Andrographolide inhibits TNFα-induced ICAM-1 expression via suppression of NADPH oxidase activation and induction of HO-1 and GCLM expression through the PI3K/Akt/Nrf2 and PI3K/Akt/AP-1 pathways in human endothelial cells

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Abstract

Andrographolide, the major bioactive component of Andrographis paniculata, has been demonstrated to have various biological properties including anti-inflammation, antioxidation, and anti-hepatotoxicity. Oxidative stress is considered a major risk factor in aging, inflammation, cancer, atherosclerosis, and diabetes mellitus. NADPH oxidase is a major source of endogenous reactive oxygen species (ROS). In this study, we used EA.hy926 endothelial-like cells to explore the anti-inflammatory activity of andrographolide. Andrographolide attenuated TNFα-induced ROS generation, Src phosphorylation, membrane translocation of the NADPH oxidase subunits p47phox and p67phox, and ICAM-1 gene expression. In the small hairpin RNA interference assay, shp47phox abolished TNFα-induced p65 nuclear translocation, ICAM-1 gene expression, and adhesion of HL-60 cells. Andrographolide induced the expression of heme oxygenase 1 (HO-1) and glutamate cysteine ligase modifier subunit (GCLM) in a time-dependent manner. Cellular glutathione (GSH) content was increased by andrographolide. shGCLM attenuated the andrographolide-induced increase in GSH content and reversed the andrographolide inhibition of HL-60 adhesion. shHO-1 showed a similar effect on andrographolide inhibition of HL-60 adhesion to shGCLM. The mechanism underlying the up-regulation of HO-1 and GCLM by andrographolide was dependent on the PI3K/Akt pathway, and both the Nrf2 and AP-1 transcriptional factors were involved. Our results suggest that andrographolide attenuates TNFα-induced ICAM-1 expression at least partially through suppression of NADPH oxidase activation and induction of HO-1 and GCLM expression, which is PI3K/Akt pathway-dependent.

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The Src family of protein tyrosine kinases includes c-Src, Fyn, York, and Yes, which are widely expressed in different tissues, whereas the other members including Lyn, Hck, Fgr, and BLK have a more restrictive distribution [10]. Because Src can activate signaling molecules such as PI3K and phospholipase C, Src is considered to be an important signaling transmitter in many cellular processes including growth, gene transcription, adhesion, and apoptosis [11]. A previous study showed that Src participates in the activation of TNF-α-induced p47phox in human airway smooth muscle cells [12]. Thus, taken together, these data suggest that proinflammatory cytokine-mediated vascular inflammation might be highly related to NADPH oxidase-derived ROS in endothelial cells.

Glutathione (GSH), a member of the cellular antioxidant defense system, assists in the clearance of excessive ROS and maintains the redox homeostasis in cells [13]. The cellular GSH level is influenced by multiple factors, and a primary determinant of the intracellular GSH level is the rate of de novo synthesis. Glutamate cysteine ligase catalyzes the rate-limiting step in GSH synthesis. The mammalian glutamate cysteine ligase holoenzyme is a heterodimer that is composed of a catalytic subunit (GCLC) and a modifier subunit (GCLM) [14]. Previous studies found that mice deficient in the Gclm or Gcf gene have a markedly reduced GSH content in aortas and liver [15,16]. Thus, the expression of GCLC and GCLM was considered to determine the cellular GSH level.

Heme oxygenase 1 (HO-1) is considered to be a phase II enzyme and antioxidant [17]. HO-1 catalyzes the rate-limiting step in heme catabolism and produces carbon monoxide, free iron, and biliverdin, which is further catabolized into bilirubin by biliverdin reductase [18,19]. Previous studies suggested that dietary phytochemicals provide chemoprevention and therapeutic potential because they induce HO-1 and thereby enhance cellular antioxidant capacity [20,21]. Several signaling molecules, such as mitogen-activated protein kinases (JNK, ERK, and p38) and PI3K/Akt, and transcriptional factors, such as activator protein 1 (AP-1) and NF-E2-related factor-2 (Nrf2), participate in the regulation of GCLC, GCLM, and HO-1 gene expression [20,22,23].

*Andrographis paniculata* (Burm. F.) Nees is a traditional herb in China, Korea, and other regions in Southeast Asia. Andrographolide is one of the major bioactive components and is the most abundant diterpenoid in the leaves of *A. paniculata* [24]. Andrographolide has been studied for its beneficial properties such as anti-inflammation, anti-cancer, and antioxidation [25–27]. Antioxidation and anti-inflammation are considered to be therapeutic strategies for prevention or treatment of atherosclerosis. The present study was undertaken to clarify the role of NADPH oxidase, GCLM, and HO-1 in the andrographolide inhibition of TNFα-induced ICAM-1 expression and the underlying mechanisms involved.

### 2. Materials and methods

#### 2.1. Reagents

Cell culture medium (RPMI-1640), RPMI-1640 without phenol red, Dulbecco’s modified Eagle’s medium (DMEM), penicillin-streptomycin solution, and 0.25% trypsin–EDTA were obtained from Gibco (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT, USA). Andrographolide was purchased from Calbiochem (Darmstadt, Germany). Human TNFα, sodium bicarbonate, HEPES, 5,5’-dithiobis(2-nitrobenzoic acid) (DTNB), LY294002, DMSO, and all other chemicals were purchased from Sigma–Aldrich (St. Louis, MO, USA). TRIzol reagent and 2,7-dichlorofluorescin diacetate (H2DCFDA) were purchased from Invitrogen (Carlsbad, CA, USA). PD98059 was purchased from Tocris Bioscience (Bristol, United Kingdom).

#### 2.2. Cell culture

The human endothelial cell line EA.hy926 was a gift from Dr. T.S. Wang, Chung Shan Medical University, Taichung, Taiwan. EA.hy926 cell line was derived by fusing human umbilical vein endothelial cells (HUVECs) with A549 human lung carcinoma cells [28]. EA.hy926 cells were cultured in DMEM supplemented with 1.5 g/l NaHCO3, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a 5% CO2 humidified incubator. Human leukemia promyelocytic cells, HL-60, were cultured in RPMI-1640 supplemented with 2.2 g/l NaHCO3, 2,603 g/l HEPES, 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37 °C in a 5% CO2 humidified incubator.

#### 2.3. Western blot analysis

After each experiment, cells were washed twice with cold PBS and were harvested in 150 µl of lysis buffer (10 mM Tris–HCl, pH 8.0, 0.1% Triton X-100, 320 mM sucrose, 5 mM EDTA, 1 mM PMSF, 1 mg/l leupeptin, 1 mg/l aprotinin, and 2 mM dithiothreitol). Cell homogenates were centrifuged at 10,000 × g for 20 min at 4 °C. The resulting supernatant was used as a cellular protein. Nuclear protein was prepared as described previously [22]. The total protein was analyzed by use of the Coomassie Plus protein assay reagent kit (Thermo Scientific Pierce Protein Research Products, Rockford, IL, USA). For Western blotting, equal amounts of cellular and nuclear protein samples were electrophoresed in sodium dodecyl sulfate (SDS)–polyacrylamide gels, and proteins were then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). Nonspecific binding sites on the membranes were blocked with 5% nonfat milk in 15 mM Tris/150 mM NaCl buffer (pH 7.4) at room temperature for 2 h. Membranes were probed with anti-Nrf2, anti-GCLM, and anti-Clathrin HC (Santa Cruz Biotechnology, Santa Cruz, CA, USA); anti-ICAM-1, anti-phospho-ERK, anti-ERK, anti-phospho-Akt (T308), anti-Akt, anti-PARP, anti-p47phox, and anti-p67phox (Cell Signaling Technology, Boston, MA, USA); anti-GCLC (Abcam, Cambridge, UK); anti-HO-1 (Calbiochem); anti-p65 (BD Bioscience, San Jose, CA, USA); and anti-β-actin (Sigma) antibodies. The membranes were then probed with the secondary antibody labeled with horseradish peroxidase. The bands were visualized by using an enhanced chemiluminescence kit (T-Pro Biotechnology, Taipei, Taiwan) and were scanned by a luminescence image analyzer (Fuji Film LAS-4000, Tokyo, Japan). The bands were quantitated with Image-Gauge software.

#### 2.4. RNA isolation and real-time PCR

Total RNA was extracted with TRIzol reagent and frozen at −70 °C until RT-PCR analysis was performed. A total of 0.6 µg RNA was used for the synthesis of first-strand cDNA by using Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) in a 20-µl final volume containing 1 µM of each deoxyribonucleotide triphosphate (dNTP Mix), 0.5 µM oligo(dT), and 40 U of RNase inhibitor. Real-time PCR was carried out in a thermocycler in a 10-µl reaction volume containing 0.01 µg cDNA, 5 µl KAPA™ SYBR® FAST qPCR Kit (Kapa Biosystems, Inc., Woburn, MA, USA), and 0.02 µM upstream and downstream primers. The primers for quantitative analyses of HO-1, GCLM, and ICAM-1 mRNA were designed by using ProbeFinder version 2.46 for human DNA from Roche Applied Science (Basel, Switzerland), and β-actin was designed by using NCBI Primer–BLAST. Primer sequences are listed in Table 1. Real-time PCR mixture samples were detected by use of the Mini Opticon™ Real-Time PCR Detection System; the thermo-cycling conditions were initiated at 95 °C for 3 min, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s and
analyzed by Bio-Rad CFX Manager™ Software version 1.5 (Bio-Rad, Hercules, CA, USA).

2.5. Subcellular fractionation

Subcellular fractionation was performed by use of the ProteoExtract® Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany). Briefly, after each experiment, cells were washed twice with cold PBS, after which cold PBS was added and the cells were scraped on ice. Cell homogenates were centrifuged at 2000 × g for 5 min at 4 °C. The supernatant was discarded, 100 μl cold extraction buffer I with 0.5 μl protease inhibitor cocktail was added to the pellet, and incubated for 15 min at 4 °C with gentle agitation. The lysate was centrifuged at 1000 × g for 10 min at 4 °C. The supernatant was collected and used as the cytosolic fraction. The pellet was resuspended in 100 μl cold extraction buffer II with 0.5 μl protease inhibitor cocktail and incubated for 30 min at 4 °C with gentle agitation. The lysate was centrifuged at 6000 × g for 10 min at 4 °C. The supernatant was used as the membrane fraction. p47phox and p67phox proteins in the two fractions were analyzed by immunoblotting.

2.6. RNA interference with small hairpin RNA (shRNA)

Lentiviral infection was performed according to the method of a previous study [29]. Two different sequences targeting human p47phox, Nrf2, JUN, GCLM, and HO-1 mRNA were chosen and purchased from National RNAi Core facility platform, Taipei, Taiwan. RNAi clones were identified by their unique number assigned by the RNAi Consortium (TRCN) as presented in Table 2. The shLuc was used for vector control targeted to luciferase and was kindly provided by Dr. J.L. Ko, Chung Shan Medical University, Taichung, Taiwan. Briefly, EA.hy926 cells (0.4 × 10^6) were plated onto 6-cm plastic culture dishes in DMEM supplemented with 1.5 g/l sodium bicarbonate, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% FBS. After 24 h of attachment, the cells were infected with packaged lentiviruses for another 24 h. On the following day, the medium was removed, and the cells were selected by using 2 μg/ml puromycin for 2 days. The cells were then passaged to 10-cm plastic culture dishes and were ready for assay.

2.7. Reactive oxygen species measurement

Detection of intracellular oxidative states was performed by using the probe H2DCFDA (Invitrogen) as described previously [30]. Briefly, cells were grown to 60–70% confluence and were then serum-starved in DMEM supplemented with 0.5% (v/v) FBS for an additional 2 days. The cells were then stabilized in serum-free DMEM without phenol red for at least 30 min before being challenged with TNFx. Cells were then incubated for 10 min with the ROS-sensitive fluorophore H2DCFDA (10 μM). Cells were immediately observed under a laser-scanning confocal microscope (Leica TCS SP2). DCF fluorescence was excited at 488 nm with an argon laser, and the evoked emission was filtered with a 515 nm long pass filter.

2.8. Cellular GSH measurement

After androgapholide treatment, cells were washed twice with cold PBS and were harvested in 20 mM potassium phosphate buffer (pH 7.0). Cell homogenates were centrifuged at 10,000 × g for 20 min at 4 °C. The resulting supernatant was used as the cell lysate. The protein content of the cell lysate was measured by using the CoomassiePlus Protein Assay Reagent Kit. Briefly, 150 μl of 5% TCA was added to 150 μl of cell lysates and centrifuged at 5000 × g for 10 min at 4 °C. The cell lysate was added to 0.4 M Tris buffer (with 0.02 M EDTA) and 0.01 M DTNB. After vortexing, the mixture was incubated at room temperature for 5 min, and the cellular GSH level was determined at 412 nm (Model 680 microplate reader, Bio-Rad).

2.9. Monocyte adhesion assay

The role of NADPH oxidase in TNFx-induced monocyte adhesion was studied by using shp47phox. Cells transfected with shp47phox were cultured in 12-well plates, were allowed to grow to 90% confluence, and were then treated with 1 ng/ml TNFx for 6 h.

Table 1
Primer sequences used for real-time PCR.

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<th>Gene</th>
<th>Accession no.</th>
<th>Forward primer</th>
<th>Reverse primer</th>
<th>Amplicon size (bp)</th>
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<td>ICAM-1</td>
<td>NM_000201</td>
<td>CACTCTCACTCTGTGACTGG</td>
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<td>HO-1</td>
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</tr>
<tr>
<td>GCLM</td>
<td>NM_002061</td>
<td>GAAGAGATATTTTTCCTCATTGAT</td>
<td>CCATCATTGATGAACTGAATT</td>
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<tr>
<td>β-Actin</td>
<td>NM_000110</td>
<td>CACAGAGGGAAGGATAGC</td>
<td>GAACAAAAGCCTTACATGCTCA</td>
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Table 2
shRNA sequences used for RNA interference.

<table>
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<th>Gene symbol</th>
<th>Accession no.</th>
<th>TRC no.</th>
<th>Responding sequence</th>
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<td>TRCN0000256333</td>
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<tr>
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<td>TRCN0000256333</td>
<td>AGGGCACACTTACAGACTTAC</td>
</tr>
<tr>
<td>shGCLM(1)</td>
<td></td>
<td>NM_002061</td>
<td>TRCN0000048489</td>
<td>CCACTAATTGTCAGTCCATT</td>
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<tr>
<td>shGCLM(2)</td>
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<td>TRCN0000267674</td>
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<tr>
<td>shNrf2(1)</td>
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<td>shJUN(1)</td>
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<td>NM_002133.2</td>
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</table>
The role of GCLM and HO-1 in the inhibition of TNFα-induced monocyte adhesion by andrographolide was studied by using shGCLM and shHO-1. Cells transfected with shGCLM or shHO-1 were cultured in 12-well plates, were allowed to grow to 90% confluence, and were then treated with 7.5 μM andrographolide for 16 h before being challenged with 1 ng/ml TNFα for another 6 h. Afterward, the monocyte adhesion assay was performed as described previously [25]. A control study showed that fluorescence is a linear function of HL-60 cells in the range of 1250–500,000 cells/well. The results are reported based on the standard curve obtained and are indicative of the number of adherent HL-60 cells per well.

2.10. Statistical analysis

Data were analyzed by using analysis of variance (SAS Institute Inc., Cary, NC, USA). The significance of the difference between mean values was determined by one-way analysis of variance followed by Tukey’s test or by a two-tailed Student’s t-test. p values < 0.05 were taken to be statistically significant.

3. Results

3.1. Effect of andrographolide on TNFα-induced ROS generation

To determine whether andrographolide attenuates TNFα-induced ROS generation, we pretreated cells with 7.5 μM andrographolide for 16 h and then challenged cells with 1 ng/ml TNFα for an additional 20 min. As shown in Fig. 1, TNFα induced ROS generation at 20 min, and pretreatment with 7.5 μM andrographolide significantly inhibited this ROS generation. N-Acetylcysteine (NAC) was used as a positive control.

3.2. TNFα-induced NADPH oxidase activation and the essentiality of NADPH oxidase in TNFα-induced ICAM-1 expression and monocyte adhesion in EA.hy926 cells

NADPH oxidase is a major source of ROS. Activation of NADPH oxidase is initiated by the membrane translocation of p47 phox, p67 phox, and Rac-1 [31]. To delineate the effect of TNFα on NADPH oxidase activation, we treated cells with 1 ng/ml TNFα for the indicated time periods. As shown in Fig. 2A, TNFα induced a peak increase in p47 phox and p67 phox membrane translocation at 15 min. We then used shp47 phox to knock down p47 phox expression to identify the role of NADPH oxidase activation in the TNFα-induced ICAM-1 expression. As shown in Fig. 2B–D, shp47 phox abolished TNFα-induced p65 nuclear translocation, ICAM-1 gene expression, and adhesion of HL-60 cells.

3.3. Andrographolide inhibits TNFα-induced Src phosphorylation, NADPH oxidase activation, and ICAM-1 expression in EA.hy926 cells

A previous study showed that Src plays an important role in mediating TNFα-induced ICAM-1 phosphorylation, which is essential for polymononuclear leukocyte adhesion [32]. To identify the effect of TNFα on Src activation, we treated cells with 1 ng/ml TNFα for the indicated time periods. As shown in Fig. 3A, TNFα induced Src phosphorylation at 5 min. We next determined whether andrographolide inhibits TNFα-induced NADPH oxidase activation. As shown in Fig. 3B, andrographolide attenuated TNFα-induced Src phosphorylation. It has been reported that ROS is

![Fig. 1. Effect of andrographolide on TNFα-induced ROS generation in EA.hy926 cells. Cells were visualized via fluorescence microscopy (scale bar = 20 μm) stained with H2DCFDA. Cells were pretreated with 7.5 μM andrographolide for 16 h or 1 mM NAC for 1 h and incubated with 10 μM H2DCFDA for 10 min before being challenged with 1 ng/ml TNFα for another 20 min. Cells treated with 0.1% DMSO for 16 h were regarded as a vehicle control (CON) (A). Quantification of the ROS levels as detected by H2DCFDA fluorescence intensities (B). Values are means ± SD of three independent experiments. Values not sharing the same letter are significantly different (p < 0.05).](image-url)
necessary for Src phosphorylation [33]. TNFα induced ROS generation, and pretreatment with 7.5 μM andrographolide significantly inhibited this generation (Fig. 1). Suppression of TNFα-induced Src phosphorylation by andrographolide is likely to be attributed to its attenuation of ROS generation. Furthermore, the membrane translocation of p47phox and p67phox was mitigated by andrographolide and the NADPH oxidase inhibitors apocynin (APO) and diphenyleneiodonium (DPI) (Fig. 3C). Moreover, we
used APO and DPI to corroborate the role of NADPH oxidase activation in TNFα-induced ICAM-1 expression. As shown in Fig. 3D and E, not only APO and DPI but also andrographolide and NAC significantly reduced TNFα-induced ICAM-1 expression. Thus, activation of Src and NADPH oxidase by TNFα is involved in the increased inflammation as evidenced by the enhanced ICAM-1 expression and HL-60 cell adhesion.

3.4. Andrographolide increases cellular GSH content and GCLM gene expression

GSH is an endogenously synthesized tripeptide thiol that plays an important role in numerous cellular processes. Andrographolide was previously found to enhance GSH content and up-regulate GCLC and GCLM expression in rat cardiomyocytes to protect against hypoxia/reoxygenation-induced injury [26]. Because we showed that andrographolide has antioxidant activity, we determined the effect of andrographolide on the cellular GSH level. We treated cells with 7.5 μM andrographolide for various time periods. As shown in Fig. 4A, andrographolide increased cellular GSH content after treatment for 24 h. As shown in Figs. 4B and C, only GCLM but not GCLC was up-regulated by andrographolide in a time-dependent manner. We therefore transfected cells with shGCLM to identify the role of GCLM in the andrographolide-induced GSH synthesis. As shown in Fig. 4D, the andrographolide-induced increase in the cellular GSH content was abolished by shGCLM.

We next examined the role of GCLM in the inhibition of HL-60 adhesion by andrographolide in the presence of TNFα by using an shGCLM knockdown model. We transfected cells with shGCLM, followed by treatment with 7.5 μM andrographolide for 16 h, and then challenged the cells with TNFα for an additional 6 h. As shown in Fig. 4E, shGCLM alleviated the andrographolide inhibition of HL-60 adhesion. Thus, these results show that andrographolide enhances cellular GSH content through up-regulation of GCLM expression and imply the importance of GCLM for the inhibition of TNFα-induced HL-60 adhesion by andrographolide.

3.5. Andrographolide induces HO-1 gene expression and inhibits TNFα-induced HL-60 adhesion

HO-1 is well-known for its cytoprotective effect against oxidative stress. Our previous study showed that andrographolide up-regulates HO-1 expression in the inflammatory condition [22]. In the present study, we determined the effect of andrographolide on HO-1 gene expression. We treated cells with 7.5 μM andrographolide for various time periods. As shown in Figs. 5A and B, treatment with andrographolide induced HO-1 mRNA and protein expression in a time-dependent manner. The role of HO-1 in the inhibition of HL-60 adhesion by
andrographolide in the presence of TNFα was examined by using an shHO-1 knockdown model. We transfected cells with shHO-1, followed by treatment with 7.5 μM andrographolide for 16 h and then challenged the cells with TNFα for an additional 6 h. As shown in Fig. 5C, shHO-1 alleviated the andrographolide inhibition of HL-60 adhesion. These results show that HO-1 plays an important role in the inhibition of TNFα-induced HL-60 adhesion by andrographolide.

3.6. The PI3K/Akt but not ERK pathway is involved in andrographolide-induced HO-1 and GCLM expression

Phytochemicals have been reported to activate signaling pathways, including mitogen-activated protein kinases (JNK, ERK and p38) and the PI3K/Akt pathways. Induction of HO-1 or GCL expression by phytochemicals was found to result from activation of the above signaling pathways, dissociation of Nrf2 from Keap1, an increase in Nrf2 nuclear translocation, or phosphorylation of c-jun, which result in the transcriptional activation of antioxidant/electrophile response element (ARE) or AP-1 sites in these responsive genes [20,34]. Therefore, to demonstrate the upstream signaling pathways involved in the andrographolide-mediated induction of HO-1 and GCLM, we studied the phosphorylation of Akt, ERK1/2, JNK, and p38. Cells were treated with 7.5 μM andrographolide for the indicated time periods. As shown in Fig. 6A, both ERK and Akt were activated by andrographolide; however, neither JNK nor p38 was activated by andrographolide (data not shown). ROS was found to participate in IL-17-dependent Akt phosphorylation in T-ALL cells in a previous study [35]. Recently, andrographolide was shown to induce early-stage ROS generation in rat VSMCs [36]. As shown in Fig. 6B, an increase in cellular ROS began at 5 min after treatment with 7.5 μM andrographolide, and a peak increase was observed at 10 min as measured by the DCF probe. This evidence supports the notion that induction of early-stage ROS generation is associated with the phosphorylation of Akt by andrographolide. To further corroborate the role of the PI3K/Akt and ERK pathways, their specific inhibitors, LY294002 and PD98059, were used. As shown in Fig. 6C–F, LY294002 attenuated andrographolide-induced HO-1 and GCLM gene expression. As shown in Fig. 6G, the andrographolide-induced increase in cellular GSH content was inhibited by LY294002. These results suggest that andrographolide induces HO-1 and GCLM gene expression and enhances GSH synthesis is through the PI3K/Akt pathway.

3.7. Nrf2 and c-jun are activated by andrographolide and are involved in the induction of HO-1 and GCLM expression by andrographolide

To elucidate the downstream target of PI3K/Akt for the induction of HO-1 and GCLM expression by andrographolide, we analyzed Nrf2 nuclear translocation and the phosphorylation of c-jun. Cells were treated with 7.5 μM andrographolide for the indicated time periods. As shown in Fig. 7A, both Nrf2 nuclear translocation and c-jun phosphorylation were increased as early as 1 h and sustained until 4 h after treatment with andrographolide. In addition, both Nrf2 nuclear translocation and c-jun phosphorylation were inhibited in cells pretreated with the PI3K inhibitor LY294002 for 1 h before being challenged with 7.5 μM andrographolide. These results show that the PI3K/Akt pathway is involved in andrographolide-induced Nrf2 nuclear translocation and c-jun phosphorylation. To further demonstrate the role of Nrf2 and c-jun in andrographolide-induced HO-1 and GCLM expression, we transfected cells with shNrf2 and shJUN. As shown in Fig. 7B–G, knockdown of either Nrf2 or c-jun abolished andrographolide-induced HO-1 and GCLM expression. These results suggest that both Nrf2 and c-jun are transcriptional factors for the andrographolide-induced HO-1 and GCLM expression.

4. Discussion

Enhancement of cellular antioxidant capacity is believed to reduce the risk of oxidative stress-mediated diseases.
Andrographolide is one of the major bioactive components and the most abundant diterpenoid in the leaves of *A. paniculata* [37]. Our laboratory has shown that andrographolide has anti-inflammatory [22] and chemopreventive [27] activities and that these activities are associated with HO-1 induction. In this study, we demonstrated that andrographolide suppressed TNFα-induced ROS generation, ICAM-1 expression, and HL-60 cell adhesion and that this inhibition was likely associated with the inhibition of NADPH oxidase activation, up-regulation of HO-1 and GCLM expression, and elevation of GSH content through the PI3K/Akt/Nrf2 and PI3K/Akt/AP-1 pathways.

Oxidative stress plays a critical role in the pathogenesis of many chronic diseases, including atherosclerosis, and is therefore an attractive therapeutic target for cardiovascular diseases (CVD). NADPH oxidase is the most important enzyme responsible for ROS generation in human vessels. Previous studies suggested a role for this enzyme in the initiation and progression of atherosclerotic disease. Deletion of NADPH oxidase 1 has a profound antiatherosclerotic effect associated with reduced ROS formation, chemokine expression, adhesion of leukocytes to vessels, macrophage infiltration, and expression of proinflammatory and profibrotic markers [38]. Inhibition of NADPH oxidase activity by its specific inhibitors VAS2870 and APO, reverses endothelial dysfunction [39]. Binding of APO to the SH3A and SH3B domains of p47phox prevents the membrane translocation of p47phox and interaction with p22phox, which results in the inhibition of NADPH oxidase system [40]. DPI, a flavoprotein inhibitor, is often used to inhibit NADPH oxidase system because of its suppression of p47phox membrane translocation [41]. In the present study, the activation of NADPH oxidase by TNFα was abrogated by shp47phox, which blocked TNFα-induced NFκB nuclear translocation, ICAM-1 expression, and HL-60 cell adhesion (Fig. 2).

TNFα is a potent proinflammatory cytokine and the plasma concentration of TNFα is elevated in several pathologies, including rheumatoid arthritis, cancer, atherosclerosis, and preeclampsia [42]. Many studies have shown that TNFα-induced expression of proinflammatory cytokines and adhesion molecules such as IL-6 and ICAM-1 is dependent on NFκB [30,43]. It has been shown that Src participates in the activation of TNFα-induced p47phox in human airway smooth muscle cells [12]. In the present study, we demonstrated that TNFα induced Src phosphorylation, activated NADPH oxidase, and increased ROS production. These effects of TNFα mentioned above were abrogated by andrographolide.

HO-1 is an inducible enzyme and accumulating evidence indicates that induction of HO-1 expression protects against various chronic diseases, including heart disease, diabetes, neurological disorders, and hypertension [44]. Several studies have addressed the role of HO-1 in atherosclerosis by using systemic modulation of either HO-1 expression or HO-1 activity, with subsequent alteration of oxidant and inflammatory parameters in the circulation. HO-1-null mice exhibit significant elevation of plasma lipid hydroperoxides [45]. Likewise, a greater macrophage infiltration in lesions and lipid deposits in rabbit abdominal aortic plaques was shown by treatment with SnPP, an HO-1 inhibitor [46]. In contrast, induction of HO-1 expression ameliorated the formation of foam cells by treatment with oxLDL [47]. Thus, evidence from both in vivo and in vitro studies supports the anti-inflammatory and anti-atherosclerotic activity of HO-1.

Induction of HO-1 and its metabolites inhibits the expression of NADPH oxidase subunits and NADPH oxidase activity. Induction of
HO-1 or treatment with bilirubin was shown to inhibit NADPH oxidase activity in both in vivo and in vitro studies [48]. Furthermore, biliverdin and bilirubin significantly inhibit angiotensin II-stimulated ROS production, NADPH oxidase activity, and NADPH oxidase 4 mRNA expression in human mesangium cells [49]. Moreover, the expression of NADPH oxidase 4, p22phox, and p47phox mRNA is down-regulated in streptozotocin-induced diabetic hyperbilirubinemic rats and db/db mice. These results implicate an inverse correlation between HO-1 induction and NADPH oxidase activation.

Glutamate cysteine ligase is a holoenzyme that is composed of GCLC and GCLM [14]. GCLC and GCLM are expressed from different genes [50]. In the present study, andrographolide up-regulates the expression of GCLM but not GCLC (Fig. 4). However, chrysirin, apigenin, and luteolin were shown to up-regulate both GCLC and GCLM in rat primary hepatocytes [20]. Chen and Kong [51] showed that GCLM is limiting in most tissues and revealed the essential role for GCLM in controlling the GSH level by using Gclm−/− mice. In addition, it has been shown that mice deficient in the Gclm gene have a markedly reduced GSH level in aorta [15]. GSH depletion precedes lipid peroxidation and atherogenesis in ApoE−/− mice [52]. In the present study, we showed that andrographolide up-regulated GCLM expression and that knockdown of GCLM reversed the andrographolide inhibition of HL-60 cell adhesion (Fig. 4E). The evidence suggests that GSH plays an important role in the prevention of atherosclerosis.

Inhibition of the abnormal induction of adhesion molecules is believed to be one of the mechanisms attributable to the protective effect of phytochemicals against CVD [22,53]. The essentiality of ICAM-1 in mediating the adhesion of monocytes to oxLDL-stimulated mouse aortic endothelium was demonstrated in a previous study [54]. In the current study, inhibition of ICAM-1 expression by andrographolide attenuated adhesion of HL-60 cells to TNFα-stimulated EA.hy926 cells. However, this inhibition was abolished by shGCLM (Fig. 4E) and shHO-1 (Fig. 5C). These results suggest the involvement of GCLM and HO-1 in the inhibition of HL-60 cell adhesion by andrographolide. In another study, silencing HO-1 attenuated the inhibition of TNFα-induced ICAM-1 expression by andrographolide [22]. Buthionine sulfoximine, a GSH synthesis inhibitor, reversed the inhibitory effect of cinnamaldehyde on TNFα-induced ICAM-1 expression [55]. These results indicated that HO-1 expression and GSH content might be associated with the suppression of TNFα-induced ICAM-1 expression and subsequent monocyte adhesion by phytochemicals.

Nrf2 and AP-1 are important transcriptional factors involved in the anti-inflammatory action of phytochemicals and are associated with the induction of HO-1, NAD(P)H:quinone oxidoreductase 1, and glutathione S-transferase [51,56]. Nrf2 interacts with its cytosolic repressor Keap1 in basal conditions. Upon stress, Nrf2 is liberated from Keap1 and subsequently translocates to the nucleus and activates the transcription of target genes through the ARE [57]. AP-1 is a hetero- or homodimer of the c-jun and c-fos families. AP-1 binds to the consensus DNA sequence, the phorbol 12-O-tetradecanoyl-13-acetate-responsive element (TRE) or the AP-1 binding site [58]. Previous studies showed that both HO-1 and GCLM promoters have ARE and AP-1 binding sites [59,60]. In the present study, andrographolide increased Nrf2 nuclear translocation and c-jun phosphorylation, and silencing Nrf2 and JUN
abrogated the andrographolide induction of HO-1 and GCLM (Fig. 7B–G). These findings suggest that both transcriptional factors are necessary for andrographolide-induced HO-1 and GCLM expression. Activation of Nrf2 and AP-1 is regulated by various kinases, such as JNK, ERK, p38, and PI3K/Akt [61]. In the present study, we showed that andrographolide activates the phosphorylation of ERK and Akt; however, by using specific inhibitors of ERK and Akt, we demonstrated that only the PI3K/Akt pathway is involved in the induction of HO-1 and GCLM expression by andrographolide (Fig. 6). The role of andrographolide in Akt phosphorylation depends on the experimental condition. Treatment of andrographolide alone induces Akt phosphorylation in cells as shown in Fig. 6A. However, andrographolide pretreatment suppresses TNFα-induced PI3K/Akt pathway [25]. This is likely that Akt phosphorylation is involved in andrographolide induction of gene expression which is important to defend against TNFα-induced deleterious effect through the PI3K/Akt pathway. Andrographolide-induced phosphorylation of c-jun is abolished by LY294002 (Fig. 7A). Although c-jun is a substrate of JNK, it is also phosphorylated by PI3K/Akt as suggested by Ding et al. [62]. This supports our finding that andrographolide induces c-jun phosphorylation through the PI3K/Akt pathway and subsequent induction of HO-1 and GCLM expression. 

Our findings in the present study are presented schematically in Fig. 8. In conclusion, we have shown that andrographolide inhibits TNFα-induced ROS generation, ICAM-1 expression, and HL-60 cell adhesion via inhibition of Src-driven NADPH complex formation, up-regulation of HO-1 and GCLM gene expression, and an increase of GSH content through the PI3K/Akt/Nrf2 and PI3K/Akt/AP-1 pathways. The antioxidant/anti-inflammatory property of andrographolide is believed to protect against oxidative stress-mediated diseases such as CVD.

Conflict of interest

The authors declare that they have no conflict of interest.

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