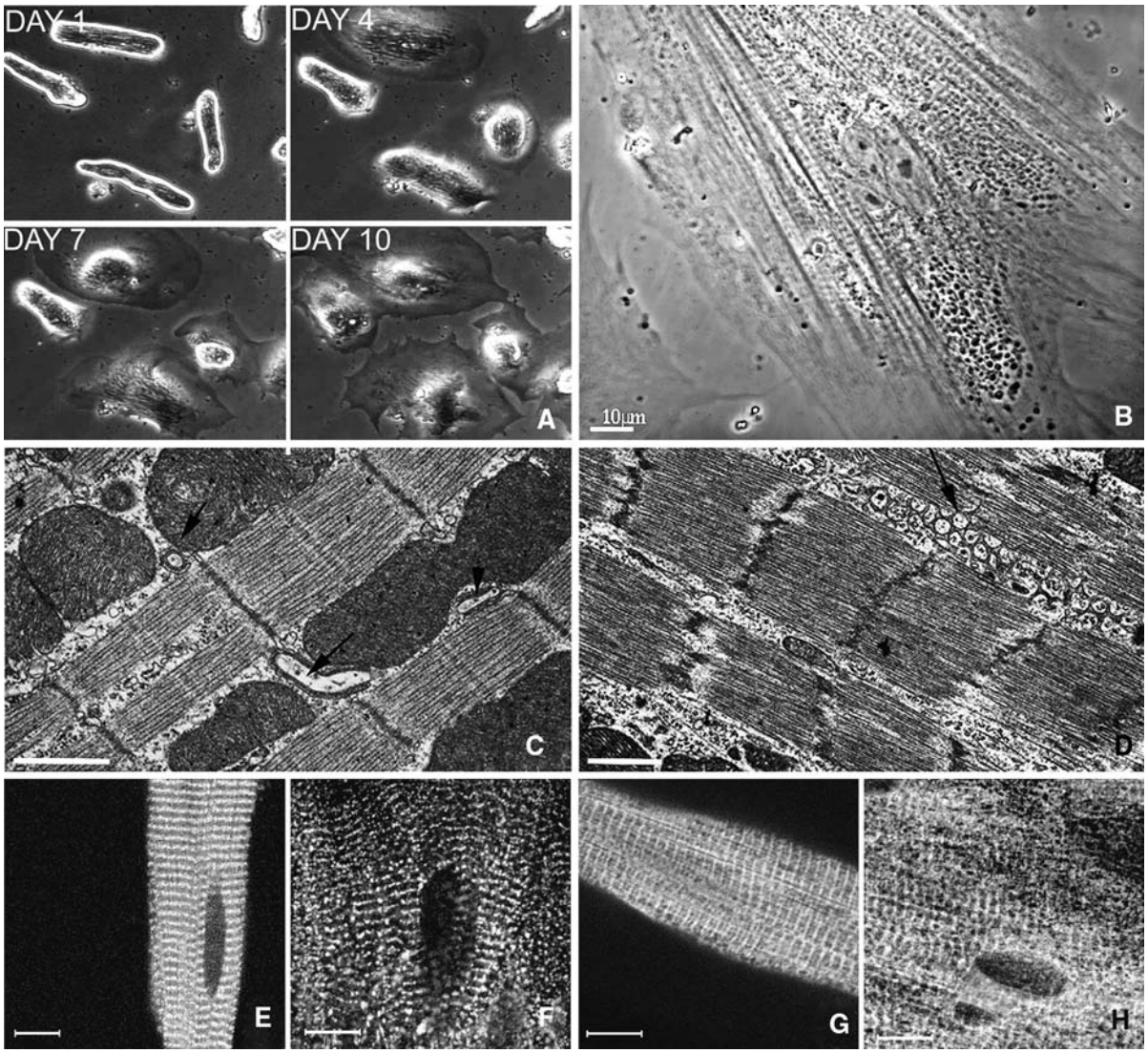

Fig. 1 Fine structure and localization of calcium-handling proteins in adult rat cardiomyocytes in the tissue, in freshly isolated cells and in long-term culture. **a**, **b** ARVM were cultured for a total of 14 days. **a** Time-lapse images of the same area of the culture were collected by phase contrast microscopy. **b** An example of a cardiomyocyte with well-discernible sarcomeric striations in phase contrast light microscopy at 14 days in culture. **c**, **d** Electron microscopy of adult rat heart tissue (**c**) or of ARVM cultured for 14 days (**d**) (*scale bar* 1 μm). *Black arrows* indicate triads or honey comb-shaped sarcoplasmic reticulum in tissue and culture, respectively. **e**–**h** ARVM cultured for 14 days (**f** and **h**) or freshly dissociated cardiomyocytes (**e** and **g**) were fixed and immunolabeled with mAb anti-RyR (**e**, **f**) or mAb anti-SERCA (**g**, **h**) (all *scale bars* 10 μm)

freshly isolated and cultured cardiomyocytes, long-term cultured ARVM were observed to contract in a regular and synchronous manner according to the imposed frequency up to 5 Hz by electrical field stimulation (Fig. 2). The beta adrenergic agonist isoproterenol (ISO) (Sigma) at 100 nM, or the bona fide myosin inhibitor 2,3-butanedione monoxime (BDM) (Sigma) at 100 nM was used as positive or negative modulator of contractility, respectively. Although BDM in the millimolar range can inhibit cardiac contractions completely, it was used in this experiment at a low concentration in order to modulate EC-coupling only to some extent. Upon treatment with BDM at 100 nM, the contractions were

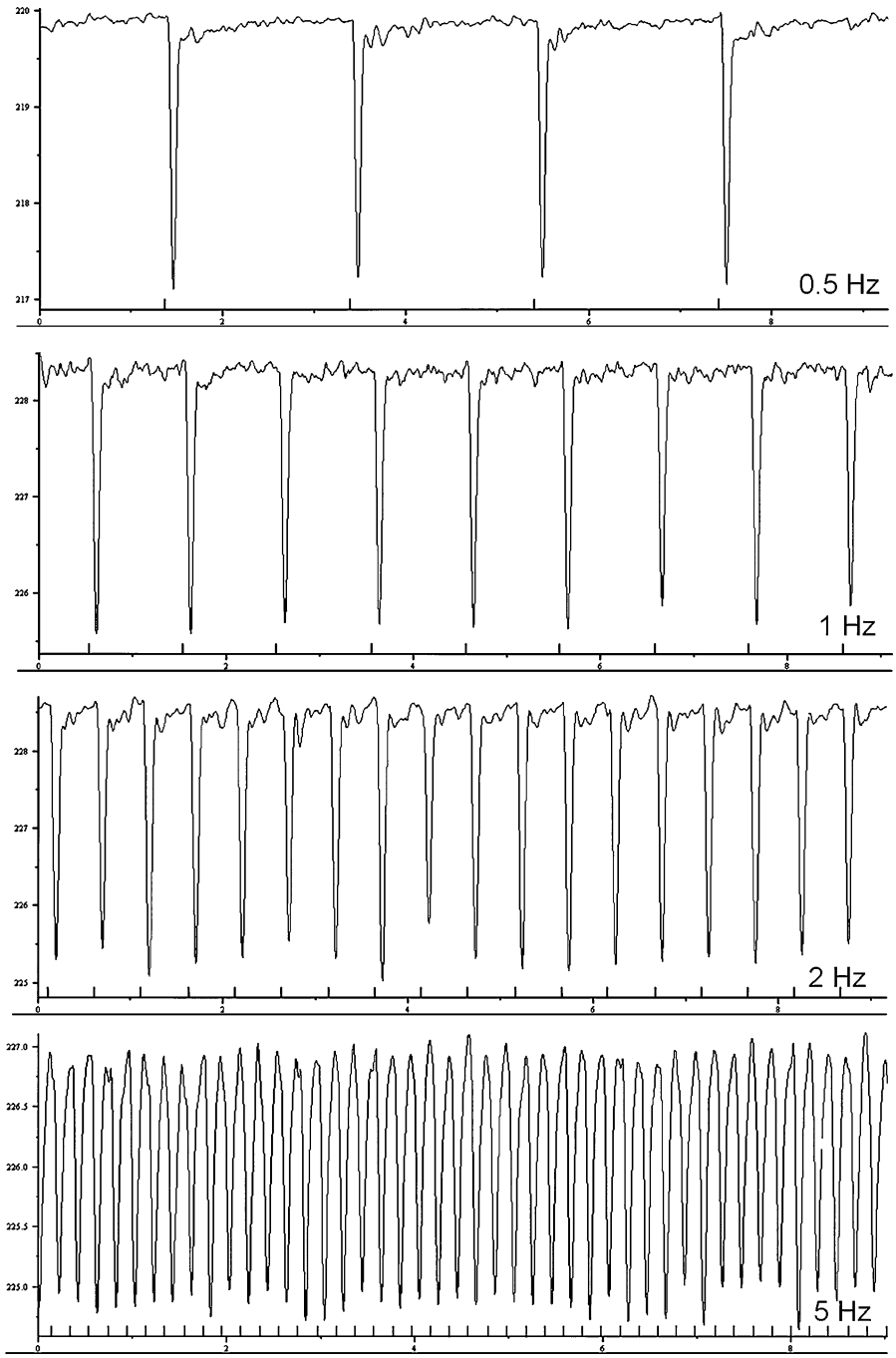

Fig. 2 Test for the stability of contractile activity in long-term cultured cardiomyocytes. ARVM cultured for 14 days were mounted in the measurement chamber and electrically stimulated at the frequency of 0.5–5 Hz. Sarcomeric contractions were recorded as described in the "Materials and methods" section. The X-axis is time (seconds) and the Y-axis is the sarcomer length (micrometer*100)

attenuated (Fig. 3a–c) in the monolayer of long-term cultured cells, similarly as in freshly isolated ARVM (data not shown). The calcium-length relationship (Fig. 3c) demonstrated, that BDM-treated cardiomyocytes required a rise in calcium before starting contraction. Additionally, BDM decreased the steepness of the relaxation trajectory in the calcium-length diagram, indicating that the coupling between cytoplasmatic calcium and sarcomere shortening was impaired. On the other hand, long-term cultured cardiomyocytes exhibited increased systolic values of cytoplasmic fura-2 ratios and faster kinetics after ISO application (Fig. 3d–f). ISO treatment caused a substantial increase in contractility, as it has been

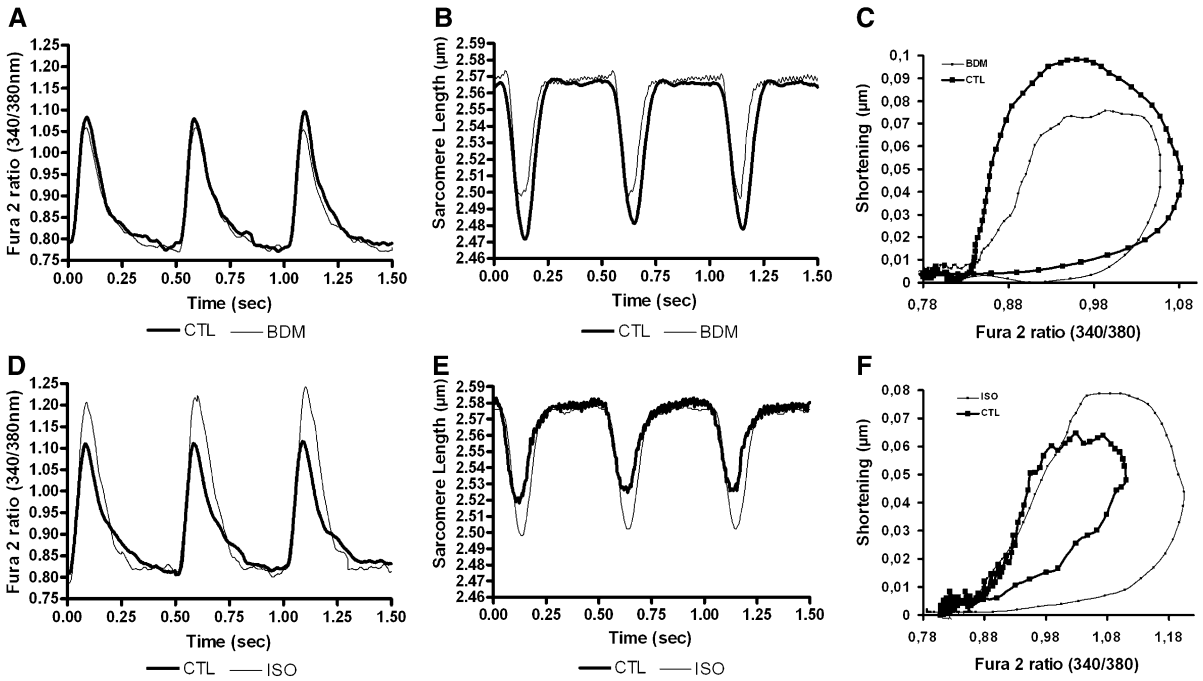

Fig. 3 Characterization of EC-coupling in redifferentiated ARVM exposed to the uncoupling agent 2,3-butanedione 2-monoxime (BDM) or to beta adrenergic stimulation by isoproterenol (ISO). ARVM were cultured for 14 days and exposed to BDM or ISO at 2 Hz field pacing. Kinetics of Fura-2 ratios (**a** BDM, **d** ISO), of contraction (**b** BDM, **e** ISO), and of the calcium-length relationship (**c** BDM, **f** ISO) are shown. *Line graphs* were plotted from averaged results of 10 cells from 2 hearts for each condition

demonstrated before in freshly isolated cardiomyocytes (Spurgeon et al. 1992).

Effects of Doxo on the viability of cardiomyocytes

Anthracyclines, which are widely used for cancer chemotherapy, have been limited in clinical use because of a dose–dependent cardiotoxicity. The endpoint of Doxo-associated cardiotoxicity most studied so far on the cellular level is cell death by apoptosis or necrosis (Sawyer et al. 1999; Kumar et al. 1999). We measured activity of mitochondrial enzymes with the MTT-assay. A decrease of the ability of the cells to convert the MTT substrate was seen in the higher range of Doxo concentrations (Fig. 1a). The LDH-assay was then used to measure the release of LDH into the cell culture medium indicating loss of plasma membrane integrity i.e. necrosis. This assay showed a sharp increase in LDH-release in the cultures at Doxo concentrations >10 μM (Fig. 1b). We then measured the percentage of TUNEL positive cells after treatment for 48 h (Fig. 4c). While ARVM were clearly undergoing apoptosis after treatment with Doxo at concentrations >10 μM, no significant increase in TUNEL positive cells was observed at the concentration of 1 and 10 μM. We then assessed several proteins by Western blotting after treatment of ARVM for 48 h (Fig. 1d). Microtubule-associated protein-1 light chain 3 beta (LC3) is involved in autophagosome formation and the amount of LC3-II is considered a marker for number of autophagosomes in cells (Gustafsson and Gottlieb 2009). Western blotting of Doxo-treated ARVM with an antibody that recognizes both LC3-I and LC3-II showed a gradual increase of LC3-II with increasing concentrations of Doxo. Detection of the myofibrillar proteins α-cardiac actin, alpha-actinin, and the intermediate filament protein desmin by Western blot showed overall no overt differences in untreated compared to Doxo-treated ARVM.

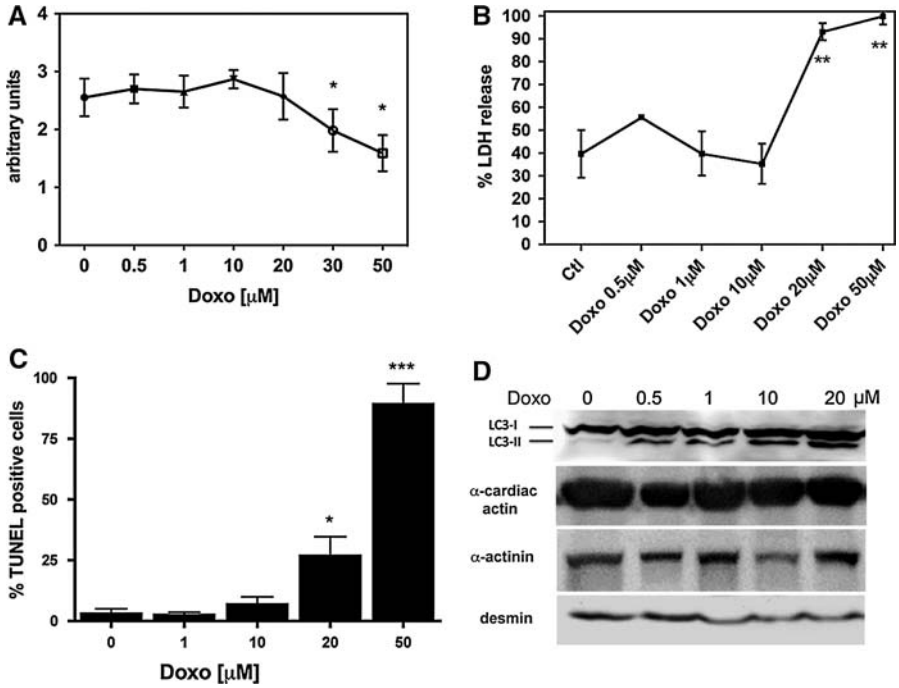

Fig. 4 Cell death and viability of ARVM exposed to different concentrations of Doxo. Cardiomyocytes were cultured for 12 days and then incubated with Doxo for 48 h. **a** MTT-assay for mitochondrial activity. **b** LDH release assay indicating loss of cell membrane integrity. **c** TUNEL-assay for DNA degradation. **d** Representative Western blots for the autophagosomal protein LC3I/II, and for the sarcomeric proteins α-cardiac actin, alpha-actinin, and desmin. $*p < 0.05$ versus untreated, $**p < 0.01$ versus untreated, $***p < 0.001$ versus untreated

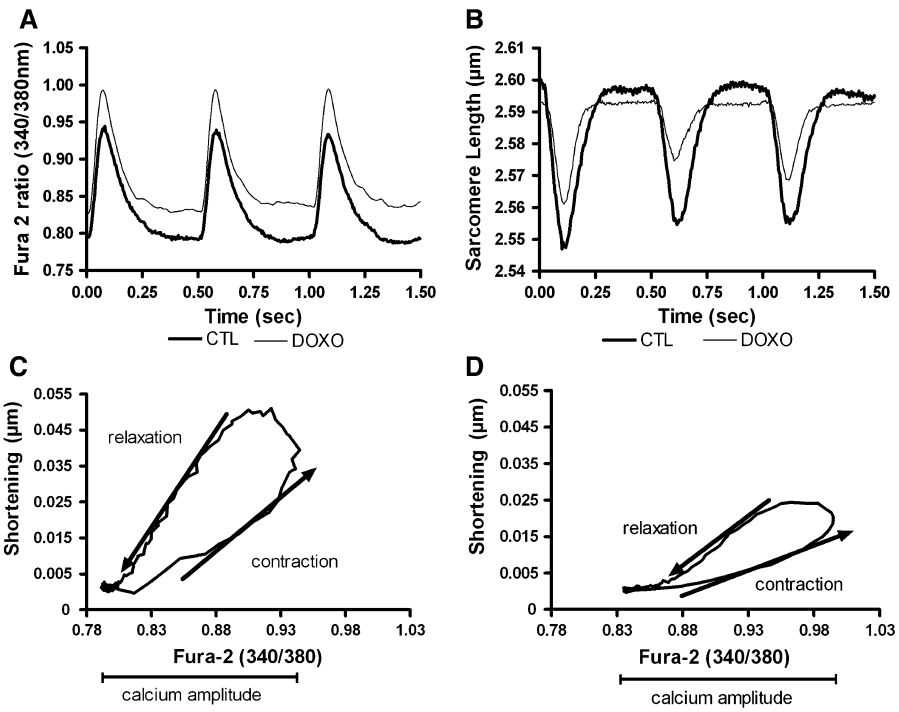

Fig. 5 Doxo induces contractile dysfunction and myofibrillar disorganization. **a**, **b** ARVM treated with 1 μM Doxo for 48 h (*thin line*) or untreated (*stronger line*) were measured for contractility (**a**) and fura-2 ratios (**b**). **c**, **d** Calcium-length diagrams demonstrating the reduction of the slope of contraction and of relaxation, as well as the myofibril sensitivity for calcium in Doxo-treated (**d**) cells compared to untreated ones (**c**). *Line graphs* were plotted from averaged results of 27 cells from 3 hearts

Impairment of EC-coupling by Doxo

Contractile defects were investigated in ARVM cultured for 10 days and treated for 48 h with Doxo at 10 μM (Fig. 5). Although systolic and diastolic calcium increased by Doxo treatment, decreases of calcium amplitude and prolongation of relaxation kinetics, as had been seen before in serum-free

cultured cells treated with Doxo (Timolati et al. 2006), were not observed (Fig. 5a). The amplitude of sarcomere shortening was reduced by Doxo (Fig. 5b). In order to assess whether uncoupling of excitation–contraction occurred, we used the calcium-length diagram (Fig. 5c, d). The effect caused by Doxo was similar to the one exerted by BDM (Fig. 3c), indicating an inefficient coupling between calcium cycling and sarcomere shortening.

Myofibrillar organization after wash-out of Doxo

Since cell death assays with different concentrations of Doxo indicated, that irreversible cell death is not observed in most ARVM at concentrations below 10 μM in our culture model, we tested the hypothesis, that the myofibrillar organization of the cells would improve after wash-out of Doxo (Fig. 6). To quantify the effect of treatments on myofibril structure, an investigator blinded to treatments counted the percentage of cells showing myofibrillar structural damage or reduction of sarcomere area at the end of a 15 days culture period after immunostaining for myomesin and F-actin. An example of the myofibrillar organization of an untreated culture and after 10 μM Doxo for 48 h is shown in Fig. 6a, b. For the wash-out experiment cardiomyocytes were cultured for 7 days, then treated with Doxo for 18 h, followed by a) two medium changes or b) a more intensive wash-out protocol with daily changes and medium

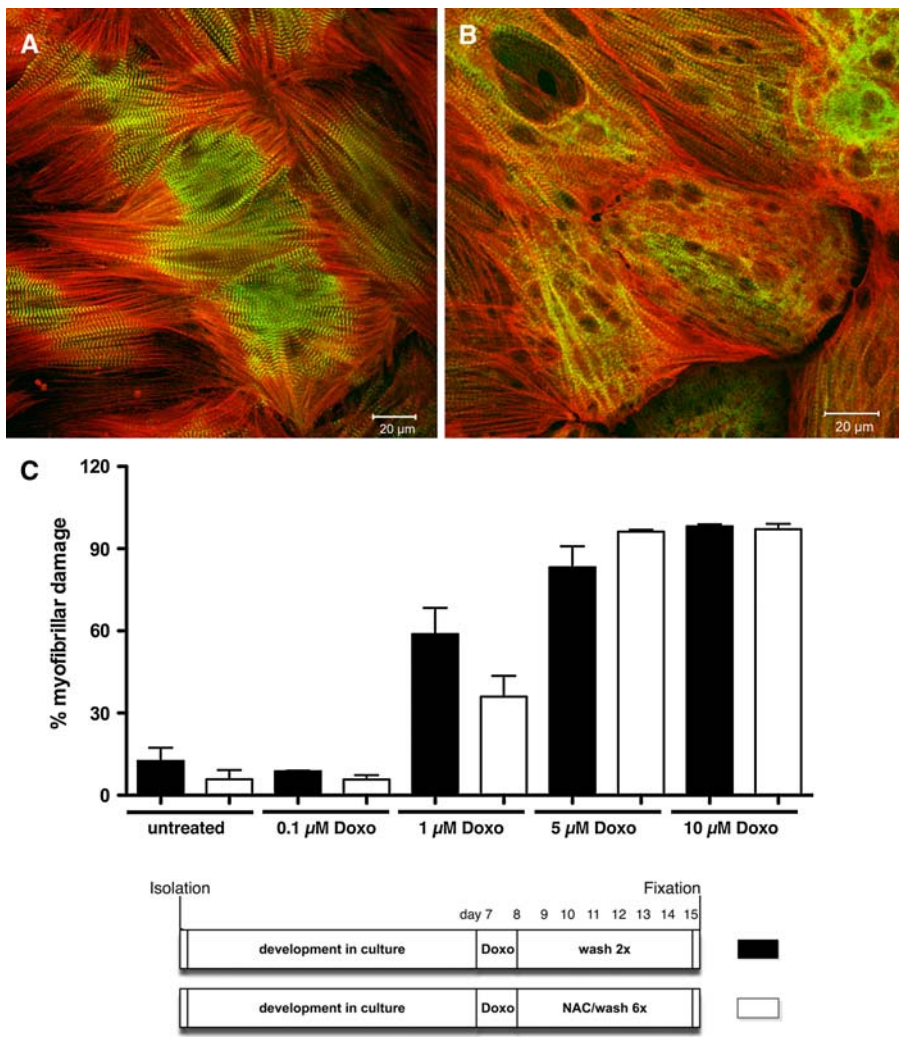

Fig. 6 Myofibrillar structural changes induced by Doxo and recovery. **a**, **b** ARVM cultured for 14 days without treatment (**a**) or after 48 h of 10 μM Doxo (**b**) were fixed and immunostained for actin (*red*) and myomesin (*green*). **c** For the recovery experiments, cardiomyocytes were cultured for 7 days, then treated with Doxo for 18 h, followed by wash-out with medium (*black bars*) or medium supplemented with *N*-acetylcystein (NAC) as indicated (*white bars*). The bar graph shows the results of three independent experiments with evaluation of 200 cells for myofibrillar changes in each condition. Overall one-way ANOVA $p < 0.0001$ (Color figure online)

including the antioxidant *N*-acetylcystein (NAC) as indicated in the treatment scheme. This procedure resulted in a trend to reduced myofibrillar damage after the intensive wash-out protocol, which was most pronounced at the concentration of 1 μM Doxo, but not at higher concentrations of the drug.

Discussion

Many observations in patients as in animal and cell culture models have repeatedly demonstrated the cardiotoxic potential of anthracyclines. There is controversy about to what extent apoptosis and other forms of cell death contribute to cardiotoxicity induced by anthracyclines in patients (Minotti et al. 2004; Gewirtz 1999). It was found in the present study that Doxo affected cell viability and the myofibrillar organization in adult cardiomyocytes, the latter phenomenon occurring already at sub-apoptotic (0–1 μM, see Figs. 4, 6) and clinically more relevant concentrations of the cardiotoxic drug. The total amounts of sarcomeric proteins such as α-cardiac actin or the intermediate filament protein desmin showed little changes after anthracycline treatment when assayed by Western blotting. This result showed, that the evident Doxo-induced damage and loss of myofibrils as visualized by immunolabeling and confocal microscopy was not accompanied by a marked decrease in total protein of those relatively long-lived sarcomeric components. Taken together, we conclude that myofibrillar morphology, but not apoptosis, can be considered a sensitive marker for myocardial stress by anthracyclines. The mechanism of this type of cellular injury is likely multifactorial including the formation of reactive oxygen species, elevation of cytoplasmic Ca^{2+} concentration and activation of intracellular proteases as well as inhibition of the mTOR signaling pathway and the cardiac transcription factor GATA4 (Lim et al. 2004; Pentassuglia et al. 2009; Timolati et al. 2006; Zhu et al. 2009). We have used myofibrillar changes as a marker for cancer therapy-associated cardiotoxicity with different classes of drugs such as paclitaxel, anti-ErbB2 antibodies, MEK-inhibitors and small tyrosine kinase inhibitors for ErbB1/ErbB2 (Pentassuglia et al. 2007, 2009; Sawyer et al. 2002).

We further investigated here, how the observed changes after cardiotoxic drug treatment affect contractility and calcium handling in the long-term culture model, which is ideally suited to study drug effects over a longer time period and to follow cytoskeleton changes in detail. However, a major concern regarding culture-induced changes of EC-coupling has been the functionality of T-tubules, which degenerate already 24–72 h after isolation in cultured adult cardiomyocytes (Spurgeon et al. 1992). These structures can be found again, although at a lesser degree of organization and more reminiscent of earlier developmental stages, in re-differentiated cells cultured in serum-containing medium (Kostin et al. 1998; Poindexter et al. 2001). Despite differences in the regulation of calcium cycling between freshly isolated and cultured adult cardiomyocytes, monolayer cells are able to follow pacing by electrical field stimulation as we have demonstrated here. Indeed, long term cultured ARVM displayed a fairly substantial sarcoplasmic network in vicinity of myofibrils, when examined at the ultrastructural level of resolution, and expressed Ca^{2+}-handling proteins in a sarcomeric distribution pattern. The cells exhibited a frequency-dependent increase of contractility and responded to beta-adrenergic stimulation. These findings show, that long-term cultured ARVM display a contractile kinetic behavior qualitatively similar to the freshly isolated cardiomyocytes. Thus, we used this model to study how cytoskeleton disorganization correlates with function in the model of anthracycline-induced cardiotoxicity in vitro. Similarly as BDM, Doxo shifted the relaxation trajectory to the right, which can be considered a crude index of the force developed by the cardiomyocytes at the level of the cross bridge interaction. Systolic calcium was increased by Doxo suggesting a compensatory mechanism, which augments calcium fluxes in order to overcome the decrease in efficiency of the contractile apparatus. This phenomenon occurred early and at concentrations of Doxo sufficient to increase the amount of the autophagosome marker LC3-II, but not to induce DNA degradation. The removal of damaged organelles is assumed to happen by macro-autophagy in mammalian cells (Gustafsson and Gottlieb 2009). The here observed increase of LC3-II in Doxo-treated cardiomyocytes indicates the formation of an increased number of autophagosomes already at lower doses of Doxo. At present, there is no clear consensus in the literature to which extent this pathway of organelle degradation

contributes to cardiac survival or to even further deterioration (Rothermel and Hill 2007). The absence of cell death under these conditions supports the notion that this type of contractile dysfunction is potentially reversible, although we cannot exclude, that the accumulation of degraded proteins and damage to genetic material might lead to cell death at a later time point.

We have assessed a possible reversibility of drug effects on myofibrillar organization in a series of experiments applying Doxo for a comparably short time and washing out the drug for 7 days. There seems to be only a limited capacity of the cells to regenerate myofibrils after Doxo-induced injury. However, the isolated cells in the in vitro model have a limited lifespan and some properties of Doxo such as DNA intercalation and aggregation in mitochondria make it difficult to model in vitro the recovery of the myocardium after anthracycline-induced injury.

We and others have observed strictly dose–dependent effects of Doxo on cellular injury in isolated adult cardiomyocytes with changes in gene expression and enzyme activity at low concentrations, but DNA degradation and loss of membrane integrity only at very high concentrations. It is therefore reasonable to conclude, that the multiple mechanisms of action that have been ascribed to the anthracyclines may be related to the utilization of different drug concentrations under varied experimental conditions. We propose that repeated and perpetual exposure to potentially cardiotoxic drugs forming reactive oxygen species leads to a sequential progression of myofibril disorganization, uncoupling of excitation–contraction and contractile failure in individual cells. At a later stage, the cumulative cardiac injury may reach an irreversible state leading to loss of cardiomyocytes. Finally, the analysis of contractile properties and myofibrillar organization of long-term cultured adult cardiomyocytes may be a useful tool to address clinically relevant mechanisms of cardiotoxicity.

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