

Effect of Three Flavonoids, 5,7,3',4'-Tetrahydroxy-3-methoxy Flavone, Luteolin, and Quercetin, on the Stimulus-Induced Superoxide Generation and Tyrosyl Phosphorylation of Proteins in Human Neutrophil

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The effect of three flavonoids, 5,7,3',4'-tetrahydroxy-3-methoxy flavone (THMF), luteolin, and quercetin, on the stimulus-induced superoxide generation and tyrosyl phosphorylation of proteins in human neutrophils were investigated. When the cells were preincubated with these flavonoids, the superoxide generation induced by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) was significantly suppressed, showing a dependence on amounts of the flavonoid. The suppressing effect of the flavonoid was THMF > luteolin > quercetin. These flavonoids also suppressed the superoxide generation induced by phorbol 12-myristate 13-acetate. In this case also, THMF was more effective than luteolin and quercetin. On the other hand, the superoxide generation induced by arachidonic acid was markedly suppressed by quercetin. The suppressing effect was quercetin >> THMF > luteolin. THMF, luteolin, and quercetin significantly suppressed tyrosyl phosphorylation of 80.1-, 58.0-, and 45.0-kDa proteins in fMLP-treated human neutrophils. The suppression depended on the concentration of the flavonoids, and the inhibition of tyrosyl phosphorylation was in parallel to that of the fMLP-induced superoxide generation, respectively. While luteolin and quercetin showed a weak hemolytic activity at 2.5 mM, THMF showed almost no hemolytic activity even at 5 mM, suggesting an advantage of THMF for its clinical use.

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Key Words: flavonoid; superoxide generation; tyrosyl phosphorylation; human neutrophil.

The dried flowers of *Gnaphalium indicum*, known in the Monterrey area in Mexico as “Gordolobo,” have been used in the Mexican folk medicine in a form of tea for the treatment of tussis and bronchitis (1). Three flavonoids, 5,7,3',4'-tetrahydroxy-3-methoxy flavone (THMF)² (2, 3), luteolin (4), and quercetin, were isolated from the methanol extracts of this plant and have been proved to have an anti-tumor promoter activity *in vivo* (5) as well as *in vitro* (6).

Recently, neutrophilic superoxide generation is known to correlate to various inflammations (7). Neutrophils play critical roles in the defense mechanism against microorganisms (8); when neutrophils are exposed to the various stimuli, one-electron reduction of molecular oxygen by NADPH oxidase leading to “respiratory burst” is induced (9, 10). *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), opsonized zymosan, phorbol 12-myristate 13-acetate (PMA), and arachidonic acid (AA) are known as the stimuli (11). We demonstrated that various compounds affected the stimulus-induced superoxide generations in human neutrophils: prolylproline, cystathionine metabolites,

² Abbreviations used: THMF, 5,7,3',4'-tetrahydroxy-3-methoxy flavone; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; PMA, phorbol-12-myristate-13-acetate; AA, arachidonic acid; KRP, Krebs-Ringer-phosphate; RBC, red blood cell.

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steroidal saponins, etc. (12–15). In many cases, the level of superoxide generation was in parallel to that of tyrosyl phosphorylation of 45.0-kDa protein in the cells.

In the present study, we investigated the effect of three flavonoids, THMF, luteolin, and quercetin, on the fMLP-, PMA-, and AA-induced superoxide generations and the tyrosyl phosphorylation of proteins in human neutrophils. We demonstrate herein their marked suppressive effect on the stimulus-induced superoxide generations.

MATERIALS AND METHODS

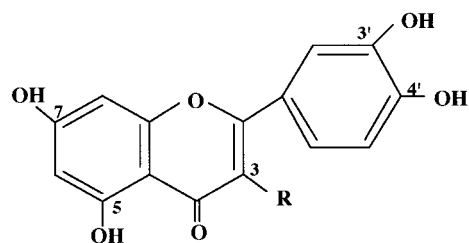
Chemicals. NADPH, ferricytochrome c (cyt. c), superoxide dismutase, fMLP, AA and PMA were purchased from Sigma Chemical Co. (St. Louis, MO). THMF was isolated from *G. indicum* according to the method of column chromatography using silica gel (16, 17). All other reagents used were of analytical grade, and were purchased from Nacalai Tesque Inc. (Osaka, Japan) unless otherwise mentioned.

Isolation of neutrophils. Polymorphonuclear leukocytes were isolated from the peripheral blood of healthy human by Ficoll-Hypaque (Flow Laboratories) density gradient centrifugation (18) and were washed twice with Krebs-Ringer-phosphate solution (KRP, pH 7.4) (12, 13). The cells were counted and resuspended in KRP at a concentration of 1×10^8 cells/ml.

Assay of superoxide generation. The superoxide generation was assayed by measuring the reduction of cyt. c at 37°C using a dual-beam spectrophotometer (Shimadzu UV-3000) under continuous stirring (12, 13). The standard assay mixture consisted of 1×10^6 cells/ml, 1 mM CaCl_2 , 20 μM cyt. c, 10 mM glucose, 0–40 μM flavonoid, and stimulus (12.5 nM fMLP, 1 nM PMA, or 10 μM AA) in a final volume of 2 ml KRP. After a preincubation for 3 min with a flavonoid, the reaction was started by adding a stimulus and the absorbance change at 550–540 nm ($\Delta A_{550-540}$) was monitored for 4 min.

Detection of tyrosyl phosphorylation of neutrophil proteins. Neutrophils (1×10^6 cells) were incubated in 1 ml of KRP containing 1 mM CaCl_2 , 10 mM glucose, 0–40 μM flavonoid, and 12.5 nM fMLP for 3 min at 37°C, then 0.5 ml of ice-cold 45% trichloroacetic acid (final concentration, 15%) was added to stop the reaction. After incubation for 30 min at 4°C, the mixture was centrifuged at 10,000g for 15 min at 4°C. The precipitate was washed twice with ice-cold diethyl ether/ethanol (1:1), dissolved in 50 μl of 62.5 mM Tris-HCl (pH 6.8) containing 2% sodium dodecyl sulfate (SDS), 0.7 M 2-mercaptoethanol, and 10% glycerol, and subjected to SDS-polyacrylamide gel electrophoresis with 12% gel (18). The electrophoresed proteins were transferred onto Immobilon-P membrane (Nippon Millipore Ltd.) using a semidry blotting apparatus for 90 min at 20 mV, and the tyrosyl phosphorylated proteins were detected using phosphotyrosine-specific monoclonal antibody (PY-20; ICN Biochemicals, Inc.), peroxidase-conjugated rabbit anti-mouse immunoglobulin G antibody (E. Y. Laboratories Inc.) and ECL Western blotting detection system (Amersham, Japan Co.) (18). Apparent molecular mass of the proteins was determined using prestained molecular weight standards (14,300–200,000 molecular weight range; Gibco-BRL).

Hemolysis measurement. Fresh blood from healthy human was collected (9 parts blood: 1 part of 3.8% sodium citrate) in plastic tubes, and red blood cells (RBCs) were separated by centrifugation at 2500 rpm for 10 min. The RBCs were washed once with 2 vol of 0.9% saline solution and then resuspended in 0.9% saline solution to give a 10% RBC suspension. A 0.25-ml measure of the RBC suspension was mixed with an equal volume of 0.9% saline solution containing



R = OCH ₃	5,7,3',4'-tetrahydroxy-3-methoxy flavone (THMF)
R = H	Luteolin
R = OH	Quercetin

FIG. 1. Chemical structure of THMF, luteolin, and quercetin.

flavonoid and then incubated at 37°C for 5 min with shaking. After being centrifuged at 1100 rpm for 5 min, 0.2 ml of the supernatant was diluted with 3.3 ml of distilled water and the absorbance was measured at 550 nm (A_{sample}). The percentage hemolysis ($H\%$) of each sample was calculated using the following equation: $H\% = A_{\text{sample}} / A_{100} \times 100$, where A_{100} is the absorbance of 100% hemolysis cells; i.e., 0.25 ml of 10% RBC suspension was incubated in 8.5 ml of distilled water (19).

RESULT AND DISCUSSION

Figure 1 shows the chemical structures of three flavonoids: THMF, luteolin, and quercetin. The effect of these flavonoids on superoxide generation in human neutrophils was investigated using fMLP, PMA, and AA as the stimuli. fMLP, PMA, and AA were used as the inducer of the receptor-mediated activation of neutrophils, activator of protein kinase C, and membrane perturber, respectively. When neutrophils were preincubated with THMF, luteolin or quercetin, the fMLP-induced superoxide generation was remarkably suppressed, depending on the concentration of flavonoid (Fig. 2). The suppression of the fMLP-induced superoxide generation by these flavonoids followed in the following order: THMF > luteolin > quercetin. In the absence of the stimulus, among these flavonoids THMF and luteolin did not induce superoxide generation (data not shown). The PMA-induced superoxide generation was also suppressed by these flavonoids in a concentration-dependent manner (Fig. 3), while the efficiencies were lower than those to the fMLP-induced superoxide generation. The suppression of the PMA-induced superoxide generation by these flavonoids followed in the following order: THMF > quercetin > luteolin. In case of the AA-induced superoxide generation, the suppressive effect of quercetin was remarkable (Fig. 4). The suppression of the AA-induced superoxide generation by these flavonoids followed in the following order: quercetin \gg THMF > luteolin. These flavonoids suppressed all the three types of superoxide generation induced by fMLP, PMA, and AA, respec-

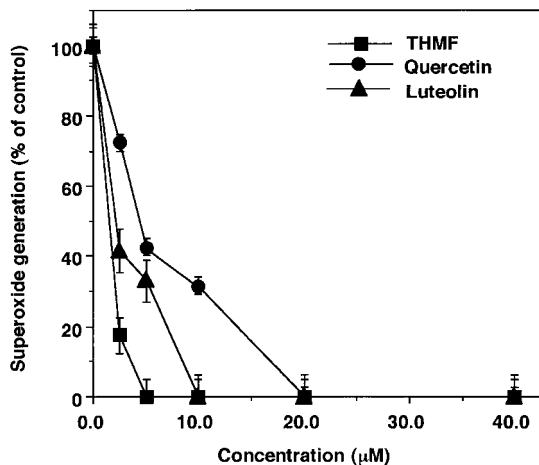


FIG. 2. Effect of THMF, luteolin, and quercetin on fMLP-induced superoxide generation in human neutrophils. The cells were preincubated with the flavonoid (0–40 μM) for 3 min prior to the addition of stimulus (12.5 nM fMLP) and the superoxide generation was measured as described under Materials and Methods. The production of superoxide in the control experiments were $8.8 \pm 0.4 \mu\text{M}$ for THMF, $7.3 \pm 1.3 \mu\text{M}$ for luteolin, and $10.2 \pm 2.1 \mu\text{M}$ for quercetin.

tively. The effects of these flavonoids on the three stimulus-induced superoxide generations were different with each other. The rates of suppression by the flavonoid on the superoxide generations were in the following order: THMF, fMLP \gg PMA > AA; luteolin, fMLP > AA > PMA; quercetin, fMLP and AA \gg PMA. These suppressive effects were observed when the neutrophils were treated with the flavonoid prior to the addition of stimulus. When 40 μM THMF was added to the reaction mixture 1 min after addition of PMA, for example, the superoxide generation was not suppressed, thus suggesting that the flavonoid does not act as a superoxide scavenger but inhibits the activation of NADPH oxidase (data not shown).

The chemical structures of three flavonoids are different in substitutive residue at the position of C-3. THMF containing $-\text{OCH}_3$ at the position of C-3 more strongly suppressed the fMLP- and PMA-induced superoxide generations than the other two flavonoids. Quercetin containing $-\text{OH}$ at the position of C-3 more strongly suppressed the AA-induced superoxide generation than the other two flavonoids. On the other hand, luteolin containing $-\text{H}$ at the position of C-3 significantly suppressed the fMLP-induced superoxide generation, but the suppression of PMA- and AA-induced superoxide generations was weak. Although the relationship between chemical structure and the suppressing effect of these flavonoids is unclear at present, it might be noteworthy that the residues linking to the position of C-3 in the flavonoids largely contribute to determining its suppressive effect on the stimulus-induced superoxide generation in human neutrophils.

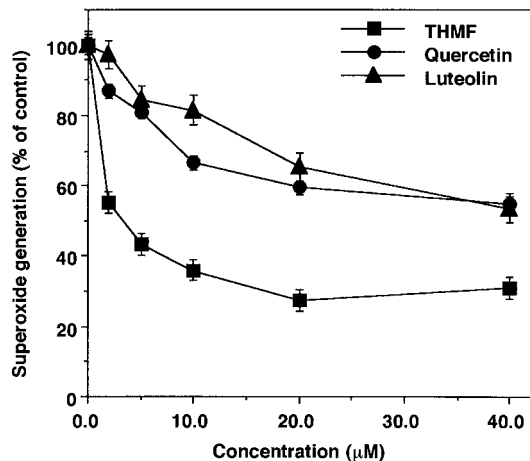


FIG. 3. Effect of THMF, luteolin, and quercetin on PMA-induced superoxide generation in human neutrophils. The assay was carried out as described in the legend to Fig. 2, except 1 nM PMA was used as the stimulus. The production of superoxide in the control experiments were $18.2 \pm 4.9 \mu\text{M}$ for THMF, $20.0 \pm 0.8 \mu\text{M}$ for luteolin, and $22.5 \pm 5.3 \mu\text{M}$ for quercetin.

In a series of our study on the superoxide generation and inflammation, we found that various compounds, such as prolylproline and cystathionine metabolites, induced tyrosyl phosphorylation of proteins in parallel to the enhancement of fMLP-induced superoxide generation in human neutrophils (12, 13). The tyrosyl phosphorylation was inhibited by genistein and herbimycin A, inhibitors of protein tyrosine kinase, suggesting the participation of protein tyrosine kinase in the mechanism for priming of human neutrophils by these compounds (13). In the present study, to gain the in-

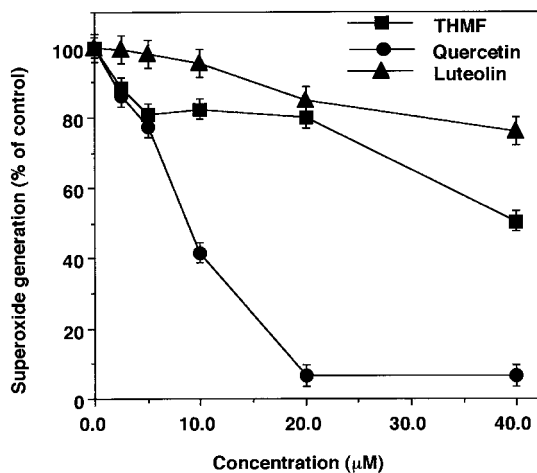


FIG. 4. Effect of THMF, luteolin, and quercetin on AA-induced superoxide generation in human neutrophils. The assay was carried out as described in the legend to Fig. 2, except 10 μM AA was used as the stimulus. The production of superoxide in the control experiments were $9.5 \pm 0.9 \mu\text{M}$ for THMF, $13.4 \pm 4.9 \mu\text{M}$ for luteolin, and $9.1 \pm 2.2 \mu\text{M}$ for quercetin.

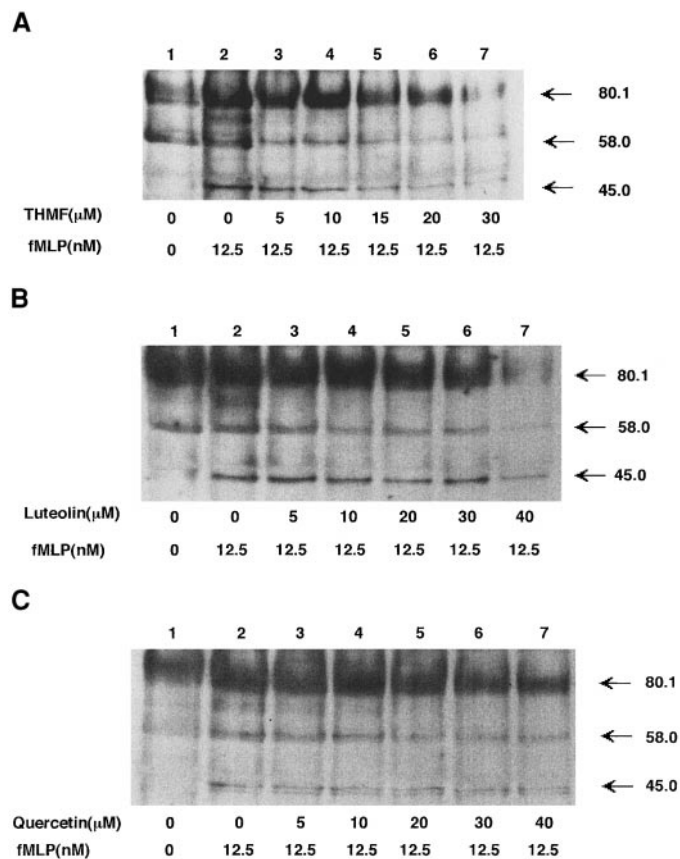


FIG. 5. Dose-dependent suppression of protein tyrosyl phosphorylation by THMF (A), luteolin (B), and quercetin (C) in fMLP-treated human neutrophils. The cells were incubated with the flavonoid in the presence or absence of 12.5 nM fMLP, and then the tyrosyl phosphorylated proteins were detected using phosphotyrosine-specific monoclonal antibody (PY20) as described under Materials and Methods. Arrows on the right side indicate apparent molecular mass of the bands, respectively.

sights into mechanism of suppression of stimulus-induced superoxide generation by the flavonoids, we examined the effect of three flavonoids on the tyrosyl phosphorylation of proteins in fMLP-treated human neutrophils. When neutrophils were incubated with fMLP, tyrosyl phosphorylation of 80.1-, 58.0-, and 45.0-kDa proteins was induced. However, in the presence of THMF, luteolin, or quercetin, the tyrosyl phosphorylation was dose-dependently suppressed (Fig. 5). These results well coincided with the change of superoxide generation level. In our previous study, prolylproline and cystathionine ketimine enhanced tyrosyl phosphorylation of 45.0-kDa protein in parallel to that of fMLP-induced superoxide generation in human neutrophils (12, 13). The flavonoids examined in this study suppressed tyrosyl phosphorylation of several other proteins. Suppression of superoxide generation via suppressing the tyrosyl phosphorylation of neutrophil

proteins may be one of the pharmaceutical effects of these flavonoids.

In conclusion, THMF, luteolin, and quercetin showed a suppressive effect on the stimulus-induced superoxide generation in human neutrophils. Therefore, a protective effect for blood vessels is expectable from these flavonoids. It is known that neutrophilic superoxide generation correlates to various inflammations especially in the dermatology field (7). Furthermore, these flavonoids have been proved to have an anti-tumor promoter activity (4, 5). Considering the possibility of clinical use, we examined the hemolytic effect of these flavonoids. All the three flavonoids showed no effect on hemolysis at 100 μ M (data not shown), which is several

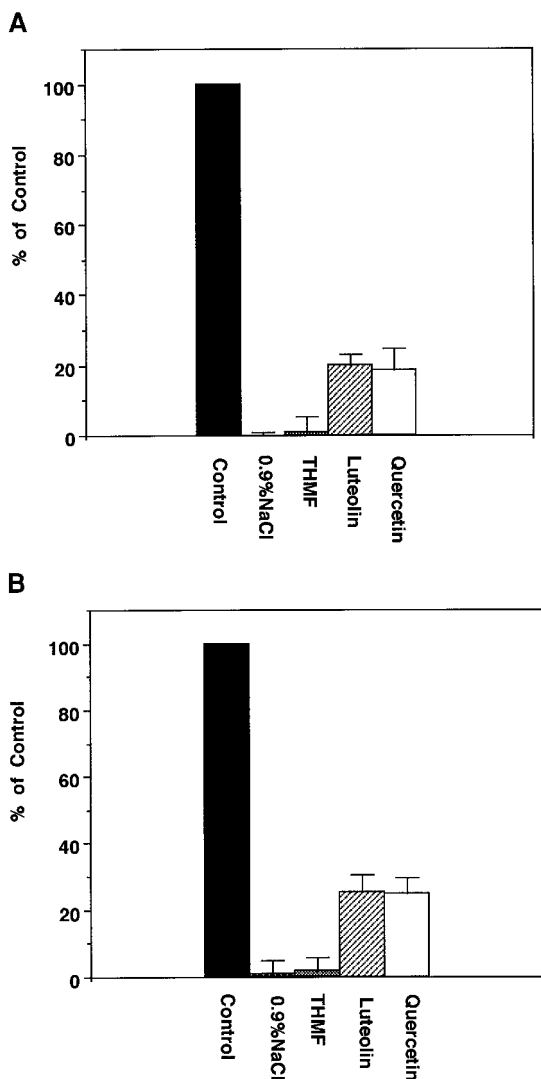


FIG. 6. Hemolytic effect of THMF, luteolin, and quercetin on human erythrocytes. The assay was carried out as described under Materials and Methods. The final concentration of the flavonoid was 2.5 mM (A) and 5 mM (B), respectively. Control means the hemolysis by distilled water, which is shown as 100%. The bars indicate mean \pm SD ($n = 5$), respectively.

times higher than the concentrations enough for the suppression of superoxide generations. As shown in Fig. 6, luteolin and quercetin showed a hemolytic activity at 2.5 mM. However, THMF showed no effect on hemolysis even at 5 mM. These results suggest that THMF has an advantage for clinical use. Further studies on the pharmaceutical functions and immunological responses of THMF may help in the development of clinical application.

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