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The inhibitory effect of phenylpropanoid glycosides and iridoid glucosides on free radical production and β2 integrin expression in human leucocytes

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Abstract

Rapid production of reactive oxygen species (ROS) and upregulation of β2 integrin by leucocytes are two important inflammatory responses in human leucocytes. To evaluate whether three phenylpropanoid glycosides (acteoside, crenatoside, and rossicaside B) and two iridoid glucosides (boschnaloside and 8-epideoxyloganic acid) identified from two medicinal plants with similar indications (*Orobanche caerulescens* and *Boschniakia rossica*) exhibited anti-inflammatory activity, their effects on *N*-formyl-methionyl-leucyl-phenylalanine (fMLP) and phorbol-12-myristate-13-acetate (PMA)-activated peripheral human neutrophils (PMNs) and mononuclear cells were examined. Pretreatment with 1–50 μM phenylpropanoid glycoside concentration-dependently diminished PMA- and fMLP-induced ROS production with IC50 values of approximately 6.8–23.9 and 3.0–8.8 μM, respectively. Iridoid glucoside was less effective than phenylpropanoid glycoside with an IC50 value of approximately 8.9–28.4 μM in PMA-activated PMNs and 19.1–21.1 μM in fMLP-activated mononuclear cells. Phenylpropanoid glycosides also effectively inhibited NADPH oxidase (NOX) and displayed potent free radical-scavenging activity, but did not interfere with pan-protein kinase C (PKC) activity. Furthermore, all compounds, except rossicaside B, significantly inhibited PMA- and fMLP-induced Mac-1 (a β2 integrin) upregulation at 50 μM but not that of fMLP-induced intracellular calcium mobilization. These drugs had no significant cytotoxicity as compared with the vehicle control. Our data suggested that inhibition of ROS production, possibly through modulation of NOX activity and/or the radical scavenging effect, and β2 integrin expression in leucocytes indicated that these compounds had the potential to serve as anti-inflammatory agents during oxidative stress.

Introduction

Orobanche caerulescens and *Boschniakia rossica* of the family Orobanchaceae have similar medical indications and are used in Taiwan folk medicine. *O. caerulescens* Stephan, a parasitic herb growing on the roots of *Artemisia* spp. (Compositae), is used as a tonic agent to treat impotence, as an anti-inflammatory agent to treat cystitis, and as a faeces softener (Chiu & Change 1983; Yang & Lu 1998). *B. rossica* (Cham. *et* Schlech.) Fedtsch. *et* Flerov., another parasitic plant growing on the root of plants of the genus *Alnus* (Betulaceae), is used as a substitute for *Cistanchis Herba*, a well-known staminal tonic agent used in Oriental medicine (Juangsu 1979). The chemical constituents of *Cistanche* species have been investigated, which led to the isolation of a number of phenylpropanoid glycosides, iridoids, iridoid glucosides, and lignans (Kobayashi et al 1985; Xiong et al 1996; Yoshizawa et al 1990). *B. rossica* and *O. caerulescens* contain large amounts of phenylpropanoid glycosides and iridoid glucosides. The main constituents of *O. caerulescens* are the phenylpropanoid glycosides acteoside (**1**) and crenatoside (**2**) (Lin et al 2004), whilst the major components of *B. rossica* are the phenylpropanoid glycoside rossicaside B (**3**) and the iridoid glucosides boschnaloside (**4**) and 8-epideoxyloganic acid (**5**) (Lin & Chen 2004).

Recently, antioxidative therapy has been adopted as a comprehensive pharmacological approach for treating inflammation-related disorders including ischaemia/reperfusion injury in organs, shock, sepsis, atherosclerosis, and cancer (Middleton et al 2000; Cuzzocrea et al 2001). One of the important strategies for antioxidative therapy is to look for natural

compounds from plants or dietary components that are capable of inhibiting oxidative stress for the prevention of reactive oxygen species (ROS)-induced cellular damage by inflammatory cells that infiltrate injured tissue. During inflammation, the production of ROS (e.g. hydrogen peroxide (H_2O_2) and superoxide anion ($O_2^{\cdot-}$)) plays an important role in the killing of microorganisms and also signals the activation of leucocytes. However, overproduction of these toxic oxygen metabolites can damage the surrounding tissues (Casimir & Teahan 1994). In addition, activated neutrophils preferentially upregulate $\beta 2$ integrin (e.g. CD11b/CD18) for firm adherence to vascular endothelium cells and subsequently transmigrate or infiltrate injured tissues where they release hydrolytic enzymes and large quantities of ROS that can lead to extensive tissue damage and immunopathogenesis (Wardlaw & Walsh 1994).

Previously, we found that phenylpropanoid glycosides isolated from *O. caerulescens* displayed an antioxidative effect against $CuSO_4$-induced low-density lipoprotein (LDL) oxidation (Chiou et al 2004). The purpose of this study was to examine whether phenylpropanoid glycosides or iridoid glucosides could suppress ROS production and $\beta 2$-integrin upregulation in activated human leucocytes so that they could be evaluated for use as anti-inflammatory agents. We set up an in-vitro model by exposing peripheral human neutrophils (PMNs) and mononuclear cells to phorbol-12-myristate-13-acetate (PMA), a direct protein kinase C (PKC) activator, and *N*-formyl-methionyl-leucyl-phenylalanine (fMLP), a receptor-mediated and G protein-coupled activator, for the induction of ROS production and upregulation of $\beta 2$ integrin. We evaluated whether phenylpropanoid glycosides (acteoside, crenatoside, and rossicaside B) and iridoid glucosides (boschnaloside and 8-epideoxyloganic acid) exhibited inflammation-modulating activity in human leucocytes.

Materials and Methods

Preparation of phenylpropanoid glycosides and iridoid glucosides

Phenylpropanoid glycosides (acteoside, crenatoside, and rossicaside B) and iridoid glucosides (boschnaloside and 8-epideoxyloganic acid) (Figure 1) were prepared as in our previous reports (Lin & Chen 2004; Lin et al 2004). For biological study, they were dissolved in dimethyl sulfoxide (DMSO) to achieve a stock concentration of 20 mM. Serial dilution in phosphate-buffered saline (PBS) to appropriate concentrations was performed from the stock immediately before use. PBS containing 0.25% DMSO was used as the vehicle control. The sample stock solution was stored at −20°C and was used within one week of preparation. For examination of the effects of these drugs, 10 μL drug solution was added to 1.0 mL cell suspension and incubated at 37°C for 20 min before the addition of PMA (Calbiochem, Germany) or fMLP (Sigma-Aldrich, USA).

Preparation of human neutrophils and mononuclear cells

Human neutrophils (PMNs) and mononuclear cells were prepared from leucocyte-concentrated whole blood obtained from a local blood centre, approved by our Institutional Review Board in accordance with international guidelines. In addition, informed consent was obtained from the subjects who participated in the study. PMNs and mononuclear cells were isolated from blood (30 mL) obtained through venipuncture from healthy adult volunteers and collected into syringes containing heparin (20 U mL^{-1} blood), followed by dextran sedimentation, Ficoll-Hypaque centrifugation, and hypotonic lysis of residual erythrocytes (Shen et al 2003). Samples so purified contained more than 95% PMNs and 85% mononuclear cells as estimated by counting 200 cells under a microscope after Giemsa staining (Sigma). In all cases where cells were pretreated with drugs, the cells were mixed with the drugs at concentrations ranging from 1 to 50 μM in Hank's buffered saline solution (HBSS) for 20 min at 37°C.

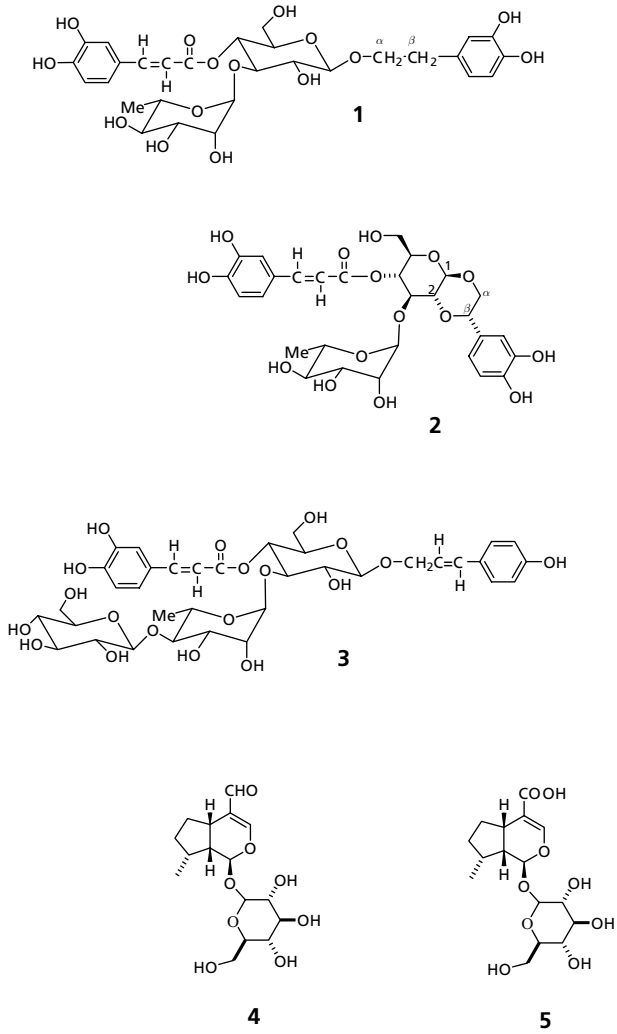

Figure 1 Chemical structures of phenylpropanoid glycosides (**1–3**): acteoside (**1**), crenatoside (**2**), and rossicaside B (**3**); and iridoid glucosides (**4–5**): boschnaloside (**4**) and 8-epideoxyloganic acid (**5**).

Measurement of PMA- or fMLP-induced ROS production in human leucocytes

ROS production was measured as detailed by Parij et al (1998). Briefly, in a bioluminescence plate (Costar, NY, USA), phenylpropanoid glycosides and iridoid glucosides were serially diluted to final concentrations ranging from 1 to 50 μM with volume of 10 μL. To each well, 50 μL PMN or mononuclear cell suspension (1×10^7 cells mL^{-1}) and 50 μL lucigenin (180 μM) solutions were added. After incubation for 20 min with test drugs, the cell suspension was triggered by adding 50 μL PMA (200 nM) or fMLP (1 μM). The chemiluminescence was monitored for 1 s every 1 min during a 30-min observation period using a Microplate Luminometer (Orion Microplate Luminometer, Berthold DS, Tforzheim, Germany). The results were presented as relative light units (RLU). Peak levels were recoded to calculate the activity of test drugs in relation to their corresponding solvent controls (0.25% DMSO). The 50% inhibitory concentration (IC50) in response to PMA- or fMLP-triggered chemiluminescence by the test drugs was calculated.

Protein kinase C (PKC) activity assay

Neutrophils (2×10^7 mL^{-1}), suspended in an ice-cold extraction buffer, were sonicated, and cytosolic cell lysate was separated by centrifugation at 100 000 *g* for 60 min at 4°C for the pan-PKC activity assay on the same day (Wang et al 2004). The protein concentration was determined with a protein assay kit (Bio-Rad, USA). PKC activity was measured with a non-radioactive protein kinase assay kit (Calbiochem, Germany) based on an enzyme-linked immunosorbent assay using a synthetic PKC pseudosubstrate and a monoclonal antibody to recognize the phosphorylated peptide. PKC phosphorylated the serine residue on the pseudosubstrate (peptide) through a Ca^{2+}/phosphatidylserine (PS)-dependent-mechanism. Ca^{2+}/phospholipid-dependent PKC activity was assayed in the presence of Ca^{2+} and PS while the negative control was measured in the presence of 20 mM EGTA or staurosporine, a PKC catalytic domain competitor. Data are expressed as OD_{492}/12 μL cell lysate from 2×10^7 cells.

Measurement of NADPH oxidase activity

NADPH oxidase activity was measured as described by Griendling et al (1994) with some modifications. Leucocytes (2×10^7 cells) were suspended in lysis buffer (20 mM potassium phosphate (pH 7.0), 1 mM ethylene-bis(oxyethylenenitrilo)-tetraacetic acid (EGTA), and a protease inhibitor fluid (Roche, Germany). The cell suspension was sonicated in five 10-s bursts at 30% power followed by cooling on ice for 30 s between each sonication. The homogenate was centrifuged twice at 800 *g* for 15 min to remove unbroken cells. The protein concentrations were determined using the Bio-Rad Bradford reagent. Cell homogenate (50 μg) was added to the wells of a bioluminescence plate. NADPH oxidase activity was measured in a 50 mM phosphate buffer, containing 1 mM EGTA, 150 mM sucrose, and 10 μM lucigenin. Various drugs were tested including 100 μM diphenyleneiodonium (DPI; an NADPH oxidase inhibitor) and 10–50 μM of the test drugs. These drugs were added to the wells of a bioluminescence plate and incubated for 20 min at 37°C in the dark. O_2^- production was stimulated with 200 μM NADPH, and chemiluminescence was monitored for 30 min, after which the AUC (area under the curve) was calculated to represent ROS production and expressed as RLU/50 μg protein.

Measurement of 1,1-diphenyl-2-picrylhydrazyl (DPPH·) radical scavenging capacity

For the DPPH· radical scavenging capacity assay, the method of Mursu et al (2005) was followed with some modifications. Drugs were diluted in methanol into a range of concentrations (10–50 μM). DPPH solution (200 μL; 200 μM in MeOH) was added to 10 μL of each diluted sample in a 96-well microplate, and the resulting solution was allowed to react for 30 min in the dark at ambient temperature. The absorbance caused by the DPPH· radical at 517 nm was determined by a microplate-spectrophotometer. The radical scavenging capacity was expressed as delta OD_{517} (ΔOD_{517}), and values were the means of three replicates. An antioxidant, Trolox, was included as a reference.

Measurement of Mac-1 (CD11b/CD18) upregulation induced by PMA or fMLP

Mac-1 expression was analysed as detailed by Shen et al (1999). Briefly, after pretreatment with test drugs for 20 min, a neutrophil suspension (2×10^6 cells mL^{-1}) was stimulated with PMA (200 nM) or fMLP (1 μM) for 20 min. The cells were then pelleted and resuspended in 1 mL ice-cold PBS containing 10% heat-inactivated fetal bovine serum (FBS) and 10 mM NaN_3. 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 sample of fluorescein isothiocyanate (FITC)-conjugated anti-Mac-1 antibody (mouse anti-human CD11b, class IgG_1; BD Biosciences Pharmingen) or a non-specific mouse antibody (class IgG_1, Sigma) as a negative control. After two washes with PBS containing 5% FBS, stained cells were resuspended in flow cytometer sheath fluid (Becton Dickinson) containing 1% paraformaldehyde and analysed by flow cytometry for Mac-1 expression. Data were expressed as the mean channel fluorescence for each sample as calculated by the CellQuest software (Becton Dickinson) on a Power Macintosh 7300/200 computer.

Estimation of cell viability

For the examination of cell viability, a method described by Ormerod (2000) was followed. Briefly, after incubation of test drugs with cells for 4 h, cell viability was determined by adding propidium iodide (PI, 10 μg mL^{-1}) and fluorescein diacetate (FDA, 100 ng mL^{-1}). After incubation with PI (for dead cell staining) and FDA (for viable cell staining) at room temperature for 10 min, the cell suspension was analysed on a flow cytometer (FACSCalibur; Becton Dickinson) by recording forward and light scatter, red (for PI) and green (for FDA) fluorescence. After gating on the scattered light to include single cells and to exclude clumps and debris, cell populations were displayed by green (viable) vs red (dead) fluorescence.

Cell viability (%) was calculated by the CellQuest software (Becton Dickinson) on a Power Macintosh 7300/200 computer. In some experiments, estimation of cell viability was further confirmed by a trypan blue exclusion assay.

Statistical analysis

All values in the text and Tables are presented as the mean ± s.e.m. Data were analysed by analysis of variance followed by post-hoc Dunnett's *t*-test for multiple comparisons. Concentration dependence was analysed by a simple linear regression analysis of response levels against concentration of the test drug, and testing the slope of the regression line against 0 by Student's *t*-test at an α level equal to 0.05.

Results and Discussion

During inflammation, ROS produced by leucocytes act as an essential bactericidal mechanism, but improper and/or overproduction of ROS mediates tissue injuries in many inflammatory disorders (Williams 1994; Nathan 2002). Lots of studies have shown that many antioxidants of botanic sources are effective anti-inflammatory drugs in many oxidative-related human diseases (Middleton et al 2000). Recently, we isolated some phenylpropanoid glycosides (e.g. acteoside) and iridoid glucosides (e.g. boschnaloside) from the Chinese herbs *O. caerulescens* and *B. rossica*, respectively, which could act as potent antioxidants (Chiou et al 2004). Various plants used in traditional medicine contain significant amounts of phenylpropanoids (Ismailoglu et al 2002). Pharmacological studies on phenylpropanoid glycosides have revealed that they can protect endothelial cells against free radical-induced oxidative stress (Chiou et al 2004), and that they exhibit anti-inflammatory (Sahpaz et al 2002), anti-nephritic (Hayashi et al 1994), and anti-hepatotoxic activity (Xiong et al 1998). On the contrary, the immunomodulating and anti-inflammatory activity of these compounds (phenylpropanoid glycosides and iridoid glucosides) in human leucocytes are not as well understood.

In this study, we have evaluated whether three phenylpropanoid glycosides (acteoside, crenatoside, and rossicaside B) from *O. caerulescens* and two iridoid glucosides (boschnaloside and 8-epideoxyloganic acid) from *B. rossica* could prevent ROS production and β2-integrin upregulation in activated human leucocytes. Our results showed that fMLP (a receptor-mediated G-protein coupling activator) and PMA (a PKC-dependent activator) induced high ROS production of up to 10–20-fold higher than that of resting cells. Pretreatment with 1–50 μM phenylpropanoid glycoside concentration-dependently diminished PMA- (Table 1) and fMLP- (Table 2) induced-ROS production by a lucigenin-amplified chemiluminescence assay. Phenylpropanoid glycosides were more potent than iridoid glucosides in the inhibition of PMA- and fMLP-induced ROS production, both of which were examined in PMNs and mononuclear cells (Tables 1, 2).

Activation of leucocytes by fMLP and PMA involved the PKC-dependent signalling pathway, and acteoside (phenylpropanoid glycoside) was reported to interact with the catalytic domain of PKC as a competitive inhibitor with respect to ATP (Herbert et al 1991). To further elucidate whether phenylpropanoid glycoside-related structures could directly inhibit PKC activity as the underlying mechanism for the prevention of PMA- or fMLP-induced ROS production in this study, an assay to determine the effect of these drugs on pan-PKC activity in whole cell lysate was performed. Our results showed that none of these compounds directly modulated pan-PKC activity (Table 3). Therefore, it was likely that these compounds modulated targets downstream of PKC activation. Moreover, NADPH oxidase (NOX) is the major ROS producing enzyme in activated leucocytes (Simons et al 1990; Van den Worm et al 2001). Therefore, NOX activity in a cell free system was evaluated. Our results demonstrated that phenylpropanoid glycosides, but not iridoid glucosides, directly inhibited NOX activity in a concentration-dependent manner in cell lysate from both neutrophils (PMNs) and mononuclear cells (Table 4). Diphenyleneiodonium (DPI, an NOX inhibitor), included as a positive control, almost completely blocked NOX activity (Table 4). This result partially accounted for the better potency of phenylpropanoid glycosides than iridoid glucosides as antioxidants (Tables 1, 2). In addition, phenylpropanoid glycosides were relatively more effective in the inhibition of fMLP (with IC50 values

Table 1 Summary of the IC50 values for the prevention of PMA-induced ROS production by three phenylpropanoid glycosides and two iridoid glucosides in human peripheral leucocytes

Drugs	IC50 (μM) in PMNs	IC50 (μM) in mononuclear cells
Acteoside	12.8 ± 7.2	9.6 ± 3.2
Crenatoside	6.8 ± 2.3	10.0 ± 4.3
Rossicaside B	5.6 ± 2.8	23.9 ± 2.9
Boschnaloside	28.4 ± 7.5	ND
8-Epideoxyloganic acid	8.9 ± 2.9	ND

Data were calculated as the 50% inhibitory concentration (IC50). Values represent the mean ± s.e.m. of five experiments performed on different days using cells from different donors. ND, values not detectable.

Table 2 Summary of the IC50 values for the prevention of fMLP-induced ROS production by three phenylpropanoid glycosides and two iridoid glucosides in human peripheral leucocytes

Drugs	IC50 (μM) in PMNs	IC50 (μM) in mononuclear cells
Acteoside	3.5 ± 0.6	8.8 ± 3.2
Crenatoside	3.0 ± 0.2	3.5 ± 0.2
Rossicaside B	3.0 ± 0.1	3.5 ± 0.2
Boschnaloside	ND	19.1 ± 11.3
8-Epideoxyloganic acid	ND	21.1 ± 2.6

Data were calculated as the 50% inhibitory concentration (IC50). Values represent the mean ± s.e.m. of five experiments performed on different days using cells from different donors. ND, values not detectable.

Table 3 Summary of the effects of acteoside-related phenylpropanoid glycosides and iridoid glucosides on PKC activity

Drugs (μM)	PKC activity (OD_{492}/12 μL cell lysate from 2×10^7 cells)
Control (PS only)	1.10 ± 0.02
+ EGTA (20 mM)	0.54 ± 0.03*
+ Staurosporine (2)	0.07 ± 0.02*
+ Acteoside (50)	1.04 ± 0.04
+ Acteoside (25)	1.07 ± 0.03
+ Crenatoside (50)	1.08 ± 0.03
+ Crenatoside (25)	0.99 ± 0.07
+ Rossicaside B (50)	1.18 ± 0.01
+ Rossicaside B (25)	0.94 ± 0.02
+ Boschnaloside (50)	0.98 ± 0.11
+ Boschnaloside (25)	0.95 ± 0.13
+ 8-Epideoxyloganic acid (50)	0.99 ± 0.03
+ 8-Epideoxyloganic acid (25)	0.99 ± 0.11

Values are the mean ± s.e.m. from three experiments performed on different days using cells lysates from different donors. PS, phosphatidylserine. *$P < 0.05$ compared with control.

Table 4 Summary of the effects of acteoside-related phenylpropanoid glycosides and two iridoid glucosides on NADPH oxidase activity

Drugs (μM)	AUC (PMNs lysate)	AUC (mononuclear cell lysate)
Basal (solvent control)	261 ± 15*	772 ± 42*
NADPH (200)	8 872 ± 75	23 928 ± 440
+ DPI (100)	363 ± 28*	860 ± 66*
+ Acteoside (50)	5432 ± 112*	18 387 ± 538*
+ Acteoside (25)	6708 ± 98*	22 034 ± 674
+Crenatoside (50)	6102 ± 62*	16 558 ± 587*
+ Crenatoside (25)	7122 ± 95*	20 252 ± 630*
+ Rossicaside B (50)	6102 ± 74*	18 533 ± 595*
+ Rossicaside B (25)	6573 ± 103*	23 506 ± 721
+ Boschnaloside (50)	8703 ± 102	23 619 ± 748
+ Boschnaloside (25)	8579 ± 98	26 366 ± 683
+ 8-Epideoxyloganic acid (50)	8638 ± 110	24 665 ± 864
+ 8-Epideoxyloganic acid (25)	8805 ± 105	25 674 ± 812

Data (RLU) were monitored for 30 min, the AUC (area under the curve) was calculated to represent ROS production. DPI, diphenyleneiodonium. Results are expressed as the mean ± s.e.m. from three experiments performed on different days using cell lysates from different donors. *$P < 0.05$ compared with NADPH alone.

of 3–9 μM) than PMA (with IC50 values of 6–23 μM) induced ROS production. This could be explained by the fact that fMLP was a relatively weaker PKC stimulator than PMA in the production of ROS (5–7- vs 10–20-fold higher than resting cells for fMLP and PMA, respectively).

To examine whether these drugs exhibited radical-scavenging ability, experiments related to free radical-scavenging activity by phenylpropanoid glycosides and iridoid glucosides were performed in a cell-free DPPH solution system. Phenylpropanoid glycosides showed effective free radical-scavenging activity, although less potent than Trolox (Table 5). Iridoid glucosides did not show free radical-scavenging activity (Table 5). On the contrary, iridoid glucosides exhibited mediocre activity in the inhibition of PMA- and fMLP-induced ROS production (with IC50 values of approximately 9–28 μM) and showed a relatively selective effect in PMA-activated PMNs and fMLP-activated mononuclear cells (Tables 1, 2). Since iridoid glucosides did not directly interfere with PKC activity or block NOX activity, determining the precise mechanism for their anti-ROS effects needs further elucidation. In this study, we included apocynin, a well-known plant-derived drug used to inhibit ROS by reducing the assembly of NOX. Apocynin significantly inhibited fMLP- and PMA-induced ROS production in PMNs with IC50 values of approximately 10 and 30 μM, respectively, revealing that phenylpropanoid glycosides were more potent than apocynin as an NOX inhibitor.

Table 5 Summary of acteoside-related phenylpropanoid glycosides and two iridoid glucosides on the 1,1-diphenyl-2-picrylhydrazyl (DPPH·) radical-scavenging capacity

Drug (μM)	DPPH· radical-scavenging capacity (ΔOD_{517}/30 min)
Trolox (50)	0.76 ± 0.01
Acteoside (50)	0.57 ± 0.03*
Acteoside (10)	0.26 ± 0.02*
Crenatoside (50)	0.56 ± 0.04*
Crenatoside (10)	0.28 ± 0.03*
Rossicaside B (50)	0.52 ± 0.02*
Rossicaside B (10)	0.17 ± 0.03*
Boschnaloside (50)	0.01 ± 0.02*
Boschnaloside (10)	0.03 ± 0.01*
8 -Epideoxyloganic acid (50)	0.01 ± 0.01*
8 -Epideoxyloganic acid (10)	0.01 ± 0.03*

Values are the mean ± s.e.m. from three individual experiments performed on different days. *$P < 0.05$ as compared with Trolox (50 μM) alone. The OD_{517} of DPPH solution (200 μM) alone was 0.72 ± 0.04.

In addition to ROS production, activated leucocytes preferentially infiltrate and accumulate at sites of injured tissues. Pivotal mechanisms underlying this process mainly depend upon the firm adhesion of the leucocyte by the upregulation of its β2 integrin, especially Mac-1 (CD11b/CD18), to counteract with its counter receptor, the intercellular adhesion molecule-1 (ICAM-1) on the surface of vascular endothelial cells (Wang & Doerschuk 2000). Since acteoside had been shown to inhibit leucocyte accumulation in the glomeruli of nephritic rats (Hayashi et al 1994), possibly through inhibition of the expression of ICAM-1 (Hayashi et al 1996), it was interesting to examine whether acteoside and other phenylpropanoid glycosides or iridoid glucosides could modulate Mac-1 upregulation in neutrophils also. Our data showed that PMA and fMLP caused marked increases in Mac-1 fluorescence (Table 6). Pretreatment with staurosporine (a PKC inhibitor)

Table 6 Prevention of PMA- and fMLP-induced β2 integrin (Mac-1) expression by phenylpropanoid glycosides and two iridoid glucosides in human peripheral leucocytes

Drugs	Mac-1 (by PMA) (drug/10 μM)	Mac-1 (by PMA) (drug/50 μM)	Mac-1 (by fMLP) (drug/50 μM)
Solvent control	218 ± 11*	218 ± 11*	218 ± 11*
PMA or fMLP only	317 ± 15	317 ± 15	285 ± 15
+ Acteoside	285 ± 12	229 ± 8*	221 ± 6*
+ Crenatoside	301 ± 9	261 ± 12*	238 ± 10*
+ Rossicaside B	315 ± 6	297 ± 11	268 ± 14
+ Boschnaloside	290 ± 14	272 ± 10*	242 ± 8*
+ 8-Epideoxyl-oganic acid	317 ± 11	238 ± 7*	225 ± 9*
+ Staurosporine (0.2 μM)	206 ± 15*	206 ± 15*	–
+ BAPTA-AM (10 μM)	–	–	211 ± 10*

Data (Mac-1 staining) were calculated as the mean channel fluorescence intensity. Values are expressed as the mean ± s.e.m. from five independent trials in each group. *$P < 0.05$ compared with PMA or fMLP only, respectively, by one-way analysis of variance followed by Dunnett's test.

or 1,2-bis-(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (acetoxymethyl)ester (BAPTA-AM; an intracellular calcium chelator) completely prevented the Mac-1 upregulation induced by PMA or fMLP, respectively. All compounds, except rossicaside B, significantly inhibited PMA- and fMLP-induced Mac-1 upregulation at 50 μM (Table 6). In addition, intracellular calcium mobilization is an important signal that mediates G-protein signalling for leucocyte activation and upregulation of integrin (Shen et al 1999). To further understand the underlying mechanism, we explored fMLP-induced mobilization of intracellular calcium in PMNs. Our results revealed that neither phenylpropanoid glycosides nor iridoid glucosides modulated intracellular calcium mobilization induced by fMLP (data not shown), indicating that direct modulation of calcium mobilization was not involved in their anti-Mac-1 expression effect.

The inhibitory effects of these drugs in human leucocytes were not due to cytotoxic effects because no significant cell death, generally less than 5% of cell death after experiments using these drugs, was observed at the concentration ranges (1–50 μM) examined as compared with the vehicle control. In conclusion, we have demonstrated that phenylpropanoid glycosides exhibited leucocyte-modulating activity, more potent than iridoid glucosides, by limiting ROS production and/or β2 integrin upregulation, possibly through modulation of NOX activity and/or radical-scavenging effect, with no significant cytotoxic effects. Thus, these drugs have the potential to be anti-inflammatory agents for the treatment of oxidative-related diseases.

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