FEBS Letters 583 (2009) 531–541

FEBS Letters
journal homepage: www.FEBSLetters.org

Targeting STAT1 by myricetin and delphinidin provides efficient protection of the heart from ischemia/reperfusion-induced injury

Tiziano M. Scarabelli [a,1], Sofia Mariotto [b,1,*], Safwat Abdel-Azeim [c,1], Kazuo Shoji [b,1], Elena Darra [b], Anastasis Stephanou [d], Carol Chen-Scarabelli [a], Jean Didier Marechal [c,e], Richard Knight [d], Anna Ciampa [b], Louis Saravolatz [a], Alessandra Carcereri de Prati [b], Zhaokan Yuan [a], Elisabetta Cavalieri [b], Marta Menegazzi [b], David Latchman [d], Cosimo Pizza [f], David Perahia [c], Hisanori Suzuki [b]

[a] Center for Heart and Vessel Preclinical Studies, St. John Hospital and Medical Center, Wayne State University School of Medicine, 22201 Moross Road, Detroit, USA
[b] Sezione di Chimica Biologica, Dipartimento di Scienze Morfologico-Biomediche, Università di Verona, Strada Le Grazie, 8, I-37134 Verona, Italy
[c] Laboratoire de Modélisation et Ingéniérie des Protéines, Université Paris-Sud, Bât 430, 91405 Orsay, France
[d] Medical Molecular Biology Unit, Institute of Child Health, University College London, London WC1N 1EH, United Kingdom
[e] Unitat de Química Física, Departament de Química, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain
[f] Dipartimento di Scienze Farmaceutiche, Università di Salerno, via Ponte Don Melillo 84084 Fisciano, Salerno, Italy

ARTICLE INFO

Article history:
Received 19 November 2008
Revised 18 December 2008
Accepted 18 December 2008
Available online 29 December 2008

Edited by Lukas Huber

Keywords:
Antioxidant flavonoid
Heart ischemia/reperfusion
STAT1
Apoptosis
Surface plasmon resonance

ABSTRACT

Flavonoids exhibit a variety of beneficial effects in cardiovascular diseases. Although their therapeutic properties have been attributed mainly to their antioxidant action, they have additional protective mechanisms such as inhibition of signal transducer and activator of transcription 1 (STAT1) activation. Here, we have investigated the cardioprotective mechanisms of strong antioxidant flavonoids such as quercetin, myricetin and delphinidin. Although all of them protect the heart from ischemia/reperfusion-injury, myricetin and delphinidin exert a more pronounced protective action than quercetin by their capacity to inhibit STAT1 activation. Biochemical and computer modeling analysis indicated the direct interaction between STAT1 and flavonoids with anti-STAT1 activity.

1. Introduction

Ischemia/reperfusion (I/R) injury leads to irreplaceable myocyte cell loss by necrosis and apoptosis with concomitant activation of signal transducers and activators of transcription 1 (STAT1) and STAT3 [1], nuclear transcriptional factors with opposing effects involved in the signal transduction pathways of a number of cytokines and growth factors [2]. STAT1 plays a critical role in the induction of cardiomyocyte apoptosis, whereas STAT3's main property is to protect cardiomyocytes from I/R injury. The result of I/R injury in part depends on the equilibrium between I/R-elicited STAT1 and STAT3 activation [3]. Recently, we have shown that epigallocatechin-3-gallate (EGCG), the main antioxidant flavonoid present in green tea leaves, protects rat hearts from I/R-induced damage by down-regulating STAT1 activation and suppressing apoptosis in cardiomyocytes without affecting STAT3 activation [4]. This strongly suggested that consumption of green tea may be able to mediate cardioprotection and enhance cardiac function during I/R injury. However, the mechanism of action underlying the selective STAT1-inhibiting activity of EGCG remains largely elusive.

Since STAT1 activation, according to some reports, may also reflect changes in the cellular redox state [5], this raises the question on the functional relationship between anti-STAT1 activity and the antioxidant capacity of EGCG and other flavonoids. This point is crucial in the future development of drugs with a specific inhibitory action on STAT1 activation.

All STATs, including STAT1 and 3, share common structural features such as a DNA-binding domain, an SH2 domain and a transactivation domain. The SH2 domain is critically involved in the recognition of, and interaction with, phosphotyrosine residues present in other protein molecules. The signal transduction pathways in which STAT1 and STAT3 are involved also share common intracellular mechanisms. STAT1 and 3 are rapidly phosphorylated by the Janus tyrosine kinases JAK 1/2 and MAP serine kinase families. Tyrosine phosphorylation is critically involved in STAT

* Corresponding author.
E-mail address: sofia.mariotto@univr.it (S. Mariotto).
[1] These four authors contributed equally to this work.

0014-5793/$34.00
doi:10.1016/j.febslet.2008.12.037

dimerization, whereas serine phosphorylation enhances DNA-binding activity of STATs, thereby regulating gene expression. STATs signaling is further regulated by common proteins such as STAMs (signal-transducing adapter molecules), StIP (STAT-interacting protein), the SH2B/Lnk/APS family, SOCS (suppressors of cytokine signaling), PIAS (protein inhibitors of activated STATs) and PTPs (protein tyrosine phosphatases) [6]. Ubiquitylation, together with sumoylation add further regulatory machineries to the STAT1 and STAT3 activation pathways. As we recently reported, I/R rapidly induces phosphorylation of tyrosine residue 701 (TYR701) and serine residue 727 (SER727) that reside in the C-terminal transactivation domain of STAT1. All these factors need to be considered in the identification of STAT1 inhibiting compounds.

In order to understand the molecular mechanisms leading to the inhibition of STAT1 activation, we first examined the protective effects of strong antioxidant flavonoids such as quercetin, delphinidin and myricetin, which are structurally highly related to EGCG, in I/R-elicited rat heart injury. Although all exerted a protective action against tissue damage, delphinidin and myricetin, which have an inhibitory action on I/R-elicited STAT1 activation as well as antioxidant activity, showed more pronounced protective effects than quercetin which did not inhibit STAT1 activation. Therefore, we further screened a number of naturally occurring antioxidant flavonoids in an in vitro cellular system to identify anti-STAT1 flavonoids and then analyzed the structural features of a few of these (delphinidin, myricetin and robinetin) to identify common characteristics responsible for their selective inhibitory activity towards STAT1. Finally, we present data indicating that anti-STAT1 flavonoids exert their action by directly interacting, with high affinity, with STAT1 at sites near the SH2 domain, thereby specifically and efficiently inhibiting I/R-induced TYR701 and SER727 phosphorylation of STAT1.

2. Materials and methods

2.1. Animal model

Male Sprague–Dawley rats were obtained from Charles River UK (Margate, UK) and were cared for in accordance with The Guidance on the Operation of the Animals (Scientific Procedures) Act 1986 and under institutional license from the Government Home Office (London, UK).

2.2. Isolation and treatment of isolated cardiac myocytes

Ventricular myocytes isolated from neonatal Sprague–Dawley rats were transferred onto six-well gelatin coated plates at a density of 10^5 cells/well. When a confluent monolayer of spontaneously beating myocytes was formed, cardiac myocytes were subjected to 4 h hypoxia and 16 h reoxygenation as previously described [7]. Delphinidin, myricetin or quercetin (10, 25 and 50 μM; Extrasynthese, Genay, France) were added to cultured myocytes 2 h prior to the hypoxic injury and during reoxygenation.

2.3. Cell death assessment in isolated cardiac myocytes

Cell death was evaluated as described previously [8,9]. Single cell suspensions were prepared and mixed with Annexin V FITC, at a final concentration of 1 μg/ml (BD Biosciences, San Jose, CA, USA), and propidium iodide, at a final concentration of 1 μg/ml (Sigma–Aldrich, St. Louis, MO, USA), in the presence of 1.8 mM calcium chloride. Cells were then incubated at room temperature for 15 min and immediately analyzed on a Beckman Coulter XL flow cytometer.

2.4. Perfusion of isolated rat hearts

Rats, weighing 250–300 g, were anesthetized by sodium pentobarbital (6 mg/kg administered intraperitoneally) and killed by decapitation. The hearts were removed, immersed in an ice-cold modified Krebs-Hensleit buffer solution and subsequently perfused by the non-recirculating Langendorff technique at a constant flow of 11 ml/min [10]. The heart rate was continuously maintained at 300 bpm by electrical pacing and the left ventricular wall was kept at a steady temperature of 37 °C.

2.5. Treatment protocols

After a period of stabilization of 30 min, the isolated hearts were randomly divided into eight groups (A, B, C, D, E, F, G and H) of at least eight hearts each. Groups A and B were aerobically perfused for 60 min, without (group A) or with the addition of delphinidin (group B1), myricetin (group B2) or quercetin (group B3) (10 μM) to the perfusate respectively. Groups C, D, E, F were exposed to 30 min of regional ischemia and 2 h of reperfusion, without (group C) or with (groups D, E and F) pretreatment with either delphinidin, or myricetin or quercetin, respectively. Group G was infused for 1 h with recombinant human interferon-γ (IFN-γ) (100 units/ml) (R&D Systems, Minneapolis, MN), with (group G2) o without (group G1) pretreatment with a specific and irreversible inhibitor of caspase-8 (Z-IEDT.fmk), at a dose of 0.07 μM (Calbiochem, EMD Biosciences Inc., San Diego, CA, USA). Group H1 received a 1 h infusion of methyl-2-cyano-3,12 dioxoolean-1,9 diene-28-oate (CDDO-Me) (0.05, 0.1, 0.5 μM), which was either given alone or preceded by treatment with the individual flavonoids at the given concentrations (groups H2, H3 and H4).

2.6. Regional ischemia

After stabilization, the left coronary artery of isolated rat hearts was surgically occluded for 30 min and subsequently reperfused for 60 min [10].

2.7. Infarct size measurement

Measurement of risk area and infarct size was performed by triphenyl-tetrazolium chloride (TTC) staining as previously reported [11]. The left ventricular (LV) infarct zone was determined by computerized planimetry and expressed as a percentage of the infarcted area within the myocardium at risk.

2.8. Left ventricular pressure

To obtain an isovolumetrically beating preparation, a latex balloon filled with saline, connected by a catheter to a Statham transducer (P 23 XL), was inserted into the left ventricle through an atriotomy and secured by a suture around the atrioventricular groove. The balloon was inflated to provide an end-diastolic pressure <1.0 mm Hg [12].

2.9. Assay of creatine phosphokinase in the coronary effluent

During each perfusion, the coronary effluent was collected at different time points in chilled glass vials and promptly assayed for CPK activity by spectrophotometry [13,14].

2.10. Caspase activity assay

Cardiac activation of caspase-3, caspase-8 and caspase-9 was evaluated in tissue extracts using commercial kits (Biovision; Mountain View, CA, USA) [8,15]. Enzyme reactions were performed

with ~300 μg of cytosolic proteins per assay in the presence of 50 μM AFC (7-amino-4-trifluoromethyl coumarin)-conjugated substrates specific for either caspase-3, -8 and -9. Samples were read in a fluorimeter equipped with a 400-nm excitation and a 505-nm emission filter.

2.11. Determination of tissue malondialdehyde (MDA) concentration

Levels of MDA were measured in cardiac tissue as described previously [11].

2.12. Preparation and pre-treatment of tissue sections

Once removed from the perfusion apparatus, the hearts were cross-sectioned from the apex to the atrioventricular groove into four 2.5 mm thick slices, which were subsequently placed in 4% formaldehyde and embedded in molten paraffin [8,10,12,14].

2.13. TUNEL

TUNEL staining was performed on myocardial sections using a commercial kit (Boehringer Mannheim; Lewes, Sussex, UK) [10,12].

2.14. Additional staining

Following TUNEL staining, a previously described multiple step immunocytochemical procedure was used [10,12]. Myocardial sections were labeled with either anti-desmin or anti-von Willebrand factor antibodies, in order to selectively identify myocytes and endothelial cells respectively. After incubation with specific secondary antibodies, the slides were counterstained with propidium iodide and finally examined by confocal fluorescent microscopy. Data are expressed as the means of 12–15 high power fields ±S.D.

2.15. MDA-MB-231 cell line

The human breast cancer cell line MDA-MB-231 was cultured in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker, Cambrex Bio Science, Belgium) supplemented with 10% fetal bovine serum (FBS; BioWhittaker, Cambrex Bio Science, Belgium), 100 UI/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 40 μg/ml gentamicin, in a humidified atmosphere of 95% air, 5% CO_2 at 37 °C.

2.16. Electrophoretic mobility shift assay

Eight micrograms of the nuclear extract prepared from MDA-MB-231 cells according to Osborn et al. [16] were incubated for 20 minutes at room temperature with $2–5 \times 10^4$ cpm of a ^{32}P-labeled double-stranded oligonucleotides containing the STAT1 binding site (sis-inducible factor-binding recognition element, SIE/m67) from the c-fos promoter (5′-gtcgaCATTTCCCGTAAATCg-3′) (Promega, Milan, Italy), in a 15 μl reaction mixture containing 20 mM Hepes, pH 7.9, 50 mM KCl, 0.5 mM dithiothreitol, 0.1 mM EDTA, 2 μg of poly(dI–dC), 1 μg of salmon sperm DNA, and 10% glycerol. Products were fractionated by electrophoresis on a non-denaturing 5% polyacrylamide gel.

2.17. Western blot analysis

Cell or tissue extracts were lysed on ice for 15 min with 20 mM HEPES, pH 7.4, 420 mM NaCl, 1% Nonidet P40, 1 mM EGTA, and 1 mM EDTA. After centrifugation for 15 min at 12 000 rpm, proteins (40 μg/lane) were fractionated by electrophoresis on 7.5% SDS–polyacrylamide gel and electroblotted onto a PVDF membrane (Immobilon P, Millipore, Bedford, MA, USA). Membranes were incubated with antibody anti-STAT1, anti-STAT3, anti-active-caspase-3; anti-FAS; anti-actin (Santa Cruz Biotechnology, CA, USA), anti-phosphoSTAT1^{Y701} (New England Biolabs, Hitchin, England) or anti-phosphoSTAT3^{Y705} (Cell Signaling Technology, MA, USA), and, after washing, with an anti-rabbit IgG-peroxidase conjugate (AmershamBiosciences, Little Chalfont, Buckinghamshire, UK). The immunoreactive proteins on the blot were detected by an enhanced chemiluminescence detection system (ECL) (Amersham Biosciences AB, Uppsala, Sweden). Following the analysis of phosphorylated STAT1 or STAT3, the blots were stripped and reprobed with anti-STAT1 or anti-STAT3 antibody.

2.18. Surface plasmon resonance analysis

Interaction of STAT1 with each flavonoid was examined by surface plasmon resonance using a BiaCore 2000 biosensor system. STAT1 protein (Biosurce, Camarillo, CA, USA), corresponding to 15 000–18 500 as resonance units (RU), was immobilized on the carboxymethylated sensor chip, CM5, by an amine coupling reaction kit (BIAcore Inc., Uppsala, Sweden) according to the manufacture's instructions. Each flavonoid in HBS-EP buffer (BIAcore Inc.) was injected into the flow cell containing immobilized STAT1. A flow cell without STAT1 was used as control. Appropriate concentration ranges of flavonoids were determined from anticipated preliminary experiments. Sensorgrams for each flavonoid were analyzed with BIA evaluation software and BIA simulation software (BIAcore Inc.).

2.19. Computer modeling

Searching for putative interaction sites was carried out by using Q-sitefinder [17] (http://www.bioinformatics.leeds.ac.uk/qsitefinder/) on the X-ray structure of STAT1 (pdb entry 1yvl.pdb [18].

Docking simulations were carried out using AutoDock v3.0.5 [19] and Gold v3.0 [20] programs which were both recently reported to be the most accurate software for this purpose [21–23]. Both docking algorithms were used with full flexibility of the ligands but with limited or no protein flexibility. The geometry of the ligands was obtained by minimization with the semi empirical method AM1 by using the Gaussian03 [24] program. ADTOOLS, the graphical interface of AutoDock was used for adding the polar hydrogens for docking calculations and for assigning the atomic charges of the protein. The protein and the ligands were prepared with Chimera-1.2255 [25] and VegaZZ 2.0.7 [26] for Gold.

Calculations with AutoDock were performed using a particularly accurate procedure using the Lamarckian algorithm with a population size of 100, a number of energy evaluations of 2.0×10^6, a generation number of 27 000, and a mutation and crossover rates of 0.02 and 0.8, respectively. The energies were evaluated by using atomic affinity potentials for each atom type of the ligand calculated by AutoGrid v3.0.5 Furthermore, the number of runs was set to 100 to explore a large number of poses of the highest affinity and the Solis and Wets algorithm was used to relax the best 10% of the obtained conformations. Gold calculations were performed using the Goldscore scoring function. The default setting parameters of the genetic algorithm were used. The best 20 orientations were analyzed in detail.

2.20. Statistics

Data are expressed as means ± S.D. Single-factor one-way analysis of variance (ANOVA) was performed for each group of treatments and significance was assumed when $P < 0.05$. Differences among means were compared within the treatment groups using Student's t-test with a significance level of $P < 0.05$. Experiments were repeated at least three times. With regard to the

hemodynamic assessment, analysis of covariance (ANCOVA), with time as the covariate and post hoc analyses, were used to test the principal component with contrast. The Bonferroni correction was then applied and P values <0.05 were considered significant.

3. Results

3.1. Cardioprotection by myricetin, delphinidin and quercetin

We first examined the effect of myricetin, delphinidin and quercetin, three strong naturally occurring antioxidant flavonoids, in neonatal cardiomyocytes under hypoxic conditions. After exposure to 4 h hypoxia followed by 16 h reoxygenation, the addition of these flavonoids, 2 h before the onset of the ischemic insult, reduced the extent of both necrotic and apoptotic cell death (Fig. 1A). The protection afforded by myricetin and delphinidin was significantly stronger than that provided by quercetin.

Secondly, to extend these findings to ex vivo conditions, we examined the effect of these flavonoids in the Langendorff perfused rat heart. Myricetin, delphinidin and quercetin, infused for 1 h before the onset of ischemia and during reperfusion, significantly reduced infarct size (Fig. 1B), as well as the post-ischemic release of creatine phosphokinase (CPK) (Fig. 1C). In line with the in vitro data, quercetin-mediated cardioprotection was significantly less than that provided by either myricetin or delphinidin.

3.2. Effect of flavonoids on hemodynamic, histological and biochemical parameters in the heart after I/R

To examine the functional implications of flavonoid-mediated cardioprotection, we also evaluated the changes in left ventricular systolic pressure (LVSP) and left ventricular end diastolic pressure (LVEDP) in the isolated rat heart exposed to I/R injury. The pre-ischemic infusion of the three flavonoids, which did not alter the baseline LVSP and LVEDP values, significantly improved the post-ischemic recovery of cardiac function, with amelioration of both LVSP and LVEDP, and was proportional to the degree of cardioprotection afforded by the three flavonoids (Fig. 2A–D).

In myocardial tissue sections from hearts infused with either myricetin, delphinidin, or quercetin, the occurrence of apoptosis was significantly reduced. Again, the reduction of apoptosis produced by myricetin or delphinidin was significantly greater than that induced by quercetin (Fig. 1D).

Since generation of reactive oxygen species (ROS) has been implicated in the induction of apoptosis not only triggering directly the mitochondrial pathway, but also leading indirectly to activation of the death-receptor-mediated pathway [27], we measured the intracellular concentration of malondialdehyde (MDA), a marker of lipid peroxidation, which occurs as a result of the damaging effects of ROS in the intact rat heart [11]. We found that MDA concentration in the area at risk of I/R hearts was approximately three times higher than in control hearts. Infusion of three flavonoids reduced MDA concentration close to control levels, confirming that free radical production had been successfully inhibited to an equivalent extent by all flavonoids. This ensures that discrepancies in antioxidant efficacy cannot account for the different degree of protection observed between the three flavonoids (Fig. 3).

3.3. Effect of flavonoids on protection against I/R-induced apoptosis in the heart

As previously stated, we have documented that cardiac ischemia results in STAT1 activation, with subsequent myocyte

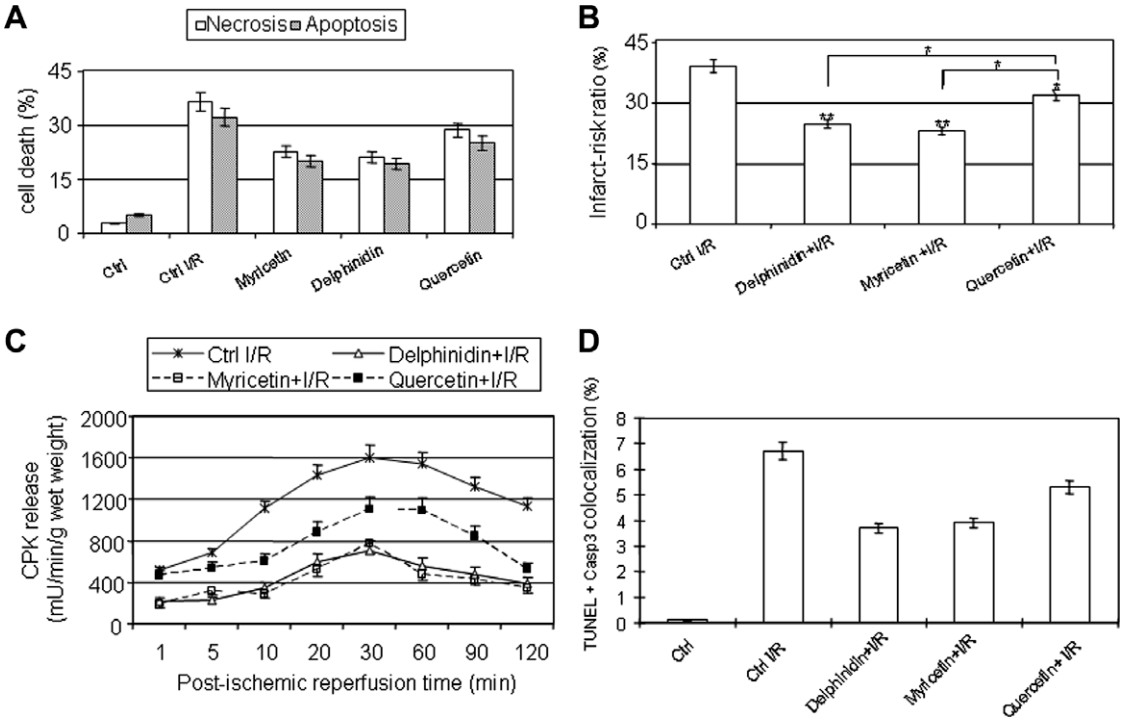


Fig. 1. Effect of delphinidin myricetin and quercetin against I/R-induced heart injury. (A) Quantification of necrosis and apoptosis by flow cytometry in primary cultures of neonatal cardiac myocytes. Values are averages of three independent experiments ±S.D. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. ischemic/reperfused control (Ctrl I/R). (B) Infarct size, expressed as a percentage of myocardial risk zone. Values are averages of three independent experiments ±S.D. (C) Post-ischemic release of CPK in I/R hearts with and without pre-treatment with flavonoids. Values are averages of three independent experiments ±S.D. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. ischemic control hearts or other ischemic/reperfused treated group, as depicted. (D) Percentages of TUNEL and cleaved active caspase-3 (Casp3) myocyte colocalization in control I/R-untreated or -I/R exposed heart (CtrI/R), and in I/R hearts pretreated ex vivo with either delphinidin, or myricetin, or quercetin. Values are averages of three independent experiments ±S.D. $^{*}P < 0.05$; $^{**}P < 0.01$ vs. ischemic control hearts or other I/R.

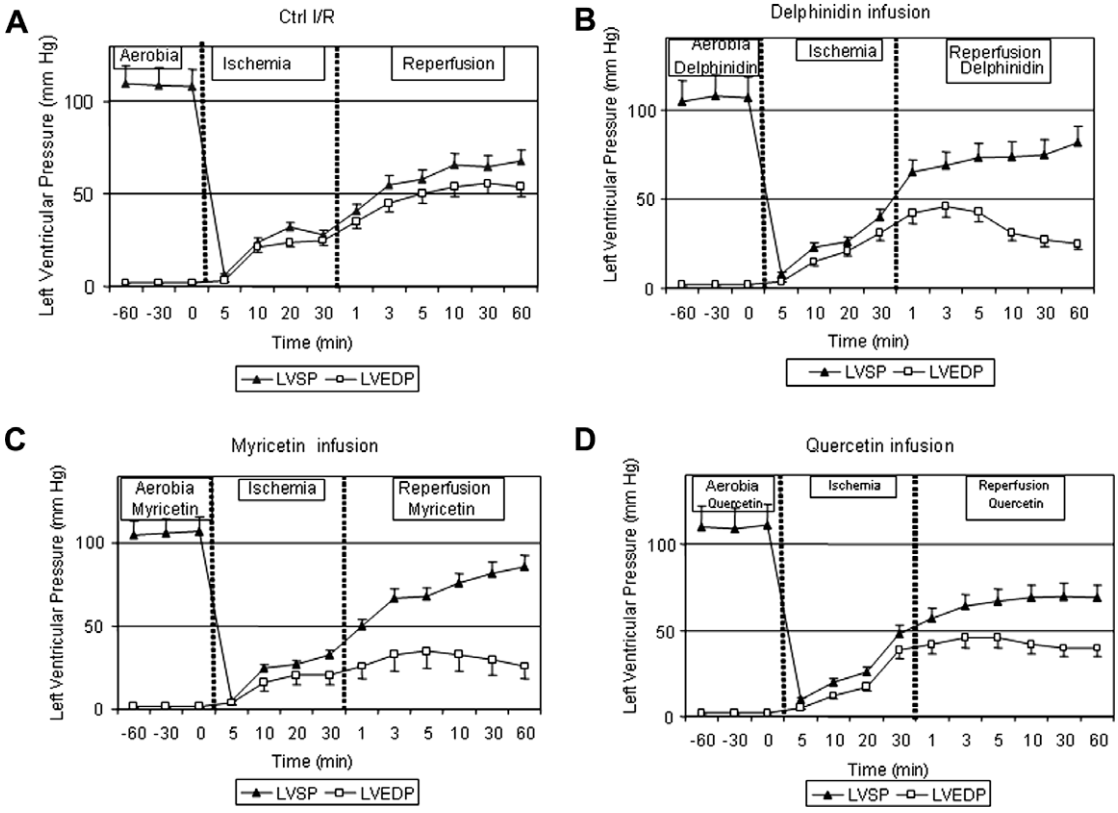


Fig. 2. Effect of delphinidin myricetin and quercetin against I/R induced heart dysfunction. Changes in left ventricular systolic pressure (LVSP) and left ventricular end diastolic pressure (LVEDP) in the control I/R (Ctrl I/R) heart (A) and in the isolated heart perfused with delphinidin (B), myricetin (C) and quercetin (D) for 1 h before undergoing I/R injury. Values are averages of three independent experiments ±S.D.

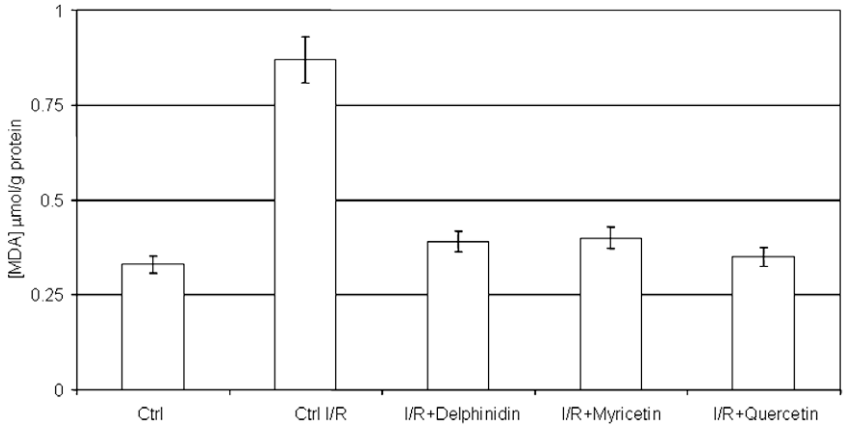


Fig. 3. Anti oxidant action of delphinidin myricetin and quercetin in the I/R treated heart. Intracellular concentration of malondialdehyde (MDA) in normoxic (ctrl) and I/R control hearts (Ctrl I/R) and in rat hearts treated for 1 hour with either delphinidin, or myricetin, or quercetin before exposure to I/R. Values are averages of three independent experiments ± S.D.

apoptosis via activation of the death-receptor-mediated pathway, following increased FAS ligand and FAS receptor expression. Myricetin and delphinidin significantly reduced the I/R-induced tyrosine phosphorylation of STAT1, though not that of STAT3. Both flavonoids also reduced substantially the expression level of FAS and the cleavage of caspase-3 (Fig. 4A and B). Conversely, infusion of quercetin reduced caspase-3 cleavage, though less dramatically than myricetin and delphinidin, but had no effect on STAT1 and STAT3 phosphorylation or FAS expression (Fig. 4C).

To further investigate the antiapoptotic mechanisms of action of delphinidin, myricetin and quercetin, we assessed the activation of caspase-8 and caspase-9 after flavonoid infusion. Caspase-9 activation was particularly pronounced in hearts exposed to I/R and was significantly reduced by all three flavonoids. Although caspase-8 activation was significantly reduced by myricetin and delphinidin, it was unaffected by quercetin (Fig. 4D), suggesting that myricetin and delphinidin inhibit apoptosis by reducing the activation of the apical caspases, while quercetin is only effective in reducing apoptosis initiated by mitochondrial injury. Together, these data are consistent with the ability of myricetin and delphinidin to inhibit the activation of STAT1 and therefore decrease the expression of components of the FAS death receptor pathway.

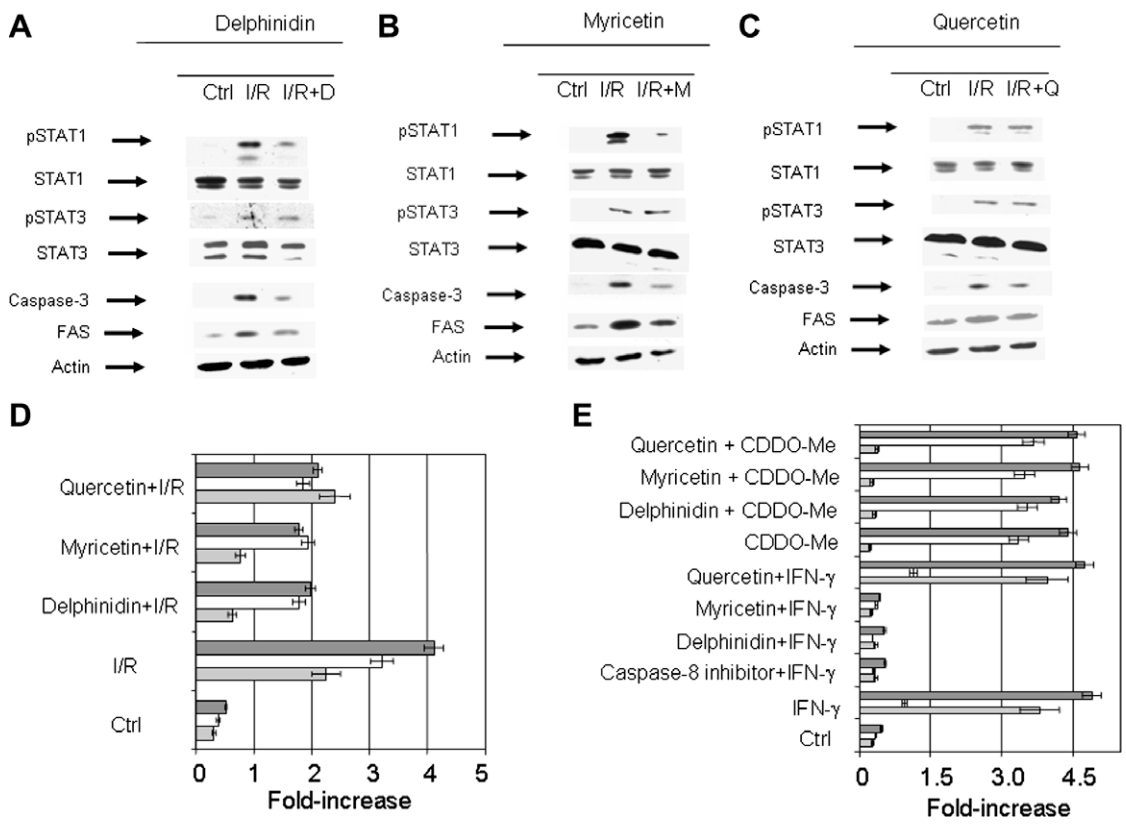


Fig. 4. Effect of delphinidin myricetin and quercetin on STAT1 activation in the I/R treated heart. (A–C) Western blot analysis of protein extracts from control, I/R control hearts and hearts treated with flavonoids. Western blots were performed with specific antibodies against STAT1 and phosphorylated STAT1 (pSTAT1); STAT3 and phosphorylated STAT3 (pSTAT3); cleaved caspase-3 and FAS. Results are representative of three independent experiments. (D) Caspase-3, (■), -8 (▩) and -9 (□) enzymatic activity in whole tissue extracts from control hearts and hearts made ischemic and reperfused with or without *ex vivo* treatment with either delphinidin, or myricetin, or quercetin. Fold-increase in caspase activity was determined by comparing fluorescence of AFC in control and treated hearts with buffer perfused control. Values are averages of three independent experiments ±S.D. $^{**}P < 0.01$ vs. ischemic control hearts. (E) Caspase-3, (■), -8 (▩) and -9 (□) enzymatic activity in whole tissue extracts from normoxic control hearts and rat hearts infused with either IFN-γ or CDDO-Me. Infusion with IFN-γ was performed alone or preceded by infusion of either a specific caspase-8 inhibitor or infusions of delphinidin, myricetin or quercetin. In another set of experiments, CDDO-Me infusion was performed alone or after infusions of delphinidin, myricetin or quercetin. Fold-increase in caspase activity was determined by comparing fluorescence of AFC in control and treated hearts with buffer perfused control. Values are averages of three independent experiments ±S.D. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. rat hearts infused with either IFN-γ or CDDO-Me.

Quercetin is correspondingly ineffective at inhibiting activation of caspase-8 but retains the ability to inhibit caspase-9 activation following mitochondrial damage due to its antioxidant properties.

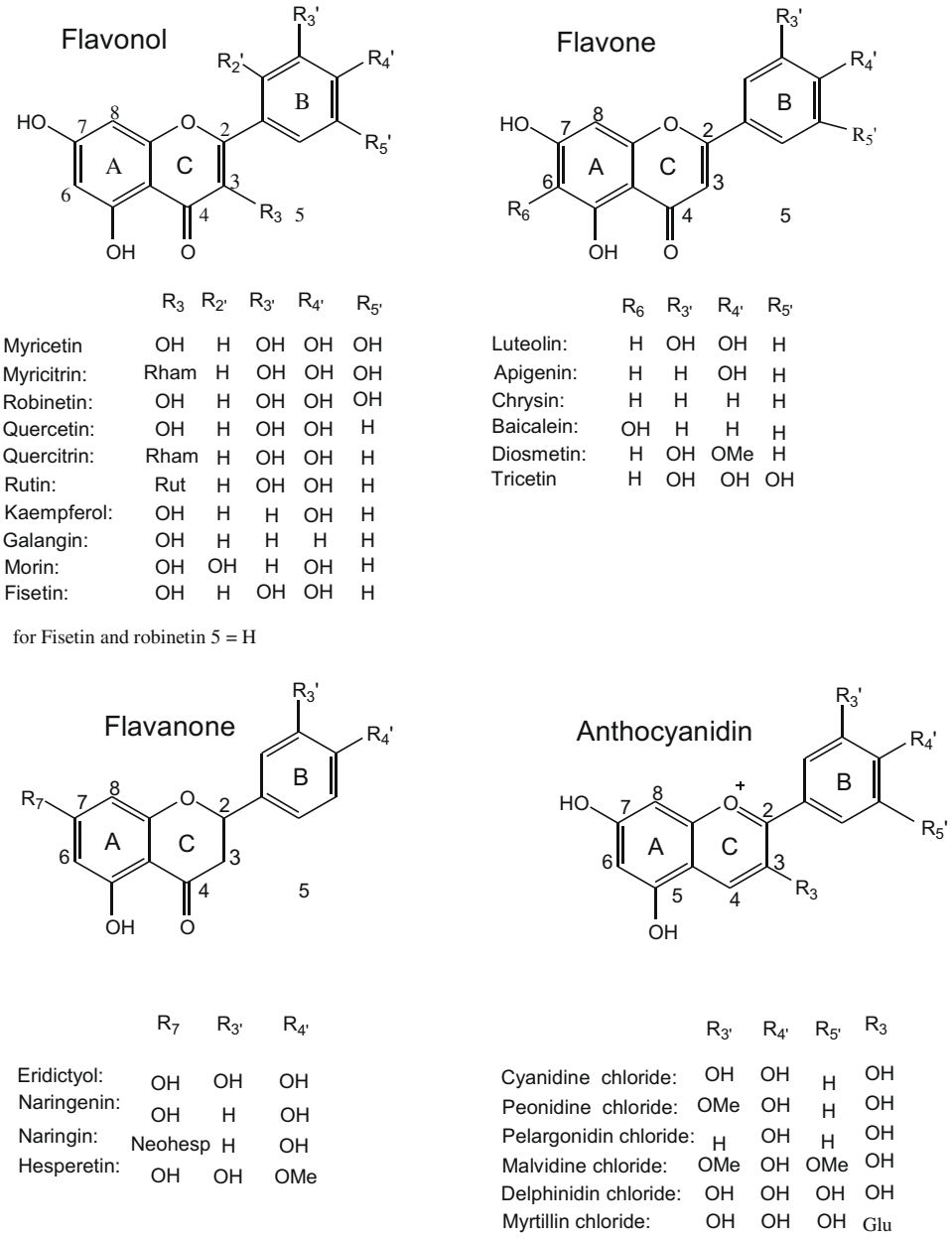
To further explore and distinguish the mechanisms of action of myricetin and delphinidin compared with quercetin, we infused IFN-γ into isolated rat hearts. While myricetin and delphinidin prevented IFN-γ-elicited activation of caspase-8, as well as caspase-9, quercetin did not affect their activation. Furthermore, none of the three flavonoids reduced caspase-9 activation following 1 h infusion of CDDO-Me, a synthetic oleanolic acid derivative that potently activates the mitochondrial pathway without enhancing free radical generation [28], suggesting that their antioxidant activity represents the main mechanism by which they inhibit activation of caspase-9 (Fig. 4E).

3.4. Structural characteristics of anti-STAT1 flavonoids

Once we had ascertained that the more pronounced protective effect of myricetin and delphinidin over quercetin was correlated with the anti-STAT1 activity of the former two, we performed a structure activity relationship study in order to identify the most important chemical groups responsible for this activity. We tested 26 flavonoids from different chemical families (Fig. 5) for their ability to inhibit STAT1 DNA-binding by electromobility shift assay (EMSA) in the cell line MDA-MB-231. Of these, and in agreement with our previous data, only myricetin and delphinidin, together with robinetin, exerted a dose-dependent inhibitory action on IFN-γ-induced STAT1 DNA-binding, with EC_{50} values of 5, 20 and 35 μM, respectively (Fig. 6A). The same three compounds also reduced IFN-γ-induced STAT1 TYR701 phosphorylation in a concentration-dependent manner without modifying the amounts of total STAT1 protein (Fig. 6B), further confirming the data from the heart studies. These results indicate the importance of the presence of three hydroxyl groups in the 3′, 4′, 5′ positions in the B ring of STAT1 inhibitory flavonoids (Fig. 5). Furthermore, the absence of the hydroxyl group in the 3 position of the C-ring as in tricetin or the glycosylation of this group as in myricitrin or myrtyllin leads to the complete loss of activity. The antioxidant nature of flavonoids is defined mainly by the presence of the cathecol B ring and of 2, 3 unsaturation in conjugation with a 4-oxo function in the C-ring [29]. Therefore, these data, in line with the in vivo results, indicate that inhibitory action of anti-STAT1 flavonoids should not be attributed simply to their antioxidant activity.

3.5. Direct interaction between anti-STAT1 flavonoids and STAT1 SPR study

Thereafter, we hypothesized that anti-STAT1 flavonoids exert their action by directly interacting with the STAT1 protein. We evaluated by surface plasmon resonance (SPR) the interaction between immobilized STAT1 and a number of flavonoids. Analysis of sensograms (Fig. 7A and B) indicates not only a direct interaction



	R_3	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$
Myricetin	OH	H	OH	OH	OH
Myricitrin:	Rham	H	OH	OH	OH
Robinetin:	OH	H	OH	OH	OH
Quercetin:	OH	H	OH	OH	H
Quercitrin:	Rham	H	OH	OH	H
Rutin:	Rut	H	OH	OH	H
Kaempferol:	OH	H	H	OH	H
Galangin:	OH	H	H	H	H
Morin:	OH	OH	H	OH	H
Fisetin:	OH	H	OH	OH	H

	R_6	$R_{3'}$	$R_{4'}$	$R_{5'}$
Luteolin:	H	OH	OH	H
Apigenin:	H	H	OH	H
Chrysin:	H	H	H	H
Baicalein:	OH	H	H	H
Diosmetin:	H	OH	OMe	H
Tricetin	H	OH	OH	OH

	R_7	$R_{3'}$	$R_{4'}$
Eridictyol:	OH	OH	OH
Naringenin:	OH	H	OH
Naringin:	Neohesp	H	OH
Hesperetin:	OH	OH	OMe

	$R_{3'}$	$R_{4'}$	$R_{5'}$	R_3
Cyanidine chloride:	OH	OH	H	OH
Peonidine chloride:	OMe	OH	H	OH
Pelargonidin chloride:	H	OH	H	OH
Malvidine chloride:	OMe	OH	OMe	OH
Delphinidin chloride:	OH	OH	OH	OH
Myrtillin chloride:	OH	OH	OH	Glu

Fig. 5. Chemical structure of natural flavonoids tested on STAT1 DNA binding activity.

between STAT1 and myricetin, delphinidin as well as robinetin, but also the presence of two putative sites of interaction on STAT1. The very low K_D value for one site of myricetin, delphinidin, and robinetin for binding to STAT1 indicated a strong affinity for this protein (Fig. 4D). These data are in line with our previous results in cell lines (Fig. 6A and B). Furthermore, all other antioxidant flavonoids examined show much lower or no affinity toward STAT1 in accordance with results described above.

3.6. Computer modeling analysis

We also analyzed the direct interaction between flavonoids and STAT1 by molecular modeling experiments with the first part of the study designed to determine the probable site(s) of interaction of the flavonoids with the protein. Indeed, neither experimental nor theoretical determinations of the position of this site(s) are presently available. Using different programs to detect binding site cavities in proteins, three major putative sites were identified. Two of the sites are located at the interface between the SH2 and the linker domains, and the third is in the DNA-binding domain. The third binding site was ignored in this study since the most stable docked orientations in this site were yet significantly higher in energy than for sites 1 and 2 (site 1 −10.90, site 2 −10.84, and site 3 −8.96 kcal/mol). This observation could be related with an earlier study reporting that EGCG blocked the phosphorylation of STAT1 for recombinant receptor with truncated DNA domain [30]. The detection of the two remaining sites (sites 1 and 2) is in agreement both with previous experimental studies [31,32] which demonstrated the importance of the SH2-domain in the STAT-JAK propagation signal and with above-mentioned results on the presence of two anti-STAT1 flavonoid-binding sites on STAT1 (Fig. 7D). The presence of these two probable binding sites at the SH2 domain is also in agreement with the findings of Mao et al. [18] who reported that the phosphopeptide pYDHPH (derived from the α chain

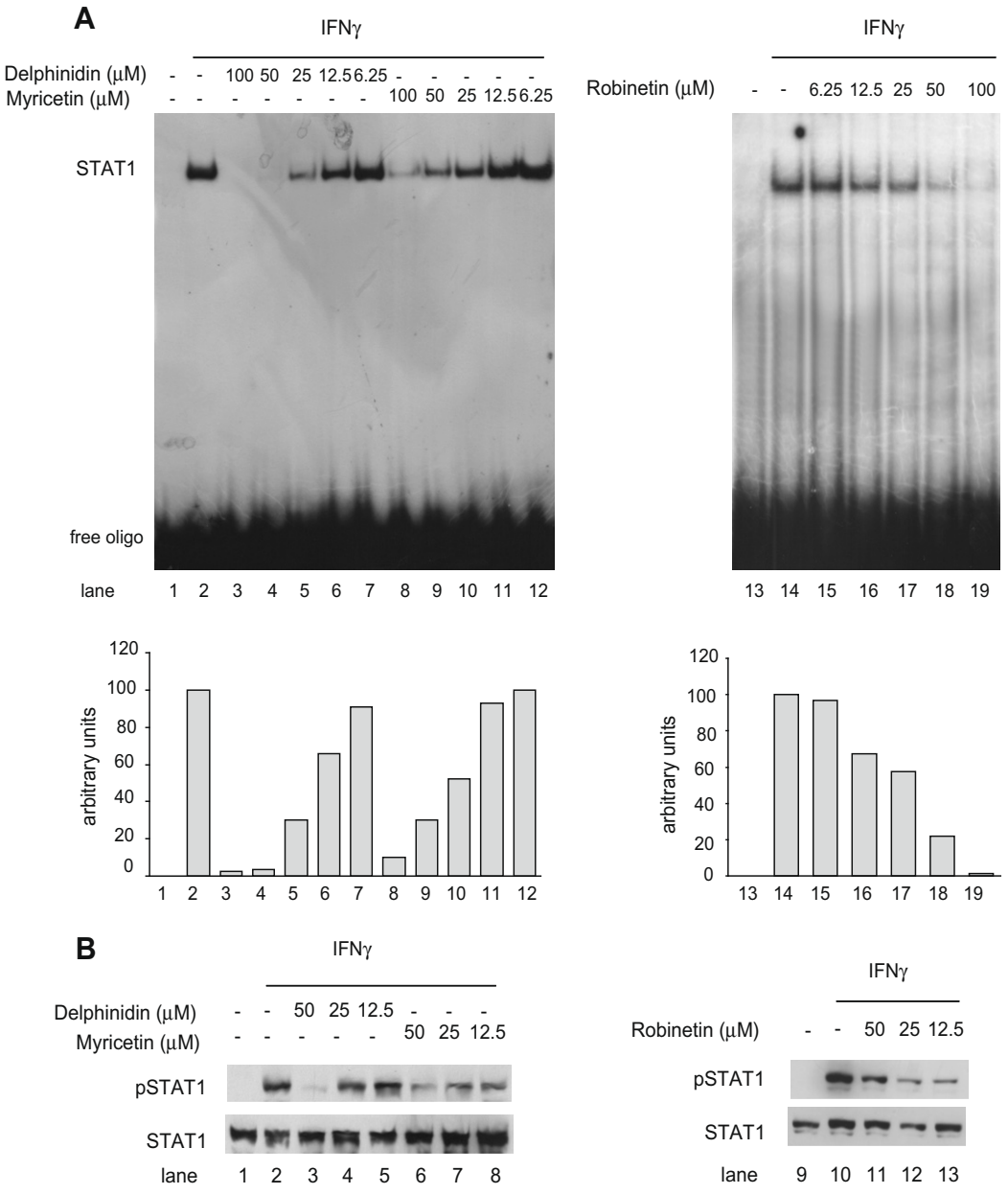


Fig. 6. Myricetin, delphinidin, and robinetin inhibit STAT1 activation. (A) Nuclear proteins of cells were analyzed by EMSA. Histograms of these gels are also presented. The gels are representative of four independent experiments. (B) Proteins of cells treated as in (A) were assessed by Western blot analysis with an anti-phosphoSTAT1 (Tyr701) antibody. Blots were stripped and reprobed with an antibody recognizing STAT1 protein. Data shown are representative of five independent experiments.

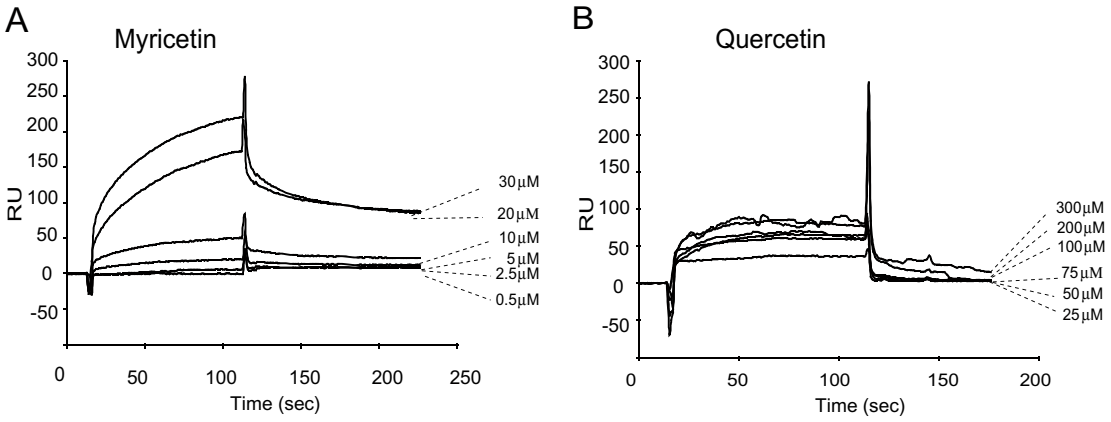
of the human IFN-γ receptor) interacts with residues 602 and 584 of STAT1 located in a flexible region of the SH2-domain.

To better understand the nature of flavonoid-STAT1 interactions at the molecular level, docking calculations were performed. Simulations predict good binding affinities for most of the ligands at both sites 1 and 2. The detailed analysis of the best predicted orientations of the ligands in the different cavities shows that the hydroxyl groups of ring B are very important for stabilizing flavonoid-STAT1 complexes which agrees with the ligand based analysis mentioned above. These groups are oriented towards the interior of the cavity either in site 1 or 2 for the three active anti-STAT1 flavonoids, and interact with residues TRP573 and GLN518 on site 1 (Fig. 7E) and HIS568 and LEU639 on site 2 (Fig. 7G). However, for non-anti-STAT1 ligands, these groups are oriented towards the exterior of the cavity, interacting with other residues – e.g. quercetin interacts with ARG586 and PHE581 in site 1 (Fig. 7F), and with SER640 in site 2 (Fig. 7H). These differences in binding orientations may explain the weaker inhibitory effect of quercetin.

4. Discussion

4.1. Role of myricetin and delphinidin in the protection of the heart from I/R-injury

Our recent reports indicated that EGCG, a potent antioxidant flavonoid present in green tea leaves, exerts an inhibitory action towards IFN-γ-elicited activation of STAT1 in a number of cell lines [33] and efficiently protects against I/R-induced injury in the heart [4], suggesting that consumption of green tea may be able to mediate cardioprotection and enhance cardiac function during I/R injury. The present study extends this notion.



C

	Dissociation Constant K_D (μM)	
Myricetin	0.103	19.5
Robinetin	0.027	8.6
Delphinidin	0.028	6.6
Quercetin	43.3	
Peonidin	ND	
Galangin	ND	
Myricetrin	634	
Tricetin	20	

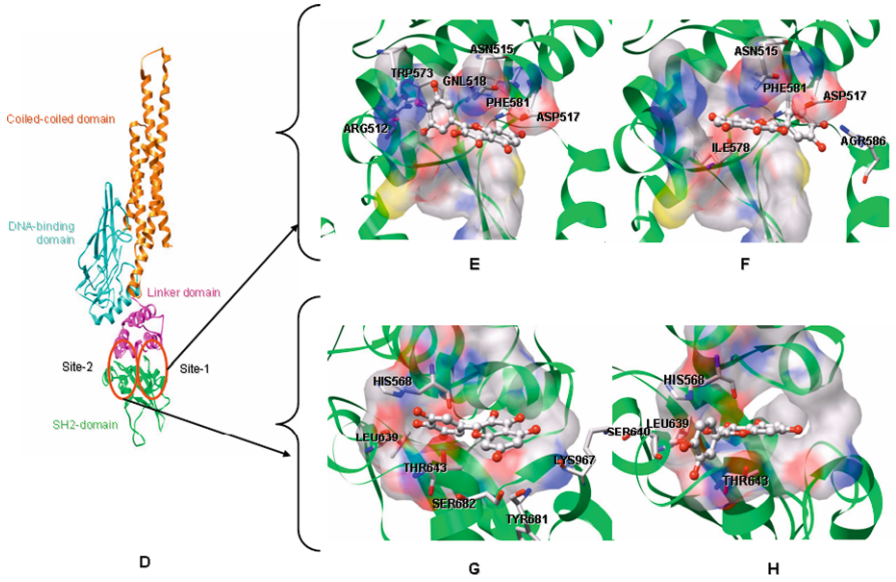


Fig. 7. Direct interaction between STAT1 and natural occurring flavonoids. (A) Example of sensograms indicating the strong interaction between STAT1 and myricetin. (B) Example of sensograms indicating the weak interaction between STAT1 and quercetin. (C) Estimated values of the dissociation constant (K_D) of the flavonoids tested. (D) Schematic view of STAT-1 without the N-domain (structure pdb reference 1YVL); the different domains as well as the two major binding sites are indicated; the locations of sites 1 and 2 are shown by the circles. (E, F) The best fit of myricetin and quercetin in site 1. Ligands are represented in stick and the protein backbone in cartoon. The surface of the protein has been drawn using polarity color code, blue positive, red negative and white hydrophobic; the residues interacting with ligands are in licorice. (G, H) The best fit of myricetin and quercetin in site 2. Ligands are represented in stick and the protein backbone in cartoon. The surface of the protein has been drawn using polarity color code, blue positive, red negative and white hydrophobic; the interacting residues are represented in licorice.

The results presented here demonstrate that all flavonoids examined (delphinidin, myricetin and quercetin), consistent with their strong antioxidant activity, are able to inhibit the intrinsic pathway of cardiomyocyte apoptosis in which mitocondrial integrity is crucial. This is in line with the widely reported beneficial action of antioxidant flavonoids towards inflammatory diseases [34]. However, the critical finding of the present study is that, compared to quercetin, myricetin and delphinidin, by attenuting I/R-induced STAT1 activation, suppress cardiomyocyte apoptosis more efficiently by selectively interfering with the extrinsic pathway in which STAT1-dependent FAS/FAS ligand expression plays a critical role. Notably, we observed a more pronounced protective effect of myricetin and delphinidin towards both heart tissue injury and hemodynamic impairment which was strictly correlated with their specific anti-STAT1 activity, suggesting that STAT1 could be a relevant target for protective treatment of I/R injury in the heart.

4.2. Mechanism of inhibition of STAT1 activation by anti-STAT1 flavonoids

It is striking that among a number of flavonoids examined in this study, only those (myricetin, delphinidin and robinetin) with a particular structural feature such as the presence of three hydroxyl groups in the B ring and one in the 3 position of the C ring, exert strong anti-STAT1 activity. Our in vitro and in vivo results indicate further that the inhibitory action of anti-STAT1 flavonoids should not be attributed solely to their antioxidant activity. Since tricetin, myricitrin and myrtyllin have no inhibitory action on IFN-γ-elicited STAT1 activation, and are unable to directly interact with STAT1, position 3 of the C-ring of anti-STAT1 flavonoids is therefore critical for their specific action. Although the docking analysis data would support this assumption, a more detailed study that takes into account the flexibility of the protein is required for a deeper understanding of the inhibitory mechanism.

The capacity of anti-STAT1 flavonoids to directly interact with STAT1 with high affinity at critical sites near the SH2 domain underlines the novel mechanism leading to the efficient, specific inhibition of STAT1 activation. In particular, the very high affinity of these flavonoids towards putative binding site 1 is in line with the specificity of their action towards the STAT1 pathway shown in this study. Indeed, STAT3, having a slightly different SH2 domain with respect to that of STAT1, was not inhibited by anti-STAT1 flavonoids in I/R injury, adding further support to the above-mentioned mechanism. This may also explain how anti-STAT1 flavonoids disturb I/R-elicited phosphorylation of both TYR701 and SER727, both of which are situated near putative anti-STAT1 flavonoid binding sites. Furthermore, the highly efficient effect of anti-STAT1 flavonoids in protecting the heart from I/R-induced injury is in line with their very high affinity towards STAT1 (especially at putative binding site 1, $K_D \approx 20$ nM). The present study may also serve as a starting point for further development of phytochemical-derived compounds capable of selectively inhibiting STAT1 activation to be tested in the prevention and/or treatment of inflammatory diseases in which STAT1 plays a critical role.

In conclusion, this report indicates that, at a molecular level, among a number of antioxidant flavonoids examined, only myricetin, delphinidin and robinetin with a common structural feature including the presence of three hydroxyl groups in the B ring and of the hydroxyl group in the 3 position of C-ring are able to interact directly with STAT1 with high affinity. Therefore, at the tissue level, these have a more pronounced protective action against I/R-induced heart injury in which STAT1 plays a critical role in activating the extrisic apoptotic pathway. The consumption of foods containing flavonoids with structural features of myricetin, delphinidin and robinetin, together with green tea containing EGCG, may represent an important new preventive treatment of cardiovascular diseases.

Acknowledgments

The authors are grateful for financial support by CARIVERONA project 2003 and CIRC (Consorzio Interuniversitario per la Ricerca Cardiovascolare) (H.S.).

References

[1] Stephanou, A., Brar, B.K., Scarabelli, T.M., Jonassen, A.K., Yellon, D.M., Marber, M.S., Knight, R.A. and Latchman, D.S. (2000) Ischemia-induced STAT-1 expression and activation play a critical role in cardiomyocyte apoptosis. J. Biol. Chem. 275, 10002–10008.
[2] Levy, D.E. and Darnell Jr., J.E. (2002) Stats: transcriptional control and biological impact. Nat. Rev. Mol. Cell Biol. 3, 651–662.
[3] Stephanou, A. (2004) Role of STAT-1 and STAT-3 in ischaemia/reperfusion injury. J. Cell Mol. Med. 8, 519–525.
[4] Townsend, P.A. et al. (2004) Epigallocatechin-3-gallate inhibits STAT-1 activation and protects cardiac myocytes from ischemia/reperfusion-induced apoptosis. Faseb J. 18, 1621–1623.
[5] Pawate, S., Shen, Q., Fan, F. and Bhat, N.R. (2004) Redox regulation of glial inflammatory response to lipopolysaccharide and interferongamma. J. Neurosci. Res. 77, 540–551.
[6] de Prati, A.C., Ciampa, A.R., Cavalieri, E., Zaffini, R., Darra, E., Menegazzi, M., Suzuki, H. and Mariotto, S. (2005) STAT1 as a new molecular target of anti-inflammatory treatment. Curr. Med. Chem. 12, 1819–1828.
[7] Simpson, P. and Savion, S. (1982) Differentiation of rat myocytes in single cell cultures with and without proliferating nonmyocardial cells. Cross-striations, ultrastructure, and chronotropic response to isoproterenol. Circ. Res. 50, 101–116.
[8] Scarabelli, T.M. et al. (2004) Minocycline inhibits caspase activation and reactivation, increases the ratio of XIAP to smac/DIABLO, and reduces the mitochondrial leakage of cytochrome C and smac/DIABLO. J. Am. Coll. Cardiol. 43, 865–874.
[9] van Engeland, M., Ramaekers, F.C., Schutte, B. and Reutelingsperger, C.P. (1996) A novel assay to measure loss of plasma membrane asymmetry during apoptosis of adherent cells in culture. Cytometry 24, 131–139.
[10] Scarabelli, T.M. et al. (1999) Quantitative assessment of cardiac myocyte apoptosis in tissue sections using the fluorescence-based tunel technique enhanced with counterstains. J. Immunol. Meth. 228, 23–28.
[11] McCormick, J. et al. (2006) Free radical scavenging inhibits STAT phosphorylation following in vivo ischemia/reperfusion injury. Faseb J. 20, 2115–2117.
[12] Scarabelli, T. et al. (2001) Apoptosis of endothelial cells precedes myocyte cell apoptosis in ischemia/reperfusion injury. Circulation 104, 253–256.
[13] Oliver, I.T. (1955) A spectrophotometric method for the determination of creatine phosphokinase and myokinase. Biochem. J. 61, 116–122.
[14] Scarabelli, T.M. et al. (2002) Urocortin promotes hemodynamic and bioenergetic recovery and improves cell survival in the isolated rat heart exposed to ischemia/reperfusion. J. Am. Coll. Cardiol. 40, 155–161.
[15] Scarabelli, T.M., Stephanou, A., Pasini, E., Comini, L., Raddino, R., Knight, R.A. and Latchman, D.S. (2002) Different signaling pathways induce apoptosis in endothelial cells and cardiac myocytes during ischemia/reperfusion injury. Circ. Res. 90, 745–748.
[16] Osborn, L., Kunkel, S. and Nabel, G.J. (1989) Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. Proc. Natl. Acad. Sci. USA 86, 2336–2340.
[17] Laurie, A.T. and Jackson, R.M. (2005) Q-SiteFinder: an energy-based method for the prediction of protein-ligand binding sites. Bioinformatics 21, 1908–1916.
[18] Mao, X. et al. (2005) Structural bases of unphosphorylated STAT1 association and receptor binding. Mol. Cell 17, 761–771.
[19] Morris, G.M., Goodsell, D.S., Halliday, R.S., Huey, R., Hart, W.E., Belew, R.K. and Olson, A.J. (1998) Automated docking using a Lamarckian genetic algorithm and empirical binding free energy function. J. Comput. Chem. 19, 1639–1662.
[20] Jones, G., Willett, P., Glen, R.C., Leach, A.R. and Taylor, R. (1997) Development and validation of a genetic algorithm for flexible docking. J. Mol. Biol. 267, 727–748.
[21] Bursulaya, B.D., Totrov, M., Abagyan, R. and Brooks 3rd, C.L. (2003) Comparative study of several algorithms for flexible ligand docking. J. Comput. Aided Mol. Des. 17, 755–763.
[22] Jenwitheesuk, E. and Samudrala, R. (2003) Improved prediction of HIV-1 protease-inhibitor binding energies by molecular dynamics simulations. BMC Struct. Biol. 3, 2.
[23] Toprakci, M. and Yelekci, K. (2005) Docking studies on monoamine oxidase-B inhibitors: estimation of inhibition constants (K(i)) of a series of experimentally tested compounds. Bioorg. Med. Chem. Lett. 15, 4438–4446.
[24] Frisch, M.J., Trucks, G.W., Schlegel, H.B., Scuseria, G.E., Robb, M.A., Cheeseman, J.R., Zakrzewski, V.G., Montgomery, J.A. Jr, Stratmann, R.E., Burant, J.C., Dapprich, S., Millam, J.M., Daniels, A.D., Kudin, K.N., Strain, M.C., Farkas, O., Tomasi, J., Barone, V., Cossi, M., Cammi, R., Mennucci, B., Pomelli, C., Adamo, C., Clifford, S., Ochterski, J., Petersson, G.A., Ayala, P.Y., Cui, Q., Morokuma, K., Malick, D.K., Rabuck, A.D., Raghavachari, K., Foresman, J.B., Cioslowski, J., Ortiz, J.V., Baboul, A.G., Stefanov, B.B., Liu, G., Liashenko, A., Piskorz, P., Komaromi, I., Gomperts, R., Martin, R.L., Fox. D.J., Keith, T., Al-Laham, M.A., Peng, C.Y., Nanayakkara, A., Gonzalez, C., Challacombe, M., Gill, P.M.W., Johnson, B., Chen, W., Wong, M.W., Andres, J.L., Gonzalez, C., Head-Gordon, M., Replogle, E.S. and Pople, J.A. (1998) Gaussian Inc., Pittsburgh, PA.
[25] Pettersen, E.F., Goddard, T.D., Huang, C.C., Couch, G.S., Greenblatt, D.M., Meng, E.C. and Ferrin, T.E. (2004) UCSF Chimera – a visualization system for exploratory research and analysis. J. Comput. Chem. 25, 1605–1612.
[26] Pedretti, A., Villa, L. and Vistoli, G. (2004) VEGA–an open platform to develop chemo-bio-informatics applications, using plug-in architecture and script programming. J. Comput. Aided Mol. Des. 18, 167–173.
[27] Ueda, S., Masutani, H., Nakamura, H., Tanaka, T., Ueno, M. and Yodoi, J. (2002) Redox control of cell death. Antioxid. Redox. Signal. 4, 405–414.
[28] Samudio, I. et al. (2006) A novel mechanism of action of methyl-2-cyano-3,12 dioxoolean-1,9 diene-28-oate: direct permeabilization of the inner mitochondrial membrane to inhibit electron transport and induce apoptosis. Mol. Pharmacol. 69, 1182–1193.

[29] Rice-Evans, C.A., Miller, N.J. and Paganga, G. (1996) Structure–antioxidant activity relationships of flavonoids and phenolic acids. Free Radic. Biol. Med. 20, 933–956.
[30] Zykova, T.A., Zhang, Y., Zhu, F., Bode, A.M. and Dong, Z. (2005) The signal transduction networks required for phosphorylation of STAT1 at Ser727 in mouse epidermal JB6 cells in the UVB response and inhibitory mechanisms of tea polyphenols. Carcinogenesis 26, 331–342.
[31] Nam, N.H., Pitts, R.L., Sun, G., Sardari, S., Tiemo, A., Xie, M., Yan, B. and Parang, K. (2004) Design of tetrapeptide ligands as inhibitors of the Src SH2 domain. Bioorg. Med. Chem. 12, 779–787.
[32] Yasukawa, H. et al. (1999) The JAK-binding protein JAB inhibits Janus tyrosine kinase activity through binding in the activation loop. Embo J. 18, 1309–1320.
[33] Menegazzi, M., Tedeschi, E., Dussin, D., De Prati, A.C., Cavalieri, E., Mariotto, S. and Suzuki, H. (2001) Anti-interferon gamma action of epigallocatechin-3-gallate mediated by specific inhibition of STAT1 activation. Faseb J. 15, 1309–1311.
[34] Middleton Jr., E., Kandaswami, C. and Theoharides, T.C. (2000) The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacol. Rev. 52, 673–751.