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Mechanisms in mediating the anti-inflammatory effects of baicalin and baicalein in human leukocytes

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Abstract

To evaluate the possible mechanisms responsible for the anti-inflammatory effects of baicalin or baicalein, phorbol-12-myristate-13-acetate (PMA)- or *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-activated inflammatory responses of peripheral human leukocytes were studied. Both baicalin and baicalein diminished fMLP- or PMA-induced reactive oxygen intermediates production in neutrophils or monocytes. Neither baicalin nor baicalein prevented the protein kinase C (PKC)-dependent assembly of the NADPH oxidase. Conversely, myeloperoxidase (MPO) activity was inhibited by baicalin or baicalein. fMLP-induced activation of leukocytes, as reflected by increased surface expression of Mac-1 (CD11b/CD18) and Mac-1-dependent neutrophil adhesion, were also inhibited by baicalin or baicalein. Furthermore, baicalein, but not baicalin, impeded fMLP- or AlF_4^--induced Ca^{2+} influx. We conclude that impairment of reactive oxygen intermediates production, through scavenging reactive oxygen intermediates by baicalin, or antagonizing ligand-initiated Ca^{2+} influx by baicalein, accounts for the inhibition of Mac-1-dependent leukocyte adhesion that confers the anti-inflammatory activity of baicalin or baicalein.

Keywords: Baicalin; Baicalein; Ca^{2+}; NADPH oxidase; Myeloperoxidase; Reactive oxygen intermediate

1. Introduction

Huangqin, *Radix scutellariae*, the dry roots of *Scutellaria baicalensis* Georgi (Lamiaceae), is officially listed in the Chinese Pharmacopoeia and is used traditionally against bacterial infections of the respiratory and gastrointestinal tract. A number of flavone derivatives had been isolated from *S. baicalensis* with baicalin (Fig. 1, upper panel) as the predominant constituent (12–17%) (Tang and Eisenbrand, 1992). The anti-inflammatory (Lin and Shieh, 1996), anti-oxidative (Hanasaki et al., 1994; Gao et al., 1999), and lipid-peroxidation preventive effects (Nagashima et al., 2000) of baicalin and its metabolite baicalein (Fig. 1, lower panel) have been reported. Recently, Kimura et al. (2001) reported that baicalein inhibits the expression of endothelial leukocyte adhesion molecule-1 (ELAM-1) and intercellular adhesion molecule-1 (ICAM-1) of cultured endothelial cells. Expression of ELAM-1 and/or ICAM-1 in human endothelial cells mediates the firm adhesion and recruitment of circulating leukocytes to tissue injury sites during inflammation (Wardlaw and Walsh, 1994). These observations indicate that drugs with anti-oxidative effect could have the potential to modulate the interaction between inflammatory target cells and peripheral leukocytes through which to display their anti-inflammatory effects.

Activation of leukocytes (e.g., neutrophil and monocyte/macrophage) during inflammation can result in the release of large amount of reactive oxygen intermediates, including superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and non-radical oxygen intermediate as host defense mechanism(s) against the invading pathogen. However, uncontrolled production of inflammatory mediators, especially reactive oxygen intermediates, by leukocytes may lead to crippling inflammatory disorders ranging from inflammatory/immune injury to myocardial infraction, neurodegeneration, ischemia/reperfusion injury, adult respiratory distress syndrome, as well as atherosclerosis (Cuzzocrea et al., 2001).

The molecular mechanism(s) mediating recruitment and accumulation of leukocytes at injured sites begins with the firm adhesion of activated leukocytes to endothelial cells by the counter receptors expressed on them [e.g., Mac-1

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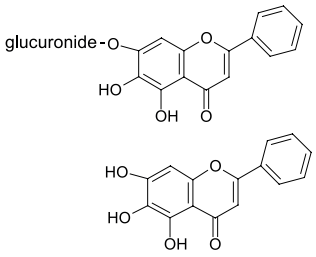

Fig. 1. Baicalin (BN, upper panel) and its aglycon baicalein (BE, lower panel).

(CD11b/CD18) and ICAM-1 for neutrophil and endothelial cells, respectively] (Huo and Ley, 2001; Saxena and Goldberg, 1994). It has been demonstrated that reactive oxygen intermediates can act as signaling molecule to modulate leukocyte Mac-1 expression and leukocyte endothelial adhesion, effects that can be diminished by superoxide dismutase and/or catalase pretreatment (Fraticelli et al., 1996; Serrano et al., 1996). Antioxidant, such as α-tocopherol, inhibits monocyte–endothelial adhesion, reactive oxygen intermediates production, and cytokine release. Such antioxidants can reduce the risk of the oxidative-modification related inflammatory diseases (Catapano, 1997; Devaraj and Jialal, 1998). Baicalin and baicalein have been shown to be anti-oxidants in cellular model (Shieh et al., 2000), cell-free system (Hanasaki et al., 1994), and in animal studies (Hara et al., 1992) suggesting that baicalin or baicalein may have potential effects on human inflammatory cells, such as neutrophil, monocyte or macrophage.

We hypothesize that the anti-inflammatory effects of baicalin or baicalein may be mediated by interfering with the reactive oxygen intermediates production and then activation and degranulation of adhesion molecules in leukocytes, leading to inhibition of leukocyte adhesion and recruitment. As mentioned above, firm adhesion of leukocytes to the endothelium is primarily Mac-1-dependent (Ragab et al., 1996; Huo and Ley, 2001), which can be enhanced by oxidant (Simms and D'amico, 1995). Therefore, *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)- or phorbol-12-myristate-13-acetate (PMA)-induced reactive oxygen intermediates production, fMLP-activated Mac-1 expression and adhesion, as well as Ca^{2+} mobilization in peripheral human leukocytes were measured to investigate the mechanism(s) in mediating the anti-inflammatory effects of baicalin or baicalein.

2. Materials and methods

2.1. Human leukocytes preparation

All experimental protocols were approved by our Institutional Review Board in accordance with international guidelines. In addition, informed consents were obtained from the subjects in those in which human participation was involved.

Human leukocytes were prepared from adult healthy volunteers as in our previous study (Shen et al., 2001). Briefly, blood samples were mixed with dextran solution to allow sedimentation of erythrocytes. The upper, leukocyte-rich layer was collected and subjected to centrifugation. The pellet was re-suspended immediately in cold lysis buffer [155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM ethylenediaminetetraacetate (EDTA), pH 7.4] to remove residual erythrocytes. The remaining leukocytes were then pelleted, washed with ice-cold phosphate buffered saline solution (PBS), and resuspended in an adequate volume of ice-cold Hank's buffered saline solution (HBSS). Alternatively, neutrophils and peripheral blood mononuclear cells were further separated by the Ficoll gradient centrifugation method followed by lysis of contaminating erythrocytes. The purified sample contained more than 95% neutrophils, as estimated by counting 200 cells under a microscope after Giemsa staining (Sigma), was used for the assay of adhesion, Mac-1 expression, Ca^{2+} mobilization, and enzyme activity determination including protein kinase(s), NADPH oxidase, myeloperoxidase (MPO), and catalase. In all cases where cells were pretreated with baicalin or baicalein, the cells were mixed with drug(s) at concentrations ranging from 1 to 100 μM in HBSS for 10 min at 37°C.

2.2. Measurement of extracellular and intracellular reactive oxygen intermediates production

Extracellular reactive oxygen intermediates generation was evaluated according to the method of Parij et al. (1998) with some modifications. fMLP- or PMA-induced extracellular reactive oxygen intermediates production by neutrophils or mononuclear cells was determined by a lucigenin-amplified chemiluminescence in the presence or absence of baicalin or baicalein. Briefly, 50 μl of baicalin or baicalein pretreated neutrophils or mononuclear cells (1×10^7/ml) were incubated at 37°C for 10 min with 25 μM lucigenin (Sigma). The cells were then immediately stimulated with fMLP (1 μM) or PMA (200 nM) in which chemiluminescence was monitored for 30 min every 1 min. The chemiluminescent signal was measured over 5 s by a microplate luminometer reader (Orion®, Germany) and represented as relative light units (RLU). Peak levels were recorded to calculate the activity of test drugs in relation to their corresponding solvent controls containing 0.005% dimethyl sulfoxide (DMSO). The 50% inhibitory doses (IC_{50}) of PMA- or fMLP-triggered chemiluminescence by test drugs were calculated using a semilog-plot transformation of the data. Alternatively, intracellular accumulation of reactive oxygen intermediates by neutrophils or monocytes was measured by a flow cytometer (FACSCalibur™; Becton Dickinson) according to our previous report (Shen et al., 1998). Briefly, neutrophils or mononuclear cells (2×10^6/ml) were preloaded with 2′,7′-dichloro-fluorescin diacetate (DCFH-DA; Molecular Probes, Eugene, OR). After labeling, cells were treated with baicalein, baicalin or other chemicals and stimulated with PMA (200 nM). Production of intracellular reactive oxygen intermediates was then determined

30 min after via gating the regions of neutrophils and monocytes from total leukocytes on a flow cytometer with measurement of emission at 525 nm (FL1) for 2′,7′-dichlorofluorescein (DCF). Data are expressed as mean channel fluorescence for each sample as described above.

2.3. Measurement of NADPH oxidase activity in subcellular fractions

Particulate fractions of neutrophils were prepared according to the method of Clark et al. (1990). Neutrophils (2×10^6/ml) were incubated for 10 min at 37 °C in the presence of either PMA (200 nM) or an equal concentration of DMSO as solvent control. After incubation, the cell suspension was centrifuged at 4 °C at $300 \times g$ for 6 min, and the pellet was resuspended in buffer containing 100 mM KCl, 3 mM NaCl, 3.5 mM $MgCl_2$, 1.25 mM EGTA, 10 mM piperazine-*N*,*N*′-bis-2-ethanesulfonic acid (PIPES), 2 mM phenylmethylsulphonyl fluoride, 33 μM leupeptin, 35 μM antipain, 10 μM chymostatin, 0.035 μM pepstatin, and 0.08 μM aprotinin, pH 7.3. The cells were disrupted using a microprobe sonicator at low power (10%) for 10 s at 4 °C, and then centrifuged at $500 \times g$ for 5 min to remove unbroken cells. The supernatant was centrifuged at 4 °C for 20 min at $115{,}000 \times g$. The supernatant was discarded and the pellet (particulate fractions) washed in buffer and recentrifuged for 20 min at $115{,}000 \times g$. NADPH oxidase activity was determined in the particulate fractions in the presence of 400 μM NADPH, and $O_2^{\cdot -}$ generation was monitored for 10 min at 25 °C as superoxide dismutase-inhibitable cytochrome *c* reduction. Baicalin or baicalein was added 10 min before addition of the NADPH (drug after PMA-activated). Alternatively, neutrophils were preincubated with baicalin or baicalein for 10 min at 37 °C before PMA-stimulation followed by cell lysed (drug before PMA-activated). Results are expressed as $O_2^{\cdot -}$ nmol/2×10^5 equivalents/10 min.

2.4. Estimation of myeloperoxidase activities

Myeloperoxidase activity was evaluated according to the method of Bani's et al. (1998) with some modification. Briefly, neutrophils (2×10^7/ml in HBSS) were stimulated with fMLP (1 μM) in the presence or absence of baicalin or baicalein. Myeloperoxidase activity was determined by mixing 0.1 ml of cell suspension with 2.9 ml of potassium phosphate buffer (PPB, 50 mM, pH 6) containing 0.19 mg/ml of *o*-dianisidine chloride and 0.0005% H_2O_2 as a substrate for myeloperoxidase. Oxidized *o*-dianisidine formed a soluble chromophore and the absorbent (OD_{460}) was determined by spectrophotometry over 2 min. Myeloperoxidase activity was expressed by subtracting the value of $OD_{460} \times 100$ at time 0 min from that of at 2 min for each sample. Data were expressed as percentage (%) of control by calculating the ratio of myeloperoxidase activity by baicalin or baicalein pretreated sample over that of fMLP alone.

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

Upregulation of Mac-1 was analyzed based on our previous study (Shen et al., 1999). Briefly, baicalin or baicalein-pretreated neutrophils or mononuclear cells (2×10^6/ml in HBSS) were stimulated with fMLP (1 μM) for 20 min. The cells were then pelleted and resuspended in 1 ml of ice-cold PBS containing 10% heat-inactivated fetal bovine serum 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, class immunoglobulin G_1 (IgG_1); BD Biosciences Pharmingen] or a non-specific mouse antibody (class IgG_1, Sigma) as a negative control. After two washes with PBS containing 5% fetal bovine serum, stained cells were resuspended in flow cytometer sheath fluid (Becton Dickinson) containing 1% paraformaldehyde and analyzed by flow cytometry for Mac-1 expression. Data are expressed as mean channel fluorescence for each sample as calculated by the CellQuest® software (Becton Dickinson) on a Power Macintosh 7300/200 computer.

2.6. Measurement of neutrophil adhesion

Adhesion of neutrophils to extracellular matrix was determined in 24-well tissue culture plates (FALCON®, USA) coated with fibrinogen as described in our previous study (Shen et al., 1999). Briefly, prior to the addition of neutrophils, the plates were incubated with 250 μl per well of human fibrinogen (50 μg/ml). The wells were washed once with HBSS, blocked with 1% bovine serum albumin, and washed twice with HBSS containing 0.1% Tween-20 and once with HBSS. Immediately prior to addition to the coated-plate, neutrophils (1×10^7/ml) were loaded with 1 μM 2′,7′-bis-(2-carboxyethyl)-5 (and -6)-carboxyfluorescein, acetoxymethyl ester (BCECF-AM) 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 BCECF-AM labeled neutrophils (5×10^5/ml in HBSS) were then added to individual wells. After stimulation with fMLP (1 μM) for 30 min at 37 °C, non-adhered cells were removed by aspiration and the wells were gently washed twice with warm PBS containing 1 mM Ca^{2+}. Adhered neutrophils were then determined by measuring the fluorescence with a fluorescent plate reader (Cytofluor 2300, Millipore®) with excitation at 485 nm and emission at 530 nm. Data are expressed as fluorescence intensity.

2.7. Protein kinase(s) activity assay

Neutrophils were resuspended (2×10^7/ml) in the ice-cold extraction buffer. The extraction buffer consisted of 50 mM Tris–HCl (pH 7.5), 50 mM EGTA, 1 mM phenyl-

methylsulphonyl fluoride, protease inhibitor cocktail (Calbiochem®, USA), and 50 mM 2-mercaptoethanol. Protease inhibitor cocktail consisted of 0.1 mM leupeptin, 1 μM pepstatin A, 5 μM bestatin, 0.08 μM aprotinin, and 1.5 μM cysteine protease inhibitor. The cell suspension was sonicated for 10 s at 4 °C for five times and the cell lysate was separated into cytosol (as cytosolic fraction) and pellet fractions by centrifugation at 100,000 × *g* for 60 min at 4 °C. The pellet was resuspending in extraction buffer with 0.1% Triton X-100, vortexed and incubated at 4 °C for 60 min. The soluble extract (as membranous fraction) and the particulate fraction were separated by centrifugation at 10,000 × *g* for 5 min. Both cytosolic and membranous extracts were assayed subsequently for kinase activity within the same day. Protein concentration was determined by protein assay kit (Bio-Red, USA). Protein kinase C (PKC) or adenosine-3′5′-cyclic monophosphate (cAMP)-dependent protein kinase (PKA) activity was measured by a non-radioactive protein kinase assay kit (Calbiochem®, Germany) which was based on an enzyme-linked immunosorbent assay (ELISA) that utilizes a synthetic PKC/PKA pseudosubstrate and a monoclonal antibody that recognizes the phosphorylated peptide. PKC or PKA phosphorylates the serine residue on the pseudosubstrate (peptide) through Ca^{2+}/phosphatidylserine-dependent- or cAMP-dependent-mechanism, respectively. Ca^{2+}/phospholipid-dependent PKC was assayed in the presence of Ca^{2+} and phosphatidylserine while the negative control was measured in the presence of 20 mM EGTA for negation of Ca^{2+} effects. In some experiments, PMA (200 nM) was added to viable neutrophils for stimulation and translocation of PKC prior to sonication, and cytosolic PKC activity from this preparation was used as another negative control as PKC was translocated to the particulate fraction. In PKA activity assay, the negative control was measured in the absence of cAMP. Data are expressed as percentage of control.

2.8. Catalase activity assay

Neutrophils were suspended (2×10^7/ml) in the ice-cold extraction buffer as stated above. Cells were sonicated for 10 s at 4 °C for three times and cytosolic fraction was separated by centrifugation at 100,00 × *g* for 20 min at 4 °C. Protein concentration was determined by protein assay kit (Bio-Red). Catalase activity was determined in the supernatants by mixing 0.1 ml of cytosolic fraction with 0.9 ml of phosphate buffer (pH 7.0) containing 0.0015% of H_2O_2 as substrate in the presence or absence of baicalin or baicalein. The decrease in the absorbance at 240 nm (OD_{240}) per minute was calculated from the initial rate using 0.0394/cm/M as the molar excitation coefficient of H_2O_2 at 240 nm (Abei, 1974). Data were expressed as percentage (%) of control (drug-free). Sodium azide (1 mM NaN_3) was included in the assay as a negative control.

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

Neutrophils were preloaded with 5 μM 1-[2-(5-carboxyoxal-2-yl)-6-amino-benzofuran-5-oxyl]-2-(2′-amino-5′-methylphenoxy-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid acetoxymethyl ester (fura 2-AM, Molecular Probes) as in our previous report (Shen et al., 1999). After baicalin or baicalein pretreatment for 5 min, 1 ml cell suspension from each sample and 1 ml HBSS containing 2 mM Ca^{2+} were transferred to individual cuvettes and gently mixed with a micromagnetic stirrer at 37 °C for 5 min before the addition of fMLP (1 μM) or AlF_4^- (10 mM NaF plus 30 μM $AlCl_3$) or thapsigargin (1 μM). The fluorescence of fura-2-loaded cells was measured by a spectrofluorometer (Hitachi F-4500) with excitation at 340 and 380 nm and emission at 510 nm. Intracellular Ca^{2+} concentration for each sample was calculated from the ratio of emission versus excitation as previously described (Grynkiewicz et al., 1985).

2.10. Baicalin, baicalein, and other chemicals

Baicalin and baicalein were purchased from Aldrich (USA). It was first dissolved in DMSO and then serially diluted in PBS immediately prior to experiments. As baicalein is relative insoluble in aqueous media, the diluted solution was kept in water bath and vortex violently to maintain solubility before use. Stock solution was used within 1 week after preparation. For examination of the effect of these drugs, 10 μl of drug solution was added to 1.0 ml leukocytes suspension and incubated at 37 °C for 10 min prior to the addition of 200 nM PMA or 1 μM fMLP (Sigma).

2.11. Statistical analysis

All values in the text and figures represent means ± S.E.M. Data were analyzed by one-way or two-way analysis of variance (ANOVA) depending on the number of experimental variables followed by post-hoc Dunnett's *t*-test for multiple comparisons. Values of $p < 0.05$ were considered significant.

3. Results

3.1. Effect of baicalin or baicalein on reactive oxygen intermediates production

To examine whether baicalin or baicalein inhibit reactive oxygen intermediates production by human neutrophils or mononuclear cells, lucigenin-amplified chemiluminescence was used to measure the extracellular accumulation of reactive oxygen intermediates. Fig. 2 shows that both baicalin and baicalein significantly inhibited the fMLP- or PMA-induced extracellular reactive oxygen intermediates accumulation in neutrophils and mononuclear cells with

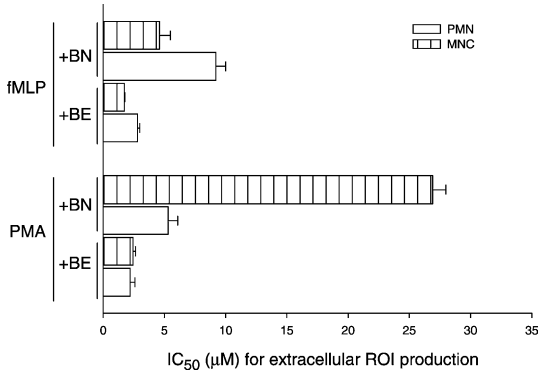

Fig. 2. Statistical summary for the IC_{50} of baicalin or baicalein on fMLP-or PMA-induced extracellular reactive oxygen intermediates (ROI) production by lucigenin-amplified chemiluminescence in neutrophils (PMN) or mononuclear cells (MNC). FMLP- (1 μM) or PMA (200 nM)-induced reactive oxygen intermediates production by neutrophils or mononuclear cells was determined in the presence or absence of 1–100 μM baicalin (BN) or baicalein (BE) with lucigenin-amplified chemiluminescence as described in Materials and methods. A 200 nM of staurosporine, a protein kinase C (PKC) inhibitor, completely prevented the lucigenin-amplified chemiluminescence. All the BN- or BE-pretreated groups, designated +BN or +BE were stimulated with fMLP or PMA. Data were expressed as 50% of inhibition (IC_{50}). Values represent the means ± S.E.M. of 6–10 experiments performed on different days using cells from different donors.

IC_{50} ranging from 2 to 26 μM. Since reactive oxygen intermediates act as signaling molecules in the regulation of inflammatory response (Shen et al., 1998; Finkel, 1998), we further determined the intracellular reactive oxygen intermediates changes via a flow cytometric method by PMA-stimulated leukocytes in the presence or absence of baicalin or baicalein. Fig. 3 summarizes the results from 6–10 experiments of PMA-stimulated accumulation of intracellular reactive oxygen intermediates (measured as DCF fluorescence) in neutrophils or monocytes, respectively. Baicalin or baicalein concentration-dependently decrease the fluorescence intensity of DCF with the 50% inhibitory concentration (IC_{50}) of 64.9 and 53.3 μM by baicalin, and 4.2 and 1.5 μM by baicalein for neutrophils and monocytes, respectively. To contrast the protein kinase C (PKC) dependency of reactive oxygen intermediates production, staurosporine, a PKC inhibitor, was also included. Staurosporine (0.2 μM) completely prevented both the extracellular and intracellular reactive oxygen intermediates production in this study (data not shown). The concentrations of these drugs used in this study were not cytotoxic to leukocytes (viability after drugs treatment >95% by trypan blue exclusion assay).

3.2. Effect of baicalin or baicalein on myeloperoxidase activity

Myeloperoxidase, an abundant component stored in azurophilic granules of neutrophils, which catalyzes the generation of hypochlorous acid (HOCl), a reactive chlorinated intermediate for bacteria killing. HOCl also induces tissue injury and accounts for most of the target oxidation that takes place in vivo. The myeloperoxidase activity in control (fMLP) sample was 19.5 ± 2.0 ($OD_{460} \times 100$). Baicalin or baicalein significantly inhibited myeloperoxidase activity in neutrophils (Fig. 4).

3.3. Effect of baicalin or baicalein on Mac-1 (CD11b/CD18) upregulation

Activation/degranulation of leukocytes can be measured by up-expression of Mac-1 (stored at specific granules). Since antioxidants can abolish Mac-1 upregulation and adhesion by neutrophils (Shen et al., 1998), we hypothesized that the de novo production of reactive oxygen intermediates by activated leukocytes may upregulate Mac-1 expression, which can be diminished by baicalin or baicalein. We measured the effect of pretreatment of baicalin or baicalein on surface expression of the Mac-1 on fMLP-

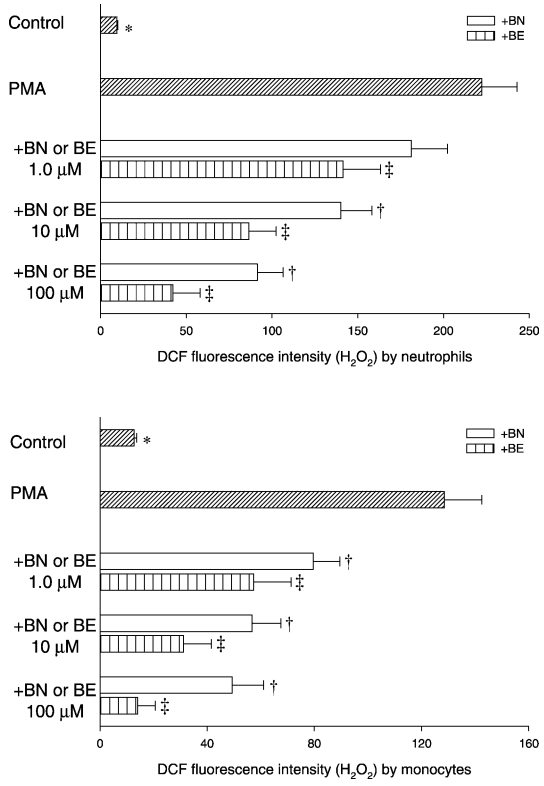

Fig. 3. Statistic summary of the effect of baicalin or baicalein on PMA-induced intracellular accumulation of reactive oxygen intermediates by flow cytometry in neutrophils (upper panel) or monocytes (lower panel). Leukocytes (2×10^6/ml) were incubated at 37 °C for 10 min with 20 μM 2′,7′dichloro-fluorescin diacetate (DCFH-DA). After labeling, cells were treated with baicalin (BN) or baicalein (BE) or other chemicals followed by PMA (200 nM) stimulation. Production of intracellular reactive oxygen intermediates was then determined 30 min after via gating the regions of neutrophils and monocytes from total leukocytes on a flow cytometer with measurement of emission at 525 nm (FL1) for DCF. Data are expressed as mean channel fluorescence for each sample. Values represent the means ± S.E.M. of 6–10 experiments performed on different days using cells from different donors. *,†,‡$P < 0.05$ as compared to samples treated with PMA alone for control, BN, or BE, respectively.

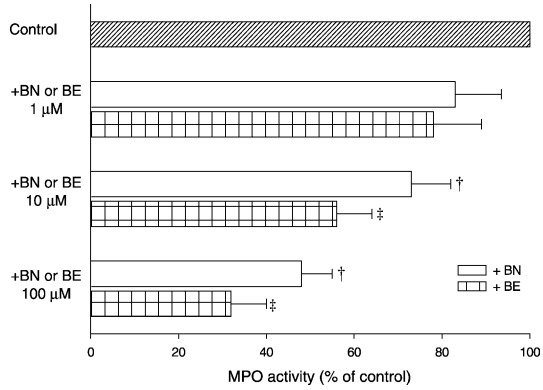

Fig. 4. Effect of baicalin or baicalein on MPO activity in neutrophils. One hundred microliters of the baicalin (BN) or baicalein (BE) pretreated cells suspension (2×10^6cells) were mixed with 2.9 ml potassium phosphate buffer containing *o*-dianisidine chloride (0.19 mg/ml) and H_2O_2 (0.0005%) as a substrate for myeloperoxidase (MPO). Oxidized *o*-dianisidine forms a soluble chromophore absorbing at wavelength of 460 nm. The absorbent (OD_{460}) was determined by spectrophotometry over 2 min. MPO activity was expressed by subtracting the value of $OD_{460} \times 100$ at time 0 min from that of at 2 min for each sample. Data were expressed as percentage (%) of control by calculating the ratio of MPO activity of drug pretreated sample over that of control (fMLP) sample. The MPO activity in control sample was 19.5 ± 2.0. Values represent the means ± S.E.M. of five experiments performed on different days using cells from different donors. †,‡$P < 0.05$ as compared to samples treated with control (fMLP) alone for BN or BE, respectively.

stimulated neutrophils or monocytes via flow cytometric analysis. fMLP caused a marked increase in Mac-1 fluorescence. An apparent leftward shift (decreased) of Mac-1 fluorescence was observed in samples pretreated with baicalin or baicalein. As showed in Fig. 5, a statistical summary of 4–6 flow cytometric experiments revealed that baicalin or baicalein significantly inhibited fMLP-induced Mac-1 upregulation by neutrophils or monocytes.

3.4. Effect of baicalin or baicalein on neutrophils adhesion

To examine whether baicalin or baicalein could inhibit Mac-1-dependent leukocyte adhesion, we established an in vitro assay system in which fMLP (1 μM) was used to induce neutrophil adhesion, a function closely related to leukocyte recruitment. Whereas untreated neutrophils displayed spontaneous adhesion with a fluorescence intensity of 217 ± 25 (Fig. 6), fMLP caused up to 170% enhancement in neutrophil adhesion relative to background levels. Pretreatment of neutrophils with baicalin or baicalein (1 to 100 μM) concentration-dependently inhibited neutrophil adhesion induced by fMLP (Fig. 6).

3.5. Effect of baicalin or baicalein on the assembly or the activity of NADPH oxidase

As reactive oxygen intermediates production by leukocytes involves the assembly of cytosolic and membrane-associated components to form an active NADPH oxidase activity, therefore, NADPH oxidase activity was determined in PMA-treated particulate fractions. Our results demonstrated that neither baicalin nor baicalein interfere with the assembly or the activity of preassembled-NADPH oxidase (Table 1). On the other hand, cromolyn, an inhibitor for the assembly but not the activity of pre-assembled NADPH oxidase (Kilpatrick et al., 1995), showed comparative result (Table 1) as in that of Kilpatrick's report.

3.6. Effect of baicalin or baicalein on protein kinase(s) activities

Protein kinase C (PKC) and adenosine-3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase (PKA) have reported to regulate the activation of leukocytes (Monk and Banks, 1991; Derian et al., 1995). We measured the activity of PKC and PKA by an enzyme-linked immunosorbent assay kit. Table 2 shows that cytosolic PKC activity was not modulated by baicalin or baicalein. In control groups, cytosolic PKC activities were reduced to 78.5 ± 2.4% or 48.0 ±

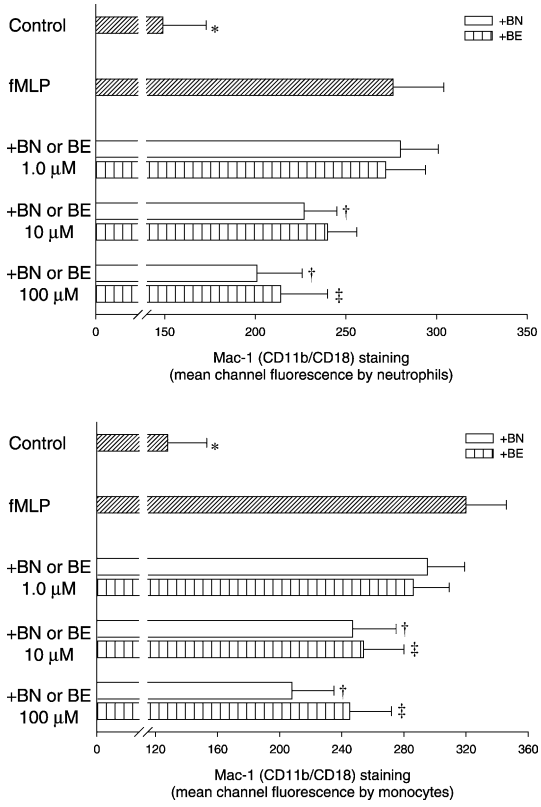

Fig. 5. Statistical summary of fMLP (1 μM)-upregulated Mac-1 expression by neutrophils (upper panel) or monocytes (lower panel) in the presence of 1–100 μM baicalin (BN) or baicalein (BE). Cells were stained in an ice-bath with anti-CD11b (Mac-1) antibody and total Mac-1 level on the cell surface was quantitated by FACSCalibur™. Control level represents samples that were neither treated with BN, BE nor stimulated with fMLP. Values represent the means ± S.E.M. of 4–6 experiments performed on different days using cells from different donors. *,†,‡$P < 0.05$ as compared to samples treated with fMLP alone for control, BN, or BE, respectively.

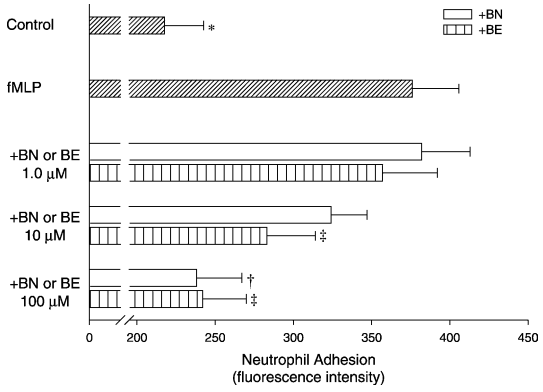

Fig. 6. Statistical summary for the inhibition of fMLP (1 μM)-induced neutrophil adhesion to extracellular matrix by baicalin or baicalein. BCECF-labeled neutrophils (5×10^5cells/ml in HBSS) were pretreated with 1–100 μM of baicalin (BN) or baicalein (BE) for 10 min at 37 °C. Cells were plated into fibrinogen-coated 24 well plates. After being stimulated with fMLP for an additional 30 min at 37 °C, non-adhered cells was washed off and adhered cells were quantified by measuring fluorescence intensity. Control represents samples that were neither treated with BN, BE nor stimulated with fMLP. Values represent the means ± S.E.M. of five experiments performed on different days using cells from different donors. *,†,‡$P < 0.05$ as compared to samples treated with fMLP alone for control, BN, or BE, respectively.

1.8% in the presence of EGTA (20 mM) or PMA, respectively. On the other hand, PKA activity was enhanced to 143% in the presence of cyclic AMP (20 μM), but neither baicalin nor baicalein modulated such PKA activity (Table 2).

3.7. Effect of baicalin or baicalein on fMLP-, ALF_4^-- or thapsigargin-induced $[Ca^{2+}]_i$ mobilization

Cytosolic Ca^{2+} fluctuation can regulate leukocyte adhesion (Lawson and Maxfield, 1995). We have previously reported that impediment to Ca^{2+} influx diminished Mac-1-dependent neutrophil adhesion (Shen et al., 1999). Therefore, effect of baicalin or baicalein on fMLP (a receptor agonist)-, AlF_4^- (a G-protein activator)- or thapsigargin (an intracellular Ca^{2+} store releaser)-induced $[Ca^{2+}]_i$ mobilization were studied. FMLP (1 μM), AlF_4^-, and thapsigargin (1 μM) triggered rapid increment in $[Ca^{2+}]_i$. Bacalein, but not baicalin, impeded the Ca^{2+}influx induced by fMLP and AlF_4^-. Intracellular Ca^{2+} store released by thapsigargin was not inhibited by baicalin or baicalein (Table 3). In our study, samples pretreated with pertussis toxin (500 ng/ml) or BAPTA-AM (10 μM) (an intracellular Ca^{2+} chelator) significantly prevented intracellular Ca^{2+} mobilization induced by fMLP/AlF_4^- or thapsigargin, respectively (data not shown).

Table 1
Effect of baicalin or baicalein on subcellular NADPH oxidase activities

	NADPH oxidase activities (O_2^- nmol/2×10^5 cells equivalents/10 min)[a]	
	Drug before PMA-activated[b]	Drug after PMA-activated[c]
Control (drug-free)	0.054 ± 0.002[a]	0.054 ± 0.002[a]
PMA	0.111 ± 0.006	0.129 ± 0.005
+BN (100 μM)	0.132 ± 0.008	0.128 ± 0.006
+BE (100 μM)	0.119 ± 0.005	0.116 ± 0.004
+CROMO (100 μM)	0.062 ± 0.012[d]	0.128 ± 0.010

[a] NADPH oxidase activities was expressed as O_2^- production in particulate fractions isolated from PMA-treated neutrophils (2×10^5 cells) via monitoring for 10 min as superoxide dismutase-inhibitable cytochrome *c* reduction at 37 °C.

[b] Neutrophils were preincubated with baicalin (BN), baicalein (BE) or cromolyn (CROMO) for 10 min at 37 °C before PMA-stimulation followed by isolation of the particulate fraction (drug before PMA-activated).

[c] BN, BE or CROMO was added 10 min before addition of the NADPH (drug after PMA-activated). Values are means ± S.E.M. from six experiments performed on different days using cells from different donors.

[d] $p < 0.05$ as compared with PMA alone.

Table 2
Effect of baicalin or baicalein on the protein kinase(s) activities

	Protein kinase(s) activity (% control)[a]	
	PKC	PKA[b]
Control	100	100
+BN (100 μM)	95.8 ± 1.7	107.8 ± 0.5
+BE (100 μM)	91.8 ± 0.2	106.0 ± 0.4
+EGTA (20 mM)	78.5 ± 2.4[c]	ND
PMA-pretreated[d]	48.0 ± 1.8[c]	ND

ND, sample not detected.

[a] Cytosolic protein of neutrophils (2×10^7/ml) were extracted for the determination of the protein kinase C (PKC) or adenosine-3′,5′-cyclic monophosphate (cAMP)-dependent protein kinase (PKA) activity in the presence of baicalin (BN) or baicalein (BE) by a non-radioactive protein kinase assay kit (Calbiochem®, Germany) based on an enzyme-linked immunosorbent assay that utilizes a synthetic PKC or PKA pseudosubtsrate and a monoclonal antibody that recognizes the phosphorylated peptide. Negative control for PKC was determined in the presence of 20 mM EGTA.

[b] In PKA activity assay, the negative control (70.0 ± 1.8%) was measured in the absence of cAMP (20 μM). Values are means ± S.E.M. from 3–5 experiments performed on different days using cells from different donors.

[c] $p < 0.05$ as compared with control, respectively.

[d] In some experiments, PMA (200 nM) was added to viable neutrophils for stimulation/translocation of PKC prior to sonication, and cytosolic PKC activity from this preparation was used as another negative control as most of the cytosolic PKC was translocated to the particulate fraction.

Table 3
Effect of baicalin or baicalein on fMLP-, AlF_4^-- or thapsigargin-induced net increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$)

	$\Delta[Ca^{2+}]_i$ (nM)[a]		
	fMLP (1 μM)	AlF_4^-	Thapsigargin (1 μM)
Agonist only	173.0 ± 12.0	168.3 ± 17.0	177.8 ± 17.5
+BN (10 μM)	166.9 ± 14.8	162.5 ± 10.6	171 ± 16.00
+BN (100 μM)	152.9 ± 13.2	162.3 ± 17.5	188 ± 17.5
+BE (10 μM)	143.6 ± 13.5	155.0 ± 14.8	174.2 ± 12
+BE (100 μM)	128.7 ± 13.6[b]	137.5 ± 14.0[b]	182 ± 12.5

[a] Net increase in $[Ca^{2+}]_i$ ($\Delta[Ca^{2+}]_i$) was calculated by subtracting control values from respective experimental values (control $[Ca^{2+}]_i$ in resting cell was 98.8 ± 9.0 nM).

[b] $p < 0.05$ as compared with samples receiving treatment of agonist alone in the presence or absence of baicalin (BN) or baicalein (BE). Values are means ± S.E.M. from 6–10 experiments performed on different days using cells from different donors.

4. Discussion

Release of reactive oxygen intermediates and reactive oxygen intermediates-related radicals by phagocytic leukocytes depends upon the activation of NADPH oxidase and myeloperoxidase. NADPH oxidase is a powerful oxidant-producing enzyme located on the surface membrane of neutrophils which generates superoxide anion ($O_2^{\cdot -}$), and then triggers the production of hydroxy radical (OH^-) and hydrogen peroxide (H_2O_2) through electron-transportation (Casimir and Teahan, 1994). Myeloperoxidase, a chloride peroxidase, generates hypochlorous acid (HOCl), a more powerful oxidant, from H_2O_2 and Cl^- (Weiss, 1989). In addition to acting as pathological factors and immunomodulating effectors, reactive oxygen intermediates may serve as signaling molecules in the activation of inflammatory cells (Shen et al., 1998; Finkel, 1998). It has been reported that quercetin, a flavonoid, inhibits the activity of NADPH oxidase and myeloperoxidase (Tauber et al., 1984; Pincemail et al., 1988). However, the effects of baicalin or baicalein, two flavonoids with structures similar to that of quercetin, on the reactive oxygen intermediates production by activated peripheral human leukocytes was not elucidated. With anti-oxidant potential, baicalin or baicalein (Fig. 1) could effectively inhibit reactive oxygen intermediates production by human leukocytes to act as anti-inflammatory agents. Our results demonstrated that baicalin and baicalein significantly impeded the reactive oxygen intermediates production and β_2 integrin-dependent adhesion by peripheral human leukocytes suggesting that drugs with anti-oxidative effect could modulate the activation of peripheral leukocytes through which to prevent their interaction with inflammatory target cells that confers the anti-inflammatory effects of these drugs.

Activation and recruitment of circulation leukocytes into inflamed site or injured tissue is an essential pathological process during inflammation (Albelda et al., 1994; Ikeda et al., 1998). Crucial mechanism(s) mediating this process (including firm adhesion and transmigration) of leukocyte closely related to the up-expression of β_2 integrin, especially Mac-1 (CD11b/CD18) (Huo and Ley, 2001; Shen et al., 2002). In the present study, leukocytes pretreated with baicalin or baicalein, at concentrations ranging from 1 to 100 μM for 10 min significantly impaired fMLP-induced Mac-1 up-expression and also inhibited neutrophil adhesion to fibrinogen-coated surface. Similar results had been reported that baicalein could inhibit the cigarette smoke condensate induced adhesion of human monocytes to endothelial cells (Kalra et al., 1994). The anti-adhesive effect of baicalin or baicalein was not due to cytotoxic effect because under these conditions there was no difference in viability between drug(s)-treated leukocytes and control cells (viability >95% at the end of experiments).

In this study, fMLP or PMA triggered remarkable extracellular and intracellular reactive oxygen intermediates production by neutrophils and mononuclear cells. Both baicalin and baicalein inhibited these reactive oxygen intermediates production with similar efficacy. Compared to other anti-oxidants, our unpublished observation revealed that tocopherol (vitamin E) or probucol (a well-known anti-lipid peroxidative drug) also inhibited the reactive oxygen intermediates production by human neutrophils with less potency (both IC_{50}>100 μM) than that of baicalein or baicalin (both IC_{50}<30 μM). Activation of leukocytes to generate $O_2^{\cdot -}$ involves the assembly of cytosolic (including p47-phox, p67-phox, p21-*rac*1, and *rho*-GDI) and membrane-associated components (gp9lphox and p22phox) to form an active NADPH oxidase enzyme complex (Clark et al., 1990; Kadri-Hassani et al., 1995; Segal, 1989). To further understand whether NADPH oxidase per se was modulated by baicalin or baicalein, the assembly of the NADPH oxidase and the activity of the pre-assembled-NADPH oxidase in membrane fractions were studied. Neutrophils are activated by PMA to trigger the assembly of the NADPH oxidase in the cell membrane. In our study, membranes isolated from PMA-triggered cells contain an active NADPH oxidase assembly and generate $O_2^{\cdot -}$ (0.111 ± 0.006 nmol/2×10^5 cells equivalents/10 min) in the presence of NADPH (400 μM), two folds higher than that of membranes isolated from resting cells. Incubation of membranes from PMA-triggered neutrophils with baicalin or baicalein had no effect on $O_2^{\cdot -}$ generation indicating that baicalin or baicalein did not inhibit the activity of the pre-assembled NADPH oxidase complex. Similar results had been reported that flavonoid (e.g., fisetin) lacking a −OH at C_2 (B-ring) exhibited comparable inhibition of oxygen consumption in whole cells but less effective as an inhibitor of NADPH oxidase as quercetin in subcellular fraction (Tauber et al., 1984). Therefore, baicalin or baicalein, with no −OH at C_2 did not directly interfere with the component(s) of the assembled NADPH oxidase nor did it interfere with the binding of NADPH. Besides, when baicalin or baicalein was added to neutrophils before isolation of the membrane fraction, $O_2^{\cdot -}$ generation was not inhibited suggesting that baicalin or baicalein also did not inhibit the assembly of an active, membrane-associated NADPH oxidase. On the other hand, cromolyn, an inhibitor for the assembly of NADPH oxidase but not the activity of pre-assembled oxidase (Kilpatrick et al., 1995), showed comparable result as in that of Kilpatrick's report.

There are several possible mechanisms that baicalin or baicalein could interfere with reactive oxygen intermediates production in addition to the NADPH oxidase. These include modulation of (1) catalase activity, which converts superoxide anion to water and oxygen molecule, (2) myeloperoxidase activity, which generates HOCl, a potent oxidant, from H_2O_2. In this study, we found NaN_3 (1 mM) completely abolished the catalase activity, but neither baicalin nor baicalein could modulate the catalase activity (data not shown). On the contrary, baicalin or baicalein significantly inhibited myeloperoxidase activity. With structure similar to quercetin that had been demonstrated

to be an effective myeloperoxidase inhibitor (IC_{50} = 3.5 μM) and HOCl scavenger (Pincemail et al., 1988), it is reasonable that baicalin or baicalein could inhibit myeloperoxidase activity in this study.

Cytosolic Ca^{2+} oscillation can serve as a signaling molecule in regulating NADPH oxidase (Suzuki et al., 1985) as well as Mac-1-dependent neutrophil adhesion (Shen et al., 1999; Lawson and Maxfield, 1995). To further elucidate the underlying mechanism(s) by which baicalin or baicalein impedes the reactive oxygen intermediates production and Mac-1-dependent neutrophil adhesion, we examined the effects of baicalin or baicalein in intracellular Ca^{2+} mobilization induced by fMLP (a receptor agonist), AlF_4^- (a G-protein activator) or thapsigargin (release Ca^{2+} from intracellular store). Our data established that baicalein (100 μM) significantly inhibited fMLP- and AlF_4^-- but not thapsigargin-induced $[Ca^{2+}]_i$ mobilization indicating that baicalein, but not baicalin, may interfere with the G-protein activation as fMLP binds to its receptor. The lack of activity of baicalin in the prevention of Ca^{2+} mobilization could be due to its lower activity than baicalein in general. This can be further noticed in this report that the IC_{50} (μM) of baicalin in suppressing reactive oxygen intermediates production by human leukocytes was relative higher than that of baicalein. Our results disagree with others' report that indicates both baicalin and baicalein can inhibit intracellular Ca^{2+} elevation through reducing phospholipase C activity in C6 rat glioma cells (Kyo et al., 1998). This could possible be due to different cellular models used. In our study, samples pretreated with pertussis toxin (500 ng/ml) or BAPTA-AM (10 μM) (an intracellular Ca^{2+} chelator) significantly prevented G protein activation or intracellular Ca^{2+} mobilization induced by AlF_4^- or thapsigargin, respectively (data not shown).

In addition to inhibition of reactive oxygen intermediates production and intracellular Ca^{2+} mobilization, many reactive oxygen intermediates-producing related enzyme(s) or protein kinase signaling pathways could be targeted by baicalin or baicalein in the regulation of reactive oxygen intermediates production and Mac-1 up-expression. For example, Shieh et al. (2000) reported that the anti-oxidative function of baicalin is mainly based on scavenging superoxide radical while baicalein was a xanthine oxidase inhibitor in cell-free system. Furthermore, protein kinase activation (e.g., PKC or PKA) and protein tyrosine kinase (PTK) is also involved in the regulation of NADPH oxidase (Clark et al., 1990), Mac-1 expression (Monk and Banks, 1991; Roubey et al., 1991), and cell activation (Huang, 1989). In this study we found that it is unlikely that baicalin or baicalein could suppress PKC or PKA activity (Table 2). In our unpublished data we observed that PD98059 [a mitogen-activated protein kinase (MAPK) kinase inhibitor], SP600125 [a c-Jun N-terminal kinase (JNK) inhibitor] and SB203580 (a p38 MAPK inhibitor), three inhibitors of MAPK family signaling pathways, all significantly impeded the intracellular reactive oxygen intermediates production by neutrophils with SB203580 being the most potent one (IC_{50} less than 10 μM). Whether PTK or MAPK pathway(s) could be modulated by baicalin or baicalein awaits further study. Mac-1 expression may also be regulated by phospholipase A_2 because inhibitors of phospholipase A_2 can inhibit the surface expression of Mac-1 (Jacobson and Schrier, 1993). Phospholipase A_2 hydrolyzes the membrane phospholipids with the release of arachidonic acid for subsequent metabolism via the cyclooxygenase and lipoxygenase pathways, two important intra- and extra-cellular mediators of inflammation (Pruzanski and Vadas, 1991). Baicalein has been shown to inhibit the prostaglandin E_2 production (Nakahata et al., 1998) and leukotriene B_4/C_4 biosynthesis (Tanno et al., 1988; Butenko et al., 1993), two down stream metabolites by cyclooxygenase and lipoxygenase, respectively. It is possible that baicalin or baicalein could influence cyclooxygenase or lipoxygenase biochemical pathways and in turn regulates reactive oxygen intermediates production and Mac-1 expression.

In conclusion, we have demonstrated the ability of baicalin and baicalein to prevent Mac-1-dependent leukocyte adhesion and that can be explained by their ability to down regulate reactive oxygen intermediates generation, possibly through scavenging reactive oxygen intermediates by baicalin or that through antagonizing ligand-initiated Ca^{2+} influx by baicalein. As effective anti-oxidant and anti-adhesive agents, baicalin and baicalein may be beneficial in the prevention of inflammatory injury through inhibition of the leukocyte activation.

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