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Dehydrosilybin attenuates the production of ROS in rat cardiomyocyte mitochondria with an uncoupler-like mechanism

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Abstract Reactive oxygen species (ROS) originating from mitochondria are perceived as a factor contributing to cell aging and means have been sought to attenuate ROS formation with the aim of extending the cell lifespan. Silybin and dehydrosilybin, two polyphenolic compounds, display a plethora of biological effects generally ascribed to their known antioxidant capacity. When investigating the cytoprotective effects of these two compounds in the primary cell cultures of neonatal rat cardiomyocytes, we noted the ability of dehydrosilybin to de-energize the cells by monitoring JC-1 fluorescence. Experiments evaluating oxygen consumption and membrane potential revealed that dehydrosilybin uncouples the respiration of isolated rat heart mitochondria albeit with a much lower potency than synthetic uncouplers. Furthermore, dehydrosilybin revealed a very high potency in suppressing ROS formation in isolated rat heart mitochondria with IC_{50}=0.15 μM. It is far more effective than its effect in a purely chemical system generating superoxide or in cells capable of oxidative burst, where the IC_{50} for dehydrosilybin exceeds 50 μM. Dehydrosilybin also attenuated ROS formation caused by rotenone in the primary cultures of neonatal rat cardiomyocytes. We infer that the apparent uncoupler-like activity of dehydrosilybin is the basis of its ROS modulation effect in neonatal rat cardiomyocytes and leads us to propose a hypothesis on natural ischemia preconditioning by dietary polyphenols.

Keywords Reactive oxygen species · Cardiomyocytes · Mitochondria · Protonophore · Respiration uncoupling · Dehydrosilybin

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Abbreviations

ANT	adenine nucleotide transporter
CAT	carboxyatractyloside
CCCP	carbonylcyanide-*m*-chlorophenyl hydrazone
DHSB	2,3-dehydrosilybin
FA	fatty acid
FCCP	carbonylcyanide-*p*-trifluoromethoxyphenyl hydrazone
ROS	reactive oxygen species
SB	silybin
SOD	superoxide dismutase
UcP	uncoupling protein

Introduction

Levels of reactive oxygen (ROS) and nitrogen species (RNS), generation of which is inherent to some intracellular processes, must be finely tuned in order to maintain their role of cell signaling molecules and to avoid damaging effects (Hensley et al. 2000; Pryor et al. 2006). Hence the modulation of ROS within a cell by intra- and extracellular means is considered a prerequisite for the longevity and sustained function of the cell (Valko et al. 2007). Intracellular ROS modulation systems are numerous and generally coordinated to maintain a fine balance of ROS levels (Valko et al. 2007). Extracellular means of ROS level modulation are multitudinous, among them a vast group of natural substances present in many plants and hence in our diet. Their antioxidant function, however, is not a simple scavenging feat as it involves the modulation of intracellular free radical defense systems as well, resulting in the perturbation of the natural balance established by the cell (Hensley et al. 2000).

Cardiomyocytes, due to their exhaustive use of mitochondria for energy generation sustaining their incessant contraction/relaxation, are continually endangered by ROS-mediated damage, particularly during ischemia reperfusion. It is one of the current research venues exploring the possibility of pre- or post-conditioning of the heart where ROS do play an important but perhaps not decisive role (Garlid et al. 2009). There are two mechanisms recognized as possible ROS limiting tools: the uncoupling of mitochondrial respiration (Brennan et al. 2006a; Brennan et al. 2006b) and the inhibition of the respiratory chain (Chen et al. 2006; Chen et al. 2007). The former mechanism has been explored by those interested in trying to discover artificial mild uncouplers taking the known as a starting point (Blaikie et al. 2006). Certain researchers may also realize that naturally occurring substances are capable of uncoupling mitochondrial respiration (Dorta et al. 2008; Ortega and Garcia 2009) and could serve as initial model compounds.

Flavonolignans silybin and dehydrosilybin, which rank amongst polyphenolic compounds, are members of a class of substances with clearly demonstrated antioxidant properties. Both substances display cytoprotective properties which are linked to their antioxidant effects (Gazak et al. 2009). The biological effects of both substances are not limited, however, to their antioxidant ability hence they are subject to more focused research with specific target proteins or structures (reviewed in (Gazak et al. 2007)).

We have been investigating the cytoprotective effects of silybin, dehydrosilybin, and a number of their derivatives and have discovered that dehydrosilybin displays uncoupler-like activity in cardiomyocytes. Dehydrosilybin decreases mitochondrial ROS formation while increasing oxygen consumption accompanied by mitochondrial membrane potential decrease. We have thus inferred that dehydrosilybin protects cardiomyocytes against ROS-mediated damage thanks to its uncoupler-like behavior.

Materials and methods

Animals

Wistar rats (250–275 g) were bred and housed in certified animal houses according to EU rules and according to the Faculty of Medicine and Dentistry, Palacký University, and the Institute of Physiology, v.v.i., licensing committee approval, in accordance with the Guide for the Care and Use of Laboratory Animals (1985), NIH, Bethesda, or European Guidelines on Laboratory Animal Care. Animal treatment and sacrifice procedure for neonatal rats was approved by the Ethical Committee for Laboratory Animal Treatment of the Faculty of Medicine and Dentistry, Palacký University.

Isolation of neonatal rat cardiomyocytes

The procedure described by Chlopcikova et al. (Chlopcikova et al. 2001) was followed. Entire hearts were isolated from 2–5 day old rats and minced in a balanced salt solution containing 20 mM HEPES, 120 mM NaCl, 1 mM NaH_2PO_4, 5.5 mM glucose, 5.4 mM KCl and 0.8 mM $MgSO_4$ (pH 7.3–7.4). The consequent trypsin digestion cells were resuspended in a medium containing Dulbecco's Modified Eagle Medium (DMEM) and a medium 199 (4:1) supplemented with horse serum (10%), fetal calf serum (5%), penicillin (100 U/ml) and streptomycin (100 μg/ml). The suspension enriched in non-adhesive myocytes was transferred to collagen I-coated culture dishes at a density of 5×10^4 cells per cm^2. The cells were incubated in 95% air and 5% CO_2 at 37 °C. The medium was removed after 72 h and replaced with a culture medium containing DMEM and medium 199 (4:1) with penicillin (100 U/ml) and streptomycin (100 μg/ml). The cultured cardiomyocytes were allowed to reach a confluence before being used experimentally. The percentage of beating myocardial cells exceeded 85–90% after 3 days in culture for each experiment.

Isolation of heart mitochondria

Rat heart mitochondria were isolated by differential centrifugation in an ice-cold isolation medium containing 180 mM KCl, 5 mM MOPS buffer, pH 7.2, 2 mM EGTA, and 0.5% BSA according to a published procedure (Vaghy et al. 1981). The final mitochondrial pellet was washed with re-suspension and centrifugation in the isolation medium lacking BSA. The protein was determined by the BCA method (Sigma).

High-resolution respirometry

Simultaneous recording of mitochondrial oxygen concentration and consumption was measured using an Oxygraph 2 k high-resolution respirometer (Oroboros, Innsbruck, Austria) supplemented with specifically optimized DatLAb analysis software. In order to assay the mitochondrial oxygen consumption, pyruvate (5 mM) *plus* malate (1 mM) *plus* succinate (5 mM) were chosen as respiratory substrates. An identical set of experiments was performed using succinate (10 mM) in the absence of complex I substrates. Mitochondria were allowed to respire at 30 °C in an assay medium containing 120 mM KCl, 5 mM K-MOPS, 1 mM K-EGTA, 0.5 mM K-phosphate, and 0.5 mM $MgCl_2$, pH 7.2. Oligomycin (1 μg/ml) was used to define the non-phosphorylating state-4 conditions.

Measurement of membrane potential in isolated rat heart mitochondria

Changes in the inner membrane potential, $\Delta\Psi_m$, were determined fluorometrically using 2 μM tetramethylrhodamine ethyl ester (TMRE; Molecular Probes), at the excitation wavelength of 556 nm (slit width 4 nm), while collecting the emission wavelength at 577 nm (slit width 4 nm) (Scaduto and Grotyohann 1999) on a Fluorolog 322 (Spex-Jobin-Yvon-Horiba) fluorometer. The experimental conditions were parallel to those used for the high-resolution respirometry.

Measurement of production of reactive oxygen species in isolated rat heart mitochondria

The production of H_2O_2 through isolated mitochondria was measured using fluorescent monitoring of oxidation of Amplex Red (5 μM) by horseradish peroxidase (0.5 μM) (Zhou et al. 1997). The fluorescence was monitored on a Fluorolog 322 (Spex-Jobin-Yvon-Horiba) fluorometer with an excitation at 570 nm (slit width 8 nm) and an emission at 585 nm (slit width 2 nm). Aliquots of H_2O_2 were used for calibration and the rates were determined with linear regressions. The experimental conditions were parallel to those used for the high-resolution respirometry.

Membrane potential evaluation in neonatal rat cardiomyocyte cultures

JC-1, a cation active dye which accumulates in mitochondria depending on their potential, was employed for transmembrane potential evaluation in entire cells. The dye displays two emission maxima: green (525 nm) and red (590 nm). The shift in fluorescence is a consequence of J-aggregate formation in the presence of high potential, the depolarization of the mitochondrial membrane is reflected in a decrease in the red/green fluorescence ratio. JC-1 displays a higher specificity for mitochondrial membrane potential that plasma membrane potential in comparison with $DiOC_6$ and rhodamine (Salvioli et al. 1997).

In order to detect membrane potential in cultures of neonatal rat cardiomyocytes, JC-1 (Molecular Probes, USA) was mixed with the culture medium and added directly to the cells for 30 min incubation at 37 °C. The medium was then decanted, the cells were washed twice with PBS and then fresh pre-warmed medium was added to the cells. Green (535 nm emission) and red (595 nm emission) JC-1 fluorescence, excitation 488 nm, was measured separately on a well plate reader (Tecan Infinite M200, Tecan, Austria), which allows for multiple readings from a single well at pre-defined positions.

Evaluation of production of reactive oxygen species in neonatal rat cardiomyocyte cultures

The formation of ROS in cell cultures was monitored by a dichlorodihydrofluorescein assay. The polar, pre-fluorescent dichlorodihydrofluorescein diacetate (H_2DCFDA, Molecular Probes) undergoes deacetylation through cytosolic esterases to form dichlorodihydrofluorescein, which reacts with ROS and gives rise to fluorescein. The fluorescence is monitored at specific excitation/emission wavelengths 488/525 nm. After the experiment, the cells were incubated with H_2DCFDA (5 nM) for 15 min in the dark. They were then washed once with PBS and scraped into PBS and sonicated to release the formed fluorescein from the cells. After centrifugation (2000×g, 10 min, 4 °C), the fluorescence in the supernatant was measured using a well-plate reader (Tecan Infinite M200, Tecan, Austria). Protein concentration was estimated using the Coommassie Blue method (Bradford 1976).

ROS production in neutrophil-like cells and the purely chemical system

HL-60 cells differentiated for 7 days in the presence of 1.25% DMSO (final density in assay 10^6 cells/mL) were transferred into a fluorescence quartz cuvette containing PBS + buffer (phosphate buffered saline, pH 7.4, containing 30 mM glucose, 1 mM $MgCl_2$, and 0.5 mM $CaCl_2$) prewarmed to 37 °C. An aqueous solution of silybin or dehydrosilybin (final concentrations 1–50 μM) or 10 U/ml of superoxide dismutase (Sigma-Aldrich) was added 5 min prior to cell stimulation. 4 μM MCLA (2-methyl-6-(4-methoxyphenyl)-3,7-dihydroimidyzo[1.2-a]pyrazin-3-one hydrochloride (Molecular Probes, USA)) was added 30 s prior to the start of measurement. The background chemiluminescence was obtained and thereafter automatically subtracted from the readings. Superoxide generation was initiated by adding 62.5 ng/ml PMA (phorbol 12-

myristate 13-acetate (Sigma-Aldrich)) 60 s after the start of the measurement. The final volume of the sample was 2 ml, the organic solvent concentration, i.e. DMSO and/or ethanol, did not exceed 0.5% in any assay. The time course of MCLA-O_2^- chemiluminescence was monitored on a luminometer equipped with a total emission mirror and a revolving cuvette holder allowing thermostating at 37 °C and continuous stirring of the sample (Perkin-Elmer LS50B, Perkin-Elmer Corp., USA). The instrument was set as follows: luminescence mode (lamp off), delay time 0.03 ms, cycle time 100 ms, gate time 90 ms, and flash count 1. The emission slit was set to 20, the emission filter to 350 nm, while the photomultiplier voltage was 775 V.

The method using the NADPH:$MnCl_2$:2-mercaptoethanol system published by Paoletti and Mocali (Paoletti and Mocali 1990) was utilized as a model of a non-cellular system generating superoxide. A DMSO solution of silybin or dehydrosilybin (final concentrations 1–50 μM) or 10 U/ml of superoxide dismutase (Sigma-Aldrich) was added to the 37 °C warm triethanolamine-diethanolamine buffer, pH 7.4, 5 min prior to 2-mercaptoethanol addition which is the start of the reaction. The decrease in absorbance at 340 nm, i.e. oxidation of NADPH, was monitored continuously for 20 min on a UV-VIS spectrophotometer (UV 2401PC, Shimadzu, Japan).

Statistical analysis

Student's t-test was used for statistical evaluation of the results, $p<0.05$ was considered statistically significant. When comparing more than two groups of data, a one-way analysis of variance (ANOVA) with a post-hoc Tukey test was applied using STATISTICA software (StatSoft).

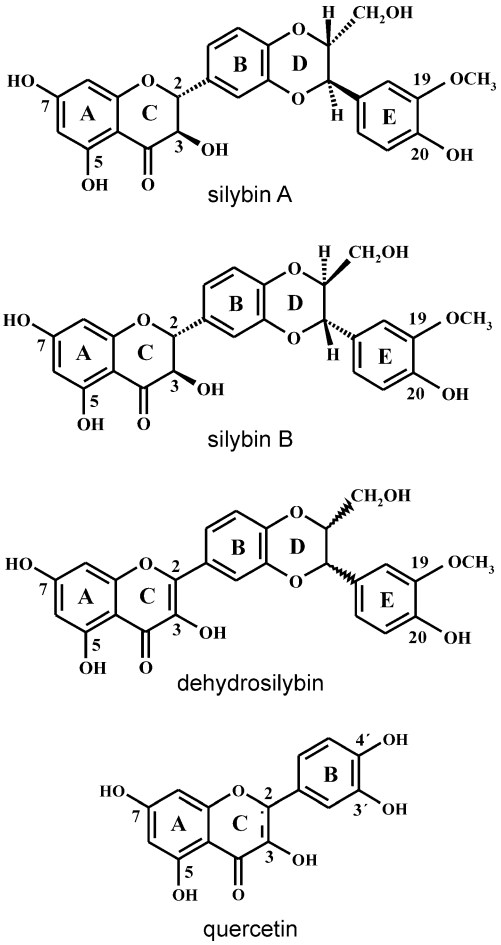

Fig. 1 The structure of silybin and dehydrosilybin. Certain important structural features and the labeling of individual aromatic rings of both tested flavonolignans are indicated in this representation. Since we used 1:1 mixture of silybins A and B in our experiments, both structures are shown. Quercetin is included for comparison

Results

The effect of silybin and dehydrosilybin on the energy status of isolated rat cardiomyocytes

Silybin and dehydrosilybin (Fig. 1) display negligible toxicity in several cell types when not exceeding 25 μM concentration. Their effect on general cellular processes, however, may vary significantly in terms of the concentration range as these substances tend to precipitate out of aqueous solutions due to their hydrophobicity, a property more pronounced in dehydrosilybin. The biological effects of both substances are multiplex as multiple target proteins or structures have been identified to date (Gazak et al. 2007). We have treated neonatal rat cardiomyocytes with micromolar concentrations of silybin and dehydrosilybin for 24 h in order to test their effects on cellular bioenergetics. Following the treatment, the cells were loaded with fluorescent dye JC-1 in order to assess the effect of the substances on mitochondrial membrane potential. Microphotographs of untreated cells revealed large populations of cells displaying both red and green fluorescence indicating the presence of high- and low-potential mitochondria. Cells treated with dehydrosilybin indicated an overwhelming presence of green JC-1 fluorescence, indicative of low membrane potential, as with treatment with artificial uncoupler CCCP (Supplemental Fig. 1). The quantification of the effect was achieved by recording the green and red fluorescence intensities, each in a separate reading, form predefined spots in each well of a culture plate and then calculating the ratio of red/green fluorescence. If the ratio is larger than one, cells possessing mitochondria with high membrane potential are more populous in that particular well.

Silybin had no significant effect on the red/green JC-1 fluorescence ratio indicating the same distribution of cells containing high potential mitochondria as control cells (Fig. 2a). In contrast, increasing concentrations of dehydrosilybin elicited a significant change in the red/green fluorescence ratio indicating a decrease in cardiomyocyte

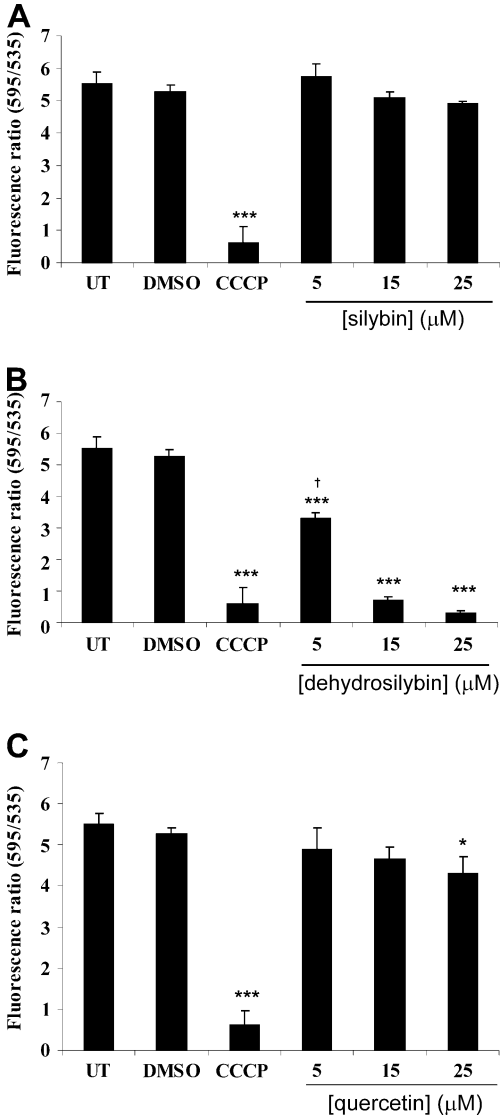

Fig. 2 Dehydrosilybin de-energizes neonatal rat cardiomyocytes in culture. The cell cultures of neonatal rat cardiomyocytes were challenged with various concentrations of silybin, dehydrosilybin, quercetin, or the artificial uncoupler CCCP. The panels show a ratio of red/green JC-1 fluorescence readings determined separately for each culture when excited at 488 nm. The cell cultures included control cells (UT), cells treated with a corresponding volume of DMSO (DMSO), and cells challenged with 2 μM CCCP (CCCP). The digits under the bars designate the micromolar concentrations of silybin (Panel A); dehydrosilybin (Panel B); and quercetin (Panel C). The full range of concentrations tested is not shown in order to preserve figure clarity. The data is the means ± SEM of four independent experiments for each substance. Statistical analysis using one-way ANOVA with post-hoc Tukey test yielded significant differences as indicated. * $p<0.05$, *** $p<0.001$ *versus* DMSO-treated cells; † $p<0.05$ *versus* CCCP treated cells

mitochondrial potential resembling that evoked by CCCP (Fig. 2b). Although less powerful than the effect of dehydrosilybin, the effect of quercetin, a flavonol with superoxide scavenging properties (Dorta et al. 2008) and the ability to uncouple respiration (Ortega and Garcia 2009), was the same (Fig. 2c).

The effect of silybin and dehydrosilybin on respiration and membrane potential of isolated rat heart mitochondria

Asserting the effect of dehydrosilybin observed in a cellular system is related to mitochondria, we have tested both compounds in an independent experiment using mitochondria isolated from rat hearts and determined the changes in oxygen consumption and membrane potential in parallel experiments upon the addition of either silybin or dehydrosilybin. Figure 3a shows that the addition of 2 μM dehydrosilybin has caused a significant increase in the oxygen consumption (*trace a*) above the control oxygen consumption with no addition (*trace b*). Contrary to the effect of dehydrosilybin, the addition of 2 μM silybin caused no changes in the oxygen consumption (*trace c*).

Figure 3b reveals that the addition of 2 μM dehydrosilybin has caused a significant increase in the TMRE fluorescence (*trace a*) above the control conditions (*trace b*), indicating a dehydrosilybin-dependent decrease of mitochondrial $\Delta\Psi_m$. The addition of 2 μM silybin was again without any effect (*trace c*). The concurrent increase in respiration and the decrease in membrane potential is consistent with dehydrosilybin causing the uncoupling of mitochondrial respiration. In all the experiments, the synthetic uncoupler FCCP was added for a comparison. The FCCP titration yielded an IC_{50}=11 +/−0.5 nM (n=3) and the saturating concentration of FCCP yielded a respiration of 90 +/−3 $nmolO_2.min^{-1}.mg^{-1}$ (n=3) (data not shown).

The experiments shown in Fig. 3 were carried out using both complex I and complex II substrates. An identical set of experiments was performed in the absence of complex I substrates with the respiration initiated by the addition of 10 mM succinate. The obtained results were identical to those shown in Fig. 3 (data not shown).

Dehydrosilybin decreases ROS formation in isolated mitochondria

While the experiments thus far indicate the uncoupler-like activity of dehydrosilybin, as opposed to uncoupling the inertness of silybin, we tested whether the resemblance to an uncoupler extends to limiting ROS formation in mitochondria, a known effect of synthetic uncouplers (Dlaskova et al. 2008). In order to determine the effects of silybin and dehydrosilybin on the production of mitochondrial ROS, we have used isolated rat heart mitochondria and determined the production of H_2O_2 using the colorimetric reaction of amplex red in the presence of horseradish peroxidase (HRP). In Fig. 4, experimental

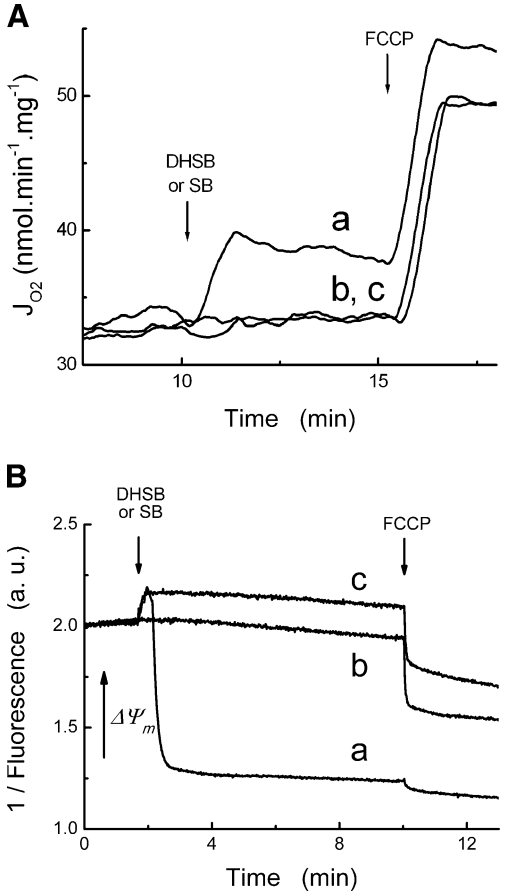

Fig. 3 The effect of dehydrosilybin and silybin on oxygen consumption and membrane potential in isolated rat heart mitochondria. Representative traces of two parallel experiments follow the changes of (**a**) oxygen consumption and (**b**) TMRE fluorescence indicating the changes in mitochondrial membrane potential. *Traces a*, addition of 2 μM dehydrosilybin resulted in both an increase in respiration and a decrease in membrane potential. *Traces b* represents a control baseline. *Traces c*, 2 μM silybin was without effect. At the end of each run, 10 nM FCCP was added for a comparison. Rat heart mitochondria (0.1 mg/ml) respired in the presence of 5 mM pyruvate, 1 mM malate, and 5 mM succinate in a medium described in the "Materials and Methods". This data is representative of more than five independent experiments

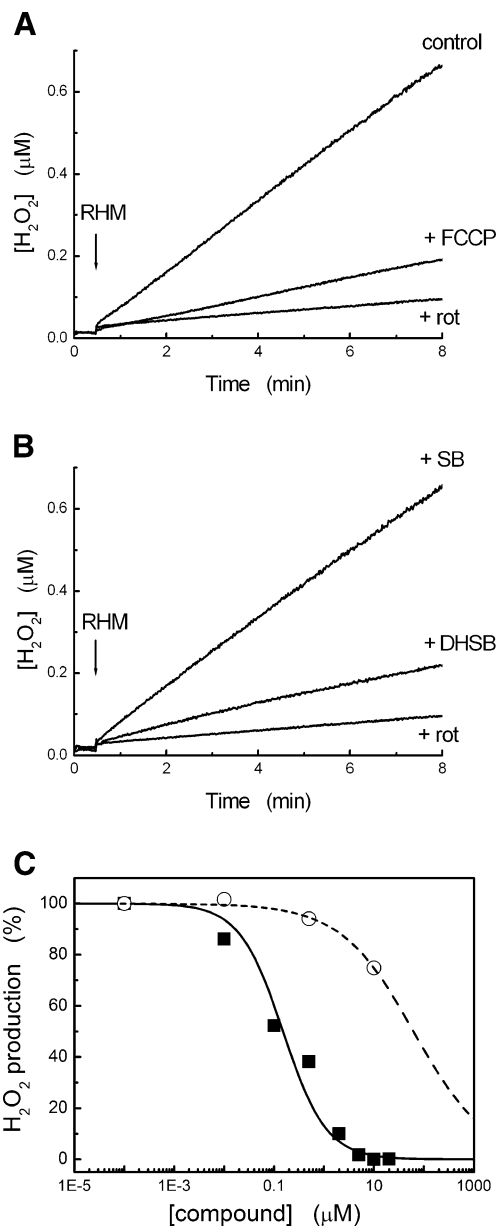

Fig. 4 The effect of dehydrosilybin and silybin on ROS formation in isolated rat heart mitochondria. **a**, Traces follow the changes in mitochondrial H_2O_2 production. Rat heart mitochondria (0.1 mg/ml) respired in the presence of 10 mM succinate ("*control*"). The addition of 5 μM rotenone resulted in nearly complete inhibition of the control H_2O_2 production ("+ *rot*"). The addition of 10 nM FCCP resulted in a substantial decrease in the control H_2O_2 production ("+ *FCCP*"). **b**, in the same experiment, the addition of 2 μM silybin caused no significant changes compared to the control H_2O_2 production ("+ *SB*"), while the addition of 2 μM dehydrosilybin resulted in a substantial decrease in the control H_2O_2 production ("+ *DHSB*"). **c**, concentration dependence of dehydrosilybin (*squares*) and silybin (*circles*)—dependent decrease of the mitochondrial H_2O_2 production. The control production of H_2O_2 was set as 100% and the rotenone-inhibited H_2O_2 production was set as 0%. The IC_{50} values are given in the "Results". This data is representative of three independent experiments

conditions using succinate in the absence of complex I substrates were chosen in order to support high ROS production by mitochondrial complex I due to a reversed electron transfer. Figure 4a shows fluorescent traces of a robust production of H_2O_2 (*trace "control"*) which is nearly completely inhibited by the addition of 5 μM rotenone (*trace "+rot"*). The addition of 10 nM FCCP reveals a large decrease in the mitochondrial H_2O_2 production (*trace "+FCCP"*), consistent with known properties of synthetic uncouplers. Figure 4b indicates that the addition of 2 μM silybin resulted in no significant decrease in the mitochondrial H_2O_2 production (*trace "+SB"*), whereas the addition of 2 μM dehydrosilybin caused about 80% decrease in the H_2O_2 production (*trace*

"+ DHSB"). Figure 4c shows dose-response curves of the effect of dehydrosilybin and silybin on the mitochondrial H_2O_2 production, yielding an IC_{50}=0.15 +/− 0.02 μM for dehydrosilybin (n=3) and approximately estimated IC_{50} of 60 μM for silybin.

In similar experiments using both complex I and complex II substrates, significantly lower control production of H_2O_2 was observed, although the dose-responses for dehydrosilybin and silybin led to qualitatively identical results to those shown in Fig. 4c (data not shown).

The low potency of dehydrosilybin and silybin to quench superoxide generated in model systems

Thus far we have demonstrated the potency of dehydrosilybin to inhibit the hydrogen peroxide mediated process, although we had to limit the possibility that the inhibitory power depends on the dehydrosilybin potency to quench ROS, particularly superoxide. We employed a cellular system consisting of DMSO-differentiated HL-60 cells that generate extracellular superoxide when challenged with fMLP or PMA (Arroyo et al. 2002). Silybin did not show a significant superoxide scavenging effect in concentrations up to 50 μM while dehydrosilybin did demonstrate inhibition of superoxide formation (Fig. 5a) with calculated IC_{50}=58.5 μM (Fig. 5b). The same lack of the superoxide scavenging effect of silybin was observed in a purely chemical system where dehydrosilybin revealed a potential to prevent the superoxide-mediated NADPH oxidation (Fig. 5c), albeit with a high IC_{50}=75.3 μM (Fig. 5d).

In light of the fact that dehydrosilybin showed weak scavenging of extracellular superoxide, we decided to address the same question when superoxide was generated intracellularly, particularly in connection with the mitochondria-driven process. We therefore used neonatal rat cardiomyocytes in order to show that the addition of rotenone to the cell culture results in ROS generation, which is inhibited by dehydrosilybin. In this case rotenone increases mitochondria-dependent ROS formation as it inhibits Complex I in a site located downstream from superoxide formation (Brandt 2006; Dlaskova et al. 2008). Using dichlorodihydrofluorescein dye, which is converted into highly fluorescent fluorescein upon reacting with ROS, we initially established that 7 μM rotenone is needed in order to evoke a significant increase in intracellular ROS formation (data not shown). Dehydrosilybin showed a concentration-dependent inhibition of the rotenone-induced ROS production (Fig. 6), while silybin revealed no ROS inhibition in the same concentration range (data not shown). Moreover, a comparison of the CCCP effect with that of dehydrosilybin on ROS formation indicates a high resemblance to their effect on cell energization (Fig. 2b).

The effect of dehydrosilybin on respiration is sensitive to carboxyatractyloside and purine nucleotides

Over the course of investigating the mechanism of the dehydrosilybin-dependent increase in mitochondrial uncoupling, we found that the dehydrosilybin-induced respiration increase can be largely prevented by carboxyatractyloside (CAT) and also to some extent by purine nucleotides, the inhibitors of the mitochondrial adenine nucleotide translocator (ANT) and uncoupling proteins (UCPs), respectively. Figure 7 summarizes the results obtained by detecting the changes in oxygen consumption of isolated rat heart mitochondria while titrating the dehydrosilybin alone (*squares*) or in the presence of 1 μM CAT (*circles*), or the presence of both 1 μM CAT and 1 mM GTP (*triangles*). Identical data was obtained when 1 mM ATP was used instead of GTP (data not shown). This data indicates that ANT, and also perhaps heart mitochondrial UCP2, participates in the dehydrosilybin-induced uncoupling and the consequent decrease in the mitochondrial production of ROS.

Discussion

Dehydrosilybin is a minor constituent of milk thistle seed extract known as silymarin which is regarded as a hepatoprotective concoction of flavonolignans (Morazzoni and Bombardelli 1995). Silymarin is still actively investigated in connection with its hepatoprotective capability (Wagoner et al. 2010), however, the investigators traditionally focus on major constituents of the extract (Polyak et al. 2010). The uncanny resemblance of dehydrosilybin-mediated de-energization of cardiomyocytes (Fig. 2b) and dehydrosilybin-mediated inhibition of intracellular ROS formation (Fig. 6) is easily explained when compared to synthetic uncouplers. The capacity of dehydrosilybin to exert the same effects as artificial uncouplers is, of course, much lower on the level of mitochondria, approximately 500-fold. On the level of intact cells, however, an only 10-fold higher concentration of dehydrosilybin is necessary in order to mimic the effect of CCCP. Dehydrosilybin and artificial uncouplers trigger cellular responses alike, while only dehydrosilybin is milder. We have therefore concluded that dehydrosilybin exhibits its antioxidant capacity by initiating uncoupler-like dissipation of the protonmotive force. When reflecting on our data, the omission of dehydrosilybin from a recent study into antiviral properties of silymarin (Polyak et al. 2010) may be tantamount to overlooking a crucial biologically active compound.

Polyphenolic compounds reportedly display antioxidant activity, the power of which is related to the structural features of each compound. When considering dehydrosi-

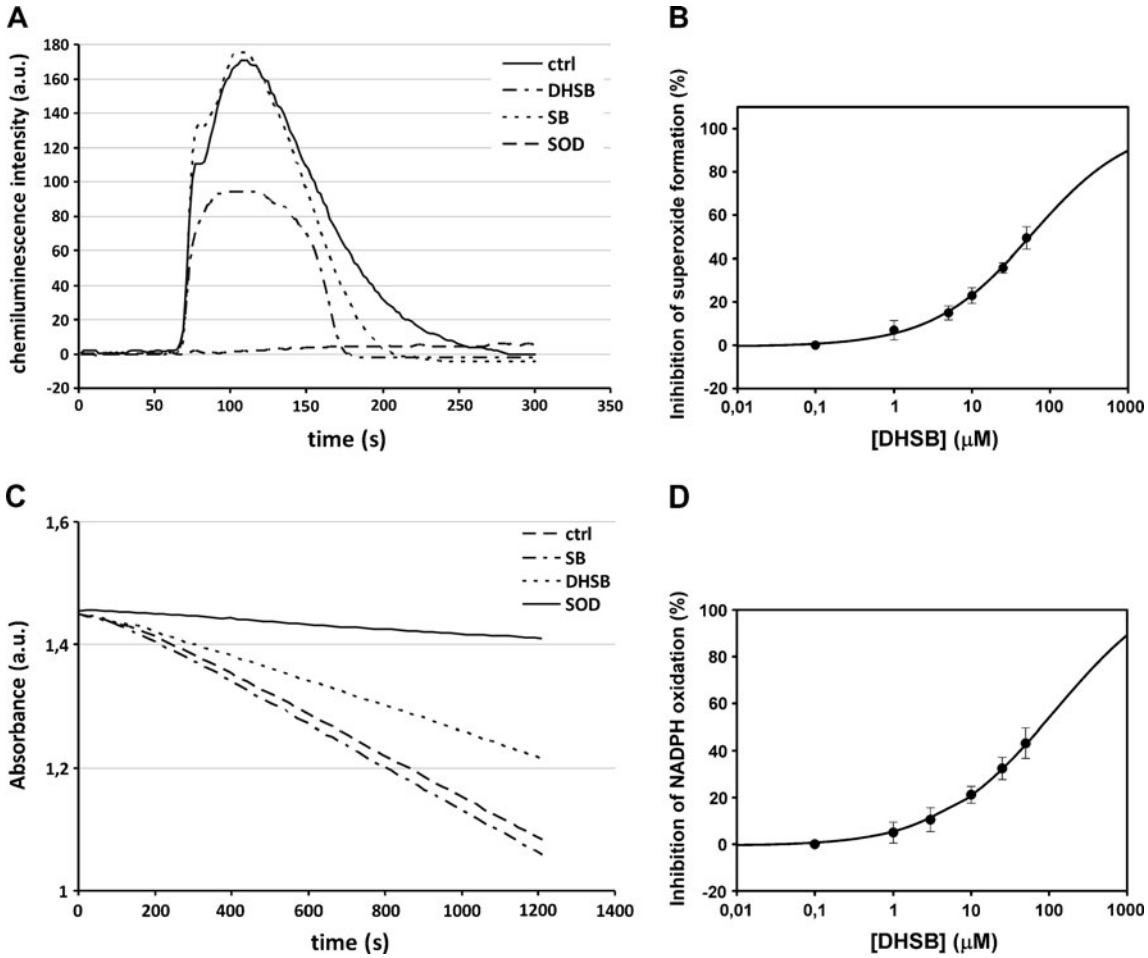

Fig. 5 The dehydrosilybin effect on superoxide production in model systems. *Panel A* Actual chemiluminescent traces of MCLA-detected superoxide production in differentiated HL-60 cells are shown. 10^6 cells/ml were stimulated by PMA alone (*solid* trace—control), or pre-incubated with 50 μM silybin (*dotted* trace—SB) or 50 μM dehydrosilybin (*dot-and-dashed* trace—DHSB) prior to PMA stimulation, or pre-incubated with 10 U/ml superoxide dismutase prior to PMA stimulation (*dashed* trace—SOD). *Panel B* Dose-response curve of dehydrosilybin titration in the system described for Panel A is shown. The area under the curve obtained with control cells stimulated by PMA alone was taken as 100%, the area under the curve obtained with cells stimulated by PMA in the presence of 10 U/ml SOD was taken as 0%. A regression analysis using 4 parameter Hill equation yielded IC_{50}=53.5 μM for dehydrosilybin. *Panel C* Actual absorbance change at 340 nm due to NADPH oxidation monitored over time is shown. Triethanolamine:diethanolamine buffer pH 7.4 contained $MnCl_2$ and NADPH, oxidation of which was initiated by 2-mercaptoethanol addition. Additions prior to the reaction start were: none (*dashed* trace—control), 50 μM silybin (*dot-and-dashed* trace—SB), 50 μM dehydrosilybin (*dotted* trace—DHSB), and 10 U/ml superoxide dismutase (*solid* trace—SOD). *Panel D* Dose-response curve of dehydrosilybin titration in the system described for Panel C is shown. The slope of the curve obtained for NADPH oxidation in the absence of other compounds was taken as 100%, the slope of the curve obtained for NADPH oxidation in the presence of 10 U/ml SOD was taken as 0%. A regression analysis using 4 parameter Hill equation yielded IC_{50}=75.3 μM for dehydrosilybin

lybin, it was shown to be a superior antioxidant in comparison with silybin (Gazak et al. 2004). This is mainly caused by the presence of an additional radical scavenging centre in its molecule (enol 3-OH group in conjugation with 4-oxo function) (Fig. 1), which has been identified as highly effective in DPPH-scavenging (ca 25 times better than silybin), but which is also an important feature for inhibition of lipid peroxidation and inhibition of ROS production in brown adipose tissue mitochondria (Gazak et al. 2009). These findings were confirmed using antioxidant assays with selectively protected derivatives of silybin and dehydrosilybin as well as with computational methods (Trouillas et al. 2008). 20-OH group of silybin is the single group of this compound, which exhibits radical scavenging properties (Trouillas et al. 2008; Gazak et al. 2009).

Previous studies by Dorta et al. focused on the aspects of energetics of mitochondria exposed to flavonoids quercetin, taxifolin, catechin, and galangin in relation to their structural features (Dorta et al. 2005) and their antioxidant activity on the Fe^{2+}/citrate-mediated membrane lipid peroxidation (Dorta et al. 2008). The studies conclude that

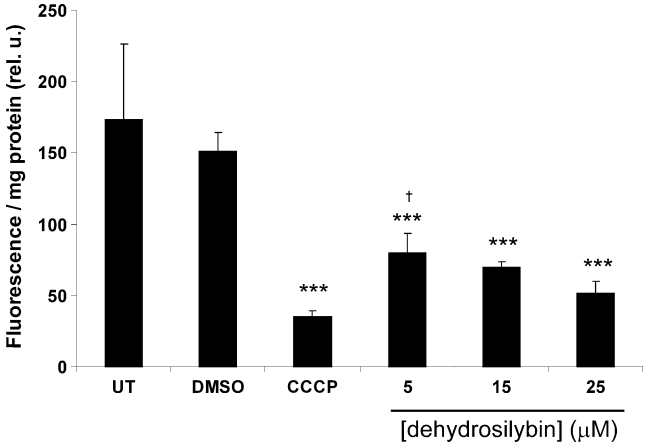

Fig. 6 The dehydrosilybin effect on rotenone-induced ROS formation in neonatal rat cardiomyocyte cultures. The production of reactive oxygen species was measured using fluorescent detection in cell lysates from neonatal rat cardiomyocytes. The bars represent the fluorescence intensity of fluorescein detected per mg of protein in cell cultures incubated in the presence of 7 μM rotenone for 1 h. The fluorescence intensity of fluorescein obtained in the absence of rotenone (background) was subtracted from each sample. Prior to the rotenone addition the cells were pre-incubated for 30 min with: no additions (UT), a corresponding volume of DMSO (DMSO), 2 μM CCCP (CCCP), and the indicated concentration of dehydrosilybin. The full range of dehydrosilybin concentrations tested is not shown in order to preserve figure clarity. The data is the means±SEM from three independent experiments. A statistical analysis using one-way ANOVA with post-hoc Tukey test yielded significant differences as indicated. * $p<0.05$, *** $p<0.001$ *versus* DMSO-treated cells; † $p<0.05$ *versus* CCCP treated cells

quercetin and galangin were more potent than taxifolin and catechin in protection against mitochondrial lipid peroxidation. The results further suggest that the 2,3-double bond in conjugation with the 4-oxo function in the flavonoid

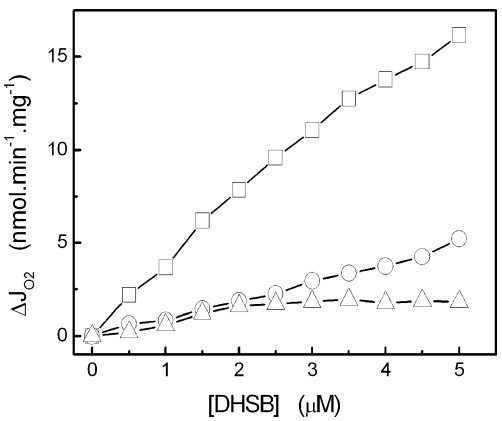

Fig. 7 Carboxyatractyloside inhibits dehydrosilybin-induced changes in respiration. Concentration dependencies of dehydrosilybin—dependent increase in respiration in the absence (*squares*) or presence of 1 μM CAT (*circles*) and 1 μM CAT *plus* 1 mM GTP (*triangles*). Rat heart mitochondria (0.1 mg/ml) respired in the presence of 5 mM pyruvate, 1 mM malate, and 5 mM succinate in a medium described in the "Materials and Methods". This data is representative of three independent experiments

structure are major determinants of the antioxidant activity of flavonoids in relation to mitochondria. The presence of an *o*-di-OH structure on the B-ring, as occurs in quercetin, favors this activity *via* superoxide scavenging, while the absence of this structural feature in galangin favors the antioxidant activity via a decrease in membrane fluidity and/or mitochondrial uncoupling (Dorta et al. 2008). Flavonoids, ranking among polyphenols, may then exert their antioxidant properties by acting in an uncoupler-like fashion apart from being ROS scavengers (Modriansky and Gabrielova 2009).

Our results indicate that dehydrosilybin is capable of cellular de-energization and also causes both an increase in mitochondrial respiration and a decrease in membrane potential in isolated intact mitochondria. These effects suggest that dehydrosilybin behaves as an uncoupler, a compound able to induce net electrophoretic H^+ transport across the inner mitochondrial membrane and thus able to uncouple the generation of the protonmotive force from the ATP synthesis. The ability of synthetic uncouplers to decrease the mitochondrial production of ROS as well as the effect of mild physiological uncoupling due to the function of specific ion carriers present in the inner mitochondrial membrane have been described in detail and are intensively studied (foa review, see (Skulachev 1998; Jezek and Hlavata 2005; Kowaltowski et al. 2009)). Extensive experimental evidence points to a decrease in mitochondrial production of ROS as a consequence of an increase in the respiratory rate and a corresponding decrease in the protonmotive force (Jezek and Hlavata 2005; Kowaltowski et al. 2009). Our data is consistent with this conclusion, indicating that dehydrosilybin-dependent uncoupling is associated with inhibition of mitochondrial ROS formation and the cytoprotective effect of dehydrosilybin against intracellular ROS.

As regards the mechanism of dehydrosilybin-dependent uncoupling and the consequent decrease in mitochondrial ROS production, our data shows that the effect of dehydrosilybin is to a great extent inhibited by CAT and further inhibited by the presence of both CAT and purine nucleotides. These results indicate that the uncoupling effect of dehydrosilybin does not mimic true uncouplers, such as CCCP or dinitrophenol, but is likely associated with the ANT and possibly other mitochondrial anion carriers, including the purine-nucleotide sensitive mitochondrial uncoupling protein UCP2. The ANT as well as UCP2 and other members of the mitochondrial solute carrier gene family are known to mediate fatty acid-dependent protonophoretic uncoupling (Garlid et al. 1996; Jaburek et al. 1999; Skulachev 1999), according to which the anionic FA head group is translocated by the carrier protein and the protonophoretic cycle is completed by the spontaneous rapid diffusion of the protonated FA through

the phospholipid bilayer. In accordance with this model, dehydrosilybin would act as an uncoupler by virtue of its ability to dissociate protons, undergo transport by the ANT in the form of anion and consequently permeate the phospholipid bilayer of the mitochondrial inner membrane as a protonated acid in order to complete the protonophoretic circle.

In light of the fact that the acidity of silybin and dehydrosilybin is somewhat similar, more important in relation to their different behavior in mitochondrial respiration is the presence of the 2,3-double bond in the structure of dehydrosilybin. Dehydrosilybin was reported as having been a compound substantially more hydrophobic than silybin. This unsaturated bond causes hyperconjugation among the A, B, and C rings, which consequently leads to the co-planarity of these rings. B and C rings in the silybin molecule, in contrast, are positioned in different planes to one other. The co-planarity of the ABC-ring system in dehydrosilybin is the possible reason for the increased hydrophobicity in comparison with silybin. This planarity could explain the ability of certain flavonols (e.g. quercetin and galangin) (Dorta et al. 2008) to influence mitochondrial respiration, as it may be responsible for a decrease in membrane fluidity. The impact of flavonols on mitochondria can vary, however, depending on the structure of their B ring. Quercetin, containing an *o*-di-OH structure on the B-ring, causes inhibition of the respiratory chain, whereas galangin, which has an unsubstituted B ring, causes uncoupling (Dorta et al. 2008). Accordingly, the difference in their action on mitochondrial respiration is in all probability related to the nature of their B ring, possibly its polarity. The similar effect of galangin and dehydrosilybin indicates, that the presence of the D ring in dehydrosilybin weakens the polarity of this part of the molecule, in comparison with quercetin, which forms part of the dehydrosilybin structure, and leads to effective uncoupling.

The ANT-mediated, dehydrosilybin-dependent protonophoretic cycle contrasts with the indicated ability of flavonoid quercetin to inhibit ANT (Ortega and Garcia 2009). Our data does not indicate that dehydrosilybin inhibits ANT, but is consistent with dehydrosilybin-dependent ANT-mediated uncoupling and the consequent decrease in the coupling efficiency of respiring mitochondria. Our preliminary data further indicates that several other structurally related flavonolignans also uncouple mitochondria through an ANT-dependent process (M. Jaburek and M. Modriansky unpublished). The possibility that dehydrosilybin and other structurally related flavonolignans are transported by ANT, UCP2 and other members of the gene family awaits further investigation.

The data presented here has led us to adjust the hypothesis put forth by Modriansky and Gabrielova (Modriansky and Gabrielova 2009) as it omits the interactions of polyphenols with inner mitochondrial membrane proteins hence leaving out an important facet of mitochondrial respiration uncoupling. Structure—activity (SAR) and quantitative structure—activity (QSAR) studies focusing on polyphenols interacting with mitochondrial carriers are likely to aid the search for evidence supporting the beneficial effects of certain foods, an indigenous source of polyphenols likely providing natural mild uncouplers of oxidative phosphorylation. We would propose that dehydrosilybin may be considered as a lead compound for synthesis of mild uncouplers potentially useful for treatment of conditions that would benefit from mild uncoupling, with these being possibly ischemic conditions (pre- and post- conditioning), obesity or metabolic syndrome (mild energy wasting).

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