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SHORT COMMUNICATIONS

Flavonoid inhibition of the human neutrophil NADPH-oxidase*

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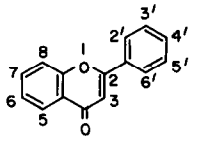
The respiratory burst of the human neutrophil results in a marked increase of enzymatic oxygen consumption subsequent to phagocytosis of particulate stimuli or by activation with soluble agonists [1]. The respiratory oxidase reduces molecular oxygen to O_2 †, and it has been characterized as a flavoprotein which preferentially utilizes NADPH as an electron donor [2]. Inhibitor studies have demonstrated a broad susceptibility of the enzyme to sulfhydryl reactive agents and disulfonic stilbene derivatives [3, 4]. The opportunity to further characterize the respiratory burst NADPH-oxidase was suggested by the recent demonstration of flavonoid inhibition of several enzyme activities of the neutrophil [5–7].

Quercetin, penta-hydroxy flavonol, is one of the most common flavonoids (flavonols being those with a hydroxy substitution in the 3-position) (Fig. 1). The flavonols are reported to possess a wide range of biological activities including inhibition of various enzymes, mutagenecity toward bacterial testing strains, and plant growth regulation [8]. Recent reports that quercetin inhibits oxygen consumption in human and guinea pig neutrophils stimulated by either soluble agonists or phagocytosable particles [5–7] prompted further investigation of the mechanisms of action of this inhibitor. In these studies, we present evidence that the flavonoids demonstrate a high degree of structural specificity in their ability to impair the normal stimulation of the respiratory burst, and that the activity of the NADPH-oxidase as studied in a broken cell preparation is directly inhibited by these agents.



Substituents	Compound
3, 7, 3′, 4′-OH	fisetin
3, 5, 7, 4′-OH	kaempferol
3, 5, 7, 2′, 4′-OH	morin
3, 5, 7, 3′, 4′-OH	quercetin

Fig. 1. Flavonoid structures.

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† Abbreviations: O_2^-, superoxide; NADPH, nicotinamide adenine dinucleotide phosphate; SOD, superoxide dismutase; HPLC, high pressure liquid chromatography; DMSO, dimethyl sulfoxide; PMA, phorbol 12-myristate, 13-acetate; IC_{50}, concentration corresponding to 50% inhibition; and P, partition coefficient.

Materials and methods

Quercetin, kaempferol, morin, nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), zymosan, cytochrome *c* (Type VI), lactate dehydrogenase kit, superoxide dismutase (SOD) and phorbol 12-myristate, 13-acetate (PMA) were purchased from the Sigma Chemical Co. (St. Louis, MO), and fisetin was purchased from the Aldrich Chemical Co. (Milwaukee, WI). The final purity of the flavonols was determined by HPLC. The HPLC system consisted of a reverse-phase column (μBondapak C18 from Waters Associates, Inc.) and a linear methanol/water (1% acetic acid) gradient from 45% methanol to 100% over 20 min. The samples were injected at 50% methanol.

The stock solutions of flavonols were made up in dimethyl sulfoxide (DMSO), and the final concentration of DMSO in any experiment did not exceed 1% (v/v). The partition coefficient of the flavonols was determined by the method reported by Hansch and colleagues [9] except that the aqueous portion consisted of the phosphate-buffered saline buffer used in the experiments with the cells. The two-phase system was mixed by vortex for 1 min and allowed to separate, and then the absorbance at 370 nm of the aqueous phase was measured.

Determination of neutrophil oxygen consumption and NADPH-oxidase activity. Neutrophils of phlebotomized venous blood from normal human donors were obtained as previously described, by Dextran sedimentation, hypotonic lysis and centrifugation in Ficoll-paque [3]. Stock solutions of PMA (1 mg/ml in DMSO) were stored at −70° and were freshly diluted in buffer. Zymosan was opsonized in autologous serum as previously described [3]. Oxygen consumption was measured using a model 53 biological oxygen monitor (Yellow Springs Instrument Co., Yellow Springs, OH) calibrated using the phenylhydrazine oxidation method, as previously described [2]. NADPH-oxidase activity was assessed in the 27,000 *g* particulate preparation from neutrophils stimulated with opsonized zymosan by the measurement of NADPH oxidation as previously described [2].

Results

Flavonoid inhibition of oxygen consumption. Net oxygen consumption was measured as the parameter of respiratory burst activity since the flavonoids non-enzymatically reduce cytochrome *c*, the standard substrate for the neutrophil O_2^- assay. The flavonoids demonstrated a high degree of structural specificity with respect to the inhibition of O_2 consumption. While morin had no significant effect on oxygen consumption of neutrophils phagocytosing opsonized zymosan, quercetin, fisetin and kaempferol exhibited a 50% inhibitory concentration (IC_{50}) on net oxygen consumption between 100 and 120 μM (Table 1). Cytotoxicity, as assessed by LDH release, was not increased significantly at these flavonol concentrations (data not shown). Although zymosan stimulation is modulated by extracellular calcium (Ca^{2+}), the inhibition by flavonoids was not due to their calcium binding capacity, since their

Table 1. Flavonoid inhibition of zymosan-stimulated neutrophil oxygen consumption

Flavonoid	IC_{50}* (μM)
Quercetin	103.6 ± 12.1 (5)
Morin	NI† (3)
Fisetin	106.0 ± 20.1 (3)
Kaempferol	120.0 ± 17.6 (2)

* The IC_{50} of the flavonoids on oxygen consumption was determined for each of several donor cells and expressed as the mean ± S.E.M. (numbers in parentheses equal the number of experiments). The control value for the series of six donors was 69.3 ± 20.6 nmoles oxygen consumed per 10^7 cells per min.

† No inhibition.

Table 2. Flavonoid inhibition of neutrophil NADPH-oxidase activity

Flavonoid	IC_{50}* (μM)
Quercetin	39.6 ± 8.7 (6)
Morin	31.5 ± 5.8 (6)
Fisetin	117.0 ± 9.3 (3)
Kaempferol	92.0 ± 18.3 (3)

* The IC_{50} of the flavonoids on NADPH-oxidase activity was determined for each of several 27,000 *g* particulate neutrophil preparations and expressed as the mean ± S.E.M. (numbers in parentheses equal the number of experiments). The control value was 4.13 ± 0.25 nmoles O_2^- per 10^7 cell equivalents per min (mean ± S.E.M.), N = 6, measured at 1 min, 21°.

effects were constant in a 10-fold molar excess of Ca^{2+} relative to the flavonoid concentration (data not shown).

The possibility that the inhibition by these compounds was, in part, dependent on the stimulus employed was suggested previously by the variable degree of inhibition seen with quercetin in guinea pig granulocytes when concanavalin A, myristic acid or the antibiotic Br-X537A was used as an agonist [6]. We compared zymosan and PMA, agents which give comparable activation of the respiratory burst [3]. Quercetin at 132 μM depressed net oxygen consumption to 42.7 ± 14.4% of control (N = 6), while PMA stimulation was inhibited 60.8 ± 22.8% (N = 4). The inhibition of quercetin was not significantly different for the two agonists which stimulate the respiratory burst via different receptors [3, 4]. This finding prompted investigation of the effects of these flavonoids on the respiratory burst enzymatic activity directly.

Flavonoid inhibition of NADPH-oxidase. We have demonstrated previously that the particulate preparation of deoxycholate-treated NADPH-oxidase, harvested from either zymosan- or PMA-stimulated cells, has similar biochemical and physical properties regardless of the stimulatory agonist and that the assay of NADPH oxidation reflects this O_2^--generating activity [2]. The effects of various flavonoids on the NADPH-oxidase were characterized in this system (Table 2). Quercetin inhibited this activity in a concentration-dependent manner with an IC_{50} of 39.6 ± 8.7 μM (N = 6). Morin, differing from quercetin by a meta-OH rather than ortho-OH in the B ring (Fig. 1), did not inhibit oxygen consumption significantly in whole cells but suppressed NADPH oxidation identically to that seen with quercetin (IC_{50} = 31.5 ± 5.8 μM, N = 6). Fisetin, lacking a —OH at C_5, and kaempferol, lacking a —OH at C_2, exhibited comparable inhibition of oxygen consumption in whole cells (Table 1) but were only one-third as potent as quercetin and morin in inhibition of NADPH-oxidase activity (IC_{50} = 90–120 μM).

Partition coefficient of quercetin and morin. The partitioning of quercetin, fisetin, kaempferol and morin between a phosphate-buffered saline solution and *n*-octanol was determined to assess the comparative hydrophobicity of the flavonoids. The partition coefficient (P) of the flavonoids in a hydrophobic solvent paralleled their ability to inhibit oxygen consumption of the stimulated neutrophil. Whereas morin was poorly partitioned in the hydrophobic phase, quercetin, fisetin and kaempferol were highly soluble. With P = flavonol (octanol)/flavonol (phosphate buffered saline), the relative octanol solubilities of morin, fisetin, kaempferol and quercetin were 1.0, 5.6, 5.6 and 15.2 respectively; log P values were: morin, 3.86; fisetin and kaempferol, 4.6; and quercetin, 5.03. Despite the structural similiarity between morin and quercetin, these compounds exhibit profound differences in hydrophobic solubility which correlated with their ability to inhibit oxygen consumption in the intact neutrophil. The results suggest that the hydrophobic solubility of these compounds may reflect their ability to permeate the cell and inhibit the respiratory burst enzymatic system.

Discussion

The flavonol quercetin has been reported to inhibit a wide variety of both membrane-bound and soluble enzymes [8, 10–12]. Recently, Long *et al.* [7] reported that quercetin had inhibitory effects on the human neutrophil membrane-bound Mg^{2+}-ATPase, the respiratory burst, and deoxyhexose transport. That the respiratory burst inhibition was mediated by effects at a post-receptor level was suggested by Romeo and colleagues [5] who reported that quercetin (but not morin and rutin) inhibits concanavalin A-stimulated oxygen uptake, without loss of lectin binding. These previous investigations suggested that there might be structural specificity in the inhibition of enzyme activity. The nature of this specificity has been demonstrated by investigating the effects of structurally related flavonols on the respiratory burst of human neutrophils and on the activity of the predominant enzyme accounting for this burst, the NADPH-oxidase [3].

Our studies have differentiated the structural requirements of inhibition of the respiratory burst in whole cell preparations (employing both soluble and particulate stimuli) and on the NADPH-oxidase. Highly specific inhibitory activity is found with hydroxylated flavonoids, but the disposition of hydroxyl groups is critical: quercetin inhibits oxygen consumption in whole cells, whereas morin does not. In a broken cell assay, however, both compounds were equipotent in their ability to inhibit the NADPH-oxidase. This finding may reflect differing capacities of quercetin and morin to permeate the cell. This hypothesis is supported by experiments measuring solubility characteristics of quercetin and morin in which the partition coefficients in octanol and phosphate-buffered saline were more than 15-fold different. The marked differences in preferential solubility in octanol of these compounds supports the hypothesis that the degree of hydrophobicity determines permeation of the cell membrane to effect inhibition of the respiratory oxidase. The lack of effect by morin in whole cells may simply reflect its exclusion from the NADPH-oxidase active site. This presumed membrane permeability difference of quercetin and morin is then not critical in the broken cell preparation since their inhibitory profile of NADPH-oxidase is the same. Similar findings were reported for impermeant sulfhydryl reactive agents and stilbene disulfonic acids [3, 4, 13].

While we have not as yet identified the chemical interac-

tion of flavonoids with the NADPH-oxidase, these studies demonstrate that inhibition of the human neutrophil respiratory burst is highly specific in regard to flavonoid structure, and this effect may be related to differing permeability characteristics. These compounds may be suitable agents for further study of the sequence of activation events to enzyme expression in this system. More broadly, the anti-inflammatory activity of the flavonoids has been observed in a variety of model systems [14] and recently extended to inhibitory activity against effector cells of the inflammatory response [5–7, 12, 15]. The structural basis for this anti-inflammatory activity has potential therapeutic importance.

In summary, the generation of the respiratory burst of the human neutrophil is dependent on intact receptor–ligand activation pathways and a flavin-dependent NADPH-oxidase. Flavonoids, a class of phenolic plant pigments, exhibit a high degree of structural specificity (differing in position or number of hydroxyl constituents) in their inhibitory effects on this system. The flavonoids quercetin, fisetin and kaempferol exhibited comparable IC_{50} values for inhibition of oxygen consumption (*ca.* 100 μM) while the structurally similar flavonol, morin, had no significant effect on whole cell oxygen metabolism, but exhibited an identical inhibitory profile to that of quercetin on the NADPH-oxidase. The ability of a particular flavonol to inhibit the respiratory burst in intact cells paralleled its hydrophobicity as measured by partition in octanol and saline. These studies demonstrate highly specific structural requirements of flavonoid inhibitory activity of the human neutrophil respiratory burst and suggest that permeability characteristics of the flavonols might determine their inhibitory activity in intact cells.

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REFERENCES

1. J. A. Badwey and M. L. Karnovsky, *A. Rev. Biochem.* **49**, 695 (1980).
2. D. R. Light, C. Walsh, A. M. O'Callaghan, E. J. Goetzl and A. I. Tauber, *Biochemistry* **20**, 1468 (1981).
3. A. I. Tauber and E. J. Goetzl, *Biochemistry* **18**, 5576 (1979).
4. A. I. Tauber and E. J. Goetzl, *J. Immun.* **126**, 1786 (1981).
5. C. Schneider, G. Berton, S. Spisani, S. Trantello and D. Romeo, *Adv. exp. Biol. Med.* **221-A**, 371 (1978).
6. G. Berton, C. Schneider and D. Romeo, *Biochim. biophys. Acta* **595**, 47 (1980).
7. G. D. Long, L. R. DeChatelet, J. T. O'Flaherty, C. E. McCall, D. A. Bass, P. S. Shirley and J. W. Parce, *Blood* **57**, 561 (1981).
8. J. W. McClure, in *The Flavonoids* (Part 2) (Eds. J. B. Harborne, T. J. Mabry and H. Mabry), p. 970. Academic Press, New York (1975).
9. T. Fujita, J. Iwasa and C. Hansch, *J. Am. chem. Soc.* **86**, 5175 (1964).
10. C. M. S. Fewtrell and B. D. Gomperts, *Nature, Lond.* **265**, 635 (1977).
11. E. M. Suolinna, R. Buchsbaum and E. Racker, *Cancer Res.* **35**, 1865 (1975).
12. E. M. Suolinna, D. R. Lang and E. Racker, *J. natn. Cancer Inst.* **53**, 1515 (1974).
13. A. I. Tauber, D. B. Brettler, E. A. Kennington and P. M. Blumberg, *Blood* **60**, 333 (1982).
14. M. Gabor, *The Anti-inflammatory Actions of Flavonoids.* Akademia Kiado, Budapest (1972).
15. E. Middleton, G. Drzewiecki and D. Krishnarao, *J. Immun.* **127**, 546 (1981).

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Depressive action of γ-aminobutyraldehyde as a precursor of γ-aminobutyric acid

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The inhibitory role of γ-aminobutyric acid (GABA) in central nervous system mechanisms is well known. Recent evidence indicates a biochemical relation between the functioning GABA system and the symptoms of Huntington's disease [1], Parkinsonism [2], and epilepsy [3]. Since GABA itself can scarcely penetrate the blood–brain barrier [4], agents have been sought which act on the GABA system and thus have the potential for altering the course of these neurological disorders [5, 6]. A major difficulty in the design of therapeutic agents acting through the GABA system is the poor brain-penetrating properties of active compounds.

A recent study in this laboratory has shown the presence in mammalian brain of an enzyme that can catalyze oxidation of 4-aminobutyraldehyde (ABAL) to GABA [7]. Subsequently, it has been reported that peripherally administered ABAL can easily penetrate the blood–brain barrier into the brain and is rapidly oxidized there to GABA [8, 9].

On the other hand, the problem of the relationship between the increase in brain GABA level and the changes in behavior of animals is still open to dispute, both as a problem of therapy and as a basic biochemical question on the role of GABA. This paper describes the depressive