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Evodiamine and Rutaecarpine Inhibit Migration by LIGHT Via Suppression of NADPH Oxidase Activation

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ABSTRACT

LIGHT acted as a new player in the atherogenesis. The dried, unripe fruit of Evodia Fructus (EF) has long been used as a traditional Chinese herbal medicine, and is currently widely used for the treatment of headache, abdominal pain, vomiting, colds and reduced blood circulation. Evodiamine and rutaecarpine are active components of EF. In this study, we investigated the inhibitory effect of evodiamine and rutaecarpine on LIGHT-induced migration in human monocytes. Evodiamine and rutaecarpine decreased the LIGHT-induced production of ROS, IL-8, monocyte chemoattractant protein-1 (MCP-1), TNF-α, and IL-6, as well as the expression of chemokine receptor (CCR) 1, CCR2 and ICAM-1 and the phosphorylation of the ERK 1/2 and p38 MAPK. Furthermore, NADPH oxidase assembly inhibitor, AEBSF, blocked LIGHT-induced migration and activation of CCR1, CCR2, ICAM-1, and MAPK such as ERK and p38 in a manner similar to evodiamine and rutaecarpine. These findings indicate that the inhibitory effects of evodiamine and rutaecarpine on LIGHT-induced migration and the activation of CCR1, CCR2, ICAM-1, ERK, and p38 MAPK occurs via decreased ROS production and NADPH oxidase activation. Taken together, these results indicate that evodiamine and rutaecarpine have the potential for use as an anti-atherosclerosis agent. J. Cell. Biochem. 107: 123–133, 2009.

KEY WORDS: LIGHT; EVODIAMINE; RUTAECARPINE; MIGRATION; ANTI-ATHEROSCLEROSIS

LIGHT [Homologous to lymphotoxins, shows inducible expression, and competes with herpes simplex virus glycoprotein D (gD) for herpes virus entry mediator (HVEM), a receptor expressed by T lymphocytes/tumor necrosis factor (TNF)-related 2 (TR2)] belongs to a member of the TNF ligand superfamily, and acts as a new player in the atherogenesis [Bobik and Kalinina, 2001]. It binds to three distinct TNF receptors: HVEM, lymphotoxin β receptor, and soluble decoy receptor 3 [Crowe et al., 1994; Murphy et al., 1998; Watts, 2005]. HVEM, which is a member of the tumor necrosis factor receptor (TNFR) superfamily, has three additional ligands: HSV surface envelope gD, lymphotoxin α (LTα), and B and T lymphocytes attenuator (BTLA) [Mauri et al., 1998; Sedy et al., 2005; Murphy et al., 2006].

It is well known that LIGHT/HVEM signaling mediates a number of T cell responses, including the induction of T cell survival, proliferation and inflammation, the expression of activation markers, the production of cytokines [Shaikh et al., 2001; Watts, 2005]. Specifically, HVEM is highly expressed in monocytes, and LIGHT/HVEM increases the production of reactive oxygen species (ROS) and nitric oxide in monocytes and neutrophils [Heo et al., 2006]. Also, LIGHT/HVEM induces intracellular calcium influx, and regulates ROS production in monocytes [Heo et al., 2007]. Moreover,

Abbreviations used: rhLIGHT, recombinant human LIGHT; HVEM, herpes virus entry mediator; PBS, phosphate-buffered saline; ROS, reactive oxygen species; BSA, bovine serum albumin; LTα, lymphotoxin α; DCF-DA, 2′,7′-dichlorofluorescin diacetate; AEBSF, 4-(2-aminoethyl) benzenesulfonyl fluoride; ICAM-1,intracellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1; CCR, chemokine receptor; MAPK, mitogen activated protein kinase.

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LIGHT/HVEM induces the migration of macrophages/monocytes and proliferation of vascular smooth muscles cells (VSMC) via mitogen activated protein kinases (MAPK) activation, and induces proinflammatory cytokines and matrix metalloproteinases [Lee et al., 2001; Wei et al., 2006]. Each is involved in atherogenesis [Bobik and Kalinina, 2001; Lee et al., 2001; Wei et al., 2006].

The dried, unripe fruit of Evodia Fructus (EF, Rutaceae, Osooyoo in Korean; Wuzhuyu in Chinese; Goshuyu in Japanese) has long been used as a traditional Chinese herbal medicine. Currently, EF is widely used for the treatment of headache, abdominal pain, vomiting, colds and reduced blood circulation [Fei et al., 2003; Takada et al., 2005]. In addition, EF can act as a stimulant for the secretion of digestive enzymes that promote appetite, and it can also induce a diuretic and analgesic effect [Iwata et al., 2005]. Evodiamine and rutaecarpine are the active components of EF. Recently, it has been reported that evodiamine and rutaecarpine exert an anti-proliferate effect in tumor cells via induction of apoptosis, including human cervical cancer [Fei et al., 2003] human lung carcinoma [Takada et al., 2005], human leukemic T-lymphocytes [Huang et al., 2004], leukemic monocytes [Lee et al., 2006] and prostate cancer cells [Kan et al., 2007]. In addition, anti-allergic effects [Shin et al., 2007], anti-inflammation in skin [Yarosh et al., 2006], and in neutrophils and microglial cells [Ko et al., 2007] in response to treatment with EF have been described. However, little is known about the effects of evodiamine and rutaecarpine on aspects of inflammation including migration and the expression of chemokine receptors such as CCR1 and CCR2 in monocytes. We suggest here that evodiamine and rutaecarpine inhibit LIGHT-induced migration via down-regulation of CCR1, CCR2, and ICAM-1 expression, IL-8, MCP-1, TNF-α, and IL-6 production and the phosphorylation of extracellular-signal regulated kinase (ERK) 1/2 and p38 MAPK in THP-1 cells. Furthermore, each of these signal networks occurred in response to the suppression of ROS production and NADPH oxidase activation.

MATERIALS AND METHODS

REAGENTS

All reagents were obtained from Sigma–Aldrich (St Louis, MO) unless otherwise indicated. Recombinant human LIGHT (rhLIGHT) was purchased from R&D systems (Minneapolis, MN) and then diluted in 0.1% BSA-PBS buffer. The CellTiter 96® Aqueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega (Madison, WI). RPMI 1640 and FBS were purchased from GibcoBRL (Grand Island, NY). ELISA kits for IL-8, IL-6, MCP-1 and TNF-α were obtained from Pierce Biotechnology (Rockford, IL). Anti-phospho-ERK 1/2, ERK 1/2, phospho-p38 and p38 mAb were purchased from Cell Signaling Technology (Beverly, MA). Anti-human ICAM-1-phycoerythrin (PE) and mouse IgG1-PE mAb were purchased from BD PharMingen (San Jose, CA). Anti-human CCR1-PE and anti-human CCR2-PE antibodies were obtained from R&D Systems (Minneapolis, MN). Anti-CCR1-HRP, Anti-CCR2-HRP, Anti-ICAM-1-HRP, anti-$p40^{phox}$, anti-phosphorylated $p40^{phox}$ (p-$p40^{phox}$), anti-mouse-HRP and anti-rabbit IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The rhLIGHT (HI-rhLIGHT) was heat inactivated by incubation at 80°C for 20 min.

CELL LINES AND CELL CULTURE

The monocyte-like cell line, THP-1 and promyelocytic leukemia, HL60 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). Both of cells were maintained in RPMI 1640 supplemented with 10% FBS, 100 μg/ml streptomycin, 100 U/ml penicillin, and 0.1% of β-mercaptoethanol at 37°C (CO_2 incubator, 5% CO_2 in humidified air).

FLOW CYTOMETRIC ANALYSIS

Cells were incubated with 20% AB-serum in PBS at 4°C for 30 min. The cells were then washed twice with FACs buffer (PBS containing 0.3% BSA and 0.1% NaN_3), after which they were incubated with appropriate fluorochrome-labeled mAbs including anti-human CCR1-PE, anti-human CCR2-PE, and anti-human ICAM-1-PE, or with isotype control mAbs at 4°C for 30 min. The samples were then analyzed by flow cytometry using a Becton Dickinson FACScan flow cytometer in conjunction with the CELL-Quest PRO software (BD Biosciences, San Jose, CA). The mean fluorescence intensity (MFI) of the fluorescence histogram was then recorded for each sample.

ROS GENERATION

Reactive oxygen species (ROS) generated by THP-1 cells were assayed using the ROS-sensitive fluorescent dye, 2′,7′-dichloro-fluorescin diacetate (DCF-DA) following a method described in our previous report [Heo et al., 2006].

ELISAS FOR CYTOKINES

Cells were pretreated with various concentrations of evodiamine and rutaecarpine for 30 min, after which they were stimulated with 100 ng/ml of rhLIGHT. Next, the cell-free supernatants were collected, after which the cytokines were assayed using ELISA kits for IL-8 and TNF-α [Heo et al., 2006, 2007].

IMMUNOBLOT ANALYSIS

THP-1 cells (1×10^7 cells/ml) were pretreated with 1 and 10 μM evodiamine and rutaecarpine for 30 min, after which they were stimulated with 100 ng/ml of rhLIGHT and then immediately lysed using lysis buffer supplemented with protease inhibitors. Immunoblot assays were then conducted as described in our previous study [Heo et al., 2008]. To prevent ROS production, cells were preincubated with the NADPH oxidase assembly inhibitor, AEBSF, for 30 min at 37°C prior to the addition of rhLIGHT [Heo et al., 2006]. In some experiments, THP-1 cells and HL60 were pretreated with 10 μM evodiamine and rutaecarpine for 10 min, after which they were stimulated with rhLIGHT.

MIGRATION

A matrigel migration assay was performed using a previously described method [Heo et al., 2008]. After the migration assay, the cells that invaded through the Matrigel and were located on the underside of the filter were subjected to hematoxylin and eosin staining (H&E staining) and then counted. Three to five invasion chambers were used per condition and the values presented were calculated by averaging the total number of cells obtained from three filters. For some experiments, cells were pretreated with the

indicated concentrations of AEBSF for 30 min at 37°C prior to stimulation with rhLIGHT.

TRANSFECTION OF p47^{phox} SIRNA

siRNA for p47^{phox} (cat No. E-180696-00-0010) and cyclophilin B control siRNA (cat No. D-001920-01-0005) were purchased from Dharmacon. THP-1 cells were transfected with siRNA using the Accell SYSTEM (Dharmacon), cultured for 48 h, and then used to measure migration. To investigate for efficiency of transfection, we used Accell Green Non-targeting siRNA (cat No. D-001950-01-05). Efficiency of transfection was over the 95% in the THP-1 cells.

STATISTICS

Data shown are the means ± SEM of at least three independent experiments. All values were evaluated by one-way ANOVA followed by Duncan's multiple range tests using the GraphPad Prism 4.0 software. Differences were considered significant at $P < 0.05$.

RESULTS

CYTOTOXICITY OF EVODIAMINE AND RUTAECARPINE

To exclude the possibility that reductions in the levels of migration, chemokine receptors, adhesion molecules, ROS and inflammatory cytokines/chemokines from the cells occurred due to direct toxicity of evodiamine and rutaecarpine on the cells, we evaluated cell toxicity using various concentrations of these compounds (0, 1, 10, 30, and 50 μM). We found that treatment with evodiamine and rutaecarpine at concentrations of 1 and 10 μM did not affect the viability of HASMC (Fig. 1). Therefore, we used these concentrations for subsequent experiments.

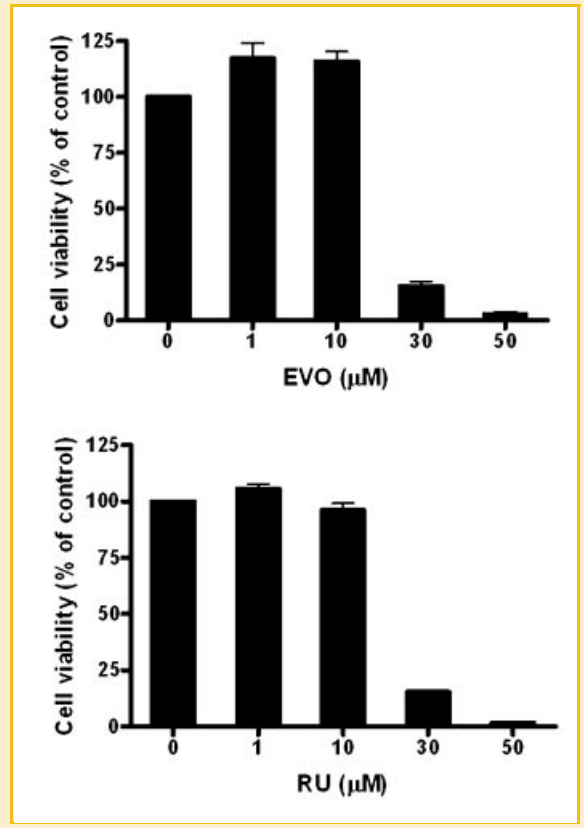

Fig. 1. The effect of evodiamine and rutaecarpine on cytotoxicity in THP-1 cells. Cells were stimulated with 0, 1, 10, 30, 50 μM EVO or RU for 24 h. Cytotoxicity was then determined by an MTS assay as described in the Materials and Methods Section. Data shown are representative of at least three independent experiments. Data represent the means ± SEM. EVO, evodiamine; RU, rutaecarpine.

EVODIAMINE AND RUTAECARPINE INHIBITS ROS PRODUCTION BY LIGHT VIA SUPPRESSION OF p40^{phox} PHOSPHORYLATION

LIGHT/HVEM increases the production of reactive oxygen species (ROS) and nitric oxide in monocytes and neutrophils [Heo et al., 2006]. To confirm that evodiamine and rutaecarpine exerted an effect on LIGHT-induced ROS production, we pre-treated THP-1 cells with various concentrations (0, 1, and 10 μM) of evodiamine and rutaecarpine for 1 h, and then stimulated them with 100 ng/ml of LIGHT. As shown in Figure 2A, ROS production increased after exposure to LIGHT, reaching levels that were 1.7-fold greater than the control values in THP-1 cells. However, treatment with either evodiamine or rutaecarpine induced a significant and dose-dependent decrease in the production of ROS in THP-1 cells (43% and 99% of LIGHT alone in response to treatment with 1 and 10 μM evodiamine, respectively; 45% and 99% of LIGHT alone in response to treatment with 1 and 10 μM rutaecarpine, respectively; Fig. 2A).

We investigated evodiamine and rutaecarpine to determine if either directly blocked NADPH oxidase activation by LIGHT. To further confirm the inhibitory effect of evodiamine and rutaecarpine against NADPH oxidase activation by LIGHT, we examined the phosphorylation state of the NADPH subunit, p40^{phox}. As shown in Figure 2B, treatment with LIGHT alone for 10 min led to a significant increase in the phosphorylation of p40^{phox} in THP-1 and HL60 cells. However, evodiamine and rutaecarpine completely inhibited the phosphorylation of p40^{phox}, while decreasing ROS production. This finding indicates that evodiamine and rutaecarpine inhibit potential NADPH oxidase activation via the down-regulation of p40^{phox} phosphorylation in THP-1 and HL60 cells that have been stimulated with LIGHT. Therefore, evodiamine and rutaecarpine inhibit ROS production by LIGHT via suppression of p40^{phox} phosphorylation.

INHIBITION OF LIGHT-INDUCED MIGRATION BY EVODIAMINE AND RUTAECARPINE OCCURS VIA THE SUPPRESSION OF NADPH OXIDASE ACTIVATION AND ROS PRODUCTION

We previously reported that the LIGHT/HVEM interaction increased ROS via induction of NADPH oxidase p47^{phox} phosphorylation (NADPH oxidase activation), and that this interaction is required for the migration of human monocytes to occur [Heo et al., 2008]. To examine the role that ROS plays in LIGHT-induced migration, we used the Accell system to transfect THP-1 cells with control siRNA and p47^{phox} siRNA prior to LIGHT stimulation. As shown in Figure 3A, the results demonstrated that LIGHT induced migration was reduced in response to inhibition by p47^{phox} siRNA, which suggests that NADPH oxidase plays a significant role in LIGHT-induced migration. The maximum inhibition of LIGHT-induced

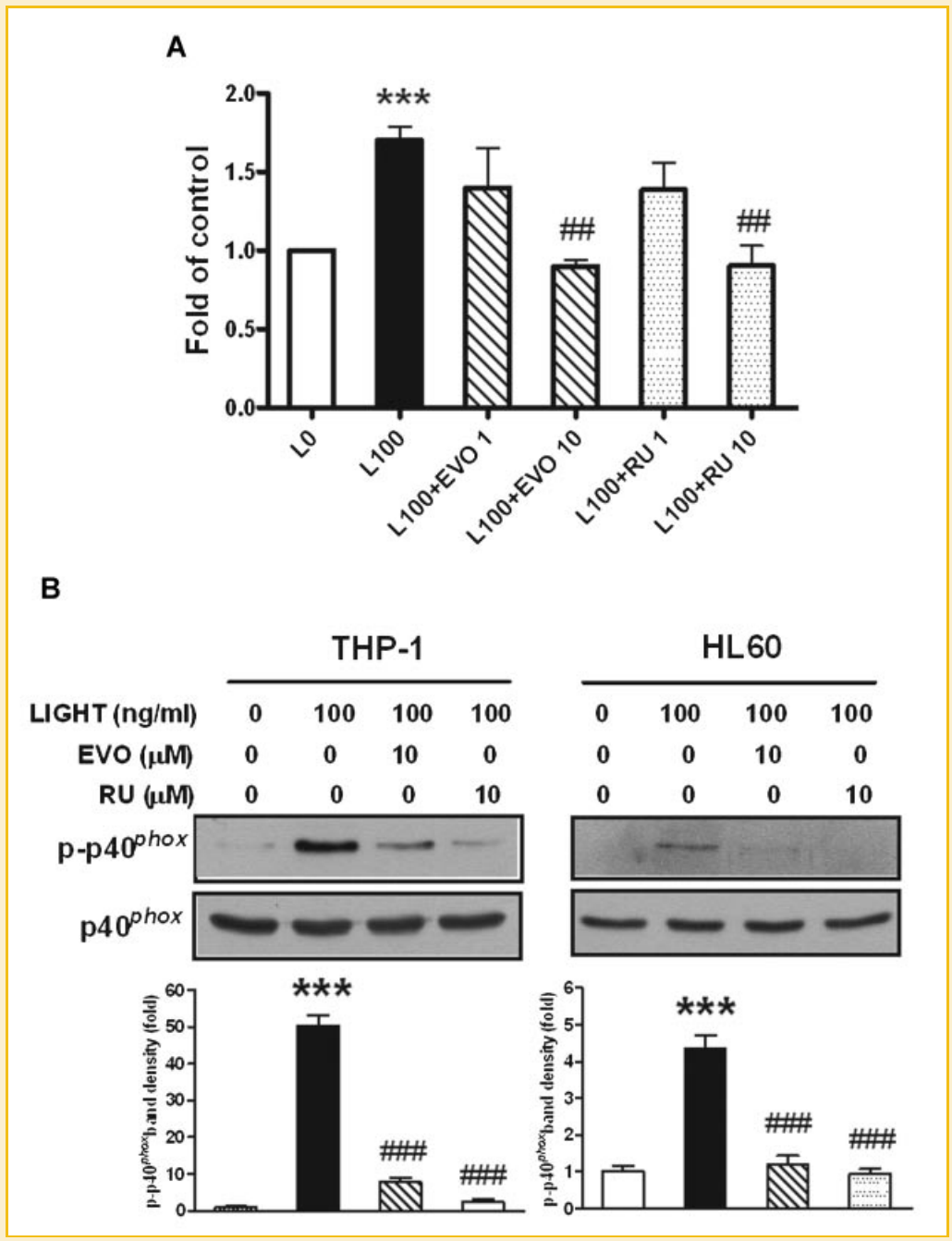

Fig. 2. Inhibitory effect of EVO and RU on LIGHT-induced ROS production. A: THP-1 cells were pre-incubated with 10 μM EVO or RU for 1 h prior to treatment with 100 ng/ml LIGHT for 45 min. To measure the ROS production, cells were stained with the ROS-sensitive fluorescent dye, DCF-DA, for 30 min. The cells were then harvested and washed, after which the percentages of ROS-positive cells were assessed by flow cytometry. B: THP-1 and HL60 cells were pre-incubated with 10 μM EVO or RU for 1 h prior to treatment with LIGHT at a concentration of 100 ng/ml for 10 min. The cell lysate was then isolated and investigated to determine if evodiamine and rutaecarpine inhibited the phosphorylation of $p40^{phox}$. The levels of phosphorylated $p40^{phox}$ ($p\text{-}p40^{phox}$) in the cell lysate were then measured by Western blot using anti-$p\text{-}p40^{phox}$ mAb to evaluate whole-cell lysate that was obtained as described in the Materials and Methods Section. Finally, the membrane was then stripped and reprobed with anti-β-actin mAb to confirm equal loading. The data shown are representative of at least three independent experiments. These data represent the means ± SEM. Significantly different from control (*) or cells treated with LIGHT alone (100 ng/ml) (#); $^{***}P < 0.001$, $^{\#\#}P < 0.01$. L, rhLIGHT; EVO, evodiamine; RU, rutaecarpine.

migration exerted by $p47^{phox}$ siRNA was 93% in LIGHT-treated THP-1 cells.

To further evaluate the effects of ROS on LIGHT-induced migration, we also pretreated THP-1 cells with the NADPH oxidase assembly inhibitor, AEBSF (0.1, 0.5 mM), prior to LIGHT stimulation. As shown in Figure 3B, LIGHT induced migration was reduced by AEBSF (up to the basal level). These findings suggest that NADPH oxidase plays a significant role in LIGHT-induced migration.

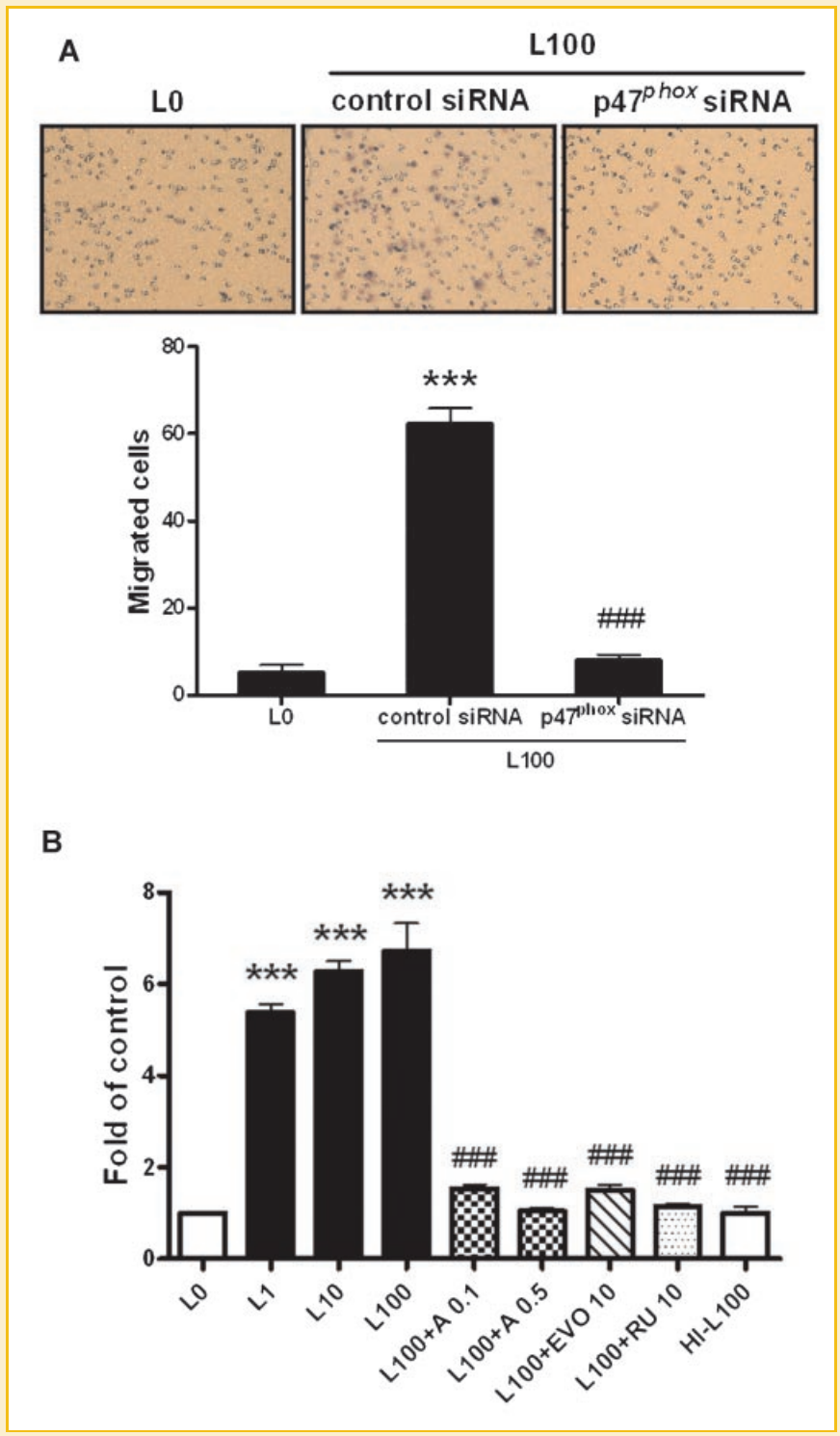

Fig. 3. Inhibitory effect of EVO and RU on LIGHT-induced migration. A: NADPH oxidase ($p47^{phox}$) was required for migration in response to LIGHT in the human monocyte, THP-1. Cells were transfected with control siRNA and $p47^{phox}$ siRNA, after which a migration assay was conducted using a Matrigel filter. The underside of the filter was then stained with hematoxylin and eosin, and the cells were counted as described in the Materials and Methods Section. B: AEBSF (ROS inhibitor), EVO and RU inhibit LIGHT-induced migration of THP-1 cells. Cells were preincubated with 0.1 or 0.5 mM AEBSF for 30 min, and then with 10 μM EVO or RU for 1 h prior to treatment with 100 ng/ml of LIGHT for 18 h. A migration assay was then conducted using a Matrigel filter as described in (A). The data shown represent the means ± SEM. Significantly different from control (*) or cells treated with LIGHT alone (100 ng/ml) (#); $^{***,\#\#\#}P < 0.001$. L, rhLIGHT; EVO, evodiamine; RU, rutaecarpine; HI100, heat inactivated rhLIGHT 100ng/ml.

To confirm that evodiamine and rutaecarpine exerted an effect on LIGHT-induced migration, we pretreated THP-1 cells with 10 μM evodiamine and rutaecarpine, and then stimulated the cells with 100 ng/ml LIGHT using a migration assay. Migration increased following exposure to LIGHT, reaching levels that were 6.7-fold greater than the control values. In addition, treatment with evodiamine and rutaecarpine led to a marked decrease in the increase in LIGHT-induced migration that occurred in THP-1 cells (Fig. 3B). Specifically, the maximum inhibition of LIGHT-induced migration exerted by evodiamine and rutaecarpine (10 μM) was 91% and 97%, respectively, in LIGHT treated THP-1 cells.

INHIBITION OF LIGHT-INDUCED CCR1, CCR2, AND ICAM-1 EXPRESSION BY EVODIAMINE AND RUTAECARPINE VIA SUPPRESSION OF ROS PRODUCTION

We also confirmed the effect of evodiamine and rutaecarpine on LIGHT-induced CCR1, CCR2 and ICAM-1 expression. To accomplish this, we pretreated THP-1 cells with 1 and 10 μM evodiamine or rutaecarpine, and then stimulated them with 100 ng/ml LIGHT for 18 h. The cells were then collected and analyzed by flow cytometry. As seen in Figures 4A and 5A, CCR1, CCR2 and ICAM-1 expression increased after exposure to LIGHT. However, treatment with evodiamine and rutaecarpine led to a marked decrease (up to the basal level) in the LIGHT-induced CCR1, CCR2, and ICAM-1 expression that was observed in THP-1 cells, and these changes occurred in a dose-dependant fashion. Moreover, THP-1 cells were collected and treated under the same conditions as described above and the lysates were then assayed for CCR1, CCR2 and ICAM-1 expression using Western blot analysis. As seen in Figure 4B, CCR1 and CCR2 expression increased after exposure to LIGHT, reaching values that were 24- and 61-fold greater than the control values, respectively. In addition, ICAM-1 expression in THP-1 cells increased after exposure to LIGHT, reaching values that were 4-fold greater than those of the control (Fig. 5B). However, treatment with evodiamine and rutaecarpine led to a marked decrease in the increased levels of CCR1 and CCR2 expression that were observed in response to treatment with LIGHT. Indeed, treatment with both of these compounds inhibited CCR1 and CCR2 expression by up to 99% in LIGHT-treated THP-1 cells (Fig. 4B). Moreover, evodiamine and rutaecarpine inhibited LIGHT-induced ICAM-1 expression by up to about 80% in LIGHT-treated THP-1 cells (Fig. 5B). These findings indicate that the rutaecarpine was more potent than evodiamine in THP-1 cells (Figs. 4 and 5). Additionally, evodiamine and rutaecarpine also significantly reduced ROS production in LIGHT treated cells (Fig. 2). To determine the mechanism by which evodiamine and rutaecarpine exerted their effects and to identify the role that ROS plays in LIGHT-induced effects including CCR1 and CCR2 expression, we pretreated THP-1 cells with the NADPH oxidase inhibitor, AEBSF (0.1, 0.5 mM), prior to LIGHT stimulation. As shown in Figure 4B, AEBSF significantly reduced the LIGHT-induced CCR1 and CCR2 expression in LIGHT-treated THP-1 cells. The CCR1 and 2 expression levels by AEBSF were similar to those by evodiamine or rutaecarpine (Fig. 4B). These results suggest that evodiamine and rutaecarpine inhibits LIGHT-induced CCR1, CCR2 and ICAM-1 expression, and that this effect is mediated by the suppression of ROS production in THP-1 cells.

INHIBITION OF LIGHT-INDUCED CYTOKINES/CHEMOKINES PRODUCTION BY EVODIAMINE AND RUTAECARPINE

Inflammatory cytokines and chemokines such as TNF-α, interleukin (IL)-6, IL-8 and monocyte chemoattractant protein-1 (MCP-1) play an important role in the mediation of inflammatory and proliferative responses and in the pathogenesis of atherosclerosis [Tipping and Hancock, 1993; Ikeda and Shimada, 2001; Ikeda et al., 2001, 2002; Fan and Watanabe, 2003; Rakesh and Agrawal, 2005]. To determine if evodiamine and rutaecarpine exerted an effect on LIGHT-induced cytokines/chemokines production, we pretreated THP-1 cells with 1 and 10 μM evodiamine or rutaecarpine for 1 h, and then stimulated them with 100 ng/ml LIGHT. The cell-free supernatants were then collected, after which the cytokines/chemokines were assayed using ELISA kits for IL-8, MCP-1, TNF-α and IL-6. As shown in Figure 6, the production of cytokines/chemokines increased significantly following exposure to LIGHT, reaching values that were greater than the control values. Moreover, treatment with evodiamine and rutaecarpine led to a marked decrease in the LIGHT-induced increase in IL-8, MCP-1, TNF-α and IL-6 production, and this change occurred in a dose-dependent manner. Indeed, the LIGHT induced production of all of the evaluated cytokines/chemokines was inhibited by over 90% (Fig. 6). Furthermore, the results revealed that treatment with rutaecarpine more effectively attenuated the production of cytokines/chemokines when compared to evodiamine. Finally, treatment with heat-inactivated rhLIGHT as a negative control had no effect on IL-8, MCP-1, TNF-α and IL-6 production (Fig. 6).

BLOCKING ROS PRODUCTION INHIBITS LIGHT-INDUCED MAPK ACTIVATION IN THP-1 CELLS

Reactive oxygen species (ROS), which are synthesized by the NADPH oxidase, serve as second messengers that activate multiple intracellular proteins and enzymes such as the epidermal growth factor receptor, c-Src, mitogen-activated protein kinases (MAPKs) and Ras. Activation of these signaling cascades and redox-sensitive transcription factors leads to the induction of many genes that play important functional roles in the physiology and pathophysiology of vascular cells [Griendling et al., 2000]. In addition, it is well known that MAPK activation is required for LIGHT-induced migration in macrophages and LIGHT-induced proliferation of vascular smooth muscle cells to occur [Wei et al., 2006]. To determine the role that ROS played in LIGHT-induced MAPKs activation, including ERK and p38 MAPK phosphorylation, we pretreated THP-1 cells with the AEBSF (0.1 and 0.5 mM), prior to LIGHT stimulation. As shown in Figure 7, Western blot analysis demonstrated that LIGHT-induced ERK and p38 MAPK phosphorylation were reduced by AEBSF. These results suggest that ROS plays a significant role in LIGHT-induced ERK and p38 MAPK phosphorylation. Moreover, blocking ROS production was found to inhibit LIGHT-induced ERK and p38 MAPK phosphorylation, and these effects were very similar to the effects of evodiamine and rutaecarpine (Fig. 7). Therefore, evodiamine and rutaecarpine likely down-regulate LIGHT-induced ERK and p38 MAPK phosphorylation via a decrease in ROS production in THP-1 cells. These findings indicate that ROS is an activator of ERK and p38 MAPK, and that it

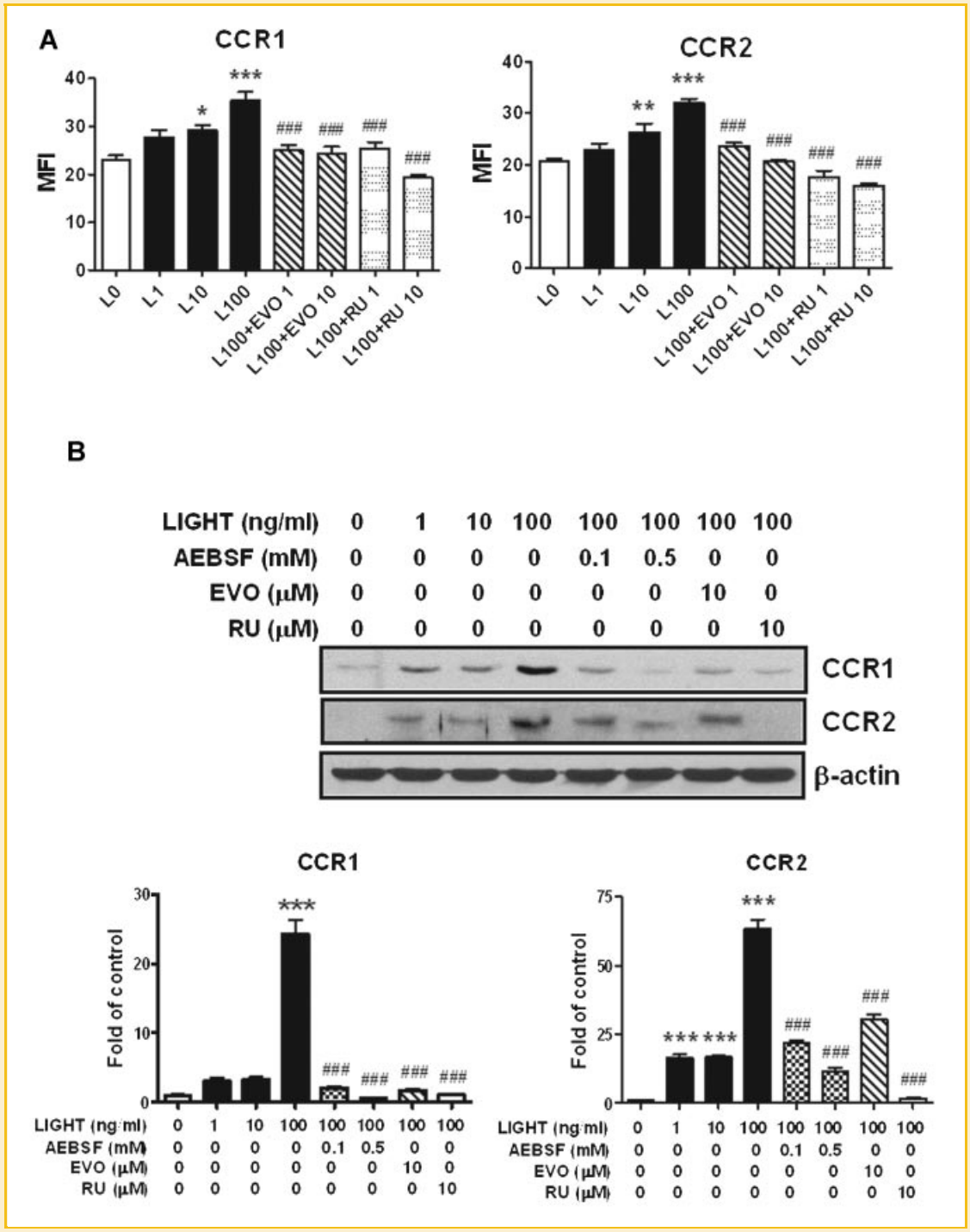

Fig. 4. Inhibitory effects of EVO and RU on LIGHT-induced CCR1 and CCR2 expression. A: Cells were preincubated with 1 or 10 μM of EVO or RU for 1 h prior to treatment with 100 ng/ml LIGHT for 18 h. Cells were then harvested and subjected to immune staining with anti-human CCR1 and CCR2 mAb. The expression of CCR1 and CCR2 was then measured by FACS analysis as described in the Materials and Methods Section. B: Cells were preincubated with 0.1 or 0.5 mM AEBSF for 30 min, and then with 10 μM EVO or RU for 1 h prior to treatment with 100 ng/ml LIGHT for 18 h. The expression of CCR1 and CCR2 was then measured by Western blot analysis as described in the Materials and Methods Section. The membrane was stripped and reprobed with anti-β-actin mAb to confirm equal loading. Lower panels show the band density of the upper panels. These data represent the means ± SEM. Significantly different from control (*) or cells treated with LIGHT alone (100 ng/ml) (#); $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***,\#\#\#}P < 0.001$. L, rhLIGHT; EVO, evodiamine; RU, rutaecarpine; A, AEBSF.

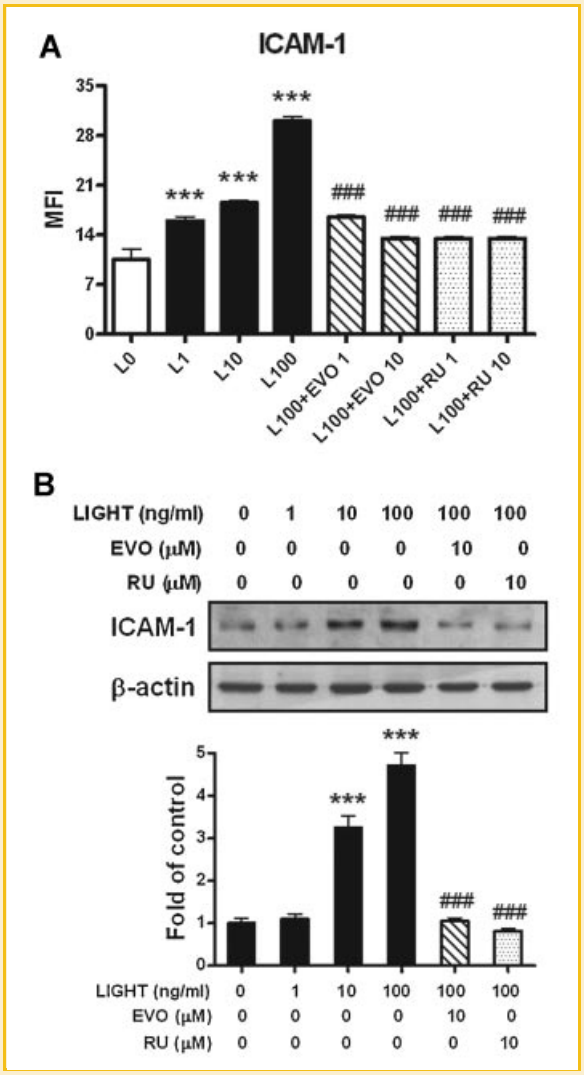

Fig. 5. Inhibitory effects of EVO and RU on LIGHT-induced ICAM-1 expression. A: Cells were preincubated with 1 or 10 μM EVO or RU for 1 h prior to treatment with 100 ng/ml LIGHT for 18 h. Cells were then harvested and subjected to immune staining with anti-human ICAM-1 mAb. The expression of ICAM-1 was then measured by FACS analysis as described in the Materials and Methods Section. B: Cells were also preincubated with 10 μM EVO and RU for 1 h prior to treatment with 100 ng/ml LIGHT for 18 h. The expression of ICAM-1 was then measured by Western blot analysis as described in the Materials and Methods Section. The membrane was stripped and reprobed with anti-β-actin mAb to confirm equal loading. Lower panels show the band density of upper panels. These data represent the means ± SEM. Significantly different from control (*) or cells treated with LIGHT alone (100 ng/ml) (#); $^{***,\#\#\#}P < 0.001$. L, rhLIGHT; EVO, evodiamine; RU, rutaecarpine.

induces migration (Fig. 3), CCR1, CCR2, and ICAM-1 expression (Figs. 4 and 5), as well as IL-8, MCP-1, TNF-α and IL-6 production (Fig. 6). Overall, these results suggest that evodiamine and rutaecarpine inhibit ERK and p38 MAPK activation via the suppression of NADPH oxidase activation and ROS production in LIGHT-treated THP-1 cells.

DISCUSSION

Abnormal growth and inflammation of VSMC plays an important role in vascular diseases, including atherosclerosis and restenosis, following angioplasty [Ross, 1995, 1999]. Interestingly, Bobik and Kalinina [2001] and Lee et al. [2001] showed that LIGHT and HVEM (TNFRSF14) are involved in various aspects of human atherogenesis such as the induction of proinflammatory cytokines or chemokines, growth factors and matrix metalloproteinases [Heo et al., 2008]. In addition, Wei et al. [2006] demonstrated that the LIGHT/HVEM interaction acted as a new player for migration of macrophages/monocytes and the proliferation of VSMC via MAPK activation, and that it was involved in atherogenesis. Previously, we reported that LIGHT-HVEM interaction induced an increase in chemokine receptors and adhesion molecules expression such as CCR1, CCR2, ICAM-1, CD11b, and CD11c, and in chemokine production such as IL-8, and accelerated the migration activity of human monocytes, and promoted the production of ROS via NADPH oxidase $p47^{phox}$ phosphorylation [Heo et al., 2006, 2008].

In this study, evodiamine and rutaecarpine were found to inhibit LIGHT-induced ROS production (Fig. 2), migration (Fig. 3B), CCR1, CCR2 and ICAM-1 expression (Figs. 4 and 5), IL-8, MCP-1, TNF-α, and IL-6 production (Fig. 6) and ERK 1/2 and p38 activation (Fig. 7). Moreover, we found that rutaecarpine more potently attenuated the effects of LIGHT in THP-1 cells than evodiamine. Furthermore, treatment with heat-inactivated LIGHT as a negative control had no effect on migration (Fig. 3B) or IL-8, MCP-1, TNF-α, and IL-6 production (Fig. 6). This negative result demonstrates that there was no contamination with endotoxins in our system.

We determined the role that ROS plays in LIGHT-induced responses, including migration, CCR1, CCR2, and ICAM-1 expression, and ERK 1/2 and p38 activation. To accomplish this, we pretreated THP-1 cells with the NADPH oxidase inhibitor, AEBSF (0.1, 0.5 mM), prior to LIGHT stimulation. As shown in Figure 3B, the results demonstrated that LIGHT-induced migration was significantly reduced by AEBSF. In addition, the results of Western blot analysis demonstrated that LIGHT-induced CCR1 and CCR2 expression was significantly reduced by AEBSF (Fig. 4). Furthermore, the ERK 1/2 and p38 activity that was induced by LIGHT was attenuated by AEBSF (Fig. 7). Moreover, we previously showed that LIGHT-induced TNF-α and IL-8 production were reduced by AEBSF in monocytes [Heo et al., 2008]. Taken together, these results suggest that ROS plays a significant role in LIGHT-induced migration, CCR1, CCR2 and ICAM-1 expression, and IL-8, MCP-1, TNF-α and IL-6 production. Heo et al. demonstrated that LIGHT-induced ROS production is induced by $p47^{phox}$ phosphorylation in monocytes [Heo et al., 2008]. Moreover, evodiamine and rutaecarpine completely inhibit ROS production by LIGHT via suppression of $p40^{phox}$ phosphorylation, as shown in Figure 2. This inhibition subsequently leads to decreased ROS production. Therefore, blocking ROS production inhibits LIGHT-induced ERK 1/2 and p38 activation in THP-1 cells, and these effects are very similar to the effects of evodiamine and rutaecarpine (Fig. 7). In addition, NADPH oxidase activation is required for LIGHT-induced migration of THP-1 cells to occur [Heo et al., 2008]. That was also showed in Figure 3A. Taken together, these results indicated that ROS is an

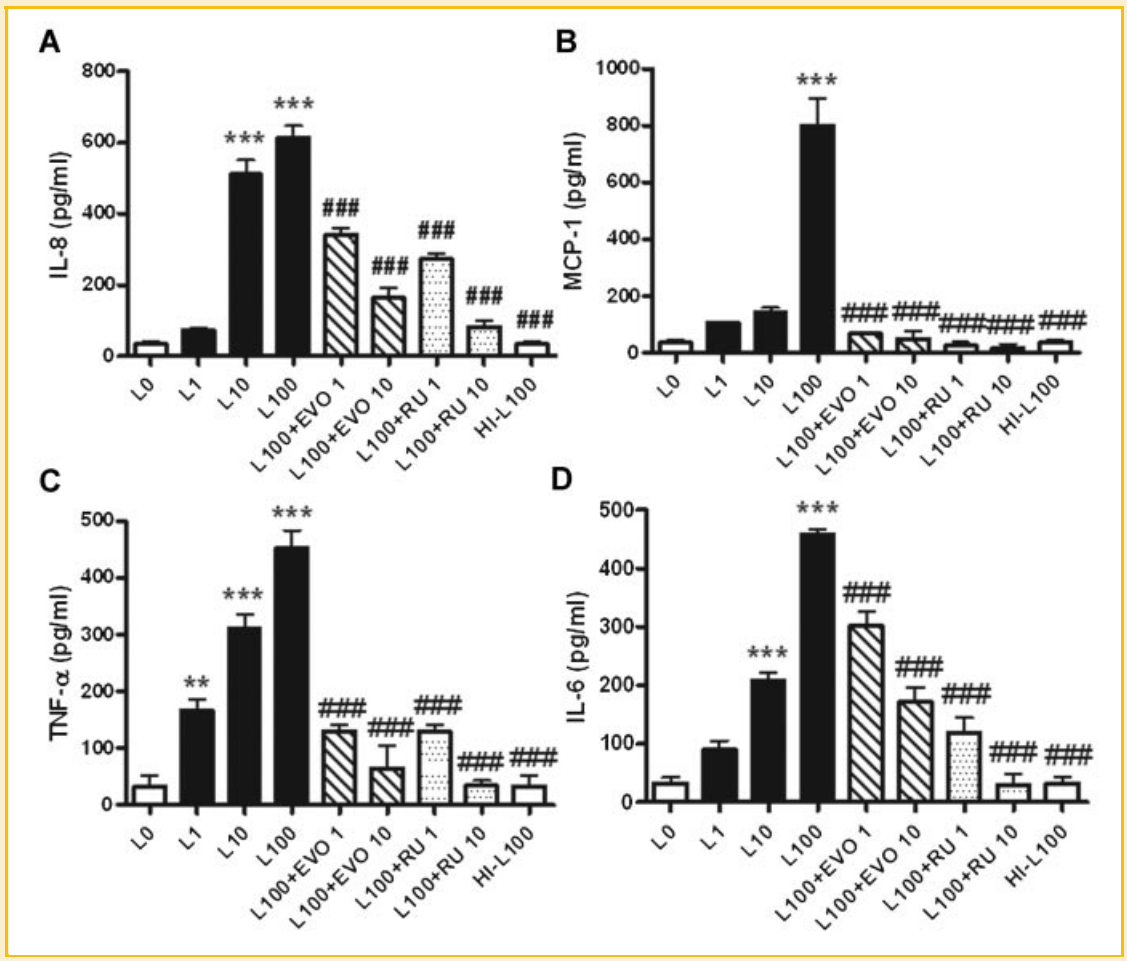

Fig. 6. Inhibitory effect of EVO and RU on LIGHT-induced IL-8, MCP-1, TNF-α and IL-6 production. THP-1cells were preincubated with 1 or 10 μM EVO or RU for 1 h prior to treatment with 100 ng/ml LIGHT for 18 h. The cell-free supernatant was then harvested and measured by ELISA as described in the Materials and Methods Section. A: IL-8 production. B: MCP-1 production. C: TNF-α production. (D) IL-6 production. These data represent the means ± SEM. Significantly different from control (*) or cells treated with LIGHT alone (100 ng/ml) (#); $^{**}P < 0.01$; $^{***,\#\#\#}P < 0.001$. L, rhLIGHT; EVO, evodiamine; RU, rutaecarpine; HI100, heat inactivated rhLIGHT 100 ng/ml.

activator of ERK and p38 MAPK, and that it induces migration (Fig. 3), CCR1, CCR2 and ICAM-1 expression (Figs. 4 and 5), and IL-8, MCP-1, TNF-α and IL-6 production (Fig. 6). These findings indicate that evodiamine and rutaecarpine inhibit potential ERK 1/2 and p38 MAPKs activation via the suppression of ROS production in LIGHT-treated THP-1 cells. Thus, evodiamine and rutaecarpine down-regulate LIGHT-induced ERK and p38 MAPK phosphorylation by decreasing ROS production in THP-1 cells (Fig. 2). In addition, these findings indicate that evodiamine and rutaecarpine inhibit potential ERK and p38 MAPK activation via the suppression of NADPH oxidase activation ($p40^{phox}$ and $p47^{phox}$ phosphorylation), and reduction of ROS production in LIGHT-treated THP-1 cells (Fig. 7). Importantly, evodiamine and rutaecarpine inhibit LIGHT-induced NADPH oxidase $p40^{phox}$ phosphorylation soon after treatment (10 min), as shown in Figure 2B. Furthermore, evodiamine and rutaecarpine were found to regulate LIGHT-induced reactions including ROS production (45 min, Fig. 2A), migration (18 h, Fig. 3B), expression of CCR1, CCR2 and ICAM-1 (18 h, Figs. 4 and 5), production of cytokines (18 h, Fig. 6), and activation of ERK1/2 and p38 MAPK (30 min, Fig. 7).

The dried, unripe fruit of EF has long been used as a traditional Chinese herbal medicine and is currently used for the treatment of headache, abdominal pain, vomiting, colds and reduced blood circulation [Fei et al., 2003; Takada et al., 2005]. In addition, EF can act as a stimulant for the secretion of digestive enzymes that promote appetite, and can also induce a diuretic and analgesic effect [Iwata et al., 2005]. Evodiamine and rutaecarpine are active components of EF. In this study, we investigated the inhibitory effect of evodiamine and rutaecarpine on LIGHT-induced migration in the human monocyte, THP-1. Evodiamine and rutaecarpine decreased the LIGHT-induced production of ROS (Fig. 2), expression of CCR1, CCR2 (Fig. 4) and ICAM-1 (Fig. 5), the production of IL-8, MCP-1, TNF-α, and IL-6 (Fig. 6), and the phosphorylation of ERK 1/2 and p38 (Fig. 7). Taken together, the results of this study indicate that evodiamine and rutaecarpine inhibit LIGHT-induced migration by decreasing the expression of chemokine receptors, adhesion

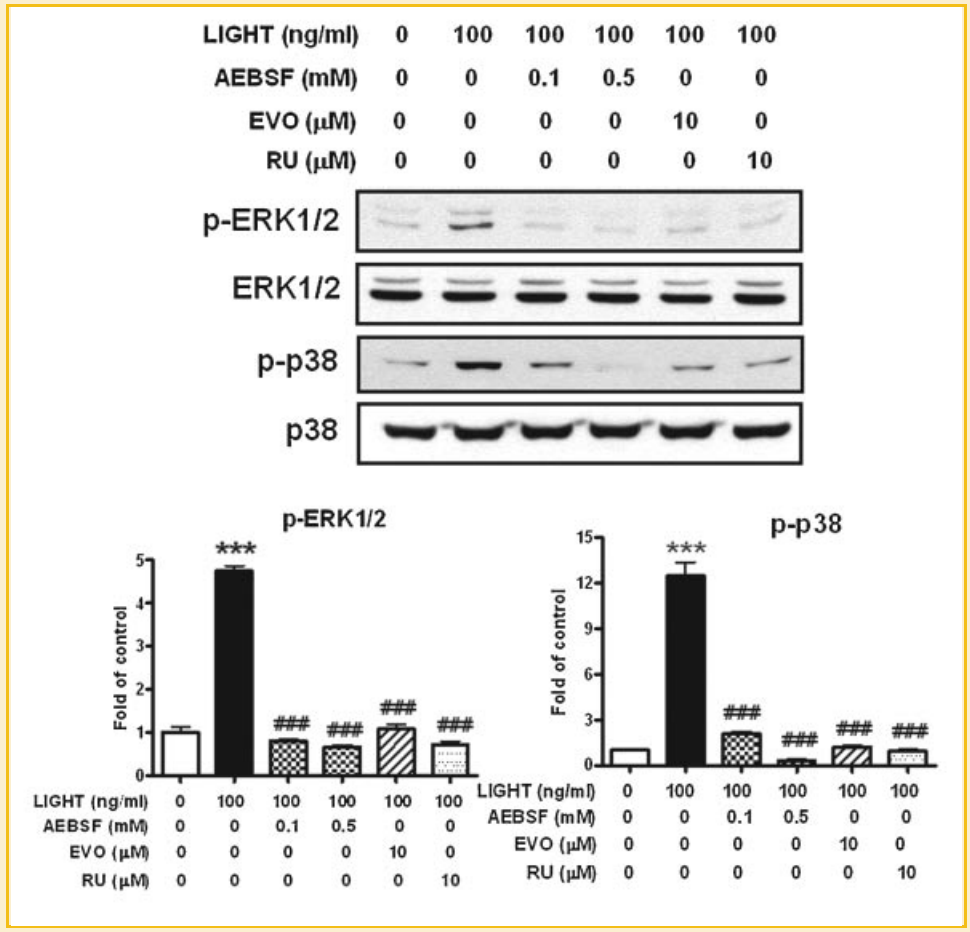

Fig. 7. AEBSF, EVO and RU inhibit LIGHT-induced ERK1/2 and p38 MAPK activation. Cells were preincubated with 0.1 or 0.5 mM AEBSF for 30 min, and then with 10 μM EVO or RU for 1 h prior to treatment with 100 ng/ml LIGHT for 30 min. The expression of phospho-EKR 1/2 (p-ERK 1/2), ERK 1/2, p-p38 and p38 were then measured by Western blot analysis as described in the Materials and Methods Section. The membrane was stripped and reprobed with anti-β-actin mAb to confirm equal loading. Lower panels show the band density of the upper panels. These data represent the means ± SEM. Significantly different from control (*) or cells treated with LIGHT alone (100 ng/ml) (#); $^{***,\#\#\#}P < 0.001$. EVO, evodiamine; RU, rutaecarpine.

molecules and cytokine/chemokine production (Fig. 3). Furthermore, treatment with AEBSF, efficiently blocked LIGHT-induced migration, CCR1, CCR2, and ICAM-1 production and the activation of MAPK such as ERK and p38. Moreover, as shown in Figure 3A, the results of this study demonstrated that LIGHT induced migration was reduced by the inhibition of $p47^{phox}$ siRNA, which suggests that NADPH oxidase plays a significant role in LIGHT-induced migration. These effects were similar to the effects of evodiamine and rutaecarpine, which indicates that the inhibitory effects of evodiamine and rutaecarpine on migration, the production of CCR1, CCR2, and ICAM-1 and the activation of MAPK such as ERK 1/2 and p38 occur via decreased ROS production and NADPH oxidase activation.

In conclusion, evodiamine and rutaecarpine inhibit LIGHT-Induced inflammatory responses such as migration, CCR1, CCR2, and ICAM-1 expression, ROS, IL-8, MCP-1, TNF-α, and IL-6 production and the activation of ERK 1/2 and p38 MAPK in THP-1 cells via the suppression of ROS production (NADPH oxidase, $p40^{phox}$ and $p47^{phox}$ activation). Taken together, these results indicate that evodiamine; rutaecarpine and EF have the potential for use as an anti-atherosclerosis agent and may be particularly useful for the prevention restenosis and inflammation following percutaneous transluminal coronary angioplasty.

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