Modulation of rabbit muscle sarcoplasmic reticulum Ca\(^{2+}\)-ATPase activity by novel quercetin derivatives

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ABSTRACT
Sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) is the pump crucial for calcium homeostasis and its impairment results in pathologies such as myopathy, heart failure or diabetes. Modulation of SERCA activity may represent an approach to the therapy of diseases with SERCA impairment involvement. Quercetin is flavonoid known to modulate SERCA activity. We examined the effect of nine novel quercetin derivatives on the activity of the pump. We found that 5-morpholinohydroxypoxyquercetin, di(prenylferuloyl)quercetin, di(diacetylcaffeoyl)-mono-(monoacetylcaffeoyl)quercetin and monoacetylferuloylquercetin stimulated the activity of SERCA. On the contrary, monochloropivaloylquercetin, tri(chloropivaloyl)quercetin, pentaacetylquercetin, tri(trimethylgalloyl)quercetin and diquercetin inhibited the activity of the pump. To identify compounds with a potential to protect SERCA against free radicals, we assessed the free radical scavenging activity of quercetin derivatives. We also related lipophilicity, an index of the ability to incorporate into the membrane of sarcoplasmic reticulum, to the modulatory effect of quercetin derivatives on SERCA activity. In addition to its ability to stimulate SERCA, di(prenylferuloyl)quercetin showed excellent radical scavenging activity.

KEY WORDS: calcium pump; scavenging; flavonoids; antioxidants

Introduction
The Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum (SR) is a particularly abundant protein, and plays a very important role in excitation-contraction coupling. Calcium is released from sarcoplasmic reticulum through calcium-release channels (ryanodine receptors) resulting in muscle contraction. Sarco/endoplasmic Ca\(^{2+}\)-ATPase (SERCA) pumps calcium back into sarcoplasmic reticulum which leads to muscle relaxation. Changes in the calcium transport have been reported to play a role in a variety of pathological processes, such as vascular diseases, diabetes, myopathy and heart failure (Hovnanian, 2007). In pathological processes that are accompanied by elevated levels of free radicals SERCA becomes inactivated. Generally, therapies aimed on improving contractility are based on increasing the activity of the pump.

Flavonoids, belonging to the group of phytoestrogens, which are rich in fruit and vegetables have been described to modulate SERCA activity (Ogunbayo et al., 2008). Quercetin (Q), the most abundant of plant flavonoids, is an excellent peroxynitrite scavenger (Haenen et al., 1997). It inhibits the release of histamine and proinflammatory cytokines (Park et al., 2008) and induces apoptosis in cancer cells by downregulation of anti-apoptic Bcl2 and upregulation of pro-apoptotic Bax protein (Duo et al., 2012). Novel quercetin derivatives were synthesized with the aim to prepare biologically active compounds which enhance antioxidative properties and/or bioavailability of quercetin. In this paper, we examined the influence of novel quercetin derivatives on the activity of SERCA and determined free radical scavenging properties of quercetin derivatives using the DPPH radical scavenging assay.

Materials and methods
Synthesis of quercetin derivatives
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 naphthoquinone derivative. The dimer of quercetin was obtained by its treatment with a metal salt at 50 °C (patent filed). Structures of quercetin derivatives are shown in Table 1.

Isolation of sarcoplasmic reticulum
SR vesicles were isolated from 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).

SR vesicles (1 mg protein per ml) were incubated with quercetin or individual quercetin derivatives at concentrations 10–150 μM for 2 min at 37 °C, pH 7.4

Ca\textsuperscript{2+}-ATPase activity
The activity of sarcoplasmic reticulum Ca\textsuperscript{2+}-ATPase was measured by the NADH-coupled enzyme assay according to Warren et al. (1974). 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\textsubscript{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) and incubated at 37 °C for 2 minutes. The reaction was started by addition of 1 mM CaCl\textsubscript{2} to the reaction mixture. The reaction rate was determined by measurement of the absorbance at 340 nm and 37 °C.

DPPH free radical scavenging assay
To investigate the radical scavenging activity of the quercetin derivatives, the ethanolic solution of DPPH (50 μM) was incubated in the presence of the compound tested (50 μg/ml) at laboratory temperature. The absorbance decrease recorded during the first 75-s interval at 518 nm, was taken as a marker of radical scavenging activity. The decrease in absorbance is expressed in Absorbance Units.

Results
Quercetin had little effect on SERCA activity up to 100 μM. We observed slight inhibition of SERCA by quercetin at 150 μM (Figure 1B). Figure 1A shows that 5-morpholinohydroxypropoxyQ, di(prenylferuloyl)Q, di(diacetylcaffeoyl)-mono(monoacetylcaffeoyl)Q and monoacetylferuloylQ stimulated SERCA activity. MonochloropivaloylQ, trichloropivaloylQ and diquercetin remarkably inhibited SERCA activity (Figure 1B). Tri(trimethylgalloyl)Q and pentaacetylQ inhibited the pump slightly.

In order to determine the radical-scavenging potential of the quercetin derivatives tested, the reactivity toward the stable free radical DPPH was measured by absorbance decrease of ethanol solution of DPPH (50 μM) containing the samples tested (50 μM) at 518 nm (Table 2). Absorbance decrease was determined during
the first 75-s interval and compared with the effect of the reference antioxidant quercetin. The antiradical activity of the quercetin derivatives is shown in Table 2 and decreases as follows: monochloropivaloylQ > quercetin > di(prenylferuloyl)Q > tri(chloropivaloyl)Q > di(diacetyl caffeoyl)-mono-(monoacetyl caffeoyl)Q > monoacetylferuloylQ > 5-morpholinohydroxypropoxyQ > pentaacetylQ > tri(trimethylgalloyl)Q.

Table 1. Structures of quercetin and its derivatives tested.

| Name                          | Substituents                        |
|-------------------------------|-------------------------------------|
| 5-morpholinohydroxypropoxyQ   | R1=\[\begin{array}{c}O ON \end{array}\] R2-5= -OH |
| di(prenylferuloyl)Q           | R1,2=\[\begin{array}{c}O O CH_3 \end{array}\] R3-5= -OH |
| di(diacetyl caffeoyl)-mono- (monoacetyl caffeoyl)Q | R1=\[\begin{array}{c}O O O \end{array}\] R2,4=\[\begin{array}{c}O O O \end{array}\] R5= -OH |
| monoacetylferuloylQ           | R1=\[\begin{array}{c}O \end{array}\] R2-5= -OH |
| pentaacetylQ                  | R1-5=\[\begin{array}{c}O CH_3 O \end{array}\] |
| quercetin                     | R1-5= -OH |
| tri(trimethylgalloyl)Q        | R1-3=\[\begin{array}{c}O O CH_3 \end{array}\] R4,5= -OH |
| monochloropivaloylQ           | R1=\[\begin{array}{c}O \end{array}\] R4-5= -OH |
| tri(chloropivaloyl)Q          | R1,4,5=\[\begin{array}{c}O \end{array}\] R2,3= -OH |

Structure of: diquercetin

\[\begin{array}{c}O OH OH OH OH OH \end{array}\]
LogP values (octanol-water partition coefficients) indicating lipophilicity and thus the ability of quercetin derivatives to incorporate into the lipid membrane of SR were calculated using ACD/Chemsketch software and are specified in Table 2. LogP values of quercetin derivatives decrease as follows: di(prenylferuloyl)Q > tri(trimethylgalloyl)Q > diqueretin > tri(chloropivaloyl)Q > di(diacetylcaffeoyl)-mono-(monoacetylcaffeoyl)Q > monoacetylferuloylQ > 5-morpholinohydroxypropoxyQ > monochloropivaloylQ > quercetin > pentaacetylQ.

### Discussion

SERCA is crucial for calcium homeostasis and its impairment results in a variety of pathologies. SERCA is a therapeutical target and its 60–80% content in SR (Engelender et al., 1995) makes it a good model for the study of its activity/properties. The aim of this study was to identify quercetin derivatives which modulate activity of SERCA. Novel quercetin derivatives may affect SERCA activity as follows: (i) incorporate into the SR membrane, change both its properties and SERCA activity; (ii) interact directly with amino acid residues of SERCA through non-covalent interactions changing SERCA conformation; (iii) autooxidize and change into reactive forms which may inhibit SERCA. SERCA is exposed to increased concentrations of a variety of free radicals such as peroxynitrite, hydrogenperoxide and hydroxyl radicals. Therefore, in addition to studying the effect of quercetin derivatives on SERCA activity, we tested free radical scavenging efficiency of quercetin derivatives using DPPH assay.

Properties of the phospholipid bilayer exert an impact on SERCA activity. It has been shown that change in bilayer thickness, fluidity and head group content of the SR membrane affects the activity of SERCA (Gustavsson et al., 2011; Li et al., 2012). SERCA activity depends on properties of the SR membrane and thus inhibition or stimulation of SERCA by quercetin derivatives may be due to changes of physico-chemical properties of the membrane. MonochloropivaloylQ and tri(chloropivaloyl)Q significantly decreased the activity of SERCA (Figure 1A). The incorporation of pivalate through an ester linkage to a bioactive molecule is an effective and established strategy for engineering a drug with improved systemic delivery of the bioactive molecule (Beaumont et al., 2003). Compared with quercetin, the pivate-containing quercetin has a higher lipophilicity and is likely to cross lipid biomembranes more efficiently. The LogP values indicate increased lipophilicity of monochloropivaloylQ and tri(chloropivaloyl)Q in comparison to quercetin (Table 2). MonochloropivaloylQ and tri(chloropivaloyl)Q may directly interact with SERCA through halogen bonding (Parisini et al., 2011), which is characterized by non-covalent interaction between halogen-bearing compounds and nucleophiles in protein backbone which contain NH and C=O groups. According to DPPH test monochloropivaloylQ is better scavenger in comparison to quercetin (Table 2), while tri(chloropivaloyl)Q showed markedly decreased scavenging activity in comparison to quercetin.

5-morpholinohydroxypropoxyQ showed stimulating effect on SERCA. Hydrophilicity of 5-morpholinohydroxypropoxyQ is slightly increased in comparison to quercetin. Thus a change of membrane properties by 5-morpholinohydroxypropoxyQ may not be the factor affecting SERCA activity. Morpholino oxygen in 5-morpholinohydroxypropoxyQ is likely to interact with the amide backbone of SERCA, which may result in its conformational change. The scavenging activity of 5-morpholinohydroxypropoxyQ was found to be negligible.

Interestingly, di(prenylferuloyl)Q has a noticeably increased logP value in comparison to all other quercetin derivatives tested (Table 2), which may indicate its extraordinary lipophilicity. Prenyl groups in

| Table 2. DPPH radical scavenging efficiency, lipophilicity of quercetin derivatives and their effect on SERCA activity. |
|---------------------------------------------------------|
| **Compound**                                             | **DPPH ΔA(518nm/75s)** | **logP (lipophilicity)** | **SERCA activity** |
|---------------------------------------------------------|
| 5-morpholinohydroxypropoxyQ                             | 0.014 ± 0.002           | 2.83 ± 1.08             | ↑                  |
| di(prenylferuloyl)Q                                     | 0.316 ± 0.015           | 15.48 ± 1.04            | ↑                  |
| di(diacetylcaffeoyl)-mono-(monoacetylcaffeoyl)Q         | 0.0425 ± 0.006          | 4.13 ± 1.34             | ↑                  |
| monoacetylferuloylQ                                    | 0.0395 ± 0.019          | 3.68 ± 1.09             | ↑                  |
| pentaacetylQ                                            | 0.0098 ± 0.003          | 0.15 ± 1.37             | ↔                  |
| quercetin                                               | 0.3859 ± 0.038          | 2.07 ± 1.31             | ↓                  |
| tri(trimethylgalloyl)Q                                  | 0.0083 ± 0.004          | 7.16 ± 1.38             | ↓                  |
| monochloropivaloylQ                                    | 0.446 ± 0.045           | 2.47 ± 1.2              | ↓                  |
| tri(chloropivaloyl)Q                                   | 0.119 ± 0.023           | 4.66 ± 1.34             | ↓                  |
| diqueretin                                              | 0.374 ± 0.057           | 6.07 ± 1.12             | ↓                  |

individual symbols: ↑-stimulation, ↓-inhibition, ↔-reaching level of untreated SERCA values for DPPH ΔA(518nm/75s) are expressed as mean ± SEM. Results are mean values ± SD from at least three experiment.
di(prenylferuloyl)Q may form hydrophobic interactions with hydrophobic amino acid residues along with hydro-
gen interaction between oxygen in prenylferuloyl moiety and NH-groups in the protein backbone of SERCA. According to its logP value, di(prenylferuloyl)Q is likely to be the most hydrophobic among all quercetin derivatives tested. Di(prenylferuloyl)Q stimulated SERCA activity and additionally showed scavenging activity comparable to quercetin (Table 2). Thus, di(prenylferuloyl)Q has the potential both to protect SERCA against free radicals and to stimulate SERCA activity.

Diacetylcaffeoyl- and monoacetylferuloyl- groups in quercetin derivatives stimulated the activity of SERCA. Di(diacetylcaffeoyl)-mono-(monoaerylcaffeoyl)Q and monoacetylferuloylQ are more lipophilic in comparison to quercetin. The negligible decrease in DPPH absorbance indicates low scavenging properties of di(diacetylcaffeoyl)- mono-(monoaerylcaffeoyl)Q and monoacetylferuloylQ. Substitution of all hydroxyl groups in the molecule of quercetin resulting in pentaacetylQ caused a slight inhibition of SERCA. The activity of SERCA in the presence of pentaacetylQ reached the level of the control at 150 μM. PentaacetylQ is a poor DPPH scavenger and its logP value is lower than that of quercetin. A low logP value indicate weak lipophilicity of pentaacetylQ, which may thus be unable to interact with the membrane. Tri(trimethylgalloyl)Q inhibited the activity of SERCA. Tri(trimethylgalloyl)Q is a lipophilic molecule (logP=7.6) and is likely to integrate into the membrane. Presence of gallic acid moiety, which by itself possesses scavenging activity, failed however to show antioxidative activity when present in tri(trimethylgalloyl)Q.

Quercetin had little effect on SERCA activity in concentrations up to 100 μM. However, we observed inhibition of SERCA by quercetin at 150 μM. The inhibitory effect of quercetin may be explained by its autooxidation. Quercetin is autoxidized in PBS buffer and gives rise to stable semiquinone radicals (Lei et al., 2008; Zhou & Sadik, 2008). The semiquinone radical may donate an electron to molecular oxygen, thus generating superoxide anion. Semiquinone radicals were shown to be cytotoxic (Metodiewa et al., 1999). Products of quercetin oxidation were demonstrated to damage cells through thiol arylation (Boots et al., 2007). The remarkable inhibitory effect of diquercetin may be explained by autooxidation and formation of semiquinones, which may decrease SERCA activity by its oxidation. The increase in inhibition of diquercetin is likely due to increased lipophilicity and/or increased formation of semiquinones in comparison to quercetin as two molecules form diquercetin. Quercetin and diquercetin have similar scavenging activities.

Inhibition of SERCA results in increased intracellular load of calcium within cells, which results in increased endoplasmic reticulum stress and apoptosis (Lam et al., 1993). Diquercetin, tri(chloropivaloyl)Q and monochloropivaloylQ showed considerable inhibition of SERCA. Inhibition of SERCA activity by quercetin derivatives may prove to be beneficial in treatment of cancer by induction of selective apoptosis in cancerous cells. On the contrary, stimulation of SERCA by di(prenylferuloyl) Q, di(diacetylcaffeoyl)-mono-(monoaerylcaffeoyl)Q, monoacetylferuloylQ and 5-morpholinohydroxypropoxyQ may be advantageous in relieving of SERCA inhibition in diseases such as myopathy and cardiovascular diseases.

Conclusion

SERCA activity modulation and elimination of consequences of oxidative stress has been recognized as an important strategy in the prevention and attenuation of diseases caused by SERCA impairment. In view of the effects of quercetin derivatives on SERCA activity, we believe that further investigations of quercetin derivatives will prove to be beneficial in identifying potential remedies for SERCA involved diseases.

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REFERENCES

Beaumont K, Webster R, Gardner I, Dack K. (2003). Design of ester prodrugs to enhance oral absorption of poorly permeable compounds: challenges to the discovery scientist. Curr Drug Metab 4: 461–485.

Boots AW, Li H, Schins RP, Duffin R, Heemskerk JWM, Bast A, Haenen GR. (2007). The quercetin paradox. Toxicol Appl Pharmacol 222: 89–96.

Duo J, Ying GG, Wang GW, Zhang L. (2012). Quercetin inhibits human breast cancer cell proliferation and induces apoptosis via Bcl-2 and Bax regulation. Mol Med Rep 5: 1453–1456.

Engelender S, Wolosker H, De Meis L. (1995). The Ca(2+)-ATPase isoforms of platelets are located in distinct functional Ca2+ pools and are uncoupled by a mechanism different from that of skeletal muscle Ca2+-ATPase. J Biol Chem 270: 20150–20155.

Gustavson M, Traseth NJ, Veglia G. (2011). Activating and deactivating roles of lipid bilayers on the Ca2+-ATPase/phospholamban complex. Biochemistry 50: 10367–10374.

Haenen GR, Paquay JB, Korthouwer RE, Bast A (1997). Peroxynitrite scavenging by flavonoids. Biochem Biophys Res Commun 236: 591–593.

Hovnanian A. (2007). SERCA pumps and human diseases. Subcell Biochem 45: 337–363.

Karlovská J, Uhríková D, Kucerka N, Teixeira J, Devínsky F, Lacko I, Balgavý P. (2013). Interdisciplinary Toxicology 7: 686–693.

Lei R, Xu X, Yu F, Li N, Liu HW, Li K. (2008). A method to determine quercetin by enhanced luminol electrogenerated chemiluminescence (ECL) and quercetin autoxidation. Talanta 75: 1068–1074.

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Li J, James ZM, Dong X, Karim CB, Thomas DD. (2012). Structural and functional dynamics of an integral membrane protein complex modulated by lipid headgroup charge. J Mol Biol 418: 379–389.

Metodiewa D, Jaiswal AK, Cenas N, Dickancaite E, Segura-Aguilar J. (1999). Quercetin may act as a cytotoxic prooxidant after its metabolic activation to semiquinone and quinoidal product. Free Radic Biol Med 26: 107–116.

Ogunbayo OA, Harris RM, Waring RH, Kirk CJ, Michelangelii F. (2008). Inhibition of the sarcoplasmic/endoplasmic reticulum Ca2+-ATPase by flavonoids: a quantitative structure-activity relationship study. IUBMB Life 60: 853–858.

Parisini E, Metrangolo P, Pilati T, Resnati G, Terraneo G. (2011). Halogen bonding in halocarbon-protein complexes: a structural survey. Chem Soc Rev 40: 2267–2278.

Park HH, Lee S, Son HY, Park SB, Kim MS, Choi EJ, Singh TSK, Ha JH, Lee MG & other authors. (2008). Flavonoids inhibit histamine release and expression of proinflammatory cytokines in mast cells. Arch Pharm Res 31: 1303–1311.

Warren GB, Toon PA, Birdsall NJ, Lee AG, Metcalfe JC. (1974). Reconstitution of a calcium pump using defined membrane components. Proc Natl Acad Sci USA 71: 622–626.

Zhou A, Sadik OA. (2008). Comparative analysis of quercetin oxidation by electrochemical, enzymatic, autoxidation, and free radical generation techniques: a mechanistic study. J Agric Food Chem 56: 12081–12091.