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Impediment to Calcium Influx and Reactive Oxygen Production Accounts for the Inhibition of Neutrophil Mac-1 Up-Regulation and Adhesion by Tetrandrine

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ABSTRACT

We studied the mechanisms by which the plant alkaloid tetrandrine (TTD) inhibits Mac-1-dependent neutrophil adhesion to fibrinogen. TTD (0.1–10 μM) significantly inhibited Mac-1 up-regulation and neutrophil adhesion, as induced by *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) or phorbol-myristate-acetate (PMA). Treatment of neutrophils with fMLP or PMA caused a rapid influx of Ca^{++} and accumulation of reactive oxygen species (ROS), both of which have been shown to enhance neutrophil adhesion via Mac-1 up-regulation. Because TTD antagonizes Ca^{++} influx and abrogates ROS, we examined the relationship between Ca^{++} influx, ROS formation, and Mac-1 expression in TTD-inhibited neutrophil adhesion. TTD alone caused a slight but statistically significant increase in $[Ca^{++}]_i$ with no effect on adhesion. In contrast, TTD as well as two Ca^{++} channel antagonists, verapamil and nifedipine, markedly diminished fMLP- and PMA-induced Ca^{++} influx, Mac-1 up-regulation, and adhesion. TTD also inhibited increases in $[Ca^{++}]_i$ and adhesion induced by the ionophore A23187 but failed to inhibit those induced by thapsigargin, an agent mobilizing Ca^{++} from intracellular stores. Thus, TTD impeded Ca^{++} influx from outward to avert neutrophil adhesion. Similarly, TTD and two ROS scavengers, superoxide dismutase and catalase, abolished ROS production, Mac-1 up-regulation, and neutrophil adhesion. Ca^{++} and ROS, therefore, represent two essential signals for Mac-1 up-regulation upon fMLP or PMA stimulation. Our data suggest that the antiadherent effect of TTD is mediated, in part, by the inhibition of Ca^{++} influx and ROS formation, resulting in suppressed up-regulation of Mac-1 and, in turn, neutrophil adhesion to fibrinogen.

Tetrandrine (TTD) is a benzylisoquinoline alkaloid isolated from the Chinese herb "Hanfangji" (*Radix Stephania Tetrandra*) that has been used as an anti-inflammatory agent both clinically (Li et al., 1981) and experimentally (Whitehouse et al., 1994). Numerous pharmacological activities have been attributed to the anti-inflammatory effects of TTD. These include the scavenging of oxygen radicals (Seow et al., 1988a), suppression of neutrophil chemotaxis and phagocytosis (Seow et al., 1988b), and inhibition of neutrophil and monocyte adhesion (Seow et al., 1986).

Neutrophil adhesion is a major event of the early inflammatory response that is characterized by the recruitment of neutrophils into areas of inflammation, which begins with the binding of these cells to endothelium followed by their transmigration into tissues (Albelda et al., 1994). Distinct phases have been characterized during neutrophil binding, including rolling, activation, and firm adhesion (Ley, 1996). The molecular basis for these phases is the up-regulation of various cell adhesion molecules that belong to three major families: the selectins (e.g., P-selectin, L-selectin, and E-selectin), the β_2 integrins (e.g., CD11/CD18), and the immmunoglobulin superfamily (e.g., intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and platelet-endothelial cell adhesion molecule-1) (Albelda et al., 1994). The selectins are important for rolling, whereas firm adhesion of neutrophils is primarily β_2 integrin-dependent (Springer and Anderson, 1986). The β_2 integrins comprise a group of heterodimeric glycoproteins, including leukocyte function-associated antigen-1 (CD11a/CD18), Mac-1 (CD11b/

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ABBREVIATIONS: BAPTA/AM, 1,2-bis-(*O*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester; CAT, catalase; DCF, 2′,7′-dichlorofluorescein; EB; ethidium bromide; FBS, fetal bovine serum; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; HBSS, Hanks' balanced salt solution; H_2O_2, hydrogen peroxide; Mac-1, macrophage adhesion molecule-1, also, CD11b/CD18; TTD, tetrandrine; PBS, phosphate-buffered saline; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species; $O^{\cdot}_2$, superoxide anion; SOD, superoxide dismutase; $[Ca^{++}]$, intracellular calcium concentration; PKC, protein kinase C; PLA_2, phospholipase A_2.

CD18), and p150,95 (CD11c/CD18), with leukocyte function-associated antigen-1 and Mac-1 being the predominant integrins on neutrophils. Although all three of these β_2 integrins may participate in the intercellular adhesive interaction, Mac-1 appears to be the major integrin responsible for the firm adhesion of neutrophils to endothelium and myriad extracellular matrix molecules (Lefer and Lefer, 1996). Increased neutrophil adhesion has been demonstrated to be an important factor in the pathogenesis of vascular injury during inflammatory processes, such as the ischemic reperfusion injury of the liver (Monden et al., 1995). Hence, therapeutic interventions targeting these phases of neutrophil adhesion may prove to be effective. For example, administration of a monoclonal antibody directed against P-selectin could reduce reperfusion-caused exasperation of vascular permeability by 36% in a skeletal muscle ischemia model (Weiser et al., 1996). Similarly, a monoclonal antibody against Mac-1 has also been shown to prevent intestine ischemic reperfusion-induced lung injury, regardless of the activation status of neutrophils (Koike et al., 1995). With its antiadhesive capability, TTD could be of particular usefulness as an anti-inflammatory agent because it may block neutrophil recruitment during the early stages of the inflammatory response. The mechanism by which TTD inhibits neutrophil adhesion, therefore, warrants further investigation.

We hypothesize that inhibition of neutrophil adhesion by TTD may be mediated by interference with the up-regulation of adhesion molecules. We used *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), a receptor-mediated neutrophil activator, and phorbol-12-myristate-13-acetate (PMA), a nonreceptor-mediated neutrophil activator, to stimulate neutrophil adhesion to a fibrinogen-coated surface as a means to investigate the antiadhesive effects of TTD. Because firm adhesion of neutrophils is primarily Mac-1-dependent (Lefer and Lefer, 1996), we examined whether TTD could influence Mac-1 up-regulation in neutrophils. Furthermore, because Mac-1 up-regulation is controlled by intracellular mechanisms including mobilization of Ca^{++} (Lawson and Maxfield, 1995) and generation of reactive oxygen species (ROS) (Simms and D'Amico, 1995), both of which could be affected by TTD (Felix et al., 1992; Cao, 1996), we studied whether TTD could inhibit Mac-1 up-regulation of neutrophils by the blockade of Ca^{++} influx and/or inhibition of ROS production.

Materials and Methods

Preparation of Neutrophils. Venous blood samples were collected in syringes containing heparin (final concentration 20 U/ml) from healthy 20- to 40-year-old volunteers of both sexes. Neutrophils were isolated by the Ficoll gradient centrifugation method as described by Boyum (1974). Briefly, blood samples were mixed with an equal volume of 3% dextran solution in 50-ml centrifuge tubes and incubated in an upright position for 20 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. Immediately after centrifugation, the pellet was resuspended in a volume of phosphate-buffered saline (PBS) equal to the starting volume of the blood. The cell suspension was then apportioned at 25 ml per tube into 50-ml centrifuge tubes, followed by layering beneath the cell suspension with 10 ml of 1.077 g/ml Ficoll solution (Histopaque 1077; Sigma Chemicals Co., St. Louis, MO). After centrifugation at 400*g* for 40 min at 20°C without the brake, the granulocyte/erythrocyte pellet was collected and the erythrocytes lysed by resuspension in 20 ml of cold 0.2% NaCl for 30 s, followed by addition of 20 ml of cold 1.6% NaCl to restore tonicity. The remaining neutrophils were collected by centrifugation, washed twice with ice-cold PBS, and resuspended in adequate volumes of ice-cold Hanks' balanced salt solution (HBSS) until further manipulation. The preparation contained more than 95% neutrophils, as estimated by counting 200 cells under microscope after Giemsa staining (Sigma). Pretreatment with TTD was performed by mixing the cells with the drug at concentrations from 0.1 to 10 μM in HBSS for 10 min at 37°C.

Measurement of Neutrophil Adhesion. Adhesion of neutrophils to extracellular matrix was performed in flat-bottom 24-well tissue culture plates (Costar, Cambridge, MA) coated with human fibrinogen (Chemicon International, Inc., Temecula, CA). Before the addition of neutrophils, the plates were incubated with 200 μl per well of fibrinogen (50 μg/ml in PBS) 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. Five hundred microliters per well of TTD-pretreated neutrophils (5×10^5 cells/ml in HBSS) were then plated into individual wells. After stimulation with fMLP (1 μM) or PMA (100 ng/ml) for 15 min at 37°C, nonadherent cells were removed by aspiration and the wells were gently washed twice with warm PBS. Adherent neutrophils were stained with 250 μl of 0.25% rose bengal solution (Sigma) for 10 min at room temperature. The staining solution was aspirated, each well washed twice with PBS, and 250 μl of ethanol/PBS (1:1) solution added to dissolve the cell-retained dye. Optical density at 570 nm (A_{570}) of each well was then determined with a microplate reader (EL311sx; Biotek Instruments, Winoski, VT) using wells containing medium alone as blanks. Data are expressed as $A_{570} \times 100$.

Measurement of Mac-1 Up-Regulation by Flow Cytometry. Expression of Mac-1 (CD11b/CD18) was analyzed as described by Endemann et al. (1996) with some modification. TTD-pretreated neutrophils were stimulated with PMA (100 ng/ml) for 15 min. The cells were pelleted and then 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 performed in an ice bath. Neutrophils were incubated for 60 min with 20 μg/ml of an anti-Mac-1 primary antibody (mouse anti-human CD11b, class IgG_1; Pharmingen, San Diego, CA) or a nonspecific mouse IgG_1 (Sigma) as a negative control. After two washes with ice-cold PBS-FBS-azide, the cells were further incubated for 30 min in the dark with 20 μg/ml of a fluorescein isothiocyanate-labeled secondary antibody (goat anti-mouse IgG_1; Serotec, Kidlington, Oxford, UK) and then washed twice with PBS containing 5% FBS. Finally, stained cells were resuspended in flow cytometer sheath fluid (Becton Dickinson, Lincoln Park, NJ) containing 1% paraformaldehyde and analyzed on a flow cytometer (FACSort; Becton Dickinson) for Mac-1 expression. Data are expressed as peak channel fluorescence for each sample as calculated using the CellQuest software (Becton Dickinson).

Determination of Intracellular Calcium Concentration ($[Ca^{++}]_i$). Before drug treatment, 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, Eugene, OR) at 37°C for 45 min, washed twice, and resuspended at 1×10^6 cells/ml in HBSS containing TTD or control vehicle. After drug treatment for 10 min, 2 ml of cell suspension from each sample was transferred to individual cuvettes and gently mixed with a micromagnetic stirrer at 37°C for 3 min before the addition of fMLP or PMA. The fluorescence of fura 2-loaded cells was measured on a spectrophotometer (Hitachi F-4500; Hitachi Instruments, San Jose, CA) with excitation at 340 and 380 nm and emission at 510 nm. $[Ca^{++}]_i$ for each sample was calculated from the ratio of

emission versus excitation as previously described (Grynkiewicz et al., 1985):

$$[Ca^{++}]_i = K \cdot (R - R_{min})(S_{f380})/(R_{max} - R)(S_{b380})$$

where K = 224 nM (fura 2 at 37°C), R_{min} = ratio value in minimal Ca^{++} conditions, R_{max} = ratio value at a maximal Ca^{++} concentration, S_{f380} = 380 nm reading in minimal Ca^{++} conditions (corrected for background), and S_{b380} = 380 nm reading in maximal Ca^{++} 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, where R_{min} and S_{f380} were determined by adding 20 mM EGTA after digitonin lysis. All measurements were performed in Ca^{++}-containing medium because no significant changes in $[Ca^{++}]_i$ could be detected under Ca^{++}-free conditions, which also has been described by others (Hong et al., 1989).

Flow Cytometric Analysis of Intracellular Superoxide Anion ($O_2^{\cdot-}$) and Hydrogen Peroxide (H_2O_2) Production. Production of $O_2^{\cdot-}$ and H_2O_2 was analyzed by a flow cytometric method described by Robinson et al. (1994) that was able to detect intracellular accumulation of ROS. Briefly, neutrophils were incubated at 37°C for 5 min with 20 mM 2′,7′-dichlorofluorescein diacetate (Molecular Probes) and for an additional 15 min with 10 mM hydroethidine (Molecular Probes). The acetate moieties of 2′,7′-dichlorofluorescein diacetate 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. Hydroethidine, on the other hand, is directly oxidized by $O_2^{\cdot-}$ to ethidium bromide (EB), which fluoresces intensely after intercalating with nucleic acids. After labeling, cells were treated with TTD and then stimulated with PMA (100 ng/ml). The cells were maintained at 37°C and production of $O_2^{\cdot-}$ and H_2O_2 was monitored every 10 min on a flow cytometer (FACSort; Becton Dickinson) by measuring emissions at 590 nm (FL2) and 525 nm (FL1) for EB ($O_2^{\cdot-}$) and DCF (H_2O_2), respectively. Data are expressed as peak channel fluorescence for each sample as described above.

TTD and Other Chemicals. TTD was purchased from Aldrich (Milwaukee, WI). Before use, the drug was dissolved in 0.1 N HCl at 10 mM and then serially diluted in PBS to adequate concentrations. The stock solution was used within 1 week of preparation. For examination of the effect of TTD, 5 μl of TTD solution was added to 500 μl of neutrophil suspension and incubated at 37°C for 10 min before the addition of 100 ng/ml PMA (Sigma). All other chemicals, including superoxide dismutase (SOD), catalase (CAT), A23187, thapsigargin, and calcium channel antagonists, were purchased from Sigma. 1,2-Bis-(*O*-aminophenoxy)ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester (BAPTA/AM) was purchased from Molecular Probes.

Statistical Analysis. Data were analyzed by one- or two-way analyses of variance depending on the number of variables in each experiment. To compare means between groups, a protected Fisher's least significant difference test was used at an α level equal to .05. Concentration dependence was analyzed by simple linear regression analysis of response levels against concentrations of TTD and testing the slope of the regression line against 0 by Student's t test at an α level equal to .05.

Results

TTD Inhibits Mac-1 Up-Regulation and Mac-1-Dependent Adhesion of Neutrophils. The anti-adhesive effect of TTD was investigated on the adhesion of fMLP- or PMA-stimulated neutrophils to a plastic surface coated with fibrinogen, an extracellular matrix to which neutrophil adhesion has been shown to be Mac-1-dependent (Everitt et al., 1996). The requirement of Mac-1 for neutrophil adhesion was established by blocking the α subunit of Mac-1, CD11b, with a specific antibody (anti-CD11b, 2 μg/ml, isotype IgG_1), which almost completely abolished neutrophil adhesion as shown previously (Shen et al., 1998). Whereas fMLP (1 μM) or PMA (100 ng/ml) caused up to 300% enhancement in neutrophil adhesion relative to background levels (measured by rose bengal staining), pretreatment of neutrophils with TTD (0.1–10 μM) dose dependently inhibited adhesion induced by these stimulators (Fig. 1). TTD alone did not affect the background neutrophil adhesion (Fig. 1). The concentrations of TTD used in this study were not cytotoxic to neutrophils (viability after TTD treatment >95% as measured by trypan blue exclusion assay).

To assess the effect of TTD on Mac-1 expression, surface levels of Mac-1 were measured on fMLP- or PMA-stimulated neutrophils with or without TTD pretreatment using a FACSort (Becton Dickinson). fMLP and PMA caused a marked increase in Mac-1 fluorescence, which was inhibited by TTD in a concentration-dependent manner (Fig. 2A). A statistical summary of the peak channel fluorescence is illustrated in Fig. 2B.

Relationship between Ca^{++} Influx, Mac-1 Expression, and TTD-Inhibited Neutrophil Adhesion. Under our experimental conditions, both fMLP and PMA triggered Ca^{++} influx. Because TTD antagonizes Ca^{++} channels (Felix et al., 1992) and cytosolic Ca^{++} fluctuation can regulate Mac-1-mediated neutrophil adhesion (Lawson and Maxfield, 1995), we studied the relationship between Ca^{++} influx and Mac-1 up-regulation in the presence or absence of TTD pretreatment. TTD markedly inhibited fMLP- or PMA-induced increase in $[Ca^{++}]_i$ (Fig. 3A) in a concentration-dependent fashion (Fig. 3B). Interestingly, in nonstimulated neutrophils, TTD caused a slight yet statistically significant increase in $[Ca^{++}]_i$ (Fig. 3B, based on Student's t test for dose dependence of TTD).

To characterize the relationship between blockade of Ca^{++}

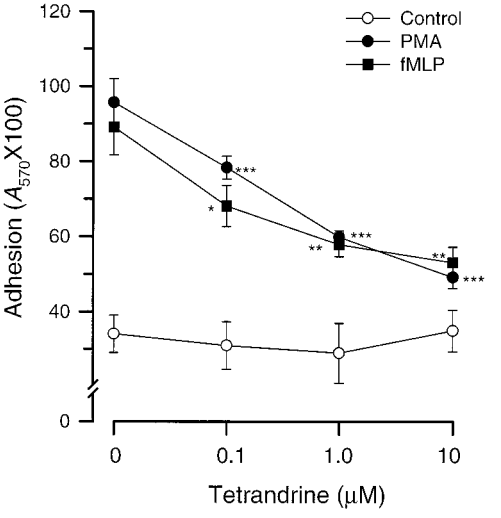

Fig. 1. Mean concentration-response curves for TTD in the inhibition of fMLP (1 μM)- or PMA (100 ng/ml)-induced neutrophil adhesion. Human neutrophils were pretreated with TTD (0.1–10 μM) for 10 min at 37°C, and then plated on fibrinogen-coated 24-well plates. After stimulation with fMLP and/or PMA for an additional 15 min at 37°C, nonadherent cells were washed off and adherent cells were stained with 0.25% rose bengal and quantified by measuring optic density at 570 nm (A_{570}). Control levels are background adhesion of neutrophils without fMLP or PMA stimulation. Values are mean and vertical lines S.E.M. of six experiments. *$p < .05$, **$p < .01$, ***$p < .001$ as compared with samples receiving fMLP or PMA alone.

influx, Mac-1 up-regulation, and adhesion to fibrinogen, the effect of TTD was compared with two calcium channel antagonists, verapamil and nifedipine. The antagonistic effect on Ca^{++} influx was comparable between TTD and these drugs (data not shown). Verapamil (10 μM) and nifedipine (10 μM) both suppressed PMA-induced Mac-1 up-regulation (Fig. 4A); however, these two Ca^{++} antagonists were less potent than TTD in the inhibition of Mac-1 expression (Fig. 4A) and adhesion (Fig. 4B).

The mechanisms underlying calcium antagonism of TTD were further investigated by examining the effect of TTD on changes in $[Ca^{++}]_i$ and neutrophil adhesion induced by calcium-mobilizing agents of two distinct classes: thapsigargin, an agonist that causes release of Ca^{++} from intracellular stores (Thastrup et al., 1989), and A23187, a calcium ionophore that produces Ca^{++} influx bypassing Ca^{++} channels. TTD failed to inhibit increases in $[Ca^{++}]_i$ and neutrophil adhesion induced by thapsigargin (1 μM) (Table 1). In contrast, the intracellular Ca^{++} chelator BAPTA/AM (10 μM) (Barritt and Lee, 1985) effectively inhibited thapsigargin-induced increase in $[Ca^{++}]_i$ and adhesion. EDTA chelated only extracellular Ca^{++} and, consequently, had no effect on thapsigargin-induced $[Ca^{++}]_i$ changes or neutrophil adhesion. Interestingly, TTD could also inhibit increase in $[Ca^{++}]_i$ and adhesion induced by a calcium ionophore A23187 that causes Ca^{++} influx bypassing Ca^{++} channels. Both BAPTA/AM and EDTA inhibited A23187-induced Ca^{++} mobilization and neutrophil adhesion (Table 1).

Relationship between Intracellular ROS Production, Mac-1 Up-Regulation, and TTD-Inhibited Neutrophil Adhesion. Reactive oxygen species ($O_2^{\cdot -}$ and H_2O_2) have recently been shown to up-regulate Mac-1 expression and enhance neutrophil adhesion (Fraticelli et al., 1996). To examine whether TTD could inhibit Mac-1 expression and, consequently, neutrophil adhesion through inhibiting ROS production, the effect of TTD was compared with that of ROS scavengers. Changes in intracellular ROS concentration were measured immediately after fMLP or PMA stimulation using a flow cytometric method (Shen et al., 1998). Both fMLP and PMA caused an immediate production of $O_2^{\cdot -}$ (measured as EB fluorescence) and H_2O_2 (measured as DCF fluorescence) up to 40 min after stimulation, which was inhibited by 10 μM TTD (Fig. 5, left). Introduction of ROS scavengers, including SOD and CAT, into the sample buffer completely abrogated PMA-induced accumulation of $O_2^{\cdot -}$ and H_2O_2 (Fig. 5, left). Both SOD and CAT markedly inhibited Mac-1 up-regulation; combination of the two reagents further

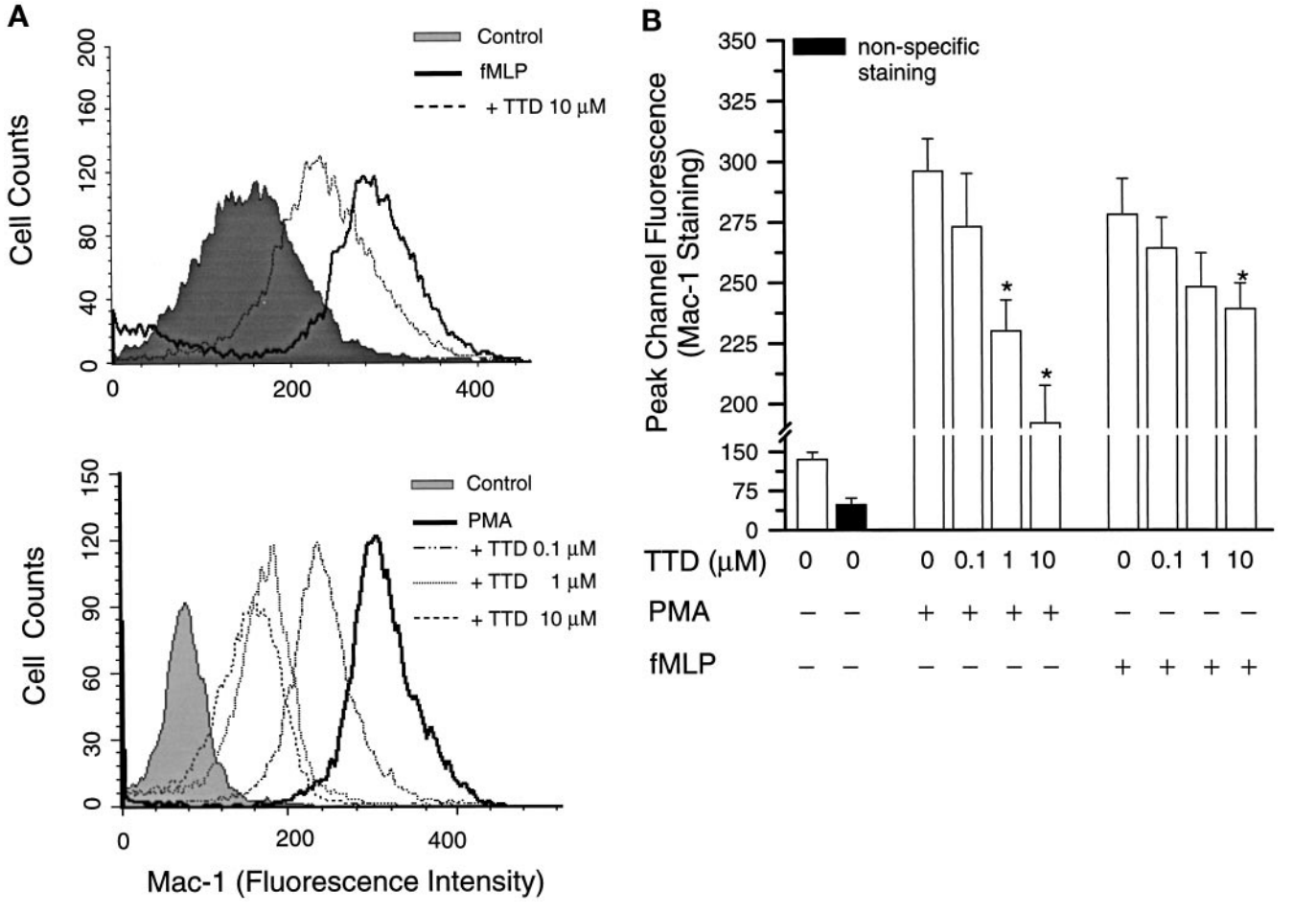

Fig. 2. Effect of TTD on fMLP (1 μM)- or PMA (100 ng/ml)-induced Mac-1 up-regulation. A, flow cytometric analysis of total Mac-1 levels on the cell surface of neutrophils. Control neutrophils received neither TTD nor fMLP or PMA treatment. All TTD-pretreated groups (+TTD) were stimulated with fMLP (upper panel) or PMA (lower panel). B, statistical summary of fMLP- or PMA-up-regulated Mac-1 expression in the presence of TTD (0.1–10 μM). Nonspecific IgG_1 (black column) was included to contrast the specificity of anti-CD11b staining. Values represent the mean of three experiments and vertical lines show S.E.M. *$p < .05$ as compared with samples receiving fMLP or PMA alone.

diminished Mac-1 expression (Fig. 6). In the presence of SOD and/or CAT, neutrophil adhesion to fibrinogen was significantly inhibited (Fig. 5, right).

Discussion

Neutrophils migrate to sites of infection or injury during inflammation. A crucial mechanism behind this process is the adhesion of neutrophils to the endothelium or extracellular matrix (Bohnsack et al., 1990). Therefore, drugs that block neutrophil adhesion should be effective as anti-inflammatory agents. In this study, pretreatment with TTD at pharmacologically applicable concentrations (0.1–10 μM) to human neutrophils for as brief as 10 min significantly impaired the adhesion of these cells to fibrinogen-coated surfaces (Fig. 1; almost complete inhibition of adhesion by 10 μM TTD). Although comparable findings have been demonstrated in previous studies (Seow et al., 1986; Li et al., 1989),

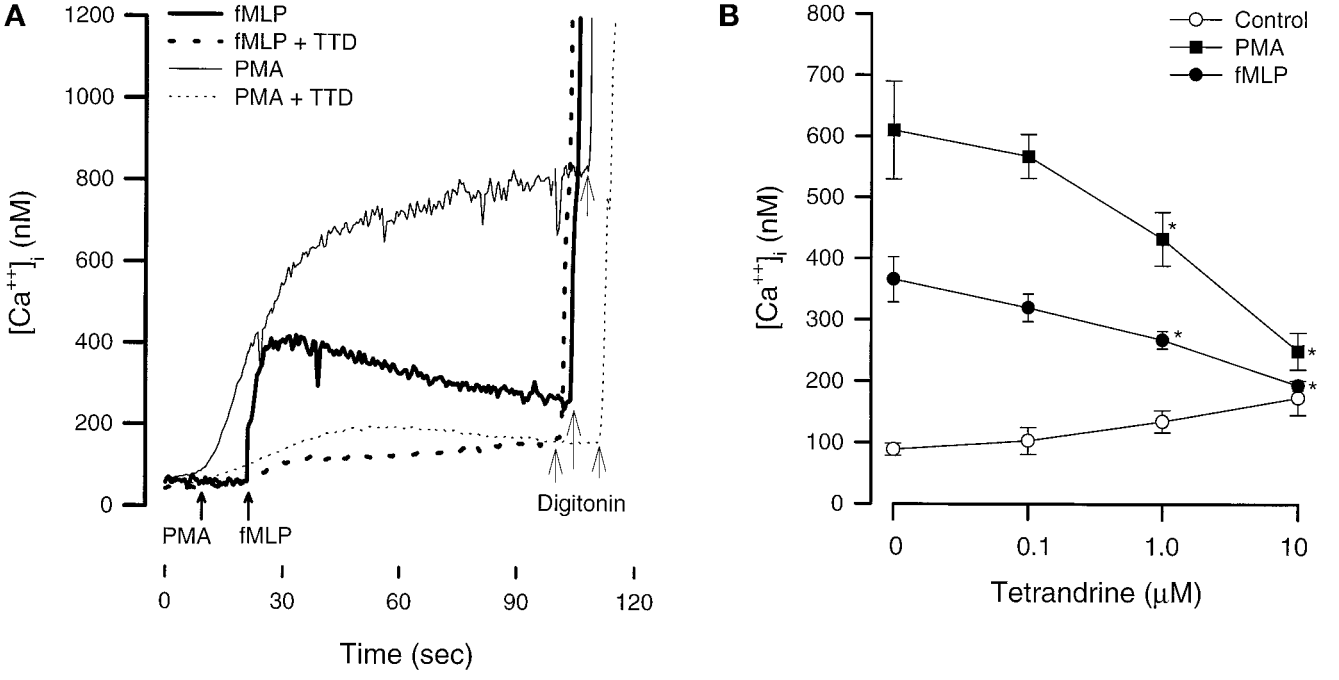

Fig. 3. Effect of TTD on fMLP (1 μM)- or PMA (100 ng/ml)-induced changes in $[Ca^{++}]_i$ of neutrophils. $[Ca^{++}]_i$ was measured as described in *Materials and Methods*. A, tracing of changes in $[Ca^{++}]_i$ triggered by fMLP or PMA in the presence (dotted lines) or absence (solid lines) of TTD. B, statistical summary of fMLP- or PMA-induced changes in $[Ca^{++}]_i$ in the presence of TTD (0.1–10 μM). The control samples received TTD alone. $^*p < .05$ as compared with samples receiving fMLP or PMA alone. Values are mean and vertical lines S.E.M. from six experiments.

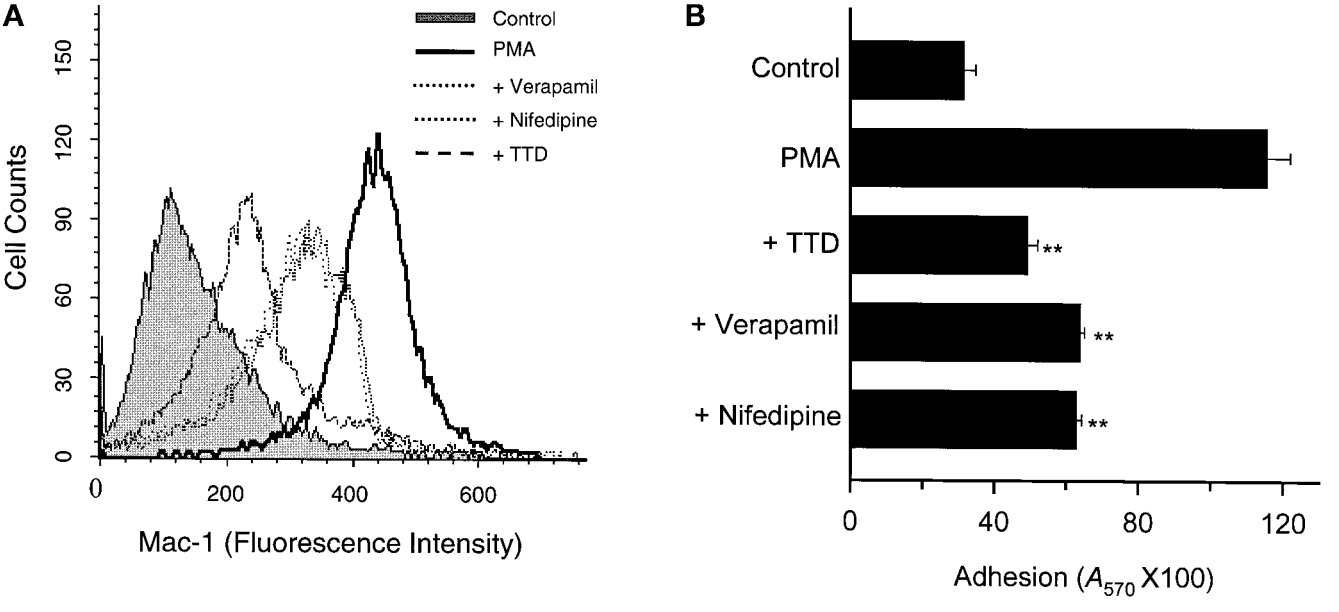

Fig. 4. Effect of calcium channel antagonists on PMA (100 ng/ml) up-regulated Mac-1 expression (A) and adhesion (B). A, Mac-1 levels were measured in the presence of calcium channel antagonists, verapamil, or nifedipine (10 μM), or TTD (10 μM). B, PMA-induced neutrophil adhesion was measured in the presence of verapamil or nifedipine (10 μM). $^{**}p < .01$ as compared with PMA-treated samples. Values are mean and vertical lines S.E.M. from four to eight experiments. The control samples received no PMA.

no mechanism was provided to show how TTD suppressed neutrophil adhesion. The results presented in this study indicate that the antiadhesive effect of TTD is, at least in part, mediated by inhibiting the up-regulation of an adhesion molecule, Mac-1, on the surface of neutrophils upon exposure to proinflammatory stimuli (Fig. 2). Furthermore, inhibition of Mac-1 up-regulation could be accounted for by the antagonizing effects of TTD on the mobilization of Ca^{++} from extracellular sources (Figs. 3 and 4 and Table 1) and the production ROS (Figs. 5 and 6). Our data suggest that TTD inhibits neutrophil adhesion through the inhibition of ROS generation and Ca^{++} influx, which, in turn, suppresses the up-regulation of Mac-1 that is required for the adhesion of neutrophils to fibrinogen.

Distinct surface receptors mediate neutrophil response to inflammatory stimuli such as fMLP (Boulay et al., 1990) and C5a receptors (Gerard and Gerard, 1991). Ligand-receptor coupling activates respiratory burst and facilitates neutrophil adhesion and migration to sites of inflammation (Casimir and Teahan, 1994). Artificial stimuli, including PMA and the calcium ionophore A23187, can also activate neutrophils through nonreceptor-mediated pathways (Casimir and Teahan, 1994). In the presence of extracellular Ca^{++} both PMA and fMLP triggered immediate and significant rise in $[Ca^{++}]_i$ (Fig. 3). Although PMA has been shown to be incapable of causing Ca^{++} mobilization in neutrophils (Barrowman et al., 1986), under certain experimental conditions others have shown different results (Zhang et al., 1995). Our data demonstrate that TTD inhibits Mac-1 up-regulation and neutrophil adhesion by impeding Ca^{++} influx through both Ca^{++} channels and ionophore-mediated pathways. Using two L-type Ca^{++} channel antagonists, verapamil and nifedipine, we found that blocking Ca^{++} flux resulted in decreased Mac-1 up-regulation and adhesion of neutrophils (Fig. 4). Because TTD inhibits both the fMLP- and PMA-induced rise in $[Ca^{++}]_i$ (Fig. 3), it is likely that Ca^{++} antagonism mediates the antiadhesive effect of TTD. These data also provide evidence that the antiadhesive effect of Ca^{++} channel antagonists can be mediated through the inhibition of Mac-1 up-regulation. The argument that TTD could inhibit Mac-1-mediated adhesion via impeding Ca^{++} influx was further substantiated by the lack of effect of TTD on Ca^{++} mobilization and neutrophil adhesion induced by thapsigargin, an agonist that causes release of Ca^{++} from intracellular pools (Table 1). Interestingly, TTD could also inhibit A23187-induced Ca^{++} influx, which has been observed in a prior study (Hong et al., 1989), and neutrophil adhesion (Table 1). These results, in addition to the findings from us and others (Leung et al., 1994) that TTD alone could induce a slight but statistically significant increase in $[Ca^{++}]_i$ (Fig. 3B), suggest that TTD may be a calcium "passage regulator" rather than a calcium "channel antagonist".

Although the Ca^{++} antagonizing capacity of verapamil and nifedipine was comparable with that of TTD (data not shown), TTD was more potent than these two agents in the inhibition of Mac-1 up-regulation and adhesion (Fig. 4). Therefore, blockade of Ca^{++} influx can only partially account for the inhibitory effect of TTD on Mac-1-mediated adhesion of neutrophils. Recently, ROS (including $O_2^{\cdot -}$ and H_2O_2) has been shown to up-regulate Mac-1 expression and enhance Mac-1-mediated neutrophil adhesion (Fraticelli et al., 1996) through increased translocation of Mac-1 from subcellular compartments to the plasma membrane (Simms and D'Amico, 1995). In this study, we demonstrated that TTD effectively inhibits generation of ROS by neutrophils (Fig. 5, left). Both SOD and catalase significantly down-regulate Mac-1 expression (Fig. 6) and neutrophil adhesion to fibrinogen (Fig. 5, right). It is noteworthy that the flow cytometric method employed for the measurement of ROS production was capable of on-line monitoring of intracellular accumulation of ROS in neutrophils. Accumulation of ROS began almost immediately after fMLP or PMA stimulation (Shen et al., 1998). Such prompt accumulation of ROS in response to proinflammatory stimuli and the inhibitory effects of ROS scavengers on Mac-1 up-regulation suggest a role for ROS as early signaling molecules participating in the regulation of neutrophil function, possibly through an autocrine-like mechanism.

In addition to the inhibition of Ca^{++} influx and ROS production, TTD may also inhibit other biochemical pathways that regulate Mac-1 expression. For example, it has been shown that TTD can inhibit purified bovine brain protein kinase C (PKC) (Matsuno et al., 1990) and that Mac-1 expression is regulated, in part, by PKC (Monk and Banks, 1991). In our observation, TTD (10 μM) appeared to inhibit Mac-1 up-regulation and neutrophil adhesion through a pan-PKC-dependent mechanism. However, TTD was much less potent than staurosporine, a broad-spectrum PKC inhibitor, in the inhibition of pan-PKC activity and had no effect on the translocation of PKCα to the cell membrane (our unpublished results). Therefore, TTD may inhibit certain isozyme(s), other than PKCα, that is(are) crucial to neutrophil adhesion. Furthermore, TTD has been shown to decrease the production of leukotriene B_4 and thromboxane B_2 (Du and Huang, 1993), two downstream metabolites of phospholipase A_2 (PLA_2). Because regulation of Mac-1 expression also involves the PLA_2 pathway (Jacobson and Schrier, 1993), it is likely that TTD suppresses Mac-1 up-regulation by influencing the activity of PLA_2. Whether TTD could affect PLA_2 activity and which PKC isozyme(s) may be inhibited by TTD and thereby

TABLE 1
Statistical summary of thapsigargin- or A23187-induced net increase[a] in $[Ca^{++}]_i$ ($\Delta[Ca^{++}]_i$) and neutrophil adhesion ($A_{570} \times 100$)

	Thapsigargin (1 μM)		A23187 (1 μM)	
	Adhesion	$\Delta[Ca^{++}]_i$ (nM)	Adhesion	$\Delta[Ca^{++}]_i$ (nM)
Agonist only	34.6 ± 2.6	168.3 ± 1.7	77.8 ± 7.5	101.7 ± 1.7
+ TTD 10 (μM)	35.6 ± 3.5	155.0 ± 1.4	35.2 ± 2.5[b]	80.0 ± 1.0[b]
+ BAPTA/AM (10 μM)	29.7 ± 1.3[b]	27.5 ± 1.4[b]	44.5 ± 1.2[b]	25.0 ± 1.0[b]
+ EDTA (5 mM)	36.9 ± 3.2	162.5 ± 1.0	30.1 ± 1.6[b]	10.0 ± 1.0[b]
Control (buffer only)	28.2 ± 0.8[b]	0	28.2 ± 0.8[b]	0

[a] Net increase in $[Ca^{++}]_i$ ($\Delta[Ca^{++}]_i$) was calculated by subtracting control values from respective experimental values (control $[Ca^{++}]_i$ in resting cell was 80.6 ± 10 nM).
[b] $p < 0.05$ as compared with samples receiving treatment of agonist alone. Values are means ± S.E.M. from five to eight experiments.

regulate Mac-1 expression are currently under investigation in our laboratory.

In conclusion, we demonstrated that TTD inhibits stimulated neutrophil adhesion to fibrinogen through the inhibition of Mac-1 up-regulation. The inhibitory effect of TTD on Mac-1 expression can be accounted for by the down-regulation of two signaling pathways for Mac-1 translocation, i.e., Ca^{++} influx and ROS generation. Because of its effectiveness as an antiadhesive agent at pharmacologically achievable concentrations, TTD may be clinically useful for prevention of inflammatory injury through inhibiting early phases of neutrophil recruitment.

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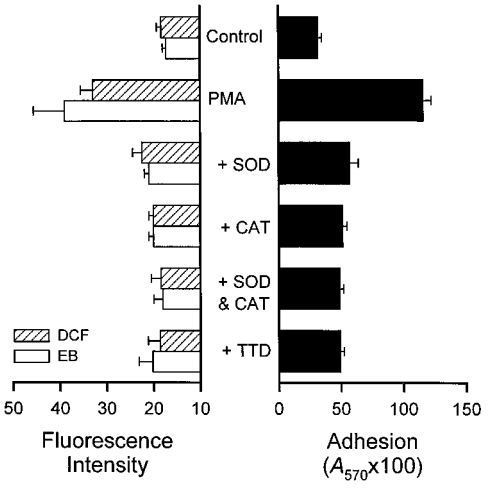

Fig. 5. Effect of ROS scavengers on PMA (100 ng/ml)-induced neutrophil adhesion and ROS production. SOD (200 U/ml) and/or CAT (500 U/ml) were used to decompose $O_2^{\cdot-}$ and H_2O_2, respectively. Values are mean and vertical lines S.E.M. from three experiments.

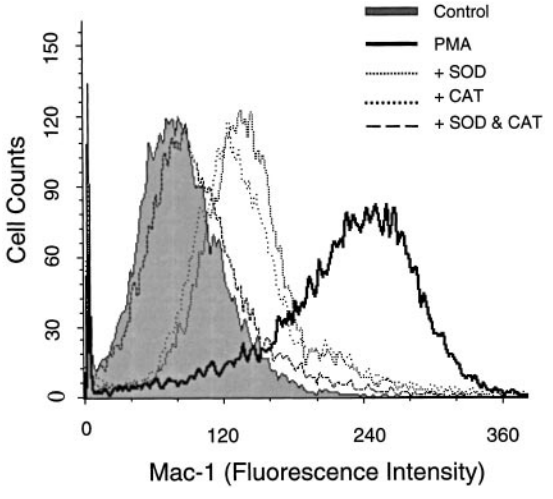

Fig. 6. Effect of ROS scavengers on PMA (100 ng/ml) up-regulated Mac-1 expression of neutrophils. Neutrophils were processed as described in *Materials and Methods* and Mac-1 expression was measured by flow cytometry in the presence or absence of SOD (200 U/ml), a scavenger of $O_2^{\cdot-}$, and/or CAT (500 U/ml), a scavenger of H_2O_2.

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