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Reduced creatine-stimulated respiration in doxorubicin challenged mitochondria: Particular sensitivity of the heart

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Abstract

Doxorubicin (DXR) belongs to the most efficient anticancer drugs. However, its use is limited by a risk of cardiotoxicity, which is not completely understood. Recently, we have shown that DXR impairs essential properties of purified mitochondrial creatine kinase (MtCK), with cardiac isoenzyme (sMtCK) being particularly sensitive. In this study we assessed the effects of DXR on respiration of isolated structurally and functionally intact heart mitochondria, containing sMtCK, in the presence and absence of externally added creatine (Cr), and compared these effects with the response of brain mitochondria expressing uMtCK, the ubiquitous, non-muscle MtCK isoenzyme. DXR impaired respiration of isolated heart mitochondria already after short-term exposure (minutes), affecting both ADP- and Cr-stimulated respiration. During a first short time span (minutes to 1 h), detachment of MtCK from membranes occurred, while a decrease of MtCK activity related to oxidative damage was only observed after longer exposure (several hours). The early inhibition of Cr-stimulated respiration, in addition to impairment of components of the respiratory chain involves a partial disturbance of functional coupling between MtCK and ANT, likely due to interaction of DXR with cardiolipin leading to competitive inhibition of MtCK/membrane binding. The relevance of these findings for the regulation of mitochondrial energy production in the heart, as well as the obvious differences of DXR action in the heart as compared to brain tissue, is discussed.

Keywords: Anthracycline; Creatine kinase; Cardiotoxicity; Isolated mitochondria; Creatine-simulated respiration

1. Introduction

Doxorubicin (DXR) and other anthracyclines are among the most potent chemotherapeutic drugs for the treatment of variety of human cancers. Cardiotoxic side effects represent, however, a serious constraint for long-term DXR chemotherapy. The molecular mechanism(s) responsible for this tissue-specific DXR-toxicity in the heart are still unclear, but molecular damage by DXR has been ascribed to oxidative stress. Cardiac injury has been related to the impairment of the essential components of myocardial energy metabolism, including the mitochondrial generation of high-energy phosphates, which provides more than 90% of the ATP used by cardiomyocytes (for a review, see [1]).

In the heart muscle, efficient adjustment of energy production to permanent, high and fluctuating demand is achieved by several means, among others by a high content of mitochondria, their specific organization and their integration into a specialized high energy phosphate transfer systems [2–5]. Mitochondrial organization contributes to the functioning of these systems via metabolic channeling and control of mitochondrial respiration by mitochondrial creatine kinase (MtCK), acting as a powerful modulator/amplifier for the regulatory action of ADP. As we could demonstrate *in vitro* [6], anthracyclines show multiple interference with MtCK, impairing several functional properties of the enzyme.

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The mitochondrial isoenzymes of CK, sarcomeric sMtCK, expressed in striated muscles including the heart, and ubiquitous uMtCK found in many other organs, mainly in the brain, are strictly localized within the cristae, as well as in the intermembrane space of mitochondria. MtCK is a peripheral membrane protein and binds to the outer leaflet of the inner mitochondrial membrane, interacting specifically with cardiolipin and even inducing the formation of specific cardiolipin membrane domains [7]. MtCK is enriched in the so-called contact sites between inner and outer membrane, where it forms functional microcompartments together with the transmembrane proteins adenine nucleotide translocator (ANT) in the inner membrane and mitochondrial porin (VDAC, voltage-dependent anion channel) in the outer membrane (reviewed in [4,8–10]). By reversible transfer of phosphoryl groups between ATP and Cr, MtCK catalyzes intramitochondrial regeneration of ADP and produces easily diffusible "high-energy" phosphocreatine (PCr). While PCr leaves the mitochondria by porin to supply the large cytosolic PCr pool, regenerated ADP is immediately re-imported by ANT into the matrix space, thus functionally coupling the MtCK reaction and mitochondrial ATP synthesis to oxidative phosphorylation. Repeated recycling of ADP stimulates the action of the respiratory chain and increases the turnover of adenine nucleotides. The privileged exchange of MtCK substrates and products between MtCK, ANT and porin is called functional coupling [3,4,9,11]. In co-operation with cytosolic CK associated to ATP-requiring reactions, it links elevated workload directly to mitochondrial ATP generation. This system is not only able to account for rapid and high changes in workload [2,5] but has additional beneficial functions. Increased respiratory rate due to intramitochondrial regeneration of ADP can prevent both free radical formation in energized mitochondria [12,13] and opening of the permeability transition pore, a trigger of apoptosis [14].

During DXR treatment, MtCK is highly exposed to the drug's toxic action as both share cardiolipin as a common binding partner in the inner mitochondrial membrane. High affinity binding of DXR to cardiolipin, shown in numerous previous studies, leads to selective accumulation of the drug in mitochondria. Thus, high local concentrations of DXR are reached in the microenvironment of MtCK, resulting in a competitive inhibition of MtCK/membrane binding [6,15–17]. As a consequence, MtCK is released from the inner mitochondrial membrane. The common location also situates MtCK at the very origin of the formation of reactive oxygen species (ROS) by redox cycling of DXR, largely mediated by mitochondrial oxidoreductive enzymes [18]. Oxidative stress is of particular danger to CK isoenzymes that have been recognized as very sensitive to loss of function by oxidation and nitration [19–24].

In our previous *in vitro* study with recombinant MtCK isoenzymes, we have shown that anthracyclines, including DXR, affect essential properties of recombinant, purified mitochondrial creatine kinase, such as its membrane binding, oligomeric state and enzymatic activity [6]. In addition, the cardiac isoenzyme sMtCK turned out to be more sensitive to the drug than the uMtCK found in brain and most other organs [6]. It was proposed that this differential sensitivity, together with high oxidative capacity of the heart and high cardiolipin content of cardiac mitochondria, could contribute to the cardiac specificity of the drug's toxic side effects. The aim of the present study was therefore to assess the effect of DXR on MtCK in its natural environment, inside the intact mitochondrion. First, we assessed Cr-stimulated respiration in isolated functionally intact heart mitochondria. There is some evidence that functional coupling is impaired by DXR [25], but the potential effects of DXR on Cr-stimulated respiration have never been investigated in detail with clinically relevant drug concentrations. Furthermore, we have compared the response of heart and brain mitochondria to such clinically relevant DXR concentrations, thus aiming at a comparison of the sMtCK and uMtCK isoforms. Finally, the kinetics of mitochondrial respiration in response to submaximal ADP concentration and in the presence or absence of physiological concentrations of Cr were analyzed in detail. The study shows that the stimulatory effect of Cr on respiration, due to metabolite channeling within the ANT-MtCK-porin complex, was rapidly abolished by DXR, an effect that was particularly pronounced in heart mitochondria. This study clearly points to Cr-stimulated respiration as an important process to be taken into account when evaluating cardiac side effects of DXR.

2. Materials and methods

2.1. Preparation of mitochondria

Mitochondria were isolated by differential centrifugation according to Dolder et al. [14] and subsequently purified by Percoll gradient. The whole procedure was performed at 4 °C. Briefly, bovine hearts were supplied by slaughter house, rat hearts and brains were freshly removed from laboratory animals (Wistar rats). Organs were placed in ice-cold isolation buffer (225 mannitol, 75 mM sucrose, 10 mM HEPES, pH 7.4) supplemented with 1 mM EDTA and 0.1% bovine serum albumin, cut with surgical scissors, and homogenized using Polytron (PT 3000, Kinematica AG) and/or Teflon potter (for brain only a Potter homogenizor was used). Homogenate (H) was centrifuged at 1000×*g* for 10 min. The resulting supernatant (H1) was centrifuged at 12,000×*g* for 10 min. Obtained in this way, the crude mitochondrial pellet (CM) was further purified by 35-min centrifugation at 100,000×*g* on a 25% Percoll gradient. Percoll was removed from the purified mitochondrial fraction (PM) by washing (centrifugation at 12,000×*g* for 10 min) twice in isolation buffer. Mitochondrial protein concentration was measured using the Bio-Rad Protein Assay with bovine serum albumin as a standard. Purity and structural integrity of mitochondrial preparation was checked by Western blotting and electron microscopy, respectively.

2.2. Chemicals

Doxorubicin hydrochloride was purchased by Fluka (Buchs, Switzerland); 10 mM stock solution was prepared as described by [6]. The disodium salts of ADP and NADP were obtained from Roche, PCr from Calbiochem, and P^1P^5-di (adenosine-5′)-pentaphosphate pentasodium salt from Sigma. All other chemicals were at least of reagent grade.

2.3. Incubation of mitochondria with doxorubicin

If not stated otherwise, mitochondria were resuspended in isolation buffer to the desired concentration (0.5–1 mg/ml) and incubated with doxororubicin 1 h at room temperature (RT) with constant shaking. Doxorubicin dose used has been always adjusted to mitochondrial protein concentration.

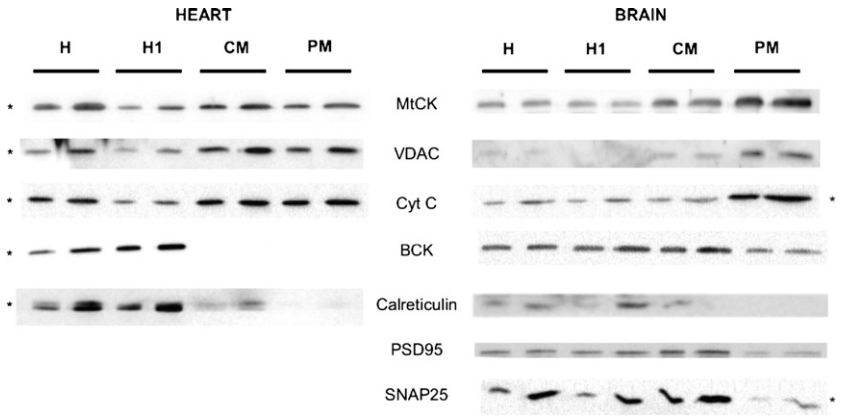

Fig. 1. Analysis of purity of mitochondrial preparations. Tissue homogenates (H, H1; prepared as described in Materials and methods), crude mitochondria (CM) and Percoll purified mitochondria (PM) from rat heart and brain were probed by immunoblotting for selected mitochondrial markers (cytochrome *c*, VDAC, MtCK), markers for possible contaminating organelles (calreticulin for endoplasmic reticulum, SNAP25 and PSD95 for synaptosomes), as well as for the presence of a cytosolic isoform of brain-type creatine kinase (BCK). Samples were loaded on the SDS-PAGE gels either in duplicates (each 10 μg) or when marked with asterisk in two different amounts (10 and 25 μg protein).

2.4. Respiratory measurements

Mitochondrial oxygen consumption was measured at 25 °C with a high performance OROBOROS oxygraph (Anton Park, Innsbruck, Austria) in respiration buffer (250 mM sucrose, 10 mM Tris/Mops, pH 7.4) supplemented with 10 mM Pi, 2 $MgCl_2$, 2 μM rotenone. ADP- and Cr-simulated respiration was analyzed in a setup described by Jacobus and Lehninger ([26]; see also [11]) with slight modifications. Resting respiration (State 4) was induced by 5 mM succinate (10 mM for brain), and subsequently stimulated by 25 μM/1 mM ADP. Stimulation of respiration by ADP was analyzed in the absence and in the presence of 10 mM Cr. At the end of the measurement cycle, ANT was blocked by addition of atractyloside (40 μM) and the level of resting respiration was checked. Alternatively, uncoupler (1μM carbonyl cyanide *m*-chlorophenylhydrazone; CCCP)-stimulated respiration was analyzed as an index of maximal electron transport capacity.

2.5. Enzymatic activity measurements

The reverse CK reaction (ATP production) was measured in a coupled photometric enzymatic assay as described earlier [27], with all reagents prepared without addition of reducing agent (β-mercaptoethanol) in order to not counteract the effects of DXR (see [6]). Values were corrected for other ADP-consuming reactions (e.g., by adenylate kinase) by subtracting activity measured in parallel in the absence of PCr, the CK-specific substrate. One International Unit of CK activity (1 U) corresponds to 1 μM of PCr transphosphorylated per min at pH 7.0 and 25 °C.

2.6. Cellulose polyacetate electrophoresis (CPAE)

CK isoenzymes were separated by native CPAE for 1 h at 150 V at room temperature [28]. Subsequently, the CK bands were visualized by an enzymatic color reaction coupled to the CK reverse reaction; the reaction mixture was supplemented with 100 μM P^1P^5-di(adenosine-5′)-pentaphosphate to inhibit interfering adenylate kinase activity.

2.7. Binding/detachment assay

Frozen in liquid N_2 and thawed mitochondria were centrifugated at 13,000 rpm for 5 min and MtCK was probed in supernatant by immunoblotting.

2.8. SDS-PAGE electrophoresis and immunoblotting

Proteins separated by SDS-PAGE were semi-dry blotted onto nitrocellulose transfer membranes (Protran, Schleicher and Schuell). After blocking, blots were immunostained with specific antibodies for 2 h at room temperature. The blocking buffer consisted of 4% milk, TBS, and 0.05% Tween 20. The primary antibodies were diluted in blocking buffer by the following factors: 1:200 for anti-VDAC1 antibody (Santa Cruz Biotechnology), 1:500 for anti-cytochrome *c* antibody (BD Biosciences Pharmingen), 1:1000 for anti-MtCK antibodies (generated and characterized previously in our laboratory [29]), anti-SNAP25 (soluble NSF attachment protein, BD Biosciences Pharmingen) and anti-PSD95 (post-synaptic density protein 95, Upstate), 1:2500 for calreticulin (BD Biosciences Pharmingen). Secondary peroxidase-coupled antibodies were diluted in blocking buffer at 1:2000 and blots were incubated for 1 h at room temperature. Blots were developed with Chemiluminescent Detection Kit (AppliChem GmbH).

2.9. Statistical analysis

Data are presented as mean ± standard deviation (S.D.). Student's *t*-test was used to compare two means. $P \leq 0.05$ was taken as significant. Experiments were performed at least three times.

3. Results and discussion

3.1. Mitochondria from the heart and brain

In vitro, muscle-specific sMtCK present in the heart was found to be more prone to oxidative damage by DXR as compared to uMtCK expressed in the brain and most other organs [6,24,30]. To examine DXR effects on the MtCK-Cr system within the mitochondria of these very organs, a procedure for their isolation and quality control was established. Quality of the mitochondrial preparations was reproducibly high, as judged from immunoblotting (Fig. 1), functional analysis (respiratory control ratio of ~5 with succinate as a substrate), as well as electron microscopy (data not shown). Both the crude mitochondrial fraction (CM) and the final Percoll preparations (PM) from the two tissues were highly enriched in mitochondria. Purification on a Percoll gradient efficiently eliminated contamination of heart CM by endoplasmic reticulum, while in the brain PM, a small contamination by synaptosomes and cytosolic BCK persisted despite repeated washing. The mitochondrial preparations from the heart and brain showed significant amounts of MtCK, but differed in their

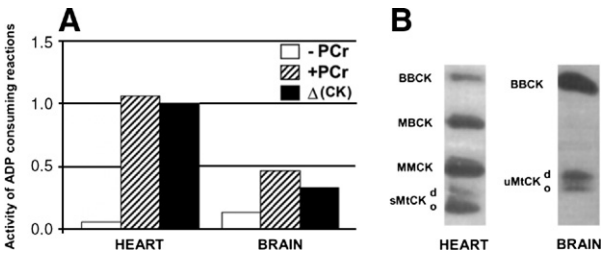

Fig. 2. CK enzymatic activity and isoform pattern in the heart and brain. (A) Specific activity of ADP-consuming reactions in rat heart and brain mitochondria. Activity measured in the absence of PCr (white bar), in the presence of PCr (hatched bar) and difference between activities measured in the presence and absence of PCr (black bar), representing genuine CK activity. All values were related to CK activity in rat heart mitochondria, which was 4.8±0.6 U/mg. (B) Distribution of total CK activity between cytosolic and mitochondrial isoforms in rat heart and brain, assessed by CPA electrophoresis. Isoforms: dimeric cytosolic CK—muscle-type MCK and brain-type BCK, forming homodimers (MM-, BBCK) and heterodimers (MBCK). Dimeric (d) or octameric (o) mitochondrial CK—sarcomeric (sMtCK) and ubiquitous (uMtCK). Heart expresses mainly MMCK and sMtCK, but less MBCK, and BBCK. Brain expresses mainly BBCK and uMtCK, but no MM- or MB-CK.

specific activities for this kinase (Fig. 2A). It has to be mentioned that also the distribution of CK activity between mitochondrial and cytosolic isoenzymes is different in these organs (Fig. 2B).

3.2. Doxorubicin affects ADP-stimulated respiration in isolated intact heart mitochondria

Respiratory analysis was performed with succinate as substrate and rat or bovine heart mitochondria. The latter, however, remained more stable and well coupled under conditions of DXR incubation. We first checked the effect of relatively low DXR concentrations (up to 300 μM DXR/mg mitochondrial protein) on respiratory states 4 and 3, the latter initiated by 1 mM ADP. At these DXR concentrations, there was no rapid effect of the drug on respiratory state 4 (Fig. 3A for 100 μM DXR). In contrast, an immediate inhibition of ADP-stimulated state 3 was observed (Fig. 3A, B), and preincubation of mitochondria with DXR for 1 h at RT increased this inhibitory effect (Fig. 3C). As seen with control mitochondria, the latter incubation had a negligible effect on respiratory control ratio, as compared to incubation on ice. The magnitude of state 3 inhibition is consistent with earlier data for pigeon and rat heart mitochondria [31,32]. DXR also affects uncoupler-stimulated respiration (Fig. 3B, see also [33]), indicating an impairment of the respiratory chain. Since our assay supplies electrons to complex II, it is likely that an inhibition of complex IV is involved in producing this effect (see [1] and literature therein). DXR inhibition was consistently higher for uncoupler- as compared to ADP-stimulated respiration, consistent with general benefits of the coupled process (see [14]). Based on these results, in further experiments mitochondria were incubated for 1 h at RT with a drug dose not exceeding 100 μM DXR/mg mitochondrial protein. Such a DXR concentration is relevant since (i) mitochondria selectively accumulate DXR to concentrations much higher than plasma levels, the latter reaching up to 2–6 μM after bolus injection [34]); (ii) the incubation mixture containing the mitochondria contains a relatively high protein concentration, and (iii) it turned out that it is rather the relation between concentrations of DXR and mitochondrial proteins that is important for DXR effects in mitochondria (data not shown and [1]).

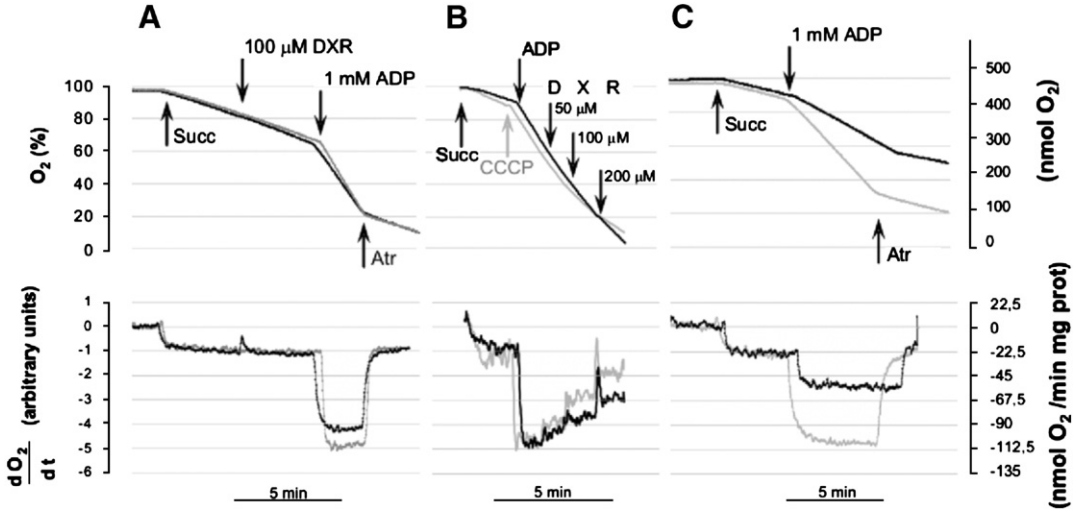

Fig. 3. Effect of DXR respiratory states 4 and 3 in heart mitochondria. Typical recordings from different experimental setups to analyze effect of DXR on respiratory states 4 and 3. (A, B) Immediate effect of DXR, (C) mitochondria were preincubated without (control, CTR) or with 100 μM DXR during 1 h at RT. (A, C) State 3 was induced by 1 mM ADP; CTR—gray trace, DXR—black trace. (B) DXR effect on state 3 respiration induced by 1 mM ADP (black trace) in comparison to uncoupled state respiration induced by 1 μM CCCP (gray trace). Upper panel: oxygen content in the measurement chamber, lower panel: corresponding derivatives, showing velocities of oxygen consumption (respiratory rates). On the left axis oxygen content is shown in percent of saturation value and the rate of oxygen consumption in arbitrary units, on the right axis both are expressed in absolute units. Bovine heart mitochondria (~0.5 mg/ml) were maintained in respiration buffer (250 mM sucrose, 10 mM Tris/Mops, pH 7.4) supplemented with 10 mM Pi, 2 mM $MgCl_2$, 2 μM rotenone. State 4 was induced by 5 mM succinate (Succ), state 3 by 1 mM ADP. At the end of measurement cycle, state 4 respiration was recovered by inhibition of ANT with 40 μM atractyloside (Atr). Mean absolute values of respiratory rate in states 4 and 3 were the following: 27±5 and 125±23 nmol O_2/min mg protein.

3.3. Doxorubicin affects Cr-stimulated respiration in heart mitochondria before inhibiting sMtCK enzymatic activity

We have compared the effect of a 1-h incubation of heart mitochondria with DXR on the kinetics of mitochondrial respiration in response to submaximal ADP concentrations (respiratory rate between states 4 and 3) in the presence and absence of Cr (Fig. 4A). The stimulatory effect of Cr at these ADP concentrations is the most significant in normally functioning mitochondria [11]. In the absence of Cr, energized mitochondria (with excess of succinate and inorganic phosphate) become already activated upon addition of submaximal ADP concentrations. The rate of oxygen consumption rapidly increases, but the respiratory burst is terminated as soon as all ADP is phosphorylated. DXR showed an effect on ADP stimulation as observed before.

In the presence of Cr, external addition of ADP leads to an immediate strong stimulation (transitory phase, Fig. 4A), followed by a prolonged activation (near steady-state phase, see arrows in Fig. 4A, lower panel). It should be noted that the transitory phase seen after ADP addition in the presence of Cr is a genuine event observed also elsewhere [26]. It does not occur after addition of the equivalent amount of solvent, and its amplitude correlates with the amount of ADP added, not with added volume. As verified by immunoblotting (Fig. 1A) and CPA electrophoresis (data not shown), there is no other contaminating CK isoform present in the cardiac mitochondrial preparations. Thus, prolonged activation corresponds to intramitochondrial ADP regeneration by the MtCK reaction, using mitochondrially produced ATP and the added Cr, as it has been shown for heart mitochondria in numerous earlier studies (for state-of-the-art reviews, see [35]). Such local channeling is due to the formation of MtCK-ANT microcompartments and the resulting high affinity of ANT for MtCK-produced ADP [4,11,14]. The experimental setup used here does not allow for exact determination of K_m (ADP) as done elsewhere [36], since externally added ADP is consumed, leading to decreasing ADP concentrations and complex respiration kinetics. In contrast, the setup avoids complex additives to the incubation medium, ADP generation by endogenous ATPases or ADP regenerating systems like hexokinase, which may all interfere themselves with DXR and thus complicate the analysis of drug effects (data not shown). However, this direct approach allows analysis of DXR effects that are exclusively revealed by the presence of ADP and Cr and that are due to endogenous mitochondrial components like the MtCK microcompartments and the respiratory chain. With bovine heart mitochondria, DXR inhibited stimulation of respiration by Cr. When quantified, this effect was present in the steady-state phase (Fig. 4B), and even more significant in the transitory phase directly following ADP addition (Fig. 4C).

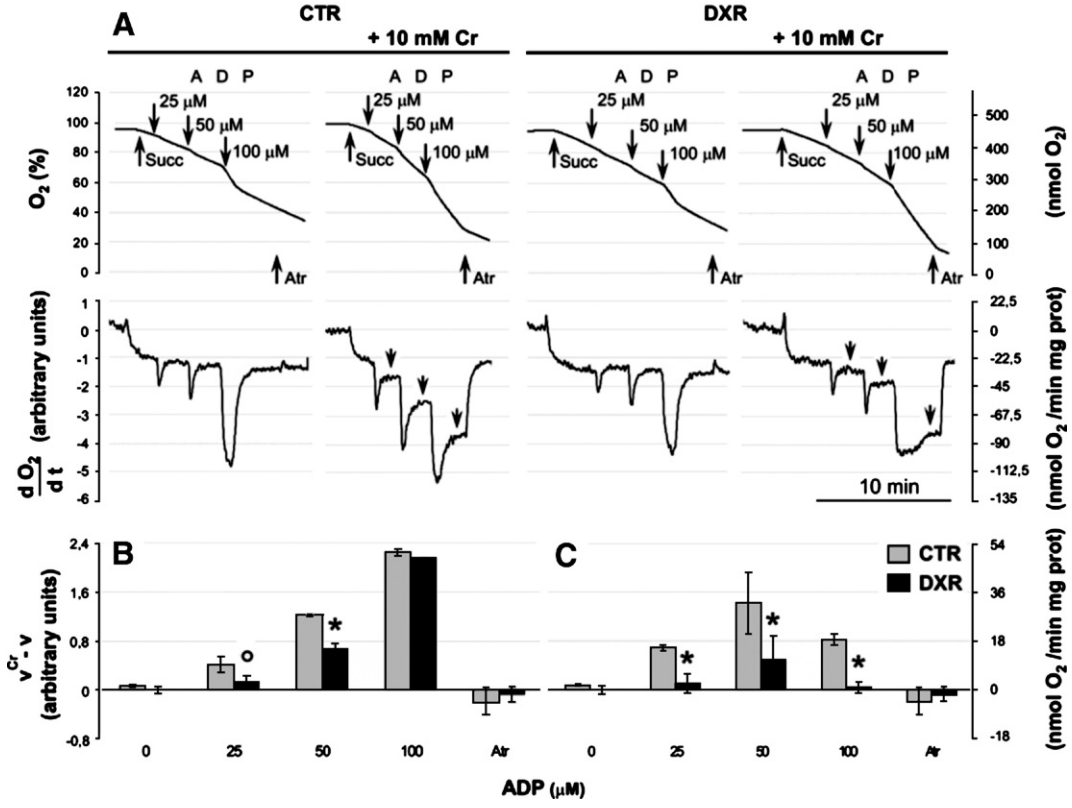

Fig. 4. Effect of DXR on Cr-stimulated respiration in heart mitochondria. (A) Representative traces of kinetics of oxygen consumption in control and DXR-treated mitochondria in response to submaximal ADP concentrations (25–100 µM) in the absence and presence of 10 mM Cr (upper panel) and corresponding derivatives, showing velocities of oxygen consumption (respiratory rates; lower panel). Bovine heart mitochondria at 0.5 mg/ml were preincubated without (control, CTR) or with 50 µM DXR during 1 h at RT. (B, C) Quantification of stimulatory effect of Cr in near steady-state phase (marked with arrow; B) and in transitory phase (peak; C), calculated as difference of the respiratory rate, v, in the presence and absence of 10 mM Cr. *$P \leq 0.05$; °$P = 0.08$ ($n = 3$–4). Other experimental details as for Fig. 3.

Similar DXR effects were observed in rat heart mitochondria, which were, however, consistently less stable at RT (not shown).

The observed inhibition of Cr-stimulated respiration occurs likely through different mechanisms. First, a more general impairment of the respiratory chain may play a role, as it occurred already during ADP-stimulated respiration (see above) and may be even more important at the higher respiratory rates induced by Cr stimulation. However, the pronounced inhibitory effect especially under conditions of Cr stimulation rather indicates a major involvement of the MtCK microcompartment, mainly a direct impairment of MtCK and its coupling to ANT. The correct functioning of MtCK largely depends on enzymatic activity and membrane binding of the octameric enzyme [3,37–39]. We have analyzed the effect of DXR on both properties (Fig. 5A–C). After 1-h incubation of bovine heart mitochondria with DXR, i.e., when Cr-stimulated respiration was already impaired, there was no decrease in MtCK enzymatic activity (Fig. 5A). A significant decrease of MtCK activity was only observed after longer exposure (several hours; Fig. 5B). The protective effect of reduced glutathione (15 mM GSH; Fig. 5B) indicated an involvement of oxidative damage. These findings are consistent with our earlier *in vitro* data with recombinant enzyme, where enzymatic inactivation of MtCK by DXR was a rather delayed process and due to oxidation of essential cysteines [6]. Although redox cycling of DXR would be more prominent in isolated mitochondria than in MtCK protein solutions due to numerous potential sites of anthraquinone reduction, the data show that MtCK inactivation in this experimental system is still a relatively slow process. This was also observed in different other model systems, where inactivation of MtCK by DXR was not immediate [9,40]. More pronounced DXR effects were only reported after activation of the drug with exogenous HRP/H_2O_2 or its coupling with iron [41,42].

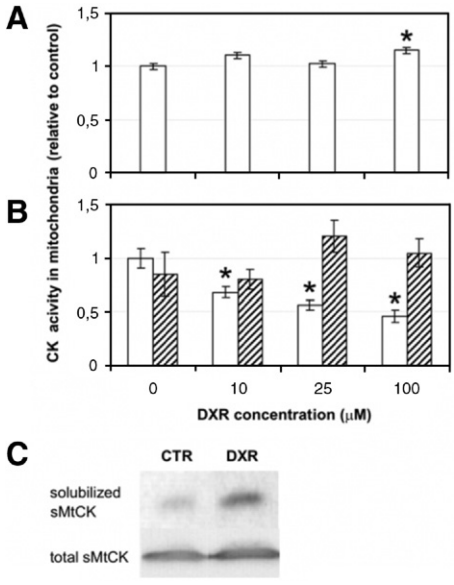

Fig. 5. Effect of DXR on enzymatic activity and membrane binding of sMtCK in heart mitochondria. sMtCK-specific activity in bovine heart mitochondria exposed to different DXR concentrations during 1 h at RT (A) and during 24 h at 4 °C (B); in panel B, mitochondria were preincubated without (control, CTR) or with DXR in absence (white bars) or in presence of 15 mM reduced glutathione (GSH; hatched bars). Control sMtCK activity in bovine heart mitochondria was 1.3±0.3 U/mg protein (n=3). (C) sMtCK probed by immunoblotting in total control and DXR-treated rat mitochondria (100 μM DXR pro 1 mg/ml mitochondrial protein during 1 h at RT) and in corresponding supernatants from the same frozen–thawed mitochondria.

In contrast, the effect of DXR on MtCK membrane binding was evident after 1 h of incubation with the drug (Fig. 5C), again similar to our earlier *in vitro* experiments [6,9]. Detachment of MtCK from mitochondrial membranes was analyzed by immunodetection of MtCK released into the supernatant after a freeze–thawing cycle (Fig. 5C). Already after 1-h incubation with DXR a significant portion of MtCK had been detached from the membranes. Such a solubilization of MtCK may render its active sites more accessible in the enzymatic assay, thus explaining the even slight but significant increase in MtCK activity after 1 h of incubation with DXR (Fig. 5A). However, detachment of MtCK reduces its coupling to ANT and can thus play a key role in the inhibition of its function in Cr stimulation of respiration. It is to note that in all experiments presented here, mitochondria were incubated with DXR in the absence of respiratory substrates. If the latter were added concomitantly with DXR, inactivation and detachment of MtCK from membranes, as well as oxidation of mitochondrial proteins as assessed by oxyblot were enhanced (data not shown). However, in our assay conditions, such a treatment affected substantially the integrity of mitochondrial membranes (data not shown) and was thus not convenient for subsequent respiratory measurements.

3.4. Differential action of DXR in brain

Under various harmful conditions, including DXR exposure, the muscle-specific sMtCK isoform has been shown to be more sensitive than the ubiquitous uMtCK with respect to different molecular properties of the enzyme [6,24,30]. Therefore, we have made an attempt to compare the DXR effects on Cr-stimulated respiration observed in the heart with those present also in brain mitochondria. Incubation of isolated rat brain mitochondria was performed both at RT and on ice to exclude an effect of incubation temperature on mitochondrial function, but no major difference was observed. In the absence of Cr, DXR showed a clear inhibitory effect on ADP-stimulated respiration, similar to that seen with heart mitochondria (Fig. 6A). In the presence of Cr, a Cr stimulation of respiration was mainly seen in the steady-state phase (see arrows in Fig. 6A). These data revealed a much lower or even no deleterious impact of DXR on Cr-stimulated respiration in brain mitochondria (Fig. 6B). This could be due to the preservation of the uMtCK-ANT microcompartment in the brain mitochondria, which is supported by the only marginal detachment of membrane-bound uMtCK of brain mitochondria by DXR (Fig. 7B) and also by our earlier *in vitro* study [6]. Similarly as in the heart, uMtCK enzymatic activity remained unchanged during 1 h of incubation with DXR (Fig. 7A). In some experiments, such an incubation slightly increased state 4 respiration as measured after inhibition

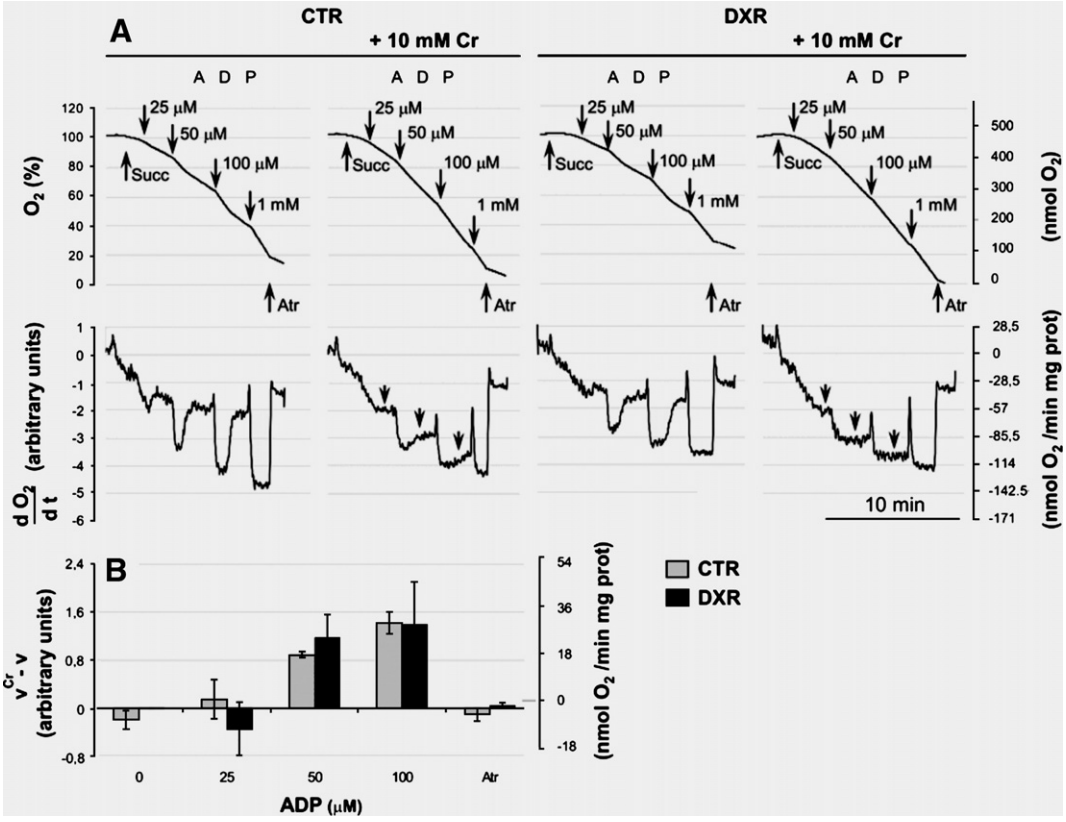

Fig. 6. Effect of DXR on Cr-stimulated respiration in rat brain mitochondria. (A) Representative traces of kinetics of oxygen consumption in control and DXR-treated mitochondria in response to submaximal ADP concentrations (25–100 µM) in the absence and in presence of 10 mM Cr (upper panel) and corresponding derivatives, showing velocities of oxygen consumption (respiratory rates; lower panel). Brain mitochondria at 1 mg/ml were incubated without (CTR) or with 100 µM DXR during 1 h at 4 °C. Mean control values of respiratory rate in states 4 and 3 were the following: 24±6 and 126±10 nmol O_2/min mg protein. (B) Quantification of stimulatory effect of Cr in near steady-state phase (marked with arrow), calculated as difference of the respiratory rate, *v*, in the presence and absence of 10 mM Cr (n=3–4). Other experimental details as for Fig. 3.

of ANT by atractyloside, possibly due to the partial permeabilization of inner membrane. Importantly, after longer incubation times (4 h with 100 µM DXR/mg protein), residual CK activity in the brain (85±8% of control) was higher than in the heart (66±9% of control), which would again argue for the better preservation of the functional integrity of the uMtCK-ANT microcompartment.

3.5. Concluding remarks

In general, comparative functional studies with intact isolated mitochondria are not straightforward, since the mitochondria from different tissue differ in many respects, as membrane permeability for substrates and ADP, apparent K_m for ADP of ANT, and repartition of respiratory control exerted by different elements of respiratory chain. Moreover, it is to note that treatment of isolated mitochondria with DXR allowed us to analyze acute effects of the drug. One can expect that chronic exposure to DXR *in vivo* will induce progression of substantial direct and radical-mediated molecular damage of MtCK (see [43]), which will further compromise MtCK functions, including energy channeling and signaling between mitochondria and cytosol [3,5], regulation of mitochondrial respiration, formation of the contact sites [44], Mt-CK-mediated inner and outer mitochondrial membrane cross-linking [45], MtCK-mediated lipid exchange between inner and outer membrane [7] and finally prevention of radical generation [13] and opening of the mitochondrial permeability pore [14], also termed "mitochon-

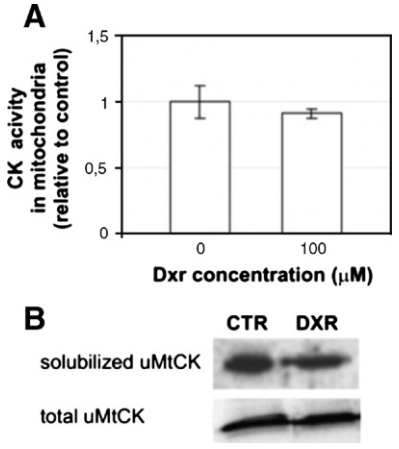

Fig. 7. Effect of DXR on enzymatic activity and membrane binding of uMtCK in brain mitochondria. (A) uMtCK-specific activity in rat brain mitochondria incubated without (control) and with 100 µM DXR pro 1 mg/ml mitochondrial protein during 1 h at RT (n=3). Absolute values of control uMtCK activity were 2.3±0.3 U/mg protein for mitochondria kept at RT in comparison to 2.0±0.4 U/mg for mitochondria kept on ice. (B) uMtCK probed by immunoblotting in total control and DXR-treated mitochondria and in corresponding supernatants from the same frozen–thawed mitochondria from rat brain (condition of incubation as in panel A).

drial permeability transition", which has been observed as a result of DXR treatment in different cardiotoxicity models [46].

In conclusion, our study clearly shows that DXR affects primarily Cr-stimulated respiration and that if reduction in Cr-stimulated respiration is not taken into account, inhibition of mitochondrial function by DXR is largely underestimated. This effect would be particularly marked in tissues expressing sMtCK, as heart and skeletal muscles. The disturbance of regulation of mitochondrial respiration by MtCK can be very harmful at elevated work and can contribute to reduced exercise tolerance, affecting up to 70% of cancer patients during and after therapy [47].

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