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INHIBITION BY QUERCETIN OF ACTIVATION OF POLYMORPHONUCLEAR LEUCOCYTE FUNCTIONS

STIMULUS-SPECIFIC EFFECTS

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Summary

The effect of some bioflavonoids on the activation of polymorphonuclear leucocyte respiration and exocytosis was examined. At 10^{-5}—10^{-4} M concentration, quercetin, but not morin and rutin, was found to inhibit the concanavalin A-induced enhancement of oxygen consumption markedly, without impairing leucocyte viability and concanavalin A binding. The inhibition could be reversed by either washing the leucocytes or adding a 10-fold molar excess of 1-anilino-8-naphthalene sulphonate. Concanavalin A-dependent cell secretion of lysozyme was also totally inhibited by 30 μM quercetin.

The effect of quercetin on the activation of leucocyte respiration appeared to be stimulus specific. In fact, at a concentration of the flavonoid (75 μM) which provided a 95% inhibition of the concanavalin A-induced stimulation, the respiratory activation produced by phospholipase C was inhibited by about 50% and that caused by myristic acid and by the antibiotic Br-X537A by less than 25%.

These data suggest that quercetin exerts its activity at specific sites of the plasma membrane of the leucocytes, and that this compound might be used to identify the membrane domain whereon different stimuli act to originate the initial stimulatory signal.

Introduction

The interaction of surface-reactive stimuli with the plasma membrane of polymorphonuclear leucocytes may induce the activation of such cell functions

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as random and oriented movement [1], secretion [2] and respiration [3].

The basic event of this type of stimulus-activation coupling is the perturbation of the molecular organization of specific membrane domains, followed by the generation of a 'signal' eventually leading to the activation of intracellular systems. These aspects of cell stimulation are the object of an extensive investigation since several years. Some knowledge has been gained on the possible nature of the activating 'signal'. For example, an elevation of cytosol free calcium concentration appears to trigger the enhancement of polymorphonuclear leukocyte directional locomotion [4,5], oxidative metabolism [6,7] and extracellular secretion of granule enzymes [8,9]. More difficult is the definition of the nature of the membrane perturbation necessary to generate the signal. In fact, cell stimulation can be obtained by exposing the leukocytes to such diverse agents as immune reactants [10—14], lectins [15], surfactants [16], fatty acids [17], endotoxin [18] and others.

A rational approach would be that of trying to identify the specific membrane domain wherefrom the initial activating signal is triggered by a certain stimulus. To this purpose it would be helpful to find substances that could interact with different membrane domains, thereby causing specific effects in relation to the type of stimulus applied. Such an approach has provided interesting clues to the elucidation of the role of other subcellular components in the regulation of plasma membrane-linked functions of polymorphonuclear leukocytes. In particular, the use of drugs preventing the assembly of microtubules or microfilaments, such as colchicine or cytochalasin B, has permitted to better understand the role of the cytoskeleton in the control of the activation of the leukocute locomotion [4,19,20], phagocytosis [20—22], secretion [23] and respiration [24].

With this concept in mind, we have investigated the effects of quercetin on the activation of some polymorphonuclear leukocyte functions induced by various plasma membrane-perturbing agents. The selection of this bioflavonoid was made on the basis of its known capacity of inhibiting membrane mediated processes, such as histamine release from mast cells [25,26], as well as the activity of membrane enzymes, such as the ATPases associated with ion transport across the plasma membrane [27,28], mitochondria [27,29—31], sarcoplasmic reticulum [25,27] and chloroplasts [32,33].

Materials and Methods

Cells. Polymorphonuclear leukocytes were isolated from the peritoneal cavity of albino guinea pigs or from the venous blood of healthy human volunteers as previously described [34] and suspended in calcium-free Krebs-Ringer phosphate buffer [34].

Oxygen uptake. This was monitored by a Clark-type oxygen electrode attached to a thermostatically controlled (37°C) plastic vessel, in which 2×10^7 leucocytes/2 ml of Krebs-Ringer phosphate buffer + 0.2 mM glucose were magnetically stirred [34].

Exocytosis. Human polymorphonuclear leukocyte suspensions (1×10^7 cells/ml) were incubated at 37°C in disposable test tubes with the appropriate reagents. After 30 min of incubation, the test tubes were centrifuged for 5 min

at 4°C and 400 × *g*. Enzyme assays were performed on the supernatant and on the cell pellet, which was suspended to the original volume and dispersed by sonication (3 mA; 10 s; Branson Sonifier), in the presence of 0.05% (v/v Triton X-100. The activity of lysozyme was determined nephelometrically by the rate of lysis of an acetone powder of the cell wall of *Micrococcus lysodeicticus* [35] and that of lactate dehydrogenase by following the oxidation of NADH at 340 nm [35].

[^{3}H]Concanavalin A binding. 5×10^6 guinea pig polymorphonuclear leukocytes in 1 ml of Krebs-Ringer phosphate buffer were incubated at 37°C with 0.25 μCi [^{3}H]concanavalin A (New England Nuclear) and 40 μg unlabeled lectin (Sigma). After 3 min of incubation, the suspension was diluted with 10 ml of ice-cold Krebs-Ringer phosphate buffer and centrifuged for 5 min at 4°C and 400 × *g*. The cell pellet was washed twice with cold Krebs-Ringer phosphate buffer and then solubilized in 10 ml of Aqualuma (Supelchem). Cell-bound radioactivity was measured by liquid scintillation spectrometry. Specific binding was determined by the difference between total binding of concanavalin A and binding obtained in the presence of 100 mM α-methyl mannopyranoside.

Glycogen aggregation. The capability of concanavalin A to aggregate glycogen was determined by a slight modification of the method of Duke et al. [36]. Briefly, the optical density at 450 nm of 0.8 mg of glycogen in 2.7 ml of Krebs-Ringer phosphate buffer was read 10 min after the addition of 0.3 ml of 1.1 mg concanavalin A/ml of buffer.

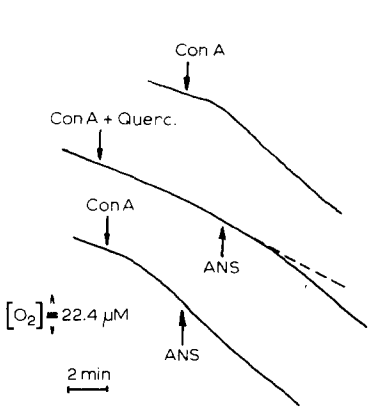
Ecto-ATPase. The (Na^+, K^+, Mg^{2+})-ATPase activity of intact guinea pig leukocytes [37] was assayed by measuring the inorganic phosphate liberated from sodium ATP (0.5 mM) by the method of Penney [38].

Reagents. Concanavalin A and phospholipase C (*Clostridium welchii*, Sigma) were dissolved in Krebs-Ringer phosphate buffer. The antibiotic Br-X537A (a gift of Dr. J. Berger, Hoffman-La Roche), myristic acid (Sigma), 1-anilino-8-naphtalene sulphonate (magnesium salt, recrystallized twice from a hot water solution of the Eastman product), and the flavonoids (Sigma) 3,3′,4′,5,7-hydroxy flavone (quercetin), 3-rhamnose-glucose, 3′,4′,5,7-hydroxy flavone (rutin), 2′,3,4′,5,7-hydroxy flavone (morin) were dissolved in dimethyl sulphoxide (Me_2SO).

Results

Quercetin, in the 10^{-5}—10^{-4} M concentration range, is a potent inhibitor of the activation of polymorphonuclear leukocyte oxygen consumption induced by concanavalin A (see Figs. 1 and 2). An identical pattern of inhibition by the flavonoid is found whether quercetin is added simultaneously with the lectin or after the stimulation has reached the maximal value [15].

Another function of polymorphonuclear leukocytes affected by quercetin is secretion of granule enzymes induced by concanavalin A. Table I shows that 30 μM quercetin reduces the release of lysozyme to control levels (the release of the cytoplasmic marker lactate dehydrogenase is not significantly different from controls in the presence of either concanavalin A or quercetin or both compounds).



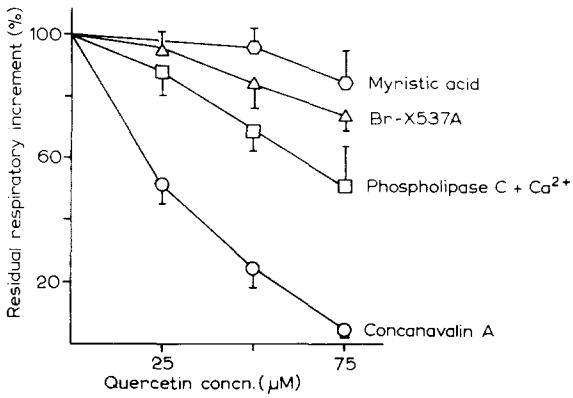


Fig. 1. Inhibition by quercetin of concanavalin A-induced stimulation of guinea pig polymorphonuclear leukocyte respiration and recovery of maximal respiration upon addition of 1-anilino-8-naphtalene sulphonate (ANS). The respiration of 2×10^7 guinea pig polymorphonuclear leukocytes/2 ml of Krebs-Ringer phosphate buffer + 0.2 mM glucose was monitored at 37° C by a Clark-type oxygen electrode. The curves shown are polarographic traces. Concanavalin A (Con A) = 80 μg/ml; quercetin = 50 μM; ANS = 0.5 mM. Interrupted line: no addition of ANS.

Fig. 2. Dose-response curves for inhibition by quercetin of stimulation of guinea pig polymorphonuclear leukocyte respiration induced by concanavalin A (80 μg/ml), phospholipase C (1 enzyme unit) + 0.5 mM Ca^{2+}, Br-X537A (20 μM) and myristic acid (50 μM). In the absence of quercetin but in the presence of the solvent Me_2SO (0.15%), these stimulants caused the following increments of respiratory rates (natoms O/min per 2×10^7 guinea pig polymorphonuclear leukocytes): 40.4 ± 6.2 (9); 55.0 ± 8.1 (3); 55.0 ± 5.9 (4); 45.7 ± 1.4 (5) (numbers of experiments indicated in brackets). The rate of respiration of resting cells was below 15 natoms O/min per 2×10^7 polymorphonuclear leukocytes. Quercetin or Me_2SO were added simultaneously with the stimulants. Bars show S.E.

Among the flavonoids tested, only quercetin exerts an inhibitory effect, whereas other related compounds such as morin and rutin have no effect on either the consumption of oxygen or exocytosis of stimulated leukocytes (data not shown).

Two types of controls exclude the possibility that quercetin impairs cell viability or irreversibly affects the capacity of the leukocytes to respond to

TABLE I

EFFECT OF QUERCETIN ON CONCANAVALIN A-INDUCED LYSOZYME SECRETION

Human blood polymorphonuclear leukocytes (10^7) were incubated at 37° C for 30 min in 1 ml of Krebs-Ringer phosphate buffer, with 0.2 mM glucose and the indicated reagents. At the end of the incubation, the cell suspensions were centrifuged and the cell-free supernatants were assayed for lysozyme activity. Data are percent of total cell-associated enzyme activity (48.7 ± 3.3 μg/10^7 cells) and are means of three determinations ± S.E. The difference between the means was analyzed by paired '*t*' statistics (n.s. = not significant).

Addition to polymorphonuclear leukocytes	Lysozyme release (%)	
Me_2SO (0.2%)	6.0 ± 0.3	$P < 0.025$
Me_2SO + concanavalin A (80 μg/ml)	12.7 ± 0.8	
Quercetin (30 μM)	5.0 ± 0.4	n.s.
Quercetin (30 μM) + concanavalin A	5.7 ± 0.3	
Quercetin (10 μM) + concanavalin A	7.7 ± 0.8	

stimuli: (i) in the presence of 100 μM quercetin the number of cells that take up the dye trypan blue (<5%) does not increase; (ii) if quercetin-treated cells are centrifuged and resuspended in fresh medium, they fully regain their sensitivity to concanavalin A stimulation. The reversibility of the quercetin action was also tested with 1-anilino-8-naphtalene sulphonate, which is known to compete with quercetin binding to chloroplast coupling factor 1 [33]. Fig. 1 shows that 1-anilino-8-naphtalene sulphonate can fully reverse the quercetin inhibitory action if used in a 10-fold molar excess. Under these conditions 1-anilino-8-naphtalene sulphonate does not modify the leukocyte respiration either at rest or stimulated by concanavalin A in the absence of quercetin.

The possibility that quercetin acts as a competitive inhibitor of concanavalin A binding to its saccharide receptors was ruled out by evaluating the [^{3}H]-concanavalin A binding to polymorphonuclear leukocytes and the lectin capability to aggregate glycogen, in the presence and in the absence of the flavonoid. As reported in Table II, both processes are not modified by 100 μM quercetin.

The study of the quercetin effects on polymorphonuclear leukocyte stimulation was subsequently extended to other stimuli known to interact with different membrane domains. Fig. 2 is a dose-response curve of quercetin action on the enhancement of the leukocytes' oxygen consumption caused by various agents (the activity of the flavonoid on the concanavalin A-induced respiratory stimulation is shown by comparison). While 75 μM quercetin inhibits the stimulatory effect of concanavalin A and phospholipase C by 95% and 50%, respectively, at the same concentration the flavonoid decreases the metabolic

TABLE II

EFFECT OF QUERCETIN ON [^{3}H]CONCANAVALIN A BINDING TO POLYMORPHONUCLEAR LEUKOCYTES AND ON CONCANAVALIN A-INDUCED GLYCOGEN AGGREGATION

(A) 5×10^6 guinea pig polymorphonuclear leukocytes were incubated at 37°C with 0.25 μCi [^{3}H]concanavalin A and 40 μg of unlabeled lectin in 1 ml of Krebs-Ringer phosphate buffer, in the absence or in the presence of quercetin (50 μM). After 3 min of incubation, the suspension was diluted with ice-cold Krebs-Ringer phosphate buffer and centrifuged. The cell pellet was washed twice with the buffer and solubilized in 10 ml of Aqualuma. Radioactivity was measured by liquid scintillation spectrometry. Data are means of two experiments. (B) The aggregation of glycogen was measured by a turbidimetric assay [37]. 0.3 ml of concanavalin A (1.1 mg/ml in 0.154 M NaCl) was added to 2.7 ml of Krebs-Ringer phosphate buffer, containing 0.8 mg of glycogen, in the absence or in the presence of quercetin. After 10 min at 37°C the absorbance was recorded; at this point α-methyl-mannopyranoside (α-MM) was added at the final concentration of 1 mM and 5 min thereafter the absorbance was again recorded. Data are means of two experiments.

(A) μg [^{3}H]concanvavalin A bound/5×10^6 leucocytes	
— Quercetin	0.92
+ Quercetin	1.14
(B) Absorbance (450 nm)	
Glycogen + concanavalin A	0.371
Glycogen + concanavalin A → α-MM	0.015
Glycogen + Quercetin + concanavalin A	0.460
Glycogen + Quercetin + concanavalin A → α-MM	0.050
Glycogen + Quercetin	0.035

TABLE III

EFFECT OF MYRISTATE AND Br-X537A ON THE INHIBITION BY QUERCETIN OF ECTO-(Na^+, K^+, Mg^{2+}) ATPase OF POLYMORPHONUCLEAR LEUKOCYTES

2×10^6 guinea pig polymorphonuclear leukocytes in 132 mM NaCl, 14 mM KCl, 1 mM $MgCl_2$ and 30 mM Hepes * (pH 7.4), were incubated at 37°C in the presence or in the absence of 0.2 mM quercetin (5 min preincubation), 80 μM myristate or 40 μM Br-X537A (the solvent Me_2SO was included in the control assays). Na-ATP was added at the concentration of 0.5 mM (final volume = 0.5 ml) and at the 2nd and 8th min of incubation an aliquot was withdrawn for P_i assay. Data are expressed as nmol $P_i/2 \times 10^6$ cells per 6 min and are means (± S.E.) of five individual experiments. The difference between the means was analized by paired '*t*' statistics (n.s. = not significant).

	Control	Myristate		Control	Br-X537A	
− Quercetin	8.75 ± 0.52	9.80 ± 0.81	n.s.	8.87 ± 0.84	9.14 ± 0.87	n.s.
+ Quercetin	5.79 ± 0.40	5.98 ± 0.33	—	5.51 ± 0.69	4.83 ± 1.13	—
	$P < 0.025$	$P < 0.05$		$P < 0.05$	$P < 0.025$	

* Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

activation induced by either the antibiotic Br-X537A or myristic acid by less than 25%.

It is unlikely that the much smaller effect of quercetin on the stimulation brought about by the antibiotic Br-X537A and by myristic acid can be ascribed to the formation of a complex between the stimulant and the flavonoid or to competition by stimulant and quercetin for the same cellular site. In fact, as shown in Table III, at the same molar ratio used in the study of oxygen consumption, quercetin inhibits the ecto-(Na^+, K^+, Mg^{2+})-ATPase of polymorphonuclear leukocytes also in the presence of either myristic acid or the antibiotic Br-X537A.

Discussion

There is a general agreement that the perturbation of the plasma membrane is a crucial event in the activation of specific functions of polymorphonuclear leukocytes. This conclusion is based on the observation that the stimulation of the leukocyte activities classically linked to phagocytosis (respiration, secretion, surface movements) is also brought about by a number of non-particulate substances capable of modifying the supramolecular organization of the cell surface [2,3].

On the basis of their chemical and/or biological properties these stimulants can be tentatively subdivided into two groups, one interacting with the more external moiety of the plasma membrane and the other with the membrane hydrophogic core. The first group includes agents that interact with surface receptors or modify the polar heads of phospholipids, such as concanavalin A [15] and phospholipase C [39], respectively. In contrast, compounds such as myristic acid [17] and the lipophilic antibiotic Br-X537A [34] very likely belong to the second group. The latter compound is known as an ionophore [40], but its rather non-specific ionic requirement in the stimulation of polymorphonuclear leukocyte respiration [34] suggests that the activity of the antibiotic Br-X537A may be more likely ascribed to its capacity of perturbing the hydrocarbon interior of the plasma membrane.

On account of the different action of quercetin on the stimulation of oxygen consumption induced by various types of membrane perturbing agents, this drug appears to be able to mainly inhibit the cell activation following the interaction of stimulants with the more external layer of the leukocyte plasma membrane. Agents that interact with the deeper hydrophobic core of the lipid bilayer, such as myristic acid and the antibiotic Br-X537A, bypass, in fact, the inhibitory action of the flavonoid.

In view of its selective inhibition of the stimulatory activity of surface-reactive agents on the leukocyte functions, quercetin might be usefully employed as a probe to establish which membrane domain is the direct target of a stimulant.

The present results also indicate that the main site of interaction of quercetin with polymorphonuclear leukocytes is in the outer leaflet of the plasma membrane and is hydrophobic in nature. Evidence in support of this conclusion is provided by the observation that 1-anilino-8-naphtalene sulphonate fully reverse the inhibition by quercetin of the concanavalin A-induced stimulation of the leukocyte respiration. Under the experimental conditions employed, 1-anilino-8-naphtalene sulphonate does not penetrate the leukocytes [41] and preferentially binds to apolar niches of the external cell surface membrane [42,43]. Furthermore, more hydrophilic flavonoids such as morin and the glycosylated quercetin derivative, rutin, have no effect on polymorphonuclear leukocyte functions.

The precise mechanism of the inhibitory action of quercetin remains to be elucidated. Recently we have reported a comparative evaluation of the stimulatory activity of tetravalent and divalent concanavalin A, which suggests that the initial triggering of the respiratory stimulation of polymorphonuclear leukocytes is in part accomplished through cross-linkage of membrane constituents [44]. We have also postulated that phospholipase C might act by a similar mechanism [44]. It is unlikely that quercetin interferes with the formation of these microaggregates of membrane constituents, since the drug exerts its inhibitory activity also when added after the onset of stimulation. Quercetin might, however, affect the generation of 'signals', such as changes in ion permeability, caused by the association of membrane constituents. This mechanism of inhibition might, for example, include an increase in efficiency of ion pumping systems localized at the plasma membrane level, as suggested by others [25—27]. One such system might be the ATP-dependent Ca^{2+} pump, that is likely to be present in the plasma membrane of polymorphonuclear leukocytes [45].

In Ehrlich ascites tumour cells quercetin promotes a rise in the cAMP levels [46]. It is unlikely that the inhibitory effects of this flavonoid, at least on the enhancement of oxygen consumption by polymorphonuclear leukocytes, is mediated by the cyclic nucleotide. Were this the case, an elevation of cyclic AMP concentration in the cytosol should depress the cell response to all the stimuli by a comparable extent. Furthermore, the inhibitory effect of quercetin on the stimulation of oxygen consumption is immediate, whereas the rise in cyclic AMP levels at least in the Ehrlich ascites tumour cells, is a delayed phenomenon. Finally, the respiratory burst of the leukocytes is independent of the intracellular levels of cyclic AMP [47,48].

Whatever is the mechanism of action of quercetin, its in vitro inhibitory effects on some stimulants of the main functions of polymorphonuclear leukocytes, such as exocytotic discharge of granule content, oxygen reduction to cytotoxic radicals and chemotaxis [49], could be very interestingly exploited for an in vivo study of the regulation of the inflammatory process. Experiments along this line are in progress.

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