ORIGINAL ARTICLE

Effects of novel quercetin derivatives on sarco/endoplasmic reticulum Ca^{2+}-ATPase activity

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Abstract

OBJECTIVES: We examined effect of novel quercetin derivatives on sarcoplasmic reticulum (SR) Ca-ATPase activity isolated from skeletal muscles and their potential to prevent injury of SERCA induced by peroxynitrite that is elevated in multiple pathological processes.
METHODS: SR was isolated by ultracentrifugation, ATPase activity of SERCA was measured by NADH-coupled enzyme assay. Sulfhydryl and carbonyl groups content was determined to test oxidation of SERCA. Conformational changes in ATP and calcium binding site were assessed using specific fluorescent labels.
RESULTS: Di(diacetylcafeoyl)-mono-(monoacetylcafeoyl) quercetin (DACQ) restored and diquercetin significantly decreased activity of SERCA in the presence of peroxynitrite. Diquercetin significantly decreased SERCA activity in absence of peroxynitrite. All tested quercetin derivatives decreased thiol group content of SR and caused change in SERCA conformation. Significant decrease of protein carbonyls was observed in SERCA treated with di(diacetylcafeoyl)-mono-(monoacetylcafeoyl) quercetin in the presence of peroxynitrite.
CONCLUSION: DACQ protected SERCA in SR against formation of carbonyls *in vitro* and protected activity of the pump against inhibition caused by peroxynitrite. However, none tested quercetin derivative did protect SERCA against conformational changes and sulfhydryl group oxidation. Diquercetin inhibited SERCA at relatively low concentrations in the presence of peroxynitrite. Diquercetin and DACQ may prove to be beneficial in treatment of cancer and inflammatory diseases, respectively.

INTRODUCTION

The sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) maintains cytosolic concentration of calcium by pumping cytosolic Ca^{2+} into the sarcoendoplasmic/endoplasmic reticulum (Aubier & Viires 1995). The SERCA pump plays two roles in muscle tonus regulation. SERCA mediates muscle relaxation by decreasing the cytosolic calcium and plays a role in restoration of calcium concentration necessary for muscle contraction. Alterations in Ca^{2+} levels cause many types of pathological

processes, including heart disease, diabetes and atherosclerosis. Oxidatively modified forms of SERCA accumulate during aging and under pathological situations accompanied by oxidative stress.

SERCA is inactivated in pathological processes that are accompanied by elevated levels of free radicals. Artherosclerosis, and other vascular inflammatory diseases, elevate the levels of reactive oxygen/nitrogen species (ROS, RNS), (Dhalla *et al.* 2000; Griendling & Fitzgerald 2003) by increasing superoxide production. Superoxide has been found to react rapidly with nitrogen oxide and form peroxynitrite. Peroxynitrite is a very strong oxidant, which oxidizes metals, lipids, DNA and proteins. SERCA inhibition by peroxynitrite was first reported by Viner *et al.* (1999). Oxidation of cysteine residues (Sharov *et al.* 2006) and nitration of tyrosines (Gutierrez-Martin *et al.* 2004) have been observed after incubation of SERCA with peroxynitrite. Oxidation of cysteines and nitration of tyrosines cause the loss of SERCA activity.

Flavonoids belonging to group of phytoestrogens that are rich in fruit and vegetables have been described to modulate SERCA activity (Ogunbayo *et al.* 2008). Quercetin has been shown to inhibit SERCA activity by preventing binding and hydrolysis of ATP (Shoshan & MacLennan 1981). Quercetin, the most abundant of plant flavonoids, is an excellent peroxynitrite scavenger (Haenen *et al.* 1997), inhibits release of histamine and proinflamatory cytokines (Park *et al.* 2008) and induces apoptosis in cancer cells by downregulation of anti-apoptic Bcl2 and upregulation of pro-apoptic Bax protein (Duo *et al.* 2012). SERCA has been reported to be inhibited by quercetin. Possible mechanism of onset of apoptosis within cancer cells is inactivation of SERCA which in turn increase an intracellular load of calcium and induces calcium-dependent mitochondria-mediated cell death. Mechanisms of apoptosis induction via SERCA inhibition have been reviewed by Denmeade and Isaacs (2005). Activation of apoptosis in cancer cells by inactivation of SERCA may be promising strategy for treatment of cancer.

SERCA activity was shown to be decreased in vascular diseases such as atherosclerosis (Tong *et al.* 2010). SERCA is injured by reactive oxygen species that are elevated in diseased blood vessels. Protection of SERCA against injury mediated by ROS may be beneficial in treatment of vascular diseases.

With the aim to prepare biologically active compounds enhancing antioxidative properties and/or bioavailability of quercetin, novel quercetin derivatives were synthesized. In this work, we have examined modulatory activities of novel quercetin derivatives as diquercetin (DQ), trichlorpivaloyl-quercetin (TCPQ), di(diacetylcafeoyl)-mono-(monoacetylcafeoyl) quercetin (DACQ) on SERCA activity. We tested activities of quercetin derivatives on SERCA activity in the absence or presence of peroxynitrite, a potent oxidant which is in excess during patological processes.

MATERIALS AND METHODS

Quercetin derivatives synthesis

Novel quercetin derivatives were prepared via selective protection procedures of quercetin and acylation with acylchlorides: The ultimate step consisted of deprotection and column chromatography. Partial alkylation and careful separation from a complex mixture produced naphtoquinone derivative. Dimer of quercetin was obtained by the treatment of quercetin with a metal salt at 50 °C (patent filed).

SR isolation

SR vesicles were isolated from the fast-twitch skeletal muscle of a New Zealand female rabbit (about 2.5 kg) according to Warren *et al.* (1974) and modified by Karlovská *et al.* (2006).

Protein concentration was determined by the method of Lowry *et al.* (1951) using bovine serum albumin as standard, and spectrophotometricaly at 280 nm in the presence of 1% of sodium dodecyl sulfate (de Foresta *et al.* 1992).

Ca-ATPase activity

The activity of sarcoplasmic reticulum Ca^{2+}-ATPase was measured by the NADH-coupled enzyme assay according to Warren *et al.* (1974) with modifications reported by Karlovská *et al.* (2006). The sarcoplasmic reticulum vesicles (12.5 μg protein per cuvette) were added to the assay mixture (40 mM HEPES pH 7.2, 0.1 M KCl, 5.1 mM $MgSO_4$, 2.1 mM ATP, 0.52 mM phosphoenolpyruvate, 1 mM EGTA, 0.15 mM NADH, 7.5 IU of pyruvate kinase, 18 IU of lactate dehydrogenase) to a final volume of 2.5 ml and incubated at 37 °C for 2 minutes. The reaction was started by addition of 1 mM $CaCl_2$ to reaction mixture. The reaction rate was determined by measurement of the absorbance at 340 nm and 37 °C.

Peroxynitrite synthesis

Peroxynitrite was synthesized according Radi *et al.* (1991). Briefly, a solution of 0.6M $NaNO_2$ was mixed at 4 °C under vigorous stirring with an equal volume of 0. 7M H_2O_2 acidified with 0.6M HCl. The reaction between H_2O_2 and $NaNO_2$ was immediately quenched by addition of an equal volume of 3M NaOH. In order to eliminate any unreacted H_2O_2, the reaction mixture was then treated under gentle agitation with manganese dioxide (7 mg/mL) for 1 hour at 4 °C. Peroxynitrite solution was split into small aliquots and frozen at –20 °C until use. Peroxynitrite concentration was measured spectrophotometrically in aliquots thawed immediately before the experiments, using an extinction coefficient of 1670 $M^{-1}.cm^{-1}$.

The concentration of peroxynitrite which induced a 50%-decrease of ATP-ase activity was found to be 100 μM (IC50). SR samples were treated with quercetin and its derivatives (10–150 μM) in absence or pres-

ence of peroxynitrite (100 μM). To assess effect of Q or its derivatives on SERCA in presence of PN, SERCA was treated with Q or its derivatives for 2 minutes. Peroxynitrite at 100 μM was then added to reactions and incubated for 2 minutes identically in all experiments. Values that were above or below IC50 indicated protective or inhibitory effect of tested compounds, respectively.

Determination of carbonyls

SR samples (1mg/ml) were derivatized with dinitrophenylhydrazine in a solution containing 10 mM dinitrophenylhydrazine, 6% SDS, 5% trifluoroacetic acid. Samples (2μg of SR proteins per lane) were subjected to SDS-PAGE, the proteins were transferred to Immobilon-PPVDF membrane (Millipore) by semi-dry electroblotting (HoeferScientific Instruments) at 15V (250 mA) for 30 min. The membrane was blocked 1 hour at room temperature and incubated with primary anti-dinitrophenyl antibody (D9659; Sigma; diluted 1:5000) overnight at 4 °C. The membrane was washed twice briefly, then 3 × 10 minutes and incubated with secondary monoclonal anti-rabbit IgG horseradish peroxidase conjugate 1 hour at room temperature (A1949; Sigma; diluted 1:10 000). After two brief and 3 × 10 minutes washes, bands were visualized by the ECL Plus Western blotting detection system (Amersham) according to the manufacturer's instructions, and the images were captured on a Kodak X-ray film using a Kodak-developer/fixer kit (Fisher). Blocking, incubations of membrane with primary and secondary antibodies were done in 3% BSA, PBST (0.1% Tween, PBS). All washing steps of membrane were done in PBST.

Labelling of Ca^{2+} ATPase with FITC

We used FITC:ATPase labeling ratio of approx. 0.5:1. SR (0.6 mg, 24 μl of SR with protein comcentration of 25 mg/ml) in 1 M KCl, 0.25 M sucrose and 50 mM Hepes, pH 8, in the volume of 35 μl. The reaction mixture was incubated with 2.5 nmol of FITC. Stock solution of FITC (6 mM) was prepared in dry dimethylformamide. The reaction mixture was left to stand at room temperature in dark for 1 h and then diluted with 250 μl of 0.2 M sucrose and 50 mM Tris-HCl pH 7. Labeled samples was left to stand 30 minutes at room temperature and stored on ice until use. Fluorescence spectra were measured on a Fluormax 4 spectrofluorimeter at 25 °C, and relative fluorescence units were recorded. Labeled protein (15 μg) was added to 1 ml of 5 mM $MgSO_4$, 100 mM KCl and 50 mM Tris-HCl buffer, pH 7, at 25 °C. EGTA and $CaCl_2$ were added from stock solutions to give total concentrations of 25 μM and 0.8 mM, respectively. Fluorescence readings were collected in the emmision range of 500–600 nm using excitation at 493 nm. Emmision readings at 517 nm were used for quantitation of fluorescence of labeled samples. Both the excitation and emmision slits were set at 3 nm.

Labelling of Ca^{2+} ATPase with NCD-4

SR ATPase was labeled with N-cyclohexyl-N0 -(4-dimethylamino-l-naphthyl) carbodiimide (NCD-4) purchased from Invitrogen, accordingto the method of Munkongeet al. (1989) and Velasco-Guillen et al.(1998). Briefly, SR (1 mg/ml) was incubated with NCD-4 (150 μM) in 100 mM KCl, 50 mM Hepes-KOH, 1 mM EGTA, 0.2 M sucrose (pH 6.2) for 3 hours at 25 °C in the dark. The reaction was quenched by adding a 75-fold volume of 40 mM Hepes-KOH, 100 mM KCI, 1 mM EGTA, 5 mM $MgSO_4$ (pH 7.0). Fluorescence intensities were measured on a Fluormax-4 spectrofluorimeter (Horiba, USA) at 25 °C using excitation at 333 nm. Emission spectra were recorded in the range of 410–450 nm. Both the excitation and emission slits were set at 3 nm. Data collected at 437 nm were used for quantitation of fluorescence in particular samples.

Sulfhydryl group determination

Oxidation of cysteine residues was monitored according to Sharov *et al.* (2006) using the maleimide-based fluorescent dye Thio-Glo1 (TG1, methyl-10-(2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl)-9-methoxy-3-oxo-3Hbenzo-[f]chromene-2-carboxylate), purchased from Calbiochem. A stock solution of TG1 (2 mM) was prepared in acetonitrile. SR samples, containing 100 μg of protein in 20 mM sodium phosphate, were incubated with 200 μM TG1 in presence of 10% (v/v) acetonitrile and 2% (w/v) SDS for 30 min at 37 °C, pH 7.4 in a final volume 100 μl. To achieve maximum labeling of SR cysteine residues, as monitored by fluorescent spectroscopy. 10 μg of TG1 labelled total SR protein was 60-fold diluted in 20 mM PBS and fluorescence emission spectra of cysteine-TG1 protein adducts were recorded by Infinite M200 Tecan spectrofluorometer using excitation and emission wavelengths of 379 and 513 nm, respectively. Glutathione was used to calibrate the absolute amount of thiols in SERCA.

Statistical data analysis

The statistical data analysis of the data was performed using one-way ANOVA test with Turkey comparison. The values are expressed as mean ± SEM. Experiments were repeated three times with at least three parallel measurements.

RESULTS AND DISCUSSION

In this paper, we study effect of quercetin or its derivatives on activity of SERCA. Quercetin and its derivatives may affect activity of SERCA by its oxidative modification and/or change of its conformation. On the contrary quercetin or its derivatives protect SERCA against peroxynitrite as quercetin was reported to scavenge free radicals. Therfore, we tested effect of Q or its derivatives on SERCA in presence of peroxynitrite. To assess effect of Q or its derivatives on SERCA we test its activity, oxidation by determination of SH groups

availability and formation of total carbonyls in SR. We used NCD-4 or FITC fluorescent labels as indicators of conformational changes in calcium, respectively ATP binding site of SERCA.

Examining SERCA in SR, we first assessed amount of SERCA in SR isolated from rabbit muscle. Total content of proteins in SR is shown in Figure 1. Content of SERCA in SR was approximately 80% which is in agreement with results reported by Martonosi (1996). Presence of SERCA in isolated SR has been confirmed also by anti-SERCA1 antibody.

Effects of quercetin and its derivatives on activity of SERCA were analysed in absence and presence of peroxynitrite (PN). To test effect of quercetin and its derivatives on SERCA in the presence of PN, we determined inhibitory concentration of peroxynitrite (IC_{50}) decreasing SERCA activity to 50% by treatment of SERCA by 0–500 μM peroxynitrite (Figure 2). 100 μM peroxynitrite was shown to inhibit SERCA activity to 50% (IC_{50}). Effect of Q or its derivatives on SERCA was tested in presence of 100 μM peroxynitrite.

In absence of PN we observed slight and negligible inhibitory effect of quercetin and DACQ, respectively (Figure 3). TCPQ and DQ markedly inhibited activity of SERCA in absence of PN. In the PN presence, Q and TCPQ showed minor effect and their inhibitory effect was observed at 150 μM (Figure 3). DQ notably decreased SERCA activity below level caused by peroxynitrite, however DACQ added at 100 μM restored SERCA activity in the presence of peroxynitrite.

To examine oxidative effect of quercetin or its derivatives on SERCA, we determined level of thiol group availability in SERCA in absence of PN. To assess protective effect of quercetin or its derivatives against thiol oxidation, thiol group content was tested in the presence of 100 μM peroxynitrite (IC_{50}). Thiol group content decreased below 20% at concentration of quercetin or its derivatives >50 μM both in absence and presence of PN (Figure 4). Protein carbonyls were determined as a marker of oxidation mediated by PN. Total carbonyls indicating oxidative damage induced by PN slightly decreased in the presence of Q, DQ and DACQ (Figure 5) in comparison to control level. The decrease of carbonyl formation at about 51 %, in the presence of DACQ and PN indicate that DACQ protects SERCA from carbonyl formation under *in vitro* conditions.

The fluorescent probe NCD-4 was used to examine structural alterations in the Ca^{2+} binding (transmembrane) domain in SERCA in the presence of quercetin or its derivatives. Peroxynitrite induced none conformational changes at 50–200 μM in absence of quercetin or its derivatives. Thus, conformational changes were measured in the presence of quercetin or its derivatives without addition of peroxynitrite. We observed a significant decrease in NCD-4 fluorescence for all tested compounds comparison to control (Figure 6a). Decrease of NCD-4 fluorescence in presence of quercetin or its derivatives indicated structural alteration in the Ca^{2+} binding site.

To test conformation change of the ATP nucleotide binding site located in cytosol, the fluorescent probe FITC, labeling a specific Lysine (Lys^{515}) residue was used. The conformational change was tested in the presence of PN and quercetin or its derivatives. We found 10% and 30% decrease (Figure 6b) in affinity of FITC to SERCA in the presence of quercetin and its tested derivatives, respectively.

We observed conformational changes for Q and all its tested derivatives. SERCA activity has been shown to be affected by lipid bilayer thickness and fluidity (Gustavsson *et al.* 2011). We propose that lipophilic character of pivaloyl moiety in trichloropivaloyl quercetin may facilitate its incorporation into lipid bilayer of SR. TrichloropivaloylQ incorporation into lipid bilayer likely change its properties which may result in decreased activity of SERCA.

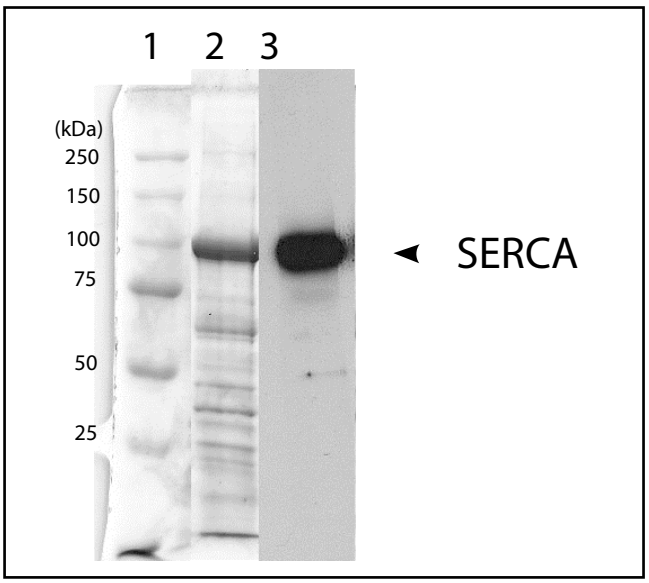

Fig. 1. Content of SERCA in SR. lane 1, protein ladder; lane 2, total protein content of SR (coomassie blue stained SDS Page); lane 3, detection of SERCA in SR total protein content using anti-SERCA1 antibody.

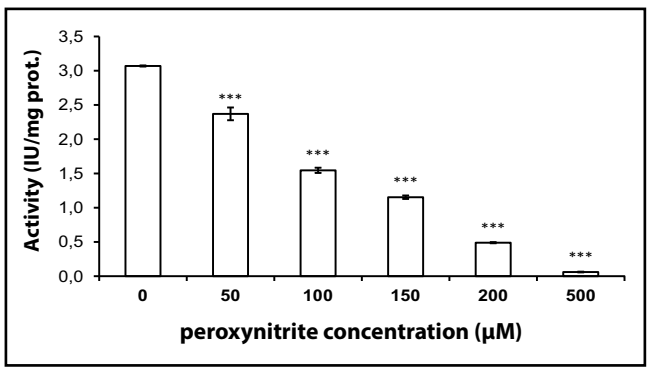

Fig. 2. Determination of PN concentration causing 50% inhibition of SERCA, (IC_{50}). Statistical significance with respect to control ***$p<0.001$.

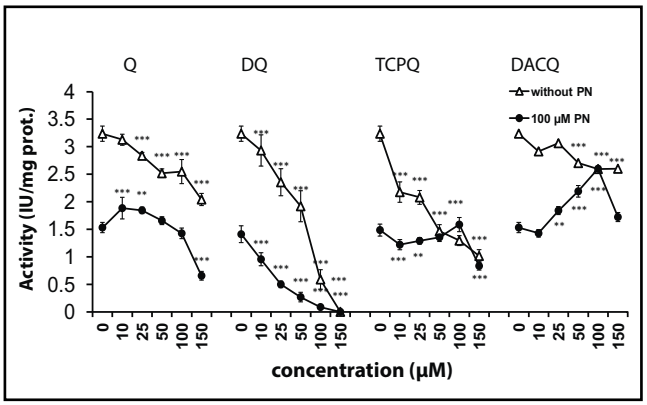

Fig. 3. Effect of quercetin or its derivatives on SERCA activity in absence (Δ) or presence (●) of 100 μM peroxynitrite. Values are mean ± SEM and represent three independent experiments with at least three parallels. Statistical significance with respect to control **p<0.01 and ***p<0.001.

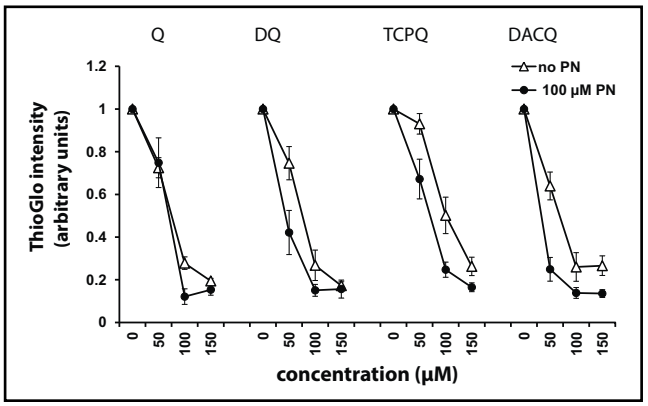

Fig. 4. Free thiol groups content of SR treated with quercetin or its derivatives in the absence (Δ) and presence (●) of 100 μM peroxynitrite. Values are mean ±SEM and represent three independent experiments with at least three parallels. All values are significantly decreased with p<0.001 in comparison to control.

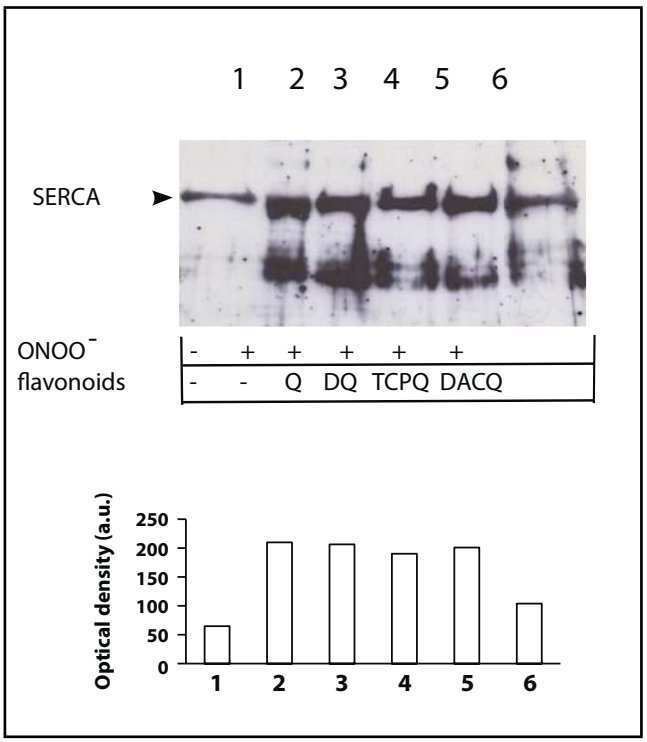

Fig. 5. Carbonyl formation. Anti-DNP antibody was used for immunoblotting. The position of SERCA and graph underneath the blot indicates density of individual bands. Density of individual bands was quantitated using ImageJ (NIH) software. The data shown are representative of three independent experiments.

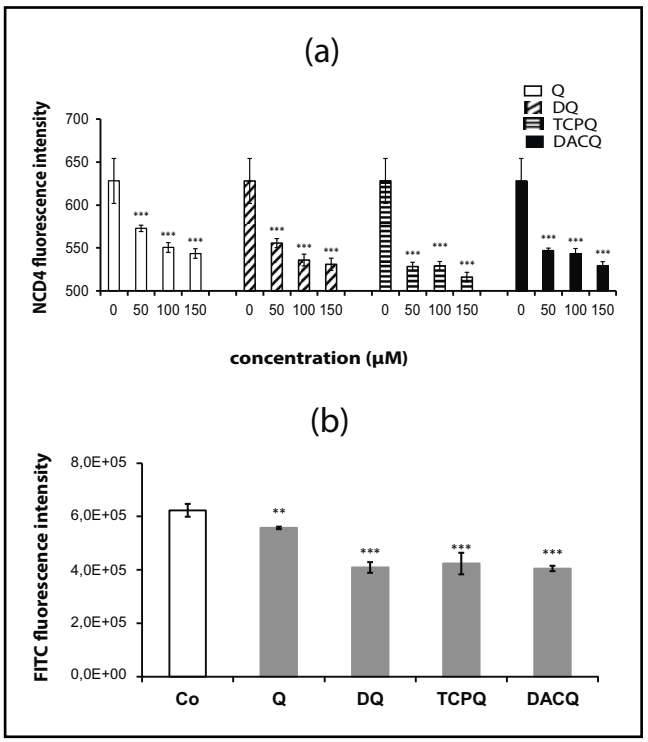

Fig. 6. Conformational changes: (a) NCD-4 and (b) FITC fluorescence was measured to identify changes in calcium respectively ATP binding site. Relative fluorescence units are shown as mean ± SEM and represent three independent experiments. NCD4 and FITC fluorescence intensity was measured in absence and presence of 100 μM PN. Effect of quercetin or its derivatives on FITC was determined at 100 μM. **p<0.01 and ***p<0.001 are significant differences with respect to control.

Presence of caffeoyl moiety in DACQ may be responsible for SERCA protection in presence of peroxynitrite. Caffeic acid has been reported to scavenge free radicals (Gulcin 2006). Protection against carbonyl formation caused by peroxynitrite supports antioxidative properties of DACQ. Decreased affinity of ATP and Ca^{2+} binding site specific label indicate changes in SERCA conformation. Thus, DACQ may protect SERCA also by change of membrane properties and/or interaction with SERCA. However, changes in SERCA conformation have to be proved by further experiments.

Summary of observed effects of Q and Q derivatives are shown in Table 1. In this study, we show notable decline in activity of DQ treated SERCA both in absence

Tab. 1. Summary of effects of quercetin and its derivatives on SERCA in presence or absence of 100 μM peroxynitrite. Arrows show the trend of values measured at increasing concentration of Q or its derivatives.

	activity		SH content		carbonyl content		ATP binding site labelling		Ca^{2+} binding site labelling	
	–PN	+PN	–PN	+PN	–PN	+PN	–PN	+PN	–PN	+PN
none	↔	↓	↔	↓	↔	↑	↔	↔	↔	↔
Q	↓	↓	↓	↓	–	↑	↓	–	↓	–
DQ	↓	↓	↓	↓	–	↑	↓	–	↓	–
TCPQ	↓	↓	↓	↓	–	↑	↓	–	↓	–
DACQ	↔	↑*	↓	↓	–	↓	↓	–	↓	–

individual symbols: ↓, reduced levels; ↑, elevated levels; ↔, unchanged levels; –, not measured; *, reaching level of SERCA activity treated with DACQ in absence of PN

or presence of PN. On the contrary, DACQ protects SERCA against inhibition caused by peroxynitrite.

Quercetin has been shown to decrease production of ROS in the cells at low concentrations, but at higher concentrations it produced peroxides in the cell culture medium and decreased thiol group content and viability of the cells (Robaszkiewicz *et al.* 2007). This is in agreement with activities of SERCA which we measured in presence of quercetin and PN (Figure 3). Quercetin was also reported to form quercetin oxidation products like semiquinone radicals or quinones during its antioxidative activity which has recently been defined as quercetin paradox (Boots *et al.* 2007). Thus, increase in inhibitory effect of diquercetin in the presence of PN may be caused by increase in formation of quercetin derived radicals as two coupled molecules of quercetin form DQ and/or hydrophobicity. Increase in carbonyl formation (Figure 5) and decrease of SH group availability supports view of prooxidative effect of Q and DQ on SERCA.

DACQ protects SERCA activity in presence of 50 and 100 μM PN despite the decrease in SERCA thiol content (Figure 3). Decrease in SH group content may correspond to oxidation of cysteine residues that are not crucial for SERCA activity. Sharov *et al.* (2006) have shown that the peroxynitrite-dependent loss of individual cysteine residues coincides with the loss of SERCA activity only for the residues at positions 674/675 and 938. No other cysteine residues have been described to correlate with protein inactivation. Sharov *et al.* (2006) also suggested that some cysteine residues, i.e. at positions 525, 498, 417, 420 and 364, may protect functionally important cysteine residues from oxidation and could be considered to be intramolecular antioxidants similar to the function ascribed to protein methionine residues (Viner *et al.* 1999; Yamashita & Kawakita 1987).

DACQ protected SERCA against protein carbonyl formation (Figure 5, lane 6) in presence of PN. Formation of protein carbonyls in cells has been proposed to arise in peroxynitrite reactions with proteins (Ischiropoulos and Mehdi 1995; Szabo *et al.* 1997), however, peroxynitrite reactions with pure proteins do not significantly yield carbonyls (Tien *et al.* 1999). Peroxynitrite has been previously shown to induce oxidation of membrane lipids resulting in production of malondialdehyde (Radi *et al.* 1991). Malondialdehyde was reported to introduce carbonyl groups into proteins (Burcham & Kuhan 1996). SERCA activity is known to be dependent on phospholipid content and structural state of the SR membrane (Lee at al., 1995; Shutova *et al.* 1999). Thus, increase in carbonylation of SERCA which we observed in lanes 3–5 (Figure 5) in comparison to control, may be caused by secondary oxidation of SERCA by products of lipid oxidation. DACQ may protect SR membrane structure and/or prevent generation of molecules arising during membrane lipid oxidation. Interestingly, DACQ increases activity of SERCA in the presence of PN, however, FITC indicates conformational change in SERCA ATP binding site. Our results are in agreement with the discrepancy between decreased FITC labeling and restored SERCA activity reaching normal level that has been previously reported by Mishima *et al.* (2009).

Diquercetin markedly inhibits SERCA activity and may prove to be beneficial in treatment of cancer as SERCA inhibitors were described to induce apoptosis (Vangheluwe *et al.* 2005). Inhibition of SERCA may lead to increase in intracellular load of calcium. Increased load of calcium has been described to induce cell death in several types of cancer cells (Lin *et al.* 2007; Sergeev *et al.* 2006). On the contrary, protection of SERCA by DACQ may be advantageous in inflammation relieve/recovery where SERCA activity is inhibited by free radicals. SERCA damaged by ROS is unable to pump calcium into ER/SR, thus causing increased intracellular content of calcium and chronically elevated calcium is a final step to cell death (Orrenius *et al.* 1991; Laporte *et al.* 2004). Protection of SERCA in cells with increased content of ROS may prevent apoptosis and necrosis of cells in tissues such as heart and veins.

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Potential Conflicts of Interest: *None disclosed.*

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