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Biochemical Pharmacology 70 (2005) 1361–1370
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

The protection of bioenergetic functions in mitochondria by new synthetic chromanols

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Received 6 June 2005; accepted 29 July 2005

Abstract

α-Tocopherol is the most important lipophilic antioxidant of the chromanol type protecting biomembranes from lipid peroxidation (LPO). Therefore, α-tocopherol and its derivatives are frequently used in the therapy or prevention of oxygen radical-derived diseases. In the present study, novel chromanol-type antioxidants (twin-chromanol, *cis*- and *trans*-oxachromanol) as well as the well-known short-chain analogue of α-tocopherol, pentamethyl-chromanol, were tested for their antioxidative potency in rat heart mitochondria (RHM). Our experiments revealed that the bioenergetic parameters of mitochondria were not deteriorated in the presence of chromanols (up to 50 nmol/mg protein). Exposure of RHM to cumene hydroperoxide and Fe^{2+} (final concentrations 50 μM each), inducing LPO, significantly affected their bioenergetic parameters which were determined in the presence of glutamate and malate (substrates of mitochondrial complex I). Alterations of the bioenergetic parameters were partially prevented in a concentration-dependent manner by preincubating RHM with antioxidants before adding the radical-generating system. In the lower concentration range, twin-chromanol turned out to be more efficient than pentamethyl-chromanol, both being far more protective than *cis*- and *trans*-oxachromanol. Measurement of protein-bound SH groups and thiobarbituric acid-reactive substances revealed that this protective effect was due to their antioxidative action. Furthermore, HPLC measurements of α-tocopherol and α-tocopheryl quinone in rat liver mitochondria demonstrated an α-tocopherol-sparing effect of twin-chromanol. In conclusion, new chromanol-type antioxidants, especially twin-chromanol, were able to improve bioenergetic and biochemical parameters of mitochondria exposed to oxidative stress.
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Keywords: Mitochondria; Bioenergetics; Lipid peroxidation; Thiol oxidation; Chromanols; Tocopheryl quinone

1. Introduction

Mitochondria are continuously exposed to a flux of reactive oxygen species (ROS) either produced by respiratory complexes or by other sources such as microsomal oxygenases and extracellular inflammatory responses [1,2]. Therefore, mitochondria possess an extensive set of enzymatic [3] and non-enzymatic [4] antioxidants to prevent their damage. This is of considerable physiological importance, since the bioenergetic function of mitochondria strongly relies on both the integrity of the membranes for maintaining membrane potential driving ATP synthesis and the proper function of electron-transfer enzymes in the inner mitochondrial membrane. Especially the latter are known to be very sensitive to changes of their membrane environment and can considerably increase the release of ROS upon degradation [5].

The protection of mitochondrial membranes from oxidative damage is guaranteed by the cooperative action of continuously recycled ubiquinol (reduced ubiquinone) and α-tocopherol. The chemical basis for this highly efficient cooperation is the reduction of the α-tocopheroxyl radical

Abbreviations: BHT, 3,5-di-*tert*-butyl-4-hydroxy-toluene; CumOOH, cumene hydroperoxide; DTNB, 5,5′-dithio-bis-(2-nitrobenzoic acid); LPO, lipid peroxidation; MPTP, mitochondrial permeability transition pore; OCA, oxachromanol; PMC, 2,2,5,7,8-pentamethyl-chroman-6-ol; RHM, rat heart mitochondria; RLM, rat liver mitochondria; ROS, reactive oxygen species; TBA, 2-thiobarbituric acid; TBARS, TBA-reactive substances; Toc, α-tocopherol; TQ, α-tocopheryl quinone; Twin, twin-chromanol (1,3,4,8,9,11-hexamethyl-6,12-methano-12H-dibenzo[d,g][1,3]dioxocin-2,10-diol)

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doi:10.1016/j.bcp.2005.07.030

by ubiquinol [6]. Due to this mechanism, only about 3–5% of α-tocopherol exists in its oxidized state (α-tocopheryl quinone) under physiological conditions [7]. However, under pathophysiological conditions, such as ischemia/reperfusion injury, this protection is not sufficient to maintain a proper mitochondrial function [8]. Therefore, a therapeutic intervention in such disorders would be of interest. Unfortunately, many clinical trials supplementing the diet of risk patients with high doses of Vitamin E (containing large amounts of α-tocopherol) were unsuccessful in preventing the progression of cardiovascular diseases [9,10]. This inefficiency could have various reasons: (i) an overestimated role of ROS in such pathophysiological events, (ii) the formation of counteracting Vitamin E degradation products, or (iii) the insufficient transport of α-tocopherol to membranes with antioxidant deficiency. Especially the latter problem could be prevented by using Vitamin E analogues which are not under the control of the physiological Vitamin E-sorting and -transporting machinery, including the α-tocopherol transfer protein [11,12] and the distribution of α-tocopherol in the body mediated by low-density lipoproteins [13].

Several attempts have been made to identify more efficient chromanol derivatives by introducing heteroatoms, such as S or N into the chromanol structure [14,15]. The major problem for such compounds is the rather unpredictable metabolic behavior of such heteroatoms, e.g. the toxicity of certain pyridine derivatives [16].

Kinetic experiments in chemical model systems have shown that newly synthesized chromanols (Fig. 1) exhibit in part much better radical-scavenging properties than α-tocopherol and its short-chain analogue pentamethyl-chromanol [17]. Twin-chromanol reacts faster with model radicals than α-tocopherol. In addition, the resulting twin-chromanoxyl radical was recycled by ubiquinol and ascorbic acid much faster than the α-tocopheroxyl radical [17].

Since the mitochondrial respiratory chain was considered as a target of ischemia/reperfusion-induced oxidative stress, the aim of the present study was to estimate the influence of the new chromanols on the bioenergetic function of isolated mitochondria. For this purpose, the antioxidative effect of new chromanols (twin-chromanol and oxachromanols) was compared with that of the well-known pentamethyl-chromanol in mitochondria oxidatively damaged by ferrous iron and cumene hydroperoxide.

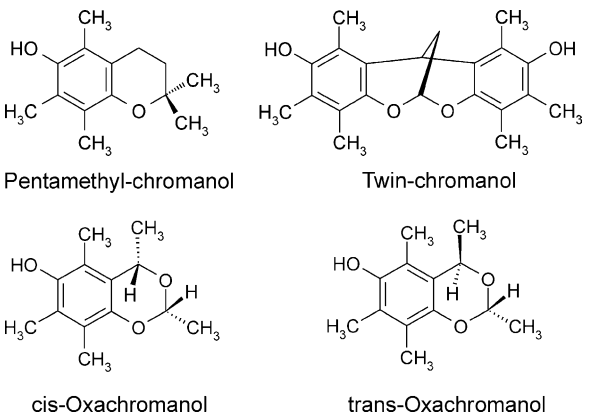

Fig. 1. Structures of chromanols under study.

2. Materials and methods

2.1. Chemicals

1,3,4,8,9,11-Hexamethyl-6,12-methano-12*H*-dibenzo [d,g] [1,3] dioxocin-2,10-diol (twin-chromanol; Twin; purity >99.8% (HPLC); elementary analysis: calculated C 74.09, H 7.11; found: C 73.97, H 7.24), *cis*- and *trans*-2,4,5,7,8-pentamethyl-4*H*-1,3-benzodioxin-6-ol (oxachromanol; OCA; *cis*-OCA: purity > 99.3% (HPLC); elementary analysis: calculated C 70.25, H 8.16; found C 70.35, H 8.05; *trans*-OCA: purity 100.0% (HPLC), elementary analysis: calculated C 70.25, H 8.16; found C 70.31, H 8.08) were synthesized as described in [18,19]. Essentially fatty acid-free BSA (fraction V), L-malic acid, rotenone, 2,2,5,7,8-pentamethyl-chroman-6-ol (PMC; purity > 97%), 3,5-di-*tert*-butyl-4-methylphenol (BHT), *n*-butanol, 1,1,3,3-tetramethoxypropane, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB), and GSH were purchased from Sigma-Aldrich Handels-GmbH (Vienna, Austria), triethanolamine-HCl, succinic acid, and L-glutamic acid from Fluka (Buchs, Switzerland); ADP (potassium salt) from Boehringer (Mannheim, Germany), desferal from Novartis (Vienna, Austria), HPLC-grade solvents and other chemicals from Merck (Darmstadt, Germany).

2.2. Preparation of mitochondria

Rat heart mitochondria (RHM) and liver mitochondria (RLM) were isolated from male Sprague–Dawley rats (Him:OFA/SPF) weighing 320 ± 14 g as described previously [20] in a buffer containing 0.3 M sucrose (0.25 M for RLM), 20 mM triethanolamine, 1 mM EGTA, 0.1 mg/ml BSA (pH 7.4, 4 °C). The protein content of the mitochondrial suspension was measured by the Biuret method using BSA as a standard [21].

2.3. Mitochondrial oxygen consumption

Respiratory parameters of RHM were determined with a Clark-type oxygen electrode of own design. Mitochondria (0.5 mg/ml) were incubated in a buffer consisting of 0.3 M sucrose, 20 mM triethanolamine, 1 mM diethylenetriaminepentaacetic acid, 4 mM KH_2PO_4 (pH 7.4, 25 °C). State 4 respiration was stimulated by the addition of 5 mM glutamate/5 mM malate (complex I substrates) or 10 mM succinate (complex II substrate) in the presence of 2 μg/ml rotenone. The latter was used to prevent electron

transport from and to complex I. The transition to state 3 respiration was induced by the addition of 200 μM ADP. To exclude any toxic effect of chromanols on intact RHM, their respiratory function was studied in the presence and absence of 25 μM PMC, Twin, *cis*- or *trans*-OCA which corresponds to 50 nmol/mg mitochondrial protein. Respiratory parameters were measured after preincubation of RHM with the respective chromanols or their solvent acetonitrile (3 min, 25 °C) followed by the addition of 0.5 mg/ml BSA.

2.4. Induction of oxidative stress

Lipid peroxidation (LPO) was induced by cumene hydroperoxide (CumOOH) and $FeSO_4$. Respiratory parameters were determined in a buffer consisting of 0.3 M sucrose, 20 mM triethanolamine, 4 mM KH_2PO_4 (pH 7.4, 25 °C). For the demonstration of antioxidative effects of chromanols, RHM (0.5 mg/ml) were preincubated in the chamber for oxygen measurements with 0, 0.5, 1, 2.5, 5, or 12.5 μM PMC, Twin, *cis*- or *trans*-OCA (2 min, 25 °C). After the addition of 50 μM CumOOH (1 min, 25 °C) followed by 50 μM $FeSO_4$ (2 min, 25 °C) for the stimulation of LPO, BSA (0.5 mg/ml) was supplemented and the respiratory parameters were immediately determined with glutamate/malate. Exactly 10 min after the initiation of LPO, i.e. after the addition of $FeSO_4$, aliquots of the samples were withdrawn for the determination of protein-bound thiol groups and 2-thiobarbituric acid (TBA)-reactive substances.

2.5. Determination of mitochondrial protein-bound thiol groups

Aliquots of mitochondrial samples (200 μl) were immediately vortex-mixed for 10 s with a solution containing trichloroacetic acid (0.3 M final concentration) and EDTA-Na_2 (10.5 mM final concentration). Proteins were allowed to precipitate for 10 min and were centrifuged for 10 min at 3.000 × *g*. Sediments were dissolved and incubated for 5 min at 25 °C in a buffer consisting of 0.2 M Tris–HCl, 10 mM EDTA-Na_2 (pH 8.5), 0.5% (w/v) SDS, and 0.1 mM DTNB. Thiol groups were determined spectrophotometrically at 412 nm according to Ellman [22] using GSH for calibration.

2.6. Determination of TBA-reactive substances (TBARS) in RHM

For termination of the radical-induced chain reaction, aliquots of mitochondrial samples (200 μl) were immediately transferred into a tube containing BHT and the iron chelator desferal (0.5 mM final concentration), vortex-mixed for 15 s, frozen and stored in liquid nitrogen (7–8 weeks). Determination of mitochondrial TBARS was adapted from a method described by Yagi [23]. For analysis, mitochondrial samples were thawed and centrifuged at 4 °C (10 min, 20.000 × *g*). The supernatants were completely removed and the pellets were resuspended in deionized water. Suspensions were precipitated with H_2SO_4 (3.3 M final concentration) and phosphotungstic acid (1% (w/v) final concentration) for 5 min at 22 °C and centrifuged at 4 °C (10 min, 2.000 × *g*). The resulting sediments were once more resuspended in H_2SO_4 and phosphotungstic acid, allowed to precipitate, and centrifuged. After complete removal of the supernatants, these sediments were suspended in 600 μl deionized water supplemented with BHT and desferal (32 μM each). The samples were mixed with 150 μl of the TBA reagent (46.5 mM TBA dissolved in a mixture of glacial acetic acid and deionized water, 1:1) and heated to 95 °C for 60 min. After cooling on ice, TBARS were extracted into 750 μl *n*-butanol by vortex-mixing for 30 s. After centrifugation (4 °C, 10 min, 2.000 × *g*), 600 μl of the butanol phase was mixed with 1400 μl butanol (22 °C) in a 3 ml quartz cuvette. Fluorescence measurements were performed on a Hitachi F4500 spectrofluorimeter at 700 V PMT voltage, 515 nm excitation (5 nm slit width), and 553 nm emission wavelengths (20 nm slit width). Concentrations of TBARS were calculated from a calibration curve with 1,1,3,3-tetramethoxypropane as standard.

2.7. Protection of rat liver mitochondria from lipid peroxidation

Mitochondria (4 mg protein per ml buffer containing 0.25 M sucrose, 20 mM triethanolamine, pH 7.4) were supplemented with 0 μM (acetonitrile only), 4 μM, 20 μM PMC or Twin. The respiration of RLM was initiated by the addition of 20 mM succinate and the oxygen concentration was monitored by a Clark-type oxygen electrode. After 1 min incubation, LPO was initiated by the addition of 400 μM CumOOH and 100 μM $FeSO_4$ in all samples (except one control experiment). From each incubation, 500 μl aliquots for HPLC measurements were taken at 0 (prior to initiating LPO), 5, 10 and 15 min. LPO was stopped by the addition of 0.5 mM desferal plus 0.5 mM BHT and samples were subsequently stored at −196 °C.

2.8. HPLC analysis of quinones and α-tocopherol in rat liver mitochondria

For each sample, 2 mg of mitochondrial protein in 0.5 ml H_2O were admixed with SDS (5 mM final concentration) prior to extraction with 1 ml ethanol/hexane (2:5). After phase separation, the organic phase was removed and evaporated to dryness. The residue was dissolved in ethanol and analyzed by HPLC on a Waters LC1 module equipped with a UV detector for measurements of quinones at 268 nm (TQ) and 275 nm (UQ). Reduced quinones and α-tocopherol were determined by an

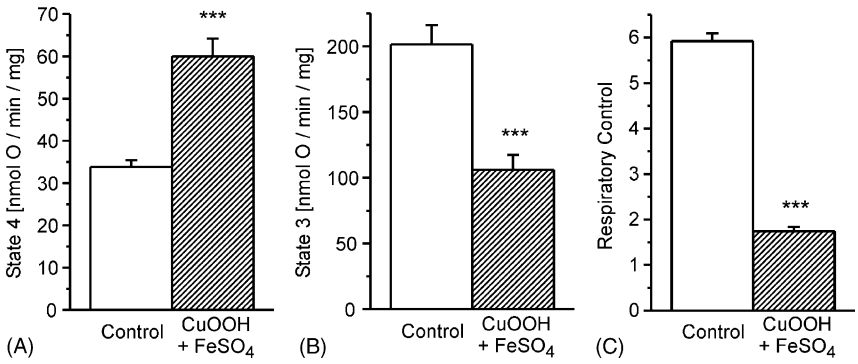

Fig. 2. State 4 respiration (A), state 3 respiration (B) and respiratory control value (C) of glutamate/malate-respiring RHM in the absence (control with acetonitrile) or presence of 50 μM cumene hydroperoxide plus 50 μM $FeSO_4$. Data are mean ± S.E.M. of six independent mitochondrial preparations, ***$p < 0.001$ in comparison to the control.

electrochemical detector (Shimadzu L-ECD-6A), which was set to a potential of +0.6 V. The column (Merck, Lichrospher 100 RP-18 (5 μm) 4 mm × 125 mm) was eluted with a mobile phase consisting of $NaClO_4$ (50 mM) dissolved in a mixture of ethanol, methanol, acetonitrile and $HClO_4$ (400:300:300:1) at 1 ml/min. Concentrations of α-tocopheryl quinone (TQ) and α-tocopherol (Toc) were calculated using an internal standard of UQ_6.

2.9. Statistics

All data are given as means ± S.E.M. of four to six independent mitochondrial preparations. Differences between two groups were analyzed by the unpaired two-tailed Student's *t*-test. For comparison of multiple groups, the one-way ANOVA followed by a post hoc Tukey HSD test was performed (SPSS 11.5). A $p < 0.05$ was considered as significant.

3. Results

To exclude any toxic effect of the novel chromanol-type antioxidants on intact RHM, their respiratory parameters were determined in the presence (25 μM final concentration) or in the absence of the respective chromanols (controls with the solvent acetonitrile). The effect of the novel chromanols was compared with that of pentamethyl-chromanol, the short-chain analogue of α-tocopherol. Mitochondrial oxygen consumption was measured in the absence and in the presence of ADP using glutamate/malate as respiratory substrates for complex I. The respiratory control value represents the ratio of state 3 to state 4 respiration. The ADP/O ratio reflects the efficiency of ATP synthesis. None of the chromanols tested significantly changed these bioenergetic parameters of RHM in comparison to controls (state 4: 27 ± 2 nmol O/min/mg; state 3: 143 ± 11 nmol O/min/mg; respiratory control: 5.41 ± 0.25, ADP/O ratio: 2.24 ± 0.5).

Also in rotenone-inhibited succinate-respiring RHM (substrate of mitochondrial complex II) no detrimental effect of the tested chromanols was observed. The respiratory parameters of chromanol-supplemented RHM (50 nmol/mg mitochondrial protein, 25 μM) were not significantly different from their respective controls which were incubated with acetonitrile only (state 4: 113 ± 4 nmol nmol O/min/mg; state 3: 322 ± 10 nmol O/min/mg; respiratory control: 2.85 ± 0.05, ADP/O ratio: 1.22 ± 0.04). A significant difference ($p < 0.05$) was only observed in the presence of Twin which slightly reduced the respiratory control value to 2.58 ± 0.06.

Bioenergetic parameters of mitochondria are very sensitive to changes of their inner membranes which can have severe consequences for controlled electron transfer and cellular ATP supply. In order to test the benefit of antioxidants in protecting mitochondrial functions, LPO was induced in mitochondrial membranes by the addition of CumOOH and $FeSO_4$ to simulate these changes. Fig. 2 shows that exposure of RHM to CumOOH and $FeSO_4$ significantly deteriorated their bioenergetic function. The dramatic impairment of respiratory control values (Fig. 2C) was caused not only by an increased state 4 respiration (Fig. 2A), but also by a decreased state 3 respiration (Fig. 2B). In addition, the pretreatment of RHM with the radical-generating system significantly reduced the ADP/O ratio in comparison to the control group (1.4 ± 0.1 versus 1.73 ± 0.03; $p < 0.05$).

This oxidative damage was partially abolished in a concentration-dependent manner by preincubating RHM with chromanols before adding the radical-generating system. The restoration of the respiratory control values (Fig. 3A) was mainly due to decreased oxygen consumption rates during state 4 respiration (Fig. 3B), and to a smaller extent to increased state 3 respiration rates in the presence of the tested chromanols (data not shown). In the lower concentration range, twin-chromanol turned out to be more efficient than PMC, both being far more protective than *cis*- and *trans*-oxachromanols (Fig. 3).

In addition to the bioenergetic function, biochemical indicators of oxidative stress in mitochondria were determined to gain insight into the protective mechanism of the chromanols tested in the present study. The mitochondrial

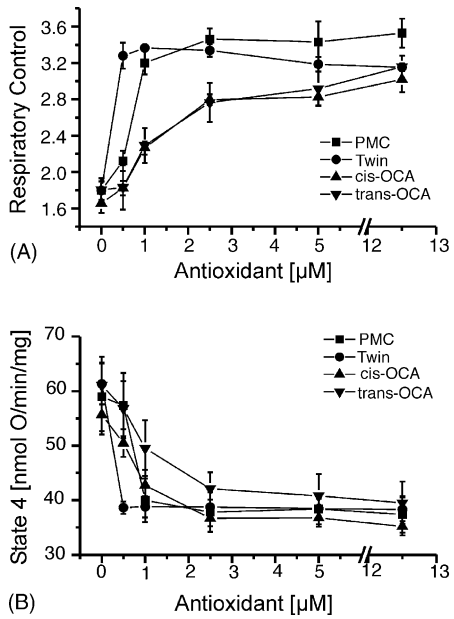

Fig. 3. Concentration-dependent improvement of the respiratory control value (A) and state 4 respiration (B) of glutamate/malate-respiring RHM, exposed to oxidative damage (50 μM cumene hydroperoxide plus 50 μM $FeSO_4$), by pentamethyl-chromanol, twin-chromanol, *cis*- or *trans*-oxachromanol in comparison to the acetonitrile control (0 μM antioxidant). Data are mean ± S.E.M. of four to six independent mitochondrial preparations.

content of protein-bound SH groups, which is an indirect indicator of protein oxidation, was significantly decreased by the exposure of RHM to the radical-generating system (Fig. 4A). A pretreatment of RHM with antioxidative chromanols partially protected mitochondrial thiol groups against oxidative stress in a concentration-dependent manner (Fig. 4B). However, only for twin-chromanol (1 and 2.5 μM) the changes were significantly different from the respective controls.

As an indicator of mitochondrial LPO, the content of thiobarbituric acid-reactive substances was determined in the presence and absence of the respective chromanols. Significantly increased amounts of TBARS were observed in membranes of oxidatively damaged RHM, treated with hydroperoxide and Fe^{2+} (Fig. 5A). The formation of TBARS in RHM was fully prevented by a preincubation of RHM with the chromanols under study, i.e., the TBARS concentrations were not significantly different from those of control-incubated mitochondria without exposure to CumOOH/$FeSO_4$ (compare Fig. 5A and B). The mitochondrial TBARS levels were significantly decreased with increasing chromanol concentrations in comparison to CumOOH/$FeSO_4$-treated RHM, which were incubated in the absence of chromanols (Fig. 5B; $p < 0.05$ for concentrations of Twin and *cis*-OCA ≥0.5 μM; concentrations of *trans*-OCA and PMC ≥2.5 μM).

Due to the high radical-scavenging efficiency of the most effective antioxidant twin-chromanol, mitochondrial α-tocopherol should be conserved by this antioxidant during oxidative stress. To test this hypothesis, mitochondria were peroxidized by CumOOH and $FeSO_4$ in the presence or absence of added chromanols (PMC or Twin). In these experiments, RLM were used instead of RHM, since Vitamin E in membranes of RLM and RHM is expected to respond in a similar manner and RLM are available in higher yields than RHM. The conversion of Toc to TQ in mitochondria was followed by HPLC analysis of membrane extracts. HPLC data shown in Fig. 6 demonstrate that PMC and Twin are similarly effective in preventing the decay of Toc (Fig. 6A) and the increase in the TQ/Toc ratio (Fig. 6B) in mitochondrial membranes. For both antioxidants, a concentration of 5 nmol/mg protein (20 μM) is required for the protective effect, whereas the lower concentration of 1 nmol/mg protein (4 μM) was insufficient.

In order to demonstrate the significance of biochemical changes for the bioenergetic function of mitochondria and their prevention by antioxidants, a correlation analysis according to Pearson between bioenergetic and biochemical parameters was performed, which included all data sets of the present study. A strong direct dependence of state 3 respiration on the content of protein-bound thiol groups was observed (Fig. 7A), as well as a strong positive correlation between the concentration of the LPO products,

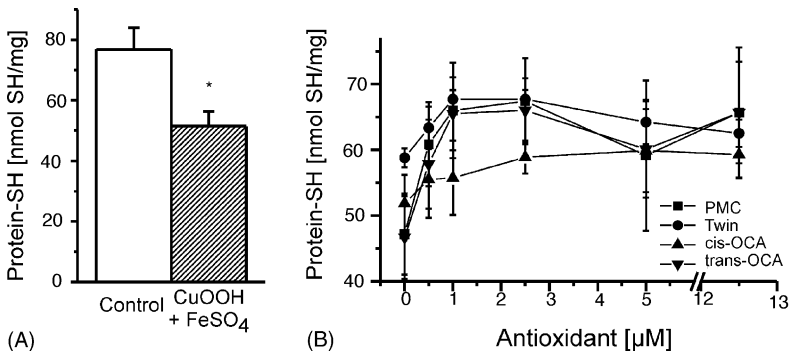

Fig. 4. Content of mitochondrial protein-bound thiol groups after exposure of RHM to 50 μM cumene hydroperoxide plus 50 μM $FeSO_4$ in comparison to the acetonitrile control (A). Concentration-dependent restoration of protein SH groups in CumOOH/$FeSO_4$-treated RHM by pentamethyl-chromanol, twin-chromanol, *cis*- or *trans*-oxachromanol (B). Data are mean ± S.E.M. of four to six independent mitochondrial preparations, *$p < 0.05$ in comparison to the control.

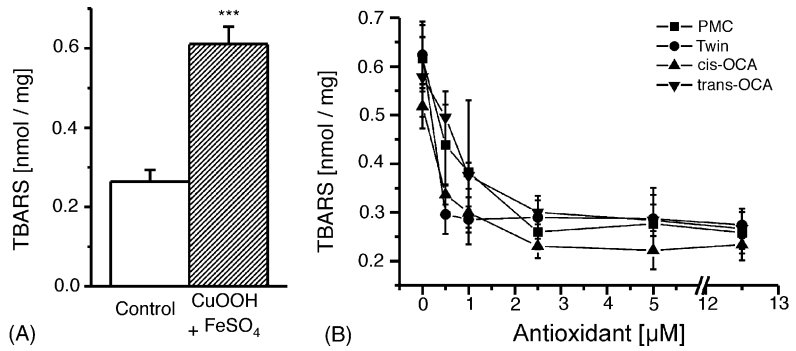

Fig. 5. Content of thiobarbituric acid-reactive substances in mitochondrial membranes after exposure of RHM to 50 μM cumene hydroperoxide plus 50 μM $FeSO_4$, in comparison to the acetonitrile control (A). Concentration-dependent prevention of TBARS formation in CumOOH/$FeSO_4$-treated RHM by pentamethyl-chromanol, twin-chromanol, *cis*- or *trans*-oxachromanol (B). Data are mean ± S.E.M. of four to six independent mitochondrial preparations, $^{***}p < 0.001$ in comparison to the control.

determined as TBARS, and the oxygen consumption during state 4 respiration (Fig. 7B). It can be seen from Fig. 7 that experimental data obtained in the presence of high antioxidant concentrations are more strongly associated with data of control experiments (without LPO), i.e. high state 3 respiration rates with high concentrations of protein-bound SH groups (Fig. 7A) and low state 4 respiration rates with low TBARS concentrations (Fig. 7B). In contrast, there was no significant correlation neither between the thiol content and state 4 respiration ($R = 0.06$) or the ADP/O ratio ($R = -0.11$), nor between the amount of TBARS and state 3 respiration ($R = 0.04$) or the ADP/O ratio ($R = -0.09$).

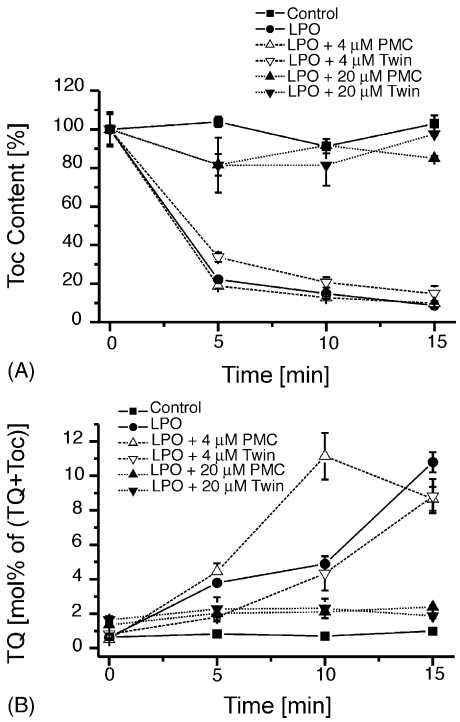

Fig. 6. Time-dependent changes of the Toc content (A: values at $t = 0$ were set to 100%) and of the molar TQ/Toc ratio (B) in mitochondrial membranes (RLM, 4 mg/ml) under control conditions, during LPO induced by CumOOH and $FeSO_4$ and the influence of PMC or twin-chromanol (4 or 20 μM). Data are mean ± S.E.M. of four independent mitochondrial extracts.

4. Discussion

4.1. Considerations on the chemical structure of the tested chromanols

The high antioxidative efficiency of tocopherols and other chroman-6-ols is based on the high rate of their reaction with lipid radicals [24] and their ability to scavenge superoxide radicals [25] leading to the formation of

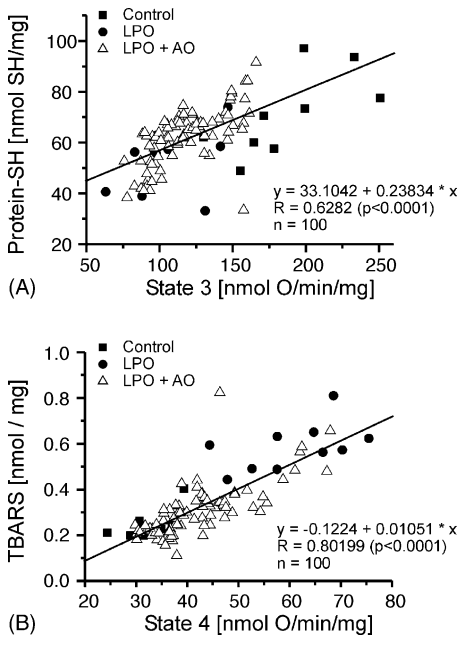

Fig. 7. Correlation between bioenergetic and biochemical parameters of RHM (A: content of protein-bound thiol groups vs. state 3 respiration; B: content of thiobarbituric acid-reactive substances vs. state 4 respiration). RHM were exposed either to control conditions (acetonitrile as vehicle) or CumOOH plus $FeSO_4$ in the absence (LPO) or presence of the tested chromanolic antioxidants (LPO + AO). *R* indicates the Pearson's correlation coefficient which was obtained from linear regression analysis.

less-reactive chromanoxyl radicals and the regeneration of the chromanols by cellular reductants (ubiquinol, GSH, ascorbic acid) [26,27]. In contrast, the disproportionation of two chromanoxyl radicals, giving rise to one molecule of the parent chromanol plus one molecule of a quinone, that cannot be converted back to the parent chromanol, irreversibly weakens the antioxidative capacity [28]. Therefore, one can expect that the antioxidative efficiency of chromanols can be increased by accelerating the reaction with lipid radicals while slowing down the disproportionation reaction of the respective chromanoxyl radicals.

The stability of the tocopheroxyl radical arises from the delocalization of the unpaired electron across the fully substituted chromanol ring system [13]. Due to structural features, new chromanols, such as the Twin and oxachromanols, were of particular interest. The unique structure of the Twin compound, possessing two chromanol moieties, suggests the formation of a quite stable chromanoxyl radical, the disproportionation of which is impeded by steric restrictions. It has recently been demonstrated that the radical-scavenging activity of Twin, which was assessed by its reaction with the stable radical 1,1-diphenyl-2-picrylhydrazyl, was comparable to that of PMC and α-tocopherol in polar solvents and much higher in the apolar solvent hexane, while the rate of the disproportionation reaction was slower by a factor of 4 (in ethanol) to 7 (in acetonitrile) [17]. Therefore, the twin-chromanoxyl radical should be efficiently recycled by cellular reductants, so that even a higher antioxidative efficiency in comparison to α-tocopherol and PMC could be anticipated.

The 3-oxachromanols are structurally similar to chromanols except C-3 being replaced by an oxygen atom. The primary oxidation product is the corresponding chromanoxyl radical. The subsequent reaction pathway, however, depends on the availability of co-reactants and is associated with the formation of a complex mixture of secondary products [18]. Kinetic experiments in model systems have shown that in comparison to α-tocopherol and PMC, the radical-scavenging activity of oxachromanols was about two to nine times lower. In contrast, the rate of the disproportionation reaction of *trans*-OCA was reduced, while that of *cis*-OCA was accelerated in comparison to α-tocopherol and PMC [17]. Considering only these kinetic data, one can assume that the tested oxachromanols are less effective than PMC. Since the kinetic experiments were performed in homogenous model systems (organic solvents), which do not take into account the distribution of antioxidants in membranes with respect to lipid radicals, tests in biological membranes (in our case mitochondria) offer a better prediction of the benefit of an antioxidant in the cell.

4.2. Exclusion of toxic side effects of chromanols on mitochondrial membranes

Several antioxidants, e.g. ascorbic acid [29,30], thiol-containing compounds [31,32], and quinones [33,34], are known to exert prooxidative effects in addition to their antioxidative action, depending on their concentration and reaction partners. Prooxidative effects of phenoxyl radicals derived from flavonoids and other polyphenolic compounds have also been described [35]. Under several conditions, even the native α-tocopherol can stimulate prooxidative processes [36].

While it has been demonstrated for chemical model systems that also S- and N-substituted chromanols are effective radical scavengers, toxicological information on these compounds is generally lacking. However, it is well known that certain cyclic N-containing molecules possess a harmful redox activity, e.g. the mitochondrial toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine [16]. S-containing compounds can cause toxic effects because of their metal-complexing activity [37].

In addition to prooxidative effects, which are often stimulated by transition metal ions, several phenol-type substances possess protonophoric properties, thereby leading to an uncoupling of mitochondrial oxidative phosphorylation and a decrease in ATP synthesis [38–40]. The tested chromanol derivatives do not deteriorate the bioenergetic function of isolated mitochondria under the conditions used. Experimental data revealed that a toxic effect can be excluded up to 50 nmol chromanol/mg mitochondrial protein, which exceeds the concentration of natural α-tocopherol in mitochondrial membranes by a factor of 40 [7].

4.3. Protection of mitochondrial membranes against lipid peroxidation by new synthetic chromanols

Lipid peroxidation, one of the main oxidative alterations in biological membranes, can be initiated by several systems, e.g. iron/ascorbate [41,42], iron/ADP/NAD(P)H [41,43], organic hydroperoxides [44,45] or thermal decomposition of azo-compounds [46,47]. The content of TBARS is usually determined as an indicator of LPO [41,42,45,48]. Although in addition to malondialdehyde also other aldehydes, which are derived from peroxidizing mitochondrial membranes, produce red-colored reaction products with TBA, malondialdehyde quantitatively represents the most important compound [42]. In the present study, RHM were oxidatively damaged by an incubation with CumOOH and Fe^{2+} which yields cumene alkoxyl radicals [49]. Although organic hydroperoxides were sufficient to stimulate LPO in liver mitochondria in the absence of transition metal ions [44,45], our preliminary experiments with heart mitochondria have shown that the bioenergetic function of isolated RHM was only weakly influenced by CumOOH alone. This discrepancy was also described by Bindoli [50]. Therefore, in the present study RHM were exposed to a combination of CumOOH and Fe^{2+} leading to increased TBARS levels in comparison to control RHM (0.61 ± 0.04 nmol TBARS/mg protein versus 0.27 ± 0.03 nmol TBARS/mg protein) which correspond fairly well to TBARS levels detected in

mitochondria after ischemia/reperfusion (0.40 ± 0.04 nmol TBARS/mg protein versus 0.25 ± 0.02 nmol TBARS/mg protein) [51].

In the present study, it was shown that the new chromanol-type antioxidants were able to protect mitochondrial membranes against CumOOH/Fe^{2+}-initiated LPO. A preincubation of mitochondrial membranes with the chromanols prior to the initiation of peroxidative processes concentration-dependently diminished TBARS formation. The novel chromanols turned out to have a higher antioxidative efficiency in comparison to pentamethyl-chromanol (Twin $\sim$ *cis*-OCA $>$ *trans*-OCA $>$ PMC), since lower concentrations were necessary for a significant decrease in TBARS levels. With increasing chromanol concentrations, all tested substances fully prevented TBARS accumulation in RHM.

By protecting lipid membranes of calcium-storing organelles from oxidative damage, new chromanols are likely to prevent a rise of the cytosolic calcium concentration, and thereby the formation of the mitochondrial permeability transition pore (MPTP). Whether antioxidants can directly prevent MPTP formation by a radical-scavenging mechanism is still not clear. There are many publications in favour of this assumption [52–54] but also conflicting reports for well-known antioxidants, such as dihydrolipoic acid [55]. Furthermore, it was shown that PMC inhibited the MPTP formation at a low concentration but promoted it at a concentration only four-times higher [52]. This rather contradicts a pure radical-scavenging mechanism during PMC-modulated MPTP formation.

4.4. Prevention of protein thiol oxidation by new synthetic chromanols

During oxidative stress alterations of mitochondrial electron transport and ATP-synthesizing proteins are expected in addition to LPO. Especially proteins containing functionally important SH groups are vulnerable to oxidative damage [46,47]. It was reported that the degree and kinetics of oxidation of protein-bound thiol groups in biomembranes depend on the type of prooxidant, the site of its action (aqueous/lipid phase) and the loss of α-tocopherol [46,47]. Takenaka et al. have shown that radicals, generated in the aqueous phase, simultaneously oxidize surface thiols of membrane proteins and α-tocopherol, while lipid-derived radicals rather attack thiols buried in the membrane after consumption of α-tocopherol [47]. In order to determine the total amount of protein-bound thiol groups, we solubilized mitochondria with the detergent SDS. The amount of protein-bound thiol groups was decreased in CumOOH/$FeSO_4$-treated RHM by 33% in comparison to control RHM, indicating that the prooxidative procedure gave rise not only to LPO but also to oxidative damage of membrane proteins. In the presence of the novel antioxidative chromanols, mitochondrial thiol groups were partially protected from oxidation. However, significant differences were observed only for lower concentrations of Twin.

4.5. Prevention of α-tocopherol oxidation by new synthetic chromanols

Accumulation of TBARS is usually accompanied or preceded by a decrease in α-tocopherol concentration in lipid membranes [41,46,47]. Paraidathathu et al. measured 5.1 nmol TBARS/mg protein after incubation of RHM from Vitamin E-deficient animals with NADPH/iron/ascorbate, while no LPO was observed under otherwise identical conditions in mitochondria from rats, that obtained a diet with normal Vitamin E content [43]. Therefore, biomembranes are assumed to be protected against LPO by lipophilic antioxidants. An enrichment of mitochondrial membranes with propylgallate, several phenolic compounds, or Vitamin E, was shown to reduce the content of LPO products in these membranes [45,48].

HPLC measurements of Toc and its first stable oxidation product, TQ, in mitochondrial membranes have shown that under our experimental conditions, Toc was rapidly consumed after initiation of oxidative stress in spite of the presence of mitochondrial substrates. This observation is in line with findings of Ham and Liebler [56]. Toc was preferentially, but not exclusively, oxidized to TQ under conditions used. For added chromanols, a threshold concentration between 1 and 5 nmol/mg protein was required under our experimental conditions to protect the endogenous mitochondrial Toc from being oxidized.

4.6. Correlation between bioenergetic and biochemical parameters

Oxidation of proteins can be associated with their functional impairment. Paradies et al. observed a marked loss of cytochrome c oxidase activity of RHM oxidatively damaged either with *tert*-butylhydroperoxide plus copper or ischemia/reperfusion [48,51]. Reinheckel et al. have found that protein oxidation in iron/ascorbate-damaged liver mitochondria occurs already before the onset of LPO and is accompanied by a functional decline of mitochondria [42]. In addition, oxidation of mitochondrial protein thiols is known to be one of the triggers of the permeability transition [44,57].

The strong dependence of state 3 respiration on the content of protein-bound thiol groups (the higher the content of reduced thiol groups, the faster the oxygen consumption during state 3 respiration) suggests an enzymatic control of electron flow rates during state 3 respiration since oxidation of proteins usually leads to an inhibition of enzymatic activities. In contrast, no significant correlation between the thiol content and state 4 respiration ($R = 0.06$) was observed. However, a strong

positive correlation between the concentration of the LPO products, determined as TBARS, and oxygen consumption during state 4 respiration (the higher the concentration of TBARS, the faster state 4 respiration) was found. State 4 respiration is generally assumed to be linked to the unspecific backflow of protons across the mitochondrial membrane. Since LPO is known to increase the proton permeability of the inner mitochondrial membrane [58], this explains the observed correlation. Furthermore, the additional oxygen consumption could be partially due to the LPO process itself. It can be expected that the additional oxygen consumption caused by the LPO process would proceed during state 3 respiration as well; however, this is obviously masked by the inhibition of mitochondrial electron flow due to protein oxidation.

Our data demonstrate that pretreatment of mitochondria with the new chromanols prior to LPO can partially restore their physiological bioenergetic and biochemical parameters.

5. Conclusions

1. Concentrations up to 50 nmol of Twin and oxachromanols/mg mitochondrial protein were without toxic side effects.
2. The mitochondrial function, which was deteriorated by oxidative stress, was improved by chromanols in the following order: Twin > PMC > *cis*-OCA ≈ *trans*-OCA.
3. Twin can protect mitochondria from oxidative damages even better than the α-tocopherol analogue pentamethyl-chromanol.

Acknowledgements

The technical assistance of Sabine Wawruschka and Werner Stamberg is gratefully acknowledged. This work was supported by the Austrian Science Fund (FWF, Grant P16244-B08).

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