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Anti-inflammatory action of ethanolic extract of *Ramulus mori* on the BLT2-linked cascade

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Mulberry tree twigs (*Ramulus mori*) contain large amounts of oxyresveratrols and have traditionally been used as herbal medicines because of their anti-inflammatory properties. However, the signaling mechanism by which *R. mori* exerts its anti-inflammatory action remains to be elucidated. In this study, we observed that *R. mori* ethanol extracts (RME) exerted an inhibitory effect on the lipopolysaccharide (LPS)-induced production of the pro-inflammatory cytokine interleukin-6 (IL-6) in Raw264.7 macrophage cells. Additionally, RME inhibited IL-6 production by blocking the leukotriene B_4 receptor-2 (BLT2)-dependent-NADPH oxidase 1 (NOX1)-reactive oxygen species (ROS) cascade, leading to anti-inflammatory activity. Finally, RME suppressed the production of the BLT2 ligands LTB4 and 12(S)-HETE by inhibiting the p38 kinase-cytosolic phospholipase A_2-5-/12-lipoxygenase cascade in LPS-stimulated Raw264.7 cells. Overall, our results suggest that RME inhibits the 'BLT2 ligand-BLT2'-linked autocrine inflammatory axis, and that this BLT2-linked cascade is one of the targets of the anti-inflammatory action of *R. mori*. [BMB Reports 2016; 49(4): 232-237]

INTRODUCTION

The mulberry tree belongs to the genus *Morus*, and it has been widely cultivated in eastern Asia and used in herbal medicines (1). Mulberry extracts contain large amounts of stilbenes, including oxyresveratrol (2,3',4,5'-tetrahydroxy-trans-stilbene; OXY) (2). OXY exhibits anti-oxidant, anti-hyperlipidemic and anti-inflammatory activities (3-5). Most studies of mulberry extracts have primarily focused on the leaves and root cortices of this tree, and little is known about the activity of twigs (called *Ramulus mori*) (6-8). Recently, several reports have suggested that *R. mori* extracts contain high levels of OXY and have anti-inflammatory properties (9-11). However, there is currently little information about the signaling mechanism underlying the anti-inflammatory activity induced by *R. mori*.

Leukotriene B_4 receptor-2 (BLT2) is a G protein-coupled receptor for pro-inflammatory lipid mediators such as leukotriene B_4 (LTB_4) and 12(S)-hydroxyeicosatetraenoic acid (12(S)-HETE) (12). Recent reports suggest that BLT2 is implicated in several inflammatory human diseases including asthma (13, 14), rheumatoid arthritis (15), cancer (16-18), and inflammatory bowel disease (19). Moreover, in inflammatory diseases, BLT2 has been closely associated with the generation of reactive oxygen species (ROS) via NADPH oxidases (NOXs) (13, 20). In a previous study, we demonstrated that the BLT2-NOX1-ROS cascade mediates LPS-induced IL-6 production (21). Despite the implications of BLT2 being an inflammatory mediator, a natural agent that inhibits BLT2 has not yet been discovered.

In this study, we prepared an ethanolic extract of *R. mori* ethanol (RME), and found that RME suppressed the LPS-induced IL-6 production by down-regulating the BLT2-linked cascade. Additionally, treatment with RME suppressed the synthesis of the BLT2 ligands LTB_4 and 12(S)-HETE by blocking a p38-cytosolic phospholipase A_2 ($cPLA_2$)-5-lipoxygenase (5-LO)/12-lipoxygenase (12-LO) signaling cascade. Together, our results suggested that the 'BLT2 ligand-BLT2'-linked autocrine axis is a target of the anti-inflammatory action of *R. mori*.

RESULTS AND DISCUSSION

RME suppresses the LPS-induced IL-6 production in Raw264.7 cells

IL-6 exerts various pro-inflammatory effects on many cell types, and high levels of IL-6 have been associated with the pathogenesis of diverse inflammatory diseases (22, 23). Here, we investigated the anti-inflammatory effects of RME on LPS-stimulated IL-6 production in Raw264.7 macrophage cells. Our initial observations had shown that IL-6 production dramatically and time-dependently increased after LPS treatment (100 μg/ml) in Raw264.7 cells (Fig. 1A and B). Before studying the anti-inflammatory activities of RME, we determined its cytotoxicity in Raw264.7 cells. Raw264.7 cells were treated with

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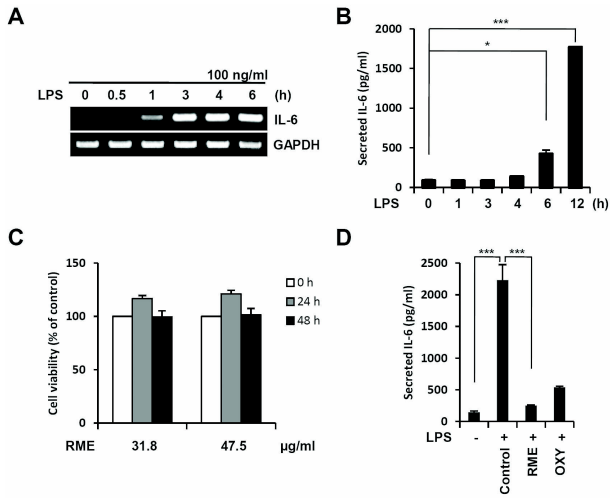

Fig. 1. RME suppresses LPS-induced IL-6 production in Raw264.7 cells. (A) Raw264.7 cells were stimulated with LPS (100 ng/ml) for the indicated times, and total RNA was subsequently subjected to RT-PCR analysis. (B) The amount of IL-6 released into the culture medium was measured by ELISA. (C) Raw264.7 cells were treated with RME (31.8 or 47.5 μg/ml) for 0 h, 24 h or 48 h. Cell viability was determined using an MTT assay. (D) Raw264.7 cells were incubated for 30 min with RME (47.5 μg/ml) or OXY (7.3 μg/ml) followed by 12 h of incubation in the presence or absence of LPS. Subsequently, the IL-6 released into the culture medium was measured. All of the quantitative data are expressed as the mean ± the SD, from three independent experiments. *P < 0.05, ***P < 0.005.

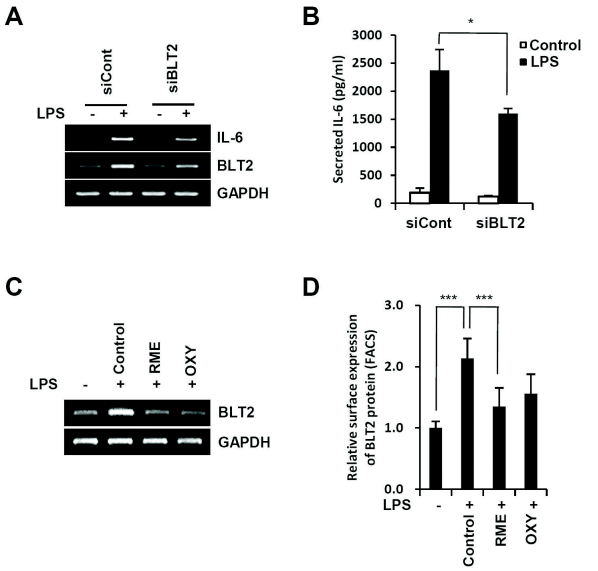

Fig. 2. RME inhibits LPS-induced BLT2 up-regulation in Raw264.7 cells. (A) Raw264.7 cells were transfected with control (siCont) or BLT2 (siBLT2) siRNAs. After 24 h, the cells were incubated in the presence or absence of LPS for 4 h, and the total RNA was isolated for RT-PCR analysis. (B) The Raw264.7 cells were transfected with control or BLT2 siRNAs, incubated for 24 h and then stimulated with LPS for 12 h, after which the IL-6 released into the cell culture medium was measured. (C) Raw264.7 cells were incubated for 30 min with RME (47.5 μg/ml) or OXY (7.3 μg/ml) and then incubated for 4 h in the presence or absence of LPS (100 ng/ml). Subsequently, total RNA was isolated and subjected to RT-PCR analysis. (D) Raw264.7 cells were incubated for 30 min with RME or OXY treatment and subsequently incubated for 12 h in the presence or absence of LPS after the cells has been subjected to FACS analysis for BLT2 protein levels. All of the quantitative data are expressed as the mean ± the SD, based on three independent experiments. *P < 0.05, ***P < 0.005.

different concentrations of RME (31.8 and 47.5 μg/ml) for 48 h and evaluated by an MTT assay. As illustrated in Fig. 1C, RME did not exhibit any cytotoxicity. Next, we examined whether RME exhibited any inhibitory effects on LPS-induced IL-6 production in Raw264.7 cells. We observed that treatment with RME clearly suppressed the LPS-induced IL-6 production in Raw264.7 cells (Fig. 1D). OXY was used as a control, and the OXY treatment also suppressed LPS-induced IL-6 production, albeit less potently than RME (Fig. 1D). Thus, these results suggest that RME exerts its anti-inflammatory effects by suppressing the IL-6 production in Raw264.7 cells.

RME inhibits LPS-induced BLT2 up-regulation in Raw264.7 cells

Recent studies have implicated the function of BLT2 as a pro-inflammatory mediator in various inflammatory diseases, such as asthma (13, 14), arthritis (15), and arteriosclerosis (24). In previous studies, BLT2 up-regulation was shown to contribute to LPS-induced IL-6 production (17, 21). Indeed, siRNA-mediated depletion of BLT2 resulted in significant inhibition of IL-6 expression in LPS-treated Raw264.7 cells (Fig. 2A and B). Therefore, we evaluated whether BLT2 is one of the targets of the anti-inflammatory activity of RME. We demonstrated that RME clearly suppressed LPS-induced BLT2 mRNA and protein expression (Fig. 2C and D). OXY was used as a control, and OXY treatment also suppressed LPS-induced BLT2 expression (Fig. 2C and D). Together, these results suggest that RME attenuates the expression of BLT2, thus contributing to the suppression of IL-6 production.

RME inhibits the LPS-induced NOX1-ROS cascade in Raw264.7 cells

ROS generation is involved in a variety of pathological and inflammatory responses. For example, inflammatory stimulants (such as LPS) induce the generation of ROS, and LPS-induced ROS generation in macrophages is dependent on NADPH oxidase (NOX) (25, 26). We previously reported that NOX-derived ROS functions as downstream mediators of BLT2 in various cell types (13, 20, 21, 27), leading us to investigate whether RME attenuates the BLT2-dependent NOX-ROS cascade in LPS-activated Raw264.7 cells. RME clearly suppressed the LPS-induced ROS generation in a concentration-dependent

manner, and exhibited anti-oxidative activity (Fig. 3A). Additionally, RME suppressed LPS-induced NOX1 up-regulation (Fig. 3B). OXY was used as a control, and OXY treatment also suppressed the LPS-induced ROS generation and NOX1 up-regulation (Fig. 3A and B). In contrast to NOX1, NOX2 and NOX4 were not up-regulated by LPS treatment (data not shown). Consistent with previous findings, NOX1 depletion by siRNA transfection resulted in significant inhibition of LPS-induced ROS generation and IL-6 expression (Figs. 3C-E). Additionally, we observed that LPS-induced NOX1 expression was greatly decreased by siRNA-mediated depletion of BLT2 in Raw264.7 cells (Fig. 3F). Together, these results suggest that RME down-regulates the BLT2-dependent NOX1-ROS cascade, thereby inhibiting IL-6 production in Raw264.7 cells.

RME inhibits LPS-induced BLT2 ligand production in Raw264.7 cells

BLT2 is activated by direct interaction with specific ligands, such as LTB_4 and 12(S)-HETE, on the cell surface (12). The synthesis of LTB_4 and 12(S)-HETE from arachidonic acid (AA) is catalyzed by 5-lipoxygenase (5-LO) and 12-lipoxygenase (12-LO), respectively (28). Previously, we have reported that the levels of LTB_4 and 12(S)-HETE are significantly increased by LPS in macrophages (21). To examine whether RME affects the production of the BLT2 ligands LTB_4 and 12(S)-HETE, we assessed the levels of these ligands. LPS-enhanced LTB_4 and 12(S)-HETE expression were markedly inhibited by RME treatment (Fig. 4A). OXY was used as a control, and OXY treatment also inhibited LPS-induced production of the BLT2 ligands, albeit less potently than RME (Fig. 4A). Additionally, we observed that RME treatment inhibited the LPS-induced up-regulation of 5-LO and 12-LO in Raw264.7 cells (Fig. 4C). cPLA2 is the rate-limiting enzyme responsible for the release of AA from membrane phospholipids (29, 30), and $cPLA_2$ activation was assessed by Ser^{505} phosphorylation, which was clearly increased by LPS treatment (Fig. 4C). Moreover, RME treatment significantly inhibited the LPS-induced stimulation of $cPLA_2$ activity and $cPLA_2$ phosphorylation in Raw264.7 cells (Fig. 4B and C). Previously, it has been suggested that p38 kinase activation is required for the LPS-induced activation of $cPLA_2$ (31).

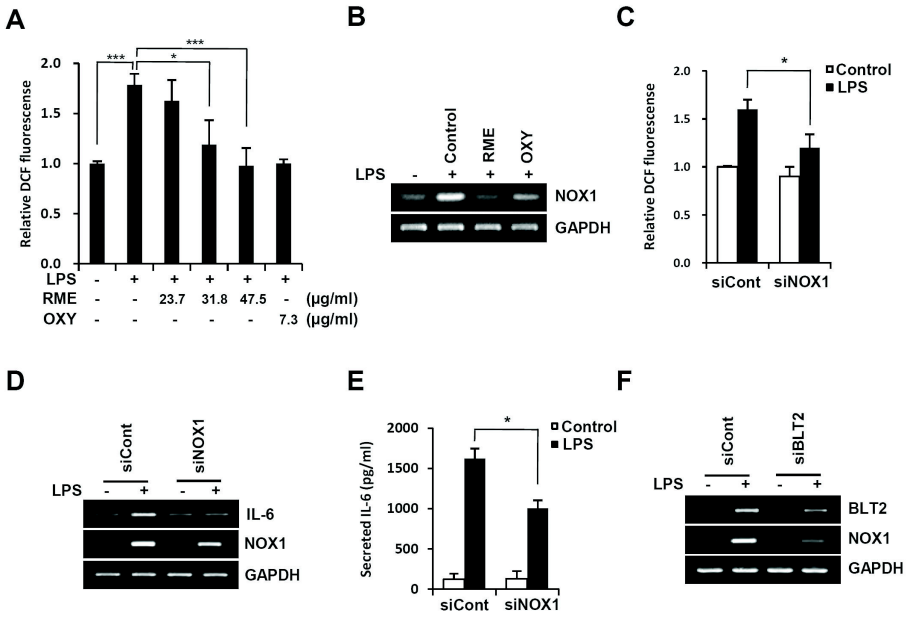

Fig. 3. RME inhibits the LPS-induced NOX1-ROS cascade in Raw264.7 cells. (A) Raw264.7 cells were incubated for 30 min with RME or OXY, followed by 1 h incubation in the presence or absence of LPS (100 ng/ml). 2',7'-dichlorofluorescein diacetate (DCF-DA; 10 μM) was added to the culture for 20 min during the final incubation, and intracellular ROS was subsequently measured using flow cytometric analysis of DCF fluorescence. (B) Raw264.7 cells were incubated for 30 min with RME (47.5 μg/ml) or OXY (7.3 μg/ml), followed by 4 h incubation in the presence or absence of LPS. Subsequently, total RNA was isolated and subjected to RT-PCR analysis. (C) The cells were transfected with NOX1 (siNOX) or control siRNA (siCont), incubated for 24 h and then stimulated with LPS for 1 h. DCF-DA was added to the culture for 20 min during the final incubation, and intracellular ROS was subsequently measured using flow cytometric analysis of DCF fluorescence. (D) Cells transfected and incubated as in panel (C) were stimulated with LPS for 4 h. Subsequently, total RNA was isolated and subjected to RT-PCR analysis. (E) Cells transfected and incubated as in panel (C) were stimulated with LPS for 12 h, and the IL-6 released into the culture medium was subsequently measured. (F) Raw264.7 cells were transfected with control (siCont) or BLT2 (siBLT2) siRNAs. After 24 h, the cells were incubated in the presence or absence of LPS for 4 h, and total RNA was then isolated and subjected RT-PCR analysis. All of the quantitative data are expressed as the mean ± the SD, of three independent experiments. *P < 0.05, ***P < 0.005.

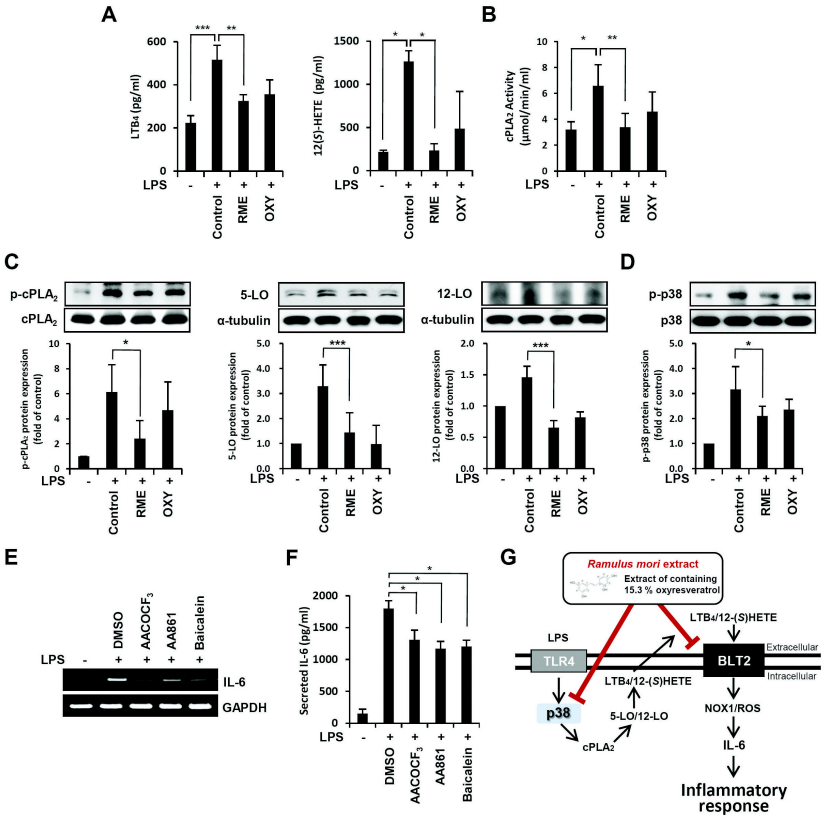

Fig. 4. RME inhibits LPS-induced BLT2 ligand production in Raw264.7 cells. (A) Raw264.7 cells were incubated with RME (47.5 μg/ml) or OXY (7.3 μg/ml) for 30 min, and further incubated for 12 h in the presence or absence of LPS (100 ng/ml). The quantities of LTB_4 (*left*) and 12(S)-HETE (*right*) in the culture supernatants were measured by ELISA. (B) Raw264.7 cells were incubated for 30 min with RME or OXY, followed by 3 h incubation in the absence or presence of LPS. Next, the cells were assayed for $cPLA_2$ activity by $cPLA_2$ assay kit. (C) Raw264.7 cells were incubated for 30 min with RME or OXY, and then incubated for 3 h in the absence or presence of LPS. The cells were then evaluated for p-$cPLA_2$, $cPLA_2$, 5-LO and 12-LO protein levels by western blot assay (densitometry data also shown). (D) Cells were treated as detailed in (C), and the cells were evaluated for p-p38 and p38 protein levels by Western blot (densitometry data also shown). (E) Raw264.7 cells were initially incubated for 30 min with $AACOCF_3$ (20 μM), AA861 (10 μM) or baicalein (20 μM), followed by 4 h incubation in the presence or absence of LPS. Subsequently, total RNA was isolated and subjected to RT-PCR analysis. (F) Raw264.7 cells were initially incubated for 30 min with $AACOCF_3$, AA861 or baicalein, and further incubated for 12 h in the presence or absence of LPS; the IL-6 released into the culture medium was then measured. (G) The schematic representation of the mechanism of action by which RME exerts its inhibitory effects on LPS-induced inflammatory responses in Raw264.7 cells. All of the quantitative data are presented as the mean ± the SD, of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.005.

We observed that RME treatment markedly suppressed the p38 kinase activation (Fig. 4D). Under these experimental conditions, LPS-induced IL-6 expression was significantly decreased by 5-LO (AA861), 12-LO (baicalein) and $cPLA_2$ ($AACOCF_3$) inhibition in Raw264.7 cells (Fig. 4E and F). Together, these results suggest that RME suppresses the LPS-enhanced production of the BLT2 ligands LTB_4 and 12(S)-HETE, by attenuating the p38-$cPLA_2$-5-LO/12-LO cascade.

In the present study, we demonstrated that RME inhibits LPS-induced IL-6 production. To analyze the detailed mode of action by which RME inhibits the LPS-induced IL-6 production, we evaluated the inhibitory effects of RME on each component (p38 kinase, $cPLA_2$, LTB_4, 12(S)-HETE, BLT2, and NOX1) in the LPS signaling mediated IL-6 production. Our results conclusively identified the 'BLT2 ligand-BLT2'-linked axis as a target for the inhibitory action of RME. RME was shown to suppress the LPS-enhanced production of the BLT2 ligands, LTB_4 and 12(S)-HETE, by attenuating the p38-$cPLA_2$-5-LO/12-LO cascade (Figs. 4A-D). Specifically, we speculate that RME targets the p38 kinase (Fig. 4D), thus attenuating the subsequent activation of downstream components ($cPLA_2$, 5-LO, and 12-LO) (Figs. 4B and C). In fact, previous studies have shown that that LPS-induced IL-6 production in macrophages is regulated through p38 kinase and p38 kinase-mediated $cPLA_2$ activation, thus pointing to p38 kinase as a principal contributor to the LPS-induced production of IL-6 in macrophages (32, 33).

Additionally, we also observed that RME suppressed the BLT2 expression levels (both the mRNA and protein levels) (Figs. 2C and D). Thus, altogether, RME appears to suppress the production of BLT2 ligands as well as the BLT2 expression.

LPS-induced IL-6 production has been suggested to play a role in the development of endotoxic or septic lung inflammation. Indeed, the production of IL-6 was shown to be a hallmark of sepsis, with high levels of this cytokine in affected individuals being associated with mortality (34, 35). Considering that BLT2 is a crucial mediator of LPS signaling-induced IL-6 production in macrophages (as summarized in Fig. 4G), our findings may point to the potential development of RME-based herbal medications being therapeutic for patients with endotoxic or septic shock. Additionally, BLT2 has been implicated in other inflammatory pathogenic conditions as well, including asthmatic airway inflammation and cancer, thus further expanding the potential clinical use of this herbal medicine as being applicable to various BLT2-associated inflammatory diseases.

In summary, our results demonstrated that RME suppresses the IL-6 production in LPS-activated Raw264.7 cells, and that the 'BLT2 ligand-BLT2'-linked autocrine inflammatory axis is one of the targets of the anti-inflammatory activity of RME. The elucidation of this mechanism provides significant insight into the anti-inflammatory activity of *R. mori*.

MATERIALS AND METHODS

Detailed information is described in online Supplementary Data.

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