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Flavonols modulate the effector functions of healthy individuals' immune complex-stimulated neutrophils: A therapeutic perspective for rheumatoid arthritis

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ABSTRACT

Rheumatoid arthritis (RA) patients usually exhibit immune complex (IC) deposition and increased neutrophil activation in the joint. In this study, we assessed how four flavonols (galangin, kaempferol, quercetin, and myricetin) modulate the effector functions of healthy individuals' and active RA patients' IC-stimulated neutrophils. We measured superoxide anion and total reactive oxygen species production using lucigenin (CL-luc)- and luminol (CL-lum)-enhanced chemiluminescence assays, respectively. Galangin, kaempferol, and quercetin inhibited CL-lum to the same degree (mean $IC_{50} = 2.5$ μM). At 2.5 μM, quercetin and galangin suppressed nearly 65% CL-lum of active RA patients' neutrophils. Quercetin inhibited CL-luc the most effectively ($IC_{50} = 1.71 \pm 0.36$ μM). The four flavonols diminished myeloperoxidase activity, but they did not decrease NADPH oxidase activity, phagocytosis, microbial killing, or cell viability of neutrophils. The ability of the flavonols to scavenge hypochlorous acid and chloramines, but not H_2O_2, depended on the hydroxylation degree of the flavonol B-ring. Therefore, at physiologically relevant concentrations, the flavonols partially inhibited the oxidative metabolism of IC-stimulated neutrophils without affecting the other investigated effector functions. Using these compounds to modulate IC-mediated neutrophil activation is a promising safe therapeutic strategy to control inflammation in active RA patients.

1. Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects many organs and tissues, in particular the peripheral joints. The RA patients' synovial membranes and synovial fluid exhibit intense lymphocyte and granulocyte infiltration; neutrophils are the most abundant cell type. Immune complexes (IC), immunoglobulin aggregates, and the rheumatoid factor deposited on the joint surface activate neutrophils via Fc receptors and trigger some neutrophil effector functions — phagocytosis, degranulation, and oxidative metabolism. Activated neutrophils release large amounts of reactive oxygen species (ROS) and proteases, which can damage the tissue or upregulate the expression of enzymes that destroy the surrounding tissues [1,2]. ROS generation by neutrophils present in RA patients' synovial fluid and bloodstream increases as compared with resting neutrophils [2–4].

Recent literature has evidenced that neutrophils are key regulators of acute and chronic inflammatory responses. Neutrophils secrete signaling mediators like cytokines, chemokines, prostaglandins, leukotrienes, granule proteins, and ROS, to deliver instructions to almost all other immune cells. It is essential that neutrophils produce

Abbreviations: ABAH, 4-aminobenzoic acid hydrazide; ANOVA, analysis of variance; AUC, area under the curve; CL, chemiluminescence; CL-luc, lucigenin-enhanced chemiluminescence; CL-lum, luminol-enhanced chemiluminescence; DMF, N,N-dimethylformamide; DMSO, dimethyl sulfoxide; DPI, diphenyleneiodonium chloride; DPPH, 1,1-diphenyl-2-picrylhydrazyl radical; FITC, fluorescein isothiocyanate; HBSS, Hank's balanced saline solution; HBSS-gel, Hank's balanced saline solution supplemented with 0.1% gelatin; IC, immune complex; IC_{50}, concentration inhibiting a biological response by 50%; LDH, lactate dehydrogenase; MPO, myeloperoxidase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; PBS, phosphate buffered saline; PI-3-K, phosphoinositide 3-kinase; RA, rheumatoid arthritis; ROS, reactive oxygen species; Tau-Cl, taurine chloramine; TMB, 3,3′,5,5′-tetramethylbenzidine; TNF, tumor necrosis factor.

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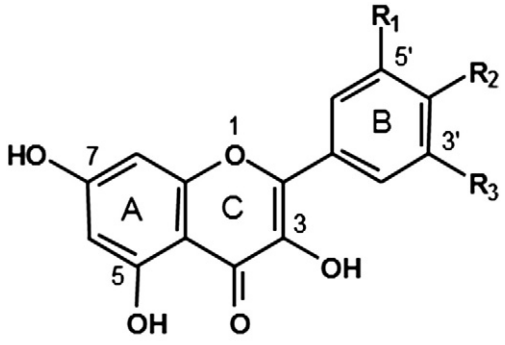
adequate amounts of ROS, to aid control of the autoimmune inflammation in patients with RA, asthma, and inflammatory bowel disease [1,5,6]. Therefore, modulating neutrophil oxidative metabolism is an interesting therapeutic strategy to treat inflammation.

To treat RA, current clinical practice relies on efficient therapeutic strategies based on the administration of glucocorticoids, non-steroidal anti-inflammatory drugs, and immunosuppressive agents. Most of these medications can directly or indirectly target neutrophil effector functions. However, the chronic use of these drugs may cause unexpected and serious side effects including stomach ulcers, hormonal imbalance, kidney and liver failure, immunodeficiency, and increased susceptibility to infection. In addition, some RA patients do not respond very well to these drug treatments [2,7]. In this context, it is important to search for new and more efficient compounds that can replace current medications or at least act as complementary agents, to reduce the effective drug dose that RA patients receive.

Natural plant products employed in human nutrition are a cheap and easily accessible source of therapeutic compounds; they also provide patients with other components that are important for their health, like fibers, minerals, and vitamins. Among natural compounds, flavonoids have emerged as promising candidates to develop potent and low-cost anti-inflammatory drugs [8]. Indeed, flavonoids are the most abundant phenolic compounds in the plant kingdom. They exhibit many biological activities both in vitro and in vivo; for example, they display antioxidant, anti-inflammatory, immunomodulatory, hepatoprotective, antiviral, and antiallergic actions [9,10].

Epidemiological data and in vivo studies in humans and different animal models have indicated that the anti-inflammatory and immunomodulatory properties of flavonoids significantly contribute to their protective effect against cardiovascular diseases and some types of cancer [8,9,11]. For instance, quercetin stabilizes the inflammatory atherosclerotic plaque by (i) affecting many steps of dendritic cell recruitment and activation induced by oxidized low-density lipoprotein and (ii) lowering asymmetric dimethylarginine levels [11]. Quercetin and other antioxidants reduce inflammation in the joints of arthritic mice, preventing joint destruction [1,12].

Screening of a set of flavonoids revealed that strong inhibition of IC-stimulated neutrophil oxidative metabolism requires the flavonol moiety (2,3-double bond in conjugation with a 4-oxo group and a 3-hydroxyl group) together with 5,7-dihydroxylation at the A-ring [13]. To exert their potent anti-inflammatory effect, flavonoids also demand these structural features [14]. On the basis of these results, we selected four flavonols for further studies: galangin, kaempferol, quercetin, and myricetin. These flavonols share the same A- and C-rings, but they differ in the degree of B-ring hydroxylation (Fig. 1). Flavonols are the most widespread subclass of dietary flavonoids. Kaempferol, myricetin, and quercetin are ubiquitous in foods; these phytonutrients exist in a wide range of fruits, vegetables, and beverages, such as onion, broccoli, apple, berries, red wine, and green tea [8,10]. Galangin occurs less frequently; it exists in high concentrations in *Alpinia officinarum*, honey, and propolis [15,16].



	R_1	R_2	R_3
Galangin	H	H	H
Kaempferol	H	OH	H
Quercetin	OH	OH	H
Myricetin	HO	OH	OH

Fig. 1. Chemical structures of the flavonols.

Our research team has investigated the antioxidant and immunomodulatory properties of kaempferol, myricetin, quercetin, and galangin. We have sought to unravel their mechanisms of action and develop appropriate liposomal carriers to improve their therapeutic effects [17–20]. These flavonols inhibit horseradish peroxidase activity [18,19], modulate IC-stimulated neutrophil oxidative metabolism in rabbits [18], and inhibit human neutrophil degranulation [17].

To continue investigating the immunomodulatory potential of the aforementioned flavonols, in this study we examined how they modulate the different steps through which IC-stimulated human neutrophils generate ROS. More specifically, we evaluated how kaempferol, myricetin, quercetin, and galangin (1) inhibit neutrophil NADPH oxidase and myeloperoxidase (MPO) activities, (2) scavenge different oxidant species, (3) affect neutrophil viability, and (4) influence neutrophil ability to phagocyte and kill microbes. We also assessed whether these flavonols modulate the neutrophil oxidative metabolism in anti-tumor necrosis factor (TNF)-α drug-treated patients with active RA who do not respond very effectively to this drug therapy.

2. Material and methods

2.1. Chemicals

Galangin (3,5,7-trihydroxyflavone), kaempferol (3,4′,5,7-tetrahydroxyflavone), quercetin (3,3′,4′,5,7-pentahydroxyflavone dihydrate), myricetin (3,3′,4′,5,5′,7-hexahydroxyflavone), camptothecin, cytochalasin B, cytochalasin D, diphenyleneiodonium chloride (DPI), 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), fluorescein isothiocyanate (FITC), luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), lucigenin (bis-*N*-methylacridinium nitrate), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), *N*,*N*-dimethylformamide (DMF), ovalbumin, propidium iodide, 3,3′,5,5′-tetramethylbenzidine (TMB), taurine, Triton X-100, and Trypan Blue were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydrogen peroxide (H_2O_2), 4-aminobenzoic acid hydrazide (ABAH), and human leukocyte myeloperoxidase (MPO, EC 1.7.1.11) were obtained from Calbiochem (Merck KGaA, Darmstadt, Germany). We acquired the following products from different suppliers: dimethyl sulfoxide (DMSO; Mallinckrodt Baker, Paris, KY, USA), Difco™ gelatin (microbiological grade; BD Biosciences, San Diego, CA, USA), ethanol (Merck KGaA, Darmstadt, Germany), RPMI 1640 medium (Cultilab, Campinas, SP, Brazil), Sabouraud's dextrose agar (Acumedia, Michigan, USA), LDH Liquiform ™ kit (Labtest Diagnostica, Lagoa Santa, MG, Brazil), and APOPTEST™-FITC apoptosis detection kit (Dako, Glostrup, Denmark).

The chemicals and solvents used in this work were of analytical grade and purchased from commercial sources. The aqueous solutions were prepared with water previously purified in a Milli-Q water system (Merck-Millipore, Merck KGaA, Darmstadt, Germany). Sterile and lipopolysaccharide-free solutions were used in all the biological assays involving neutrophils.

2.2. Patients and healthy subjects

The study included twenty-six healthy volunteers, who met the criteria described by Paula et al. [21], and twelve patients with RA that has remained active despite anti-TNF-α drug (infliximab) therapy.