ORIGINAL ARTICLE

# Modulating effect of flavonoids and their derivatives on sarcoplasmic reticulum $Ca^{2+}$-ATPase oxidized by hypochloric acid and peroxynitrite

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**Abstract**

**OBJECTIVES:** Effect of rutin and its lipophilic derivatives on $Ca^{2+}$-ATPase of sarcoplasmic reticulum (SERCA) oxidized by hypochloric acid and peroxynitrite was investigated to examine the role of flavonoids in SERCA activity modulation.
**METHODS:** $Ca^{2+}$-ATPase activity was measured spectrophotometrically at 37 °C using NADH-coupled enzyme pyruvate kinase/lactate dehydrogenase assay. SERCA was oxidized by HOCl (3 min) or $ONOO^-$ (30 s) after previous treatment with flavonoids (2 min) at 37 °C. Lipophilic rutin derivatives were prepared by lipase-catalyzed esterification of flavonoids with fatty acids.
**RESULTS:** Both hypochloric acid (HOCl) and peroxynitrite ($ONOO^-$) decreased ATPase activity concentration-dependently with $IC_{50}$ of 50±10 μmol/l and 150±15 μmol/l, respectively. Rutin was found to have a protective effect on SERCA activity in both oxidation systems in the concentration range 5 – 250 μmol/l. Lipophilic rutin derivatives (rutin oleate, rutin linoleate, rutin linolenate) exerted inhibitory effect on ATPase activity both in the presence and absence of oxidants.
**CONCLUSION:** The results suggest that selective lipophilization of the flavonoid skeleton may represent a useful tool for SERCA activity modulation.

**Abbreviations:**

## INTRODUCTION

$Ca^{2+}$-ATPase from sarco/endoplasmic reticulum (SERCA) is a major calcium-regulating protein responsible for $Ca^{2+}$ homeostasis within cells and contractility in cardiac and skeletal muscle (East, 2000). SERCA is sensitive to redox regulation and oxidative stress (Csordás & Hajnóczky, 2009). Excessive exposure to reactive oxygen (ROS) and nitrogen (RNS) species induces an increase of cytosolic $Ca^{2+}$ concentrations causing disruption of $Ca^{2+}$ homeostasis (Suzuki *et al.* 1997).

Peroxynitrite and hypochloric acid belong to reactive species that have been detected under conditions of inflammation *in vivo*. They are associated with various pathophysiological processes, including ischemia/reperfusion, atherosclerosis, rheumatoid arthritis, skeletal and cardiac muscle dysfunction (Pullar *et al.* 1999; Soszyński *et al.* 2002). HOCl attacks mainly lysine residues of

$Ca^{2+}$ – calcium ions
$Ca^{2+}$-ATPase – calcium-dependent adenosine triphosphatase
HOCl – hypochloric acid
$IC_{50}$ – half maximal inhibitory concentration
$ONOO^-$ – peroxynitrite anion
ROS/RNS – reactive oxygen/nitrogen species
SERCA – skeletal muscle sarco/endoplasmic reticulum $Ca^{2+}$-ATPase
SR – sarcoplasmic reticulum

proteins resulting in formation of chloramine species (Zavodnik *et al.* 2001). Furthermore, it causes oxidation of thiol groups, tryptophan residues and changes of membrane fluidity (Zavodnik *et al.* 2001). The most potent effects of peroxynitrite appear to be thiol modifications, which either affect the function of signaling systems or result in the production of tissue-derived donors of NO. $ONOO^-$ readily interacts with thiols to cause thiol oxidation or the formation of nitrated ($RSNO_2$) or nitrosated (RSNO) thiols (Radi *et al.* 1991). Peroxynitrite induces tyrosine nitration of proteins and alters both calcium channels and calcium pumps (Gutierrez-Martin, 2004).

Flavonoids, due to many biological activities including antioxidant properties and the ability to interact with proteins, appear to be potential SERCA activity modulators. They are known to directly scavenge both ROS and RNS (Middleton *et al.* 2000). There is not much information available to date on flavonoid effect on $Ca^{2+}$-ATPase activity. We carried out selective modification of rutin skeleton via esterification with fatty acids with purpose of lipophilization of the initial rutin molecule. We studied the modulating effect of rutin and lipophilic rutin derivatives on SERCA activity both in the presence and absence of oxidation systems represented by hypochloric acid and peroxynitrite.

## MATERIAL AND METHODS

Rutin esters were synthesized via lipase-catalyzed esterification of rutin with oleic, linoleic or linolenic acid in 2-methylbutan-2-ol at 60 °C. Molar ratio rutin:fatty acid was adjusted to 1:5. Direct esterification reaction was initiated with immobilized lipase from *Candida antarctica* (0.25 g). The water content in solvent was kept lower than 2% with molecular sieves (150 g/l). Rutin derivatives were isolated by column chromatography on silica gel using ethyl acetate/methanol 7:3 (v/v) as mobile phase. All compounds tested were dissolved in DMSO to final concentration 5 – 250 μmol/l before use. Rutin and other chemicals were of analytical purity purchased from Sigma Aldrich (USA). Peroxynitrite was synthesized according to Radi *et al.* (1991). Solution of 0.6 mol/l $NaNO_2$ was mixed at 4°C under stirring with an equal volume of 0.7 mol/l $H_2O_2$ acidified with 0.6 mol/l HCl. Immediately, an equal volume of 3 mol/l NaOH was added. The excess of $H_2O_2$ was removed with $MnO_2$ (7 mg/ml) for 1 h at 4 °C.

SR vesicles were isolated from the fast twitch white muscle of a New Zealand female rabbit (2.5 kg). Enzyme activity of SR $Ca^{2+}$-ATPase was measured by NADH-coupled enzyme assay outlined by Warren *et al.* (1974) and modified by Karlovska *et al.* (2006). The SR vesicles (final concentration 12.5 μg protein/cuvette) were added to the assay mixture (40 mmol/l Hepes (pH 7.2), 0.1 mol/l KCl, 5.1 mmol/l $MgSO_4$, 2.1 mmol/l ATP, 0.52 mmol/l phosphoenolpyruvate, 1 mmol/l EGTA, 0.15 mmol/l NADH, 7.5 IU of pyruvate kinase, 18 IU of lactate dehydrogenase) and incubated at 37 °C for 15 min. The reaction was started by the addition of $CaCl_2$ (final concentration 1 mmol/l). The reaction rate was determined by measuring the decrease of NADH absorbance at 340 nm, at 37 °C. SR vesicles (1 mg protein/ml) were oxidized by HOCl (10–250 μmol/l) for 3 min or $ONOO^-$ (10–250 μmol/l) for 30 s at 37°C, pH 7.4. When HOCl was used, the reaction was stopped by addition of cysteine (1 mmol/l). SR vesicles were incubated at 37 °C, pH 7.4 for 2 min with rutin and individual rutin derivatives before oxidation.

Partition coefficients were calculated using Molinspiration software available online at www.molinspiration.com. All data were expressed as means±SD (standard deviation of the mean) of the indicated number of observations. Statistical comparison between groups was performed using the one-way ANOVA followed by Bonferroni test for mutual comparison among samples. Differences among means were considered significant when $p < 0.05$.

## RESULTS AND DISCUSSION

Treatment of SR vesicles with both oxidants used caused concentration-dependent loss of $Ca^{2+}$-ATPase activity with a decrease of the rate of ATP hydrolysis by SERCA. The concentrations required for half inhibition of SERCA activity were found to be 50±10 μmol/l and 150±15 μmol/l for HOCl and $ONOO^-$, respectively. Inactivation of SERCA by HOCl is mainly caused by oxidation of critical cysteine and lysine residues of the protein (Radi *et al.* 2002). In addition to thiol group oxidation, peroxynitrite is responsible for tyrosine nitration of SERCA, resulting in changes in the catalytic activity of enzymes, altered cytoskeletal organization, and impaired cell signal transduction. It is thus considered a central aspect of peroxynitrite-mediated cytotoxicity (Pacher *et al.* 2007).

Some flavonoids have been reported to be able to initiate apoptosis, especially in tumor cells, via inhibition of $Ca^{2+}$-ATPase activity and elevation of cytosolic $Ca^{2+}$ concentrations (Ogunbayo *et al.* 2008). The mechanism of the enzyme inhibition lies in the interaction of flavonoids with both the ATP-binding site and a hydrophobic region of ATP-binding proteins (Pérez-Victoria *et al.* 2000). Structure-activity analysis revealed that the

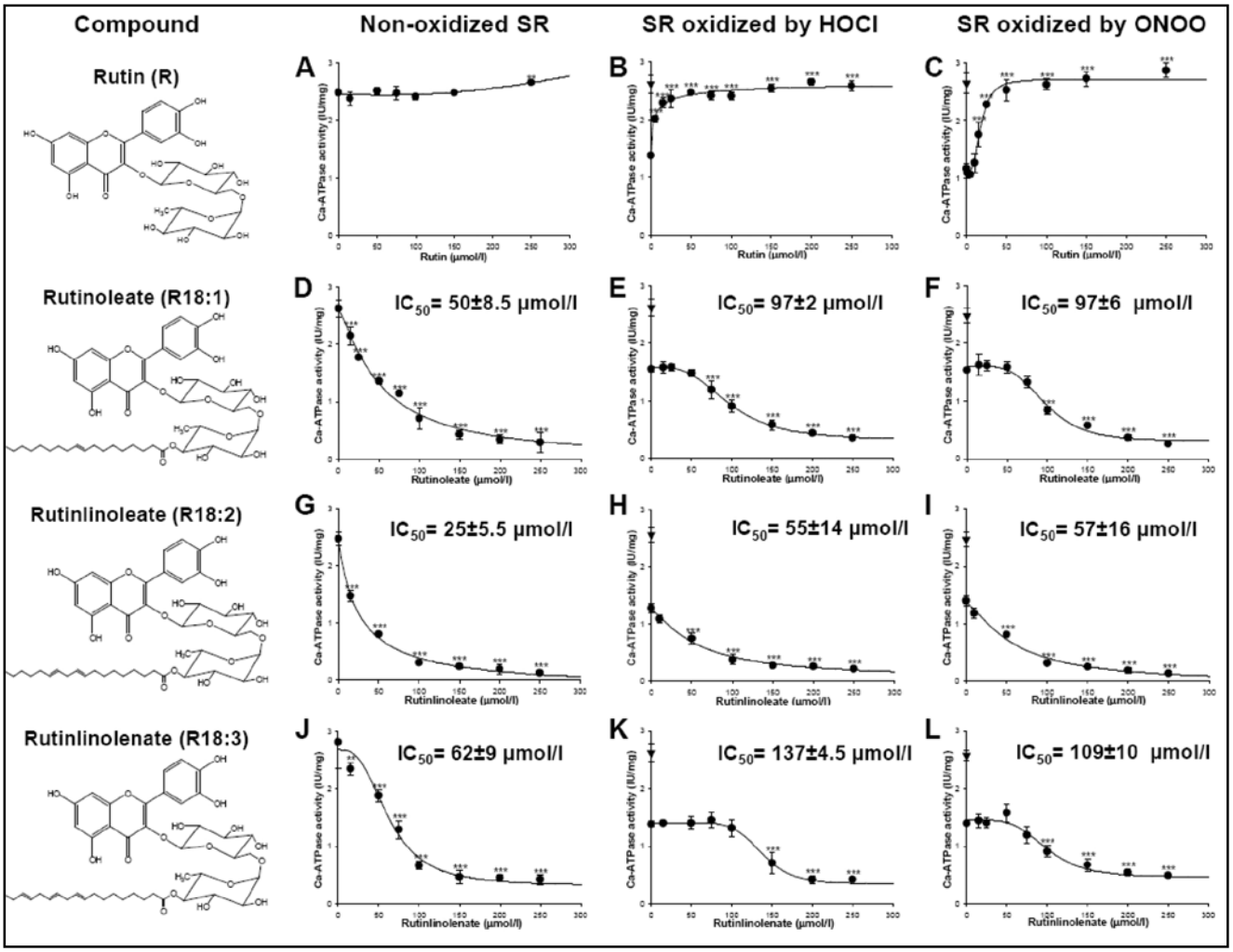


**Fig. 1:** Modulating effect of rutin and its lipophilic derivatives on SR $Ca^{2+}$-ATPase activity. SR vesicles were treated at 37°C in phosphate buffer (pH = 7.4) with different concentrations of rutin, rutin oleate, rutin linoleate and rutin linolenate: (A,D,G,J) in the absence of oxidants, (B,E,H,K) in the presence of 50 µmol/l HOCl and (C,F,I,L) in the presence of 150 µmol/l $ONOO^-$. Values are means±SD of 3 independent measurements with at least 2 parallels. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ are significant differences between control and samples.

inhibitory potency of flavonoids was dependent on the number of hydroxyl groups (Ogunbayo *et al.* 2008).

Rutin is a flavonol glycoside with a wide range of pharmacological activities, such as antioxidant, anti-inflammatory, vasoactive, antibacterial and anticarcinogenic properties (Calabro *et al.* 2005). According to our knowledge, no studies have been reported so far focusing on the rutin effect on SERCA. We examined rutin and its lipophilic esters for their *in vitro* effect on $Ca^{2+}$-ATPase activity both in the presence and absence of oxidants. ***Figure 1*** summarizes the results. Rutin derivatives esterified with unsaturated fatty acids with increasing number of unsaturated bonds (18:1 – 18:3) manifested themselves as strong inhibiting agents of $Ca^{2+}$-ATPase activity in the micromolar concentration range (Fig 1D,G,J). However, it is hard to make any conclusions on the relationship between the degree of unsaturation and inhibitory effect on SERCA activity due to the small number of rutin derivatives involved in this study.

We propose that the increased hydrophobicity of rutin derivatives could facilitate their incorporation into phospholipid bilayer with fatty acid chain, thus changing membrane properties, potentially through interaction with annular lipids, which are known to keep α-helices of SERCA in the conformational shape, ensuring correct helix tilting. Correct anchoring of the helices at the lipid-water interface is important for the functioning of $Ca^{2+}$-ATPase (Adams *et al.* 1998). Flavonoid derivatives bound in the phospholipid bilayer may interact with SERCA affecting conformation by creating pressure on the trans-membrane region of the protein and changing membrane thickness. A change in membrane thickness affects the trans-membrane region of SERCA through a realignment of helices 4, 5, 6 and 8, destroying effectively the $Ca^{2+}$ binding site region (Lee *et al.* 1995). Hydrophilic rutin (log P = -1.06) with low affinity to the SR membrane was found to possess a slightly stimulating effect on enzyme activity, unlike hydrophobic esters (log P = 6.93, 6.20 and 5.19 for R18:1, R18:2 and R18:3, respectively) with

inhibitory properties. Similarly, Strosova *et al.* (2006) observed inhibitory effect of Pycnogenol, a standardized extract of flavonoids, on SERCA activity. Effect of pyridoindole antioxidants on oxidative impairment in rats with adjuvant arthritis was previously evaluated (Strosová *et al.* 2008). Rutin alone slightly increased the activity of $Ca^{2+}$-ATPase (Fig 1A) and intensively prevented SERCA from oxidative stress induced by both HOCl ($EC_{50}$= 4.5±2 μmol/l) and $ONOO^-$ ($EC_{50}$= 18±1.5 μmol/l) (Fig 1B,C). On the contrary, lipophilic rutin derivatives inhibited $Ca^{2+}$-ATPase activity. Interestingly, in the presence of oxidants, the decrease of $Ca^{2+}$-ATPase activity induced by lipophilic derivatives was not so intensive (Fig 1E,F,H,I,K,L).

We suggest that the differences in the behavior of rutin and its derivatives could be explained by their binding to different sites of SERCA. While rutin probably preferentially binds to the nucleotide domain – in the ATP binding site of SERCA, its lipophilic esters with their higher affinity for the SR membrane incorporate into the lipid bilayer, changing physico-chemical properties of the membrane, which are essential for the functioning of SERCA.

While unmodified flavonoids with a protective effect on SERCA could be useful in anti-inflammatory defense, lipophilic derivatives, due to their strong inhibitory properties on $Ca^{2+}$-ATPase activity, may represent therapeutic agents for apoptosis induction in antitumor therapy.

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