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Quercetin binds to calcineurin at a similar region to cyclosporin A and tacrolimus

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ABSTRACT

Quercetin, the primary dietary flavonol, exerts a strong inhibitory effect on calcineurin (CN), a unique Ca^{2+}/calmodulin-dependent serine/threonine protein phosphatase. Using fluorescence spectroscopy (FS) we showed quercetin strongly bound to calcineurin catalytic subunit (CNA) with a ratio of 1:1; we also showed that calcineurin regulatory subunit (CNB) weakened this binding. In addition, the secondary structure of CNA was much tighter in the presence of quercetin. An FS study with CNA truncated mutant CNAa showed that the binding area for quercetin was reduced to the catalytic domain of CNA. Furthermore, fluorescence resonance energy transfer (FRET) results and molecular docking indicated three potential binding sites for quercetin, which were located at a region between the active centre of CNA and the CNB binding domain, a similar binding area to that of cyclosporin A and tacrolimus. Interestingly, this region was also important for CN substrate recognition.

1. Introduction

Quercetin (3,3′,4′,5,7-pentahydroxyflavone) is a typical flavonoid ubiquitously present in vegetables and fruits, and its antioxidant effect is suggested as being beneficial for human health (Wach, Pyrzyńska, & Biesaga, 2007). In our previous work, we found quercetin also exerted a strong inhibitory effect on calcineurin (CN) (Wang, Zhou, Lei, & Wei, 2010). Calcineurin is a unique serine/threonine protein phosphatase (PP2B), regulated by Ca^{2+} and calmodulin (CaM). As a heterodimer, CN consists of a catalytic subunit (CNA) and a regulatory subunit (CNB). CNA contains a catalytic domain (CNAa), a CNB-binding domain (BBH), a CaM-binding domain (CBD) and an auto-inhibitory domain (AI) (Klee, Ren, & Wang, 1998). Besides many biological processes, clinical interest in CN has continued to focus on T cells and other cells of the immune system, especially on CN-nuclear factor of activated T cells (NFAT) signalling, which is the principal target of the widely prescribed immunosuppressive drugs, cyclosporin A (CsA) and tacrolimus (also called FK506) (Cardenas, Sanfridson, Cutler, & Heitman, 1998; Kincaid, 1995; Liu et al., 1991; Rusnak & Mertz, 2000).

After administration, CsA and FK506 first need to bind their respective immunophilin, cyclophilin A (CyPA) and FK506-binding protein (FKBP), to form immunophilin–immunosuppressant complex (Liu et al., 1991); then the complex binds to CN and shows strong inhibition to CN activity. It has been reported these two immunophilin–immunosuppressant complexes share a hydrophobic groove formed at the junction of CNA and CNB for binding with a few distinct recognition elements (Griffith et al., 1995; Huai et al., 2002; Jin & Harrison, 2002; Kissinger et al., 1995).

Compared with CsA and FK506, quercetin is also a non-competitive CN inhibitor when assayed with a phosphopeptide substrate (Kissinger et al., 1995; Wang et al., 2010). However, inhibition of quercetin to CN is immunophilin-independent and the interaction between them is unclear. Does quercetin also bind to CN like kaempferol, as we reported previously (Lei, Qi, Jia, Lin, & Wei, 2009)? If so, which part does it bind to and in which way does it inhibit CN activity? Are they the same as cyclosporin A and FK506? Answering these questions is not only significant for greater understanding of the roles of quercetin in human health, but also important for developing new CN inhibitors.

2. Materials and methods

2.1. Materials

Quercetin was from National Institute for the Control of Pharmaceutical and Biological Products (NICPBP, China). CNA, CNAa, CNB, the four one-Trp-containing mutants of CNA (each of them contained only one tryptophan, whilst the other three tryptophans in CNA were all mutated to phenylalanines), were named CNA–W134 (W232F, W342F, W352F), CNA–W232 (W134F, W342F, W352F), CNA–W342 (W134F, W232F, W352F) and CNA–W352 (W134F, W232F, W342F) according to the position of tryptophan. They were expressed and purified as we described previously; construction of the one-Trp-containing mutants of CNA was also included (Lei et al., 2009; Wei & Lee, 1997).

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2.2. Fluorescence spectroscopy (FS) measurements

Proteins were dissolved in 0.05 M Tris/HCl buffer pH 7.4. By mixing protein solution with different concentration of quercetin in dimethyl sulphoxide (DMSO), a series of solution were obtained containing 2 μM protein with different concentrations of quercetin in 2%(v/v) DMSO.

Fluorescence spectra at wavelengths from 305 to 430 nm were recorded on a Fluoro Max-2 Fluorimeter (Jobin Yvon-Spex; Lonjumeau, France) using the excitation wavelength of 295 nm at 4 °C. Excitation and emission bandwidths were 5 nm.

2.3. Circular dichroism spectra (CD) measurement

CD measurements were carried out on a Jasco J-810 spectropolarimeter (Jasco, Tokyo, Japan) in a cell with a 10-mm path-length at 4 °C in 0.05 M Tris/HCl buffer (pH 7.4). Spectra were collected at a scan speed of 200 nm/min and response time of 1 s. Each spectrum was the average of three scans from 190 to 250 nm. The mean residue ellipticity $[\theta]$ in degree $cm^2\ dmol^{-1}$ was related to $\Delta\epsilon$ in $M^{-1}\ cm^{-1}$ by $\Delta\epsilon = [\theta]/3298$. The fraction contents of secondary structures were calculated using CDPro software (Sreerama, Venyaminov, & Woody, 1999; Sreerama & Woody, 2000).

2.4. UV absorbance spectra of quercetin

The UV absorbance spectra of quercetin were recorded on a Cintra-10e UV–Vis spectrometer (GBC, Braeside, Australia) from 220 to 450 nm at room temperature. The reference solution was 0.05 M Tris–HCl with 2% (v/v) DMSO at pH 7.4.

2.5. Fluorescence resonance energy transfer (FRET)

Based on Förster's non-radiative energy transfer theory, whenever the emission spectrum of the donor overlaps with the absorption spectrum of the acceptor, fluorescence resonance energy transfer (FRET) occurs (Förster & Sinanoglu, 1966; Zhang et al., 2007). Here we use each one-Trp-containing CNA mutant as the donor and quercetin always as the acceptor. The relation between energy transfer efficiency E and the distance (r) between acceptor (quercetin) and donor (each tryptophan residue in CNA) is

$$E = 1 - F/F_0 = R_0^6/(R_0^6 + r_6). \quad (1)$$

F_0 and F are the fluorescence intensity of the donor in the absence and presence of acceptor, respectively. R_0, the Förster distance or critical distance, at which efficiency of transfer is 50%, is calculated from

$$R_0^6 = 8.8 \times 10^{-25}\ K^2\ N^{-4} \Phi J. \quad (2)$$

The fluorescence quantum yield Φ of CNA–W134, CNA–W232, CNA–W342 and CNA–W352 in the absence of quercetin is 0.050, 0.034, 0.012 and 0.096 correspondingly as measured before (Lei et al., 2009); K^2 is the spatial orientation factor (assumed to be 2/3); N is the refractive index of the medium (assumed to be 1.336) (Cyril, Earl, & Sperry, 1961); J as the overlap integral between each mutant fluorescence emission spectrum and quercetin absorption spectrum, can be obtained by

$$J = \sum F(\lambda)\epsilon(\lambda)\lambda^4 \Delta\lambda / \sum F(\lambda)\Delta\lambda, \quad (3)$$

Here $F(\lambda)$ is the fluorescence intensity of protein at wavelength λ, $\epsilon(\lambda)$, with unit of $cm^{-1}\ M^{-1}$, is the molar absorption coefficient of quercetin at the same wavelength λ. Therefore, the distance (r) between quercetin and each tryptophan in CNA can be calculated.

2.6. Molecular docking

Autodock 4.0 was used for the docking studies. Protein Data Bank (PDB) entry 1AUI (CN) was chosen as rigid receptor and quercetin was treated as flexible. Non-polar hydrogen atoms were merged and five rotatable bonds were detected with Autodock tools (1.4.5). Gasteiger charges and solvation parameters were also added. Using the Autogrid program, with 1.0 Å spacing, grid box's numbers of points in X, Y, Z dimensions were 64, 58, and 92 separately, the box was centred on the macromolecule. Docking simulations were performed using the Lamarckian genetic algorithm (LGA), other search and docking parameters were default. Number of GA runs was set at 50 (Bikádi & Hazai, 2008). In the end, docking results were compared with the potential binding area from the FRET study. All figures were rendered by PyMOL (DeLano, 2002).

3. Results

3.1. Quercetin directly bound to CNA without any co-factor

Interaction of quercetin and CNA was monitored following the quenching of relative fluorescence intensity of CNA. First, 2% DMSO did not affect determination of CNA–inhibitor interaction. With stoichiometric addition of quercetin, CNA fluorescence intensity quenched gradually and the wavelength maxima also decreased slightly (344–340 nm) (Fig. 1A). The quenching mechanism could be analysed according to Stern–Volmer quenching plot (Lakowicz, 1991) in Eq. (4)

$$F_0/F = 1 + k_q \tau_0 [Q] = 1 + K_D [Q], \quad (4)$$

Here F_0 and F represent fluorescence intensities in the absence and presence of quencher, $[Q]$ is concentration of quencher, k_q is the bimolecular quenching constant and τ_0 (assumed to be 10^{-8} s) (Eftink, 1991) is the lifetime of the fluorophore without quencher. K_D, as the Stern–Volmer dynamic quenching constant, was determined by linear regression of a plot of F_0/F versus $[Q]$ (Fig. 1B). Resultantly, k_q was calculated to be 9.8 $(\pm 0.5) \times 10^{12}\ M^{-1}\ s^{-1}$ ($n = 3$, relative coefficient $r > 0.99$), larger than the maximum diffusion collision quenching rate constant of various quenchers with the biopolymer, which is $2.0 \times 10^{10}\ M^{-1}\ s^{-1}$ (Zhang et al., 2007), so the fluorescence quenching should be static quenching (complex formation) instead of dynamic collision quenching. For static quenching, the binding constant K_A and the binding sites n for the reaction between quercetin and the protein can be obtained by the plot of $\log [(F_0 - F)/F]$ against $\log [Q]$ based on Eq. (5) (Fig. 1C).

$$\log[(F_0 - F)/F] = \log K_A + n \log [Q], \quad (5)$$

K_A and n turned out to be 1.7 $(\pm 1.0) \times 10^6\ M^{-1}$ ($n = 3$, relative coefficient $r > 0.99$) and 1.2 (±0.2), respectively, i.e., quercetin bound to CNA strongly with a ratio of 1:1.

3.2. Changes of CNA conformation induced by quercetin binding

The slight blue shift in Fig. 1A reflected the microenvironment of the tryptophan residue, which was affected by quercetin binding. To determine the changes of CNA conformation after interaction with quercetin, CD spectra of free CNA and CNA–quercetin were shown in Fig. 1D and percentages of CNA secondary structure were shown in Table 1. Obviously, the addition of equimolar quercetin caused much increase of α-helix in CNA (from 23.7% to 40.1%), suggesting the secondary structure of the protein was much tighter with quercetin binding.