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CaMKII-dependent SR Ca leak contributes to doxorubicin-induced impaired Ca handling in isolated cardiac myocytes

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Abstract

Objective—Doxorubicin (DOX) is one of the most effective chemotherapeutic agents, but cardiotoxicity limits DOX therapy. Although the mechanisms are not entirely understood, reactive oxygen species (ROS) appear to be involved in DOX cardiotoxicity. Ca/calmodulin dependent protein kinase II (CaMKII) can be activated by ROS through oxidation and is known to contribute to myocardial dysfunction through Ca leakage from the sarcoplasmic reticulum (SR).

Rationale—We hypothesized that CaMKII contributes to DOX-induced defects in intracellular Ca ([Ca]_i) handling.

Methods—Cardiac myocytes were isolated from wild-type (WT) adult rat hearts and from mouse hearts lacking the predominant myocardial CaMKII isoform (CaMKIIδ^{-/-}, KO) vs. WT. Isolated cardiomyocytes were investigated 30 min after DOX (10 μmol/L) superfusion, using epifluorescence and confocal microscopy. Intracellular ROS-generation ([ROS]_i) and [Ca]_i handling properties were assessed. In a subset of experiments, KN-93 or AIP (each 1 μmol/L) were used to inhibit CaMKII. Melatonin (Mel, 100 μmol/L) served as ROS-scavenger. Western blots were performed to determine the amount of CaMKII phosphorylation and oxidation.

Results—DOX increased [ROS]_i and led to significant diastolic [Ca]_i overload in rat myocytes. This was associated with reduced [Ca]_i transients, a 5.8-fold increased diastolic SR Ca leak and diminished SR Ca content. ROS-scavenging partially rescued Ca handling. Western blots revealed increased CaMKII phosphorylation, but not CaMKII oxidation after DOX. Pharmacological CaMKII inhibition attenuated diastolic [Ca]_i overload after DOX superfusion and led to partially restored [Ca]_i transients and SR Ca content, presumably due to reduced Ca spark frequency. In

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Disclosures

None

line with this concept, isoform-specific CaMKII δ -KO attenuated diastolic $[Ca]_i$ overload and Ca spark frequency.

Conclusions—DOX exposure induces CaMKII-dependent SR Ca leakage, which partially contributes to impaired cellular $[Ca]_i$ homeostasis. Pharmacological and genetic CaMKII inhibition attenuated but did not completely abolish the effects of DOX on $[Ca]_i$. In light of the clinical relevance of DOX, further investigations seem appropriate to determine if CaMKII inhibition could reduce DOX-induced cardiotoxicity.

Introduction

The anthracycline doxorubicin (DOX) is one of the most potent and frequently used chemotherapeutic agents in modern oncology [1–3]. DOX is used to treat various cancers, including sarcoma, breast, ovary and lung cancer, as well as lymphoma and leukemia [4]. Unfortunately, the clinical use of this highly effective substance is limited by its cardiotoxic side effects, such as arrhythmias and acute and late-onset toxic cardiomyopathy [1,5–7]. Recent epidemiologic studies in childhood cancer survivors reveal that even low level DOX treatment (<250 mg/m²) are sufficient to induce toxic cardiomyopathy [8]. In contrast to the clinical need to develop potent pharmacological protectors against DOX-dependent cardiomyopathy, the underlying pathological mechanisms are not entirely understood. Among others, induction of apoptosis, cross-linking of DNA, inhibition of topoisomerase II as well as impaired $[Ca]_i$ handling have been discussed [2,9]. DOX is well-known to induce direct drug-dependent Ca loss from the sarcoplasmic reticulum (SR) [9]. However, whether other modulators of SR Ca release might further amplify DOX-dependent SR Ca release has not been completely answered.

Reactive oxygen species (ROS) are critically involved in the development of the cardiotoxic side effects of DOX [10–13]. ROS are defined as highly reactive oxygen-based chemical compounds, such as hydrogen peroxide or superoxide anion, which can disturb cellular homeostasis [14]. In higher concentrations, they are known to induce apoptosis, but on a lower level, they function as a cellular second messenger, interfering with $[Ca]_i$ handling of cardiomyocytes [15,16]. For example, ROS can oxidate thiol groups of cysteines forming disulfide bridges, which can then alter the function of cardiac ion channels, transporters, and Ca pumps [15,17–20].

It has recently been shown that the Ca/calmodulin-dependent protein kinase II (CaMKII) can be activated by oxidation of methionine residues at positions 281 and 282. As a consequence, activation of CaMKII is prolonged, regardless of the concentration of the Ca/calmodulin complex [21]. ROS-dependent modification of CaMKII activity is most likely to have a great impact on cardiac myocyte (patho-)physiology, since CaMKII, a multifunctional serine/threonine kinase, acts as central signal in $[Ca]_i$ handling of myocytes [22]. Among its many targets are the sarcoplasmic reticulum (SR) Ca ATPase (SERCA2a), phospholamban (PLB), the L-type Ca channel (LTCC) [23], and the cardiac ryanodine receptor (RyR2) [22]. Elevated CaMKII activity contributes to cardiac hypertrophy and heart failure [24]. CaMKII-dependent diastolic SR Ca loss is favored by RyR2 hyperphosphorylation that causes decreased SR Ca content and impaired contractility [25]. In light of the recently appreciated link between CaMKII activation and elevated ROS [21] an involvement of CaMKII in the pathogenesis of DOX-induced cardiac failure seems plausible. Our study aims to investigate the basic role of CaMKII in acute effects of DOX on cardiomyocytes.

Materials and Methods

Cardiac myocyte isolation

We isolated single ventricular cardiac myocytes from rat and mouse hearts. For the former, adult 6 months old wildtype Wistar rats were anaesthetized with isoflurane (Abbott, Wiesbaden, Germany). Afterwards, hearts were carefully excised and mounted on a Langendorff-perfusion apparatus, which was retrogradely perfused with an isolation solution containing 1.25 mmol/L Ca to proof vitality, and subsequently perfused for 20 min with nominal Ca free solution. Next, the heart was perfused with an enzyme solution containing (in mmol/L) NaCl 137, KCl 5.4, $\text{MgSO}_4 \times 7 \text{H}_2\text{O}$ 1.2, $\text{Na}_2\text{HPO}_4 \times 12 \text{H}_2\text{O}$ 1.2, glucose 15 (all Merck, Darmstadt, Germany), HEPES 20 (Sigma Aldrich, Munich, Germany), collagenase (230 U/mL, Biochrom AG, Berlin, Germany) and proteinase (0.5 U/mL, Sigma Aldrich) as well as Ca (0.1 mmol/L, Fluka, Munich, Germany). After digesting the extracellular matrix up to the point of visible tissue solution, the heart was transferred into a glass basin and the atria were carefully separated from the ventricles and discarded. Subsequently, the ventricles were cut with preparatory scissors into small pieces of tissue. The cell suspension was shortly slewed in a water quench and afterwards filtered with a nylon gaze. After sedimentation of the tissue, the enzyme solution was removed and replaced with 10 mL M199 medium (Sigma Aldrich), taurine 62.5 mg (Sigma Aldrich), D,L-carnitine 10 g (Sigma Aldrich), creatinine 65.5 mg (Sigma Aldrich), 100 $\times$ pen/strep 0.1 mL (Biochrom AG), 100 $\times$ L-glutamine 0.1 mL (Biochrom AG). Mouse cardiac myocytes isolation was performed similarly. Hearts were carefully excised from mice specifically lacking the CaMKII δ isoform (CaMKII δ -KO) [26]. Matching WT littermates served as control. Perfusion solution for mice contained (in mM) NaCl 113, KCl 4.7, KH_2PO_4 0.6, $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$ 0.6, $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ 1.2, NaHCO_3 12, KHCO_3 10, HEPES 10, Taurine 30, 2,3-butanedione 10, glucose 5.5 and phenol red 0.032 and was perfused for 4 minutes at 37°C (pH 7.4) via a Langendorff apparatus as described previously [26,27]. Then, 7.5 mg/mL liberase 1 (Roche Diagnostics, Mannheim, Germany), trypsin 0.6%, and 0.125 mmol/L CaCl_2 were added to the perfusion solution for another 3 min. After similar preparation of the mouse hearts as it has been described for rat hearts, Ca reintroduction was performed carefully through stepwise increasing [Ca] from 0.1 to 0.8 mmol/L.

Assessment of Ca handling properties and [ROS] using epifluorescence microscopy

As previously reported [27,28], we used a Nikon Eclipse TE2000-U inverted microscope which was provided with an fluorescence detection system for assessment of Ca handling properties and ([ROS]_i, ION OPTIX, Milton, MA, USA). In terms of ROS detection, myocytes were loaded with 10 $\mu\text{mol/L}$ of the ROS sensitive fluorescent dye CM-H₂DCFDA [14] (Invitrogen, Carlsbad, CA, USA) for 20 min in darkness and excited at 485 $\pm$ 15 nm. Emitted fluorescence was collected at 535 $\pm$ 20 nm for only 10 s every min to prevent photooxidation of the dye. For Ca measurements, isolated cardiac myocytes were loaded with Fura-2 AM (Invitrogen) at 10 $\mu\text{mol/L}$ and incubated for 15 min at room temperature in darkness. Cells were excited at 340 nm and 380 nm and the emitted fluorescence was collected at 510 nm. Ca transient amplitude was assessed as the 340/380 nm fluorescence ratio (F_{340nm}/F_{380nm} in ratio units, r.u.) after subtraction of background fluorescence at each excitation wavelength [28]. Myocytes were field-stimulated at 20 $\pm$ 4 V at 1 Hz for ~5 min until steady-state conditions were achieved. Myocytes were superfused with an experimental solution containing (in mmol/L) NaCl 140, KCl 4, glucose 10, HEPES 5, MgCl_2 1, CaCl_2 1.2 (rat) or 1 (mouse) at 37°C (pH 7.4). Red light transillumination was used to visualize myocytes sarcomeres. SR Ca load was evaluated by Ca transient amplitudes induced by rapid application of caffeine (10 mmol/L, Sigma Aldrich) [29]. The “caffeine-spritz” was applied after pausing field stimulation for exactly 1 s using a 21 g needle which was positioned directly next to the cell of interest. Upon continuous

superfusion of caffeine, SR Ca storage function is blocked and Na/Ca-exchanger (NCX) is mainly responsible for Ca elimination (by 93%) [29].

Assessment of Ca spark frequency using confocal microscopy

Fluo-4 (10 $\mu\text{mol/L}$, Invitrogen) loaded rat cardiac myocytes were mounted on a laser scanning confocal microscope (Zeiss LSM 5 PASCAL, Göttingen, Germany) to assess spontaneous diastolic Ca release events from the SR (Ca sparks) [25,28]. Myocytes were excited via an argon laser (at 488 nm) and emitted fluorescence was collected at 505 nm through a long-pass emission filter. Cells were superfused with normal Tyrode's solution (see above) and stimulated at 0.5 Hz. Ca sparks were analyzed with the use of Zeiss software.

Scavenging ROS and pharmacological inhibition of CaMKII

For some studies we used the ROS scavenger drug melatonin (MEL, Sigma Aldrich, Germany) at a concentration of 100 $\mu\text{mol/L}$ [30]. CaMKII-inhibition was ensured by incubating the cells for 15 min with the CaMKII inhibitors KN-93 or the control agent KN-92 (both at 1 $\mu\text{mol/L}$) [31] or autocamtide-2 related inhibitory peptide (AIP, 1 $\mu\text{mol/L}$) [25].

Assessment of CaMKII protein expression and phosphorylation

CaMKII protein expression was detected using antibodies, which were kindly provided by Dr. Bers (UCD, USA). CaMKII phosphorylation levels were detected using a phosphospecific CaMKII-antibody from Affinity BioReagents (Golden, CO, USA). Oxidized CaMKII was detected using an immune serum against oxidized M281/M282 of CaMKII (ox-CaMKII) [21].

Statistics

Data are presented as mean $\pm$ S.E.M. Student's t-test was used for Fig. 2B, 3B, 4B&C, 5B–D, 6B&D, 7C, 8B, 9B and 10B&C as wells as for Table 1 and 2. Two-way analysis of variance (ANOVA) for repeated measurements combined with Student-Newman-Keuls post hoc test were performed for Fig. 1, 2C and 7D. Values of $P<0.05$ were considered as statistically significant.

Results

DOX exposure increases $[\text{ROS}]_i$ in isolated cardiac myocytes

Fig. 1 shows an increasing ROS-fluorescence in DOX-treated myocytes as compared to vehicle-treated control myocytes. While it is notable that there is no significant difference in $[\text{ROS}]_i$ at the starting point of the experiment between both groups ($P=\text{n.s.}$), DOX (10 $\mu\text{mol/L}$, $n=6$) increased $[\text{ROS}]_i$ by 97.0 ± 2.8 r.u./min, as compared to 66.5 ± 10.5 r.u./min in control group ($n=5$) over a ~25 min experimental time. These data suggest increased myocyte ROS is generated by DOX in our model.

DOX decreases $[\text{Ca}]_i$ transient amplitudes and induces diastolic $[\text{Ca}]_i$ overload

Fig. 2A shows original $[\text{Ca}]_i$ transients, which were obtained upon the first 15 min of DOX exposure. While control cells revealed only a slight decrease in $[\text{Ca}]_i$ transient amplitudes, DOX treated cells showed dramatically decreased $[\text{Ca}]_i$ transient amplitudes and increased diastolic $[\text{Ca}]_i$ levels. Average data show significantly decreased $[\text{Ca}]_i$ transients after 30 min of DOX exposition (from 0.21 ± 0.02 , $n=9$, to 0.12 ± 0.01 r.u., $n=5$, Fig. 2B). In contrast, $[\text{Ca}]_i$ transients in control cells were not significantly decreased ($n=8$).

Diastolic $[Ca]_i$ rose in a time dependent manner ($P<0.05$, Ctrl $n=8$ vs DOX $n=9$, Fig. 2C). While diastolic $[Ca]_i$ levels were almost unchanged in the untreated control group (0.01 ± 0.01 r.u.), we found significantly increased diastolic $[Ca]_i$ levels after 30 min DOX exposure (0.07 ± 0.03 r.u.). Despite dramatically elevated diastolic $[Ca]_i$ levels, $[Ca]_i$ transient decline was not affected by DOX. Based on these findings we hypothesized that increased SR Ca release caused elevated diastolic $[Ca]_i$ levels [9], a concept we examined as a next step.

DOX enhances diastolic SR Ca loss and decreases SR Ca content

Fig. 3A shows original Ca spark images obtained in control and DOX treated cells. Average data in Fig. 3B confirm a dramatically elevated Ca spark frequency upon DOX exposure, which was elevated by ~5.8-fold from 0.13 ± 0.07 sparks per $100\ \mu\text{m}^{-1}\text{s}^{-1}$ in the control group ($n=41$) to 0.75 ± 0.20 sparks per $100\ \mu\text{m}^{-1}\text{s}^{-1}$ upon $10\ \mu\text{mol/L}$ DOX treatment ($n=34$, $P<0.05$ vs. Ctrl). While Ca spark amplitude and Ca spark duration were not significantly different between groups (Table 1), SR Ca loss, as calculated by the product of Ca spark frequency, amplitude, duration, and width was elevated from 100 ± 50 under control conditions to 536 ± 164 upon DOX demonstrating enhanced SR Ca loss by DOX.

Our data showing increased SR Ca loss by DOX are consistent with findings by Abramson et al. [9], SR Ca content was reduced to 57% in the presence of DOX ($P<0.05$, Fig. 4A&C). We interpreted the caffeine-induced decrease in the $[Ca]_i$ transient to suggest that NCX-function was accelerated (Table 2, $P<0.05$), while the tendency for increased fractional release of SR Ca, from $44.5\pm8.4\%$ to $63.7\pm10.4\%$ ($P=0.1$, Fig. 4B), to indicate a modest increase in RyR2 opening.

Melatonin attenuates impairment of Ca handling upon DOX exposure

To test whether elevated $[ROS]_i$ contribute to impaired $[Ca]_i$ handling after DOX exposure, we performed experiments using the ROS scavenger Mel. The use of Mel significantly reduced Ca spark frequency as shown in the original line scan images in Fig 5A and by the average data in Fig 5B ($P<0.05$). Decreased SR Ca loss was sufficient to partly maintain SR Ca content despite DOX exposure (Fig 5C, $P<0.05$) and resulted in attenuated diastolic $[Ca]_i$ (to $104\pm3\%$ in Mel treated group, $n=8$ as compared to $116\pm3\%$ in the untreated group, $n=7$, $P<0.05$, Fig 5D) These findings point to a role for ROS in the DOX-dependent effects on $[Ca]_i$ handling.

CaMKII phosphorylation is elevated after 30 min of DOX treatment

CaMKII is a typical mediator of enhanced SR Ca leak and was recently shown to become modified by elevated ROS levels in addition to its well-known Ca-dependency [21]. Therefore, CaMKII might further amplify the effects of DOX on cardiac Ca handling. Indeed, Western blot analysis revealed an elevated phosphorylation status after 30 min of DOX exposure (Fig. 6A) by $59\pm12\%$ compared to Ctrl ($n=6$ each, $P<0.05$, Fig. 6B). The data was normalized to CaMKII expression. Interestingly, ox-CaMKII was slightly, but not significantly increased ($P=0.16$, Fig. 6C&D).

CaMKII inhibition protects against loss of $[Ca]_i$ homeostasis by DOX

Since we found CaMKII to be activated upon DOX treatment, we tested whether CaMKII inhibition might attenuate the detrimental effects of DOX on $[Ca]_i$ handling. Fig. 7A depicts original epifluorescence traces in the presence of the CaMKII inhibitor KN-93 and its inactive analogue KN-92 [31]. In the KN-92 control group, $[Ca]_i$ transient amplitudes decreased in step with diastolic $[Ca]_i$ overload, similar to the effects of DOX alone. However, CaMKII inhibition using KN-93 induced a partial maintenance of $[Ca]_i$ transients

and significantly attenuated diastolic $[Ca]_i$ overload. Summary data revealed an attenuated increase in diastolic $[Ca]_i$ levels in CaMKII inhibited cells as compared to control group ($P<0.05$, Fig. 7D).

The $[Ca]_i$ transient amplitudes (Fig. 7C) were significantly decreased in DOX-exposed cells treated with KN-92 after 30 min of DOX exposure, similar to the effect of DOX alone (from 0.22 ± 0.03 r.u., $n=10$, to 0.05 ± 0.01 r.u., $n=6$). In CaMKII inhibited cells, however, this effect could be significantly attenuated despite DOX exposure. The $[Ca]_i$ transient amplitudes were slightly decreased after 30 min of DOX exposure from 0.16 ± 0.02 to 0.11 ± 0.04 ($n=8$). $[Ca]_i$ transient relaxation kinetics were unaffected by CaMKII-inhibition ($P=n.s.$, data not shown).

CaMKII inhibition reduces Ca spark frequency and partially restores SR Ca content after DOX

While SR Ca reuptake was unaffected by CaMKII-inhibition after DOX exposure, the line scanning images show decreased SR Ca release, as measured by reduced Ca spark occurrence after CaMKII inhibition (using AIP, Fig. 8A). Summary data (Fig. 8B) showed a reduced Ca spark frequency to only 0.42 ± 0.12 sparks per $100\ \mu\text{m}^{-1}\text{s}^{-1}$ (DOX+AIP) as compared to 0.85 ± 0.14 sparks per $100\ \mu\text{m}^{-1}\text{s}^{-1}$ in the group treated solely with DOX ($n=55$ vs. $n=72$ respectively, $P<0.05$).

CaMKII inhibition partially maintained SR Ca content whereas NCX-function was still enhanced. While CaMKII inhibition reduced SR Ca loss to 49% (Fig. 8), it increased SR Ca content by 168% as compared to the control group (Fig. 9A). Summary data in Fig. 9B confirm SR Ca content to be significantly elevated in the KN-93 treated group (0.27 ± 0.04 r.u. vs 0.1 ± 0.01 r.u. in the group treated with KN-92, $P<0.05$). However, it should be noted that impaired Ca handling (Fig. 8B) was not abolished in CaMKII-inhibited cells, because Ca spark frequency was still increased by 3.2-fold as compared to control conditions. This may point to the pathophysiological relevance of CaMKII-independent DOX effects such as direct DOX- or ROS-dependent SR Ca release [9,15,17].

Diastolic $[Ca]_i$ overload is attenuated in CaMKII δ -KO myocytes

To further test our hypothesis that CaMKII is involved in impaired $[Ca]_i$ handling upon DOX exposure, we used CaMKII $\delta^{-/-}$ mice. First, we confirmed that cardiac myocytes from WT mouse hearts also showed diastolic $[Ca]_i$ overload upon DOX-exposure. We found that diastolic $[Ca]_i$ is also time-dependently increased following 12 min of DOX treatment ($P<0.05$ using One-Way-ANOVA, data not shown), indicating that this phenomenon is similar in mouse and rat cardiac myocytes. Interestingly, myocytes lacking the CaMKII δ isoform [26] showed significantly reduced SR Ca loss (Fig 10 A) to only 0.11 ± 0.03 sparks per $100\ \mu\text{m}^{-1}\text{s}^{-1}$, $n=196$ as compared to 0.31 ± 0.06 sparks per $100\ \mu\text{m}^{-1}\text{s}^{-1}$, $n=193$ in the control group (Fig. 10B, $P<0.05$). The reduced frequency of Ca sparks in CaMKII $\delta^{-/-}$ myocytes resulted in attenuated diastolic $[Ca]_i$ overloading after 12 min of DOX exposure (down to $108\pm3\%$, $n=9$ vs. $124\pm5\%$, $n=7$ in WT control animals, Fig 10C, $P<0.05$). These results suggest that CaMKII amplifies DOX-dependent SR Ca release.

Discussion

The current study confirms the detrimental effects of DOX on cardiac Ca handling and identifies CaMKII-dependent SR Ca leak as an important contributor to DOX-induced loss of $[Ca]_i$ homeostasis. CaMKII inhibition leads to attenuated SR Ca leak and reduction of the detrimental effects of DOX on intracellular Ca cycling. Therefore, it is possible that CaMKII inhibition may be beneficial in the setting of DOX-induced cardiomyopathy.

DOX increases ROS and impairs cardiac ECC via increased SR Ca leak

We observed a severe impairment of cardiac ECC after DOX treatment, as previously shown [10,11], DOX induced diastolic $[Ca]_i$ overload in a time-dependent manner and decreased intracellular $[Ca]_i$ transient amplitudes, both of which could contribute to clinically observed heart failure [1,5–7]. SR Ca content was dramatically decreased upon DOX exposure, which can be caused by either i.) decreased SR Ca reuptake or ii.) increased SR Ca release. The former seems unlikely, since we failed to detect a prolongation of relaxation in our model, in contrast to previously published studies [10]. Of note, Ca elimination during diastole occurs mostly, but not exclusively via SR Ca reuptake. In fact, trans-sarcolemmal Ca extrusion also plays an important role [29]. We observed increased NCX function upon DOX exposure, which would tend to accelerate Ca elimination and might have compensated for decreased SR Ca reuptake. However, in rat cardiomyocytes, Ca elimination during diastole mainly depends on SR Ca reuptake and only to a small extent on trans-sarcolemmal extrusion via NCX [29,32]. Thus, in our model, NCX function should be of rather minor importance for Ca elimination. Consequently, we presumed that the unimpaired relaxation in our rat cardiac myocytes reflects preserved SR Ca reuptake.

Increased SR Ca loss could be causal for SR Ca depletion by DOX, and previous work by Abramson et al. strongly support this assumption [9]. Indeed, a ~5.8 fold increase in Ca spark frequency could be detected despite depressed SR Ca content, which under physiological circumstances would cause reduced Ca spark frequency [29]. Our current results therefore also point to a dramatically increased SR Ca leak upon DOX-exposure, which has been previously suspected to cause cardiac myocyte malfunction [25,33,34].

It is important to point out that DOX was reported to increase $[ROS]_i$ [2,10–13]. Consistent with this concept, we observed a strong and continuous increase in $[ROS]_i$ upon DOX-exposure. However, oxidation of CM-H₂DCFDA by light can lead to an overestimation of $[ROS]_i$. Therefore, we confirmed the relevance of elevated $[ROS]_i$ upon DOX by reinvestigating Ca handling using the ROS scavenger drug Mel [30]. Indeed, using Mel, we found Ca handling to be partly maintained despite DOX. This finding suggests the generation of ROS upon DOX exposure.

Elevated oxidative stress increases the probability that ROS-sensitive proteins are likely to be affected. For instance, ROS can activate CaMKII [21]. CaMKII is a central signal in cardiac Ca handling and a typical promoter of diastolic SR Ca leak [24,25,28,35]. CaMKII activation is further favored by elevated diastolic Ca levels. Therefore, we examined CaMKII activation upon DOX treatment in a next step.

DOX increases CaMKII phosphorylation while CaMKII inhibition partially improves Ca handling properties upon DOX exposition

CaMKII is crucial for cardiac excitation-contraction coupling [22]. Among its targets are sarcolemmal ion channels such as the LTCC [35] or the Na-channel [36] but also SR associated proteins, including SR Ca reuptake regulating PLB [27] as well as the RyR2, which are responsible for SR Ca release [37]. From a pathophysiological point of view, CaMKII-dependent RyR2 modification and subsequent CaMKII-dependent SR Ca release are highly relevant because RyR2 hyperphosphorylation causes diastolic Ca loss from the SR, which then in turn impairs Ca transients and contractility. In our DOX-model with elevated oxidative stress and diastolic Ca overload, CaMKII-dependent effects on the RyR2 are likely to be enhanced [21]. Indeed, Western blots detecting phosphorylated and oxidized CaMKII suggest CaMKII can become activated. However, since we failed to detect a significantly increased oxidized CaMKII (at least after 30 min DOX exposition) but found clearly increased CaMKII phosphorylation, a Ca-dependent activation of CaMKII seems to

be the initial trigger for CaMKII activation upon DOX. Regarding our current results, significantly elevated ROS-levels were not found within the first 15 minutes of DOX exposure. This might imply that other, Ca-dependent mechanisms might have caused CaMKII activation during that initial phase. One potential mechanism by which CaMKII could have been Ca-dependently activated might have been the Ca release from the SR upon direct interaction of DOX with RyR2 [9]. Unpublished data from our lab using the lipid bilayer technique strongly support a greatly enhanced open probability of RyR2 in case of DOX presence (already at 1 $\mu\text{mol/L}$). This direct, DOX-dependent SR Ca release [9] might therefore have caused an initial Ca-dependent CaMKII-activation (leading to increased CaMKII phosphorylation). Our data showing increased phosphorylation but not oxidation after 30 min of DOX exposure would favor an activation cascade based on CaMKII autophosphorylation. However, CaMKII-phosphorylation then appears to be followed by additional oxidation, which may further amplify CaMKII-activation.

CaMKII-activation after DOX amplified detrimental effects on cardiac Ca handling. CaMKII inhibition was sufficient to partially restore SR Ca content and maintain $[\text{Ca}]_i$ transient amplitudes. Surprisingly, while CaMKII inhibition is generally associated with slowed relaxation [27] due to a reduced phosphorylation status of PLB, we failed to detect significant effects on relaxation by CaMKII inhibition in our DOX model. It is therefore unlikely that increased SR Ca reuptake is causal for restored SR Ca content upon CaMKII inhibition. Consequently, we interpret the data and literature taken together to suggest that reduced SR Ca leak to be the main cause for partial CaMKII-mediated rescue of $[\text{Ca}]_i$ handling. The SR Ca leak could be reduced but not diminished upon CaMKII-inhibition (still increased by 3.2-fold), which likely is due to direct effects of DOX itself or ROS on Ca handling proteins such as the RyR2 [9,15,17]. Nevertheless, CaMKII inhibition was able to attenuate diastolic Ca overload. These findings suggest that CaMKII dependent effects on RyR may represent an additional amplifier of DOX effects, which should be carefully considered in addition to the well-known effects of ROS and DOX directly on RyR2 [9,17].

This assumption might be further stressed by our finding that cardiac myocytes, isoform-specifically lacking CaMKII δ [26], also showed reduced SR Ca loss resulting in attenuated diastolic Ca overload upon DOX. Moreover, it may point to a potential protective role for CaMKII-inhibition upon DOX exposure. It is interesting to consider that CaMKII expression is increased in animal and human heart failure [25]. Therefore detrimental DOX effects are likely to become even more relevant in diseased hearts where CaMKII inhibition is known to be beneficial [21,25,26,28, 38–40].

Pathophysiological implications, perspectives and limitations

DOX is a highly effective chemotherapeutic agent that is an active member of the pharmacopeia [1,3]. So far, neither an effective nor a causal therapy of its cardiotoxic side effects could be identified [2,5]. This might be due to the fact that the pathological mechanisms remain unclear. In our current project we have studied the acute effects of DOX on Ca handling and CaMKII activation. It is important to state that there is some clinical evidence in the literature for early-onset DOX-induced cardiomyopathy. For instance, Krischer et al. found in a pediatric study population (the Pediatric Oncology Group), that cardiac impairment became clinically apparent within less than one year in almost 90% of all cases of the children affected by the cardiotoxic side effects in the long-run, developing e.g. congestive heart failure [6]. Ganame et al. observed children under anthracycline therapy had echocardiographically confirmed reduction in left ventricular function within two hours after DOX [41]. These reports highlight the need to clarify the acute molecular mechanisms leading to acute DOX-dependent cardiac failure. Eschenhagen et al. pointed out that the consequences of an anthracycline therapy are still a serious, perhaps underappreciated issue in modern cardiology [42]. Mulrooney et al. could show that

especially childhood cancer survivors are affected by this severe side effect [8]. From a clinical point of view, this group is particularly interesting because its outcome could be largely improved over the last decades [43], but is now affected by lethal cardiac side effects. Therefore, these patients would substantially profit from a causal protective therapeutic approach. Future studies are warranted to clarify the molecular mechanisms underlying DOX induced cardiac failure to eventually find a causal therapy for affected patients.

Conclusions

In conclusion, the current study shows a severe impairment of cellular Ca homeostasis upon DOX and identifies a CaMKII-dependent SR Ca leak to contribute to these adverse effects in addition to direct effects of DOX on SR Ca leak. Most importantly, CaMKII inhibition (either pharmacologically or genetically) partly reduced the effects of DOX on diastolic SR Ca loss and therefore improved $[Ca]_i$ handling to some extent. CaMKII-inhibition might be beneficial in anthracycline-induced heart failure. In light of the clinical relevance of this subject, further investigations seem appropriate.

Highlights

- The chemotherapeutic agent doxorubicin (DOX) is known for its cardiotoxicity.
- Reactive oxygen species (ROS) appear to be involved.
- Ca/calmodulin dependent kinase II (CaMKII) can be activated by Ca and ROS.
- CaMKII contributes to DOX-induced SR Ca leak and disturbed myocyte Ca handling.
- CaMKII inhibition offers a therapeutic approach against DOX-induced cardiotoxicity.

Non-standard Abbreviations and Acronyms

a.u.	arbitrary units
CaMKII	Ca/calmodulin-dependent protein kinase II
Ctrl	control
DOX	doxorubicin
ECC	excitation-contraction coupling
KO	CaMKII δ -KO
LTCC	L-type Ca channels
Mel	Melatonin
NCX	Na/Ca exchanger
PLB	phospholamban
ROS	reactive oxygen species
RyR2	ryanodine receptor type 2 (SR Ca release channel)
SERCA2a	sarcoplasmic reticulum Ca ATPase
SR	sarcoplasmic reticulum

r.u. ratio units (F 340 nm/ F380 nm)

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Fig. 1. ROS concentration is elevated upon DOX treatment

Using the fluorescent dye CM-H₂DCFDA, oxidation-sensitive fluorescence was significantly elevated upon DOX exposition at a concentration of 10 $\mu\text{mol/L}$ (n=6, black squares) compared to Ctrl group (n=5, white squares). # indicates significance vs. Ctrl. using Student-Newman-Keuls post hoc test.

Fig. 2. DOX exposure decreases Ca transient amplitudes and induces Ca overload

Fig. 2A shows decreased Ca transient amplitudes upon DOX exposition (10 $\mu\text{mol/L}$) in line with elevated diastolic Ca over a time period of 15 min. Average data for B: Ca transient

amplitudes and C: diastolic Ca. * indicates significance using paired t-test, # indicates significance vs. Ctrl. using Student-Newman-Keuls post hoc test.

Fig. 3. DOX exposure induces diastolic SR Ca leakage

A: Diastolic spark frequency is elevated by ~5.8 fold as compared to Ctrl during DOX treatment (10 $\mu\text{mol/L}$). B: Average data for A. *indicates significance using student's t-test.

Fig. 4. DOX treatment decreases SR Ca content

Fig. 4A demonstrates reduced SR Ca content after 30 min of DOX exposure. Average data for B: fractional release and C: SR Ca content. *indicates significance using student's t-test.

Fig. 5. ROS scavenging attenuates SR Ca loss upon DOX

Fig. 5A shows original line scans demonstrating decreased SR Ca loss in case of ROS scavenging using 100μmol/L Melatonin (right panel). Average data for B: Ca spark frequency, C: SR Ca content and D: diastolic Ca overloading after 30 min of DOX exposure. * indicates significance using student's t-test.

Fig. 6. DOX activates CaMKII

Fig. 6A shows original Western Blots demonstrating increased CaMKII phosphorylation status after 30 min DOX exposure (10 μ mol/L). B: Average data for A. C depicts slightly increased ox-CaMKII upon DOX exposition. D: Average data for C. * indicates significance using student's t-test.

A

B**+KN-92 upon 10 μ mol/L DOX** **+KN-93 upon 10 μ mol/L DOX**

C **D**

Fig. 7. CaMKII inhibition prevents diastolic Ca overload and maintains Ca transient amplitudes upon DOX treatment

Fig. 7A depicts original Ca recordings showing decreased Ca transient amplitudes and diastolic Ca overload upon DOX exposure (KN-92, upper panel), but not in case of CaMKII-inhibition (KN-93, lower panel). Fig. 7B shows expanded original Ca transients upon DOX exposure (upper panel) and upon simultaneous CaMKII inhibition (KN-93, lower panel). Average data for C: Ca transient amplitudes and D: Diastolic Ca levels. * indicates significance using paired t-test. # indicates significance vs. Ctrl. using Student-Newman-Keuls post hoc test.

Fig. 8. CaMKII inhibition reduces diastolic SR Ca loss upon DOX exposure

Fig. 8A shows reduced Ca spark frequency in case of CaMKII inhibition (AIP, lower panel) despite DOX exposure. Fig. 8B: Average data for A. *indicates significance using student's t-test.

Fig. 9. CaMKII inhibition restores SR Ca content despite DOX exposure

Fig. 9A shows increased SR Ca content in case of CaMKII inhibition (KN-93, lower panel) despite DOX exposure. Fig. 9B: Average data for A. *indicates significance using student's t-test.

Fig. 10. Genetic CaMKII δ -KO decreases SR Ca loss

Fig. 10 A shows decreased SR Ca spark frequency in cardiac myocytes from CaMKII δ -KO mouse. Average data for B: Ca spark frequency and C: diastolic Ca upon 12 min DOX exposure in KO myocytes (light grey) vs WT control cells. *indicates significance using student's t-test.

Tab. 1

Ca spark properties of Ca sparks obtained upon a 15 min DOX exposure period in 40 control and 34 DOX (10 μ mol/L) treated rat cardiac myocytes. Sampling frequency of the confocal microscope was ~1.3 kHz. Though Ca spark frequency is elevated upon DOX treatment, spark qualities such as spark duration and spark amplitude are unaltered.

	Spark Duration (ms)		Spark Amplitude (F/F ₀)	
	Ctrl	10 μ mol/L Dox	Ctrl	10 μ mol/L Dox
	86.80	56.22	1.20	1.33
	52.01	111.63	1.38	1.27
	72.50	45.23	1.30	1.20
	65.54	61.87	1.30	1.34
	80.65	127.31	1.30	1.24
		122.38		1.49
		99.33		2.12
		124.94		1.31
		42.61		1.25
		109.00		1.30
		100.90		1.28
		57.16		1.34
		94.44		1.25
		60.17		1.60
		24.38		1.36
		86.86		1.25
		100.83		1.36
		120.22		1.34
		43.37		1.42
Mean	71.5	83.62	1.30	1.37
SEM	6.06	7.57	0.03	0.05
t-Test		0.44		0.43

Tab. 2

τ_{Ca} of caffeine transients is shown to be reduced after 30 min of 10 $\mu\text{mol/L}$ DOX exposition in rat cardiac myocytes indicating increased NCX function.

τ_{Ca} of Caffeine Transients (NCX Function)			
	Ctrl	1 $\mu\text{mol/L}$ DOX	10 $\mu\text{mol/L}$ DOX
	2.674	3.130	0.594
	2.689	1.058	0.469
	2.625	0.926	2.272
	2.828	3.413	0.781
	3.735	2.596	2.303
Mean	2.671	2.225	1.214
SEM	0.294	0.520	0.344
t-Test			0.009