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Biochemical Pharmacology 67 (2004) 2251–2262
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Prevention of macrophage adhesion molecule-1 (Mac-1)-dependent neutrophil firm adhesion by taxifolin through impairment of protein kinase-dependent NADPH oxidase activation and antagonism of G protein-mediated calcium influx

Yea-Hwey Wang[a], Wen-Yen Wang[b], Jyh-Fei Liao[a], Chieh-Fu Chen[a,d], Yu-Chang Hou[c], Kuo-Tong Liou[f], Yueh-Ching Chou[g,h], Jung-Hsiung Tien[g], Yuh-Chiang Shen[d,e,*]

[a]*Institute of Pharmacology, School of Medicine, National Yang-Ming University, Taipei, Taiwan*
[b]*Departments of Surgery, Tao-Yuan General Hospital, Department of Health, Taiwan*
[c]*Chinese Medicine, Tao-Yuan General Hospital, Department of Health, Taiwan*
[d]*National Research Institute of Chinese Medicine, Taipei, Taiwan*
[e]*Institute of Biological Sciences, College of Life Science, National Chung-Hshing University, Taichung, Taiwan*
[f]*Department of Chinese Martial Arts, Chinese Culture University, Taipei, Taiwan*
[g]*Department of Pharmacy, Taipei Veterans General Hospital, Taipei, Taiwan*
[h]*School of Pharmacy, Taipei Medical University, Taipei, Taiwan*

Received 30 September 2003; accepted 10 February 2004

Abstract

Taxifolin has been reported to down-regulate the expression of intercellular adhesion molecule-1 (ICAM-1), a receptor-mediating firm adhesion with β2 integrin (e.g., Mac-1) expressed on leukocytes. To evaluate whether taxifolin could modulate Mac-1-dependent firm adhesion by neutrophils, and the possible mechanism(s) underlying its anti-inflammatory action, its effects on *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol-12-myristate-13-acetate (PMA)-activated peripheral human neutrophils were studied. Pretreatment with taxifolin (1–100 μM) concentration-dependently diminished fMLP- or (PMA)-induced Mac-1-dependent firm adhesion and upexpression of surface Mac-1. Mobilisation of intracellular calcium and production of reactive oxygen species (ROS) signal the upexpression of Mac-1 and firm adhesion by neutrophils. Taxifolin impeded the calcium influx induced by fMLP (a receptor-mediated activator) or AlF_4^- (a G protein-mediated activator). Taxifolin also effectively inhibited the fMLP- or PMA-induced ROS production with 50% inhibitory concentration (IC_{50}) less than 10 μM, possibly through impairing the activation of NADPH oxidase, a major ROS-generating enzyme in neutrophils, by restricting the activation of p38 mitogen-activated protein kinase (p38 MAPK) and protein kinase C (PKC). In conclusion, we propose that impairment of ROS production by NADPH oxidase through interfering with p38 MAPK- and/or PKC-dependent signals, and antagonism of G protein-mediated calcium influx may account for the inhibition of Mac-1-dependent neutrophil firm adhesion that confers taxifolin the anti-inflammatory activity.

Keywords: Calcium; Mac-1 (CD11b/CD18); NADPH oxidase; p38 Mitogen-activated protein kinase; Protein kinase C; Taxifolin

1. Introduction

Plant flavonoids have long been reported to inhibit the functions of human inflammatory cells, possibly through modulation of enzyme systems related to inflammatory responses and/or scavenging of reactive oxygen radicals. Of these, quercetin is the most documented [1]. Taxifolin (Fig. 1), with structure similar to that of quercetin, has been

Abbreviations: fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; Mac-1, macrophage adhesion molecule-1; p38 MAPK, p38 mitogen-activated protein kinase; PMA, phorbol-12-myristate-13-acetate; PKC, protein kinase C; ROS, reactive oxygen species

* Corresponding author. Tel.: +886-2-28201999x9101; fax: +886-28264266.
E-mail address: yuhcs@nricm.edu.tw (Y.-C. Shen).

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doi:10.1016/j.bcp.2004.02.020

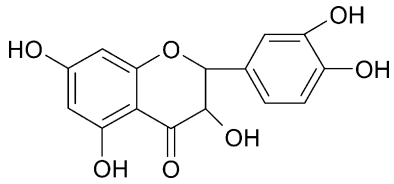


Fig. 1. Chemical structure of taxifolin.

shown to exhibit anti-inflammatory effects in protection against oxidative cellular injury in rat peritoneal macrophage [2] and human endothelial cells [3]. Recently, Bito et al. reported that taxifolin is the most potent flavonoids screened for inhibiting the expression of intercellular adhesion molecule-1 (ICAM-1) in human epidermal keratinocytes [4]. Expression of ICAM-1 on inflammatory target cells (e.g., epidermal cells, endothelial cells) mediates the activation and firm adhesion of circulating neutrophils to the injured tissue through interaction with β2 integrin expressed on neutrophils [5]. Drugs with antioxidative or anti-inflammatory effects could modulate upexpression of Mac-1 (CD11b/CD18), a dominant β2 integrin in activated-human neutrophils [6,7]. These observations suggest that impairing the interaction between peripheral neutrophils and their target cells by potential drugs could prevent further activation of these inflammatory cells and confer drugs with anti-inflammatory effects. Thus, the effects of taxifolin on surface Mac-1 expression and Mac-1-dependent firm adhesion by neutrophils as well as the possible mechanism(s) in mediating its anti-inflammatory effect were investigated in this study.

Activation of neutrophils during inflammation can result in the release of large amounts of ROS including superoxide anion ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) by NADPH oxidase, a powerful oxidant-producing enzyme assembled on the surface of activated-neutrophils [8]. Other generators of ROS in neutrophils include myeloperoxidase (MPO) and cyclooxygenase (COX) [9,10]. Although ROS plays an essential role in the host defence mechanism(s) against pathogen invasion, uncontrolled production of these ROS by leukocytes may lead to crippling inflammatory disorders such as myocardial infarction, neurodegeneration, ischemic/reperfusion (I/R) injury, adult respiratory distress syndrome as well as atherosclerosis [11]. The molecular mechanism(s) in mediating these inflammatory disorders begin with the recruitment and accumulation of leukocytes, especially neutrophils, at injury sites via firm adhesion of these activated leukocytes to endothelial cells through the counter receptors expressed on them (e.g., Mac-1 and ICAM-1 for neutrophils and endothelial cells, respectively) [12,13].

In this study, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP, a receptor-mediated activator)- or phorbol-12-myristate-13-acetate (PMA, a direct PKC activator)-induced Mac-1 upexpression and firm adhesion in human neutrophils were performed as an in vitro model to elucidate whether prevention of Mac-1 upexpression and firm adhesion by neutrophils accounts for the possible mechanism(s) underlying the anti-inflammatory effect of taxifolin. To further investigate which signalling pathway(s) may be targeted by taxifolin, especially for the ROS production, its effects on mitogen-activated protein kinase (MAPK)- and protein kinase C (PKC)-dependent NADPH oxidase activation, and fMLP- or G protein-mediated calcium influx were elucidated.

2. Materials and methods

2.1. Human neutrophils preparation

All experimental protocols were approved by our Institutional Review Board in accordance with international guidelines. In addition, knowledgeable consents were obtained from the subjects who participated in this study. Human neutrophils were prepared from blood obtained through venipuncture from adult healthy volunteers and collected into syringes containing heparin (20 U/ml blood). Blood samples were mixed with equal volumes of 3% dextran solution in a 50-ml centrifuge tube and incubated in an upright position for 30 min at room temperature to allow sedimentation of erythrocytes. The upper, leukocyte-rich layer was collected and subjected to centrifugation at 250 × *g* for 15 min at 4 °C. Neutrophils and peripheral blood mononuclear cells (PBMC) were then separated by the Ficoll gradient centrifugation method followed by lysis of contaminating erythrocytes [14]. Samples so purified contained more than 95% neutrophils as estimated by counting 200 cells under a microscope after Giemsa staining (Sigma Chemical Co., MO, USA). In all cases where neutrophils were pretreated with taxifolin, the cells were mixed with the drug(s) at concentrations ranging from 1 to 100 μM in Hank's buffered saline solution (HBSS) for 20 min at 37 °C.

2.2. Measurement of neutrophil firm adhesion

Firm adhesion of neutrophils to extracellular matrix was determined in 24-well tissue culture plates (FALCON®) coated with fibrinogen as in our previous study [7]. Prior to the addition of neutrophils, the plates were incubated with 250 μl per well of human fibrinogen (50 μg/ml in phosphate buffered saline (PBS); Chemicon International, Inc.) for 2 h at 37 °C. The wells were washed once with HBSS, blocked with 1% BSA (Sigma) in HBSS for 1 h at 37 °C, and washed twice with HBSS containing 0.1% Tween 20 (Sigma) and once with HBSS. Immediately prior to being added to the coated plates, the neutrophils (1×10^7 ml^{-1}) were loaded with 1 μM 2′,7′-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Molecular Probe) in HBSS for 20 min at 37 °C and then washed twice with 10 ml of HBSS without Mg^{2+} or Ca^{2+}. Two hundred microliter per well of taxifolin-pretreated

(for 20 min) BCECF-AM-labelled neutrophils (5×10^5 ml^{-1} in HBSS) were then added to each wells. After stimulation either with a receptor agonist, fMLP (1 μM), or a PKC activator, PMA (100 ng/ml), for 40 min at 37 °C, non-adherent cells were removed by aspiration and the wells were gently washed twice with warm PBS containing 1 mM Ca^{2+}. Adhered neutrophils were then determined by measuring the fluorescence with a fluorescent plate reader (Cytofluor 2300, Millipore®) with excitation at 485 nm and emission at 530 nm. Data are expressed as fluorescence intensity.

2.3. Measurement of Mac-1 (CD11b/CD18) upregulation by flow cytometry

Upregulation of Mac-1 was analysed as in our previous study [7]. In brief, taxifolin-pretreated samples (2×10^6 ml^{-1} in HBSS) were stimulated with fMLP (1 μM) or PMA (100 ng/ml) for 15 min. The cells were then pelleted and resuspended in 1 ml of ice-cold PBS containing 10% heat-inactivated fetal bovine serum (FBS) and 10 mM sodium azide (NaN_3). For staining of Mac-1, all subsequent steps were carried out on ice-bath. Cells were incubated in the dark for 60 min with a proper aliquot of fluorescein isothiocyanate (FITC)-conjugated anti-Mac-1 antibody (mouse-anti-human CD11b, class IgG_1; BD Biosciences Pharmingen) or a non-specific mouse antibody (class IgG_1, Sigma) as a negative control. After two washes with PBS containing 5% FBS, stained-cells were resuspended in flow cytometer sheath fluid (Becton Dickinson) containing 1% paraformaldehyde and analysed by flow cytometry for Mac-1 expression. Data are expressed as mean channel fluorescence for each sample as calculated by the CellQuest® software (Becton Dickinson) on a Power Macintosh 7300/200 computer.

2.4. Determination of intracellular calcium concentration ($[Ca^{2+}]_i$)

Neutrophils were preloaded with 5 μM 1-[2-(5-carboxyoxal-2-yl)-6-amino-benzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxy-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (fura 2-AM; Molecular Probes) at 37 °C for 45 min, washed twice and resuspended at 2×10^6 ml^{-1} in calcium-free HBSS containing taxifolin or control vehicle. After pretreatments for 5 min, 1 ml of cell suspensions from each sample and 1 ml of HBSS containing 2 mM Ca^{2+} were transferred to individual cuvettes and gently mixed with a micromagnetic stirrer at 37 °C for 5 min before the addition of fMLP (1 μM) or a G protein activator AlF_4^- (10 mM NaF plus 30 μM $AlCl_3$) or thapsigargin (1 μM), a releaser of calcium from intracellular store. The fluorescence of fura-2-loaded cells was measured by a spectrofluorometer (Hitachi F-4500) with excitations at 340 and 380 nm and emission at 510 nm. Intracellular calcium concentration for each sample was calculated from the ratio of emission versus excitation as previously described [7].

2.5. Measurement of reactive oxygen species (ROS) generation

Reactive oxygen species generation was evaluated according to our previous method [15]. In brief, in white 96-well, flat-bottom EIA/RIA microplate (Costar), apocynin (a NADPH oxidase inhibitor) or taxifolin was serially diluted to final volumes of 10 μl. To each well, 50 μl of neutrophils suspension (1×10^7 cells/ml) and 50 μl of luminol (45 μM) or lucigenin (180 μM) solutions were added. Neutrophils were triggered by adding 50 μl of PMA (20 nM) or fMLP (1 μM), and chemiluminescence was monitored every 1 min for 1 s during a 30-min observation period using a microplate luminometer reader (Orion®) and represented as relative light units (RLU). Peak levels were recoded to calculate the activity of test drugs in relation to their corresponding solvent controls (0.25% DMSO). The 50% inhibitory concentration (IC_{50}) of PMA- or fMLP-triggered chemiluminescence by test drugs was calculated using a semilog-plot transformation of the data.

2.6. Measurement of NADPH oxidase activity in subcellular fractions

Particulate fraction of neutrophils was prepared according to our previous report [15]. In brief, neutrophils (2×10^6 ml^{-1}) were incubated for 10 min at 37 °C in the presence of either PMA (100 ng/ml) or an equivalent concentration of DMSO. After incubation, the cell suspension was centrifuged at 4 °C at 300 × *g* for 6 min, and the pellet was resuspended in sample buffer containing 100 mM KCl, 3 mM NaC1, 3.5 mM $MgC1_2$, 1.25 mM EGTA, 10 mM PIPES, 2 mM PMSF, 33 μM leupeptin, 35 μM antipain, 24 μg/ml chymostatin, 0.035 μM pepstatin, 0.08 μM aprotinin and pH 7.3. The cells were disrupted using a microprobe sonicator at low power (10%) three times for 10 s at 4 °C. Intact cells were removed by centrifugation at 500 × *g* for 5 min. The resulting supernatant was centrifuged at 4 °C for 20 min at 115,000 × *g* after which the supernatant was discarded and the pellet (particulate fractions) was washed in sample buffer and recentrifuged again. Following the removal of the supernatant, the pellet was gently resuspended in assay buffer without protease inhibitors. NADPH oxidase activity was determined in this particulate fractions in the presence of 400 μM NADPH by monitoring $O_2^{-\bullet}$ generation for 20 min at 25 °C via SOD-inhibitable cytochrome *c* reduction assay. Taxifolin was added to the PMA-assembled particulate fractions 20 min before the addition of NADPH (for subcellular NADPH oxidase activity study). Alternatively, neutrophils were preincubated with taxifolin, staurosporine (a PKC inhibitor) or cromolyn, an inhibitor for

oxidase assembly [16], for 20 min at 37 °C before PMA stimulation (for the study of the assembly of NADPH oxidase). Data are expressed as $O_2^{-\bullet}$ nmol per 2×10^6 cell equivalents per 20 min.

2.7. Western immunoblotting analysis for p38 mitogen-activated protein kinase

To further examine the effect of taxifolin on the activation of p38 MAPK, phospho-p38 was analysed by Western immunoblotting assay. Neutrophils (10^7 cells in 1.5 ml HBSS) were stimulated with PMA (100 ng/ml) for 10 min or fMLP (1 μM) for 3 min at 37 °C in the presence or absence of taxifolin (10–50 μM). After washing once with ice-cold PBS, cells were resuspended in lysis buffer consisting of Tris–HCl pH 7.4 (50 mM), 2-mercaptoethanol (50 mM), phenylmethyl sulfonyl fluoride (1 mM), leupeptin (1 mg/ml), NaF (1 mM), Na_3VO_4 (1 mM), EDTA (2 mM) and EGTA (5 mM), and were sonicated on ice for three 10-s cycles. After centrifugation at $18{,}000 \times g$, 4 °C for 30 min, supernatant was collected and protein concentration was determined by using a BCA protein assay kit (Pierce). Equal amount of protein (40 μg) from different treatments were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 10% mini-gels. The proteins were then electro-transferred to a hydrophobic polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Pharmacia). The membrane was preblocked with 5% non-fat milk in PBS containing 0.05% Tween 20 (PBST) at 4 °C for 1 h with agitation. After three 15-min washes with PBST, the membrane was incubated overnight at 4 °C with an antibody against p38 MAPK or phospho-p38 MAPK (mouse-anti-human, isotype: IgG; BD Pharmingen) at a dilution of 1:1000. After three additional washes with PBST, the membrane was incubated with a second antibody (goat-anti-mouse IgG conjugated with horseradish peroxidase; Amersham, England) for 1 h at room temperature. The immuno-blot on the membrane was visible after developing with an enhanced chemiluminescence (ECL) system (Amersham).

2.8. Protein Kinase C activity assay

Neutrophils were resuspended to a concentration of 2×10^7 ml^{-1} in ice-cold extraction buffer [17]. The extraction buffer consisted of 50 mM Tris–HC1 (pH 7.5), 50 mM EGTA, 1 mM phenylmethylsulphonyl fluoride, protease inhibitor cocktail (Calbiochem) and 50 mM 2-mercaptoethanol. Protease inhibitor cocktail consisted of 0.1 mM of leupeptin, 1 μM of pepstatin A, 5 μM of bestatin, 0.08 μM of aprotinin and 1.5 μM of cysteine protease inhibitor. The cell suspension was sonicated for 10 s at 4 °C for five times. The cell lysate was separated into the cytosol (as cytosolic fraction) and pellet fractions by centrifugation at $100{,}000 \times g$ for 60 min at 4 °C. The pellet was solubilised by resuspending in extraction buffer with 0.1% Triton X-100, vortexed and incubated at 4 °C for 60 min. The solubilised extracts and the particulate fraction were separated by centrifugation at $10{,}000 \times g$ for 5 min. Both cytosolic and particulate fractions were assayed subsequently for kinase activity on the same day. Protein concentration was determined with a protein assay kit (Bio-Red). PKC activity was measured with a non-radioactive protein kinase assay kit (Calbiochem). This kit was based on an enzyme-linked immunosorbent assay (ELISA) that used a synthetic PKC pseudosubstrate and a monoclonal antibody that recognised the phosphorylated peptide. PKC phosphorylates the serine residue on the pseudosubstrate (peptide) through a Ca^{2+}/phosphatidylserine (PS)-dependent-mechanism. Ca^{2+}/phospholipid-dependent PKC activity was assayed in the presence of Ca^{2+} and PS while the negative control was measured in the presence of 20 mM EGTA. In some experiments, PMA (100 ng/ml) was added to live neutrophils for stimulation/translocation of PKC prior to sonication, and cytosolic PKC activity from this preparation was used as a negative control as most of the cytosolic PKC had translocated to the particulate fraction. Data are expressed as $100 \times OD_{492}/12$ μl of cell lysate from 2×10^7 cells.

2.9. Reactive oxidant-scavenging assay

Xanthine oxidase catalyses the oxidation of xanthine to produce uric acid as the final product, during this reaction both superoxide anion and hydrogen peroxide are produced [18]. The generation of these oxidants was performed in a 1 ml volume of PBS containing xanthine (160 μM) and xanthine oxidase (20 mM). The reaction was monitored both by the reduction of cytochrome *c* (0.5 mg/ml) at 550 nm and the production of uric acid at 290 nm over 5 min using a spectrophotometer (Hitachi). The reduction of cytochrome *c* by superoxide was inhibited by the addition of 100 U of superoxide dismutase (SOD) to the reaction mixture.

2.10. Taxifolin and other chemicals

(±)-Taxifolin was purchased from OXIS. It was first dissolved in dimethyl sulfoxide (DMSO) at 20 mM as a stock solution and then serially diluted in PBS immediately prior to experiments. Aliquot stock solution was stored at −20 °C and was used within 1 week after preparation. For examination of the effects of these drugs, 10 μl of drug solution was added to 1.0 ml of neutrophils suspension and incubated at 37 °C for 20 min prior to the addition of PMA (Calbiochem) or fMLP (Sigma). For the study of protein kinases the following inhibitors were used including GF109203x (Upstate), rottlerin (Upstate), staurosporine (Calbiochem), PD98059 (Tocris), SP600125 (Tocris), SB203580, SB202190 (Tocris). Other chemicals, except where indicated, were purchased from Sigma.

2.11. Statistical analysis

All values in the text and figures are given as means ± S.E.M. Parametric data were analysed by one-way or two-way analyses of variance (ANOVA) depending on the number of experimental variables followed by post hoc Dunnett's *t*-test for multiple comparisons. Values of $P < 0.05$ were considered significant.

3. Results

3.1. Effect of taxifolin on neutrophil firm adhesion and Mac-1 (CD11b/CD18) upregulation

To test whether taxifolin could interfere neutrophil firm adhesion, we measured the effect of taxifolin pretreatment on fMLP- or PMA-induced neutrophil firm adhesion to a fibrinogen-coated surface. FMLP (1 μM) or PMA (100 ng/ml) triggered remarkable increases in firm adhesion to three- or four-fold the basal level, respectively (Fig. 2). Taxifolin concentration-dependently impeded the firm adhesion induced by fMLP or PMA (Fig. 2). Pretreatment with 0.5 μg/ml of pertussis toxin (a G protein inhibitor) or 0.2 μM of staurosporine (a PKC inhibitor) completely prevent the fMLP- or PMA-induced firm adhesion, respectively. Furthermore, pretreatment with 25 μM of SB203580 (a p38 MAPK inhibitor) or 10 μM of apocynin (a NADPH oxidase inhibitor) also significantly reduced the fMLP- or PMA-induced firm adhesion around 30%.

Since Mac-1 mediates the firm adhesion of neutrophil to extracellular matrix [6], we further examined the effect of taxifolin on the Mac-1 upexpression induced by these activators. PMA and fMLP caused a dramatic increase in Mac-1 fluorescence (Fig. 3A). A leftward shift of the Mac-1 fluorescence curve was observed in samples pretreated with 100 μM of taxifolin (Fig. 3A). A statistical summary of 4–6 flow cytometric experiments illustrated significant inhibition of fMLP- or PMA-induced Mac-1 upregulation by taxifolin in a concentration-dependent manner (Fig. 3B). Pretreatment with 0.5 μg/ml of pertussis toxin or 0.2 μM of staurosporine significantly prevent the fMLP- or PMA-induced Mac-1 upexpression, respectively (data not shown).

The concentrations of these drugs used in this study were not cytotoxic to neutrophils because cell viability after drugs treatment was always more than 95% as estimated by the trypan blue exclusion. Moreover, cell viability was further compared by detection of the lactate dehydrogenase (LDH) activity using a cytotoxicity detection kit [17] or by staining dead/dying cell with propidium iodide and viable cells with fluorescein diacetate, followed by analysis on a flow cytometer [17], and comparable results were observed by these two methods.

3.2. Effect of taxifolin on fMLP- or AlF_4^--induced $[Ca^{2+}]_i$ mobilisation

Cytosolic calcium fluctuation can regulate leukocyte adhesion [19]. We had previously reported that impediment to calcium influx diminished Mac-1-dependent neutrophil

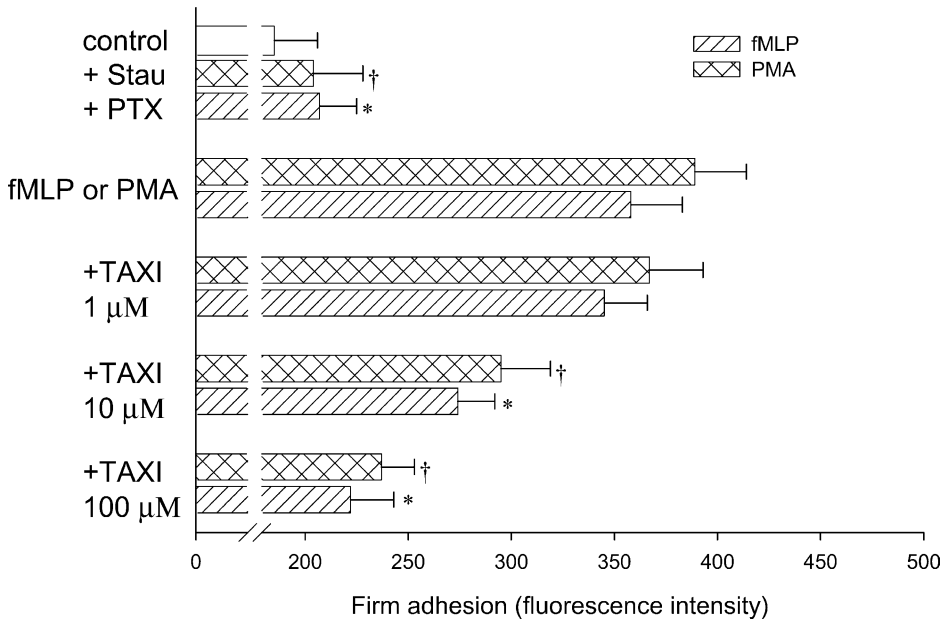
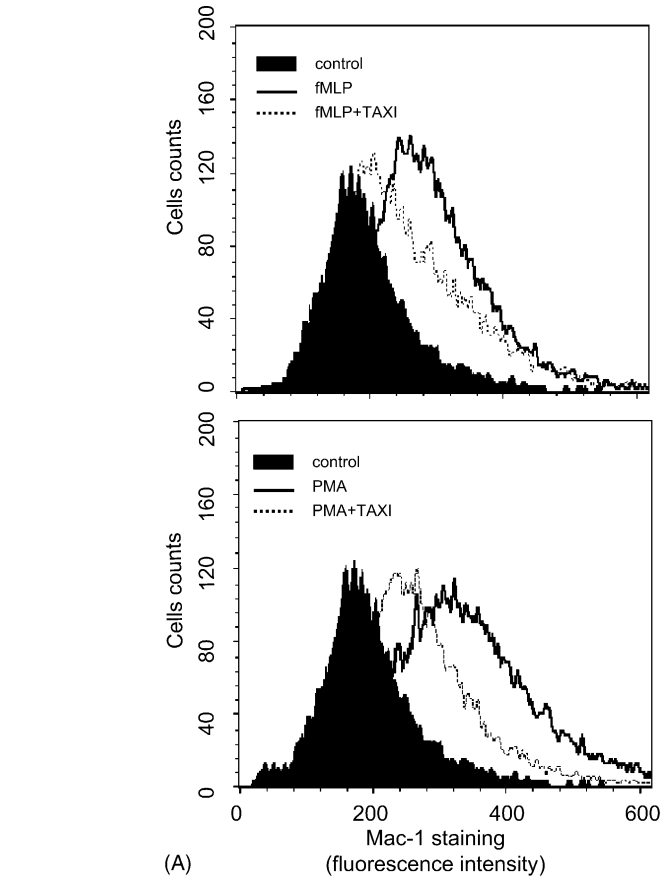


Fig. 2. Effect of taxifolin on fMLP- or PMA-induced firm adhesion by human peripheral neutrophils. Neutrophils were isolated and loaded with BCECF-AM (1 μM). BCECF-labelled neutrophils (5×10^5 cells/ml) were pretreated with 1–100 μM of taxifolin (TAXI), 0.2 μM of staurosporine (Stau, a PKC inhibitor) or 0.5 μg/ml of pertussis toxin (PTX) for 10 min then plated into fibrinogen-coated 24-well plates followed by stimulating with fMLP (1 μM) or PMA (0.1 μg/ml) for 30 min. Non-adherent cells were removed and firm adherent of neutrophils were determined by measuring the fluorescence by a fluorescent plate reader. Data are expressed as fluorescence intensity. Values are means ± S.E.M. from four to six experiments performed on different days using cells from different donors. $^{*,\dagger}P < 0.05$ as compared to samples treated with fMLP or PMA alone, respectively.



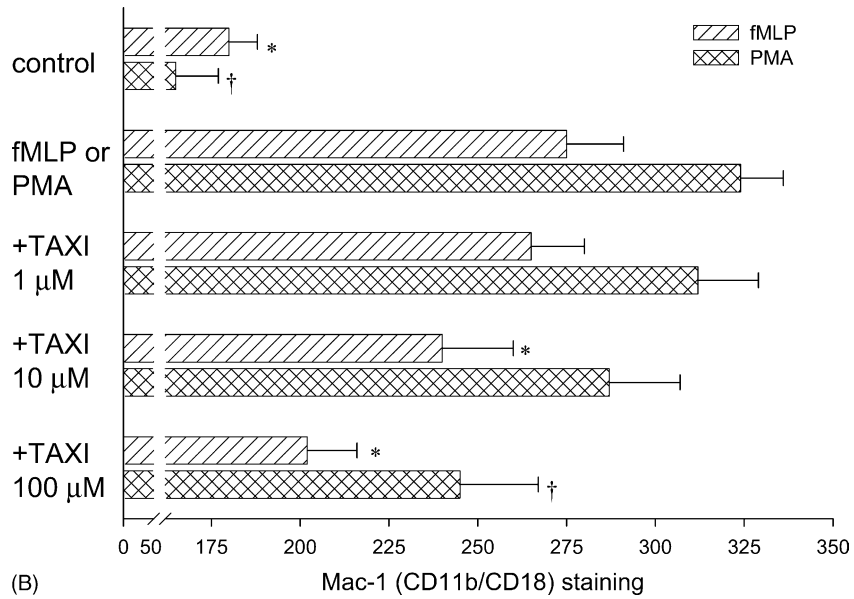


Fig. 3. Effect of taxifolin on fMLP- or PMA-induced Mac-1 (CD11b/CD18) upregulation. (A) Flow cytometric analysis of a representative experiment. fMLP (1 μM)- or PMA (100 ng/ml)-stimulated neutrophils in the presence or absence of 100 μM of taxifolin (TAXI), were stained in an ice-bath with anti-CD11b (Mac-1) antibody and total Mac-1 level on the cell surface was quantitated by FACSCalibur™. Control level represents samples that were neither treated with taxifolin nor stimulated with fMLP or PMA. All the TAXI-pretreated groups, designated '+TAXI', were stimulated with fMLP (upper panel) or PMA (lower panel). (B) Statistical summary of fMLP- or PMA-upregulated Mac-1 expression by neutrophils in the presence of 1–100 μM of taxifolin (TAXI). Values are means ± S.E.M. from four to six experiments performed on different days using cells from different donors. $^{*,\dagger}P < 0.05$ as compared to samples treated with fMLP or PMA alone, respectively.

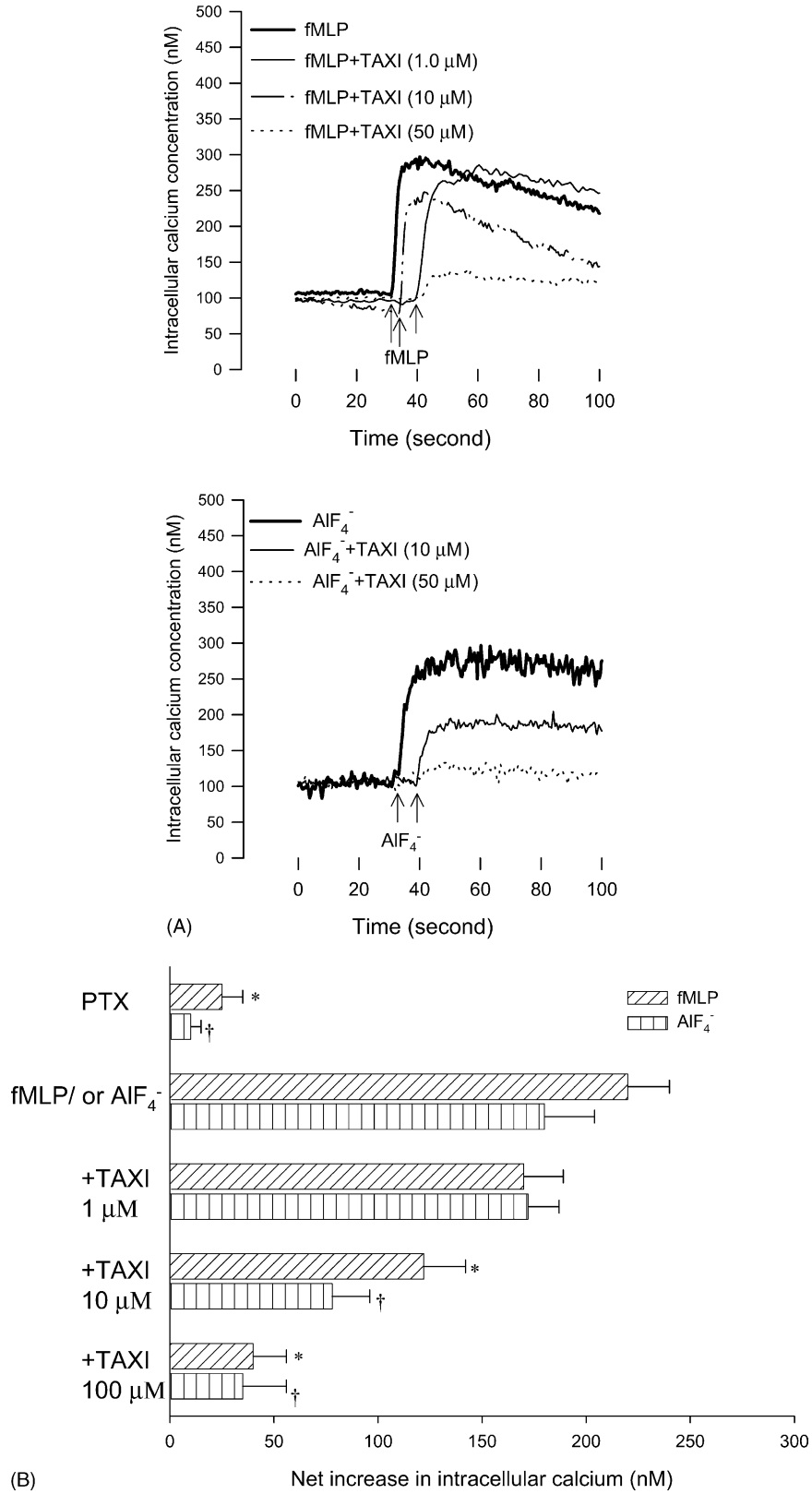


Fig. 4. Effect of taxifolin on fMLP- or AlF_4^--induced changes in $[Ca^{2+}]_i$. Net increase in intracellular calcium ($[Ca^{2+}]_i$) was measured as described in Section 2. (A) Tracing of changes in $[Ca^{2+}]_i$ triggered by fMLP (1 μM) or AlF_4^- (10 mM NaF plus 30 μM $AlCl_3$) in the presence or absence of 1–50 μM of taxifolin (TAXI). (B) Statistical summary of fMLP- or AlF_4^--induced changes in $[Ca^{2+}]_i$ in the presence of TAXI (1–100 μM). For G protein study, sample was pretreated with 0.5 μg/ml of pertussis toxin (PTX) at 37 °C for 2 h. Values represent the means ± S.E.M. of 5–10 experiments performed on different days using cells from different donors. $^{*,\dagger}P < 0.05$ as compared to samples treated with fMLP or AlF_4^- alone, respectively.

adhesion [7]. In this study, fMLP (a receptor-mediated agonist) and AlF_4^- (a G protein-mediated activator) triggered rapid increases in intracellular calcium ($[Ca^{2+}]_i$; Fig. 4). Taxifolin concentration-dependently impeded the calcium influx induced by fMLP or AlF_4^- (Fig. 4B). Intracellular calcium store released by thapsigargin (1 μM) was not modulated by taxifolin (data not showed).

3.3. Effect of taxifolin on ROS production in human neutrophils

Mobilisation of intracellular calcium mediates the ligand-initiated signalling for the ROS production by neutrophils [20] and antioxidants abolishes Mac-1 upregulation and firm adhesion in neutrophil [6]. We hypothesised that the de novo production of ROS by activated neutrophils may upregulate Mac-1 expression, which could be diminished by taxifolin. To examine whether taxifolin inhibited ROS production by human neutrophils-, lucigenin- or luminol-enhanced chemiluminescence was performed to measure the extracellular or total ROS accumulation [21]. Fig. 5 shows that both PMA- and fMLP-induced ROS production were significantly inhibited by taxifolin with IC_{50} less than 10 μM. Apocynin (Apo), a NADPH oxidase inhibitor [22,23] included as positive control, showed comparable effect as that of taxifolin in the inhibition of fMLP- or PMA-induced ROS production (Fig. 5).

3.4. Effect of taxifolin on the assembly of NADPH oxidase

ROS production by neutrophils depends on the assembly of cytosolic (e.g., p47-phox, p67-phox,) and membrane-associated components (e.g., gp91 and p22) to form an active NADPH oxidase, a process regulated by protein kinase [24]. Therefore, NADPH oxidase activity was determined in particulate fractions prepared from cells pretreated with taxifolin followed by PMA activation (for the assembly of NADPH oxidase) or in PMA-activated particulate fraction in the presence of taxifolin for the study of the preassembled-NADPH oxidase activity. Our results demonstrated that taxifolin not only interfered with the assembly of NADPH oxidase but also the activity of preassembled NADPH oxidase (Table 1). Comparable results were observed with cromolyn (100 μM), a drug had been reported to inhibit the assembly of NADPH oxidase but not the activity of a preassembled oxidase in human neutrophils [16].

3.5. Effect of taxifolin on the protein kinase C activity

Protein kinase C had been revealed to regulate the phosphorylation and translocation/assembly of p47-phox to NADPH oxidase [24,25]. We measured the PKC activity by an enzyme-linked immunosorbent assay. Table 2 shows that cytosolic PKC activity was inhibited by taxifolin. In the negative control groups, PKC activity was reduced to 37 or 67% in the presence of 0.2 μM of staurosporine (a PKC inhibitor) or 20 mM of EGTA (for negation of calcium-dependent effect), respectively. In some experiments in which PMA (100 ng/ml) was added to viable neutrophils, cytosolic PKC activity was reduced to 52% as most of the cytosolic PKC was translocated to the particulate fraction resulting from PMA activation.

3.6. Effect of taxifolin on the p38 MAPK activity

Phosphorylation of the NADPH oxidase component, e.g., p67-phox, by p38 MAPK plays an important role

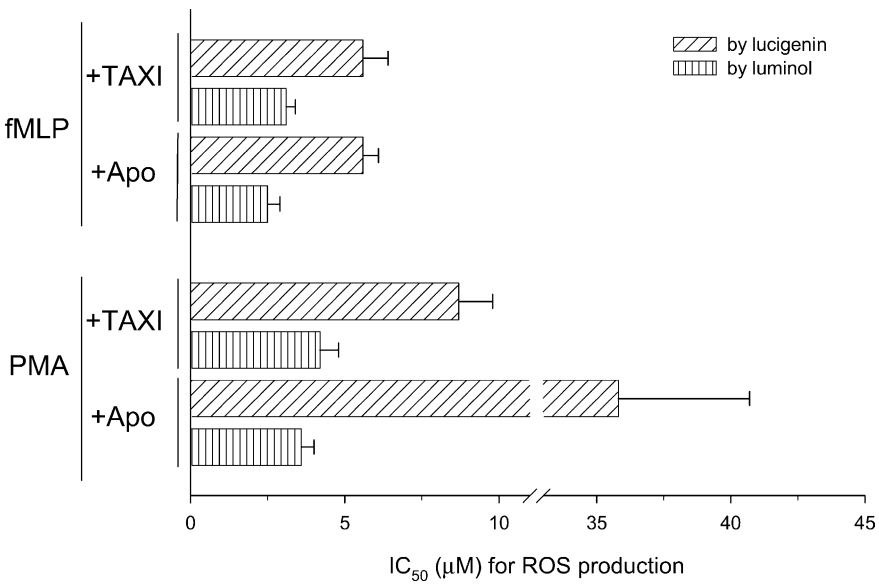


Fig. 5. Effect of taxifolin on fMLP- or PMA-induced ROS production. PMA (20 nM)- or fMLP (1 μM)-induced ROS by neutrophils was determined in the presence or absence of 1–100 μM of taxifolin (TAXI) via a luminol- or lucigenin-enhanced chemiluminescence. Data are expressed as 50% inhibitory concentration (IC_{50}). Values represent the means ± S.E.M. of 10–15 experiments performed on different days using cells from different donors.

Table 1
Summary of the effects of taxifolin on subcellular NADPH oxidase activity

	NADPH oxidase activities[a] ($O_2^{-\bullet}$ nmol per 2×10^6 cell equivalents per 20 min)	
	Drug added before PMA-activation[b]	Drug added after PMA-activation[c]
Control (drug free)	0.47 ± 0.03[d]	0.47 ± 0.03[d]
PMA alone	1.57 ± 0.31	1.29 ± 0.25
+TAXI (10 μM)	0.96 ± 0.25[d]	1.21 ± 0.12
+TAXI (100 μM)	0.64 ± 0.12[d]	0.76 ± 0.14[d]
+Cromolyn (100 μM)	0.64 ± 0.11[d]	1.24 ± 0.26
+Stau (0.2 μM)	0.39 ± 0.05[d]	N.D.

[a] NADPH oxidase activity was measured as $O_2^{-\bullet}$ production in particulate fractions isolated from PMA-treated neutrophils via monitoring for 20 min as SOD-inhibitable cytochrome *c* reduction at 37 °C.

[b] Neutrophils preincubated with taxifolin (TAXI), cromolyn or staurosporine (Stau) for 20 min at 37 °C were stimulated with PMA (100 ng/ml), then cells were sonicated and particulate fraction (2×10^6 cell equivalents per well) were used for the assay of oxidase activity (drug added before PMA activation).

[c] Alternatively, the membrane-associated well-assembled oxidase (by PMA) was mixed with TAXI or cromolyn for 20 min before addition of the NADPH (drug added after PMA activation). Values are means ± S.E.M. from six experiments performed on different days using cells from different donors.

[d] $P < 0.05$ as compared with PMA alone. N.D., sample not detected.

during neutrophil activation by several agonists [26]. We examined whether taxifolin could prevent the fMLP- or PMA-induced phosphorylation of p38 MAPK. FMLP or PMA induced a maximal phosphorylation of p38 MAPK at 3 or 10 min, respectively (Fig. 6). Pretreatment with taxifolin concentration-dependently prevented the phosphorylation of p38 MAPK without modulating the expression of endogenous p38 MAPK.

Table 2
Summary of the effects of taxifolin on the PKC activity

	PKC activity[a] ($100 \times OD_{492}$/12 μl of cell lysate from 2×10^7 cells)
Control (drug free)	306.1 ± 24.8
+TAXI (10 μM)	240.9 ± 20.2[c]
+TAXI (100 μM)	175.1 ± 9.6[c]
+EGTA (20 mM)	205.8 ± 14.1[c]
+Stau (0.2 μM)	114.2 ± 10.1[c]
PMA preactivated[b]	165.3 ± 17.5[c]

[a] Cytosolic fraction of neutrophils (2×10^7 cell/ml) were extracted for the determination of the protein kinase C (PKC) activity in the presence of taxifolin (TAXI) by a non-radioactive protein kinase assay kit (Calbiochem) based on an enzyme-linked immunosorbent assay that used a synthetic PKC pseudosubstrate and a monoclonal antibody that recognised the phosphorylated peptide. Negative control for PKC activity assay was determined in the presence of 0.2 μM staurosporine (Stau) or 20 mM EGTA.

[b] In some experiments, PMA (100 ng/ml) were added to live neutrophils for stimulation/translocation of PKC prior to sonication and centrifugation, and cytosolic PKC activity from this preparation was used as another negative control as most of the cytosolic PKC was translocated to the particulate fraction (PMA preactivated). Values are means ± S.E.M. from three to five experiments performed on different days using cells from different donors.

[c] $P < 0.05$ as compared to control.

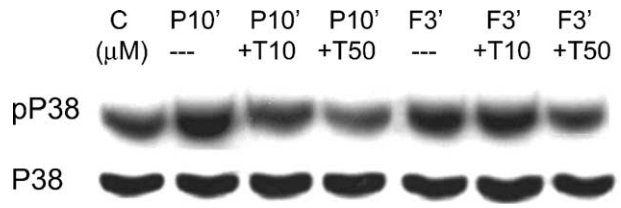


Fig. 6. Effect of taxifolin on the phosphorylation of p38 MAPK. Neutrophils were stimulated with 100 ng/ml of PMA for 10 min (P10′) or 1 μM of fMLP for 3 min (F3′) at 37 °C in the presence or absence of 10 or 50 μM of taxifolin (T10 or T50, respectively). Control group (C) received vehicle (0.25% DMSO) only. After treatment, cells were sonicated in lysis buffer. Equal amount of protein (40 μg) from different treatments were subjected to SDS–PAGE using 10% mini-gels. After electro-transferred to a PVDF membrane, the membrane was preblocked with 5% non-fat milk in PBS containing 0.05% Tween 20 at 4 °C for 1 h followed by incubation overnight at 4 °C with an antibody against p38 MAPK (P38) or phospho-p38 MAPK (pP38). After incubated with a second antibody for 1 h at room temperature, the immuno-blot on the membrane was visible after developing with an enhanced chemiluminescence (ECL) system. Experiment was repeated at least for three times and comparable result was observed.

3.7. Oxidant-scavenging effect by taxifolin on xanthine oxidase free radical generation system

To test whether taxifolin has a ROS-quenching activity in cell free system, the xanthine oxidase-dependent free radical generation was performed. Superoxide anion production by this system, as measured by cytochrome *c* reduction, increased around 12-fold as compared to that in the presence of SOD (100 U/ml). Taxifolin significantly decreased the superoxide anion production at relative high concentration (50 μM) but not at low concentration (10 μM; Table 4). Production of uric acid was neither inhibited by SOD nor by taxifolin indicating that xanthine oxidase per se was not inhibited by taxifolin or SOD. Similar results by SOD had been reported previously [18].

4. Discussion

In the present study, pretreatment with taxifolin (1–100 μM) for 20 min significantly impaired fMLP- or PMA-induced firm adhesion (Fig. 2) and Mac-1 upexpression in neutrophils (Fig. 3). Our previous report demonstrated that the expression of Mac-1 (CD11b/CD18) and Mac-1-dependent neutrophil firm adhesion was enhanced by ROS produced in activated neutrophils and was prevented by anti-oxidants [6] or by NADPH oxidase inhibitor [27]. This observation suggests that ROS can act as a signalling molecule in modulating leukocyte Mac-1 expression and leukocyte-endothelial adhesion. We therefore examined whether ROS production was impaired by taxifolin. Both PMA and fMLP triggered marked intracellular and extracellular ROS production in neutrophils that

could be quantified by a lucigenin (extra)- or luminol (both intra and extra)-enhanced chemiluminescence assay [17]. Taxifolin effectively inhibited both PMA- and fMLP-induced ROS production with IC_{50} less than 10 μM (Fig. 5) illustrating that a non-receptor-related mechanism may mediate its anti-oxidant effect. Similar results had indicated a concentration-dependency by taxifolin in the inhibition of ROS production with mechanism(s) of action remained unclear [28]. Therefore, we undertook the present study to elucidate the possible mechanism(s) in mediating the prevention of ROS production by taxifolin.

Cytosolic calcium serves as an important ligand-mediated signal in the regulation of NADPH oxidase [29] as well as Mac-1-dependent neutrophil adhesion and ROS production [19]. We have previously demonstrated that both modulation of PKC-dependent activation [30] or impediment to calcium influx [7] can account for the inhibition of Mac-1-dependent neutrophil firm adhesion. To further elucidate the underlying mechanism(s) by which taxifolin impede the ligand (fMLP)-induced ROS production and Mac-1-dependent neutrophil firm adhesion, we examined the effects of taxifolin on intracellular calcium ($[Ca^{2+}]_i$) mobilisation and neutrophil adhesion induced by fMLP, AlF_4^- or thapsigargin. Our data established that taxifolin significantly inhibited fMLP- and AlF_4^--induced $[Ca^{2+}]_i$ mobilisation (Fig. 4) and neutrophil firm adhesion but not that induced by thapsigargin (data not shown) indicating that taxifolin may interfere with G protein activation as fMLP binds to its receptor. In this study, samples pretreated with pertussis toxin (a G protein inhibitor) or BAPTA-AM (an intracellular calcium chelator), introduced to contrast the specificity of G protein activation and intracellular calcium mobilisation by AlF_4^- or thapsigargin, both significantly antagonised agonist's effect, respectively.

Production of ROS by neutrophil predominantly comes from the activation of NADPH oxidase to generate $O_2^{-\bullet}$ that involves the assembly of cytosolic components (e.g., p47-phox, p67-phox) and membrane-associated components (gp91-phox and p22-phox) to form an active NADPH oxidase enzyme complex [31,32]. To further understand whether the assembly of NADPH oxidase or the activity of membrane-associated well-assembled oxidase could be modulated by taxifolin, its effects on the NADPH oxidase activity in particulate fractions were studied. Particulate fraction isolated from PMA-treated cells contained an active NADPH oxidase assembly and generated $O_2^{-\bullet}$ three-fold more than resting cells (drug free) in the presence of NADPH (400 μM). Treatment of taxifolin before PMA activation concentration-dependently inhibited the NADPH oxidase activity in the particulate fraction (Table 1). Comparable result was observed in sample pretreated with cromolyn, an inhibitor for the assembly of NADPH oxidase [16]. In high concentration (100 μM), taxifolin also impeded the well-assembled NADPH oxidase (Table 1).

Phosphorylation and translocation of cytosolic component, e.g., p47-phox, to surface membrane by PKC is crucial for the activation and assembly of NADPH oxidase [33]. Taxifolin and staurosporine both significantly inhibited the PKC activity (Table 2) and prevented the activation of NADPH oxidase (Table 1) suggesting that taxifolin may interfere with the PKC-dependent pathway, possibly the phosphorylation/translocation of p47-phox resulting in suppression of the assembly of NADPH oxidase, in turn the ROS production by neutrophils. Furthermore, incubation of PMA-assembled particulate fractions with taxifolin showed significant decrease on $O_2^{-\bullet}$ generation indicating that taxifolin could also modulate the activity of the preassembled NADPH oxidase complex (Table 1). It had been reported that quercetin, a flavonoid, inhibits oxygen consumption in whole cells and acts as an inhibitor of NADPH oxidase in subcellular fractions [34] but the mechanisms of actions have remained unknown. In this study, we demonstrated that taxifolin, with structure similar to quercetin, inhibited the activity of assembled NADPH oxidase and the PKC-dependent assembly of an active, membrane-associated NADPH oxidase. It is likely that taxifolin could suppress the activity of PKC (Table 2) to reduce ROS production (Fig. 5), albeit less potent than PKC inhibitors (Table 3). Besides its inhibitory activity of enzymes, taxifolin also exhibited quenching activity of oxidants in relative high concentration (50 μM; Table 4).

In addition to the PKC-dependent pathway, the intracellular signalling pathways leading to its phosphorylation/activation of NADPH oxidase in neutrophils may also involve a PKC-independent pathway. It has been reported that p38 MAPK and MEK (the MAPK/ERK kinase) all

Table 3
Summary of the IC_{50} of some protein kinase inhibitors on the ROS production

	IC_{50} (μM) in PMA-induced[a] ROS production	IC_{50} (μM) in fMLP-induced ROS production
PKC inhibitors		
GF109203x	0.2 ± 0.1	0.4 ± 0.2
Staurosporine	0.9 ± 0.2	0.04 ± 0.02
Rottlerin	1.5 ± 0.2	0.2 ± 0.1
MAPK inhibitors		
PD98059	832 ± 170	359 ± 71
SB600125	351 ± 55	73 ± 13
SB203580	17 ± 2	7 ± 1
SB202190	24 ± 1	4 ± 1

[a] PMA (20 nM)- or fMLP (1 μM)-induced ROS production was determined via a lucigenin-enhanced chemiluminescence in human neutrophils in the presence of different protein kinase C (PKC) inhibitors including two general PKC inhibitors (GF109203x, staurosporine) and a PKCδ inhibitor (rottlerin), or mitogen activated protein kinase (MAPK) inhibitors including PD98059 (a MEK inhibitor), SP600125 (a *c-Jun* N-terminal kinase inhibitor), and SB203580 or SB202190 (two p38 MAPK inhibitors). Data are expressed as 50% inhibitory concentration (IC_{50}). Values represent the means ± S.E.M. of 10–15 experiments performed on different days using cells from different donors.

Table 4
Summary of the oxidants-scavenging effect by taxifolin on the xanthine/oxidase system

Treatments	Superoxide production[a] (A550 nm/min)	Uric acid production (A290 nm/min)
Xanthine/xanthine oxidase	0.145 ± 0.003	0.058 ± 0.001
+SOD (100 U/ml)	0.012 ± 0.007[b]	0.056 ± 0.003
+TAXI (10 μM)	0.125 ± 0.007	0.055 ± 0.002
+TAXI (50 μM)	0.094 ± 0.003[b]	0.058 ± 0.009

[a] Superoxide production was monitored by the reduction of cytochrome *c*. Cytochrome *c*, xanthine and xanthine oxidase were incubated at 25 °C in the presence of superoxide dismutase (SOD) or taxifolin (TAXI). The reduction of cytochrome *c* was monitored by the increase in absorbance at 550 nm (A550 nm). Uric acid production was monitored at 290 nm (A290 nm). Values are means ± S.E.M. from four individual experiments performed on different days.

[b] $P < 0.05$ as compared with xanthine/xanthine oxidase alone.

participate in the generation of second messengers that triggers the activation of NADPH oxidase in neutrophil [26,35]. In this study, we demonstrated that only p38 MAPK inhibitors (SB203580 and SB202190), not other MAPK inhibitors, could significantly impede the ROS production in human neutrophils (Table 3), but were 20- to 30-fold less potent than that of PKC inhibitors (Table 3). Immuno-blotting study revealed that fMLP- or PMA-induced phosphorylation of p38 MAPK was concentration-dependently prevented by taxifolin suggesting that p38 MAPK-mediated signalling pathway was modified by taxifolin (Fig. 6).

There are several other possible targets in regulating ROS production in neutrophils could be interfered with by taxifolin in addition to the NADPH oxidase. These include modulation of (1) MPO activity, which generates potent oxidants HOCl from H_2O_2; (2) catalase (CAT) and/or glutathione peroxidase (GPX) activity, which convert superoxide anion to water and oxygen and (3) COX, another enzyme in generating oxygen radical [10]. We found neither CAT nor GPX activities were modulated by taxifolin (data not shown). In contrast, taxifolin significantly inhibited COX or MPO activity (data not shown). Furthermore, Mac-1 expression may also be upregulated by phospholipase A_2 (PLA_2), as inhibitors of PLA_2 can inhibit the surface expression of Mac-1 [36]. PLA_2 releases arachidonic acid for subsequent metabolism via the COX or lipoxygenase pathways, two important intra- and extracellular mediators of inflammation [37]. Since taxifolin also inhibited COX activity, it is possible that taxifolin could modulate ROS production and Mac-1 expression via modulating COX activity.

In conclusion, our data suggest that down-regulation of ROS generation through interference with p38 MAPK- and PKC-dependent activation of NADPH oxidase as well as antagonism of ligand-initiated calcium influx accounts for the prevention of Mac-1-dependent neutrophil firm adhesion by taxifolin. As an efficient anti-oxidative and anti-adhesive agent, taxifolin may be useful for the prevention of injury that mainly characterises ROS production and neutrophil activation/adhesion as inflammatory mediators.

Acknowledgments

This study was supported in part by grants 91-21231R and 92-212332, and NSC-92-2320-B-077-003, from Tao-Yuan General Hospital, Department of Health, and National Science Council, Taiwan, Republic of China, to W.-Y. Wang, Y.-C. Shen and C.-F. Chen, respectively.

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