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Andrographolide prevents oxygen radical production by human neutrophils: possible mechanism(s) involved in its anti-inflammatory effect

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1 We have reported that andrographolide (ANDRO), an active component of *Andrographis paniculata*, inhibits inflammatory responses by rat neutrophils. To further elucidate the possible mechanism(s) underlying the ANDRO's effect, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced adhesion and transmigration of isolated peripheral human neutrophils were studied.

2 Pretreatment with ANDRO (0.1–10 μM) concentration-dependently prevented fMLP-induced neutrophil adhesion and transmigration. We further examined the up-expression of surface Mac-1 (CD11b/CD18), an essential integrin mediated in neutrophil adhesion and transmigration. ANDRO pretreatment significantly decreased fMLP-induced up-expression of both CD11b and CD18.

3 Accumulation of reactive oxygen species (ROS) as well as quick intracellular calcium ($[Ca^{++}]_i$) mobilization induced by fMLP displays two important signalling pathways in regulating the up-expression of Mac-1 by neutrophils. That ANDRO pretreatment diminished fMLP-induced production of H_2O_2 and $O_2^{\cdot-}$, but failed to block that of $[Ca^{++}]_i$ mobilization suggested that the ROS but not $[Ca^{++}]_i$ signalling could be modulated by ANDRO.

4 To clarify whether ROS production impeded by ANDRO could be an antagonism of fMLP binding, phorbol-12-myristate-13-acetate (PMA), a direct protein kinase C (PKC) activator, was introduced to activate ROS production. PMA triggered remarkable ROS production and adhesion, and were partially reversed by ANDRO. This indicated that a PKC-dependent mechanism might be interfered by ANDRO.

5 We conclude that the prevention of ROS production through, at least in part, modulation of PKC-dependent pathway could confer ANDRO the ability to down-regulate Mac-1 up-expression that is essential for neutrophil adhesion and transmigration.

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Keywords: Adhesion; andrographolide; calcium; H_2O_2; Mac-1 (CD11b/CD18); *N*-formyl-methionyl-leucyl-phenylalanine (fMLP); neutrophil; $O_2^{\cdot-}$; phorbol-12-myristate-13-acetate (PMA); transmigration

Abbreviations: ANDRO, andrographolide; BAPTA/AM, 1,2-bis-(O-aminophenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester; BCECF/AM, 2′,7′-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein, acetoxymethyl ester; DCF, 2′,7′-dichlorofluorescein; DCFH, 2′,7′-dichlorofluorescin; EB; ethidium bromide; FBS, foetal bovine serum; fMLP, *N*-formyl-methionyl-leucyl-phenylalanine; Fura 2/AM, 1-[2-(5-carboxyoxal-2-yl)-6-aminobenzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxy)-ethane-*N,N,N′,N′*-tetraacetic acid acetoxymethyl ester; HBSS, Hanks' buffered saline solution; HE, hydroethidine; H_2O_2, hydrogen peroxide; Mac-1, macrophage adhesion molecule-1, also, CD11b/CD18; $O_2^{\cdot-}$, superoxide anion; PKC, protein kinase C; PMA, phorbol-12-myristate-13-acetate; ROS, reactive oxygen species

Introduction

Andrographolide (ANDRO) is a bicyclic diterpenoid lactone isolated from leaves of *Andrographis paniculata* (Lu *et al*., 1981), a Chinese official herbal medicine used as an anti-inflammatory drug for the treatment of laryngitis, diarrhoea, and rheumatoid arthritis. We have previously reported that ANDRO may be beneficial in rats suffered with endotoxaemia by diminishing the production of nitric oxide (NO) through inhibiting the expression of inducible nitric oxide synthase (Chiou *et al*., 1998; 2000). Production of NO and reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$), are important toxic metabolites involved in the intracellular killing of microorganisms. Uncontrolled production of these radicals by phagocytes could lead to tissue injury during inflammation. For example, it is known that exaggeratory ROS production by neutrophils plays an important pathological factor for many inflammatory disorders, such as in the induction of ischaemic and reperfused injury (Ferrari *et al*., 1991). Recently, we have demonstrated that ANDRO can prevent the ROS production and adhesion by isolated rat neutrophils (Shen *et al*., 2000). However the mechanism(s) of action remained unclear.

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Proinflammatory stimuli, such as platelet activating factor (PAF) and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) generated from tissue injury could prime neutrophil to upregulate cell-surface glycoproteins (e.g., fMLP receptor and Macrophage adhesion molecule-1 (Mac-1)) and enhance inflammatory responses (e.g., adhesion, ROS production, and degranulation) (Pabst, 1994). Therefore, neutrophils are more likely to adhere to endothelium during inflammation and, subsequently, infiltrate into site(s) of tissue injury where they release hydrolytic enzymes and large amounts of ROS to induce tissue damage (Albelda *et al*., 1994; Williams, 1994). This physiology course comprises distinct phases including rolling, activation, firm adhesion, and transmigration of neutrophils (Ley, 1996). Specific interaction of various cell adhesion molecules expressed on neutrophil and endothelium provides a molecular explanation for these phases. These adhesion molecules fall into three major superfamilies: the selectins and their mucin ligands, the integrins, and their extracellular matrix or immmunoglobulin superfamily ligands (Brown, 1997). The selectins are important for neutrophil rolling while firm adhesion and transmigration of neutrophils are primarily β_2 integrin dependent (Arfors *et al*., 1987; Werr *et al*., 2000). The β_2 integrins comprise a group of heterodimeric glycoproteins with Mac-1 (CD11b/CD18) being the predominant form elevated on neutrophils during inflammation (Dreyer *et al*., 1989). Therefore, prevention of Mac-1 mediated adhesion and/ or transmigration by neutrophils is a potential target for drug to control inflammation. Further, accumulation of ROS as well as intracellular calcium ($[Ca^{++}]_i$) mobilization have been demonstrated to be two important signalling pathways in regulating the Mac-1 dependent neutrophil adhesion by neutrophils (Shen *et al*., 1999). Also, it has been shown that ROS could modulate leukocyte Mac-1 expression and leukocyte endothelial adhesion could be diminished by antioxidants (Serrano *et al*., 1996). Given the antioxidant potential of ANDRO (Choudhury & Poddar, 1984; Shen *et al*., 2000), whether ANDRO could inhibit neutrophil infiltration through prevention of Mac-1 up-expression, therefore, warrants further investigation.

We hypothesized that the anti-inflammatory effect of ANDRO might be mediated by interference with the ROS production and/ or calcium mobilization, leading to impairments of Mac-1 dependent neutrophil adhesion and transmigration. To accomplish the investigation, fMLP-induced adhesion and transmigration, as well as ROS production and $[Ca^{++}]_i$ mobilization by isolated peripheral human neutrophils were performed as an *in vitro* model in the present studies. As remarked above, adhesion and transmigration of neutrophils are mainly Mac-1 dependent; therefore, Mac-1 expression on the surface of neutrophils was particularly examined.

Methods

Preparation of human neutrophils

Preparation of human neutrophils was obtained by venipuncture from adult healthy volunteers and collected into syringes containing heparin (20 U ml^{-1} 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 30–40 min at room temperature to allow sedimentation of erythrocytes. The upper, leukocyte-rich layer was then collected and subjected to centrifugation at $250 \times g$ 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 layering 8 ml of 1.077 g ml^{-1} Ficoll solution (Histopaque 1077; Sigma Chemicals Co., St. Louis, MO, U.S.A.) beneath the cell suspension, using a pipette. After centrifugation at $400 \times g$ 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 cold lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM ethylenediaminetetraacetate (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 indicated where neutrophils was pretreated with ANDRO, the cells were mixed with drug at concentrations ranging 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 determined in 24-well tissue culture plates (FALCON®, NJ, U.S.A.) coated with fibrinogen as our previous study (Shen *et al*., 1999). Prior to the addition of neutrophils, the plates were incubated with 250 μl per well of human fibrinogen (50 μg ml^{-1} in PBS; Chemicon International, Inc., CA, U.S.A.) for 2 h at 37°C. The wells were washed once with HBSS, blocked with 1% BSA (Sigma, U.S.A.) in HBSS for 1 h at 37°C, and washed twice with HBSS containing 0.1% Tween-20 (Sigma, U.S.A.) and once with HBSS. Immediately prior to addition to the coated-plate, neutrophils (1×10^7 cells ml^{-1}) were loaded with 1 μM 2′,7′-bis-(2-carboxyethyl)-5(and-6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) (Molecular Probe, Inc., Eugene, OR, U.S.A.) in HBSS for 20 min at 37°C and then washed twice with 10 ml HBSS without Mg^{++} or Ca^{++}. Two hundred microliters per well of ANDRO-pretreated BCECF-AM labeled neutrophils (5×10^5 cells ml^{-1} in HBSS) was then added to individual wells. After stimulation with 1 μM fMLP (as a receptor-mediated activator) or 100 ng ml^{-1} PMA (as a non receptor-mediated activator) for 30 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^{++}. Adherent 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.

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, U.S.A.) were pre-coated with human fibrinogen (20 μg ml^{-1}, 100 μl). Immediately prior to add to the upper chamber of fibrinogen-coated inserts, 100 μl per well of BCECF-labelled neutrophils (5×10^5 cells ml^{-1} in HBSS) was treated with ANDRO (0.1 – 10 μM) for 10 min at 37°C. After incubation with fMLP (1 μM) in the lower chambers for 90 min at 37°C, fluorescence intensity in the lower chambers (represent migrated neutrophils) was quantitated with a fluorescent plate reader (Cytofluor 2300, Millipore®) with excitation at 485 nm and emission at 530 nm. Data are expressed as fluorescence intensity.

Measurement of Mac-1 upregulation by flow cytometry

Expression of Mac-1 (CD11b/CD18) was analysed as our previous study (Shen *et al*., 1999). Briefly, ANDRO-pretreated neutrophils were stimulated with fMLP (1 μM) for 15 min. The cells were then pelleted and resuspended in 1 ml ice-cold PBS containing 10% heat-inactivated foetal 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 (FITC)-conjugated anti-Mac-1 antibody (mouse anti-human CD11b or CD18, class IgG_1; Pharmingen, San Diego, CA, U.S.A.) 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% of paraformaldehyde and analysed on a flow cytometer (FACSort; Becton Dickinson) 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 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 analysed on a flow cytometer (FACSort; Becton Dickinson) according to our previous work (Shen *et al*., 1998). Briefly, neutrophils were incubated at 37°C for 5 min with 20 μM 2′, 7′-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Inc., Eugene, OR, U.S.A.) and for an additional 15 min with 10 μM of 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 ANDRO (0.1 – 10 μM) or staurosporine (200 nM) for 10 min and stimulated with fMLP (1 μM) or PMA (100 ng ml^{-1}). Production of $O_2^{\cdot-}$ and H_2O_2 were then determined 60 min after on a flow cytometer (FACSort; Becton Dickinson) 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 calcium concentration ($[Ca^{++}]_i$)

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, Eugene, OR, U.S.A.) at 37°C for 45 min, washed twice and resuspended at 2×10^6 cells ml^{-1} in calcium free HBSS containing ANDRO, BAPTA/AM or control vehicle. After drugs pretreatment for 10 min, 1 ml of cell suspension from each sample was mixed with 1 ml of HBSS containing 2 mM Ca^{++}. Samples were then transferred to individual cuvettes and gently mixed with a micromagnetic stirrer at 37°C for 5 min before addition of fMLP (1 μM) or thapsigargin (0.16 μM). 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 previously described (Shen *et al*., 1999):

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

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), 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.

ANDRO and other chemicals

ANDRO (andrographolide) were purchased from Aldrich (Milwaukee, U.S.A.). It was first dissolved in dimethylsulphoxide at 10 mM and then serially diluted in PBS immediately prior to experiments. Thapsigargin, digitonin, NH_4Cl, $KHCO_3$, EDTA (ethylene diamine tetraacetate), EGTA (ethylen glycol-bis-β-aminoethyl ether tetraacetate) staurosporine, and Hanks' buffered saline solution (HBSS) were purchased from Sigma (U.S.A.). Stock solution of ANDRO was used within 1 week after preparation. For examination of the effect of these drugs, except indicated, 10 μl of drug solution was added to 1.0 ml neutrophils suspension and incubated at 37°C for 10 min prior to the addition of 1 μM fMLP or 100 ng ml^{-1} PMA (Calbiochem, U.S.A.).

Statistical analysis

All values in the text and figures represent means ± s.e.m. Data were analysed by one-way analysis of variance (ANOVA) followed by *post-hoc* Dunnett's *t*-test for multiple comparison. Concentration dependency was analysed by simple linear regression analysis of response levels against concentrations of drug and testing the slope of the regression line against 0 by Student's *t*-test. Values of $P<0.05$ were considered significant.

Results

Effects of ANDRO on fMLP-induced neutrophil adhesion and transmigration

We established an *in vitro* assay system in which fMLP (1 μM) was used to induce neutrophil adhesion and transmigration, functions underlying neutrophil infiltration, to examine whether ANDRO could inhibit neutrophil infiltration. In the adhesion assay, whereas untreated neutrophils displayed spontaneous adhesion with a fluorescence intensity of 171 ± 18, fMLP caused up to 300% enhancement in neutrophil adhesion relative to background levels (Figure 1). Pretreatment of neutrophils with ANDRO (0.1 – 10 μM) concentration-dependently inhibited fMLP-induced neutrophil adhesion (Figure 1). Similar results were also observed in the transmigration assay (Figure 1). Neutrophils displayed spontaneous transmigration with a fluorescence intensity of 179 ± 6, fMLP caused up to 400% enhancement in neutrophil transmigration relative to background levels (Figure 1). ANDRO alone did not influence spontaneous neutrophil adhesion or transmigration. At these concentrations (0.1 – 10 μM), ANDRO were not cytotoxic to neutrophils (viability after drugs treatment >95% by trypan blue exclusion assay at the end of the experiments).

Effect of ANDRO on fMLP-induced Mac-1 (CD11b/CD18) upregulation

Neutrophils adhesion and extravasation have been shown to be mainly depend on upregulation of Mac-1 (CD11b/CD18) (Everitt *et al*., 1996; Werr *et al*., 2000). We further examined whether ANDRO could inhibit neutrophil adhesion/ transmigration by down regulation of Mac-1 expression. Surface levels of Mac-1 expression by fMLP-stimulated neutrophils in the presence or absence of ANDRO was measured by flow cytometry to assess the effect of ANDRO on Mac-1 expression. fMLP caused a marked increase in Mac-1 fluorescence and an apparent shifting-to-the-left of Mac-1 fluorescence was observed in samples pretreated with 10 μM of ANDRO (Figure 2a). A statistical summary revealing that ANDRO significantly inhibited fMLP-induced Mac-1 upregulation is illustrated in Figure 2b ($P<0.05$, $n=4$).

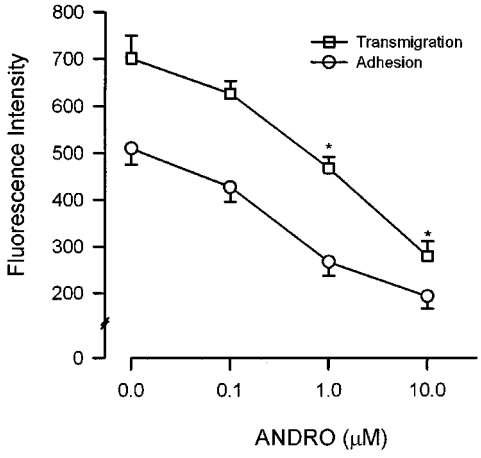


Figure 1 Mean concentration-response curves for andrographolide (ANDRO) in the inhibition of fMLP (1 μM)-induced neutrophil adhesion and transmigration. BCECF-labeled neutrophils were pretreated with ANDRO (0.1 – 10 μM) for 10 min at 37°C. For adhesion assay, cells were plated into fibrinogen-coated 24 well plate. After stimulated with fMLP for an additional 30 min at 37°C, non-adherent cells was wash off and adherent cells were quantified by measuring fluorescence intensity. For transmigration assay, cells were plated into upper chamber of fibrinogen-coated inserts. After stimulated with fMLP in the lower chamber for an additional 90 min at 37°C, transmigrated cells in the lower chambers were quantified by measuring fluorescence intensity. Values are mean and vertical lines s.e.m. of six experiments. *, † $P<0.05$, as compared to samples receiving fMLP alone for transmigration and adhesion, respectively.

ANDRO inhibits intracellular ROS ($O_2^{\cdot -}$ and H_2O_2) production

ROS (e.g., $O_2^{\cdot -}$ and H_2O_2) has been reported to upregulate Mac-1 expression and enhance neutrophil adhesion that could be abolished by antioxidants (Serrano *et al*., 1996; Fraticelli *et al*., 1996). We hypothesized that the *de novo* production of ROS by neutrophils may serve to upregulate Mac-1 expression that could be impeded by ANDRO. A flow cytometric method enables us to on line measure intracellular ROS accumulation by fMLP-stimulated neutrophils in the presence or absence of ANDRO. 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 (Figure 3a), while the results of six experiments are summarized (Figure 3b). ANDRO concentration-dependently decreased the fluorescence intensity of EB and DCF induced by fMLP (Figure 3b, $P<0.05$, $n=6$).

ANDRO does not inhibit $[Ca^{++}]_i$ mobilization

We have previously reported that impediment to calcium influx diminished Mac-1 dependent neutrophil adhesion (Shen *et al*., 1999). Also, other's report showed that cytosolic calcium fluctuation could regulate neutrophil migration (Lawson & Maxfield, 1995). Therefore, effects of ANDRO in fMLP-induced Ca^{++} influx as well as thapsigargin-released Ca^{++} from intracellular storage (Thastrup *et al*., 1989) were determined. fMLP (1 μM) triggered rapid increment in $[Ca^{++}]_i$ which was significantly impeded by verapamil (1 μM), a nonspecific calcium channel blocker (Figure 4, $P<0.05$, $n=5$), but could not been blocked by ANDRO (Figure 4, $P>0.05$, $n=5$). ANDRO also failed to block the $[Ca^{++}]_i$ increment induced by thapsigargin (0.16 μM) that was diminished by BAPTA/AM (10 μM), an intracellular calcium chelator (Barritt & Lee, 1985) (Figure 4).

ANDRO inhibits PMA-induced ROS ($O_2^{\cdot -}$ and H_2O_2) production and adhesion

To further clarify whether inhibition of ROS production by ANDRO could be an antagonism of fMLP binding, PMA, a direct PKC activator, was used to activate NADPH oxidase bypassing the receptor. PMA (100 ng ml^{-1}) triggered obvious ROS production and adhesion that were almost completely abolished by staurosporine (200 nM), a non-specific PKC inhibitor (Figure 5). ANDRO pretreatment significantly impeded PMA-induced ROS production and adhesion (Figure 5) indicating a PKC-dependent mechanism might be interfered by ANDRO.

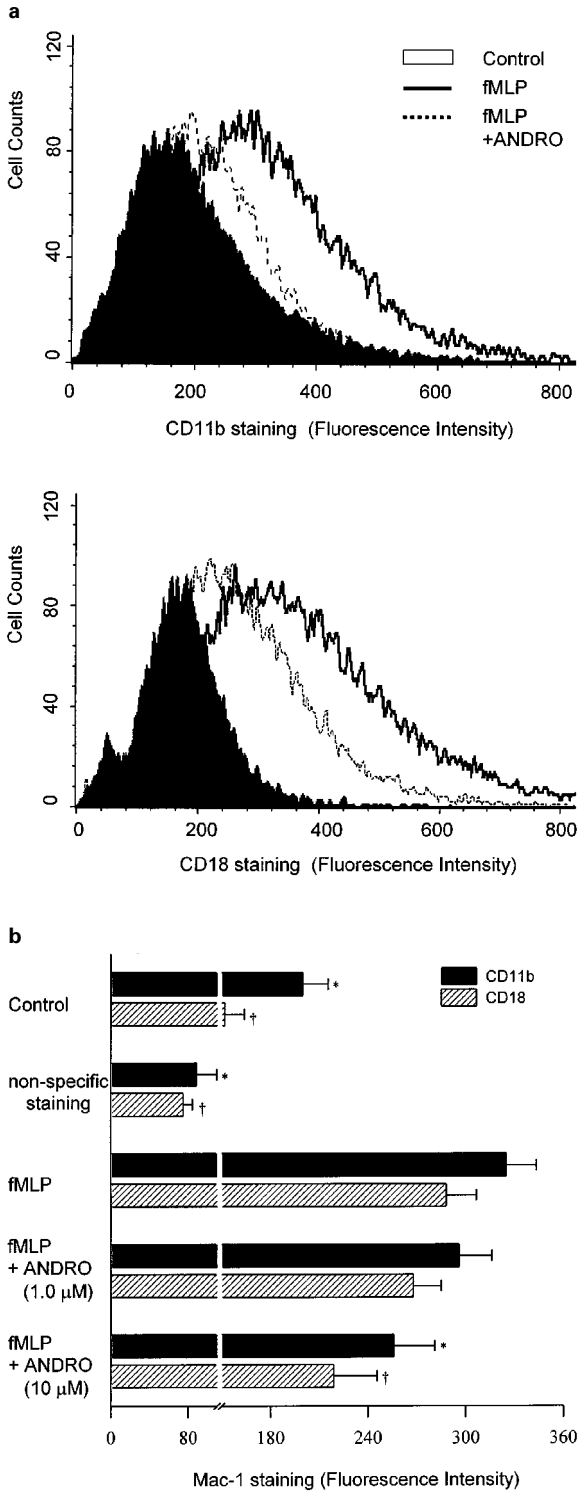


Figure 2 Effect of ANDRO on fMLP (1 μM)-induced Mac-1 upregulation. (**a**) Flow cytometric analysis of total Mac-1 levels on the cell surface of neutrophils. Control neutrophils received neither ANDRO nor fMLP treatment. ANDRO-pretreated sample, designated 'fMLP+ANDRO', has been stimulated with fMLP. (**b**) Statistical summary of fMLP-upregulated Mac-1 expression in the presence or absence of ANDRO (1–10 μM). Non-specific IgG_1 was included to contrast the specificity of anti-CD11b or CD18 staining. Values represent the mean of four experiments and horizontal lines show SEM. *, † $P<0.05$, as compared to samples receiving fMLP alone for CD11b and CD18 staining, respectively.

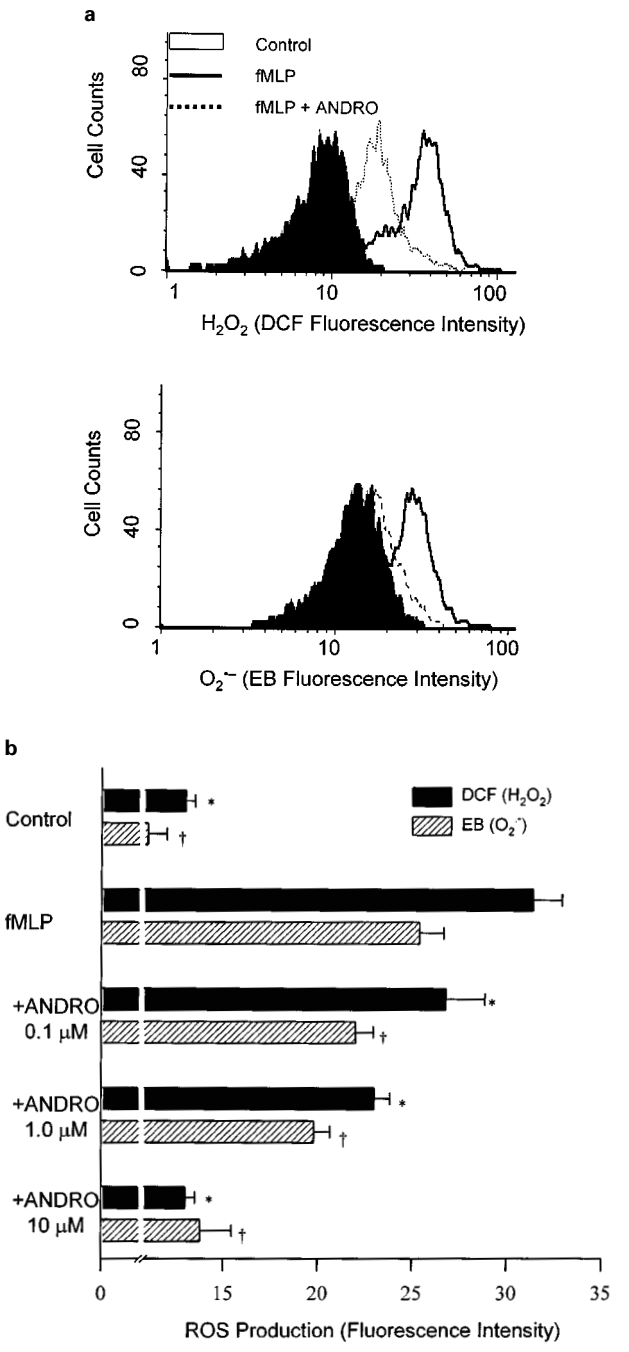
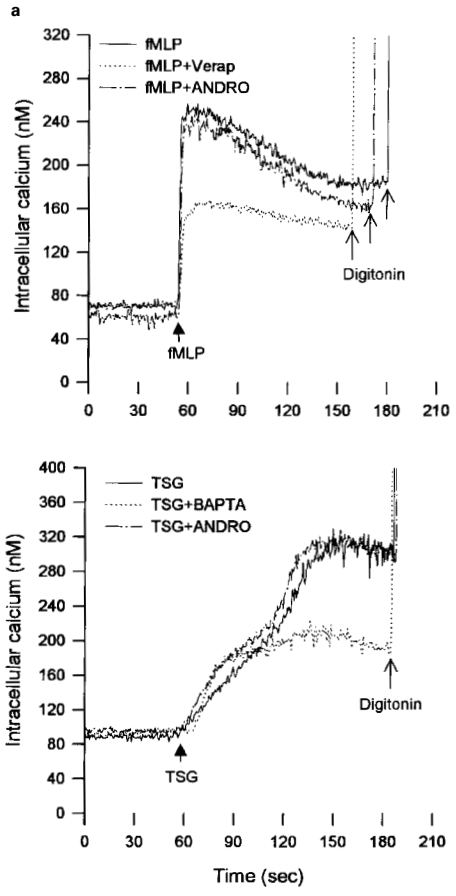


Figure 3 Effect of ANDRO on fMLP (1 μM)-induced neutrophils ROS (H_2O_2 and $O_2^{\cdot-}$) production. (**a**) Flow cytometric analysis of H_2O_2 (DCF fluorescence, upper panel) and $O_2^{\cdot-}$ (EB fluorescence, lower panel) production. Control neutrophils received neither ANDRO nor fMLP treatment. ANDRO (0.1–10 μM)-pretreated sample, designated 'fMLP+ANDRO', has been stimulated with fMLP for 60 min. (**b**) Statistical summary of fMLP-induced H_2O_2 and $O_2^{\cdot-}$ production in the presence of ANDRO (10 μM). Values are mean and horizontal lines s.e.m. from six experiments. *, † $P<0.05$, as compared to sample receiving fMLP alone for DCF and EB fluorescence intensity, respectively.

Discussion

The recruitment of circulating neutrophil to inflamed site or tissue injury is an essential course during inflammation



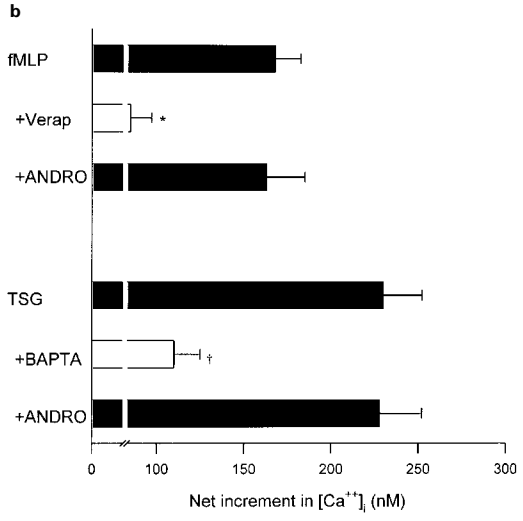


Figure 4 Effect of ANDRO on fMLP (1 μM) or thapsigargin (0.16 μM)-induced changes in intracellular calcium concentration ($[Ca^{++}]_i$) of neutrophils. $[Ca^{++}]_i$ was measured as described in Methods. (**a**) tracing of changes in $[Ca^{++}]_i$ triggered by fMLP (upper panel) or thapsigargin (TSG) (lower panel) in the presence or absence of ANDRO (10 μM). 1 μM verapamil (Verap) and 10 μM BAPTA/AM (BAPTA) were included as calcium channel blocker and intracellular calcium chelator, respectively. (**b**) Statistical summary of fMLP or TSG-induced changes in $[Ca^{++}]_i$ in the presence of ANDRO (10 μM). Net increase in $[Ca^{++}]_i$ was calculated by subtracting control values from respective experimental values (control $[Ca^{++}]_i$ in resting cell was 76 ± 12 nM). *, † $P<0.05$, as compared to samples receiving fMLP or TSG alone. Values are mean and vertical lines s.e.m. from five experiments.

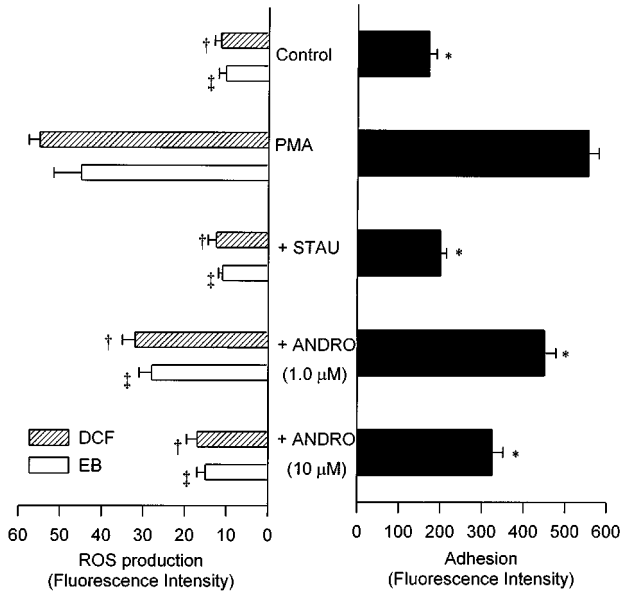


Figure 5 Effect of ANDRO on PMA-induced neutrophils ROS (H_2O_2 and $O_2^{\cdot -}$) production and adhesion. Flow cytometric analysis of H_2O_2 (DCF fluorescence) and $O_2^{\cdot -}$ (EB fluorescence) production, and neutrophil adhesion (BCECF fluorescence) were measured as described in Methods. Control neutrophils received neither ANDRO nor PMA treatment. A PKC inhibitor, staurosporine (200 nM)- and ANDRO (1–10 μM)-pretreated samples, designated as 'PMA + STAU' and 'PMA + ANDRO', respectively, had been stimulated with PMA (100 ng ml^{-1}). Values are mean and horizontal lines s.e.m. from three experiments. *, †, ‡ $P<0.05$, as compared to sample receiving PMA alone for BCECF (adhesion), DCF, or EB fluorescence intensity, respectively.

(Albelda *et al*., 1994). Crucial steps underlying this process include attachment, rolling, firm adhesion, and finally transmigration of neutrophils (Butcher, 1991). Thus, drug that can prevent neutrophils from adhesion and/or transmigration should be useful as an anti-inflammatory drug. In the present study, neutrophils pretreated with 0.1–10 μM of ANDRO for 10 min significantly impaired neutrophil adhesion and transmigration. These effects of ANDRO were not due to cytotoxicity because under these conditions there was no significant difference in viability between drug-treated neutrophils and control cells (viability >95% at the end of the experiments). To further elucidate the mechanism(s) involved in ANDRO's effects, in consideration of the pivotal role of Mac-1 (CD11b/CD18) in neutrophil adhesion and transmigration (Albelda *et al*., 1994; Werr *et al*., 2000), we examined the effect of ANDRO in Mac-1 expression on cell surface. ANDRO significantly prevented fMLP-induced Mac-1 upregulation indicating that the inhibition of Mac-1 upregulation on neutrophil membrane might be involved in mediating the anti-adhesion and anti-transmigration effects of ANDRO.

Mac-1 expression has been reported to be up-regulated by ROS and Mac-1-mediated neutrophil accumulation and adhesion could be diminished by anti-oxidants (Serrano *et al*., 1996; Fraticelli *et al*., 1996). ANDRO decreased the fMLP-induced ROS ($O_2^{\cdot -}$ and H_2O_2) production indicating that ANDRO might serve to act as a ROS scavenger through which in turn down-regulate Mac-1 expression and then neutrophil adhesion/transmigration. It has been reported that ROS production induced by fMLP closely relates to calcium-

dependent priming of neutrophil which if blocked interferes with ROS production (Lew *et al*., 1984). Besides, cytosolic Ca^{++} fluctuation could regulate Mac-1-mediated neutrophil adhesion (Lawson & Maxfield, 1995; Perry *et al*., 1993). Our previous finding confirmed that antagonism of calcium influx could diminish Mac-1 dependent neutrophil adhesion (Shen *et al*., 1999). In this study, fMLP caused prompt and prominent $[Ca^{++}]_i$ increment which was diminished by a classic calcium channel antagonist, verapamil, but not by ANDRO. Furthermore, thapsigargin triggered significant Ca^{++} release from intracellular stores that was effectively suppressed by BAPTA/AM (10 μM), an intracellular Ca^{++}-chelator, but ANDRO again failed to inhibit the thapsigargin-induced $[Ca^{++}]_i$ increment. These results indicate that ANDRO inhibit fMLP-induced ROS production might mediate a calcium independent pathway.

ROS production in neutrophils could be through coupling of inflammatory stimuli to their relative receptor(s) to activate PKC-dependent phosphorylation of the cytosolic component(s) of NADPH oxidase for activation of this enzyme (Casimir & Teahan, 1994). On the contrary, artificial stimuli such as fluoride, PMA and other phorbol esters, the calcium ionophore A23187, and opsonized latex beads also can activate the NADPH oxidase through direct PKC activation (Castagna *et al*., 1982). To further elucidate whether ROS prevention by ANDRO could be antagonism of fMLP binding, PMA was introduced to activate NADPH oxidase bypassing the receptor. PMA triggered obvious ROS production and adhesion that were significantly impeded by ANDRO indicating a (PKC)-dependent mechanism might be interfered by ANDRO. Our prior studies confirmed that ROS scavengers such as superoxide dismutase (SOD) and catalase significantly down regulated PMA-induced ROS production as well as Mac-1 expression and neutrophil adhesion to fibrinogen coated surface (Shen *et al*., 1998; 1999). We found accumulation of $O_2^{\cdot-}$ and H_2O_2 began almost immediately after stimulation (Shen *et al*., 1998). The rapid accumulation of $O_2^{\cdot-}$ and H_2O_2 in response to stimuli and our observation that Mac-1 upregulation 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 strengthened by the observation that ROS can act as second messengers in activation of ligand-stimulated NF-κB, various PKC family members, and mitogen-activated protein kinase (MAPK), as well as tyrosine kinases/phosphatase (Finkel, 1998). Thus, we suggest that ROS could regulate neutrophil functions through second messenger mechanism(s).

Although the DCF technique for the measurement of ROS production has been criticized extensively over the last two years (Rota *et al*., 1999), we had reported that DCFH-DA is a relative specific probe for the on-line monitoring of the accumulation of intracellular H_2O_2 (Shen *et al*., 1999). In the report (Shen *et al*., 1999), we showed that catalase (500 U ml^{-1}) can completely prevent the increase of DCF fluorescence level *in vivo*. The major problems regarding the use of DCFH to measure H_2O_2 production raised by Mason RP (Rota *et al*., 1999) are: (1) the deacetylation of DCFH-DA, by esterase, can produce H_2O_2 to enhance DCF fluorescence and (2) the disproportional $O_2^{\cdot-}$ forms H_2O_2 which, in the presence of peroxidase activity (1 μM HRP), will oxidize more DCFH to DCF with self-amplification of the fluorescence. In our experiments, the DCFH-DA and HE loaded cells (neutrophils) had been monitored for the basal H_2O_2 and $O_2^{\cdot-}$ production, respectively, and the DCF and EB fluorescence did not increase significantly over 60 min. This results imply that the production of H_2O_2 (or $O_2^{\cdot-}$) by esterase (or peroxidase), *in vivo*, can be ignored. For the self-amplification of DCF fluorescence by HRP-catalyzed DCFH oxidation to DCF in Mason's report, it is questionable whether the peroxidase concentration *in vivo* can reach to the relative high level (1 μM HRP) as used in Mason's study. To further strengthen the inhibition of ROS production by ANDRO, the 'SOD-inhibitable reduction of cytochrome C' had been compared to the flow cytometric technique using HE as a probe for the measurement of $O_2^{\cdot-}$ production in our laboratory. Our unpublished results show that the flow cytometric method is relative sensitive, reproducible, and possible of on-line monitoring of the ROS production than the cytochrome *c* reduction assay, although ANDRO showed comparable results by the latter method in our unshown data.

It is likely that other biochemical pathway(s), in addition to inhibition of ROS production, could be regulated by ANDRO for the Mac-1 expression. For example, blocking the synthesis of arachidonates by phospholipase A_2 (PLA_2) inhibitors could inhibit the surface expression of Mac-1 (Jacobson & Schrier, 1993) and prostaglandin F_2, oxidative modification of arachidonic acid, could induce β_2 integrin-mediated rapid adhesion of neutrophils (Fontana *et al*., 2001). Although ANDRO had been shown not to interfere the biosynthesis of eicosanoids, downstream metabolites of arachidonate (Amroyan *et al*., 1999), further studies should be focused on the effect of ANDRO in the signaling of cyclic nucleotide induced by the eicosanoids. Besides, it is noted that MAPK pathways play central roles 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 ANDRO in the regulation of Mac-1 dependent neutrophil adhesion and transmigration needs further research and is currently under investigation in our laboratory.

In conclusion, we have established that the anti-inflammatory effect of ANDRO could be explained by its ability to inhibit neutrophil adhesion/transmigration through suppression of Mac-1 upregulation. The inhibitory effect of ANDRO on Mac-1 expression could be mediated by down regulation of ROS production *via* a PKC-dependent but calcium independent mechanism. As effective anti-adhesive and anti-transmigration drug at pharmacological concentrations (0.1–10 μM), ANDRO may be useful for the improvement of inflammatory disorders by limiting the early phases of neutrophil infiltration.

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References

ALBELDA, S.M., SMITH C.W. & WARD, P.A. (1994). Adhesion molecules and inflammatory injury. *FASEB J.,* **8,** 504 – 512.

AMROYAN, E., GABRIELIAN, E., PANOSSIAN, A., WIKMAN, G. & WAGNER, H. (1999). Inhibitory effect of andrographolide from Andrographis paniculata on PAF-induced platelet aggregation. *Phytomedicine,* **6,** 27 – 31.

ARFORS, K.E., LUNDBERG, C., LINDBOM, L., LUNDBERG, K., BEATTY, P.G. & HARLAN, J.M. (1987). A monoclonal antibody to the membrane glycoprotein complex CD18 inhibits polymorphonuclear leukocyte accumulation and plasma leakage *in vivo*. *Blood,* **69,** 338 – 340.

ATTA, U.R., HARVEY, K. & SIDDIQUI, R.A. (1999). Interleukin-8: An autocrine inflammatory mediator. *Curr. Pharmaceutical. Design,* **5,** 241 – 253.

BARRITT, G.J. & LEE, A.M. (1985). Effects of electrical stimulation and an intracellular calcium chelator on calcium movement in suspensions of isolated myocardial muscle cells. *Cardiovasc. Res.,* **19,** 370 – 377.

BROWN, E. (1997). Neutrophil adhesion and the therapy of inflammation. *Semin. Hematol.,* **34,** 319 – 326.

BUTCHER, E.C. (1991). Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell,* **67,** 1033 – 1036.

CASIMIR, C.M. & TEAHAN, C.G. (1994). The respiratory burst of neutrophils and its deficiency. In: *Immunopharmacology of Neutrophils*. ed. Hellewell, P.G. & Williams, T.J. pp. 27 – 54. London: Academic Press.

CASTAGNA, M., TAKAI, Y., KAIBUCHI, K., SANO, K., KIKKAWA, U. & NISHIZUKA, Y. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.,* **257,** 7847 – 7851.

CHIOU, W.F., CHEN, C.F. & LIN, J.J. (2000). Mechanisms of suppression of inducible nitric oxide synthase (iNOS) expression in RAW 264.7 cells by andrographolide. *Br. J. Pharmacol.,* **129,** 1553 – 1560.

CHIOU, W.F., LIN, J.J. & CHEN, C.F. (1998). Andrographolide suppresses the expression of inducible nitric oxide synthase in macrophage and restores the vasoconstriction in rat aorta treated with lipopolysaccharide. *Br. J. Pharmacol.,* **125,** 327 – 334.

CHOUDHURY, B.R. & PODDAR, M.K. (1984). Andrographolide and kalmegh (Andrographis paniculata) extract: in vivo and in vitro effect on hepatic lipid peroxidation. *Methods Find Exp. Clin. Pharmacol.,* **6,** 481 – 485.

DREYER, W.J., SMITH, C.W., MICHAEL, L.H., ROSSEN, R.D., HUGHES, B.J., ENTMAN, M.L. & ANDERSON, D.C. (1989). Canine neutrophil activation by cardiac lymph obtained during reperfusion of ischemic myocardium. *Circ. Res.,* **65,** 1751 – 1762.

EVERITT, E.A., MALIK, A.B. & HENDEY, B. (1996). Fibronectin enhances the migration rate of human neutrophils *in vitro*. *J. Leukoc. Biol.,* **60,** 199 – 206.

FERRARI, R., CECONI, C., CURELLO, S., CARGNONI, A., ALFIERI, O., PARDINI, A., MARZOLLO, P. & VISIOLI, O. (1991). Oxygen free radicals and myocardial damage: protective role of thiol-containing agents. *Am. J. Med.,* **91,** 95S – 105S.

FINKEL, T. (1998). Oxygen radicals and signaling. *Curr. Opin. Cell. Biol.,* **10,** 248 – 253.

FONTANA, L., GIAGULLI, C., MINUZ, P., LECHI, A. & LAUDANNA, C. (2001). 8-Iso-PGF_2 alpha induces beta 2-integrin-mediated rapid adhesion of human polymorphonuclear neutrophils: a link between oxidative stress and ischemia/reperfusion injury. *Arterioscler. Thromb. Vasc. Biol.,* **21,** 55 – 60.

FRATICELLI, A., SERRANO, C.V.J., BOCHNER, B.S., CAPOGROSSI, M.C. & ZWEIER, J.L. (1996). Hydrogen peroxide and superoxide modulate leukocyte adhesion molecule expression and leukocyte endothelial adhesion. *Biochim. Biophys. Acta,* **1310,** 251 – 259.

JACOBSON, P.B. & SCHRIER, D.J. (1993). Regulation of CD11b/CD18 expression in human neutrophils by phospholipase A_2. *J. Immunol.,* **151,** 5639 – 5652.

KRULL, M., KLUCKEN, A.C., WUPPERMANN, F.N., FUHRMANN, O., MAGERL, C., SEYBOLD, J., HIPPENSTIEL, S., HEGEMANN, J.H., JANTOS, C.A. & SUTTORP, N. (1999). Signal transduction pathways activated in endothelial cells following infection with Chlamydia pneumoniae. *J. Immunol.,* **162,** 4834 – 4841.

LAWSON, M.A. & MAXFIELD, F.R. (1995). Ca^{++}-and calcineurin-dependent recycling of an integrin to the front of migrating neutrophils. *Nature,* **376,** 75 – 79.

LEW, P.D., WOLLHEIM, C.B., WALDVOGEL, F.A. & POZZAN, T. (1984). Modulation of cytosolic-free calcium transients by changes in intracellular calcium-buffering capacity: correlation with exocytosis and O_2^- production in human neutrophils. *J. Cell. Biol.,* **99,** 1212 – 1220.

LEY, K. (1996). Molecular mechanisms of leukocyte recruitment in the inflammatory process. *Cardiovasc. Res.,* **32,** 733 – 742.

LU, X.L., ZHANG, S.L. & WANG, Z.S. (1981). Analysis of andrographolide compounds. I. Ion pair high performance liquid chromatographic analysis of andrographolide derivatives. *Acta. Pharmaceutica Sinica,* **16,** 182 – 189.

PABST, M.J. (1994). Priming of Neutrophils. In: *Immunopharmacology of Neutrophils*. ed. Hellewell, P.G. & Williams, T.J. pp. 195 – 221. London: Academic Press.

PERRY, I., BUTTRUM, S.M. & NASH, G.B. (1993). Effects of activation on adhesion of flowing neutrophils to cultured endothelium: time course and inhibition by a calcium channel blocker (nitrendipine). *Br. J. Pharmacol.,* **110,** 1630 – 1634.

ROTA, C., CHIGNELL, C.F. & MASON, R.P. (1999). Evidence for free radical formation during the oxidation of 2′-7′-dichlorofluorescin to the fluorescent dye 2′-7′-dichlorofluorescein by horseradish peroxidase: possible implications for oxidative stress measurements. *Free Radic Biol. Med.,* **27,** 873 – 881.

SERRANO, C.V.J., MIKHAIL, E.A., WANG, P., NOBLE, B., KUPPUSAMY, P. & ZWEIER, J.L. (1996). Superoxide and hydrogen peroxide induce CD18-mediated adhesion in the postischemic heart. *Biochim. Biophys. Acta,* **1316,** 191 – 202.

SHEN, Y.C., CHEN, C.F. & CHIOU, W.F. (2000). Suppression of rat neutrophil reactive oxygen production and adhesion by diterpenoid lactone andrographolide. *Planta Medica,* **66,** 314 – 317.

SHEN, Y.C., CHEN, C.F., WANG, S.Y. & SUNG, Y.J. (1999). Impediment to calcium influx and reactive oxygen production accounts for the inhibition of neutrophil Mac-1 up-regulation and adhesion by tetrandrine. *Mol. Pharmacol.,* **55,** 186 – 193.

SHEN, Y.C., SUNG, Y.J. & CHEN, C.F. (1998). Magnolol inhibits Mac-1 (CD11b/CD18)-dependent neutrophil adhesion: relationship with its antioxidant effect. *Eur. J. Pharmacol.,* **343,** 79 – 86.

TANDON, R., SHA'AFI, R.I. & THRALL, R.S. (2000). Neutrophil β_2-integrin upregulation is blocked by a p38 MAP kinase inhibitor. *Biochem. Biophy. Res. Commun.,* **270,** 858 – 862.

THASTRUP, O., DAWSON, A.P., SCHARFF, O., FODER, B., CULLEN, P.J., DROBAK, B.K., BJERRUM, P.J., CHRISTENSEN, S.B. & HANLEY, M.R. (1989). Thapsigargin, a novel molecular probe for studying intracellular calcium release and storage. *Agents Actions,* **27,** 17 – 23.

WERR, J., ERIKSSON, E.E., HEDQVIST, P. & LINDBOM, L. (2000). Engagement of β_2 integrins induces surface expression of β_1 integrin receptors in human neutrophils. *J. Leukoc. Biol.,* **68,** 553 – 560.

WILLIAMS, F.M. (1994). Role of neutrophils in reperfusion injury. In: *Immunopharmacology of Neutrophils*. ed. Hellewell, P.G. & Williams, T.J. pp. 245 – 257. London: Academic Press.

YAMAMORI, T., INANAMI, O., NAGAHATA, H., CUI, Y. & KUWABARA, M. (2000). Roles of p38 MAPK, PKC and PI3-K in the signaling pathways of NADPH oxidase activation and phagocytosis in bovine polymorphonuclear leukocytes. *FEBS Lett.,* **467,** 253 – 258.

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