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Report

Cyanidin-3-Glucoside Attenuates aP2 Expression Independent of PPRE-Mediated Transcription in Macrophage Cells

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Cyanidin-3-glucoside (C3G) is a major anthocyanin present in various berries and pigmented grains and has been reported to exhibit antioxidant and anti-inflammatory activities. In the present study, we examined the effects of C3G on inflammatory responses in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages, with particular attention to adipocyte protein 2 (aP2), a lipid-associated protein implicated in inflammation. Treatment with C3G at concentrations up to 50 μM did not affect cell viability in the presence or absence of LPS. Quantitative real-time PCR analysis demonstrated that C3G significantly suppressed LPS-induced expression of aP2, as well as the pro-inflammatory cytokines monocyte chemoattractant protein-1 (MCP-1) and interleukin-6 (IL-6). Furthermore, luciferase reporter assays revealed that C3G inhibited LPS-induced aP2 promoter activity in a manner independent of the peroxisome proliferator-activated receptor response element (PPRE)-like site. These findings indicate that C3G attenuates macrophage inflammatory responses through the suppression of aP2 expression and pro-inflammatory cytokine production, suggesting a novel molecular basis for the anti-inflammatory action of C3G.

Key words cyanidin-3-glucoside, anthocyanins, inflammation, RAW264.7, aP2, cytokines

INTRODUCTION

Cyanidin-3-glucoside (C3G) is a naturally occurring anthocyanin widely distributed in dark-colored plant-derived foods, including black rice, black beans, and haskap berries. As a bioactive flavonoid, C3G has attracted pharmacological interest due to its reported antioxidant and anti-inflammatory properties, as well as beneficial effects on glucose and lipid metabolism.[1-4] However, despite these reported bioactivities, the molecular mechanisms underlying the anti-inflammatory actions of C3G remain incompletely understood.

Macrophages are key effector cells in innate immunity and play a central role in the development of inflammatory responses associated with infectious stimuli and metabolic disorders. Activation of macrophages by lipopolysaccharide (LPS) induces the production of pro-inflammatory mediators, including monocyte chemoattractant protein-1 (MCP-1), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), and contributes to chronic inflammation observed in obesity and related metabolic diseases.[5-7]

Peroxisome proliferator-activated receptors (PPARs) are ligand-activated nuclear receptors that regulate genes involved in lipid metabolism, glucose homeostasis, and inflammatory signaling. Among the PPAR isoforms, PPARγ plays a pivotal role in metabolic regulation and controls the expression of adipocyte protein 2 (aP2), also known as fatty acid–binding protein 4 (FABP4).[8-13] aP2 functions as a lipid-binding protein linking metabolic pathways to inflammatory responses and is expressed in macrophages as well as adipocytes, suggesting that aP2 contributes to macrophage-driven inflammation and the pathogenesis of metabolic disorders.[13]

In the present study, we investigated the effects of C3G on aP2 expression and inflammatory mediator production in LPS-stimulated RAW 264.7 macrophages to clarify the molecular mechanisms underlying C3G-mediated regulation of macrophage activation and its pharmacological relevance as a bioactive compound with potential therapeutic utility in inflammation associated with metabolic disorders. While previous studies have primarily focused on the antioxidant and anti-inflammatory properties of C3G, little attention has been paid to its direct transcriptional regulation of aP2. Here, we provide a novel perspective by demonstrating that C3G suppresses both aP2 gene expression and aP2 promoter activity, suggesting that its anti-inflammatory effects are mediated,

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at least in part, through a previously unrecognized transcriptional regulatory mechanism that is distinct from the currently accepted antioxidant signaling pathways.

MATERIALS AND METHODS

Material Cyanidin-3-glucoside-chloride (C3G) was purchase from Nagarasience (Gifu, Japan). Lipopolysaccharides (LPS) was from Escherichia coli 0111:B4 was from Sigma-Aldrich. Dulbecco's modified Eagle's medium (DMEM) was from Sigma-Aldrich.

Cell Culture and Viability The RAW 264.7 macrophage cell line was grown in DMEM supplemented with 10% FBS, penicillin (100U/ml), and streptomycin sulfate (100 μg/ml) in 5% CO_2. Cells were seeded in 96-well plates at $1x10^4$ cells per well in 100 μL of culture medium and allowed to adhere overnight. Compounds were added to the medium, and the cells were incubated for a future 24 h in the presence of absence of LPS. Cell proliferation was measured using an MTT assay kit (Roshe diagnostics) as described before.[14)]

RNA Isolation and Quantitative PCR The conditions for RT-PCR reactions and the data analysis were performed as described previously.[14)] The primer sequences used for real-time PCR are as follows, aP2: 5'-CATGGCCAAGCCCAACAT-3'and 5'-CGCCCACTTTGAAGGAAATC-3', IL-6: 5'-CCACGGCCTTCCCTACTTC-3' and 5'- TTGGGAGTGGTATCCTCTGTGA-3', MCP-1:5'-CCACTCACCTGCTGCTACTCAT-3' and 5'- TGGTGATCCTCTTGTAGCTCTCC-3' β-actin: 5 -CAGCCTTCC TTCTTGGGTATG G-3 and 5 -CTGTGTTGGCATAGAGGTCTTTAC G-3.

Plasmid Construction and Luciferase Reporter Assay The aP2-Luc (SV40-) reporter vector was generated from aP2 luciferase (addgene.org Plasmid #8858) by restriction enzyme digestion and subsequent ligation steps. The ΔaP2-Luc (SV40−) vector was generated by deleting the site (−518 to −320) containing the PPRE-like sequence from the aP2-Luc (SV40-) plasmid. For reporter assay, the cells were seeded in 24 well plates, and next day cells were transfected with aP2-Luc (SV40-) or PPRE X3-TK-Luc (addgene.org Plasmid #1015) using TransIT LT-1 reagent (Takara). Five hours after transfection, cells were treated with the indicated concentration of C3G. Cells were assayed for luciferase activity using the dual luciferase assay system (Promega) for 24 h after treatment of C3G and LPS.

Statistical Analysis The results are presented as the means of at least triplicate determinations ± standard deviation. Differences between two groups were evaluated using Tukey's multiple comparison test. Values of $p<0.05$ were considered significant.

RESULTS

Effect of C3G on RAW264.7 Cell Viability The cytotoxic effects of cyanidin-3-glucoside (C3G) on RAW264.7 macrophages were first evaluated in the presence or absence of lipopolysaccharide (LPS). As shown in Fig. 1A and Fig. 1B, treatment with C3G over a wide concentration range did not significantly affect cell viability under either basal conditions or following stimulation with LPS (100 ng/mL). These results indicate that C3G does not exert detectable cytotoxic effects on RAW264.7 cells at the concentrations used in subsequent experiments (Fig. 1A and B).

Suppression of LPS-Induced aP2 Expression by C3G Next, the effect of C3G on adipocyte protein 2 (aP2) expres-

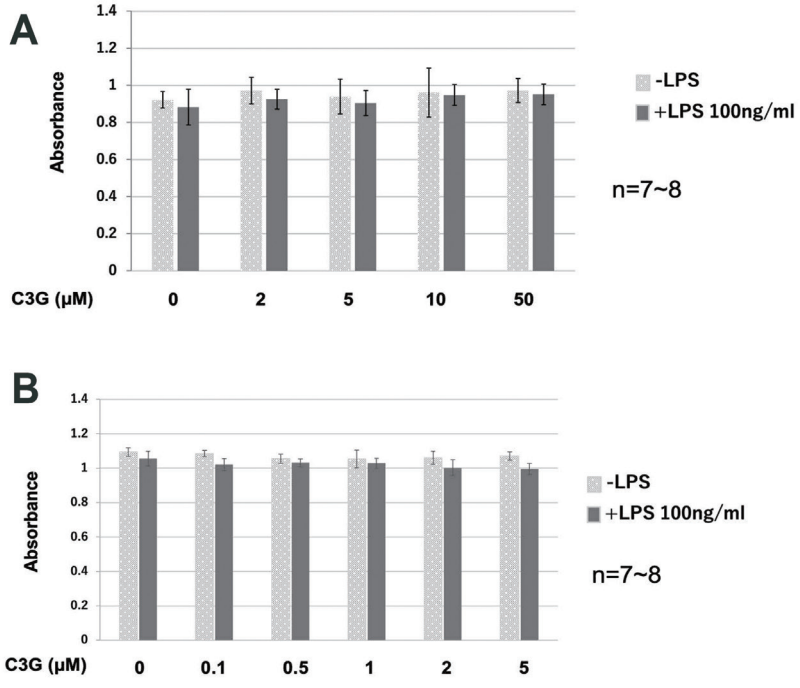

Fig. 1. Effect of Cyanidin-3-Glucoside (C3G) on RAW264.7 Cell Viability

RAW264.7 macrophages were treated with the indicated concentrations of C3G in the presence or absence of LPS (100 ng/ml). Cell viability was assessed as described in Materials and Methods. (A) Effects of C3G (0-50 μM) on cell viability with or without LPS (100 ng/ml). (B) Effects of C3G (0-5 μM) on cell viability in the presence of LPS (100 ng/mL). Data are expressed as mean ± S.D. (n=7~8). No significant cytotoxicity was observed at any concentration tested.

sion in LPS-stimulated RAW264.7 cells was examined. As shown in Fig. 2, LPS stimulation markedly increased aP2 expression compared with unstimulated controls. Treatment with C3G significantly attenuated LPS-induced aP2 expression, with significant suppression observed at concentrations between 2 and 5 μM. C3G with higher concentrations of 10μM did not show significant suppressive effect.

Inhibitory Effects of C3G on aP2 Promoter Activity To investigate whether C3G regulates aP2 expression at the transcriptional level, luciferase reporter assays were performed using an aP2 promoter construct. As shown in Fig. 3B, LPS stimulation increased aP2 promoter activity, whereas C3G treatment significantly suppressed this activity at the concentration of 0.5 μM. To further clarify the involvement of the peroxisome proliferator-activated receptor response element (PPRE)-like site, a deletion construct lacking the PPRE-like site (ΔaP2 promoter) was examined. As shown in Fig. 3C, ΔaP2 promoter was also activated by LPS stimulation and C3G also significantly suppressed ΔaP2 promoter activity, indicating that the inhibitory effect of C3G on aP2 transcription is independent of the PPRE-like site.

Lack of Effect of C3G on PPRE-Driven Transcriptional Activity To further assess whether C3G directly modulates PPAR-mediated transcriptional activity, a PPRE (×3) reporter construct, which is containing three tandem copies of the PPAR response element (PPRE), was examined. As shown in Fig. 4, LPS stimulation activated PPRE (×3) reporter in RAW264.7 cells. However, C3G treatment did not significantly alter PPRE-driven luciferase activity in LPS-stimulated RAW264.7 cells. These results suggest that C3G does not directly inhibit aP2 transcriptional activity through the PPRE and that its suppressive effect on aP2 expression is mediated through PPRE-independent mechanisms.

Effects of C3G on Pro-Inflammatory Cytokine Expression Finally, the effects of C3G on the expression of pro-inflammatory cytokines were examined. As shown in Fig. 5, LPS stimulation significantly increased the mRNA expression levels of interleukin-6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1). Treatment with C3G significantly suppressed the LPS-induced expression of both IL-6 and MCP-1. These results indicate that C3G attenuates inflammatory cytokine production in activated macrophages.

DISCUSSION

In the present study, we demonstrated that C3G attenuates inflammatory responses in LPS-stimulated RAW264.7 macrophages through aP2 expression and pro-inflammatory cytokine production. Importantly, these effects were observed at concentrations of C3G that did not affect cell viability, indicating that the anti-inflammatory actions of C3G are not attributable to cytotoxic effects.

To elucidate the mechanism underlying the suppression of aP2 expression, we examined the effects of C3G on aP2 promoter activity. Luciferase reporter assays revealed that C3G inhibited aP2 promoter activity not only in the full-length promoter construct but also in a deletion construct lacking the PPRE-like site. Furthermore, C3G did not suppress transcriptional activity driven by a PPRE reporter construct. These results indicate that the inhibitory effect of C3G on aP2 transcription occurs independently of PPAR-mediated signaling through the PPRE.

Furthermore, C3G significantly suppressed aP2 expression at 1-5 μM, but no effect was observed at higher concentrations. In addition, in the promoter activity assay, an inhibitory effect was observed at lower concentrations of 0.5 μM-1 μM, but no effect was observed at higher concentrations. This is because the promoter activity assay expression level is different from that of full aP2.

aP2 expression is increased by elevated PPARγ, which is thought to be due to the binding of transcription factors to the PPRE site.[8,10,12] However, the downregulation of aP2 expression by C3G was not related to the PPRE-like site. The increase in aP2 expression due to LPS stimulation is thought to be due to a mechanism similar to that of other inflammatory cytokines. In other words, it may be due to a different mechanism from the increase in PPARγ caused by fat accumulation.

The present findings extend these observations by identifying aP2 as a downstream target of C3G in macrophages and by demonstrating that C3G suppresses aP2 expression via a PPRE-independent transcriptional mechanism. Although the precise transcriptional regulators involved remain to be identified, it is plausible that C3G modulates aP2 expression through alternative promoter elements or transcription factors responsive to inflammatory stimuli. Future studies should aim to identify the transcription factors involved in C3G-mediated regulation.

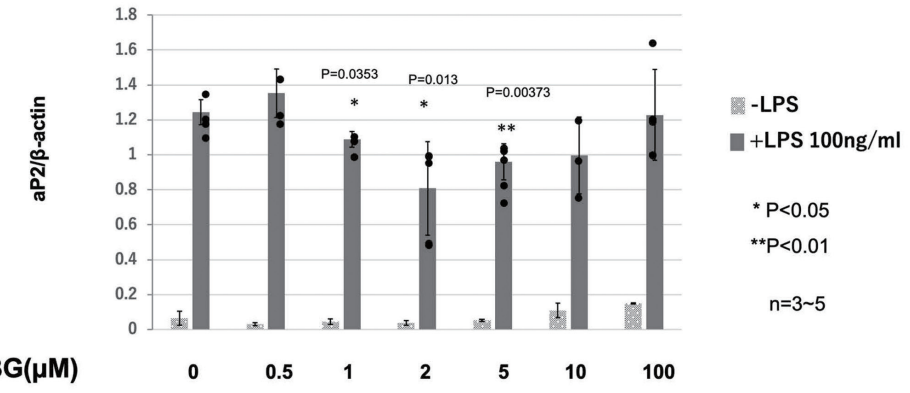

Fig. 2. Suppression of LPS-Induced aP2 Expression by C3G in RAW264.7 Cells

RAW264.7 macrophages were pretreated with the indicated concentrations of C3G and then stimulated with LPS (100 ng/mL) after 1h incubation. After 24 h, aP2 expression was analyzed and normalized to β-actin. Data are expressed as mean ± S.D. (n=3~5). *$p < 0.05$, **$p < 0.01$ versus LPS-treated control.

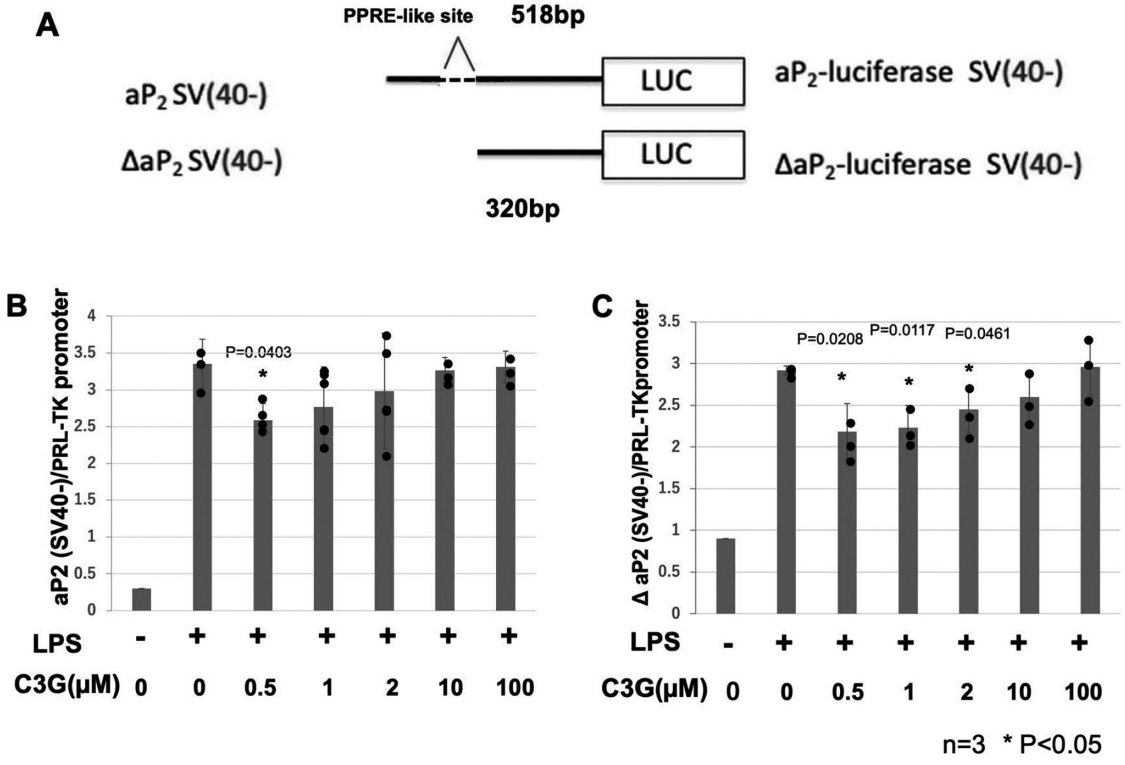

Fig. 3. Effects of C3G on aP2 Promoter Activity

(A) Schematic representation of the aP2 promoter and ΔaP2 promoter constructs. RAW264.7 cells were transfected with (B) the aP2 promoter construct or (C) the ΔaP2 promoter construct lacking the PPRE-like site and then treated with the indicated concentrations of C3G, followed by LPS (100 ng/ml) stimulation. After 24 h incubation, luciferase activity was measured and normalized to Renilla luciferase activity. Data are expressed as mean ± S.D.(n=3). *p < 0.05 versus LPS-treated control.

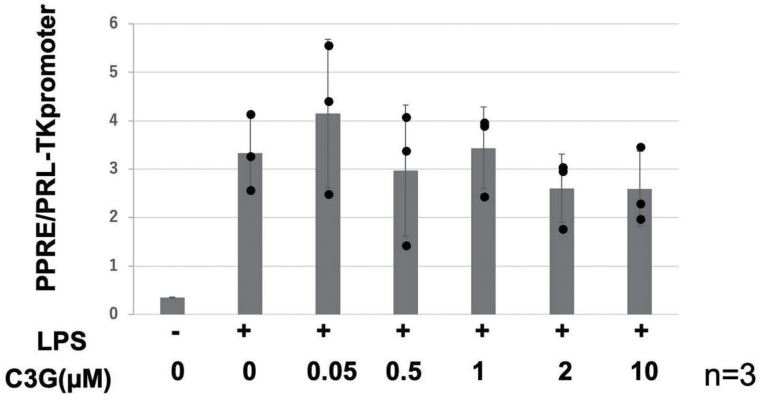

Fig. 4. Effect of C3G on PPRE-Driven Transcriptional Activity

RAW264.7 cells were transfected with a PPRE (×3) luciferase reporter construct and treated with the indicated concentrations of C3G in the presence of LPS. Luciferase activity was measured and normalized to Renilla luciferase activity. Data are expressed as mean ± S.D. (n=3). C3G did not significantly affect PPRE-driven transcriptional activity.

In addition to suppressing aP2 expression, C3G significantly reduced LPS-induced expression of the pro-inflammatory cytokines IL-6 and MCP-1 in our results. Given the established role of these cytokines in macrophage-driven inflammation, the concurrent suppression of aP2 and inflammatory mediators suggests that regulation of aP2 expression may contribute to the anti-inflammatory effects of C3G in RAW macrophages. The present findings indicate that C3G inhibits IL-6 and MCP-1 expression at concentration that do not significantly affect aP2 expression, suggesting that C3G initially suppresses inflammatory signaling through aP2-independent mechanisms. One possible explanation is that low concentration of C3G directly modulate inflammatory signaling pathways, such as NF-kB or MAPK, thereby rapidly reducing cytokine gene expression. At higher concentrations, C3G additionally suppresses aP2 transcription, which may contribute to sustained inhibition of inflammatory responses, chronic inflammation.

In conclusion, the present study demonstrates that C3G suppresses inflammatory responses in LPS-stimulated RAW macrophages through inhibition of aP2 expression independ-

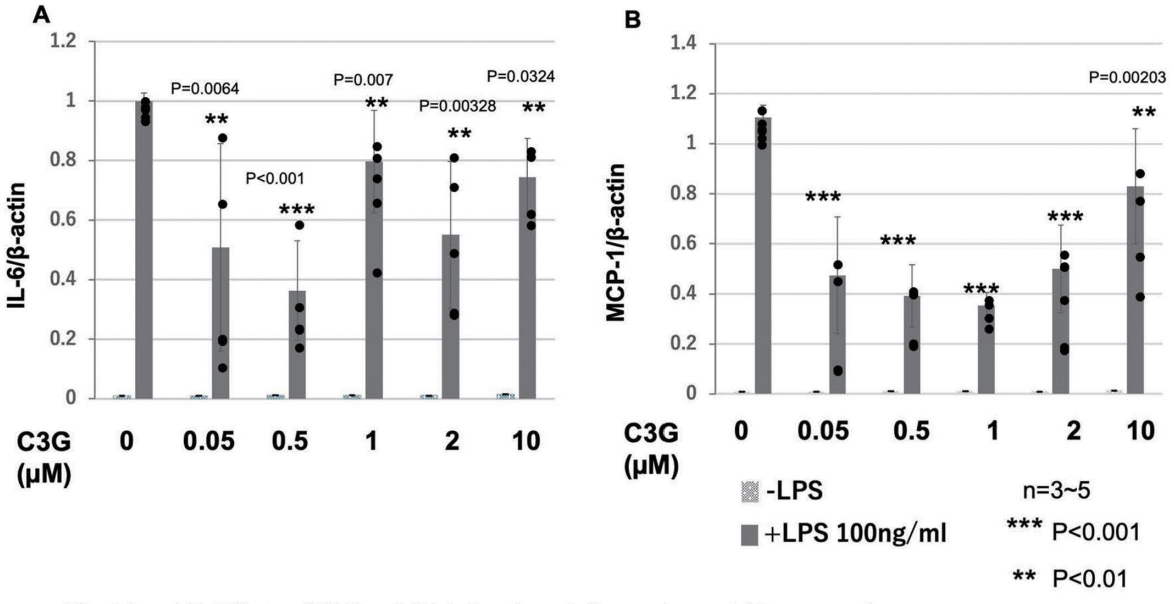

Fig. 5. Effects of C3G on LPS-Induced Pro-Inflammatory Cytokine Expression

RAW264.7 macrophages were treated with the indicated concentrations of C3G and stimulated with LPS. mRNA expression levels of IL-6 (A) and MCP-1 (B) were determined by quantitative real-time PCR and normalized to β-actin. Data are expressed as mean ± S.D. (n=3~5). *p < 0.05 versus LPS-treated control.

ent of PPRE-mediated transcriptional regulation. These findings provide novel pharmacological insight into the molecular mechanisms underlying the anti-inflammatory actions of C3G and suggest its potential utility as a bioactive compound for the modulation of inflammation associated with metabolic disorders. Further investigations using additional immune cell types and animals are required to establish the generalizability of C3G-mediated immunomodulatory effects.

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Conflict of interest The authors declare no conflict of interest.

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