Elastase Release by Stimulated Neutrophils Inhibited by Flavonoids: Importance of the Catechol Group

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Pathogenesis of chronic inflammatory diseases is associated with excessive elastase release through neutrophil degranulation. In the present study, inhibition of human neutrophil degranulation by four flavonoids (myricetin, quercetin, kaempferol, galangin) was evaluated by using released elastase as a biomarker. Inhibitory potency was observed in the following order: quercetin > myricetin > kaempferol = galangin. Quercetin, the most potent inhibitor of elastase release also had a weak inhibitory effect on the enzyme catalytic activity. Furthermore, the observed effects were highly dependent on the presence of a catechol group at the flavonoid B-ring. The results of the present study suggest that quercetin may be a promising therapeutic agent in the treatment of neutrophil-dependent inflammatory diseases.

Key words: Neutrophil, Flavonoids, Elastase, Structure-Activity Relationship

Introduction

Neutrophils, cells from the innate immune system, play an important role in host defense against fungal and bacterial infections (McPhail *et al.*, 1992). Recognition of humoral stimuli by these cells triggers a set of effector functions, including phagocytosis and proteolytic enzyme releases. Neutrophil elastase, the major secreted product of activated neutrophils, is stored in the azurophilic granules and has been suggested to be the essential enzyme in the degradation of foreign proteins during the phagocytotic process. On the other hand, elastase might escape from cells and cause severe damage to macromolecules in cell membranes and the extracellular space, including proteoglycans, elastin and fibronectin, due to its unspecific activity (Cohen, 1994; Johansson *et al.*, 2002).

Cytokines, endotoxins, platelet aggregation factor (PAF), and formyl-methionyl-leucyl-phenylalanine (fMLP) can also stimulate elastase release by neutrophils, which is physiologically controlled by specific endogenous inhibitors such as α-1-antitrypsin. The imbalance between elastase and its inhibitors is implicated in many inflammatory diseases, like rheumatoid arthritis, respiratory distress syndrome, pulmonary emphysema, cystic fibrosis, and acute lung injury (Mohr and Wessinghage, 1978; McGuire *et al.*, 1982; Lee and Downey, 2001). In the last decades, recognition of elastase as a promising therapeutic target in chronic inflammatory diseases has considerably increased scientific interest in the discovery of new inhibitors of the enzymatic release (Mitsuhashi *et al.*, 1999; Yoshimura *et al.*, 2003).

Medicinal plants have long been used in folk medicine for alternative treatments of chronic inflammatory processes of diverse origins (Calixto *et al.*, 2003). The modulatory activity of secondary metabolites isolated from natural sources, especially flavonoids and sesquiterpene lactones, on neutrophil functions has been investigated (Kanashiro *et al.*, 2004, 2006; Taleb-Contini *et al.*, 2006). Inhibition of cyclooxygenases, lipoxygenases, phospholipase A_2, and xanthine oxidase, which are involved in the inflammatory process, has been assumed as the result of flavonoid immunomodulatory properties (revised by Middleton *et al.*, 2000). However, there are few studies investigating the flavonoid biological effects on neutrophil elastase release and structural features related to such effects remain controversial.

Thus, the aim of this study was to investigate the immunomodulatory effects of four flavonoids (galangin, kaempferol, quercetin, myricetin, Fig. 1) on elastase release by stimulated hu-

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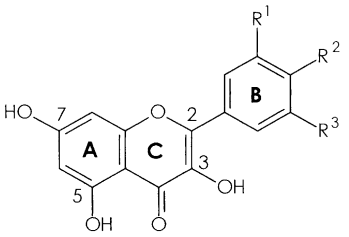

	R^1	R^2	R^3
Galangin	H	H	H
Kaempferol	H	OH	H
Quercetin	OH	OH	H
Myricetin	OH	OH	OH

Fig. 1. Chemical structures of the flavonoids tested.

man neutrophils. Inhibitory effects on the catalytic activity of the released enzyme were also investigated. In addition, the relationship between flavonoid chemical structures and biological activities was discussed, as well as the relevance of the flavonoid B-ring hydroxylation pattern.

Material and Methods

Chemicals

Formyl-methionyl-leucyl-phenylalanine (fMLP), cytochalasin B (CB), quercetin, myricetin, galangin and kaempferol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dimethyl-sulfoxide (DMSO) (Merck-Schuchardt, Hohenbrunn, Germany) and the elastase peptide substrate *N*-succinyl-Ala-Ala-Val-*p*-nitroanilide (SAAVNA) (Merck KGaA, Darmstadt, Germany) were the other chemicals used. All chemicals were dissolved in DMSO.

Isolation of human neutrophils

Blood from healthy human donors was drawn by venous puncture into Alsever solution (v/v) as anticoagulant. Neutrophils were isolated by the modified method of Lucisano and Mantovani (1984) (Kabeya *et al.*, 2002). Cell pellets suspended in Hank's balanced salt solution (HBSS) containing 0.1% gelatin (w/v) (HBSS-gel) were $> 90\%$ viable as determined by the Trypan Blue exclusion test and 80–90% were neutrophils. The local Research Ethics Committee (protocol HCRP 10097/2002) approved the experimental procedure.

Elastase release assay

Enzyme release was measured according to Johansson *et al.*, (2002) with some modifications. Briefly, neutrophils (2×10^5 cells/well) were incubated in 96-well microplates with CB (1 μmol/L) and flavonoid solution (0.5–10 μmol/L) or DMSO (positive control) at 37 °C for 10 min. The substrate SAAVNA (1 mmol/L) was added to the wells and degranulation triggered by adding fMLP (1 μmol/L). After incubation for 30 min at 37 °C the cleavage yellow product *p*-nitroaniline (*p*-NA) was quantified spectrophotometrically at 405 nm. Incubation of cells plus medium was considered as negative control, and DMSO at a final content of 0.1% (v/v) had no inhibitory effect on elastase release by the stimulated neutrophils.

Inhibition of catalytic activity

This assay was conducted according to Johansson *et al.* (2002) with some modifications. Briefly, neutrophils (2×10^5 cells) were incubated with CB (1 μmol/L) at 37 °C for 10 min. Degranulation was triggered by adding fMLP (1 μmol/L) and after incubation (37 °C for 30 min) the reaction tubes were centrifuged (1500 rpm, 37 °C, 10 min). The elastase-containing supernatant was mixed with DMSO (positive control) or quercetin, at the highest concentration used in the elastase release assay (10 μmol/L). The negative control was the supernatant of non-stimulated neutrophils. Substrate (SAAVNA, 1 mmol/L) was added to all samples and release of the yellow product, *p*-NA, quantified spectrophotometrically at 405 nm during 30 min.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) followed by the Dunnet's *post-hoc* test. Absorbance values were expressed as means ± standard error of the mean (SEM). The compared values were considered significantly different when $P < 0.05$. Data analysis was performed using the GraphPad Prism software (GraphPad Software, San Diego, CA, USA).

Results

Effect of flavonoids on elastase release

Catalytic activity of the elastase released by human neutrophils was evaluated by spectrophotometric measurement of the coloured end product

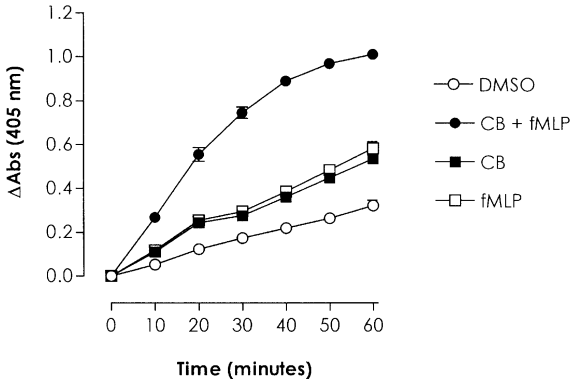

Fig. 2. Kinetics of elastase release from human neutrophils after addition of DMSO (vehicle) (open circle), cytochalasin B (CB, closed square), fMLP (open square), cytochalasin B plus fMLP (closed circle) and substrate (SAAVNA) to all samples. Product *p*-NA released into the reaction medium was measured spectrophotometrically at 405 nm. Data shown are means ± SEM ($n = 3$).

p-nitroaniline (*p*-NA). Fig. 2 shows the typical kinetics of *p*-NA formation by neutrophils treated with cytochalasin B (CB) and stimulated with the chemotactic peptide fMLP. Absorbance values increased linearly with incubation time up to 30 minutes; after this time, the rate of *p*-NA formation slowed down. In controls, containing the vehicle (DMSO), fMLP or CB alone, absorbance increase was slower.

Flavonoid effects on elastase release were evaluated at the final concentration range of 0.5 to 10 μmol/L. Quercetin showed a concentration-dependent inhibitory effect above 2 μmol/L, which was significantly different from the control (DMSO) (Fig. 3). Myricetin also inhibited elastase release at the highest concentration tested (10 μmol/L). Kaempferol and galangin, on the other hand, did not show a significant inhibitory effect under the assessed conditions. Flavonoids yellow colour did not interfere with the final absorbance of the reaction (data not shown).

Effect of quercetin on the catalytic activity

The inhibitory effect of the flavonoid quercetin on elastase catalytic activity was evaluated by adding 10 μmol/L of this compound (the highest concentration tested in the previous assay) to the supernatant of neutrophils treated with CB and further stimulated with fMLP. Under the experimental conditions used, quercetin showed a weak but significant inhibitory effect on the enzyme catalytic activity (Fig. 4).

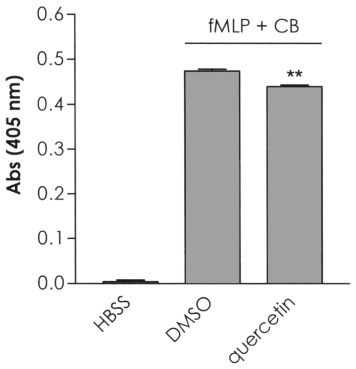

Fig. 4. Inhibition of elastase catalytic activity by quercetin at 10 μmol/L. Quercetin was added to the supernatant of fMLP-stimulated neutrophils, previously treated with cytochalasin B. The enzyme activity was measured by *p*-NA release from the cleaved substrate SAAVNA. Data are expressed as means ± SEM ($n = 3$). HBSS, Hank's balanced salt solution (supernatant from unstimulated cells). Significant difference is indicated by asterisks: ** $P < 0.01$ *vs.* DMSO (vehicle, control) (ANOVA and Dunnet's *post-hoc* test).

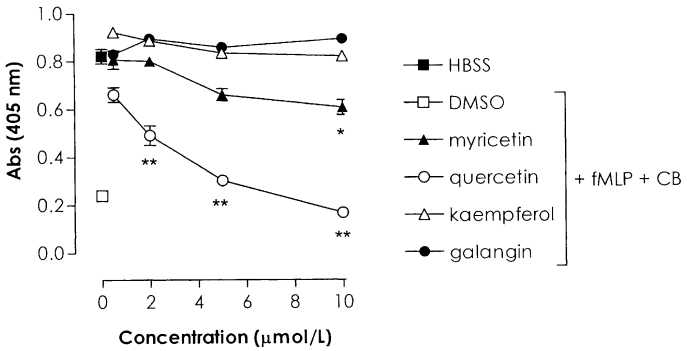

Fig. 3. Concentration-dependent inhibitory effects of the flavonoids myricetin, quercetin, kaempferol and galangin on elastase release by fMLP-stimulated human neutrophils. Elastase activity was detected by *p*-NA release from the substrate SAAVNA. Data are expressed as means ± SEM ($n = 3$). Significant differences are indicated by asterisks: * $P < 0.5$, ** $P < 0.01$ vs. DMSO (vehicle, control) (ANOVA and Dunnet's *post-hoc* test). HBSS, Hank's balanced salt solution (unstimulated cells).

Discussion

Recent *in vitro* studies have been performed to investigate the relationship between the flavonoid chemical structure and biological activity in neutrophil effector functions (Kanashiro *et al.*, 2004; Taleb-Contini *et al.*, 2006). These studies have revealed that the number and position of free hydroxy groups and the C2–C3 double bond at the C-ring are important structural features for the compounds modulatory activities on oxygen-dependent neutrophil functions as oxidative burst (Kanashiro *et al.*, 2004). However, there are few studies investigating the relationship between the biological activity and chemical structure of flavonoids in oxygen-independent neutrophil functions, such as degranulation and proteolytic enzyme release.

In the present study, the effect of four flavonoids on both the elastase release by neutrophils and the enzyme catalytic activity was evaluated by a colorimetric assay. This multitarget functional assay has been proposed as a suitable method for the fast screening of natural compounds (Johansson *et al.*, 2002). Our results showed that quercetin, bearing a catechol group at the B-ring, was the most effective inhibitor of elastase release. Introduction of an additional hydroxy group at the B-ring, yielding myricetin, significantly decreased its inhibitory potency. Both kaempferol and galangin, not bearing a catechol group, were not significantly active under the conditions assessed. Taken together, these results suggest that the catechol group is a specific structural requirement for the high inhibitory effect in neutrophil degranulation.

The results of the present study also demonstrated a weak, but significant, inhibitory effect of quercetin on elastase catalytic activity. This biological property has already been reported for compounds isolated from natural sources, including flavonoids (Meloni *et al.*, 1995) and sesquiterpene lactones (Siedle *et al.*, 2002). Meloni *et al.* (1995) demonstrated that the flavonoid 3′-hydroxyfarrerol may act as a reversible, non-competitive inhibitor of the neutrophil elastase activity. Interestingly, flavonoids chemical structure is similar to isocoumarins, which have also been described as serine proteinase inhibitors (Harper *et al.*, 1985).

In addition to the potential deleterious effect of released elastase, the excessive free radical generation by neutrophils through the oxidative burst is an important contributor to the pathology of inflammatory chronic diseases. Weiss (1989) reported that neutrophil-derived elastase and free radicals together can induce tissue damage by a synergic mechanism in which the free radicals may inactivate endogenous elastase inhibitors, thereby allowing elastase to attack tissues.

Thus, it is suggested that quercetin may be useful in the treatment of many inflammatory diseases in which both oxygen-dependent and independent neutrophil functions are involved in tissue damage.

In addition, the present work suggests that quercetin can be of therapeutic value in the treatment of excessive proteolysis during neutrophil-mediated inflammation. Furthermore, in order to correlate the present structure-activity study with flavonoid immunosuppressive effects, additional experiments in different cellular and enzymatic systems are in progress.

Acknowledgements

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