J. Biochem. 2010;147(2):185–190 doi:10.1093/jb/mvp163

JB THE JOURNAL OF BIOCHEMISTRY

Inhibition of calcineurin by quercetin *in vitro* and in Jurkat cells

Received September 14, 2009; accepted September 23, 2009; published online October 29, 2009

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Calcineurin (CN), the Ca^{2+}/calmodulin (CaM)-dependant protein phosphatase, is an integral enzyme involved in activation of T cells. It is also the target of various inhibitors such as cyclosporine A (CsA) and FK506 both of which have been widely used as immunosuppressants. We show that the novel CN inhibitor, quercetin (QC), associates with CN both *in vitro* and in Jurkat cells, and that it causes non-competitive inhibition of phosphatase activity. Unlike CsA and FK506, QC does not require a matchmaker protein for CN inhibition. It acts directly on the catalytic domain and its inhibitory effect was increased by the presence of CNB. Using semi-quantitative and real-time RT–PCR, we show that QC inhibits IL-2 gene expression in activated Jurkat cells. The physiological inhibitory activity of QC together with its hypotoxicity suggests that it may be an effective immunosuppressant.

Keywords: calcineurin/inhibitor/Jurkat cell/quercetin/RT–PCR.

Abbreviations: CN, Calcineurin; CaM, Calmodulin; CsA, Cyclosporine A; FK506, Tacrolimus; QC, Quercetin; CNB, Calcineurin B subunit; CNA, Calcineurin A subunit; BBH, Subunit B binding domain; CBD, Calmodulin binding domain; AID, autoinhibitory domain; NFAT, nuclear factor of activated T cells; AP1, activator protein-1; MEF2, myocyte enhancer factor-2; FBS, fetal bovine serum; CNAa, calcineurin A Subunit catalytic domain; CNAab, truncate mutant of calcineurin A subunit; CNAabc, truncate mutant of calcineurin A subunit; BSA, bovine serum albumin; p-NPP, p-nitrophenyl phosphate; DTT, dithiothieitol; EGTA, ethyleneglycol bis(2-aminoethyl ether)tetraacetic acid; RT–PCR, Reverse Transcription Polymerase Chain Reaction; IC50, the half maximal inhibitory concentration; IL-2, interleukin-2; TNF-α, tumor necrosis factor-α; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; cDNA, complementary DNA.

Calcineurin (CN), also known as protein phosphatase 2B, is a widely distributed protein phosphatase regulated by calcium and calmodulin (CaM) (*1*). It is a heterodimer consisting of a 60-kDa catalytic subunit (CNA) and a 19-kDa calcium-binding regulatory subunit (CNB). CNA can be further divided into a catalytic domain and three regulatory domains, namely a CNB-binding domain (BBH), a CaM-binding domain (CBD) and an autoinhibitory domain (AID). The catalytic domain is responsible for the dephosphorylation of substrates, whereas the regulatory domain is involved in regulating the enzyme activity in the presence of CNB and CaM (*2*, *3*). All of the domains contribute to enzyme activity (*4*, *5*).

CN is involved in multiple cellular processes (such as T-cell activation) and controls the synthesis of several cytokines by dephosphorylating a family of transcription factors known as NF-ATs (nuclear factor of activated T cells). Once dephosphorylated by CN, NFATs translocate to the nucleus, where, in cooperation with other transcription factors such as AP-1, MEF2 and GATA, they induce the expression of target genes, including cytokine genes that regulate the immune response (*6*, *7*). Thus, many CN inhibitors are immunosuppressants (*8*).

A number of compounds have been demonstrated to have inhibitory activity against CN, including cyclosporin A (CsA), FK506, microcystin LR, dibefurin, PD 144795, endothal derivatives, cantharidin, metal-ligating phosphonates (*9*), gossypol (*10*) and so on. CsA and FK506 are the two most effective and best-known natural inhibitors of CN. They are widely used to prevent rejection after organ transplantation (*11*). However, the use of these drugs is associated with a number of significant side effects including nephrotoxicity, hypertension, hyperlipidaemia and neurotoxicity, and this has limited their therapeutic use (*12*, *13*). Thus, we have sought to develop CN inhibitors that have effective immunosuppressive activity but are less toxic.

In this study, we evaluated a novel CN inhibitor, quercetin (QC). We determined its *in vitro* inhibitory activity in ^{32}P-labelled protein phosphatase assays, and its *in vivo* potency in Jurkat cells. We also investigated its possible inhibitory mechanism. Our results show that QC is a potentially effective immunosuppressive agent with the advantage of low toxicity.

Materials and methods

Chemicals and cells

QC was purchased from Sigma-Aldrich (St Louis, MI, USA), RII peptide was from BioMoL Research Laboratories (Philadelphia, PA, USA) and [γ-^{32}P] ATP from Beijing Furi Biologic Engineering Corp. The catalytic subunit of cAMP-dependent protein kinase was obtained from Promega Chemical Corp (Madison, WI, USA). All reagents were of the highest quality available.

Jurkat cells were cultured in RPMI1640 (GIBCO-BRL, Life Technologies, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 μg ml^{-1} streptomycin

and 100 U ml^{-1} penicillin at 37°C in humidified air containing 5% CO_2.

Expression and purification of CNA and deletion mutants

The deletion mutants of CNA used were CNAa (catalytic domain only), CNAab (catalytic domain and BBH domain) and CNAabc (catalytic domain, BBH domain and long CBD domain) (Fig. 1A). Strains expressing CNA and its deletion mutants were from our laboratory, and the enzymes were expressed and purified as described (*14*). Protein purity was analysed by SDS–PAGE.

Assay of phosphatase activity and enzyme kinetics using ^{32}P-labelled protein substrate

Enzyme activity was determined using an assay described in detail previously (*15*). A 20 mM QC stock solution was prepared in dimethylsulphoxide. Reactions were initiated by adding 10 µl of RII peptide (40 µM) to 10 µl of diluted enzyme solution containing QC at the required concentrations. Reactions were performed at 30°C for 10 min and terminated by addition of 180 µl H_3PO_4 solution (83.3 mM). The released ^{32}P was quantified by liquid scintillation spectrometry. The same vehicle without QC was used as matched control. Phosphatase activities are presented in the form of phosphate activity (percentage of control). Kinetic analyses with/without QC were performed in the above conditions with 2.5, 5, 10, 20, 40 and 80 mM RII substrate.

Assay of phosphatase activity using p-NPP

Assays using *p*-nitrophenyl phosphate (*p*-NPP) as substrate were performed in 1 mM $CaCl_2$, 0.5 mM $MnCl_2$, 2 µM CaM, 2 µM CNB, 1 mM DTT, 0.1 mg ml^{-1} BSA, 50 mM Tris–HCl (pH 7.4) at 4°C (*15*). QC was added as indicated. After addition of CNA, the solutions were pre-incubated for 10 min at 4°C before initiating the reaction by adding *p*-NPP to a final concentration of 20 mM. A sample without QC was used as matched control. The enzyme activity was monitored in a spectrophotometer (Cintra 10e, GBC) at 410 nm. Phosphatase activities are presented as percentage of control activity.

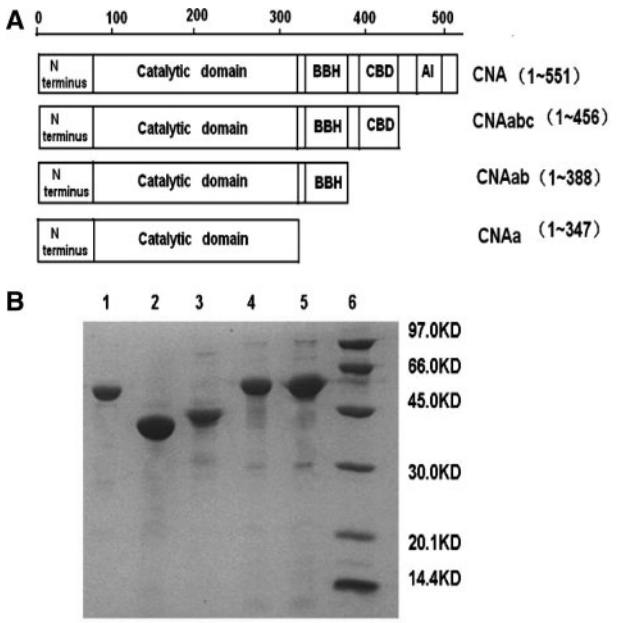

Fig. 1 Identification of proteins. (A) Schematic representation of CNA and its domain deletion mutants. (B) SDS–PAGE analysis of the purified proteins. (The proteins were run on a 14% acrylamide gel and stained with Coomassie Blue. Lane 1, CNAabc; lane 2, CNAa; lane 3, CNAab; lanes 4 and 5, CNA; lane 6, protein markers.)

Treatment, lysis and phosphatase assay of Jurkat cells

Jurkat cells were grown and lysed as described in (*16*) except that the cells were incubated for 24 h. Phosphatase assays were performed as described (*17*). Cell lysates (treated with QC or untreated) were diluted to the appropriate ratio (v/v) in the 2 × assay buffer (100 mM Tris–HCl, pH 7.5, 200 mM NaCl, 12 mM $MnCl_2$, 1 mM DTT, 200 nM okadaic acid and either 1 mM $CaCl_2$ or 20 mM EGTA). Reactions in 10 µl samples were carried out by mixing with 10 µl of RII peptide (40 µM, dissolved in milliQ water) at 30°C for 10 min. CN activity was calculated as the difference in phosphatase activity in $CaCl_2$ versus EGTA. CsA was added in the same way as a positive control. Phosphatase activities are presented in the form of percentage of control phosphate activity.

RNA extraction and semi-quantitative RT–PCR analysis

Jurkat cells were grown as described above. The whole RNA was isolated and semi-qualitative RT–PCR was performed as described, with appropriate modification (*18*). β-Actin levels were determined as an endogenous control. The whole RNA was reverse transcribed into cDNA using oligo $(DT)_{15}$ as the primer. The primer pairs for IL2 and β-actin were constructed as shown in Table 1, and 35(β-actin) and 38(IL-2) cycles of PCR were preformed in a DNA Thermal Cycler (Eppendorf) using the following conditions: denaturation at 95°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s. The PCR products were separated on 1.2% agarose gels and visualized by ethidium bromide staining. The gels were observed and photographed under ultraviolet light.

Real-time RT–PCR analyses

Jurkat cell culture and whole RNA isolation were carried out as for the semi-quantitative RT–PCR analysis. Real-time PCR was performed with SYBR Green I (Molecular Probes) as fluorescent dye, as recommended in the literature (*19*), and expression of the IL-2 gene was normalized to that of β-actin as an internal control. cDNAs were analysed using the following conditions: 5 min at 95°C and 50 cycles of 15 s at 95°C and 15 s at 60°C, in an ABI PRISM 7500 Sequence Detector System (Applied Biosystems, Foster City, CA, USA). Every sample was tested in triplicate and the data were analysed with the ABI PRISM Sequence Detector Systems software.

Data analysis

OriginPro 7.0 statistical software was employed to analyse the data and draw plots. One-way analysis of variance followed by least significant difference *post hoc* tests was used to determine the statistical significance of differences between means. All results are representative of three independent experiments.

Results

Expression and purification of CNA and its truncated derivatives

CNA and truncated derivatives were expressed, purified by a series of chromatographic steps and analysed

Table 1. Primer and probe sequences for semi-quantitative and real-time PCR.

Primers	Semi-quantitative	Real-time PCR
Forward (IL2)	GTCACAAACAGTGCACCTACTTC	TGTCACAAACAGTGCACCTACTTC
Reverse (IL2)	TGATATTGCTGATTAAGTCCCTG	TGTGGCCTTCTTGGGCATGT
Forward (β-actin)	CCTCTGACGTCCATCATCTA	GTGACAGCAGTCGGTTGGAG
Reverse (β-actin)	ATCTTCTGCTGCCGTCGCTT	AGTGGGGTGGCTTTTAGGAT

by SDS–PAGE (Fig. 1B). The proteins were all electrophoretically pure.

Inhibition of CN by QC

QC inhibited CN activity *in vitro* with either *p*-NPP or RII peptide as substrate. The IC_{50} values were $11.2 \pm 2\,\mu M$ (RII phosphopeptide, Fig. 2A) and $281.8 \pm 4\,\mu M$ (*p*-NPP, Fig. 2B), respectively. These results differ from those with CSA and FK506, which inhibit dephosphorylation of RII peptide but stimulate the phosphatase activity of CN towards *p*-NPP (*11*). In the RII peptide-based assay, kinetic analysis indicated that inhibition by QC was non-competitive (Fig. 2C).

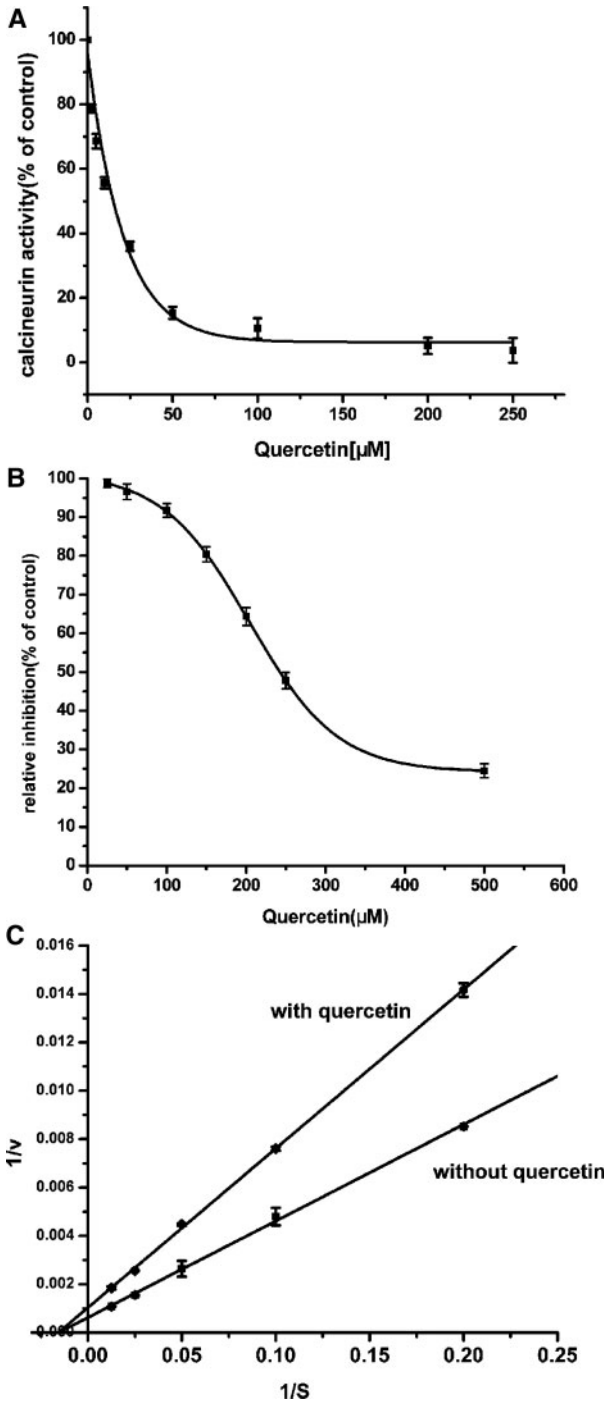

Fig. 2 Inhibitory effects of quercetin on phosphatase activity and its kinetics. (A) Phosphatase activity was measured with 20 μM RII peptide as substrate and is presented as nanomoles of phosphate released/mg of protein/min. (B) 20 mM *p*-NPP as substrate. Data are expressed as nanomoles of phosphate released/mg of protein/min. (C) Kinetic analysis with/without 12 μM QC were performed with RII as substrate at 2.5 mM, 5 mM, 10 mM, 20 mM, 40 mM and 80 mM. All data are means ± SD of three independent experiments. Further details may be found in the 'Materials and methods' section.

Inhibition of CNA and the truncated mutants by QC

The results in Fig. 3A demonstrate inhibition of CNA and its truncated derivatives by QC. All the enzymes were diluted to 10 nM in the final assay buffer. QC inhibited CNAabc with an IC_{50} of 9.8 μM, similar to that for CNA. However, the IC_{50} for CNAab was 41 μM, and it was even higher for CNAa ($IC_{50} = 192.8\,\mu M$).

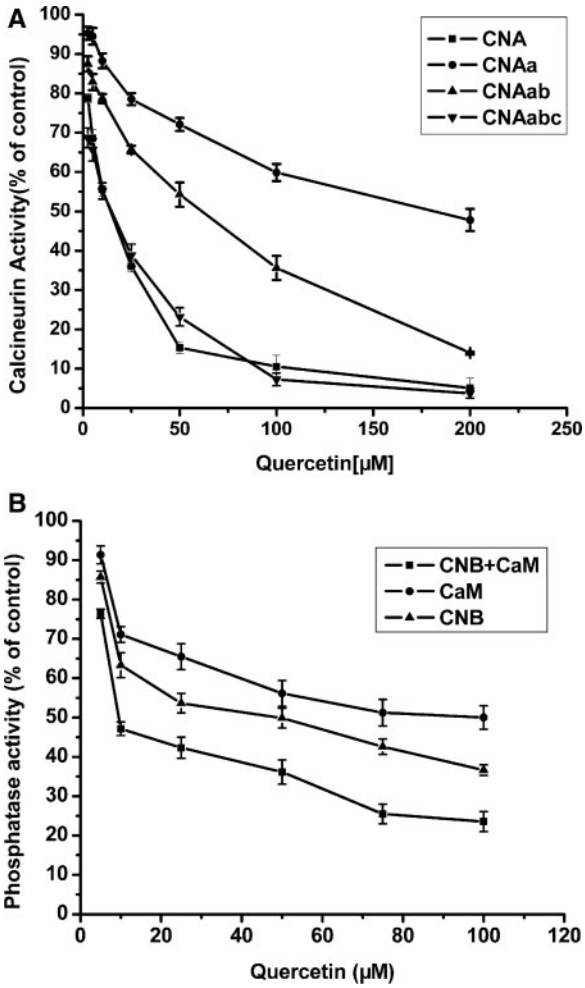

Fig. 3 Investigation of the mechanism of inhibition of CN by quercetin. (A) Inhibition of CNA and its truncated derivatives: CNAa, CNAab CNAabc by QC. CNA and its derivatives were diluted to 10 nM in the final assay buffer. Phosphatase activity of CNAa (circle), CNAab (triangles up), CNAabc (triangles) and CNA (squares) were measured at the indicated QC concentrations. (B) Inhibition of phosphatase activity by QC in the presence of CaM (circle), CNB (triangles up), CaM and CNB (squares). CNA was diluted to 15 nM in the final assay buffer. All data are means ± SD of three independent experiments. Further details may be found in the 'Materials and methods' section.

Comparison of inhibition by QC in the presence of CaM, CNB and CaM plus CNB

The protein phosphatase activity of CN is dependent on CaM and CNB, but CNB is more important for CN activity (*19*). We, therefore, investigated the inhibitory effect of QC on CNA under the circumstances of CaM and CNB. CNA was diluted to 15 nM in the final assay buffer in each case. As shown in Fig. 3B, the extent of inhibition of CNA by QC was differently affected by the presence of CaM, CNB or CaM plus CNB. Their IC_{50} values, respectively, were 113.5 μM, 61.7 μM and 16.8 μM. Evidently, inhibition of CNA is less sensitive to QC in the absence of CaM or CNB. Compared with CaM, CNB is more important for inhibition by QC.

Inhibition on CN by QC in Jurkat cells

We further investigated inhibition of CN by QC in Jurkat cells, human T-cell leukaemia cells that contain CN (*6*). The level of CN protein in the cells exposed to 40 μM QC was the same as in the control samples but the phosphatase activity was reduced by ~60% (Fig. 4A).

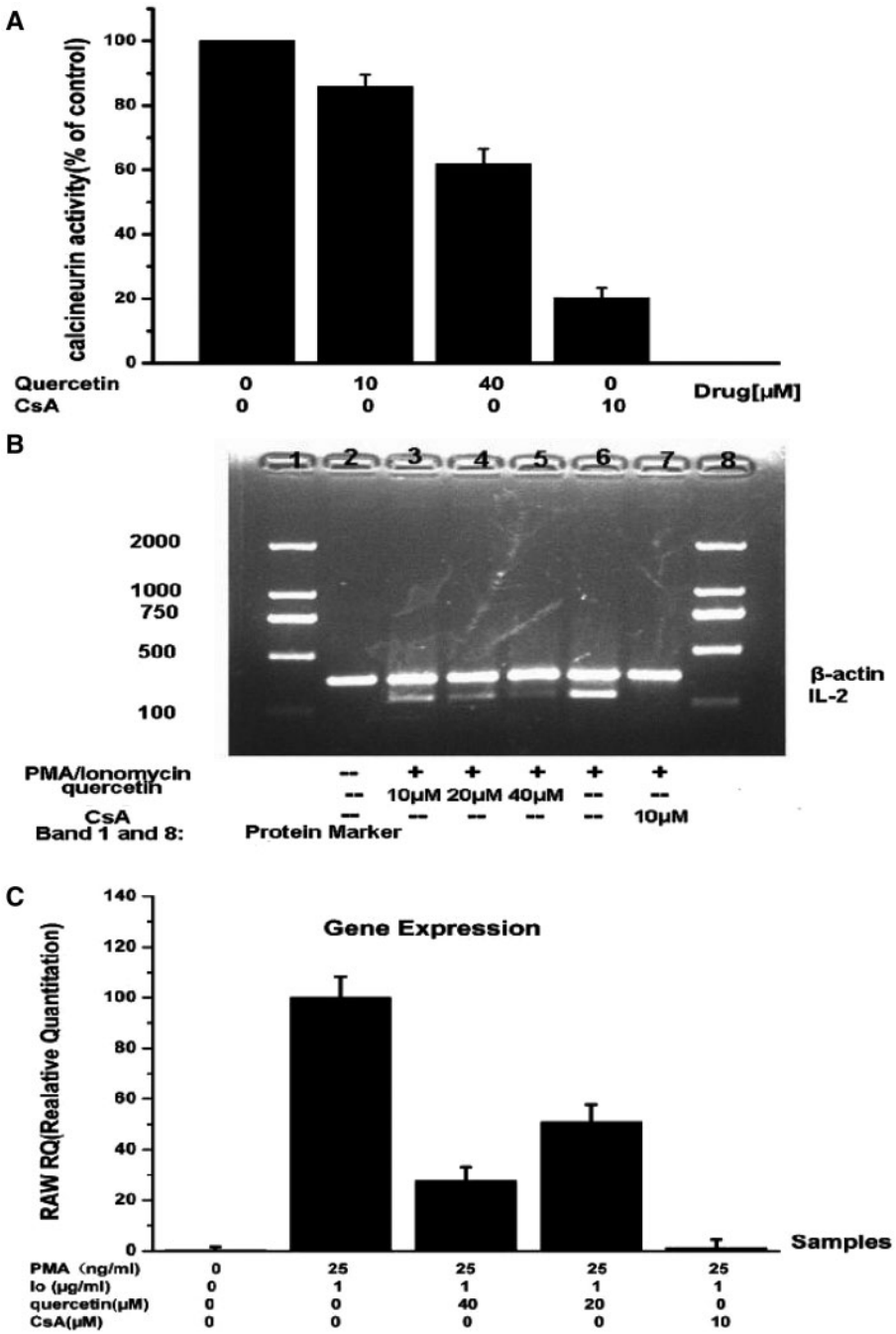

Fig. 4 Effect of quercetin on Jurkat cells. Protein concentrations in the lysates of drug-treated and control cells were equivalent. Samples were analysed in triplicate. (A) Effect of QC on CN activity. (B) Effect of QC on IL-2 mRNA expression assayed by semi-qualitative RT–PCR. (C) Effect of QC on IL-2 mRNA expression assayed by real-time RT–PCR. Further details may be found in the 'Materials and methods' section.

Inhibition of IL-2 gene expression by QC in activated Jurkat cells

The effect of QC on IL-2 mRNA levels was evaluated by semi-qualitative and real-time RT–PCR in Jurkat cells stimulated with phorbol-12-myristate-13-acetate (PMA)/ionomycin (Io). As shown in Fig. 4B and C, QC, like CsA, strongly inhibited PMA/Io-induced IL-2 expression in a dose-dependent manner.

Discussion

Nowadays, the main method of immunosuppression during organ transplantation and the treatment of autoimmune diseases is inhibition of CN activity with CsA or FK506. Unfortunately, long-term therapy with these anti-CN drugs can have major adverse effects, such as nephrotoxicity, hypertension, hyperlipidaemia and neurotoxicity (*9*). Thus, it is essential to find and characterize novel immunosuppressants with less severe side effects. During the screening for effective and noncompetitive inhibitors of CN, a natural inhibitory compound of flavonol, Quercetin (3,4′,5,7-pentahydroxyflavonel) was identified. Unlike CsA and FK506, QC inhibited CN activity with both *p*-NPP and RII peptide as substrates in the absence of the matchmaker protein (*20*).

In order to investigate the mechanism of inhibition of phosphatase by QC, we compared inhibition of CNA with that of its truncation mutants (CNAabc, CNAab and CNAa). The results showed that QC acts directly on the catalytic domain. Although the inhibition of CNAa by QC was very weak, it was not abolished. The fact that inhibition is non-competitive suggests that the binding site for QC may not be located in the catalytic centre, but in some other part of the catalytic domain. Our results also indicate that deletion of AID in CNA did not enhance the inhibitory potency of QC. In other words, AID may not be involved in the inhibition of CN by QC.

Previous results showed that CNB endows CN with the requisite molecular structure for interaction with the immunosuppressant drug CsA (*21*). Our present findings also demonstrate that CNB affects the inhibition by QC. First, inhibition of CNA by QC was dramatically decreased in the absence of CNB. Second, as is well known, BBH is the domain that links CNB and CNA, so that removal of BBH prevents CNB from acting; we observed in our experiments that if BBH, CBD and AID were removed from CNA, QC had a significantly reduced inhibitory effect on the enzyme activity; on the other hand, if only CBD and AID were removed, and BBH was retained, there was only a slight reduction in the inhibitory activity. These results also demonstrate the importance of CNB in inhibition by QC.

CN is reported to be inactivated by oxidative processes and can be reactivated by DTT and protected from inactivation by ascorbate (*22*). Inhibition of phosphatase activity by QC was unaffected by high concentrations (5 mM) of ascorbate or of DTT in our hands (data not shown). This result suggests that QC does not inactivate CN by a redox mechanism.

QC showed immunosuppressive activity in a mice splenocyte proliferation assay in response to concanavalin A (Con A, a special T lymphocyte mitogen) and lipopolysaccharide (LPS, a special B lymphocyte mitogen). The assay of ConA-induced T lymphocyte and LPS-induced B-lymphocyte proliferation were generally used for assessment of the effects of chemicals on the T- and B-lymphocyte activities, both of them are very important to immunity. In our experiments, QC significantly suppressed Con A- and LPS-stimulated splenocyte proliferation *in vitro* in a concentration-dependent manner (data not shown). This result might be significant, at least in part, for the inhibition of QC in cellular immune response.

CN activity by dephosphorylating a family of transcription factors known as NFATs is necessary for the synthesis of several cytokines. IL-2 is one of these cytokines and is considered to be an immune modulator (*23*). It is essential that IL-2 regulates T-cell proliferation and survival for correct homeostasis in the immune system (*24*). By inhibiting CN activity, CSA and FK506 prevent the nuclear translocation of NFAT secondary to its dephosphorylation, thereby suppressing T-cell activation (*25*). To clarify the effect of QC on the immune system, IL-2 production was measured in activated Jurkat cells, and the results indicated that QC inhibited PMA/Io-induced IL-2 gene expression in a dose-dependent manner. One the other hand, this study also authenticated the presumption that QC might partly affect immune responses by modulating T-cell responses.

QC is a common flavone in nature, present in various vegetables, fruits, seeds and beverages. The safety of QC, in terms of genotoxicity and mutagenicity, both *in vivo* and *in vitro*, has been evaluated in numerous short- and long-term studies in animals and humans. Current evidence indicates that QC, at its estimated intake levels, does not adversely affect health (*26*). QC has a wide range of biological activities. These include anti-oxidant, anti-bacterial and anti-viral actions, as well as anti-angiogenic and anti-allergic effects, and analgesic, hepatoprotective, cytostatic, apoptotic, estrogenic and anti-estrogenic properties (*27*, *28*). It also has potential anti-inflammatory activity as an inhibitor of the proinflammatory cytokine TNF-α (*29*). It reduces severe adjuvant arthritis (AA) signs in rats and is a potential anti-inflammatory therapeutic and preventive agent, targeting the inflammatory response of macrophages, which are activated by antigen-specific T-cell-derived lymphokines during the immune response (*30*). Combined with these pharmacological characteristics, our present studies suggest that QC is a novel CN inhibitor with immunosuppressive activity.

In conclusion, our work demonstrates that QC is able to inhibit CN activity *in vitro* and in Jurkat cells. Inhibition is non-competitive and the binding site of QC on CN may be located in the catalytic domain. A role for this compound in regulating the immune response is suggested by its effect on IL2 expression. All these data suggest that it is a potential immunosuppressant. Compared with other immunosuppressant agents used in patients, QC has many

advantages, including numerous biological, pharmacological effects and hypotoxicity.

Acknowledgements

We acknowledge Professor Jing Luo and Dr Li Tong for valuable suggestions.

Funding

National Natural Science Foundation of China; the National Important Basic Research Project and China International Science and Technology Cooperation Project.

Conflict of interest

None declared.

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