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Multiple Interference of Anthracyclines with Mitochondrial Creatine Kinases: Preferential Damage of the Cardiac Isoenzyme and Its Implications for Drug Cardiotoxicity

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ABSTRACT

Anthracyclines are among the most efficient drugs of cancer chemotherapy, but their use is limited by a significant risk of cardiotoxicity, which is still far from being understood. This study investigates whether impairment of mitochondrial creatine kinase (MtCK), a key enzyme in cellular energy metabolism, could be involved in anthracycline cardiotoxicity. We have analyzed the effects of three anthracyclines, doxorubicin, daunorubicin, and idarubicin, on two MtCK isoenzymes, sarcomeric/cardiac sMtCK and ubiquitous uMtCK, from human and chicken. Using surface plasmon resonance, gel filtration, and enzyme assays, we have quantified properties that are of basic importance for MtCK functioning in vivo: membrane binding, octameric state, and enzymatic activity. Anthracyclines significantly impaired all three properties with differences in dose-, time-, and drug-dependence. Membrane binding and enzymatic activity were already affected at low anthracycline concentrations (5–100 μM), indicating high clinical relevance. Effects on membrane binding were immediate, probably because of competitive binding of the drug to cardiolipin. In contrast, dissociation of MtCK octamers into dimers, enzymatic inactivation and cross-linking occurred only after hours to days. Different protection assays suggest that the deleterious effects were caused by oxidative damage, mainly affecting the highly susceptible MtCK cysteines, followed by generation of free oxygen radicals at higher drug concentrations. Enzymatic inactivation occurred mainly at the active site and involved Cys278, as indicated by experiments with protective agents and sMtCK mutant C278G. All anthracycline effects were significantly more pronounced for sMtCK than for uMtCK. These in vitro results suggest that sMtCK damage may play a role in anthracycline cardiotoxicity.

Anthracyclines are among the most efficient drugs of cancer chemotherapy, but a significant risk of cardiotoxicity limits their use (Olson and Mushlin, 1990; Singal et al., 1997). The molecular mechanisms of anthracycline cardiotoxicity are still far from being clear. Cardiac injury has been related to the impairment of mitochondrial functions, such as respiratory rate, and generation of high-energy phosphates. Numerous mechanisms for inactivation of the cardiac mitochondrial respiratory chain by anthracyclines have been proposed, such as generation of free radicals, interaction with mitochondrial DNA, disruption of cardiac gene expression, alteration of calcium exchange, lipid peroxidation inducing disturbance of mitochondrial membranes, and cardiomyocyte apoptosis (Singal et al., 1997; Minotti et al., 1999; Arola et al., 2000; Horenstein et al., 2000).

This study focuses on an alternative mechanism that could lead to heart energy depletion: the impairment of different key properties of mitochondrial creatine kinase (MtCK). Cells and tissues with high or fluctuating energy demands, like heart and brain, use creatine and isoenzymes of creatine kinase (CK) to cope with high ATP requirements (Wallimann et al., 1992). CK, catalyzing the reversible transphosphorylation between ATP and phosphocreatine (PCr), is able to stock the "high energy" of ATP in form of PCr and, vice versa, to use PCr to replenish cellular ATP pools. The interplay between dimeric cytosolic and mainly octameric mitochondrial CK isoenzymes, referred to as CK/PCr-circuit, represents an "energy buffer" and also provides an "energy shuttle" bridging sites of energy generation with sites of energy consumption (Wallimann et al., 1992). Two MtCK isoen-

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Some preliminary results were reported in abstract form: Tokarska-Schlattner M, Wallimann T, and Schlattner U (2000) Surface plasmon resonance studies (BiaCore) of protein-membrane interactions using mitochondrial creatine kinase. *Biophys J* **78**(Suppl):410A, and Tokarska-Schlattner M, Wallimann T and Schlattner U (2000) In vitro effect of the anthracycline drugs on MtCK isoform functions. *Eur J Cell Biol* **79**(Suppl 52):178.

ABBREVIATIONS: MtCK, mitochondrial creatine kinase; CK, creatine kinase; PCr, phosphocreatine; sMtCK, sarcomeric MtCK; uMtCK, ubiquitous MtCK; β-ME, β-mercaptoethanol; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; SPR, surface plasmon resonance; CL, cardiolipin; PC, phosphatidylcholine; TSAC, transition state analog complex; SOD, superoxide dismutase; ROS, reactive oxygen species.

zymes are expressed in a tissue-specific pattern: sarcomeric MtCK (sMtCK), restricted to heart and skeletal muscles, and ubiquitous MtCK (uMtCK), found in different organs and tissues like brain, kidney, and skin (Wyss et al., 1992). MtCK shows high affinity to the outer surface of the inner mitochondrial membrane and its major negatively charged phospholipid, cardiolipin (Cheneval et al., 1989). The enzyme is located in mitochondrial cristae and the peripheral intermembrane space, where it forms complexes with porin in the outer and adenylate translocator in the inner membrane (Schlattner et al., 1998). These complexes function in the vectorial export of energy equivalents and probably also in the regulation of the mitochondrial permeability transition involved in apoptosis. An essential prerequisite for both of these functions is the membrane-bound, octameric state of MtCK (O'Gorman et al., 1997; Khuchua et al., 1998).

Effects of anthracyclines on MtCK have never been investigated systematically. However, several lines of evidence suggest the CK/PCr system, in particular MtCK, to be important targets of anthracycline toxicity. Decreased CK activity after treatment with doxorubicin alone or in combination with horseradish peroxidase was observed with rat heart and cardiomyocyte cultures (DeAtley et al., 1999), heart homogenates, or purified cytosolic CK (Miura et al., 2000). Anthracycline treatment reduced transcriptional rate of cytosolic CK and adenylate translocator, as well as PCr levels (Olson and Mushlin, 1990; Minotti et al., 1999). Doxorubicin, exhibiting high affinity to cardiolipin, was shown to diminish binding of MtCK to this phospholipid (Cheneval et al., 1989) and, after complexation with iron, to decrease enzymatic activity of sMtCK in isolated mitochondria (Miura et al., 1994). Involvement of MtCK in anthracycline toxicity is also suggested by the fact that MtCK is a prime target of damage by peroxynitrite and oxygen radicals (Stachowiak et al., 1998; Koufen et al., 1999), which are known to be generated by anthracyclines (Olson and Mushlin, 1990; Weinstein et al., 2000). Impairment of MtCK by radicals is recognized as a factor decreasing cardiac performance in many pathologies (Wyss et al., 1992; Soboll et al., 1999).

Some earlier reports focused on specific aspects of doxorubicin-treated sMtCK of vertebrate animals. Here, we present a comprehensive, quantitative in vitro study that compares the effect of different anthracyclines (doxorubicin, daunorubicin, and idarubicin) on both types of purified human MtCK isoenzymes and includes all properties of the enzyme known to be of basic importance for its functioning in vivo: membrane binding, octameric state, and enzymatic activity (Schlattner et al., 1998). We show that all these properties of MtCK are impaired by anthracyclines and that sMtCK compared with uMtCK is the more susceptible isoenzyme because of its slightly different molecular structure (Fritz-Wolf et al., 1996; Eder et al., 2000). The data indicate a link between sMtCK damage and anthracycline cardiotoxicity.

Materials and Methods

Proteins and Chemicals. Human and chicken sMtCK, human uMtCK, and a C278G mutant of chicken sMtCK (Furter et al., 1993) were expressed in *Escherichia coli* and purified to homogeneity as described by Schlattner et al. (2000). Purified proteins were stored at −80°C at 3 to 6 mg/ml in a buffer containing 50 mM NaP_i, pH 7.0, 150 mM NaCl, 0.2 mM EDTA, and 2 mM β-mercaptoethanol (β-ME). Before use, dilutions were made in 10 mM TES, pH 7.0, containing 50 mM NaCl. Unless stated otherwise, experiments were performed at β-ME concentrations not exceeding 50 μM. Daunorubicin, idarubicin, and doxorubicin (all in hydrochloride form) were a kind gift of Pharmacia (Milan, Italy). Doxorubicin was also purchased from Aldrich (Buchs, Switzerland). Stock solutions of anthracyclines at 10 mM were prepared in water, aliquoted, and kept frozen until use. Further dilutions were made in 10 mM TES, pH 7.0, and 50 mM NaCl. Egg yolk phosphatidylcholine was from Lipid Products (South Nutfield, UK); avidin, cardiolipin, and superoxide dismutase (EC 1.15.1.1, from bovine erythrorocytes) were from Sigma (Buchs, Switzerland), and all other chemicals from Fluka (Buchs, Switzerland).

Binding Studies with Surface Plasmon Resonance (SPR). Large unilamellar vesicles (liposomes) with a diameter of approximately 160 nm were produced by a combination of freeze/thawing and extrusion techniques and checked by electron microscopy (Schlattner and Wallimann, 2000a). Liposomes containing 83.9% (w/w) phosphatidylcholine (PC), 16% (w/w) cardiolipin (CL), and 0.1% (w/w) *N*-((6-(biotinoyl)amino) hexanoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine (composition mimicking the inner mitochondrial membrane) or 99.9% (w/w) PC and 0.1% (w/w) *N*-((6-(biotinoyl)amino) hexanoyl)-1,2-dihexadecanoyl-*sn*-glycero-3-phosphoethanolamine were stored at 4°C and used within 2 days. Binding of MtCK to model lipid membranes was measured using surface plasmon resonance (SPR) spectroscopy with a BiaCore 2000 (BiaCore, Uppsala, Sweden) according to Schlattner and Wallimann (2000a,b). To evaluate the effect of anthracyclines on binding of MtCK to PC or PC/CL vesicles, a premixture of MtCK and anthracycline drug incubated for 20 min at room temperature was injected onto the liposomes immobilized on the avidin-coated CM5 chip surface. Anthracyclines influenced the SPR signal during association phase of MtCK by dramatic refractive index mismatches and additional binding to the liposomes. Therefore, MtCK binding was determined as the amount of protein that remained bound after 230 s of dissociation phase (injection of buffer). At lower anthracycline concentrations (≤100 μM), the end-dissociation SPR signal of anthracyclines was negligible compared with the MtCK signal. At higher anthracycline concentrations (>100 μM), data were corrected for the signal from bound anthracyclines, which was determined in separate SPR measurements with the drug alone using the same PC/CL vesicles. All data were corrected for background binding to avidin or PC vesicles.

Analysis of MtCK Oligomers with Gel Filtration Chromatography. The distribution of MtCK oligomeric species was determined by gel filtration chromatography with a calibrated Superose 12 column (Amersham Biosciences, Uppsala, Sweden) as described by Schlattner and Wallimann (2000b).

Enzymatic Activity. MtCK samples in volume of 100 to 200 μl at 50 μg/ml (250 μg/ml for electrophoretic studies) were preincubated with anthracyclines at room temperature in the darkness up to 7 days; as a control, MtCK was preincubated with anthracycline vehicle (buffer). If not stated otherwise, experiments were performed at β-ME concentration not exceeding 50 μM. The reverse reaction of MtCK (ATP production) was measured in a spectrophotometric assay as described by Schlattner et al. (2000) with all reagents prepared without addition of β-ME. Protectants such as β-ME (0.5–2 mM), transition state analog complex substrates (TSAC; 4 mM ADP, 5 mM $MgCl_2$, 20 mM creatine, and 50 mM KNO_3), or superoxide dismutase (SOD; 100 μg/ml) were added to MtCK samples before incubation with anthracyclines. Reactivation of MtCK activity by β-ME was studied by addition of 5 mM or 10 mM β-ME to inactivated MtCK and incubation for 15 min or 48 h as indicated in figure legends. Enzymatic activity of control samples (incubated without anthracyclines) is normalized relative to control activity at the beginning of the experiment (time 0). If not stated otherwise, enzymatic activity of anthracycline-treated samples is normalized relative to activity of control at the corresponding time point and, where applicable, incubated with the same protectant (β-ME, TSAC). Thus,

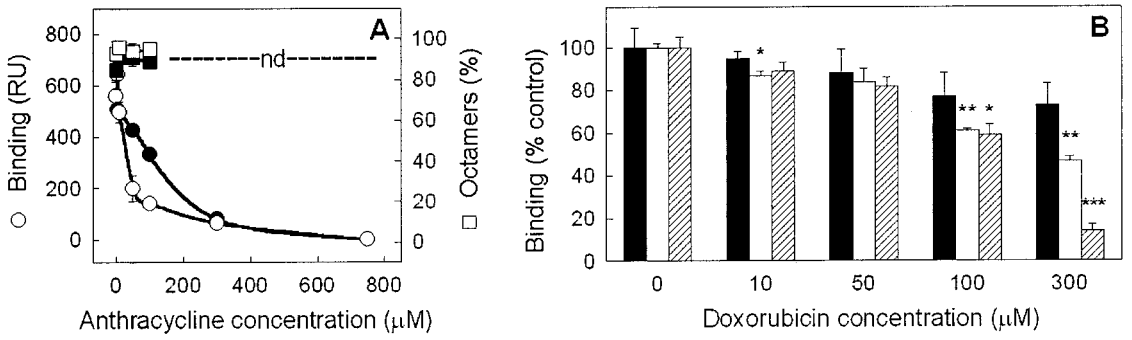

Fig. 1. Effect of doxorubicin and idarubicin on membrane binding and octameric state of MtCK. A, concentration-dependence of anthracycline effects on membrane binding (circles) and octamer content (squares) of chicken sMtCK: doxorubicin (filled symbols), idarubicin (open symbols). B, effect of doxorubicin on membrane binding of different MtCK isoenzymes: human uMtCK (■), human sMtCK (□), and chicken sMtCK (▨). MtCK was incubated with anthracycline for 20 min at room temperature and subsequently used for surface plasmon resonance spectroscopy to determine binding of MtCK to PC/CL vesicles (expressed in arbitrary response units, RU) or for gel filtration chromatography to analyze the distribution of the oligomeric species (for details, see *Materials and Methods*). Data are given as mean ± S.E.M. ($n \geq 3$). In A, the MtCK octamer content at high anthracycline concentrations could not be quantified (nd). In B, * indicates a significant difference in anthracycline-induced inhibition of membrane binding between human uMtCK and human or chicken sMtCK.

traces of control samples reveal the spontaneous inactivation of the enzyme, whereas traces of anthracycline-treated samples show exclusively activity changes caused by drug treatment.

SDS-Gel Electrophoresis. MtCK samples at 250 μg/ml were denaturated in nonreducing sample buffer. Proteins (3.2 μg/lane) were loaded onto a 7.5, 10, and 12% standard SDS polyacrylamide gel and separated at 200 V for 45 min.

Statistics. Means, SEM, and statistical probability based on Student's *t* test were calculated with Excel 97 software (Microsoft Corporation, Redmond, WA). For comparison of means, significance levels are given as *, $p < 0.05$; **, $p < 0.01$; and ***, $p < 0.001$; † is used for a second comparison.

Results

Effect of Doxorubicin and Idarubicin on MtCK/Cardiolipin Interaction. Membrane binding was quantitated by SPR spectroscopy using PC/CL vesicles mimicking the inner mitochondrial membrane compared with pure PC vesicles. Anthracyclines alone already interacted with CL (data not shown). Because the SPR signal was much higher than expected for binding of such low-molecular-weight compounds ($M_r \sim 600$), the drugs may have also induced changes in the lipid bilayer and/or the form of the vesicles. Doxorubicin bound more efficiently to CL-containing vesicles than idarubicin (end-dissociation levels 2.5–10 times higher), whereas idarubicin showed faster on and off rates than doxorubicin. Simultaneous injection of a premixture of anthracyclines and chicken sMtCK resulted in a dose-dependent inhibition of MtCK binding to PC/CL vesicles (Fig. 1A). Lineweaver-Burk analysis in the range of 12.5 to 300 μg/ml chicken sMtCK and 10 to 100 μM anthracycline revealed that inhibition is competitive with idarubicin being slightly more inhibitory than doxorubicin. This may be related to the faster on-rate of idarubicin, which allows more efficient competition with MtCK for CL when coinjected simultaneously as in our experiments. The two types of MtCK isoenzymes differed significantly at anthracycline concentrations ≥100 μM, because membrane binding of sMtCK was more inhibited compared with uMtCK (Fig. 1B).

Effect of Doxorubicin and Idarubicin on MtCK Oligomeric State. Octamers are the functional units of MtCK. Destabilization of the octamer may affect the functioning of MtCK directly and/or indirectly through the low affinity of dimeric MtCK to mitochondrial membranes (Schlattner and Wallimann, 2000a). The octamer/dimer ratio of MtCK was analyzed by gel filtration chromatography in parallel to SPR measurements. Incubation of MtCK with 5 to 100 μM doxo- or idarubicin for 20 min did not significantly change the oligomeric state of MtCK (Fig. 1A). After incubation with 300 to 750 μM anthracycline for 20 min, octamer and dimer peaks were no longer resolved and thus could not be quanti-

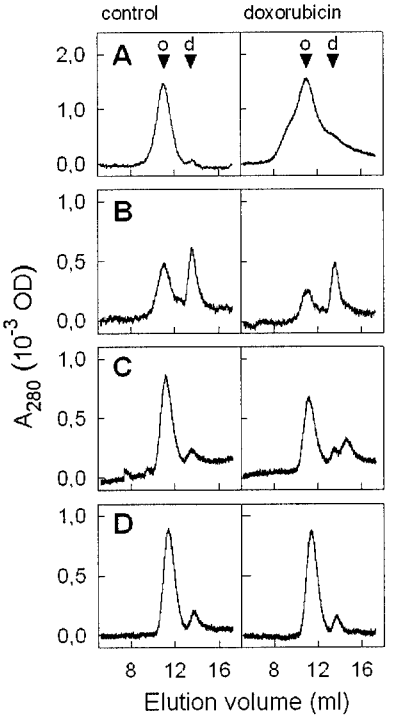

Fig. 2. Effect of doxorubicin on the oligomeric state of MtCK isoenzymes. Gel filtration chromatograms of octameric MtCK incubated without (control, left) or with doxorubicin (right). The effects of the following treatments were analyzed: incubation of chicken sMtCK with 300 μM doxorubicin for 20 min (A); incubation with 100 μM doxorubicin for 7 days of chicken sMtCK (B), human sMtCK (C), and human uMtCK (D). Oligomeric species were separated by gel filtration chromatography with running buffer lacking β-ME. The elution volume of octamers (o) and dimers (d) is indicated. Note the broad peak (A, right), indicating cross-link-products, and the small peak to the right of the dimer peak (C, right), corresponding to the MtCK monomers.

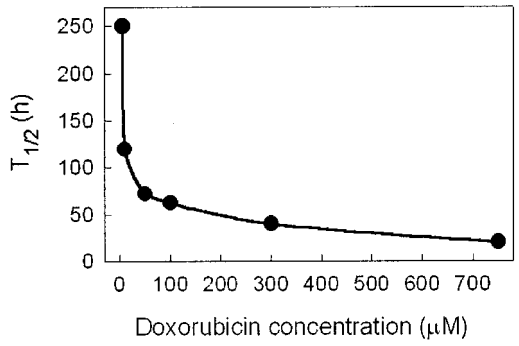

Fig. 3. Concentration-dependence of $t_{1/2}$ for enzymatic inhibition by doxorubicin. The time needed to decrease enzymatic activity of chicken sMtCK by 50% was determined for different doxorubicin concentrations. Data represent means from at least three experiments.

fied. However, the broad peak seen in the chromatograms indicated an increased dimer content and additional MtCK species in the range M_r 40 to 400 (Fig. 2A, right), probably consisting of MtCK monomers and cross-linking products thereof. After long-term exposition (7 days), even moderate anthracycline concentrations (100 μM) resulted in an enhanced dimerization of octameric MtCK compared with controls (Fig. 2, B-D). Dimerization was more pronounced for sMtCK (Fig. 2, B and C) compared with uMtCK (Fig. 2D). Human sMtCK also gave an additional peak at the molecular weight of monomers. After 7 days, no other additional molecular weight species were observable, but a lower recovery indicated that MtCK cross-link products might have aggregated and did not enter the column.

Effect of Doxorubicin, Daunorubicin, and Idarubicin on MtCK Activity. Incubation of MtCK with 5 to 750 μM anthracycline resulted in a slow, dose-dependent decrease of enzymatic activity; the time needed for half inhibition ($t_{1/2}$) was on the order of days (Fig. 3). The inactivation time course was determined for all three wild-type isoenzymes and chicken sMtCK mutant C278G (Fig. 4), where the reactive Cys278 was exchanged against glycine (Furter et al., 1993). Anthracycline treatment of 50 or 250 μg/ml MtCK gave qualitatively similar inhibition patterns, whereas activity of control samples was not markedly diminished even after several days. At 5 to 100 μM doxorubicin, sMtCK was more affected than uMtCK (Fig. 4, A-C). IC_{50} values calculated after 120 h were 10 ± 1 μM for chicken sMtCK, 9 ± 2 μM for human sMtCK, and 51 ± 8 μM for human uMtCK (n = 3 in each case). The exquisite sensitivity of sMtCK in comparison to uMtCK was also observed for dauno- and idarubicin (results not shown). In contrast to wild-type enzyme, enzymatic activity of C278G mutant remained almost unchanged at lower drug concentration (50 μM), whereas it was also lost at 750 μM doxorubicin (Fig. 4D). As shown in Fig. 5, anthracyclines differed in the concentration-dependence and the extent of their inhibitory effect. In the case of all three MtCK isoenzymes, dauno- and idarubicin induced an almost maximal activity decrease at concentrations < 100 μM, whereas doxorubicin achieved this only at concentra-

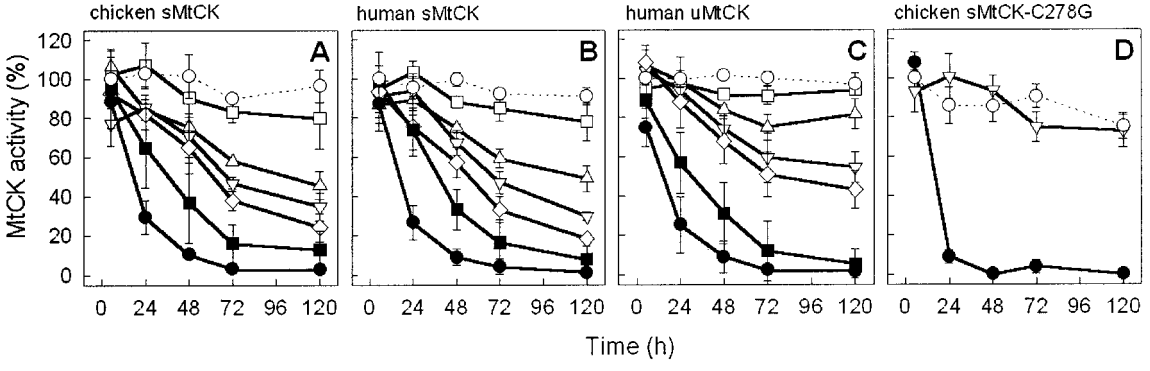

Fig. 4. Time course of inactivation of MtCK isoenzymes by doxorubicin. Chicken sMtCK (A), human sMtCK (B), human uMtCK (C), and chicken sMtCK (D) C278G mutant were treated with the following concentrations of doxorubicin: 0 μM (○, control), 5 μM (□), 10 μM (△), 50 μM (▽), 100 μM (◇), 300 μM (■) and 750 μM (●). Note that activities of samples incubated without doxorubicin (control; dotted line) were expressed relative to the activity measured at the beginning of the experiment; activities of doxorubicin treated samples (continuous line) are expressed relative to the activity of control sample at the same time point of the experiment. Data are given as mean ± S.E.M. ($n \geq 3$).

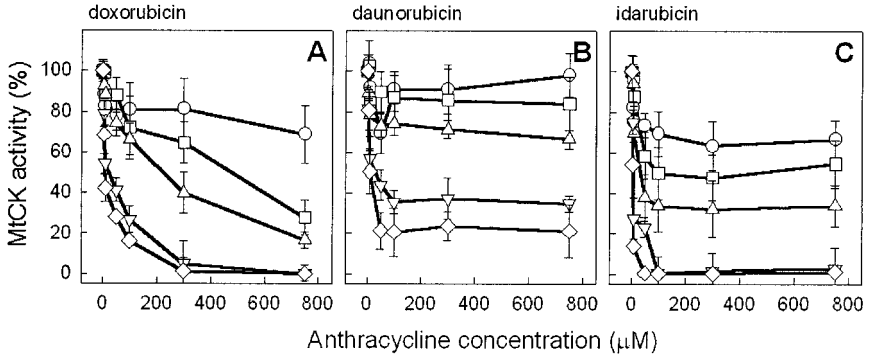

Fig. 5. Concentration-dependence of sMtCK inactivation by anthracyclines. The effects of doxorubicin (A), daunorubicin (B) and idarubicin (C) on the enzymatic activity of human sMtCK were measured after 5 h (○), 24 h (□), 48 h (△), 72 h (▽), and 120 h (◇) of incubation. Data are given as mean ± S.E.M. ($n \geq 3$).

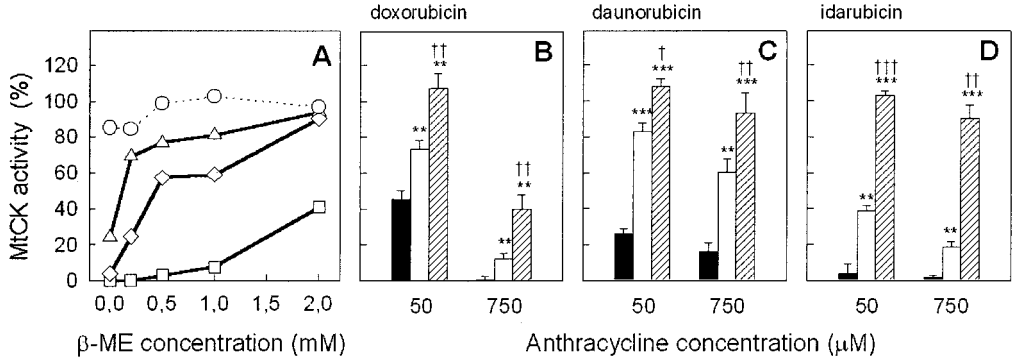

Fig. 6. Protective and reversal effects of β-mercaptoethanol against anthracycline-induced MtCK inactivation. A, Concentration-dependence of the protective effect of β-ME. Human sMtCK was incubated for 7 days in presence of different β-ME concentrations (0–2 mM) without anthracyclines (control; ○) or with 750 μM anthracyclines: doxorubicin (□), idarubicin (◇), and daunorubicin (△). Note that activities of samples incubated with β-ME in absence of anthracyclines (control; dotted line) were expressed relative to the activity measured at the beginning of the experiment; activities of anthracycline-treated samples (continuous line) are expressed relative to the activity of control sample at the same time point of the experiment. B to D, comparison of protective and reversal effect of β-ME. Human sMtCK was incubated with doxo- (B), dauno- (C), and idarubicin (D) at 50 and 750 μM for 7 days, either without addition of β-ME (■), with 5 mM β-ME added after 5 days of incubation (reversal effect; □), or in presence of 2 mM β-ME during the entire incubation period (protective effect; ▨). Data are given as mean ± S.E.M. ($n \geq 3$); ∗, significant difference between the anthracycline effect in absence and in presence of protective or reversal treatment; †, significant difference between protective and reversal effects of β-ME.

tions ≥ 300 μM. However, doxo- and idarubicin were able to abolish MtCK activity entirely, whereas daunorubicin resulted in only partial inactivation (≤ 80%).

We further analyzed potential mechanisms and target residues of anthracycline-induced enzymatic inactivation. The strong reducing agent β-ME protected enzymatic activity of MtCK to an extent depending on the applied drug concentration, the time point of β-ME addition (Fig. 6), and the MtCK isoenzyme. Already at low β-ME concentrations (e.g., 0.2 mM), considerable protection of human sMtCK was achieved against dauno- and idarubicin but not against doxorubicin (Fig. 6A). β-ME recovered enzymatic activity of all examined MtCK isoenzymes, either when permanently present in the incubation mixture (protective effect) or when added to entirely or partially inactivated samples (reversal effect; Fig. 6, B-D), albeit to lesser extent. β-ME (2 mM) protected almost 100% of enzymatic activity at 50 μM anthracycline, but only 40 to 90% at 750 μM. We conclude that anthracycline treatment at lower concentrations results in a partially reversible oxidation of MtCK, indicating the participation of cysteine residues; an additional nonreversible damage occurs at higher anthracycline concentrations. TSAC (Milner-White and Watts, 1971), which specifically shields the active site of CK, prevented MtCK inactivation to variable extent, depending on the type of anthracycline (Fig. 7) as well as the MtCK isoenzyme. Protection was most efficient in case of doxorubicin and human uMtCK (data not shown).

The protective effects of β-ME and TSAC suggested oxidative modifications at the active site involving the highly reactive Cys278. The role of this cysteine was analyzed with the C278G mutant that still shows about 5% residual enzymatic activity under control conditions (Furter et al., 1993). The residual activity was decreased by only 20% after treatment with 50 μM doxorubicin for 5 days (Fig. 4D), whereas activity of the wild-type chicken sMtCK diminished by more than 60% (Fig. 4A), indicating that this inactivation is caused mainly by modification of Cys278. However, treatment with 750 μM doxorubicin resulted in a drastic loss of C278G mutant activity, suggesting that further cysteines and/or other residues were modified at high anthracycline concentrations. Such additional damage could be caused by indirect effects of anthracyclines, mediated by the generation of free radicals as a result of redox cycling of the anthraquinone molecules. The presence of reactive oxygen species (ROS) such as superoxide anions was confirmed by adding SOD to the incubation mixture (Fig. 8). SOD exerted a remarkable protective effect on human sMtCK treated with 750 μM doxorubicin, whereas it had practically no effect at 50 μM doxorubicin.

Formation of High-Molecular-Weight Cross-Link Species Accompanying MtCK Inactivation. We performed nonreducing 12% SDS-polyacrylamide gel electrophoresis of samples treated with 50 μM (data not shown) or 750 μM doxorubicin (Fig. 9). At both concentrations, MtCK isoenzymes showed a monomer band at $M_r \sim 45$ and an

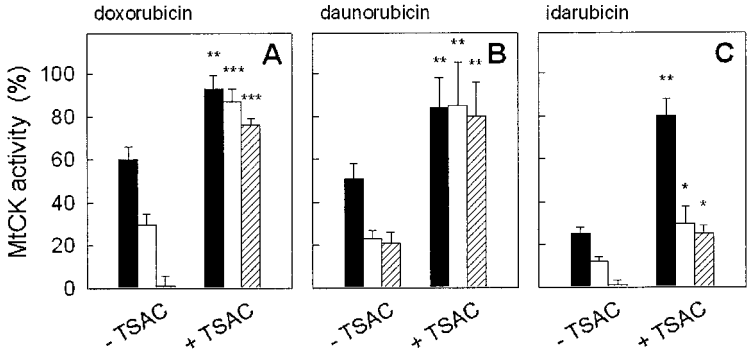

Fig. 7. Protective effect of TSAC against anthracycline-induced MtCK inactivation. Human sMtCK was incubated with (A) doxorubicin, (B) daunorubicin and (C) idarubicin at 10 μM (■), 100 μM (□), or 750 μM (▨) in the absence or presence of TSAC substrates for 4 days. Data are given as mean ± S.E.M. ($n \geq 3$). ∗, significant difference between the anthracycline effect in absence and in presence of TSAC substrates. No significant effect of TSAC on the activity of control samples was observed (see Fig. 9B; lane 5d and 5T).

additional higher molecular weight band situated below that of octameric MtCK (data not shown). The latter increased in intensity in parallel to enzyme inactivation during a time course of 7 days. Treatments that preserved enzymatic activity, namely incubation with β-ME and TSAC, almost prevented the formation of the higher molecular weight band, whereas a limited reactivation of MtCK by 10 mM β-ME only partially diminished the higher molecular weight band. These data suggest that cross-linked aggregates were caused mainly by reversible intermolecular disulfide bridges between MtCK monomers. However, because C278G mutant protein showed the same doxorubicin-induced cross-link products, this cysteine was not essential for cross-linking (data not shown). In this case, the protective effect of TSAC is rather explained by dimerization of octameric MtCK (Schlattner and Wallimann, 2000b) that reduces the probability of crosslinks between dimers.

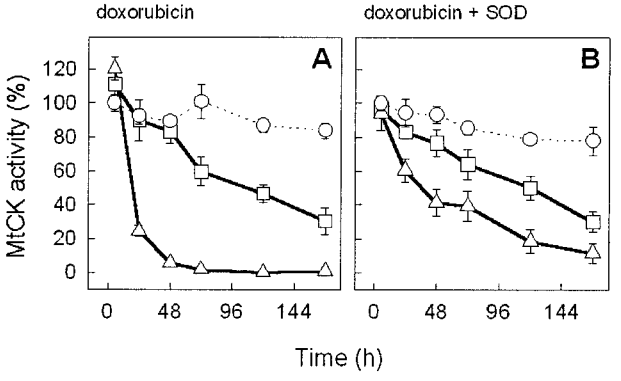

Fig. 8. Protective effect of superoxide dismutase against anthracycline-induced inactivation of MtCK. Human sMtCK was incubated with 0 μM (control, ○), 50 μM (□) and 750 μM (△) doxorubicin in the absence (A) and in the presence (B) of superoxide dismutase (SOD, 100 μg/ml). Note that activities of samples incubated with SOD in absence of doxorubicin (control; dotted line) were expressed relative to the activity measured at the beginning of the experiment; activities of doxorubicin treated samples (continuous line) are expressed relative to the activity of control sample measured at the same time point of the experiment. Data are given as mean ± S.E.M. ($n \geq 3$).

Discussion

The present in vitro study evaluates the potential role of MtCK damage in anthracycline-induced cardiotoxicity. We have found that doxorubicin, daunorubicin, and idarubicin exert deleterious effects on recombinant MtCK isoenzymes, affecting properties essential for enzyme functioning: membrane binding, octameric structure, and enzymatic activity. Cardiac sMtCK was more susceptible to anthracycline-induced damage than uMtCK, found in most other organs and tissues, suggesting a possible involvement of sMtCK damage in anthracycline cardiotoxicity.

At low and moderate anthracycline concentrations (≤100 μM), we observed immediate inhibition of MtCK binding to cardiolipin-containing vesicles, similar to earlier reports (Cheneval et al., 1989; Vacheron et al., 1997); sMtCK was affected more than uMtCK. Inhibition was competitive, probably because MtCK and anthracyclines share cardiolipin as the main binding partner in mitochondrial membranes (Nicolay et al., 1984; Goormaghtigh et al., 1990). Further anthracycline-induced MtCK damage occurred after longer incubation times and/or at higher drug concentrations (>100 μM) and included octamer dissociation, enzymatic inactivation, and finally monomerization, cross-linking and aggregation. In all cases, sMtCK was the most susceptible isoenzyme. Damage at lower anthracycline concentrations was caused mainly by oxidation of sulfhydryl groups as indicated by protective and reversal effects of β-ME, whereas the protective effect of SOD at higher drug concentrations point to additional damage by ROS (e.g., superoxide anion). CK is known to be particularly sensitive to oxidative and radical injury (Stachowiak et al., 1998; Koufen et al., 1999) as well as inactivation by thiol-specific reagents (Furter et al., 1993). Anthracyclines, because of their quinone structure and intrinsic electrophilicity, are potent redox-active agents (Feng et al., 1999; Bolton et al., 2000). Thus, anthracyclines would oxidize MtCK thiols (direct effect) and generate ROS that contribute to further damage (indirect effect). The limiting step for redox cycling in our in vitro system would be the accessibility of molecular oxygen. Because we used neither

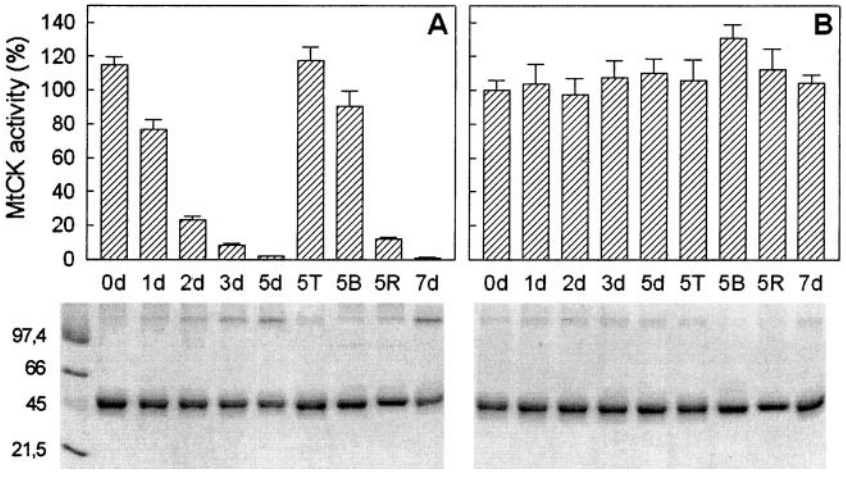

Fig. 9. Time course of formation of higher molecular weight species during MtCK inactivation. Enzymatic activity (top) and polypeptide pattern in nonreducing SDS-polyacrylamide gel electrophoresis (bottom) of human sMtCK incubated with 750 μM doxorubicin (A) or control (B) was followed for 7 days. After 5 days, the effects of protective treatments with TSAC substrates (lane 5T), 2 mM β-ME (lane 5B), or reversal treatment with 10 mM β-ME for 15 min (lane 5R) were determined. Note that all enzymatic activities are given as relative values expressed as percentage of control activity at the beginning of the experiment. Activity data are given as mean ± S.E.M. ($n \geq 3$). Polypeptide pattern was reproduced in the other two experiments.

iron complexes nor peroxidase/H_2O_2 activation of anthracyclines (Miura et al., 1994, 2000), redox cycling in our system was probably less efficient and inactivation was therefore rather slow.

MtCK contains seven (uMtCK) or eight cysteines (sMtCK; Fig. 10). Cys278 situated near the active site of the enzyme is well accessible, highly reactive, and important for full enzymatic activity (Furter et al., 1993). It was identified as the main target of oxidative and radical-induced inactivation (Stachowiak et al., 1998; Koufen et al., 1999; Koufen and Stark, 2000; Miura et al., 2000). In our study, oxidation of Cys278 was responsible for MtCK inactivation at lower drug concentrations (<100 μM) but was not essential for disulfide bond formation that led to intermolecular cross-linking. Thus, MtCK inactivation and cross-linking are at least partially independent events. As suggested by earlier studies (Vacheron et al., 1997; Koufen et al., 1999), octamer destabilization after long-term anthracycline treatment may also involve ROS-mediated modifications of cysteines like Cys358 near the C-terminal membrane-interaction site (Schlattner et al., 1998).

The examined anthracyclines differ in their side effects, with doxorubicin being the most cardiotoxic in clinical trials (Jain, 2000). Our in vitro studies indicate that doxorubicin may also induce major damage to MtCK in vivo. Under reducing conditions typical for the intracellular environment, doxorubicin caused markedly higher enzymatic inactivation than daunorubicin and idarubicin, whereas the latter two were more inhibitory in absence of β-ME. Under these conditions, their higher hydrophobicity compared with doxorubicin (Gallois et al., 1998) possibly led to increased selectivity for Cys278, which is situated in a hydrophobic patch. Because doxorubicin binds more efficiently to cardiolipin-containing membranes, its local concentration in mitochondria will be increased and it will be more inhibitory for MtCK/membrane interaction. The stronger short-term effect of idarubicin compared with doxorubicin seen in our in vitro assay were probably caused by the faster on-rate of this drug, because we used coinjection of drug and MtCK.

An important outcome of our study is the significantly higher sensitivity of sMtCK to anthracycline treatment. Similar differences between sMtCK and uMtCK were observed in vitro for peroxynitrite-induced inactivation and octamer-destabilization of the enzyme (S. Wendt, U. Schlattner and T. Wallimann, unpublished observations). In vivo, a decreased octamer/dimer ratio of sMtCK was found in animal models of ischemia (Soboll et al., 1999). The different susceptibility of MtCK isoenzymes can be explained by the known molecular properties (Schlattner and Wallimann, 2000b) and molecular structures of MtCKs (Fritz-Wolf et al., 1996; Eder et al., 2000). Sarcomeric MtCK can recruit fewer binding sites on cardiolipin-containing membranes than uMtCK, probably because of a different primary structure of the C terminus. sMtCK also forms octamers that are less stable because it lacks numerous polar interactions present at the dimer/dimer interfaces of uMtCK (Schlattner et al., 2000). Although both isoenzymes share a number of conserved cysteine residues, including Cys278 and Cys358, sMtCK exposes one cysteine more than uMtCK and may therefore be more prone to oxidative modifications (Fig. 10).

Different mechanisms were proposed to account for the heart-specific anthracycline toxicity. These include the relatively high amount of cardiolipin in heart mitochondria, the scarcity of antioxidants and radical scavengers, the exquisite presence of mitochondrial NADH dehydrogenase in heart as a site of anthraquinone reduction, or the disruption of cardiac-specific gene expression by doxorubicin (Horenstein et al., 2000). Many studies on drug action used relatively high drug concentrations, which rapidly affect vital functions. We have observed effects on sMtCK already at clinically relevant concentrations, which are in the range of 5 to 30 μM for isolated heart mitochondria (Sokolove, 1994; Gewirtz, 1999). For in vitro experiments with purified MtCK, as in our study, clinically relevant concentrations are probably much higher, because the enzyme is bound to mitochondrial membranes, which specifically accumulate anthracyclines to rather high levels, as seen in our binding experiments.

Our data suggest an alternative, but not exclusive mechanism, that could contribute to the selective cardiotoxicity of anthracyclines. The drugs would affect preferentially the membrane-bound octameric state and the enzymatic activity of cardiac sMtCK, which are both essential for its function in the CK/PCr energy circuit and in regulating mitochondrial permeability transition (O'Gorman et al., 1997; Khuchua et al., 1998). In heart, relying mainly on oxidative metabolism, with mitochondria occupying up to 40% of cellular volume, high amounts of sMtCK are expressed. As shown in an earlier study, sMtCK plays an especially important role in the control of mitochondrial respiration in oxidative muscles (Kay et al., 2000). Thus, impairment of this isoenzyme in

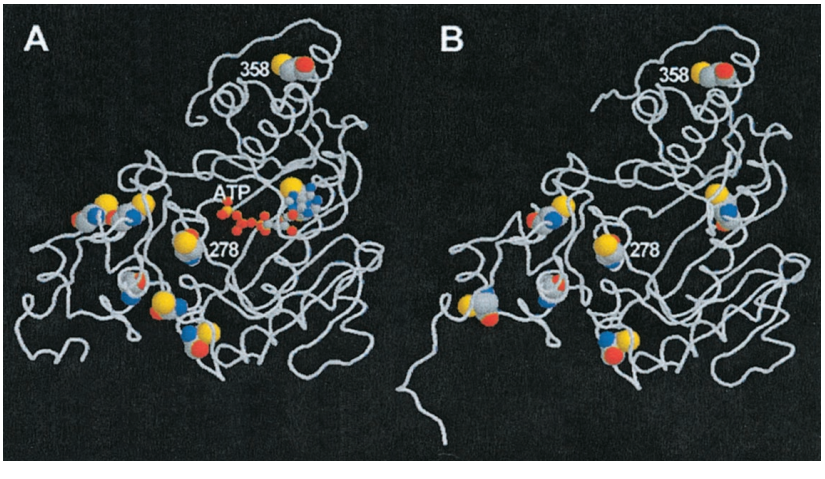

Fig. 10. Location of cysteine residues in the molecular structure MtCK. Chicken sMtCK (A) (Fritz-Wolf et al., 1996) and human uMtCK (B) (Eder et al., 2000) are shown in backbone representation. All cysteines are depicted as spacefill models and ATP present in chicken sMtCK as a ball-and-stick model. Cys278 and Cys358 are indicated. (Figure prepared with RasMol 2.6, R. Sayle, GlaxoSmithKline Research and Development, Greenford, Middlesex, UK).

heart would have the most serious consequences, including poor energetic state of the cells and possibly also increased apoptotic cell death (Arola et al., 2000). This could contribute significantly to the toxicity as well as the tissue-specificity of the toxic effects. Our model is supported by the lack of severe anthracycline toxicity in liver (Papadopoulou et al., 1999), an organ lacking MtCK, but also in kidney and brain, organs that express high levels of uMtCK. Brain is normally protected by blood-brain barrier, but drugs can cross the barrier under specific administration regimens (Bigotte and Olsson, 1989). The differential response of MtCK isoenzymes to anthracyclines may also have relevance for the therapeutic action of the drug. The higher resistance of uMtCK may explain in part why tumors overexpressing this isoenzyme are renowned for their especially poor prognosis. Functional uMtCK could confer an improved resistance against energy failure and apoptotic elimination of cancer cells via the postulated regulation of mitochondrial permeability transition (O'Gorman et al., 1997). Resistance to apoptosis is the principal reason for resistance of cancers to chemotherapy or radiation (Bold et al., 1997).

We have presented evidence that, among other mechanisms responsible for anthracycline cardiotoxicity, a compromised CK/PCr system can play an important role. The present study demonstrates the detrimental effects of anthracyclines on important properties of MtCK and the higher sensitivity of cardiac sMtCK compared with uMtCK. Impairment of sMtCK functions, together with anthracycline-induced damage to other components of cellular energy transduction [adenylate translocator (see Goormaghtigh et al., 1990), cytosolic CK (DeAtley et al., 1999)] and changes in gene expression (cytosolic muscle-type CK, adenylate translocator; see Minotti et al., 1999) could seriously compromise the energetic state of the heart. In this case, CK-targeted cardioprotectants may become useful clinical tools to alleviate anthracycline cardiotoxicity.

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