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THE BIOFLAVONOID QUERCETIN INHIBITS NEUTROPHIL DEGRANULATION, SUPEROXIDE PRODUCTION, AND THE PHOSPHORYLATION OF SPECIFIC NEUTROPHIL PROTEINS

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Summary: Quercetin, a C-kinase antagonist, inhibits neutrophil degranulation and superoxide production induced by f-met-leu-phe, solid phase IgG, zymosan treated serum and a phorbol ester (PMA). Quercetin is more effective in inhibiting degranulation (IC_{50} = 20uM) than superoxide production (IC_{50} = 80uM). Neutrophil activation by PMA is accompanied by the phosphorylation of neutrophil proteins of 205, 170, 130, 91, 77, 67, 56, 47, 39, 34, 27, and 20 kilodaltons; quercetin also inhibits the phosphorylation of these proteins. Dose-response studies indicated that phosphorylation of the 67 kilodalton protein was particularly sensitive to inhibition by quercetin at concentrations that also inhibit neutrophil degranulation and superoxide production. These results suggest that phosphorylation of the 67 kilodalton protein may be an important intracellular reaction associated with neutrophil activation.

Neutrophils are activated by a variety of agonists through interaction with specific receptors on the surface of the plasma membrane. Activated neutrophils release proteases and produce potent oxygen metabolites which play essential roles in host defense but which also contribute to tissue destruction in a number of acute and chronic inflammatory disorders (1). Although the intracellular reactions linking receptor-agonist interaction with neutrophil degranulation and superoxide production are not well understood, activation of certain protein kinases, including a calcium/phospholipid dependent kinase (C-kinase), is considered an important aspect of the signal transduction process (2-5). These kinases phosphorylate specific cellular proteins and enzymes to alter their activities resulting in the propagation of the intracellular signals which trigger cellular

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activation. Several studies have reported proteins which are phosphorylated upon activation of neutrophils with the agonists PMA and f-met-leu-phe (2-8). However, these studies have provided widely disparate results, due to a failure in some studies to control for proteolysis of the phosphoproteins, and have not delineated which of the numerous phosphoproteins play important roles in cellular activation.

Quercetin, a bioflavonoid, inhibits, possibly due to its effect on the C-kinase, the function of human neutrophils (9-12) and the growth of certain tumor cell lines (13-15). Because the C-kinase is considered to be important in neutrophil activation (2-4), we were interested in further evaluating the effects of quercetin on neutrophil function to determine if it could be used to correlate the inhibition of the functional aspects of neutrophil activation with inhibition of the phosphorylation of specific neutrophil proteins.

MATERIALS AND METHODS

Materials: Quercetin, f-met-leu-phe, phorbol myristic acetate (PMA), Cohn Fraction II, bovine serum albumin (BSA), and ferric cytochrome C were purchased from Sigma Chemical Co., St. Louis, Mo. Human IgG was purified from Cohn fraction II as previously described (16). ^{32}P-inorganic phosphate (8mCi/ml) was purchased from Amersham Corp., Arlington Heights, Ill. Stock solutions of quercetin were prepared in DMSO and were diluted 1:1 with water prior to addition to the reaction mixtures. The maximum concentrations of DMSO in the reaction mixtures was always less than 0.5% (v/v); control experiments established that this level of DMSO has no effect on the functional assays utilized in this study.

Blood was collected by venipuncture from healthy adult volunteers and neutrophils were isolated by established procedures (16). For functional assays neutrophils (5×10^6) were incubated (30 min, 37°) in 96 well microtiter plates coated with IgG or in BSA coated wells containing either f-met-leu-phe (FMLP), zymosan treated serum (ZTS), or PMA. Upon completion of the incubation period, the wells were aspirated; the aspirates were individually centrifuged (300 xg, 10 min), and the cell free supernatants assayed for lactoferrin and elastase by radioimmunoassay (16) to measure degranulation. Superoxide production was measured by following the reduction of ferric cytochrome C with spectrophotometric procedures (17). Each sample assayed for superoxide production was standardized after incubation such that each contained identical amounts of DMSO and quercetin.

To identify phosphoproteins, the cellular pools of ATP and phosphoproteins were labeled with ^{32}P as described by White et al (2). Neutrophils (10^8 cells/ml) dispersed in phosphate free buffer (2) were incubated (90 min, 37°C) in the presence of ^{32}P-inorganic phosphate (0.5 mCi/ml). The ^{32}P-labelled neutrophils were then washed twice in phosphate free buffer and activated as described above for the functional assays. The reactions were terminated by addition of diisopropylfluorophosphate (DFP; 1

mM) followed by sodium dodecyl sulfate (SDS; 1%) and immediate incubation (2 min) in a boiling water bath. SDS gel electrophoresis was conducted using the buffer system described by Laemmli (18) on 5-15% linear gradient polyacrylamide gels; following electrophoresis the dried gels were autoradiographed on Kodak XAR film.

RESULTS

Quercetin inhibited PMA induced degranulation and superoxide production. Figure 1 shows that fifty percent inhibition of degranulation was obtained with 20 uM quercetin; however, fifty percent inhibition of superoxide production required a higher dose of 80 uM quercetin. At the highest concentration tested (250 uM) quercetin inhibited greater than 90% of the degranulation activity but only 70% of superoxide production. Inhibition of degranulation could be reversed by washing the cells in quercetin free media (data not shown). Figure 2 shows that quercetin was also effective in inhibiting neutrophil degranulation induced by the agonists f-met-leu-phe, zymosan treated serum, and solid phase IgG. Concentrations of quercetin which

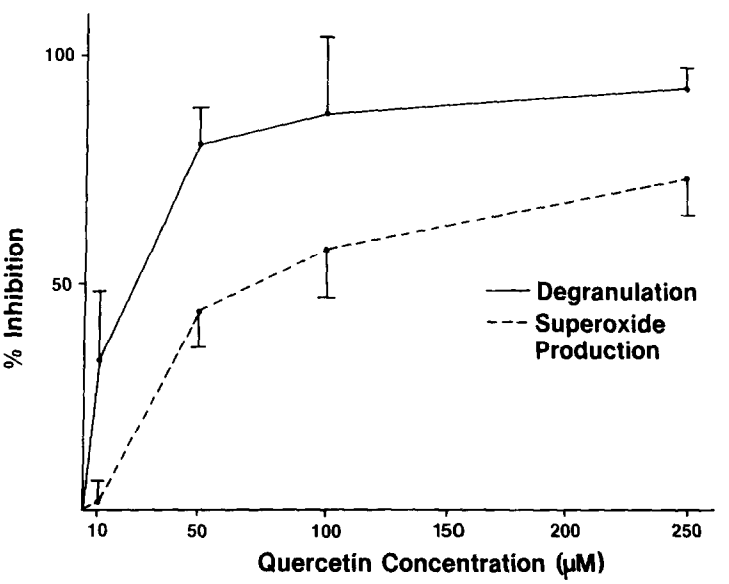

Figure 1. Inhibition of neutrophil degranulation and superoxide production by quercetin. Neutrophils (5 x 10^6/ml) were preincubated (30 min, 37°C) with the indicated concentrations of quercetin. The cells were then aliquoted (250 ul) into wells of microtiter plates, PMA (1 ug/ml) was added, and the cells were incubated for an additional 30 minutes at 37°C; degranulation was measured by assaying the released lactoferrin; superoxide production was measured spectrophotometrically as described in "Materials and Methods". Each point is the mean of six different determinations; the standard deviation is indicated by the bars.

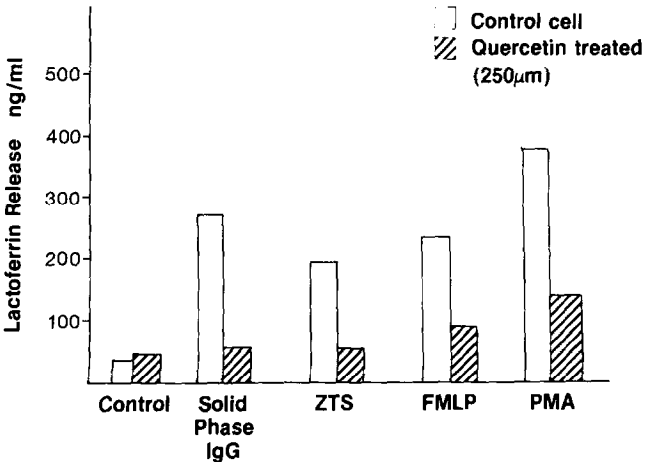

Figure 2. Effects of quercetin on neutrophil degranulation induced by various agonists. Neutrophils (5 x 10^6/ml) were pre-incubated (30 min, 37°C) with (open bars) and without (stripped bars) quercetin (250 uM). Aliquots (250ul) of the cells were then transferred to wells of microtiter plates containing either no addition (control), coated with IgG (5 ug/well), zymosan treated serum (ZTS; 10% v/v), FMLP (1 uM), or PMA (100ng/ml) and incubated for 30 minutes at 37°C. Degranulation was measured by assaying the cell free supernatants for lactoferrin as described in "Materials and Methods". Each bar indicates the mean of four experiments.

inhibited degranulation by these mediators are essentially identical to those noted to inhibit PMA induced degranulation. Zymosan treated serum and f-met-leu-phe mediated superoxide production were also inhibited by quercetin in concentrations similar to those observed to inhibit PMA induced superoxide production (data not shown). Neutrophil viability, based upon trypan blue exclusion and the lack of lactate dehydrogenase activity in the cell supernatants, was not affected by quercetin concentrations ranging up to 500 uM.

Preliminary experiments indicated that there was a marked degradation of phosphoproteins during the preparation of neutrophils for SDS gel electrophoresis making it impossible to determine which proteins were phosphorylated in response to neutrophil stimulation and if the phosphorylation reactions were inhibited by quercetin. A brief (15 sec) treatment of the cells with the serine protease inhibitor DFP prior to the addition of SDS blocked this degradation and provided reproducible phosphoprotein patterns. The addition of other protease inhibitors including o-phenanthroline, N-ethylmaleimide, EGTA, and soybean trypsin inhibitor to

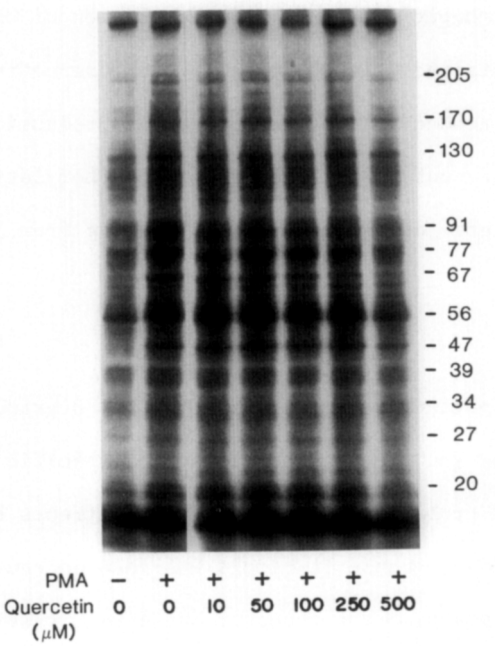

Figure 3. Effect of quercetin on PMA induced phosphorylation of neutrophil proteins. Neutrophil (3 x 10^7/ml) which had been labeled with ^{32}P as described in "Materials and Methods" were preincubated (15 min, 37°C) in various concentrations of quercetin as indicated in the figure and activated with PMA (100ng/ml) for 30 min. at 37°C. The cells were treated with DFP, dissolved in SDS, and subjected to electrophoresis and autoradiography as described in "Materials and Methods".

DFP-treated cells resulted in no further change in the protein and phosphoprotein patterns (data not shown).

Figure 3 shows that in the unstimulated neutrophil approximately 30 proteins incorporate ^{32}P under our labelling conditions. When ^{32}P-labelled neutrophils were activated with PMA, proteins of 205, 170, 130, 91, 77, 67, 56, 47, 39, 34, 27, and 20 kilodaltons either became phosphorylated or increased their level of ^{32}P-labelling. Activation with f-met-leu-phe (not shown) resulted in the phosphorylation of an identical set of proteins with the exception of a protein of 47 kilodaltons and several proteins between 53 and 56 kilodaltons. In cells pretreated with quercetin there was a decrease in the labelling of phosphoproteins in both the unstimulated neutrophils and in the cells stimulated with f-met-leu-phe and PMA. Phosphorylation of the 67 kilodalton protein appears particularly sensitive to inhibition by quercetin. Dose-response studies (data not shown) indicated

that fifty percent inhibition of the phosphorylation of the 67 kilodalton protein could be obtained with a dose of 70 uM quercetin, a value very similar to the dose needed to inhibit superoxide production and degranulation (figure 1). Fifty percent inhibition of the phosphorylation of the other proteins required higher doses of quercetin ranging from 250-500 uM.

DISCUSSION

Quercetin is a potent inhibitor of neutrophil degranulation, superoxide production and the phosphorylation of specific neutrophil proteins. The compound probably does not exert its effects by interfering with ligand-receptor binding because it blocks PMA-induced neutrophil activation; PMA bypasses the plasma membrane receptor and directly stimulates the C-kinase. A more likely possibility is that quercetin directly inhibits the activation of various neutrophil kinases which are necessary for neutrophil activation. Studies by other investigators using various cell types have indicated that quercetin inhibits the C-kinase (19), tyrosine kinases (14,20), and phosphorylase kinase (20). In addition, quercetin binds to the Ca^{2+}/ calmodulin complex (21,22) and studies in our laboratory indicate that quercetin inhibits the calmodulin stimulated activity of a Ca^{2+}-dependent phosphodiesterase; thus, quercetin may also inhibit calmodulin-dependent phosphorylation reactions important for neutrophil activation. The inhibitory effects of quercetin on protein phosphorylation are probably not related to the cAMP-dependent protein kinase; quercetin does not inhibit cAMP-dependent kinase (20,23,24).

Using the serine protease inhibitor DFP, we have been able to define a stable pattern of neutrophil phosphoproteins, similar to that noted by Andrews et al (5), who also used DFP. Pontremoli et al (25,26) were able to bypass this problem by fractionating neutrophils prior to analysis and noted a pattern of phosphoproteins nearly identical to ours. Most important is our observation that phosphorylation of a 67 kilodalton neutrophil protein is inhibited by quercetin at concentrations similar to those needed to inhibit

degranulation and superoxide production. This suggests that phosphorylation of the 67 kilodalton protein may be intimately linked with neutrophil activation. Previous reports have indicated that several cytosolic proteins, including one with a molecular weight of 65 kilodaltons, were phosphorylated by a calpain activated C kinase and have suggested that these phosphoproteins may be involved in degranulation (25,26). Further delineation of the regulatory mechanisms which control the phosphorylation state of the 67 kilodalton protein and its role in neutrophil function will undoubtedly provide new insights into the molecular events involved in neutrophil activation.

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