ECa 233 Suppresses LPS-Induced Proinflammatory Responses in Macrophages via Suppressing ERK1/2, p38 MAPK and Akt Pathways

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A current anti-inflammatory agent often targets the prevention of inflammatory disorder development. The standardized Centella asiatica ECa 233 extract has been previously reported for anti-inflammatory effect. This study aimed to investigate its anti-inflammatory effect and mechanisms of ECa 233 in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages, through 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, nitric oxide (NO) assay, reactive oxygen species (ROS) production assay, enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. Our results found that ECa 233 significantly inhibited LPS-stimulated pro-inflammatory mediators production including ROS, NO and prostaglandin E2 (PGE2), and pro-inflammatory cytokines, including tumor necrosis factor (TNF-α) and interleukin (IL)-1β without cytotoxicity. In addition, ECa 233 downregulated not only the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), but also the activation of nuclear factor-kappa B (NF-xB), activated protein kinase B (Akt), extracellular signal-regulated kinase (ERK1/2) and p38 mitogen-activated protein kinases (MAPK) induced by LPS. The inhibition of LPS-induced inflammation due to ECa 233 offered an opportunity as a tentatively potential candidate for the prevention and treatment of inflammatory diseases.

Key words Centella asiatica; cyclooxygenase-2; inducible nitric oxide synthase; reactive oxygen species; nuclear factor-kappa B

INTRODUCTION

Human diseases such as cancer, allergy, infectious diseases, atherosclerosis, and autoimmune disorders are considered as a result of chronic inflammation. Lipopolysaccharide (LPS), a major component in an outer membrane of Gram-negative bacteria, plays a significant role to negotiate inflammation. Following LPS activation, macrophage releases numerous inflammatory signaling molecules including reactive oxygen species (ROS), nitric oxide (NO), interleukin-1 beta (IL-1β), tumor necrosis factor-alpha (TNF-α), prostaglandin E2 (PGE2) and cyclooxygenase to promote inflammatory responses. Oxygen metabolism, mainly ROS, is well known as a primary action in the pathogenesis of inflammation, and ROS becomes increasingly essential in inflammatory disorder progression. Mitogen-activated protein kinase (MAPK), comprises of the extracellular signal-regulated kinase (ERK), p38 and c-Jun N-terminal kinase (JNK), have controlled the mechanism of LPS-stimulated inflammatory responses in macrophages. An increasing number of studies indicated a vital role of MAPKs in mediating the activation of nuclear factor kappa B (NF-xB) signal transducing pathway, and a role of MAPKs in inflammatory correlated diseases including Alzheimer disease, rheumatoid arthritis, and autoimmune diseases. Activated protein kinase B (Akt), also termed as serine/threonine kinase B, plays an essential role in order to initiate the cellular process including apoptosis, migration of cell, survival of the cell, and also the stimulation of NF-xB. Therefore, the anti-inflammatory agent screening tool has paid much attention to the downregulation of these inflammatory mediators.

Centella asiatica (L.) Urban (Apiaceae) has been widely used in folk medicine in Southeast Asia. Its major bioactive composition is madecassoside and asiaticoside. The pharmacological activity of C. asiatica has been shown to exhibit antioxidant, anti-inflammatory, neuroprotective and wound healing effects. A difficulty of the crude extracts of C. asiatica is its compound variation, leading to inconsistency of pharmacological responses. An attempt to establish a pharmacological standard of the crude extract as a standardized extract of C. asiatica ECa 233 has been made to circumvent this problem. ECa 233 contained madecassoside and asiaticoside in concentration at least 80%. The proportion of madecassoside and asiaticoside were kept in 1.5 ± 0.5, and wound healing effects were kept in 1.5 ± 0.5. Previous work has addressed the beneficial effect of ECa 233 on wound healing and skin inflammation (unpublished data); however, the effect on inflammatory responses remains an issue of ambiguity. Therefore, the present study aims to investigate the anti-inflammatory effect of ECa 233 and the mechanism of action against LPS-induced macrophages.

MATERIALS AND METHODS

Cell Culture and Reagent RAW264.7 cell was purchased from the American Type Culture Collection (Manassas, VA, U.S.A.). RAW264.7 cells were grown in completed Dulbecco's modified Eagle's medium (DMEH) (Gibco, OK, U.S.A.) con-
RAW264.7 cells were treated with 0, 0.1, 1, 10, 100 and 1000 µg/mL of ECa 233 for 24, 48 and 72 h. Values are expressed as mean ± standard error of the mean (S.E.M.) for four independent experiments. The difference among groups were performed by using one-way ANOVA. *p < 0.01 compared to the normal control group.

RAW264.7 macrophages were exposed with LPS (1 µg/mL) in the presence or absence of various concentrations of ECa 233 (0.1, 1, 10 and 100 µg/mL) and dexamethasone (1 µg/mL) for 24 h. Intracellular ROS production was determined by DCFH-DA assay and the cytotoxicity was measured by using MTT assay. Values are expressed as mean ± S.E.M. for four independent experiments. The difference among groups were performed by using one-way ANOVA. *p < 0.001 compared to the LPS group.

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to examine COX-2 and iNOS protein expression, the cells (1 × 10⁶ cells/plate) were treated with LPS (1 µg/mL) in the presence or absence of 0.1, 1, 10 and 100 µg/mL of ECa 233 and dexamethasone (1 µg/mL) for 24 h. For p38, ERK1/2, Akt and NF-κB protein expression, RAW264.7 cells (1 × 10⁶ cells/plate) were treated with LPS (1 µg/mL) for 1 h in with or without ECa 233 (0.1, 1, 10 and 100 µg/mL) and dexamethasone (1 µg/mL). RAW264.7 macrophages were prepared in RIPA buffer containing protease inhibitor cocktail on ice for 30 min. The protein concentration in each collected sample was assessed by the Bradford protein assay (Bio-Rad, CA, U.S.A.). Proteins (75 µg) were subjected to 8–10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes. The membranes were blocked and incubated with primary antibody against Akt (1:1000), NF-κB (1:1000), ERK1/2 (1:1000), p38 (1:1000), iNOS (1:1000), and COX-2 (1:200) overnight. Then, they were incubated with HRP-conjugated secondary antibodies for 1 h at room temperature. Specific bands were visualized by chemiluminescence (Supersignal West Pico; Pierce Biotechnology, IL, U.S.A.).

**Statistical Analysis** All experiment data were presented as mean ± standard error of results. One-way ANOVA was utilized for testing the differences of the mean values between three or more groups and pairwise comparison between groups was used by Tukey post hoc test. Values of p < 0.05 were considered to be statistically significant.

**RESULTS**

**Effect of ECa 233 on RAW264.7 Cell Viability** We first determined an effect of ECa 233 on the viability of RAW264.7 macrophages by using MTT assay. As shown in Fig. 1, ECa 233 at a concentration (0.1–100 µg/mL) did not suppress the viability of RAW264.7 macrophages after 24, 48 and 72 h treatment but significant decrease of cell viability was shown by ECa 233 at 1000 µg/mL after 72 h. These results indicated that ECa 233 at 0.1–100 µg/mL was not toxic to the RAW264.7 macrophages.

**Effect of ECa 233 on LPS-Induced ROS Production in RAW264.7 Macrophages** Treatment of RAW264.7 macrophages with LPS alone caused a significant increase in an intracellular ROS approximately 197.65% of control (Fig. 2A). After treatment with ECa 233 at all concentration (0.1–100 µg/mL), the generation of ROS was significantly decreased (Fig. 2A). We further determined the cytotoxicity of LPS in the presence or absence of 0.1, 1, 10 and 100 µg/mL of ECa 233 and dexamethasone (1 µg/mL) by MTT assay. The results showed that the cell viability was not significantly decreased by treatment with ECa 233 and dexamethasone for 24 h in the presence of 1 µg/mL of LPS (Fig. 2B). These results indicate that the suppression of ROS was not due to chemically activated cytotoxicity.

**ECa 233 Inhibits LPS-Induced NO and PGE₂ Generation, iNOS and COX-2 Protein Expression in RAW264.7 Macrophages** NO level significantly increased after the
treatment of RAW264.7 macrophages with LPS (1 µg/mL) alone. Interestingly, ECa 233 at 1, 10 and 100 µg/mL and dexamethasone (1 µg/mL) induced a significant decline in the nitrite content, an index of NO in LPS-induced RAW264.7 macrophages (Fig. 3A). We further determined the production of PGE2 level in LPS-stimulated RAW264.7 macrophages. The PGE2 production was found to be decreased after the administration of ECa 233 (10 and 100 µg/mL) and dexamethasone (Fig. 3B). Upon the stimulation of RAW264.7 macrophages with LPS, the expressions of iNOS and COX-2 were significantly upregulated as demonstrated in Figs. 3C and D. ECa 233 at 1, 10 and 100 µg/mL and dexamethasone significantly diminished the expression of iNOS in the LPS-stimulated RAW264.7 macrophages (Figs. 3C, D). Besides, ECa 233 at the concentrations 10 and 100 µg/mL and also dexamethasone could suppress COX-2 expression in the LPS-stimulated RAW264.7 macrophages (Figs. 3C, D). These results suggest that the anti-inflammatory activity of ECa 233 via the reduction of PGE2 and NO production, and subsequently inhibited COX-2 and iNOS protein expression.

**ECa 233 Suppressed LPS-Induced TNF-α and IL-1β Level in RAW264.7 Macrophages** We next attempted to examine an inhibitory effect of ECa 233 towards the proinflammatory cytokines in the LPS-induced RAW264.7 macrophages. As shown in Figs. 4A and B, IL-1β and TNF-α secretion significantly increased upon the administration with 1 µg/mL of LPS in RAW264.7 macrophages. All concentration of ECa 233 (0.1–100 µg/mL) and dexamethasone have significantly inhibited the release of IL-1β and TNF-α in LPS stimulated RAW264.7 macrophages (Figs. 4A, B). Therefore, this result implied that ECa 233 could diminish the inflammatory response in LPS-induced RAW264.7 macrophages by suppressing the proinflammatory cytokines IL-1β and TNF-α production.

**ECa 233 Suppressed LPS-Induced Inflammation via Inhibiting p38 and ERK1/2 Signaling in RAW264.7 Macrophages** An involvement of the p38 and ERK1/2 pathway in the LPS-induced inflammation was examined. In RAW264.7 macrophages treated with LPS, the phospho-ERK1/2 and phospho-p38 levels were significantly increased after 1 h of treatment without affecting the total protein level (Figs. 5A, B). Interestingly, treatment with ECa 233 (10 and 100 µg/mL) and dexamethasone significantly suppressed LPS-induced increasing levels of phospho-ERK1/2 and phospho-p38 (Figs. 5A, B), confirming the anti-inflammatory effects of ECa 233 on LPS-induced inflammation by suppressed ERK1/2 and p38 MAPK signaling pathway.

**Effect of ECa 233 on Akt and NF-κB Signaling Pathway in LPS-Induced RAW264.7 Macrophages** The activation of Akt and NF-κB is critically affected in the inflammation progression and involves in the inflammatory cytokines production. We thus examined the effect of ECa 233 on the...
expressions of Akt and NF-κB in LPS-induced RAW264.7 macrophages. As shown in Figs. 6A and B, the expression of p-Akt and p65 NF-κB proteins increased in LPS-stimulated RAW264.7 macrophages. Interestingly, ECa 233 (10 and 100 µg/mL) and dexamethasone significantly decreased the elevating level of p-Akt and p65 NF-κB proteins expression in LPS-stimulated RAW264.7 macrophages (Figs. 6A, B). These results indicated the anti-inflammatory effects of ECa 233 via the suppression of Akt and NF-κB inflammatory signaling pathway.

DISCUSSION

Chronic inflammation is closely related to the initiation and progression of many diseases such as cancer, arteriosclerosis, diabetes, obesity, and neurodegenerative diseases. During inflammation, inflammatory mediators including ROS, NO, PGE₂, IL-1β, and TNF-α were released after LPS-stimulated macrophages against the invading pathogens. In addition, prolonged energizing of the macrophage may cause severe life-threatening disorders. Accordingly, to inhibit abnormal activation and to maintain macrophages within their appropriate range of activity are essential for the prevention of chronic inflammatory diseases due to macrophages. An inhibitory effect of ECa 233 on the inflammatory mediators via the Akt, ERK1/2 and p38 MAPK pathways in LPS-activated RAW264.7 macrophages were thus examined.

It is widely known that endotoxin of bacteria activates the production of inflammatory negotiators including PGE₂, COX-2, NO, iNOS and proinflammatory cytokines (for example, TNF-α and IL-1β), which has a primary role during inflammatory responses. An innate immunity normally handles inflammatory response via prevention of invasive pathogen into the host. Still, overproduction of inflammatory mediators as well as their excessive activity could lead to various adverse effects such as septic shock, atherosclerosis, cancer and rheumatoid arthritis though. Moreover, the pharmacological inhibition of the inflammatory mediators is crucial in order to control the inflammatory response. Furthermore, PGE₂ production and expression of COX-2 were proposed as another therapeutic alternative in inflammatory disease treatment. By using RAW264.7 macrophages, we examined whether ECa 233 attenuated the inflammatory reaction through diminishing PGE₂ generation and expression of COX-2. Intriguingly, we established that ECa 233 successfully attenuated the COX-2 protein expression in LPS-induced mac-
Besides, we also found that ECa 233 decreased the LPS-induced PGE\(_2\) generation in macrophages, which may be possibly due to the abolition of COX-2 at protein levels. Similar to COX-2, iNOS is a crucial molecule of inflammatory for NO synthesis.\(^{26}\) Based on our report, administration with ECa 233 remarkably restricted the release of NO by downregulating the iNOS expression at protein levels. Consequently, our study proved that treatment with ECa 233 inhibited the release of TNF-\(\alpha\) and IL-1\(\beta\) in LPS-induced macrophages. Potential inhibitory effects of ECa 233 related to the suppression of NF-\(\kappa\)B, ERK1/2, p38 and Akt pathways in LPS-activated macrophages. In this manner, we additionally examined the downregulatory effects of ECa 233 on NF-\(\kappa\)B signaling pathway and that the major valuable pathway in order to control the inflammatory response. The inflammatory negotiator’s expression, including cytokines involved during inflammation progression, was regulated by NF-\(\kappa\)B transcription factor.\(^{27,28}\) Besides NF-\(\kappa\)B signal transduction pathway, ECa 233 exhibited suppressive effects on Akt, ERK1/2 and p38 MAPK signaling occurrence. Akt signal transduction pathway was reported to be responsible for the proinflammatory molecules expression through the NF-\(\kappa\)B activation in LPS-triggered cell through numerous studies.\(^{29,30}\) For that reason, inhibition of the Akt phosphorylation has been identified as a primary key to handle inflammatory diseases. Hence, the stimulation of Akt, possibly correlated with NF-\(\kappa\)B activation in LPS-stimulated cells, could provide the generation of inflammatory signaling molecules.\(^{31}\) Significantly, this study found that Akt phosphorylation was remarkably decreased by the treatment

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**Fig. 6. Effect of ECa 233 on Akt (A) and NF-\(\kappa\)B (B) Signaling Pathway in LPS-Induced RAW264.7 Macrophages**

RAW264.7 cells were treated with LPS (1 \(\mu\)g/mL) for 1 h in with or without ECa 233 (0.1, 1, 10 and 100 \(\mu\)g/mL) and dexamethasone. The expression of Akt and NF-\(\kappa\)B protein were measured by using Western blot. Values are expressed as means ± S.E.M. (n = 4). *\(p<0.001\) compared to the normal control group, **\(p<0.001\) compared to the LPS group, #\(p<0.01\) compared to the LPS group.

**Fig. 7. Proposed Anti-inflammatory Mechanism of ECa 233**

ECa 233 inhibits LPS-induced inflammation by suppressing the Akt and NF-\(\kappa\)B pathways, leading to the downregulation of MAPKs.
of ECa 233 in LPS-triggered macrophages. Likewise Akt, the MAPKs signaling pathway was observed to control the inflammatory mediators’ expression by regulating the activity of NF-κB transcription in the LPS-activated cells.\(^3\,\text{,}\,^3\text{0}\,\text{,}\,^3\text{1}\,\text{,}\,^3\text{2}\) The unauthorised stimulation of MAPKs signal transduction pathway has been demonstrated to increase the possibility of chronic inflammatory diseases due to the inflammatory mediators’ activation.\(^2\,\text{,}\,^2\text{3}\) Moreover, intracellular signaling pathways including ERK1/2, p38 MAPKs together with NF-κB activation.\(^3\text{2}\,\text{,}\,^3\text{3}\) Furthermore, previous reports have also demonstrated that C. asiatica with anti-inflammatory properties were capable of inhibiting the LPS-triggered the secretion of cytokines through abolishing the activity of MAPKs signaling in macrophages.\(^3\text{0}\,\text{,}\,^3\text{4}\) Combined all supportive statements together led to our established part in the stimulation of inflammatory cytokines production including ERK1/2, p38 MAPKs together with NF-κB.

**Conclusion**

Our present study demonstrated the anti-inflammatory effect of ECa 233 in LPS-induced RAW264.7 macrophages through the selective suppression of ERK1/2, p38 MAPK, Akt and NF-κB signaling pathway (Fig. 7). ECa 233 possessed therapeutic beneficial for inflammatory disorders; however further investigations on other cellular models such as human macrophages are still needed to explore for the additional information on the specific molecular mechanisms responsible for the anti-inflammatory effect of ECa 233. In addition, further studies are required to explore in vivo and clinical trials efficacy thereafter ECa 233 could be developed as an effective anti-inflammatory agent candidate.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


