0026-895X/01/6005-1083–1090$3.00
MOLECULAR PHARMACOLOGY
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Mol Pharmacol 60:1083–1090, 2001

Vol. 60, No. 5
949/940423
Printed in U.S.A.

Anti-Inflammatory Effects of the Partially Purified Extract of Radix *Stephaniae tetrandrae*: Comparative Studies of Its Active Principles Tetrandrine and Fangchinoline on Human Polymorphonuclear Leukocyte Functions

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Received March 13, 2001; accepted August 6, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

We hypothesized that prevention of neutrophil from activation may underlie the myocardial protective effect of the specially processed extract of radix *Stephaniae tetrandrae* (SPRST). Inflammatory responses in isolated peripheral human neutrophils were studied in the presence or absence of SPRST. SPRST (1–10 μg/ml) concentration-dependently prevented *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)- or leukotriene B_4 (LTB_4)-induced neutrophil adhesion and transmigration. Comparable results were also observed in neutrophils pretreated with fangchinoline (Fan) or tetrandrine (Tet), two active components in SPRST. It has been reported that neutrophil adhesion/transmigration is mainly Mac-1 (CD11b/CD18)-dependent and could be modulated by reactive oxygen species (ROS) production. SPRST, Tet, and Fan diminished fMLP- or LTB4-induced Mac-1 up-regulation and ROS production. SPRST, Fan, Tet, and verapamil impaired fMLP-induced rapid intracellular alkalization, an essential mechanism for neutrophil ROS production, and $[Ca^{2+}]_i$ increment, suggesting that a calcium dependent pathway might be involved. Direct G protein activation by AlF_4^- also triggered $[Ca^{2+}]_i$ increment and adhesion that could be abolished by pertussis toxin and were partially reversed by SPRST, Fan, and Tet. These results reveal that inhibition of neutrophil adhesion and transmigration may account for SPRST's myocardial protective effect. This effect of SPRST may be mediated by component(s) in addition to Tet and Fan because combination of 0.1 μg/ml of Tet and Fan did not mimic the effect of SPRST. We conclude that SPRST exerts anti-inflammatory effects by interfering with ROS production and Ca^{2+} influx through G protein modulation to prevent Mac-1 up-regulation in neutrophil activation.

Radix *Stephaniae tetrandrae*, dry roots of *Stephaniae tetrandrine* S. Moore (Menispermaceae), is officially and traditionally used as an analgesic and anti-hypertension drug in China. The main chemical constituents in radix *Stephania tetrandrae* are tetrandrine (Tet) and fangchinoline (Fan) (Tang and Eisenbrand, 1992). Tet is the best characterized as a Ca^{2+}-entry blocker (Felix et al., 1992); it exhibits numerous pharmacological activities, including modulating cardiovascular disorders (Huang and Hong, 1998) and anti-tumor (DeConti et al., 1975) and anti-inflammatory effects (Shen et al., 1999). Fan was shown to be less potent than Tet as a vasodilator and calcium channel blocker (Kim et al., 1997). Fan also exhibits antioxidant (Ma et al., 1992), anti-inflammatory effects in the mouse ear edema model (Choi et al., 2000), and proinflammatory cytokines released by human peripheral monocyte (Onai et al., 1995).

We have demonstrated that the partially purified extract of *S. tetrandrae* containing 10% Tet produces a cardioprotective effect equipotent with that of Tet on the isolated ischemia/reperfused (I/R) rat heart but circumventing the side effects of verapamil (Yu et al., 2001). However, the mechanism(s) of action have remained unclear. It is well known that activation and transmigration of neutrophils to infarct myocardium play a crucial role in the myocardial I/R injury (Williams, 1994) and neutrophil infiltration has been emphasized as an essential pathological factor contributing to the induction of myocardial I/R injury (Engler et al., 1986). Infiltration of neutrophils into tissue injury begins with the bind-

This study was supported, in part, by Grants NSC89-2113-M-077-007 (C.-J.C.), NSC89-2320-B-077-003 (W.-F.C.), and NSC89-2320-B-077-014 (C.-F.C.) from the National Science Council, Taiwan, the Republic of China.

ABBREVIATIONS: Fan, fangchinoline; Tet, tetrandrine; I/R, ischemia/reperfused; ROS, reactive oxygen species; SPRST, the specially processed extract of radix *Stephaniae tetrandrae*; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; LTB_4, leukotriene B_4; Mac-1, macrophage adhesion molecule-1 (also, CD11b/CD18); PBS, phosphate-buffered saline; HBSS, Hanks' buffered saline solution; BCECF/AM, 2′,7′-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein, acetoxymethyl ester; FBS, fetal bovine serum; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DCF, 2′,7′-dichlorofluorescein; EB, ethidium bromide; $O_2^{\cdot-}$, superoxide anion; ANOVA, analysis of variance; PTX, pertussis toxin.

ing of neutrophils to the endothelium, followed by their extravasation into tissues (Albelda et al., 1994). This physiology comprises distinct phases, including rolling, activation, firm adhesion, and transmigration (Ley, 1996). A molecular explanation for these phases involves specific interactions of various cell adhesion molecules expressed on neutrophil and endothelium. These fall into three major superfamilies: (1) the selectins and their mucin ligands; (2) the integrins; and (3) their extracellular matrix or immmunoglobulin superfamily ligands (Brown, 1997). Whereas the selectins are important for rolling, firm adhesion and transmigration of neutrophils are essentially β_2 integrin-dependent (Arfors et al., 1987; Werr et al., 2000). The β_2 integrins comprise a group of heterodimeric glycoproteins; CD11b/CD18 (Mac-1) is the principal form elevated on neutrophils during myocardial I/R activation (Dreyer et al., 1989). Thus, prevention of Mac-1 mediated adhesion and/or transmigration of neutrophil into the site of tissue injury is a potential target for drugs to control inflammation. Besides, it has been demonstrated that ROS could modulate leukocyte Mac-1 expression and leukocyte endothelial adhesion, and both could be diminished by antioxidants (Serrano et al., 1996). Furthermore, antagonizing Ca^{2+} influx could impair Mac-1 dependent neutrophil adhesion (Perry et al., 1993).

In this study, we confirmed that a specially processed extract of *S. tetrandrae* (SPRST), containing only 1.3% Tet and 0.7% Fan, inhibited the neutrophil adhesion and transmigration. We hypothesized that interference with the upregulation of adhesion molecules may be involved in the effect. As remarked above, adhesion and transmigration of neutrophils is Mac-1 dependent and could be modulated by ROS and calcium mobilization. Therefore, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or leukotriene B_4 (LTB_4) induced adhesion and transmigration as well as ROS production and Ca^{2+} mobilization by neutrophils were analyzed to investigate the effects of SPRST. In particular, Mac-1 expression on the surface of neutrophils was examined.

Materials and Methods

Human Neutrophils Isolation. Preparation of human neutrophils was obtained by venipuncture from adult healthy volunteers and collected into syringes containing heparin (20 U/ml of blood). Neutrophils were isolated by the Ficoll gradient centrifugation method, followed by lysis of contaminating erythrocytes. Briefly, blood samples were mixed with an equal volume of 3% dextran solution in a 50-ml centrifuge tube and incubated in an upright position for 30 to 40 min at room temperature to allow sedimentation of erythrocytes. The upper, leukocyte-rich layer was then collected and subjected to centrifugation at 250*g* for 15 min at 4°C. After centrifugation, the pellet was resuspended immediately in a volume of phosphate-buffered saline (PBS) equal to the starting volume of blood. The cell suspension was then apportioned, 6 ml per tube, into 15-ml centrifuge tubes, followed by laying 8 ml of Ficoll solution (Histopaque 1077; Sigma Chemical Co., St. Louis, MO) beneath the cell suspension, using a pipette. After centrifugation at 400*g* for 40 min at 20°C without brake, the upper (PBS) and lower (Ficoll) layers were carefully removed, leaving the granulocyte/erythrocyte pellet. To remove residual erythrocytes, the pellet was resuspended in 10 ml of cold lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA, pH 7.4). The remaining neutrophils were then pelleted, washed twice with ice-cold PBS, and resuspended in an adequate volume of ice-cold Hanks' buffered saline solution (HBSS) until further manipulation. The preparation contained more than 95% neutrophils, as estimated by counting 200 cells under a microscope after Giemsa staining (Sigma). In all cases except the indicated where neutrophils were pretreated with SPRST, Tet, or Fan, the cells were mixed with drug(s) at concentrations ranging from 1 to 10 μg/ml in HBSS for 10 min at 37°C.

Measurement of Neutrophil Adhesion. Adhesion of neutrophils to extracellular matrix was determined in 24-well tissue culture plates (BD Falcon, Franklin Lakes, NJ) coated with fibrinogen as our previous study (Shen et al., 1999). Before the addition of neutrophils, the plates were incubated with 250 μl of human fibrinogen per well (50 μg/ml in PBS; Chemicon International, Inc., Temecula, CA) for 2 h at 37°C. The wells were washed once with HBSS, blocked with 1% bovine serum albumin (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 before addition to the coated-plate, neutrophils (1×10^7 cells/ml) were loaded with 1 μM 2′,7′-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM; Molecular Probes, Eugene, OR) in HBSS for 20 min at 37°C and then washed twice with 10 ml HBSS without Mg^{2+} or Ca^{2+}. Two hundred microliters per well of drug-pretreated neutrophils labeled with BCECF-AM (5×10^5 cells/ml in HBSS) was then added to individual wells. After stimulation with fMLP (1 μM) or LTB_4 (0.1 μM) for 15 min at 37°C, nonadherent cells were removed by aspiration and the wells were gently washed twice with warm PBS containing 1 mM Ca^{2+}. Adherent neutrophils were then determined by measuring the fluorescence with a fluorescent plate reader (Cytofluor 2300; Millipore, Bedford, MA) with excitation at 485 nm and emission at 530 nm. Data are expressed as fluorescence intensity.

Measurement of Neutrophil Transmigration. Transmigration of neutrophils was quantified as described previously (Krull et al., 1999) with some modification. Briefly, 6.5-mm-diameter Transwell inserts of 5-μm pore size (Corning Costar, Cambridge, MA) were precoated with human fibrinogen (20 μg/ml, 100 μl). One hundred microliters per well of BCECF-labeled neutrophils (5×10^5 cells/ml in HBSS) were treated with SPRST, Tet, or Fan for 10 min at 37°C immediately before adding to the upper chamber of fibrinogen-coated inserts. Then, fMLP (1 μM) or LTB4 (0.1 μM) was added to the lower chambers and incubated with cells in the upper inserts for 60 min at 37°C. Fluorescence intensity in the lower chambers (represent migrated neutrophils) was quantified with a fluorescent plate reader (Cytofluor 2300) with excitation at 485 nm and emission at 530 nm. Data are expressed as fluorescence intensity.

Measurement of Mac-1 Up-Regulation by Flow Cytometry. Expression of Mac-1 (CD11b/CD18) was analyzed as our previous study (Shen et al., 1999). Briefly, SPRST-pretreated neutrophils were stimulated with fMLP (1 μM) or LTB_4 (0.1 μM) 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. For staining of Mac-1, all subsequent steps were carried out in an ice bath. Cells were incubated in the dark for 60 min with a proper aliquot of fluorescein isothiocyanate-conjugated anti-Mac-1 antibody (mouse anti-human CD11b, class IgG_1; BD PharMingen, San Diego, CA) or a nonspecific 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 (BD Biosciences, San Jose, CA) containing 1% of paraformaldehyde and analyzed on a flow cytometer (FACSort; BD Biosciences) for Mac-1 expression. Data are expressed as mean channel fluorescence for each sample as calculated by the CellQuest software (BD Biosciences) on a Power Macintosh 6100/66 computer.

Flow Cytometric Analysis of Intracellular ROS Production. Intracellular production of $O_2^{\cdot-}$ and H_2O_2 were measured as ROS production in this study and analyzed on a flow cytometer (FACSort) as described previously (Shen et al., 1998). Briefly, neutrophils (1×10^6 cells/ml) were incubated at 37°C for 5 min with 20 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes) and for an additional 15 min with 10 μM hydroethidine (Molecular Probes). The acetate moieties of DCFH-DA are cleaved off intracellularly by

esterases, liberating the membrane impermeable 2′,7′-dichlorofluorescin, which fluoresces when oxidized to 2′,7′-dichlorofluorescein (DCF) by H_2O_2; hydroethidium, on the contrary, can be directly oxidized by $O_2^{\cdot-}$ to ethidium bromide (EB), which fluoresces after intercalating with nucleic acids. After labeling, cells were pretreated with SPRST or other chemicals for 10 min and stimulated with fMLP (1 μM). Production of $O_2^{\cdot-}$ and H_2O_2 was then determined 30 min after on a flow cytometer (FACSort) by measuring emission at 525 nm (FL1) for DCF and 590 nm (FL2) for EB. Data are expressed as mean channel fluorescence.

Determination of Intracellular pH. The method described by Boyer & Hedley (1994) was followed. Briefly, cells were loaded with BCECF-AM (2 μg/ml) at 37°C for 30 min, washed twice, and resuspended at 1×10^6 cells/ml in HBSS. After pretreatment with drug(s) for 10 min, fMLP (1 μM) was added to cells suspension and incubated at 37°C in 5% CO_2. Samples were measured by flow cytometry (FACSort) at the time as indicated in the figure. Fluorescence intensity of BCECF at 525 to 535 nm is pH dependent with greater intensity at higher pH. In order to make measurements of pH_i, a ratio was taken between a pH-dependent fluorescence intensity at 525 nm (FL1) and a pH-independent fluorescence intensity at 640 nm (FL3). The value obtained is therefore independent of such factors as photobleaching, cell thickness, and instrument stability, as well as nonuniform loading or leakage of the dye. For calibration samples, the pellet was resuspended in high $[K^+]$ buffers made by mixing appropriate volumes of solution 1 (130 mM KH_2PO_4, 20 mM NaCl) and solution 2 (110 mM K_2HPO_4, 20 mM NaCl) to give buffers with a range of known pH values between 6.5 and 7.8. Before measurement of pH_i of calibration samples (2–3 min), 1 μg/ml nigericin (Sigma), a H^+/K^+ ionophore, was added to allow the ratios of intracellular to extracellular potassium ion concentration ($[K^+]_i$ and $[K^+]_e$) and that of intracellular to extracellular hydrogen ion concentration ($[H^+]_i$ and $[H^+]_e$) to become equal (i.e., $[K^+]_i/[K^+]_e = [H^+]_i/[H^+]_e$).

For if $[K^+]_i$ and $[K^+]_e$ are equal, then $[H^+]_i$ will be equal to $[H^+]_e$, hence pH_i can be estimated simply by measuring pH_e. A calibration curve of fluorescence ratio to pH was performed for each experiment individually over a pH range of 6.5 to 7.8. Data are expressed as pH value of individual samples.

Determination of Intracellular Calcium Concentration. Before drug treatment, neutrophils were preloaded with 5 μM fura 2-AM (Molecular Probes, Eugene, OR) at 37°C for 45 min, washed twice, and resuspended at 2×10^6 cells/ml in calcium-free HBSS containing SPRST, Tet, Fan, or control vehicle. After drug treatment for 10 min, 1 ml of cell suspension 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 addition of fMLP (1 μM), LTB_4 (0.1 μM), or AlF_4^- (10 mM NaF plus 10 μM $AlCl_3$). The fluorescence of fura-2–loaded cells was measured on a spectrofluorometer (Hitachi F-4500) with excitation 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 described previously (Shen et al., 1999): $[Ca^{2+}]_i = K \cdot (R - R_{min})\,(S_{f380}) / (R_{max} - R)\,(S_{b380})$, where: K = 224 nM (Fura-2 at 37°C), R_{min} is the ratio value in minimal Ca^{2+} conditions, R_{max} is the ratio value at a maximal Ca^{2+} concentration, S_{f380} = 380 nm reading in minimal Ca^{2+} conditions (corrected for background), S_{b380} = 380 nm reading in maximal Ca^{2+} conditions (corrected for background). R_{max} and S_{b380} were obtained at the end of a measurement by permeabilizing the cells with 0.2% digitonin, and R_{min} and S_{f380} were determined by adding 20 mM EGTA after digitonin lysis. All measurements were performed in Ca^{2+}-containing medium, because no significant changes in $[Ca^{2+}]_i$ could be detected under Ca^{2+}-free conditions.

SPRST and Other Chemicals. SPRST, Tet, and Fan were isolated and purified by Professor Chou in our Institute (Chou et al., in press). It was first dissolved in 5N HCl at 10 mg/ml and then serially diluted in PBS immediately before experiments. Stock solution was used within 1 week after preparation. For examination of the effect of these drugs, except where indicated, 10 μl of drug solution was added to 1.0 ml of neutrophil suspension and incubated at 37°C for 10 min before the addition of fMLP (1 μM) or LTB_4 (0.1 μM; Sigma). For G protein study, cells were incubated for 2 h at 37°C with 500 ng/ml pertussis toxin (Calbiochem) before the addition of AlF_4^{2-} (10 mM NaF plus 10 μM $AlCl_3$) or other inducers.

Statistical Analysis. All values in the text and figures represent means ± S.E.M. Data were analyzed by one- 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. Concentration dependence was analyzed by simple linear regression analysis of response levels against concentrations of drug(s) and testing the slope of the regression line against 0 by Student's *t* test. Values of $p < 0.05$ were considered significant.

Results

SPRST and Its Active Components Tet and Fan Inhibit Neutrophil Adhesion and Transmigration. To examine whether SPRST and/or its active components Tet and Fan could inhibit neutrophil infiltration, we established an in vitro assay system in which fMLP (1 μM) or LTB_4 (0.1 μM) was used to induce neutrophil adhesion and transmigration, functions underlying neutrophil infiltration. In the adhesion assay, whereas untreated neutrophils displayed spontaneous adhesion with a fluorescence intensity of 206 ± 18, fMLP or LTB_4 caused up to 200% enhancement in neutrophil adhesion relative to background levels (Fig. 1). Pretreatment of neutrophils with SPRST, Tet, or Fan dose-dependently inhibited fMLP- or LTB_4-induced neutrophil adhesion (Fig. 1). Combination of 1 or 10, but not 0.1 μg/ml Tet and Fan further attenuated neutrophil adhesion (data not shown). Similar results were also observed in the transmigration study (Fig. 2). Untreated neutrophils displayed spontaneous transmigration with a fluorescence intensity of 254 ± 14 (Fig. 2). SPRST, Tet, or Fan alone did not influence spontaneous neutrophil adhesion or transmigration (ANOVA, $p > 0.05$). The concentrations of these drugs used in this study were not cytotoxic to neutrophils (viability after drugs treatment > 95% by trypan blue exclusion assay).

SPRST, Tet, and Fan Inhibit Mac-1 (CD11b/CD18) Up-Regulation. Neutrophil adhesion to the extracellular matrix has been shown to mainly depend on up-regulation of Mac-1 (CD11b/CD18) (Everitt et al., 1996), and β_2 integrins may serve to regulate neutrophil extravasation (Werr et al., 2000). Therefore, we examined whether SPRST, Tet, or Fan could inhibit neutrophil adhesion and/or transmigration by virtue of down-regulation of Mac-1. To assess the effect of these drugs on Mac-1 expression, we measured surface levels of Mac-1 on fMLP- or LTB_4-stimulated neutrophils with or without drug(s) pretreatment by flow cytometric analysis. fMLP or LTB_4 caused a marked increase in Mac-1 fluorescence, whereas an apparent shift to the left of Mac-1 fluorescence was observed in samples pretreated with SPRST (10 μg/ml) (Fig. 3A). A statistical summary revealing Tet and Fan, as well as SPRST, significantly inhibited fMLP- or LTB_4-induced Mac-1 up-regulation was illustrated in Fig. 3b ($p < 0.05$, n = 3–5).

SPRST, Tet, and Fan Inhibited Intracellular ROS ($O_2^{\cdot-}$ and H_2O_2) Production. It has been shown that ROS (e.g., $O_2^{\cdot-}$ and H_2O_2) could up-regulate Mac-1 expression and enhance neutrophil adhesion that could be abolished by anti-

oxidants (Fraticelli et al., 1996; Serrano et al., 1996). Therefore, we hypothesized the de novo production of ROS by neutrophils may participate in Mac-1 up-regulation that could be diminished by SPRST. We used a flow cytometric method to measure intracellular ROS production in fMLP-stimulated neutrophils in the presence or absence of SPRST. A representative experiment by fMLP-stimulated accumulation of intracellular H_2O_2 (measured as DCF fluorescence) and $O_2^{\cdot-}$ (measured as EB fluorescence), respectively, was illustrated in Fig. 4A, and the results of five experiments are summarized in Fig. 4B. SPRST, Tet, and Fan concentration-dependently decreased the fluorescence intensity of EB and DCF induced by fMLP (Fig. 4B, $p < 0.05$, $n = 5$–8).

SPRST, Tet, and Fan Limited fMLP-induced Intracellular pH Alkalization. ROS production induced by fMLP is a calcium sensitive event (Lew et al., 1984) and accompanied by transient cytosolic alkalization to maintain the activity of NADPH oxidase (Henderson and Meech, 1999). In this study, we observed that fMLP induced a rapid and profound alkalization of pH_i over 60 min (Fig. 5). Pretreatment with 10 μg/ml of SPRST, Tet, or Fan as well as verapamil (10 μM) significantly limited the cytosolic alkalization induced by fMLP (ANOVA, $p < 0.05$, $n = 5$) indicating that a calcium dependent pathway may be modulated by these drugs.

Relationship between Ca^{2+} Influx and SPRST-Inhibited Neutrophil Adhesion. In addition to modulating ROS

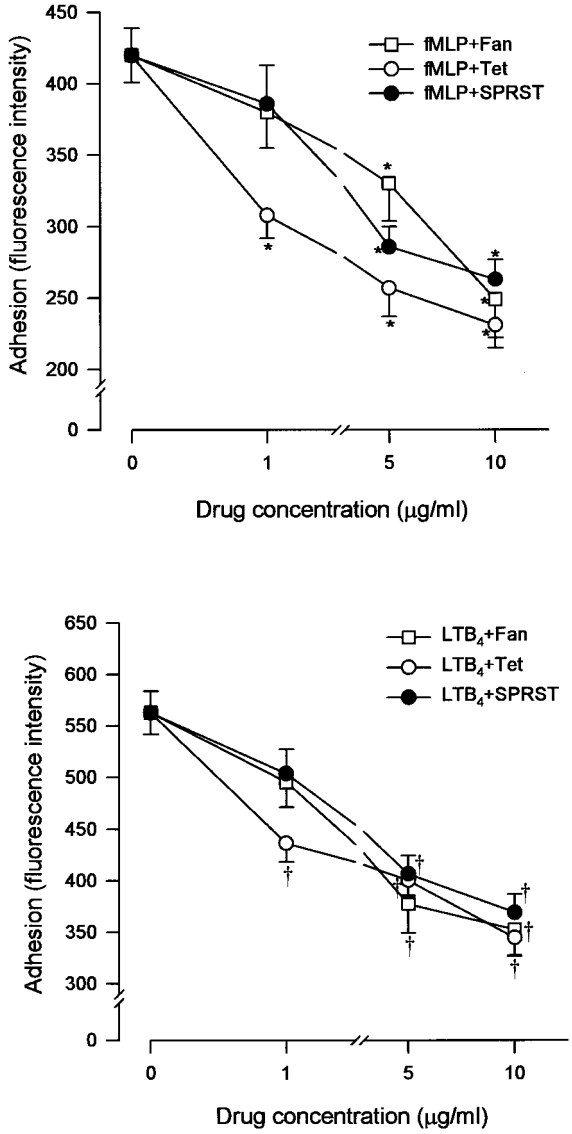

Fig. 1. Mean concentration-response curves for SPRST, Tet, or Fan in the inhibition of fMLP- or LTB_4-induced neutrophil adhesion. Neutrophils (1×10^7/ml) were loaded with 1 μM BCECF-AM for 20 min at 37°C and washed twice. BCECF-labeled neutrophils (5×10^5/ml) were then pretreated with 1 to 10 μg/ml of SPRST, Tet, or Fan for 10 min at 37°C, and plated onto fibrinogen-coated 24-well plates. After stimulating with 1 μM fMLP (top) or 0.1 μM LTB_4 (bottom) for an additional 15 min at 37°C, nonadherent cells were washed off and adherent cells were quantified by measuring fluorescence intensity. Values are mean ± S.E.M. ($n = 6$). *,†$p < 0.05$, compared with samples receiving fMLP or LTB_4 alone, respectively.

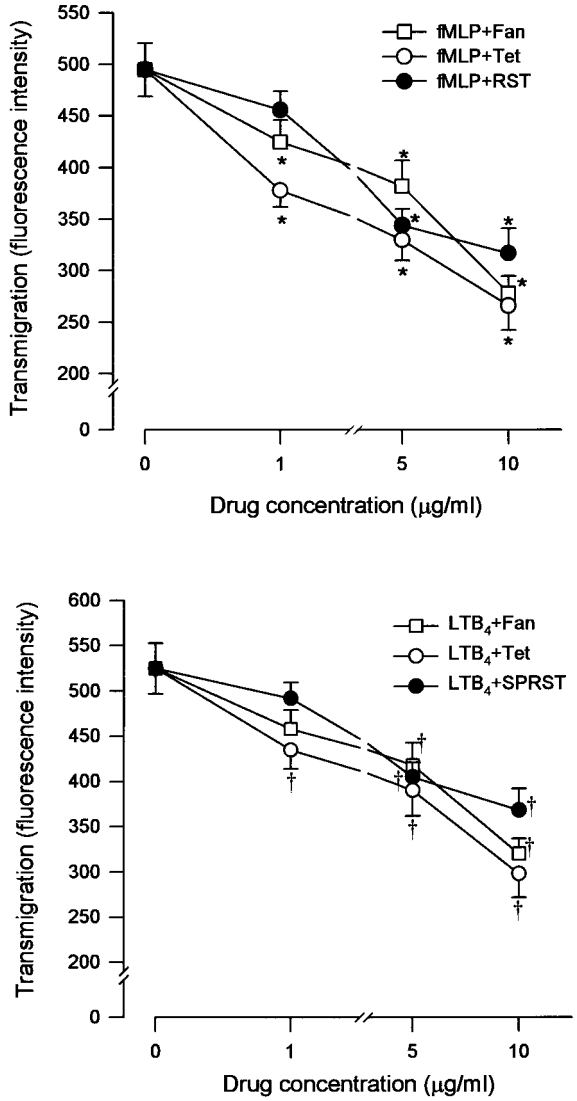

Fig. 2. Mean concentration-response curves for SPRST, Tet, or Fan in the inhibition of fMLP- or LTB_4-induced neutrophil transmigration. Neutrophils (1×10^7/ml) were loaded with 1 μM BCECF-AM for 20 min at 37°C and washed twice. BCECF-labeled neutrophils (5×10^5/ml) were pretreated with 1 to 10 μg/ml of SPRST, Tet, or Fan for 10 min at 37°C, and then plated onto upper chamber of fibrinogen-coated inserts. After stimulating with 1 μM fMLP (top) or 0.1 μM LTB_4 (bottom) in the lower chamber for an additional 60 min at 37°C, transmigrated cells in the lower chambers were quantified by measuring fluorescence intensity. Values are mean ± S.E.M. ($n = 6$). *,†$p < 0.05$, compared with samples receiving fMLP or LTB_4 alone, respectively.

production, cytosolic calcium fluctuation could also regulate neutrophil migration (Lawson and Maxfield, 1995), and we have previously reported that impediment to calcium influx diminished Mac-1 dependent neutrophil adhesion (Shen et al., 1999); therefore, effects of SPRST, Tet, and Fan in Ca^{2+} mobilization were determined. Calcium influx could be triggered by receptor-coupled activation or by direct G protein activation. To elucidate the possible targets of these drugs, fMLP/LTB_4 (receptor-mediated) or AlF_4^- (direct G protein-mediated) induced calcium mobilization was performed. fMLP or LTB_4 triggered rapid increase in $[Ca^{2+}]_i$ which was abolished by pertussis toxin (PTX) pretreatment, and were inhibited in a concentration-dependent manner by SPRST, Tet, or Fan (Fig. 6, $p < 0.05$, $n = 4$–8). AlF_4^--induced $[Ca^{2+}]_i$ increment and neutrophil adhesion were also significantly inhibited by PTX and concentration-dependently decreased by SPRST, Tet, or Fan (Fig. 7, $p < 0.05$, $n = 5$). SPRST was as potent as Tet or Fan in antagonizing Ca^{2+} mobilization or adhesion induced by AlF_4^- (Fig. 7, ANOVA, $p > 0.05$).

Discussion

In the present study, neutrophils pretreated with 1 to 10 μg/ml of SPRST for 10 min significantly impaired neutrophil adhesion (Fig. 1) and transmigration (Fig. 2). Same concentration of Tet and Fan, two active components in SPRST, displayed similar results as SPRST (Fig. 1; Fig. 2). In neutrophils pretreated with 0.1 μg/ml of Tet or Fan (SPRST containing 1.3% Tet and 0.7% Fan), neither single drug treatment nor combination of Tet and Fan could attenuate neutrophil adhesion or transmigration (data not shown), indicating that elements in addition to Tet and Fan might be involved in mediating the effect of SPRST. The antiadhesive and transmigration prevention effects of SPRST were not caused by cytotoxicity because under these conditions, there was no difference in cell viability between SPRST-treated neutrophils and control cells (viability > 95% at the end of the experiments). To further elucidate the mechanism(s) involved in the anti-inflammatory effects of SPRST, in view of the importance of Mac-1 (CD11b/CD18) in neutrophil adhesion (Albelda et al., 1994) and transmigration (Werr et al.,

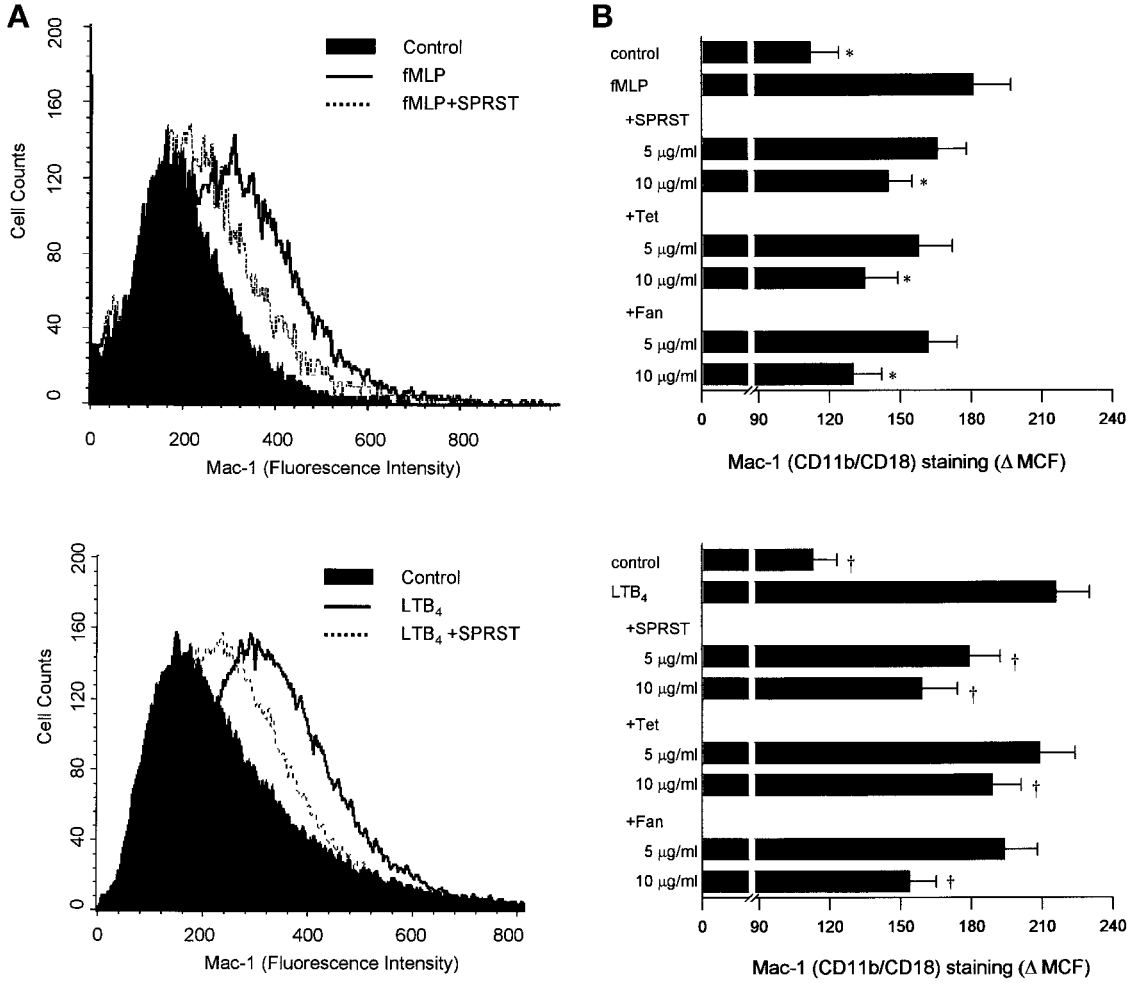

Fig. 3. Effects of SPRST, Tet, or Fan on fMLP- or LTB_4-induced Mac-1 up-regulation. A, flow cytometric analysis of total Mac-1 levels on the cell surface of neutrophils. Control neutrophils received neither SPRST nor fMLP/LTB_4 treatment. SPRST (10 μg/ml)-pretreated sample, designated 'fMLP+SPRST' or 'LTB_4+SPRST', were stimulated with 1 μM fMLP (top) or 0.1 μM LTB_4 (bottom). B, statistical summaries of fMLP- (top) or LTB_4- (bottom) up-regulated Mac-1 expression in the presence or absence of 5–10 μg/ml of SPRST, Tet, or Fan. Net increase in mean channel fluorescence (ΔMCF) was calculated by subtracting the MCF value from sample receiving nonspecific IgG_1 staining (70 ± 12). The control value is 112 ± 12 (top) or 113 ± 10 (bottom). Values represent the mean ± S.E.M. of ΔMCF ($n = 3$–5) experiments. *,† $p < 0.05$, compared with samples receiving fMLP or LTB_4 alone, respectively.

2000), we further examined the effect of SPRST on cell surface expression levels of Mac-1. SPRST, Tet, and Fan significantly prevented fMLP or LTB_4-induced Mac-1 up-regulation (Fig. 3) revealing that the antiadhesive and transmigration prevention effects of these drugs were, at least in part, mediated by inhibition of the Mac-1 up-regulation on neutrophil membrane.

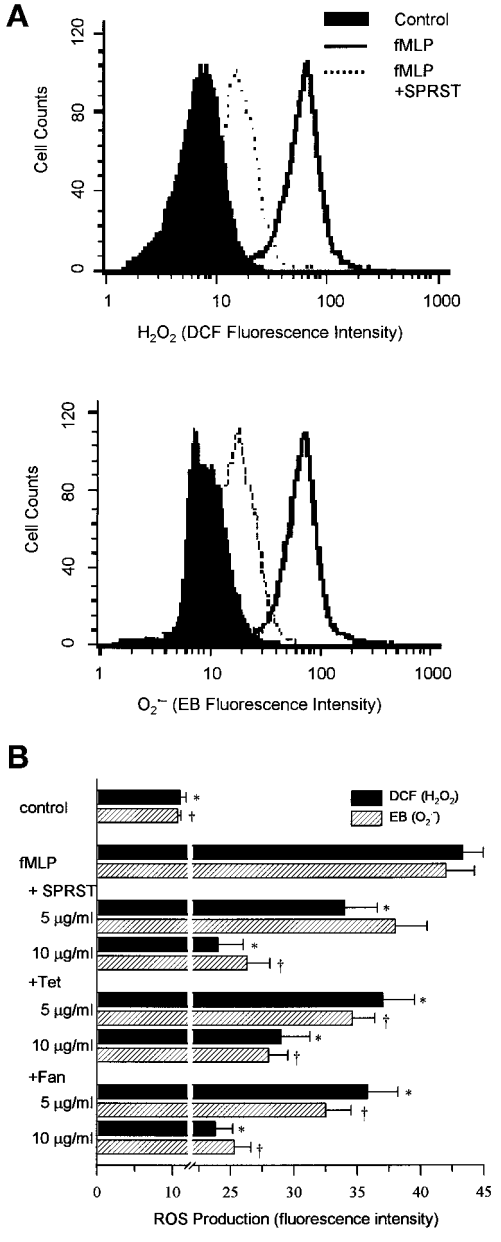

Fig. 4. Effects of SPRST, Tet, or Fan on fMLP-induced ROS (H_2O_2 and $O_2^{\cdot-}$) production. Neutrophils (1×10^6/ml) were incubated at 37°C for 5 min with DCFH-DA (20 μM) and for additional 15 min with hydroethidium (10 μM). After labeling, cells were pretreated with 5 to 10 μg/ml SPRST or other chemicals for 10 min and stimulated with fMLP (1 μM). Production of $O_2^{\cdot-}$ and H_2O_2 were then determined 30 min later by flow cytometry. A, flow cytometric analysis of H_2O_2 (DCF fluorescence, upper panel) and $O_2^{\cdot-}$ (EB fluorescence, bottom) production. Control neutrophils received neither SPRST nor fMLP treatment. SPRST (10 μg/ml)-pretreated samples, designated 'fMLP+SPRST', were stimulated with fMLP. B, statistical summaries of fMLP- induced H_2O_2 and $O_2^{\cdot-}$ production in the presence of 5 to 10 μg/ml of SPRST, Tet, or Fan. The control values are 11.0 ± 0.8 and 10.7 ± 0.4 for DCF (H_2O_2) and EB ($O_2^{\cdot-}$), respectively. Values are mean ± S.E.M. (n = 5–8). *,† $p < 0.05$, compared with samples receiving fMLP alone for DCF (H_2O_2) or EB ($O_2^{\cdot-}$), respectively.

It has been reported that ROS enhanced Mac-1 up-regulation and antioxidants diminished Mac-1-mediated neutrophil accumulation and adhesion after ischemia and reperfusion (Fraticelli et al., 1996; Serrano et al., 1996). In this study, ROS ($O_2^{\cdot-}$ and H_2O_2) production induced by fMLP was diminished by SPRST as well as Tet and Fan (Fig. 4). This indicates that SPRST, Tet, and Fan may act as ROS scavengers through which to down-regulate Mac-1 expression and then neutrophil adhesion/transmigration. Our prior studies confirmed that antioxidants (superoxide dismutase and catalase) significantly down-regulated ROS production as well as Mac-1 expression and neutrophil adhesion to fibrinogen (Shen et al., 1998, 1999). The flow cytometric method used in this study for the measurement of ROS production enabled on-line monitoring of the intracellular accumulation of $O_2^{\cdot-}$ and H_2O_2 in neutrophils. We found accumulation of $O_2^{\cdot-}$ and H_2O_2 began immediately after stimulation (data not shown). Thus, the rapid accumulation of $O_2^{\cdot-}$ and H_2O_2 in response to stimulation and our observation that Mac-1 up-regulation could be inhibited by ROS scavengers (Shen et al., 1999) suggest that ROS are early signaling molecules involved in the regulation of neutrophil function. This argument is further intensified by Finkel's observations (1998) that ROS can act as second messengers in the activation of ligand-stimulated nuclear factor-κB, various protein kinase C family members, and mitogen-activated protein kinase as well as tyrosine kinases/phosphatase. Thus, we suggest that ROS could regulate neutrophil functions through second messenger mechanism(s).

ROS production by neutrophil through activation of membrane-bound NADPH oxidase is accompanied by transient cytosolic alkalization to maintain the activity of this enzyme (Henderson and Meech, 1999). In this study, fMLP induced a rapid and intense intracellular alkalization (Fig. 5). Comparable findings had been reported by Coakley et al. (2000). Verapamil as well as SPRST, Tet, and Fan limited the

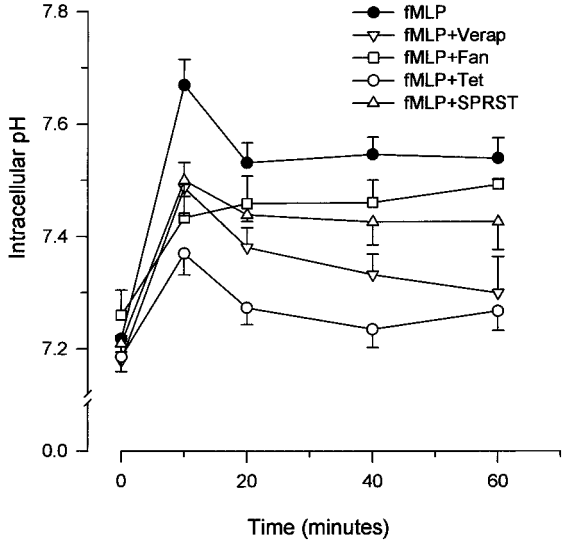

Fig. 5. Mean time-response curves for SPRST, Tet, or Fan in the inhibition of fMLP-induced intracellular alkalization (pH_i). Neutrophils (1×10^6/ml) were loaded with BCECF-AM (2 μg/ml) for 30 min at 37°C and washed twice. BCECF-loaded neutrophils were pretreated with10 μg/ml of SPRST, Tet, or Fan as well as 10 μM verapamil (Verap) for 10 min at 37°C. After stimulating with fMLP (1 μM), pH_i was measured by flow cytometry as described under *Materials and Methods* at the time indicated in the figure. Values are mean ± S.E.M. (n = 5).

prompt cytosolic alkalization (Fig. 5), indicating a calcium-dependent pathway mediated fMLP-induced alkalization that could regulate ROS production. This is further illustrated by the observation that ROS production induced by fMLP is related to calcium-dependent priming of neutrophil, which, if blocked, interferes with ROS production (Lew et al., 1984). We found fMLP and LTB_4 trigger prompt and prominent $[Ca^{2+}]_i$ increment, and both could be diminished by SPRST, Tet, and Fan (Fig. 6). Thus, modulation of calcium mobilization could be the possible target by these drugs. To elucidate the possible target by SPRST, AlF_4^-, a direct G protein activator, induced calcium influx was introduced to contrast the receptor (fMLP or LTB_4)-mediated calcium mobilization. SPRST, Tet, and Fan concentration-dependently impaired AlF_4^--induced calcium influx as well as neutrophil adhesion (Fig. 7). Therefore, G protein could be modulated by SPRST. Because SPRST, containing 1.3% Tet and 0.7% Fan, was as potent as Tet and Fan in the inhibition of AlF_4^--induced calcium influx and neutrophil adhesion (Fig. 7, ANOVA, $p > 0.05$) indicated component(s) in addition to Tet and Fan mediated the inhibitory effect of SPRST.

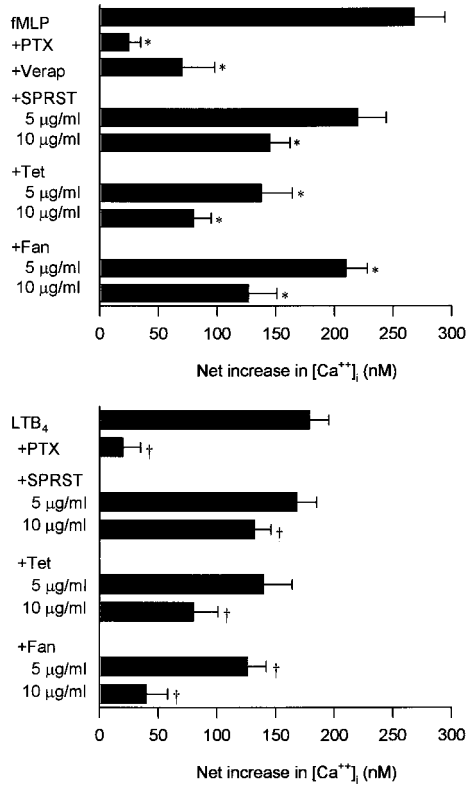

Fig. 6. Effects of SPRST, Tet, or Fan on fMLP- or LTB_4-induced changes in intracellular calcium concentration ($[Ca^{2+}]_i$). Neutrophils (2×10^6/ml) were preloaded with fura 2-AM (5 μM) at 37°C for 45 min and washed twice with HBSS (calcium free). After drug treatments with 5 to 10 μg/ml of SPRST, Tet, or Fan as well as 10 μM verapamil (Verapa) for 10 min, 1 ml of cell suspension from each treatment was mixed with equal volume of HBSS (with 2 mM Ca^{2+}) and transferred into individual cuvettes. For G protein study, sample was pretreated with 500 ng/ml of PTX at 37°C for 2 h. Samples were gentle mixed with a micromagnetic stirrer at 37°C for 5 min before addition of 1 μM fMLP (top) or 0.1 μM LTB_4 (bottom). $[Ca^{2+}]_i$ was measured on a spectrofluorometer as described under *Materials and Methods*. Net increase in $[Ca^{2+}]_i$ was calculated by subtracting control values from respective experimental values (control $[Ca^{2+}]_i$ in resting cell was 108 ± 16 nM). Values are mean ± S.E.M. (n = 4–8). *,† $p < 0.05$, compared with samples receiving fMLP or LTB_4 alone, respectively.

In addition to inhibition of ROS production and Ca^{2+} mobilization, SPRST may also inhibit other biochemical pathways that could regulate Mac-1 expression. For example, Mac-1 expression may be regulated by phospholipase A_2, which catalyzes the synthesis of arachidonate, because phospholipase A_2 inhibitors can inhibit the surface expression of Mac-1 (Jacobson and Schrier, 1993). Tet has been shown to decrease the production of prostaglandin E_2 and leukotriene $C_4/D_4/E_4$, downstream metabolites of arachidonate (Teh et al., 1990). It is likely that this biochemical pathway may be targeted by SPRST and Mac-1 expression could be regulated in turn. Furthermore, it is noted that mitogen-activated protein kinase pathways play a central role in regulating a wide range of inflammatory responses, including activation of NADPH oxidase (Yamamori et al., 2000), migration of neutrophils (Atta et al., 1999) as well as β_2 integrin expression (Tandon et al., 2000). Whether these biochemical pathways are targets of SPRST in the regulation of Mac-1 dependent neutrophil adhesion and transmigration awaits further research and is currently under investigation in our laboratory.

In conclusion, we have demonstrated that inhibition of neutrophil adhesion and transmigration through suppression of

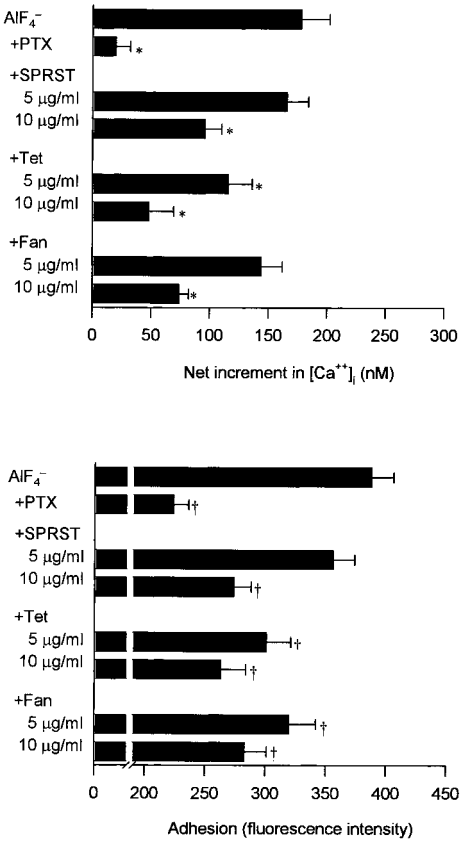

Fig. 7. Effects of SPRST, Tet, or Fan on AlF_4^--induced changes in intracellular calcium concentration ($[Ca^{2+}]_i$) and neutrophil adhesion. Fura 2-AM– or BCECF-AM–labeled neutrophils were pretreated with 5 to 10 μg/ml of SPRST or other chemicals at 37°C for 10 min. For G protein study, sample was pretreated with 500 ng/ml of PTX at 37°C for 2 h before the addition of AlF_4^- (10 mM NaF plus 10 μM $AlCl_3$), a direct G protein activator. AlF_4^--induced changes in $[Ca^{2+}]_i$ (top) and neutrophil adhesion (bottom) were measured as described under *Materials and Methods*. Untreated neutrophils displayed spontaneous adhesion with a fluorescence intensity of 218 ± 22. Values are mean ± S.E.M. (n = 5). *,† $p < 0.05$, as compared with samples receiving AlF_4^- alone for $[Ca^{2+}]_i$ and neutrophil adhesion, respectively.

Mac-1 up-regulation could account for the cardioprotective effect of SPRST. The inhibitory effect of SPRST on Mac-1 expression could be mediated by down-regulation of ROS production and intracellular Ca^{2+} mobilization through, at least in part, G protein modulation. The effect of SPRST per se can be attributed to component(s) in addition to Tet and Fan because no significant effect was observed by combination low dose (0.1 μg/ml) of Tet and Fan. As effective antiadhesive and transmigration-preventing drugs at pharmacological concentrations (1–10 μg/ml), SPRST, along with its active components Tet and Fan, may be clinically beneficial for the amelioration of I/R injury by limiting the early phases of neutrophil activation.

Acknowledgments

We thank Yu-Ling Chen for her technical assistance in isolation and purification of SPRST, Tet, and Fan. The invaluable assistance in language editing and revision of this manuscript by Dr. Andrew Y. Shum of the Department and Institute of Pharmacology, the National Yang-Ming University, Taipei, Taiwan, ROC, is gratefully acknowledged.

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