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Mitochondrion 4 (2004) 49–57

Mitochondrion

www.elsevier.com/locate/mito

Higher respiratory rates and improved creatine stimulation in brain mitochondria isolated with anti-oxidants

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Received 5 March 2004; received in revised form 1 June 2004; accepted 2 June 2004

Abstract

We tested the effect of an anti-oxidant mixture on respiration in isolated rat brain mitochondria. Mitochondria were isolated in mannitol/sucrose/EGTA/BSA ± SCAVEGR™ anti-oxidants (*S*OD, *ca*talase, *v*itamin *E*, vitamin E acetate, and *g*lutathione *r*educed). TBARS were reduced by greater than 40% with SCAVEGR. Respiration driven by ADP showed a two-fold higher V_{max} and a 15% higher respiratory control ratio when mitochondria were prepared with SCAVEGR. SCAVEGR also stabilized the octameric state of mitochondrial creatine kinase and thus improved creatine-stimulated respiration. These results suggest that significant improvements in brain mitochondrial function are obtained by isolation in the presence of an anti-oxidants mixture.

Keywords: Anti-oxidant; Brain mitochondria; Creatine kinase; Respiration; ROS

1. Introduction

Mitochondria are often isolated from tissue to study their structure and function. However, a major obstacle is preserving structural and functional integrity of the mitochondrial preparation during the purification procedure and thus to avoid in situ artifacts. Isolated mitochondria are detached from the cytoskeleton, diluted into an aqueous solution, and their molecular components may be damaged. Different improvements of standard isolation protocols have been proposed to avoid such problems, e.g. addition of osmotically active compounds (Gellerich et al., 1996), but no critical analysis of anti-oxidants is available so far.

In vivo, mitochondria are notorious generators of reactive oxygen (ROS) and nitrogen (RNS) species. Therefore, oxidation of lipids and proteins may constitute a problem during isolation of mitochondria, especially since certain anti-oxidative compounds may be lost or inactivated during this process. ROS originate directly or indirectly from leaks in the respiratory chain that yield reactive superoxide. This species can dismutate to form hydrogen peroxide which can further react to the hydroxyl radical, a potent reactive species. Alternatively, mitochondrial

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1567-7249/$20.00
doi:10.1016/j.mito.2004.06.001

superoxide may react with nitric oxide to form the very potent oxidant peroxynitrite. It is now well established that NO is synthesized inside mitochondria by a mitochondrial isoenzyme of nitric oxide synthase (Ghafourifar and Richter, 1997; Elfering et al., 2002). ROS and RNS species potentially interfere with a large number of proteins and important processes in mitochondria. Certain components however, appear more sensitive. For example, nitric oxide preferentially modifies SH-groups in complexes II and IV of the respiratory chain (Lizasoain et al., 1996). With peroxynitrite, mitochondrial creatine kinase (MtCK) has been identified as a prime target that is damaged even before the respiratory chain is affected (Stachowiak et al., 1998b). MtCK is situated in the mitochondrial intermembrane space, where it binds to the inner membrane and has direct access to ATP exported by the adenine nucleotide translocator (Stachowiak et al., 1998a). A detailed analysis has shown that peroxynitrite modifies specific MtCK residues that are crucial for enzymatic activity and the proper octameric state of the enzyme (Dolder et al., 2002, 2003; Wendt et al., 2003). Mitochondrial membranes are more vulnerable if they contain more unsaturated lipids that are more susceptible to degradation by ROS. Since brain mitochondria contain higher levels of C22 poly-unsaturated lipids as compared to more commonly studied liver and heart mitochondria (Barzanti et al., 1994), they may be especially sensitive to ROS. In fact, it is known that isolated brain mitochondria, in contrast to those from heart or liver, remain well-coupled for a much shorter period of time.

Despite these issues, methods for isolation of brain mitochondria generally follow those for liver and heart without use of anti-oxidants. Based on the requirement for anti-oxidants during the radical-generating procedure of isolating primary neurons from the brain (Brewer et al., 1993), we have tested here whether anti-oxidants can improve structural and functional parameters of isolated rat brain mitochondria. Lipid soluble anti-oxidants such as vitamin E (alpha-tocopherol) and its acetate conjugate with increased bioavailability (Kamal-Eldin and Appelqvist, 1996) may be needed to prevent generation of proton-conducting fatty acids as well as aldehyde by-products. Superoxide dismutase would detoxify the superoxide anion, while glutathione peroxidase with its substrate glutathione, together with catalase, would detoxify hydrogen peroxide. Since each of these five anti-oxidants was essential for high survival of embryonic neurons during isolation (Brewer et al., 1993), all of them were included in the cocktail during the isolation of brain mitochondria (SCAVEGR-mixture: *S*OD, *ca*talase, *v*itamin *E*, vitamin E acetate, and *g*lutathione *r*educed). Analysis of lipid peroxidation, overall organelle morphology, succinate-driven respiration, and MtCK-dependent creatine-stimulated respiration revealed that, except ultrastructure, all parameters were improved by addition of anti-oxidants to the isolation medium.

2. Materials and methods

2.1. Isolation of brain mitochondria and synaptosomes

Chemicals were obtained from Sigma unless stated otherwise. Mitochondria and synaptosomes were isolated from cortices of adult Fisher rat brains (Rehncrona et al., 1979). The cortices were removed and each half placed into a separate dish in either ice-cold (A) 1 mg/ml bovine serum albumin fatty acid free in homogenization buffer (225 mM mannitol, 75 mM sucrose, 0.1 mM EGTA, 10 mM HEPES, pH 7.4) or (B) SCAVEGR™ anti-oxidants in homogenization buffer. SCAVEGR (available at www.BrainBitsLLC.com) contains superoxide dismutase (2.5 μg/ml), catalase (5 μg/ml), vitamin E (1 μg/ml), vitamin E acetate (1 μg/ml) and glutathione reduced (1 μg/ml) with albumin (1 mg/ml). The hemispheres were minced into 2 mm^3 pieces. After discarding the wash solution, the tissue was incubated with subtilisin A (0.67 mg/ml) in 7.5 ml buffer A or B (with SCAVEGR) on ice for 1 min. Next, the mixtures were diluted with 10 ml homologous buffer and homogenized with 10 strokes in separate 30 ml glass tubes with Teflon pestles (Fisher Scientific) at 4 °C. The homogenates were poured into centrifuge tubes along with a rinse of 3.5 ml buffer. Homogenates were centrifuged for 3 min at 4500 rpm (2500 × *g*). The supernatants were saved while the pellets were resuspended in 10 ml

homologous buffers and recentrifuged. The supernatants were combined and centrifuged for 8 min at 10,000 rpm (12,000 × *g*). The pellets were resuspended in 20 ml homologous buffers, combined with 5 ml Percoll (Amersham Biosciences, Piscataway, NJ), mannitol (40 mg/ml) and sucrose (25 mg/ml). These mixtures were centrifuged for 35 min at 38,000 rpm (100,000 × *g*). The white synaptosome layer was recovered from the top into 30 ml PBS (osmolarity adjusted to 351 mOsm for TBAR assays) or buffer A without albumin (respiration assays). The lower brown mitochondria layer was similarly recovered. Synaptosomes and mitochondria were centrifuged for 10 min at 9000 rpm (10,000 × *g*) to remove the Percoll. Each pellet was resuspended into 0.6 ml PBS (for TBAR) or buffer A without albumin (for respiration). Protein content was determined using the Bradford reagents (Biorad, Hercules, CA) in a uQuant spectrophotometer (Bio-Tek Instruments, Winooski, VT).

2.2. Thiobartituric acid reactive (TBAR) lipids (Lauderback et al., 2001)

Triplicate 200 μl blank samples of PBS or aliquots of each sample were precipitated with 0.4 ml trichloroacetic acid and 1 μM butylated hydroxytoluene to inhibit additional oxidation. The samples were centrifuged at 6000 rpm for 5 min 0.4 ml of the supernatant was incubated with 0.2 ml thiobarbituric acid (0.335% in 50% glacial acetic acid) for 1 h at 100 °C in plastic Eppendorf tubes with a needle puncture hole in the cap. Following incubation, the samples were cooled to room temperature. 0.4 ml butanol was added to each tube, vortexed, and then centrifuged for 30 s. Triplicate aliquots of 100 μl were removed from the top organic phase of each tube and placed in a 96-well plate. The fluorescence was measured on a Gemini SpectraMax plate reader (Molecular Devices, Sunnyvale, CA) with excitation at 518 nm and emission at 588 nm.

2.3. Respiration

Mitochondria were diluted to 0.2 mg/ml in 2 ml respiration buffer containing 225 mM mannitol, 75 mM sucrose, 10 mM Hepes (pH 7.4), 5 mM $MgCl_2$, 10 mM NaH_2PO_4, 5 μM rotenone to inhibit complex 1, 5 IU/ml hexokinase with 10 mM glucose to maintain constant ADP concentrations, and 5 mM succinate to supply electrons into complex 2 of the respiratory chain. Creatine (20 mM) was added as indicated from solid. Respiration was measured at 25 °C in parallel compartments of a high resolution Oroboros respirometer (Innsbruck, Austria) stirred at 500 rpm. The rate of oxygen consumption before addition of mitochondria was subtracted from all measurements. Respiration in state 4 was determined before addition of ADP. Titration of ADP in four concentration increments of 2.5 μM, followed by three increments of 5 μM and one of 50 μM was used to determine the apparent K_m and V_{max} of ADP for respiration by double reciprocal plots (rate of respiration vs. ADP concentration). Respiratory control ratios were calculated as the ratio of respiration in the presence of succinate with saturating ADP (maximal ATP synthesis) divided by that in the absence of ADP (no ATP synthesis).

2.4. Native gel electrophoresis for oligomers of creatine kinase

Samples from several preparations of mitochondria were frozen. Blue native gel electrophoresis was conducted according to Schagger et al. (1994). Briefly, mitochondrial suspensions were thawed and incubated for 1 h at 4 °C with an equal volume of 200 mM Na_2HPO_4 (pH 9.0) to extract the brain isoform of mitochondrial creatine kinase, uMtCK. The samples were centrifuged for 10 min at 14,000 × *g*, saving the supernatant. The pellet was resuspended in the same buffer, incubated again and recentrifuged. Twenty micrograms of protein from the combined supernatants were applied to a 6–16% gradient polyacrylamide gel and electrophoresed at 4 °C. Proteins in the gel were electrotransferred in a semidry transfer unit (Biorad) to an Immobilon filter, blocked for 1 h with 4% non-fat dry milk in Tris-buffered saline (TBS) and incubated for 1 h at room temperature with a 1:1000 dilution of rabbit anti-uMtCK in TBS-milk (Schlattner et al., 2002). After rinsing four times for 5 min in TBS-milk, the filter was incubated for 1 h with a 1:5000 dilution of goat anti-rabbit IgG conjugated to peroxidase (Calbiochem). After rinsing four times for 5 min in milk, the creatine kinase dimer and octamer bands

were detected by chemiluminescence with Renaissance reagent (Amersham) and X-ray film. Films were scanned and analyzed for density of bands with Image-Pro + software (Media Cybernetics, Silver Spring, MD).

2.5. Electron microscopy

Samples of mitochondria and synaptosomes prepared with or without anti-oxidants were centrifuged for 30 s at 13,000 rpm, fixed for 45 min in 2.5% glutaraldehyde, postfixed with 2% OsO_4, dehydrated, embedded in Epon/Araldite and sectioned. Sections of mitochondria were stained with uranyl acetate and lead citrate and examined in a Joel 100CX electron microscope at 80 kV accelerating voltage and film magnification of 13,000.

3. Results

To evaluate the effect of an anti-oxidant medium during the homogenization and isolation of brain mitochondria, mitochondria were isolated with and without SCAVEGR anti-oxidants into a common medium without anti-oxidants. The effect of SCAVEGR on lipid oxidation products that are reactive with thiobarbituric acid (TBARS) were determined for Percoll gradient-purified mitochondria, as well as for the fraction of synaptosomes that also contain mitochondria from the same purification step (Fig. 1). Both the isolated mitochondria and synaptosomes were reduced in their TBAR levels by over 40% by including SCAVEGR anti-oxidants during the isolation procedure. The yield of mitochondria was 17% larger with SCAVEGR in the isolation, but this difference was not statistically significant for an n of only three preparations (Table 1). The yield is subject to considerable variability in the choice of how much material is removed from the gradient, especially since the boundary of the lighter synaptosomes and the brown mitochondria is indefinite.

The functional consequence of less oxidized mitochondrial lipids was evaluated by respirometry. Fig. 2 shows the analysis of the rate of oxygen consumption as a function of ADP concentration.

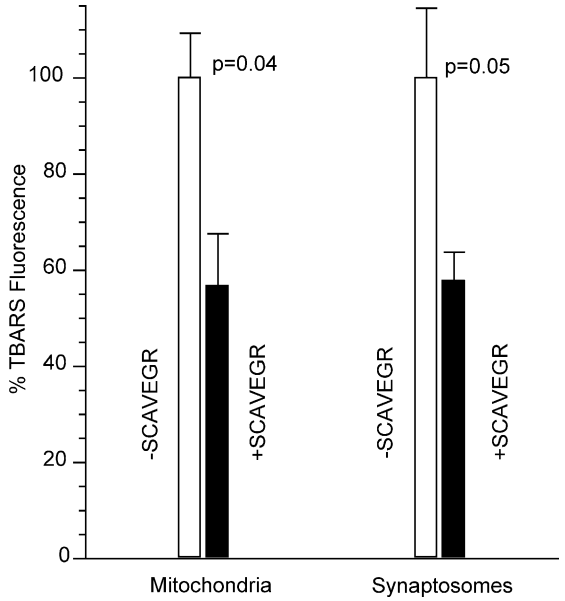

Fig. 1. SCAVEGR anti-oxidants reduce membrane lipid peroxidation. Lipid oxidation was measured as thiobarituric acid reactive material (TBAR) in mitochondria and synaptosomes isolated with (solid) and without (open) SCAVEGR anti-oxidants. To reduce inter-animal variability, values were normalized to readings from mitochondria minus SCAVEGR of each preparation. The absolute averages for the three preparations minus SCAVEGR were 0.368 fluorescence units/μg protein for mitochondria and 0.139 fluorescence units/μg protein for synaptosomes.

Table 1
Summary of yield and respiratory characteristics from three preparations of mitochondria with and without SCAVEGR anti-oxidants ($n = 3$ preparations)

	Control	+SCAVEGR	Ratio SCAVEGR/ control
Yield of mitochondria (mg protein/brain)	5.3 ± 0.4	6.2 ± 0.6	1.17
Respiration K_m for ADP (μM)			
− Cr	29 ± 7	62 ± 11	2.1 ($P = 0.05$)
+ Cr	29 ± 3	20 ± 3	0.7
Ratio (+Cr/−Cr)	1.0	0.3 ($P = 0.02$)	
V_{max}(nmolO_2/min mg)	125 ± 62	262 ± 45	2.1 ($P = 0.05$)
Respiratory control ratio	4.7 ± 0.3	5.4 ± 0.5	1.15 ($P = 0.04$)

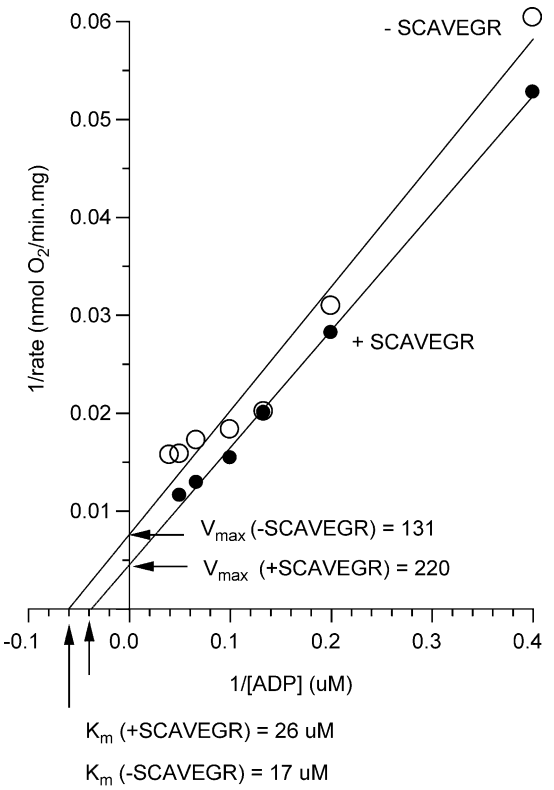

Fig. 2. SCAVEGR anti-oxidants reduce respiratory K_m for ADP. SCAVEGR anti-oxidants (solid circles) causes about a two-fold increase in V_{max} of respiration compared to without SCAVEGR (open circles). Measurements were done with brain mitochondria energized with succinate and stimulated with 2.5–75 μM ADP. Results are representative of three experiments averaged in Table 1.

From the extrapolated intercept with the *y*-axis, the V_{max} was two-fold higher for mitochondria isolated with SCAVEGR, compared to those without (Table 1). The intercept on the *x*-axis shows that the apparent K_m (ADP) in the absence of creatine was modestly increased by SCAVEGR treatment (Fig. 2), with an average two-fold increase for three experiments to 62 μM, in the absence of creatine (Table 1). In the presence of creatine, the apparent K_m (ADP) remained unchanged at 29 μM for mitochondria isolated without SCAVEGR, while it was significantly reduced from 62 to 20 μM when using SCAVEGR (Table 1). The respiratory control ratio in the presence of saturating ADP (maximal ATP synthesis) divided by that in the absence of ADP (no ATP synthesis) was significantly increased by 15% for mitochondria isolated in the presence of SCAVEGR reaching levels of 4.8–6.3 (Table 1).

Electron microscopy of mitochondrial preparations prepared with SCAVEGR and without revealed no major differences in morphology (Fig. 3). In both cases, cristae were condensed and abundant with intact outer membranes. There was significant contamination of these brain mitochondrial preparations with synaptosomes containing mitochondria and numerous 30–40 nm diameter electron-lucent vesicles. In the synaptosome fraction, electron microscopy also revealed no ultrastructural differences between samples prepared with and without anti-oxidants.

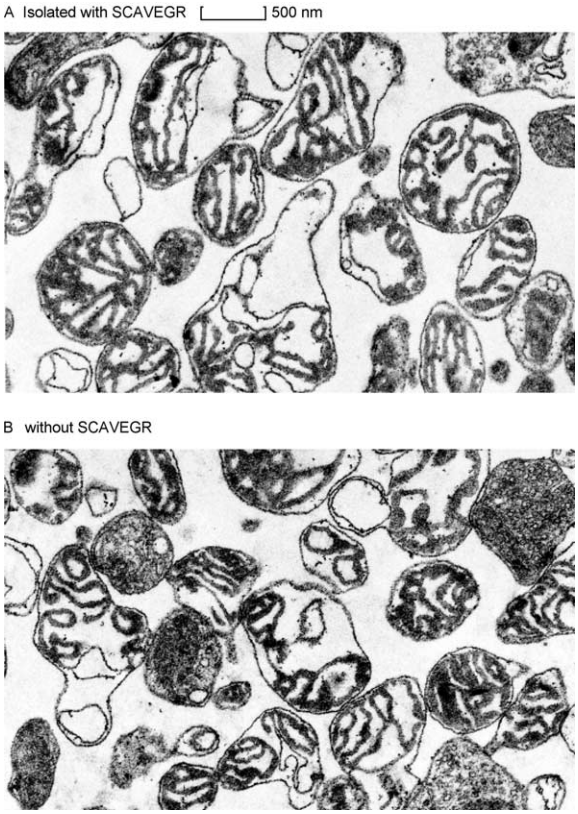

Fig. 3. SCAVEGR anti-oxidants do not affect mitochondrial ultrastructure. Electron micrographs of mitochondria isolated (A) with SCAVEGR and (B) without SCAVEGR from one preparation.

Nitrogen radicals produced by mitochondria are known to cause disassembly of the active octamer of mitochondrial creatine kinase (uMtCK) into dimers (Stachowiak et al., 1998a,b). To analyze the effect of SCAVEGR on uMtCK, the isoform of MtCK found in brain, we performed native gel electrophoresis that resolves octamers and dimers of uMtCK, followed by blotting and immunodetection with anti-uMtCK antibodies. Fig. 4 shows that higher levels of the active uMtCK octamer remained after mitochondrial isolation in the presence of SCAVEGR. Mitochondria from three preparations showed an average 8.7-fold densitometric ratio of octamer to dimer for isolations with SCAVEGR and a ratio of 2.9 without ($P < 0.05$).

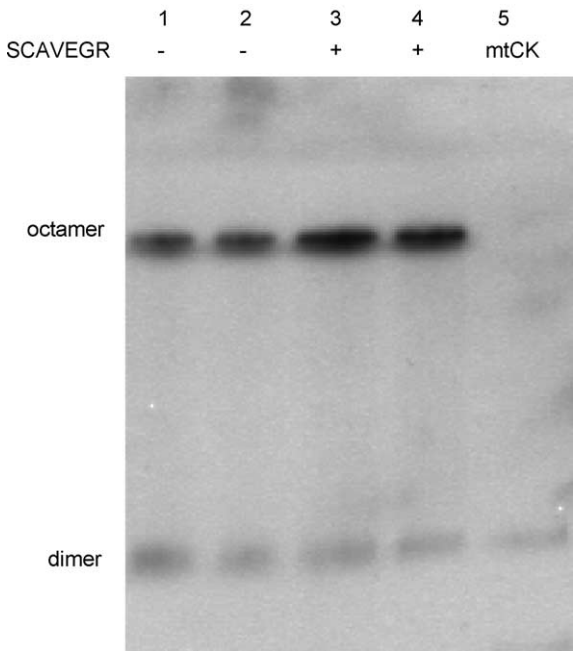

Fig. 4. SCAVEGR anti-oxidants preserve the octamer active state of ubiquitous mitochondrial creatine kinase (uMtCK) in isolated mitochondria. uMtCK octamers and dimers were visualized by native gel electrophoresis and immunoblot detection with anti-uMtCK antibodies. Lanes 1 and 2, two preparations of mitochondria isolated without SCAVEGR; lanes 3 and 4, two preparations of mitochondria isolated with SCAVEGR; lane 5, dimer form of purified recombinant mitochondrial creatine kinase. Representative of a total of five preparations. Note also the higher recovery of immunodetectable uMtCK with SCAVEGR (lanes 3 and 4), as well as the dissociation of recombinant marker uMtCK into dimers due to dilution (Schlattner and Wallimann, 2000).

4. Discussion

A 40% reduction in oxidized lipids measured as TBARs was achieved by homogenization and isolation of brain mitochondria in a mixture of five anti-oxidants and albumin. Functionally, these mitochondria isolated with SCAVEGR have a two-fold higher V_{max} and a 15% improvement in respiratory control ratio, compared to mitochondria isolated with albumin without anti-oxidants. Furuno et al. (2001) found that the addition of albumin to isolated rat liver mitochondria suppressed the permeability transition and prevented respiratory run down during storage on ice, probably by adsorption of free fatty acids and aldehydes released from damaged mitochondria in the process of isolation and from ceramides (Siskind et al., 2002). Free fatty acids are known to act as uncouplers that would reduce the respiratory control ratio (Arslan et al., 1984). Both our anti-oxidant cocktail and control treatments contained albumin, indicating an additional benefit from the five anti-oxidants. Gnaiger et al. (2000) reported use of a preservation medium for rat heart mitochondria consisting of 16 ingredients, including anti-oxidants 20 μM vitamin E and 3 mM glutathione, 10 and 1000 times higher than our SCAVEGR preparation. No comparisons were made to common respiratory characteristics without anti-oxidants. Friberg et al. (1999) added 2 mM glutathione during isolation of brain mitochondrial without effect on their swelling assay. Together, these anti-oxidants could inhibit damage from reactive oxygen species produced by mitochondria or by damage to other organelles during the isolation process. Since mitochondrial electron transport should be low at 4 °C and in the absence of substrates, other sources of reactive oxygen species such as disrupted lysosomes and peroxisomes should be considered for inhibition by SCAVEGR.

The apparent Michaelis–Menten rate constant, K_m, for ADP of respiration in brain mitochondria that we measured was 29 μM in the absence of SCAVEGR and 62 μM for isolations in the presence of SCAVEGR. These values are similar to those reported by Costa et al. (1997) of 30 μM for liver and 52 μM for heart mitochondria under similar assay conditions without SCAVEGR. In mitochondria containing MtCK, addition of creatine to the respiration buffer generally lowers K_m (ADP) due to additional,

intramitochondrial regeneration of ADP by creatine kinase. However, under our standard isolation conditions lacking anti-oxidants, apparent K_m for ADP of brain mitochondria appears independent of creatine. In contrast, for mitochondria isolated with anti-oxidants, the K_m for ADP decreases over three-fold to 20 μM upon addition of creatine. Thus, SCAVEGR maintains creatine-stimulated respiration, while this stimulation seems to be lost quite rapidly in mitochondria isolated with the standard procedure. This suggests that either mitochondrial creatine kinase or its coupling to adenine nucleotide transporter is affected by oxidation in the standard isolation medium. This conclusion, i.e. protection of MtCK from oxidation by SCAVEGR, is supported by the enhanced octamer form that we observe for mitochondria isolated with anti-oxidants. In contrast to dimers of MtCK, the octamer strongly binds to the mitochondrial porin (Schlattner et al., 2001) and membranes (Stachowiak et al., 1998b), and is functionally associated with adenine nucleotide translocator (Khuchua et al., 1998). Octameric MtCK is therefore considered as the physiologically active oligomeric form. It is known as a prime target for inactivation by free radicals (Koufen et al., 1999) and peroxynitrite (Stachowiak et al., 1998b), causing dimerization by oxidation of tryptophan residues at the dimer–dimer interface (Wendt et al., 2003).

Brookes et al. (1998) added increasing amounts of peroxynitrite to rat brain mitochondria. Small additions stimulated non-phosphorylative state 4 respiration due to increased proton leak, which could be inhibited by a vitamin E analogue that inhibits oxidation of mitochondrial membrane lipids. Larger amounts of peroxynitrite inhibited respiration. An internal source of peroxynitrite could be generated by activation of the mitochondrial nitric oxide synthase (Bates et al., 1995; Ghafourifar and Richter, 1997). Richter et al. (1997) reported the effects of inhibition of cytochrome oxidase by nitric oxide and its normoxic by-product, peroxynitrite, to stimulate calcium release from mitochondria (reviewed further by Richter, 1997). Other mitochondrial enzymes commonly known to be more sensitive to oxidative inhibition include aconitase (Bulteau et al., 2003) and complex I NADH-ubiquinone oxido-reductase (Murray et al., 2003; Paradies et al., 2002).

The increased V_{max} with anti-oxidants may be partially attributed to the reduced glutathione present in SCAVEGR. McStay et al. (2002) reported that the adenine nucleotide transporter (ANT) has critical thiol groups that control the mitochondrial permeability transition. Reduced thiols allow protection of the permeability transition by ADP above 10 μM, but after treatment with 500 μM *N*-ethylmaleimide, required ADP above 200 μM to inhibit. Depletion of glutathione increases oxyradicals and stimulates the permeability transition (Armstrong and Jones, 2002). Another anti-oxidant link comes from studies of the lipid aldehyde, 4-hydroxy-2-nonenal (HNE) that would be measured in our TBARS assay. HNE inhibits rat brain mitochondria at complexes I, II and most significantly at complex III (Picklo et al., 1999). In another study of anti-oxidants, mice given oral vitamin E showed in their mitochondria reduced generation of the superoxide anion radical (Lass and Sohal, 2000). Lipid peroxidation was lower and reduced glutathione levels increased in guinea pig heart after supplementation with vitamin E (Rojas et al., 1996). Thus, there is abundant support in the literature for the need for anti-oxidants for normal mitochondrial function.

We have isolated mitochondria in the presence of Cu/Zn-superoxide dismutase to replace the normal cytoplasmic enzyme that converts the superoxide anion to the less toxic hydrogen peroxide. However, a recent report presents evidence for a mitochondrial localization of Cu/Zn SOD, in addition to the Mn-SOD in the mitochondrial matrix (Okado-Matsumoto and Fridovich, 2001). Our anti-oxidant mixture also contains catalase to degrade hydrogen peroxide. Catalase has recently been shown to reduce the permeability transition in brain mitochondria (Maciel et al., 2001).

In summary, mitochondria prepared with SCAVEGR anti-oxidants display a 40% reduction in oxidized lipids, and a two-fold higher respiratory V_{max}. Although differences were not seen by electron microscopy, the anti-oxidants protected creatine-stimulated respiration and the octamer native form of mitochondrial creatine kinase, a sensitive measure for oxidative damage. Together, these results suggest that brain mitochondria are sensitive to reactive oxygen species, but significant improvements in mitochondrial function are

obtained by isolation in the presence of a mixture of anti-oxidants that also promotes neuron survival in culture. Since mitochondria from other sources also contain considerably more unsaturated lipids than other membranes, their isolation in anti-oxidants may also be beneficial.

Acknowledgements

We thank Else Zanolla for assistance with electron microscopy and Dr Max Dolder for methodological advice. This work was supported in part by Swiss Natl. Science Foundation (31-62024.00) to T.W. and U.S., Novartis Foundation for Biomedical Research to U.S., and NIH AG13435 to G.B.

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