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 B4 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 A2 (cPLA2)-5-lipoxygenase (5-LO)/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 R. 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 B4 receptor-2 (BLT2) is a G protein-coupled receptor for pro-inflammatory lipid mediators such as leukotriene B4 (LTB4) 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 LTB4 and 12(S)-HETE by blocking a p38-cytosolic phospholipase A2 (cPLA2)-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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different concentrations of RME (31.8 and 47.5 μg/ml) for 48 h and evaluated by an MTT assay. 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 LTB₄ and 12(S)-HETE, on the cell surface (12). The synthesis of LTB₄ 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 LTB₄ and 12(S)-HETE are significantly increased by LPS in macrophages (21). To examine whether RME affects the production of the BLT2 ligands LTB₄ and 12(S)-HETE, we assessed the levels of these ligands. LPS-enhanced LTB₄ 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 cPLA₂ activation was assessed by Ser⁵⁰⁵ phosphorylation, which was clearly increased by LPS treatment (Fig. 4C). Moreover, RME treatment significantly inhibited the LPS-induced stimulation of cPLA₂ activity and cPLA₂ 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 cPLA₂ (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.
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 cPLA2 (AACOCF3) inhibition in Raw264.7 cells (Fig. 4E and F). Together, these results suggest that RME suppresses the LPS-enhanced production of the BLT2 ligands LTB4 and 12(S)-HETE, by attenuating the p38-cPLA2-5-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, cPLA2, LTB4, 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, LTB4 and 12(S)-HETE, by attenuating the p38-cPLA2-5-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 (cPLA2, 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 cPLA2 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. morii.

MATERIALS AND METHODS

Detailed information is described in online Supplementary Data.

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REFERENCES

1. Nomura T (2001) [Chemistry and biosynthesis of prenyl-
flavonoids]. Yakugaku Zasshi 121, 535-556
2. Zhou J, Li SX, Wang W et al (2013) Variations in the levels of mulberroside A, oxyresveratrol, and resveratrol in mul-
berries in different seasons and during growth. Scientific World Journal 2013, 380692
3. Lee HS, Kim DH, Hong JE, Lee JY and Kim EJ (2015) Oxyresveratrol suppresses lipopolysaccharide-induced in-
flammatory responses in murine macrophages. Hum Exp Toxicol 34, 808-818
4. Lorenz P, Roychowdhury S, Engelmann M, Wolf G and Horn TF (2003) Oxyresveratrol and resveratrol are potent antioxidants and free radical scavengers: effect on nitro-
sative and oxidative stress derived from microglial cells. Nitric Oxide 9, 64-76
5. Hwang D, Jo SP, Lee J, Kim JK, Kim KH and Lim YH (2015) Anti-hyperlipidaemic effects of oxyresveratrol-containing Ramulus morii ethanol extract in rats fed a high-cholesterol diet. Journal of Functional Foods 19, 353-362
6. Eo HJ, Park JH, Park GH et al (2014) Anti-inflammatory and anti-cancer activity of mulberry (Morus alba L.) root bark. BMC Complement Altern Med 14, 200
7. Wang W, Zu Y, Fu Y and Efferth T (2012) In vitro anti-
oxidant and antimicrobial activity of extracts from Morus alba L. leaves, stems and fruits. Am J Chin Med 40, 349-356
8. Lim HJ, Jin HG, Woo ER, Lee SK and Kim HP (2013) The root barks of Morus alba and the flavonoids constituents inhibit airway inflammation. J Ethnopharmacol 149, 169-
175
9. Zhang Z, Jin J and Shi L (2008) Protective function of cis-mulberroside A and oxyresveratrol from Ramulus morii against ethanol-induced hepatic damage. Environ Toxicol Pharmacol 26, 325-330
10. Zhang Z and Shi L (2010) Anti-inflammatory and analgesic properties of cis-mulberroside A from Ramulus morii. Fitoterapia 81, 214-218
11. Xu L, Yang F, Wang J, Huang H and Huang Y (2015) Anti-diabetic effect mediated by Ramulus morii polysaccharides. Carbohydr Polym 117, 63-69
12. Yokomizo T, Kato K, Hagiya H, Izumi T and Shimizu T (2001) Hydroxyeicosanoids bind to and activate the low affinity leukotriene B4 receptor, BLT2. J Biol Chem 276, 3049-3056
13. Cho KJ, Seo JM, Lee MG and Kim JH (2010) BLT2 is upregulated in allergen-stimulated mast cells and mediates the synthesis of Th2 cytokines. J Immunol 185, 6329-6337
14. Cho KJ, Seo JM, Shin Y et al (2010) Blockade of airway in-
flammation and hyperresponsiveness by inhibition of BLT2, a low-affinity leukotriene B4 receptor. Am J Respir Cell Mol Biol 42, 294-303
15. Mathis SP, Jala VR, Lee DM and Haribabu B (2010) Nonredundant roles for leukotriene B4 receptors BLT1 and BLT2 in inflammatory arthritis. J Immunol 185, 3049-3056
16. Park GS and Kim JH (2015) LPS Up-Regulates ICAM-1 Expression in Breast Cancer Cells by Stimulating a MyD88-
BLT2-ERK-Linked Cascade, Which Promotes Adhesion to Monocytes. Mol Cells 38, 821-828
17. Park GS and Kim JH (2015) Myeloid differentiation pri-
mary response gene 88-leukotriene B4 receptor 2 cascade mediates lipopolysaccharide-potentiated invasiveness of breast cancer cells. Oncotarget 6, 5749-5759
18. Park J, Park SY and Kim JH (2016) Leukotriene B4 re-
ceptor-2 contributes to chemoresistance of SK-OV-3 ovar-
ian cancer cells through activation of signal transducer
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Geun-Soo Park, et al.

and activator of transcription-3-linked cascade. Biochim Biophys Acta 1863, 236-243
19. Singh VP, Patil CS, Jain NK, Singh A and Kulkarni SK (2003) Effect of nimesulide on acetic acid- and leukotriene-induced inflammatory bowel disease in rats. Prostaglandins Other Lipid Mediat 71, 163-175
20. Kim GY, Lee JW, Ryu HC, Wei JD, Seong CM and Kim JH (2010) Proinflammatory cytokine IL-1beta stimulates IL-8 synthesis in mast cells via a leukotriene B4 receptor 2-linked pathway, contributing to angiogenesis. J Immunol 184, 3946-3954
21. Lee AJ, Cho KJ and Kim JH (2015) MyD88-BLT2-dependent cascade contributes to LPS-induced interleukin-6 production in mouse macrophages. Exp Mol Med 47, e156
22. Galasy C (2006) Interleukin-6 and chronic inflammation. Arthritis Res Ther 8 Suppl 2, S3
23. Kwon DJ, Bae YS, Ju SM, Youn GS, Choi SY and Park J (2014) Salicortin suppresses lipopolysaccharide-stimulated inflammatory responses via blockade of NF-kappaB and JNK activation in RAW 264.7 macrophages. BMB Rep 47, 318-323
24. Hoyer FF, Albrecht L, Nickenig G and Muller C (2012) Selective inhibition of leukotriene receptor BLT-2 reduces vascular oxidative stress and improves endothelial function in ApoE-/- mice. Mol Cell Biochem 359, 25-31
25. Maitra U, Singh N, Gan L, Ringwood L and Li L (2009) IRAK-1 contributes to lipopolysaccharide-induced reactive oxygen species generation in macrophages by inducing NOX-1 transcription and Rac1 activation and suppressing the expression of antioxidative enzymes. J Biol Chem 284, 35403-35411
26. Kim JS, Yeo S, Shin DG et al (2010) Glycogen synthase kinase 3beta and beta-catenin pathway is involved in toll-like receptor 4-mediated NADPH oxidase 1 expression in macrophages. FEBS J 277, 2830-2837
27. Kim EY, Seo JM, Kim C, Lee JE, Lee KM and Kim JH (2010) BLT2 promotes the invasion and metastasis of aggressive bladder cancer cells through a reactive oxygen species-linked pathway. Free Radic Biol Med 49, 1072-1081
28. Powell WS, Gravel S, Khanapure SP and Rokach J (1999) Biological inactivation of 5-oxo-6,8,11,14-eicosatetraenoic acid by human platelets. Blood 93, 1086-1096
29. Funk CD (2001) Prostaglandins and leukotrienes: Advances in eicosanoid biology. Science 294, 1871-1875
30. Leslie CC (1997) Properties and regulation of cytosolic phospholipase A2. J Biol Chem 272, 16709-16712
31. Qi HY and Shellhammer JH (2005) Toll-like receptor 4 signaling regulates cytosolic phospholipase A2 activation and lipid generation in lipopolysaccharide-stimulated macrophages. J Biol Chem 280, 38969-38975
32. Uozumi N, Kita Y and Shimizu T (2008) Modulation of lipid and protein mediators of inflammation by cytosolic phospholipase A2alpha during experimental sepsis. J Immunol 181, 3558-3566
33. Wang X, Xue H, Xu Q et al (2008) p38 kinase/cytosolic phospholipase A2/cyclooxygenase-2 pathway: a new signaling cascade for lipopolysaccharide-induced interleukin-1beta and interleukin-6 release in differentiated U937 cells. Prostaglandins Other Lipid Mediat 86, 61-67
34. Rau S, Kohn B, Richter C et al (2007) Plasma interleukin-6 response is predictive for severity and mortality in canine systemic inflammatory response syndrome and sepsis. Vet Clin Pathol 36, 253-260
35. Song R, Kim J, Yu D, Park C and Park J (2012) Kinetics of IL-6 and TNF-alpha changes in a canine model of sepsis induced by endotoxin. Vet Immunol Immunopathol 146, 143-149