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Inhibition of *N*-Formylmethionyl-Leucyl-Phenylalanine-Stimulated Tyrosine Phosphorylation and Phospholipase D Activation by Quercetin in Rabbit Neutrophils

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ABSTRACT. We investigated the effect of bioflavonoid quercetin on tyrosine phosphorylation and phospholipase D (PLD, EC 3.1.4.4) activation in rabbit peritoneal neutrophils stimulated by *N*-formylmethionyl-leucyl-phenylalanine (fMLP). The quercetin dose-dependently inhibited degranulation and superoxide production in fMLP-stimulated neutrophils. A strong inhibitory effect of quercetin on the tyrosine phosphorylation of several proteins (40, 42, 43, 45, 46 and 75 kDa) was observed when the neutrophils were pretreated with different concentrations of quercetin. Furthermore, quercetin inhibited mitogen activated protein kinase (MAP kinase) and PLD activation induced by fMLP in a dose-dependent manner. The reduction in PLD activity was 30% at 0.1 μM and 70% at 100 μM of quercetin. These results suggest that impairment of neutrophil functions by quercetin may be due, at least in part, to inhibition of tyrosine phosphorylation and PLD activation. BIOCHEM PHARMACOL 53;10:1503–1510, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. quercetin; bioflavonoids; neutrophils; superoxide production; phospholipase D; tyrosine phosphorylation

Flavonoids are a group of naturally occurring plant compounds recognized to have antiallergic, antiviral, antiproliferative and anticarcinogenic activities as well as affecting some aspects of the mammalian enzyme system [1–3]. Quercetin, one of the most common bioflavonoids, is cited as a very active compound having the capacity to inhibit protein kinase C (PKC), protein tyrosine kinase (PTK) and phospholipase A_2 (PLA_2) activities in different types of cells [1, 4–6]. In neutrophils, quercetin has been reported to be a potent inhibitor of degranulation and superoxide production as well as an inhibitor for some enzymes involved in arachidonic acid metabolism [1, 2, 5, 7]. Quercetin is thought to interfere with superoxide generation by inhibiting enzyme activities that participate in NADPH oxidase activation [3, 8, 9] or by scavenging the superoxide anions [9]. However, the precise mechanism of the inhibitory effect of quercetin on neutrophils is not fully understood.

Neutrophils participate in the defense response against invading agents, playing a key role in the processes of killing and regulation of responses of host defense cells. Following the binding of chemoattractants to specific receptors, neutrophils are stimulated to secrete granule contents containing degradative enzymes and antimicrobial agents, and activate the NADPH oxidase to generate toxic metabolites of oxygen [10–13]. Recently, it has been shown that tyrosine phosphorylation is involved in processes such as superoxide production, adherence and chemotaxis in human neutrophils [14, 15]. A rapid increase in the phosphotyrosine content of several proteins is observed in neutrophils after stimulation with different chemotactic stimulants [11, 14–17]. The treatment of cells with different concentrations of tyrosine kinase inhibitors (erbstatin and genistein) resulted in a dose-dependent inhibition of oxygen species production by human neutrophils, providing evidence for the possible participation of some tyrosine kinase in this process in neutrophils. The participation of phospholipase D (PLD, EC 3.1.4.4) in degranulation and superoxide production was proposed by our and other groups [18, 12, 13]. Phosphatidic acid (PA), a product of the breakdown of phosphatidylcholine by PLD, is thought to be involved in the respiratory burst in human neutrophils [13].

On the basis of these reports on neutrophil signaling, we investigated the effect of quercetin on tyrosine phosphorylation and PLD activation induced by *N*-formylmethionyl-leucyl-phenylalanine (fMLP) stimulation to gain more

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Abbreviations: CB, cytochalasin B; ERK1, extracellular signal-regulated kinase 1; fMLP, *N*-formylmethionyl-leucyl-phenylalanine; MAP kinase, mitogen activated protein kinase; PA, phosphatidic acid; PAF, platelet-activating factor; PBut, phosphatidylbutanol; PKC, protein kinase C; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D; PTK, protein tyrosine kinase; Querc, quercetin; SOD, superoxide dismutase; TBS-T, Tris-buffered saline (10 mM Tris-HCl, pH 7.4, 150 mM NaCl and 0.2% Tween 20).

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insight into the mechanism underlying inhibition of neutrophil functions by flavonoids. We suggest that suppression of physiological functions such as degranulation and superoxide production by quercetin might at least in part be due to inhibition, of a different intensity, of tyrosine phosphorylation of specific proteins and PLD activation in fMLP-activated neutrophils. The different pattern of inhibition obtained provides evidences for the existence of multiple pathways of PLD regulation in rabbit neutrophils.

MATERIALS AND METHODS

Chemicals and Materials

Quercetin and glycogen (from oyster, type II) were purchased from Nacalai Tesque Co. (Kyoto, Japan), *p*-Nitrophenyl-*N*-acetyl-β-D-glucosaminide, fMLP, horse heart cytochrome c, superoxide dismutase (SOD) and cytochalasin B (CB) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [1-O-^{3}H]lyso platelet-activating factor (PAF), anti-rabbit IgG horseradish peroxidase, sheep anti-mouse IgG horseradish peroxidase-conjugated antibody and ECL detection reagent were from Amersham Int. (Buckinghamshire, England). Antiphosphotyrosine antibody (monoclonal IgG2bk) and anti-extracellular signal-regulated kinase 1 (anti-ERK1) were from UBI (Lake Placid, NY, USA) and Transduction Lab. (Lexington, KY, USA) respectively. Immobilon polyvinylidene difluoride used in the Western blotting experiments was from Millipore Corp. (Bedford, MA, USA). Phosphatidylbutanol (PBut) standard was prepared from egg PC by using crude cabbage PLD according to the modified method of Yang as described previously [18, 19].

The stock solution of quercetin was prepared in DMSO, the final concentration of which was adjusted to 0.2% (v/v) in all experiments. Other reagents were of highest analytical grade.

Preparation and Radiolabeling of Rabbit Neutrophils

Neutrophils were harvested from rabbit peritoneum 6 to 10 hr after the injection of 300–400 mL of sterile 0.2% (w/v) glycogen in saline solution. Contaminating erythrocytes were removed by hypotonic lysis and the cells were washed twice and resuspended in HEPES buffer (134 mM NaCl, 5 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 4.7 mM KCl, 1.2 mM $MgSO_4$, 10 mM Hepes/NaOH (pH 7.4), 5 mM glucose and 0.1% BSA). The purity was assessed by counting the cells using Turk's solution (>95%) and the cells were keep on ice until use. $CaCl_2$ (final concentration at 1 mM) was added to the assay buffer just before the preincubation. For the PLD experiments, the washed cells were labeled with [^{3}H]lysoPAF (0.5 μCi/mL) for 2 hr at 37° under a 5% CO_2/air atmosphere. Nearly 90% of the total radioactivity incorporated into the lipid fraction was present in the PC fraction.

Degranulation Assay

Neutrophils (5×10^7 cells/mL) suspended in 200 μL Hepes buffer containing 1 mM Ca^{2+} were preincubated for 5 min at 37° with quercetin or vehicle (DMSO), for 5 more min with CB (0.5 μg/mL) and then stimulated with fMLP (1 μM). After 5 min, the reaction was terminated with addition of 750 μL of ice-cold PBS in the reaction medium and centrifugation at 8000 g/3 sec. The amount of enzyme released was determined by using chromogenic substrate *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide. It was calculated as the percentage of β-*N*-acetyl-glucosaminidase (EC 3.2.1.30) in neutrophils determined by lysis with 0.12% Triton X-100, according to a method described previously [18, 19].

Superoxide Generation Assay

The quantitative determination of superoxide species was performed by measuring SOD-inhibitable cytochrome c reduction as described by Markert *et al.* 1984 and Kanaho *et al.* 1993 [20, 21]. Neutrophils (1×10^7 cells/assay) were preincubated for 5 min at 37° with or without different concentrations of quercetin and with CB (0.5 μg/mL) for more 5 min, and then incubated with cytochrome c (1.5 mg/mL) and fMLP (1 μM) for a further 10 min. The reaction was terminated by placing the tubes on ice for 10 min and centrifuging at 2000 × *g*/5 min. The superoxide generation was quantified as that part of the change in absorbance (at 550 nm) of cytochrome c that was abolished in the presence of SOD (120 units/mL). The amount of O_2^- nmol generated in 1 mL of reaction mixture was calculated from the formula: O_2^- (nmol) = 47.7 × absorbance.

Tyrosine Phosphorylation

Neutrophils (2×10^7 cells/mL) were stimulated as described in the degranulation assay method. The reaction was terminated with ice-cold PBS buffer containing 20 mM β-glycerophosphate, and solubilized with ice-cold lysis buffer (1% Triton X-100, 0.1% SDS, 1% sodium desoxicholate, 50 mM NaCl, 25 mM Hepes/NaOH (pH 7.4), 10 mM NaF, 1 mM Ortho-vanadate, 0.2 mM molybdate, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 10 μg/mL leupeptin). After the insoluble materials were removed by centrifugation (14000 × *g*/20 min), the soluble proteins (80 μg) were mixed with SDS-PAGE sample buffer (2% SDS, 5% glycerol, 100 mM dithiothreitol (DTT), 50 mM Tris/HCl and 0.02% bromophenol blue) and then boiled for 5 min. The proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and electrotransferred onto Immobilon membranes. After blocking with Tris-buffered saline plus Tween-20 (TBS-T) (10 mM Tris, pH 7.4, 150 mM NaCl and 0.2% Tween 20) containing 5% of chicken egg albumin for 2 hr, the membranes were incubated for 1 hr with antiphosphotyrosine IgG2bk monoclonal antibody or anti-ERK1 (1:500). Then, the

membranes were washed three times with TBS-T and were reacted for an additional hour with sheep anti-mouse IgG horseradish peroxidase-conjugated antibody (1:4000). The immunoblots were detected by using enhanced chemiluminescence reagents (ECL-Amersham), and the bands were quantified by a scanning densitometry (Densitometer AE-6900-E, ATTO, Tokyo, Japan).

Phospholipase D Assay

PLD activity was determined by measuring [^{3}H]PBut formation in the presence of butanol. The [^{3}H]lyso PAF-labeled neutrophils (2×10^7 cells/mL) were suspended in Hepes buffer, and the stimulation of neutrophils followed the same procedure as in the degranulation essay, differing only in that 0.3% (v/v) of butanol was added at the same time as the agonist. The reaction was terminated with 1 mL of ice-cold PBS and subsequent centrifugation at 1000 g/5 min, followed by addition of 1.5 mL of MeOH, 0.75 mL of PBS and 1.5 mL of $CHCl_3$/MeOH (2:1). The extraction of lipids was carried out as described previously [19]. The lipids were separated by TLC using the upper phase of ethyl acetate, isooctane, acetic acid and water (13:2:3:10, v/v) as solvent system. The plates were exposed to iodine vapor and the silica gel spots corresponding to PBut were scraped off and its radioactivity measured in a liquid scintillation counter (Beckman LS 6500, Fullerton, CA, USA).

RESULTS

Effect of Quercetin on Neutrophil Functions Induced by fMLP

We have examined the effects of flavonoid pretreatment on enzyme release elicited by fMLP. As shown in Fig. 1A, quercetin dose-dependently inhibited β-glucosaminidase release induced by fMLP (10^{-6} M) in the presence of CB (0.5 μg/mL). The flavonoid only weakly inhibited degranulation, quercetin inhibited enzyme release at 10 μM and 100 μM by almost 20 and 35%, respectively. Although CB is required for lysosomal enzyme release induced by fMLP, the inhibitory effect of quercetin on enzyme release was suppressed by addition of a high concentration of CB. In the presence of 5 μg/mL of CB, degranulation was only weakly inhibited (15% of inhibition) even at 100 μM quercetin (data not shown).

The effect of quercetin on the production of superoxide species was examined as inferred by reduction of cytochrome c. Peak superoxide production was observed at 1 min and sustained for at least 15 min (data not shown). Figure 1B shows the inhibition of oxygen species production by different concentrations of quercetin. At 1 and 10 μM quercetin, the suppression of superoxide production was 20 and 50%, respectively. When the cells were pretreated with 100 μM quercetin, the amount of SOD-inhibitable superoxide production was reduced to below control levels. Thus, quercetin inhibition of superoxide species production induced by fMLP stimulation was more evident than degranulation inhibition.

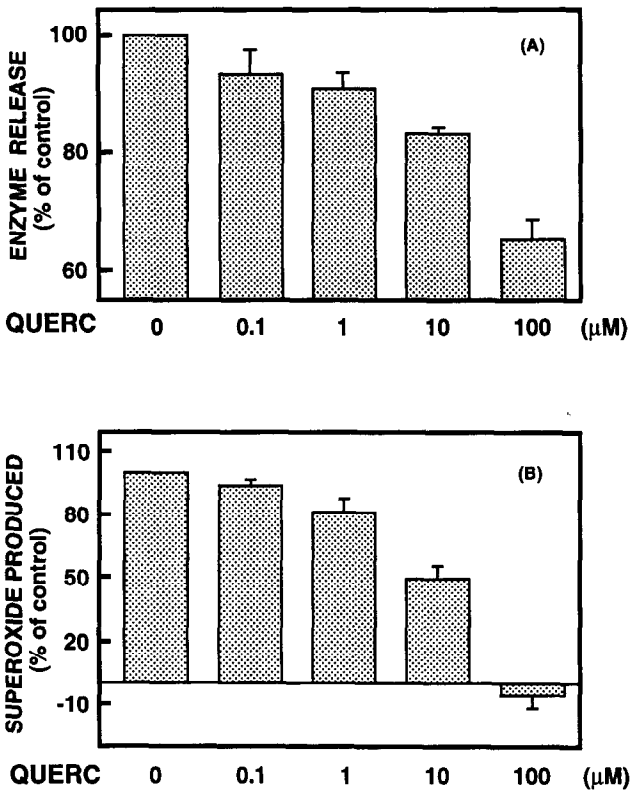

FIG. 1. Effect of quercetin (QUERC) on degranulation and superoxide production elicited by fMLP. (A)Neutrophils (1×10^7 cells) were preincubated for 5 min with the indicated concentrations of quercetin, subsequently incubated with 0.5 μg/mL of CB and stimulated for 5 min with 1 μM fMLP. The amount of released enzyme was measured with p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate. Data are expressed as a percentage inhibition of the total amount of released enzyme in the absence of quercetin (39.69% ± 2.39). Results are mean ± SD and are representative of four separate experiments performed in duplicate. (B)Neutrophils (1×10^7 cells) were preincubated for 5 min in the absence or presence of indicated concentrations of quercetin, treated for 5 min with 0.5 μg/mL of CB, and then reacted for a further 10 min with cytochrome c (1.5 mg/mL) and 1 μM fMLP. O_2^- generation was quantified as the change in cytochrome c absorbance at 550 nm of that abolished by SOD (120 units/mL). Data are expressed as a percentage of the control obtained from the stimulated cells in the absence of quercetin. The control rate (100% of control) corresponds to 23.96 ± 1.52 nmol of superoxide/min per 1×10^7 cells for stimulated cells. Data are mean ± SD of three experiments done in duplicate.

Effect of Quercetin on Tyrosine Phosphorylation

Treatment of neutrophils with tyrosine kinase inhibitors was reported to suppress superoxide production in fMLP-stimulated human neutrophils [11, 14], suggesting that protein tyrosine phosphorylation may be involved in neutrophil activation. We have examined which proteins are phosphorylated in response to fMLP stimulation by immunoblotting with antiphosphotyrosine antibody. Fig. 2A

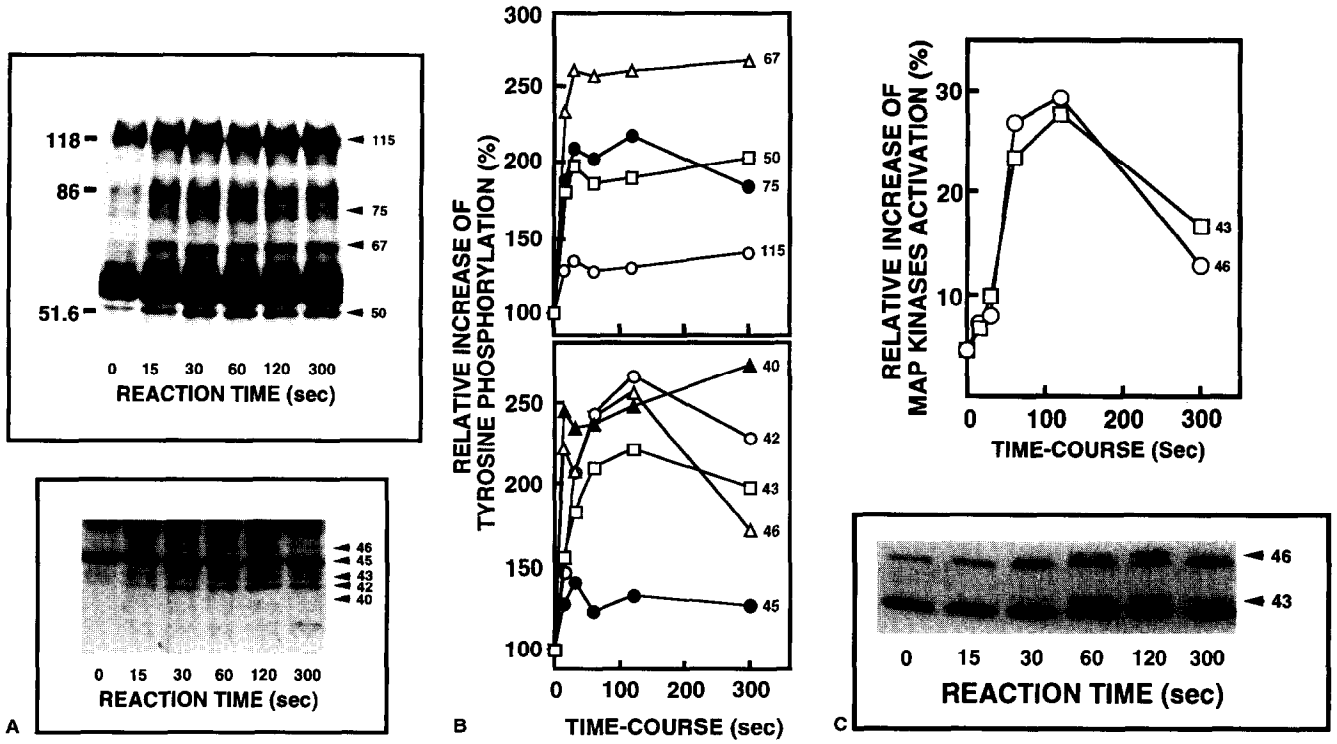

FIG. 2. Time-course of tyrosine phosphorylation in fMLP-stimulated neutrophils. (A) Neutrophils (2×10^7 cells) were preincubated for 5 min at 37°, subsequently incubated with CB (0.5 μg/mL for 5 min), and then stimulated with fMLP (1 μM) for indicated periods. After reaction, the samples were boiled in SDS sample buffer and the lysates subjected to 10% SDS/PAGE and then immunoblotted with antiphosphotyrosine IgG2bk antibody. The standard proteins used were myosin (203 kDa), β-galactosidase (118 kDa), bovine serum albumin (86 kDa), ovalbumin (51.6 kDa), and carbonic anhydrase (34.1 kDa). Data show a representative experiment of three separate experiments with similar results. (B) Densitometric scans of tyrosine-phosphorylated proteins obtained in (A). Data are expressed as a percentage increase of the control. (C) Immunoblot showing the time-course of MAP kinase activation detected by reaction with ERK1 antibody (lower panel) and densitometric analysis of immunoblot showing the increase in the band shift of 43 and 46 KDa MAP kinase proteins (upper panel).

shows a representative time-course of tyrosine phosphorylation induced by fMLP. Exposure of CB-pretreated neutrophils to 1 μM fMLP induced phosphorylation of several proteins. These proteins displayed distinct time-dependent increases in tyrosine phosphorylation. As shown in Fig. 2B, some proteins (45, 50, 67 and 115 KDa) showed phosphorylation peaks between 30 and 60 sec while others (42, 43, 46 and 75 KDa) showed maximum phosphorylation at 2 min. The 40 KDa protein showed a rapid phosphorylation response, with the first peak at 30 sec; however, the level of phosphorylation showed constant increase for at least 5 min following stimulation.

Because some isoforms of mitogen activated protein (MAP) kinases (40~46 KDa) are regulated by phosphorylation of both tyrosine and serine/threonine residues [22–24], we used the antibody against ERK1 to verify whether some of the tyrosine-phosphorylated proteins in our experiments were MAP kinase. The antibody reacts with the peptide sequence corresponding to positions 325–345 of human ERK1, but is known to react to some extent with ERK2 according to the product specification. In unstimulated neutrophils, two bands (42 and 45 KDa) were perceptible by reaction with ERK1 antibody, with the 42 KDa protein band being more evident than the 45 KDa protein. When stimulated by fMLP, these proteins showed a small upward shift in their location. The tyrosine-phosphorylated proteins with 43 and 46 KDa proteins were corresponded to the shifted band of 42 and 45 KDa MAP kinases, respectively. The time-dependence of stimulation of these proteins revealed a very similar time-dependence with tyrosine phosphorylation, showing a peak activation at 2 min and decreasing thereafter (Fig. 2C).

The pretreatment of neutrophils for 10 min with quercetin inhibited tyrosine phosphorylation of several proteins such as 40, 42, 43, 45, 46 and 75 KDa proteins (Fig. 3A). The densitometric analysis of Fig. 3A shows a different pattern of inhibition of some proteins by quercetin (Fig. 3B). The 40 KDa protein exhibited high sensitivity to quercetin, with 0.1 μM of quercetin inhibiting nearly 55% of tyrosine phosphorylation. The inhibition observed at 10 μM quercetin reached almost 70% as compared with control level. Conversely, other proteins such as 75 KDa showed low sensitivity to flavonoid: 15 and 30% inhibition were noted when the cells were pretreated with 0.1 and 10 μM, respectively. Under the assay conditions, the inhibitions of 43, 45 and 42 KDa protein phosphorylation evidenced a very similar dose-dependency of quercetin, with only a small difference in the intensity of these

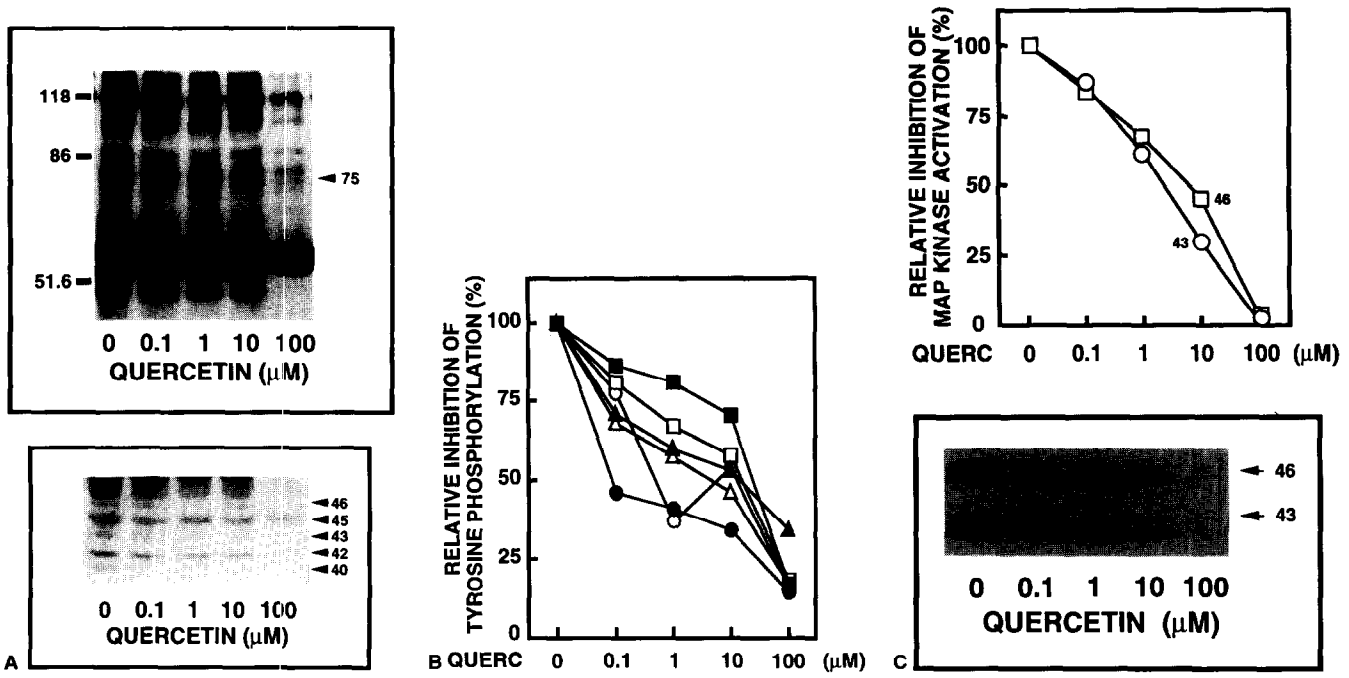

FIG. 3. Inhibition of fMLP-stimulated tyrosine phosphorylation by quercetin. Cells were incubated for 5 min in the presence of the indicated concentrations of quercetin, subsequently incubated for 5 min with 0.5 μg/mL CB and stimulated for 5 min with 1 μM fMLP. (A) Immunoblot with IgG2bk antibody showing the inhibition of tyrosine phosphorylation by quercetin. Data shown are representative of three independent experiments. (B) Densitometric scans of the immunoblotted membrane obtained in (A). Data are expressed as percentage inhibition of the control. ● 40 kDa; △ 42 kDa; □ 43 kDa; ▲ 45 kDa; ○ 46 kDa; ■ 75 kDa. (C) Immunoblot (lower panel) and densitometric pattern of immunoblot (upper panel) of inhibition of MAP kinase activation by different concentrations of quercetin. The immunoblot was reacted with anti-ERK1 antibody, and show the representative pattern of three separate experiments. The control represents the fMLP-stimulated MAP kinase activation obtained in the absence of inhibitor.

inhibitions, i.e., 43, 47 and 54% inhibition at 10 μM quercetin, respectively. Although the 46 KDa displayed strong inhibition at 1 μM of quercetin (63% inhibition), the inhibition observed at 10 μM of flavonoid was not increased.

Treatment with different concentrations of quercetin caused a very similar dose-dependent inhibition of 43 and 46 kDa proteins in the immunoblots treated with anti-ERK1 antibody (Fig. 3C). The inhibition of MAP kinase activity by quercetin was calculated as the decrease in the band shift in poliacrilamide gel observed in stimulated neutrophils without treatment with flavonoid. The 46 kDa showed a different pattern of inhibition from those obtained in the tyrosine phosphorylation (Fig. 3A and B): when treated with 1 μM of quercetin, the inhibition was *ca.* 30%, increasing to 60% at 10 μM.

Effect of Quercetin on PLD Activation Induced by fMLP

PLD activation has been reported to be functionally linked to lysosomal enzyme release and superoxide production induced by fMLP in neutrophils [12, 13, 18]. PLD activation by chemoattractants appears to be rapid, with apparent peak activity at 1 min, and is sustained for at least 5 min after stimulation (Fig. 4A). As shown in Fig. 4B, quercetin inhibited [^{3}H]PBut formation induced by 1 μM fMLP in a dose-dependent manner. The PLD activation mechanism in fMLP-stimulated neutrophils appears to be more sensitive to inhibition by bioflavonoids than degranulation. Quercetin pretreatment caused reductions in PLD activation of 30% of control at 0.1 μM; when a high concentration (100 μM) was used, the inhibition of [^{3}H]PBut formation was approximately 70%. As was the case in degranulation experiments, CB was required for PLD activation in fMLP-stimulated neutrophils. The inhibition of PLD activation by quercetin was dependent on the CB concentration: 1 μM quercetin inhibited [^{3}H]PBut formation at 0.5 μg/mL CB, but not at 5 μg/mL CB (data not shown).

DISCUSSION

We have investigated the mechanism of inhibition of neutrophil functions by quercetin. Previous studies have indicated that flavonoids interfere with degranulation and superoxide production in neutrophils by inhibiting PKC and PLA_2 activity [1, 8, 3]. However, recent studies have shown that besides the classical chemoattractant receptor signaling involving phospholipase C (PLC)-PKC, protein phosphorylation by tyrosine kinase is also implicated in neutrophil signaling. Naccache *et al.* [25] and Kusunoki *et al.* [14] reported that tyrosine phosphorylation of the 40

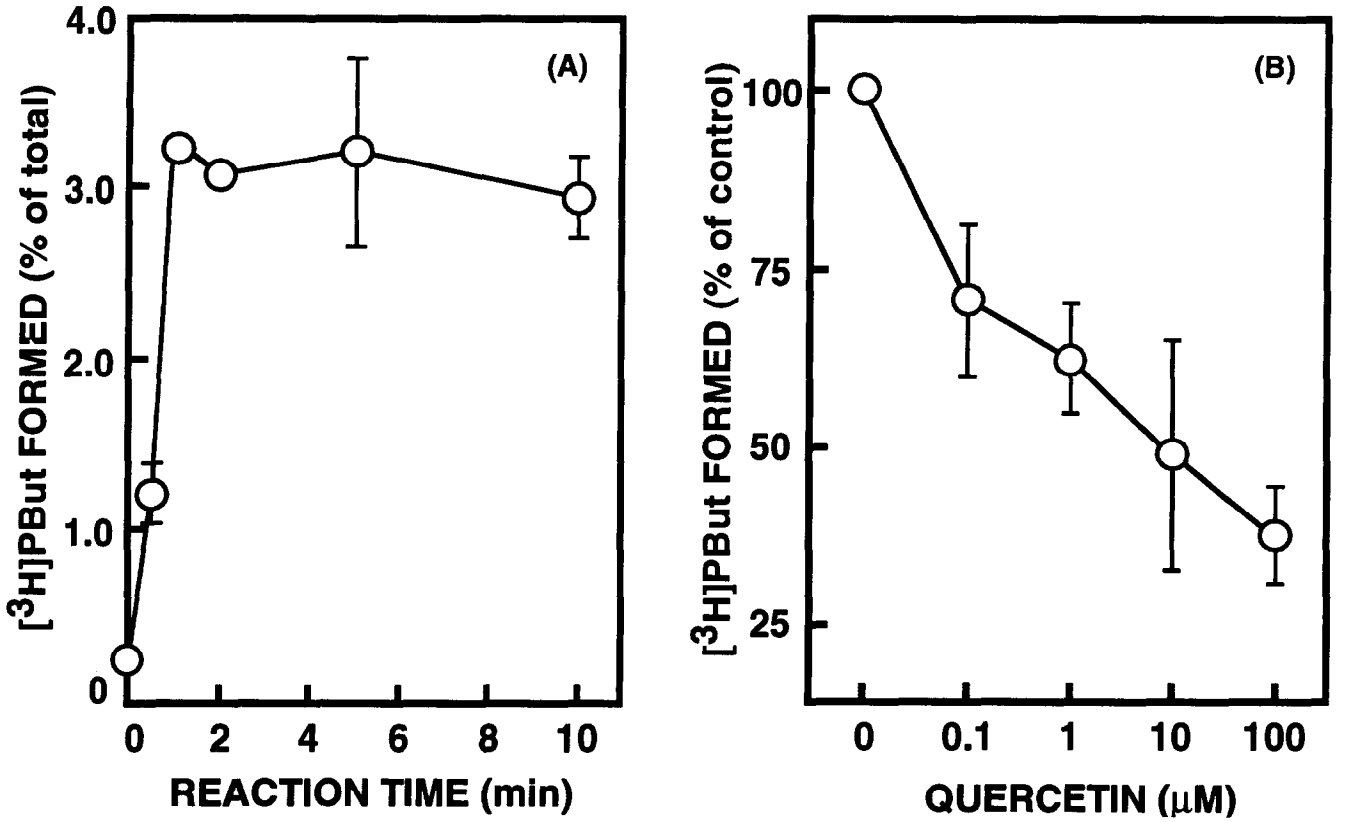

FIG. 4. PLD activation in fMLP-stimulated neutrophils. (A) Time-course of PLD activation by fMLP. The [^{3}H]lysoPAF-labeled neutrophils (2×10^7 cells) were pre-incubated with 0.5 μg/mL CB for 5 min and stimulated for indicated periods with 1 μM fMLP in presence of 0.3% BuOH. After the reaction was terminated, [^{3}H]PBut formation was determined. Data show mean ± SD from two experiments performed in duplicate, and are expressed as percentage of the total lipids. (B) Inhibition of fMLP-stimulated PLD activity by quercetin. [^{3}H]lysoPAF-labeled neutrophils (2×10^7 cells) were incubated for 5 min in the presence of indicated concentrations of quercetin, subsequently incubated for 5 min with 0.5 μg/mL CB and stimulated for 5 min with 1 μM fMLP. After reaction, the lipids were extracted and the amount of [^{3}H]PBut formed was measured after separation by thin-layer chromatography (TLC). Data are expressed as percentage of PLD activity in fMLP-stimulated cells in absence of quercetin (1.644 ± 220 dpm). Data are mean ± SD of four different experiments each performed in duplicate.

KDa protein appears to be an important element in the regulation of human neutrophil functions because it always occurs when the cells are stimulated with different agonists; moreover, its phosphorylation is inhibited when the neutrophils are pretreated with inhibitors of superoxide production. However, under our experimental conditions, phosphorylation of the 40 KDa protein was very weak compared with other proteins. This difference may be due to the different antiphosphotyrosine antibody used or the different phosphoprotein content in rabbit and human neutrophils. Quercetin inhibited tyrosine phosphorylation of the 40 KDa protein by more than 70% at 10 μM. On the contrary, the inhibitions of degranulation or superoxide production by quercetin were weak, i.e., 20 and 50% even at 10 μM, respectively. These results show that there is no direct correlation between the 40 KDa protein tyrosine phosphorylation and these physiological events.

The 43 and 46 KDa proteins, which were identified to be activated MAP kinase using anti-ERK 1 antibody, displayed marked increases in phosphorylation when stimulated with fMLP. The tyrosine phosphorylation of the 42~46 KDa proteins as well as the MAP kinase activity observed in the gel band shift were inhibited by treatment with quercetin, suggesting that the tyrosine phosphorylation of 43 and 46 KDa proteins may be related to superoxide production. These data are consistent with Dusi and colleagues [24], who suggested that activation of MAP kinase might be correlated with superoxide generation in human neutrophils.

Of special interest was the observation that the inhibition profile by quercetin observed in PLD activation showed similarity with the inhibition of 45 KDa protein phosphorylation (30 and 50% at 0.1 and 10 μM of quercetin). Furthermore, this protein exhibits maximal peak phosphorylation at 30 sec (Fig. 2B) and is also correlated with the activation of PLD, which peaks at 1 min (Fig. 4A). These data suggest that tyrosine phosphorylation of the 45 KDa protein may regulate the activation of PLD in fMLP-stimulated neutrophils. However, further experiments are needed to confirm this hypothesis.

PLD was shown to participate in degranulation and superoxide production [18, 26]. In this study, we have demonstrated that preincubation with quercetin caused a greater inhibition of PLD activation than degranulation

inhibition in fMLP-stimulated neutrophils, suggesting that PLD activation may not be directly involved in degranulation in fMLP-stimulated neutrophils. On the other hand, the inhibition of superoxide production by quercetin was quite similar to PLD inhibition. These results indicate that PLD has a greater implication in superoxide production than in the degranulation process.

The different inhibition profiles in different neutrophil functions in which PLD is considered to be involved lead us to assume differential mechanisms in fMLP-stimulated PLD activation. Trudel *et al.* [27] have revealed divergent signaling pathways in granulocytic HL60 cells by using vanadate, the tyrosine phosphatase inhibitor. Vanadate was found to elicit an NADPH-dependent respiratory burst and actin polymerization, but not lysozyme release nor calcium-induced secretion, suggesting that protein tyrosine phosphorylation may not be involved in the regulatory mechanism of secretion in granulocytes. Under our experimental conditions, pervanadate (100 μM), but not orthovanadate (0.01, 0.1 and 1 mM), stimulated superoxide generation in intact neutrophils (data not shown). β-*N*-acetyl-glucosaminidase release was found to be small but significant, occurring only when the cells were treated with pervanadate (100 μM), but not with orthovanadate. These data suggest a small but significant participation of tyrosine phosphorylation in the degranulation processes.

In the present experiment, although PLD activation was largely inhibited by treatment with quercetin, inhibition of degranulation was unexpectedly low. Upon stimulation with chemoattractants, CB-pretreated neutrophils show high PLD activation in a PKC-independent process [28, 29]. Recent studies have reported the existence of isoforms of mammalian PLDs which are thought to be regulated by different regulatory mechanisms [30, 31]. Several lines of evidence have indicated that PLD is activated by various mechanisms such as PTK, PKC and small G-proteins (ARF and Rho) [32]. Given the evidence presented above, it is tempting to speculate the existence of different PLD isoforms in neutrophils which show distinct regulatory mechanisms in degranulation and superoxide production.

In summary, we have shown that several cellular events stimulated by fMLP were inhibited by quercetin in a dose-dependent manner in rabbit neutrophils. These events include PLD activation, tyrosine phosphorylation and MAP kinase activation. We suggest that impairment of neutrophil functions such as degranulation and superoxide production by flavonoids can be at least in part explained as the consequence of inhibition of tyrosine phosphorylation of specific proteins involved in the activation of these processes. The different profiles of inhibition in neutrophil functions suggest different extents of participation of tyrosine phosphorylation in the regulation of these functions in neutrophils.

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