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Annatto Tocotrienol Attenuates NLRP3 Inflammasome Activation in Macrophages

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Abstract

Accumulating evidence suggests that aberrant innate immunity is closely linked to metabolic diseases, including type 2 diabetes. In particular, activation of the NOD-like receptor family pyrin domain–containing 3 (NLRP3) inflammasome and subsequent secretion of interleukin 1β (IL-1β) are critical determinants that precipitate disease progression. The seeds of annatto (*Bixa orellana L.*) contain tocotrienols (T3s), mostly (>90%) in the δ form (δT3). The aim of this study was to determine whether annatto T3 is effective in attenuating NLRP3 inflammasome activation in macrophages. Our results showed that annatto δT3 significantly attenuated NLRP3 inflammasome by decreasing IL-1β reporter activity, IL-1β secretion, and caspase-1 cleavage against lipopolysaccharide (LPS) followed by nigericin stimulation. With regard to mechanism, annatto δT3 *1*) reduced LPS-mediated priming of the inflammasome and *2*) dampened reactive oxygen species production, the second signal required for assembly of the NLRP3 inflammasome in macrophages. Our work suggests that annatto δT3 may hold therapeutic potential for delaying the onset of NLRP3 inflammasome–associated chronic metabolic diseases. *Curr Dev Nutr* 2017;1:e000760.

Introduction

Inflammasomes are gatekeepers of the innate immune system that sense dangerous molecular patterns of microbial infection for the production of the proinflammatory cytokine IL-1β. The inflammasome is also activated by endogenous damage–associated molecular patterns, such as cholesterol crystals, ATP, FFAs, and ceramides, resulting in propagation of inflammation to systemic levels (1). Inflammasome activation has been implicated in various inflammatory diseases. In particular, the activation of the NOD-like receptor family pyrin domain–containing 3 (NLRP3) inflammasome has been recognized as a molecular culprit that exacerbates chronic inflammatory diseases such as diabetes (2, 3). The activation of the NLRP3 inflammasome requires 2-step signaling: *1*) the priming step for NF-κB signaling through pattern recognition receptors, resulting in transcriptional activation for inflammasome scaffold proteins and pro–IL-1β, and *2*) the post-transcriptional step that activates the assembly of the inflammasome through reactive oxygen species (ROS) production, leading to proteolytic cleavage of caspase-1 for IL-1β secretion (4). Accordingly, bioactive molecules that inhibit inflammasome priming or suppress inflammasome assembly signals would be effective in mitigating NLRP3 inflammasome activation and IL-1β production.

Annatto (*Bixa orellana L.*), also known as achiote, is an indigenous plant in South America. The seeds of annatto have been used as a traditional medicine to cure infection as well as a food additive for orange coloring (5). Annatto seeds are a unique source of naturally occurring tocotrienol (T3), a member of the family of unsaturated vitamin E. Annatto T3 is almost exclusively found in the δ isoform (δT3), whereas δT3 is a relatively minor fraction compared with αT3 and γT3 in other sources of T3 (e.g., palm and rice bran oil) (6). There is increasing evidence that annatto δT3 exerts health benefits against inflammation (7), but its immunomodulatory function is unknown. We previously showed that γT3, an unsaturated

Keywords: annatto, delta-tocotrienol, NLRP3 inflammasome, IL-1β, ROS production

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Supplemental Methods are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://cdn.nutrition.org.

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Abbreviations used: iGLuc, IL-1β–Gaussia luciferase fusion construct; iJ774, J774 macrophage stably expressing iGLuc reporter construct; LPS/Ng, LPS followed by nigericin; NLRP3, NOD-like receptor family pyrin domain–containing 3; ROS, reactive oxygen species; T3, tocotrienol.

form of vitamin E, suppresses NLRP3 activation in murine macrophages and leptin receptor knockout mice, thereby alleviating the symptoms of type 2 diabetes (8). γT3 and δT3 possess similar molecular characteristics and exert strong potency in downregulating inflammation and oxidative stresses compared with αT3 (7, 9). Given the high availability and easy preparation of δT3 from annatto plants, it is of interest to determine whether δT3 is capable of suppressing NLRP3 inflammation for therapeutic application. The aim of this study was to determine whether annatto δT3 inhibits the NLRP3 inflammasome and to compare its efficacy with palm γT3. Herein, we report that annatto δT3 is a bioactive dietary source to suppress NLRP3 inflammasome activation.

Methods

Annatto δT3 (90% δT3) was provided by American River Nutrition, and γT3 (>90%) was provided by Carotech. The experimental details are shown in **Supplemental Methods**.

All of the data are presented as means ± SEMs. The data were statistically analyzed by using either Student's *t* test or 1-factor ANOVA with Tukey's multiple-comparison tests. $P < 0.05$ was regarded as significant. All of the analyses were performed with GraphPad Prism 6 (version 6.02).

Results

Annatto δT3 inhibits NLRP3 inflammasome activation in J774 macrophage stably expressing iGLuc reporter construct (iJ774) macrophages

To conduct the NLRP3 inflammasome reporter assay, iJ774 macrophages that stably overexpress an inflammasome reporter (hereafter referred to as iJ774; **Figure 1**A) (10) were pretreated with 1, 2.5, and 5 μM δT3 or vehicle control (DMSO). The NLRP3 inflammasome was stimulated by priming with LPS followed by nigericin (LPS/Ng). Pretreatment with 1–5 μM δT3 significantly decreased inflammasome reporter activity in a dose-dependent manner compared with the control (Figure 1B). Consistently, δT3 pretreatment markedly lowered IL-1β secretion in medium in iJ774 macrophages (Figure 1C). IL-1β–Gaussia luciferase fusion construct (iGLuc) protein is secreted from macrophages upon caspase-1 cleavage of pro–IL-1β. Confirming the NLRP3 inflammasome activation, treatment

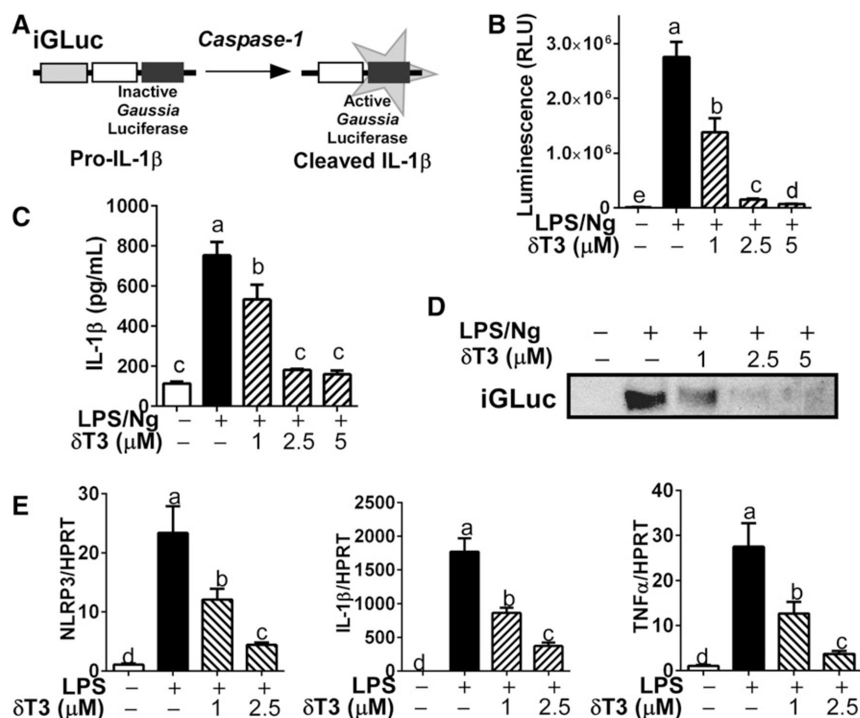

FIGURE 1 δT3 inhibited NLRP3 inflammasome activation. iJ774 macrophages were pretreated with δT3 (0, 1, 2.5, and 5 μM), and then stimulated with LPS/Ng. (A) Structure of the iGLuc (NLRP3 inflammasome and caspase activity reporter construct). (B) Relative Gaussia luciferase activity measured by luminometer. (C) IL-1β secretion in medium quantified by ELISA. (D) Released iGLuc fusion protein after caspase-1 activation. (E) RAW 264.7 macrophages were pretreated (1 or 2.5 μM) and stimulated with LPS (100 ng/mL) for 1 h. mRNA expression of *Nlrp3*, *Il1b*, and *Tnfa* were quantified by qPCR. Values in panels B, C, and E are means ± SEMs; n = 6–7. Means not sharing a common letter differ, $P < 0.05$ (1-factor ANOVA). Results shown in panel D are representative of triplicate samples. HPRT, hypoxanthine-guanine phosphoribosyltransferase; iGLuc, IL-1β–Gaussia luciferase fusion construct; iJ774, J774 macrophage stably expressing iGLuc reporter construct; LPS/Ng, LPS followed by nigericin; NLRP3, NOD-like receptor family pyrin domain–containing 3; RLU, relative luminescence unit; *Tnfa*, tumor necrosis factor α; δT3, δ-tocotrienol.

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with δT3 of >1 μM abolished iGLuc and IL-1β secretion in the medium (Figure 1C, D).

In our experimental setting, NF-κB activation occurs through Toll-like receptor 4 (TLR4) signaling, a pattern recognition receptor sensing LPS (11). To determine whether dose-dependent inhibition of the NLRP3 inflammasome by δT3 relies on the NF-κB priming step, we examined the effects of δT3 on NF-κB downstream target genes in RAW macrophages. qPCR results showed that mRNA gene expression of *Nlrp3*, tumor necrosis factor α (*Tnfa*), and *Il1b* was significantly decreased in a dose-dependent manner (Figure 1E). Taken together, these data show that annatto δT3 is effective in inhibiting LPS/Ng-mediated NLRP3 inflammasome activation by effectively attenuating the NF-κB priming step.

δT3 is effective in blocking inflammasome priming and assembly

To further understand the mechanism, we investigated the role of annatto δT3 on inflammasome priming and ROS production, an

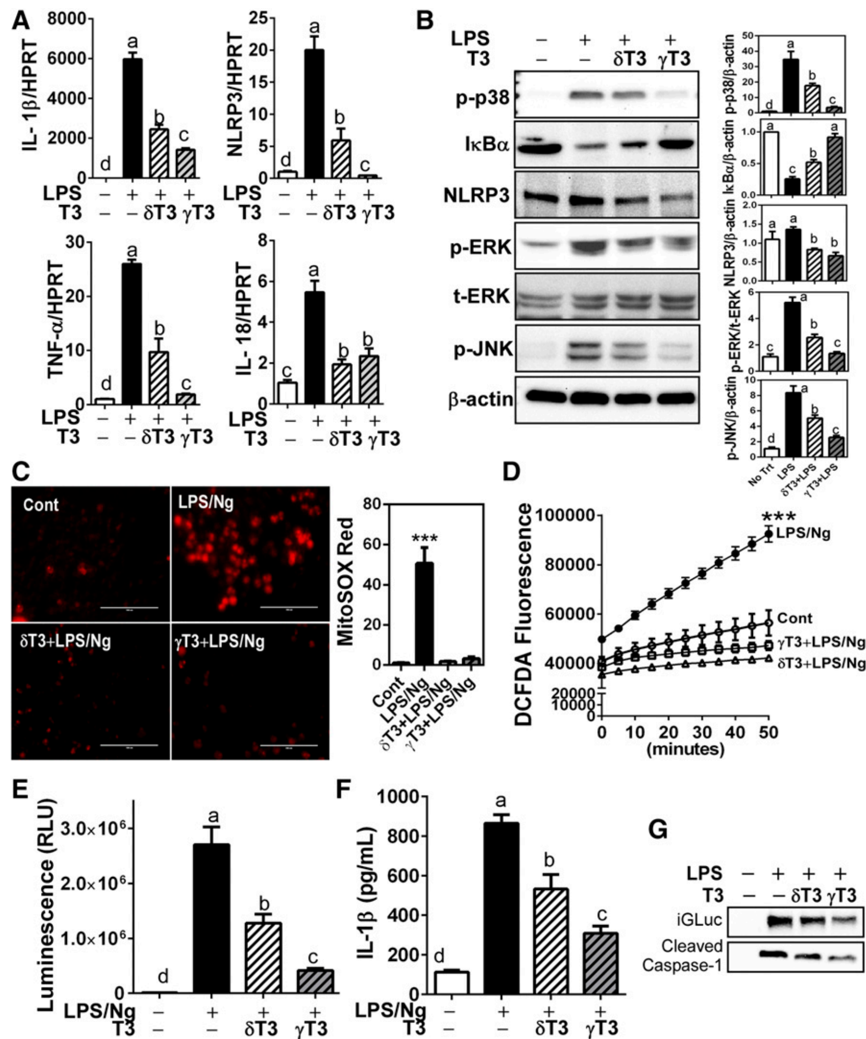

FIGURE 2 δT3 inhibits NLRP3 inflammasome priming and ROS production in macrophages. RAW (A–D) or iJ774 (E–G) macrophages were pretreated with 1 μM of either δT3 or γT3, then stimulated with LPS alone (A, B) or LPS/Ng (C–G). (A) Proinflammatory gene expression of *Nlrp3*, *Il1b*, *Tnfa*, and *Il18* by qPCR analysis (n = 6). (B) Western blot analysis of MAPKs of p-p38, p-ERK, p-JNK, IκBα degradation, and NLRP3. Relative intensity was quantified by Image J software (NIH; n = 3). (C) ROS production by MitoSOX Red (left panels) and quantification of relative fluorescence intensity (right panel; n = 5). (D) Cellular ROS production detected by DCFDA fluorescence (n = 8). (E) Relative Gaussia luciferase activity (n = 6). (F) IL-1β secretion in medium quantified by ELISA (n = 6). (G) Released iGLuc fusion protein and cleaved caspase-1 by Western blot analysis. Results are shown as means ± SEMs. Means not sharing a common letter differ, $P < 0.05$ (1-factor ANOVA). Panels C and D: ***$P < 0.001$ compared with control (Student's t test). Cont, control; DCFDA, 2,7-dichlorofluorescin diacetate; HPRT, hypoxanthine-guanine phosphoribosyltransferase; iGLuc, IL-1β–Gaussia luciferase fusion construct; iJ774, J774 macrophage stably expressing iGLuc reporter construct; IκBα, inhibitor of κB; LPS/Ng, LPS followed by nigericin; NLRP3, NOD-like receptor family pyrin domain–containing 3; p-ERK, phosphorylated ERK MAPKinase; p-JNK, phosphorylated JNK MAPKinase; p-p38, phosphorylated p38 MAPKinase; RLU, relative luminescence unit; ROS, reactive oxygen species; t-ERK, total levels of ERK MAPKinse; *Tnfa*, tumor necrosis factor α; Trt, treatment; T3, tocotrienol; δT3, δ-tocotrienol; γT3, γ-tocotrienol.

assembly signal, and compared its efficacy with 1 μM palm γT3. Pretreatment with 1 μM annatto δT3 significantly decreased LPS (100 ng/mL)-mediated mRNA expression of *Nlrp3*, *Tnfa*, *Il18*, and *Il1b* compared with the control, but to a lesser extent than 1 μM palm γT3 (**Figure 2**A). In parallel, annatto δT3 treatment significantly reduced the following: *1*) LPS-mediated MAPK phosphorylation of phosphorylated ERK MAPKinase (p-ERK), phosphorylated p38 MAPKinase (p-p38), and phosphorylated JNK MAPKinase (p-JNK); *2*) degradation of inhibitor of κB (IκBα), a surrogate marker for NF-κB activation; and *3*) protein concentrations of NLRP3, the scaffold of inflammasome, but a lesser degree than γT3-treated cells (Figure 2B).

Next, we examined whether δT3 and γT3 exert different potency in attenuating ROS production, a common event required for second signaling for NLRP3 assembly (12–14). LPS/Ng stimulation caused a significant increase in ROS production, measured by MitoSOX Red (Molecular Probe) fluorescence. ROS production was dampened by both δT3 and γT3 (Figure 2C). To further quantify ROS quenching rate, RAW 264.7 macrophages were preloaded with 2,7-dichlorofluorescin diacetate (DCFDA), a dye that emits fluorescence upon oxidation by ROS. Consistent with the MitoSOX results, DCFDA fluorescence was significantly suppressed by pretreatment of either δT3 or γT3 to the nonstimulated concentrations (Figure 2D).

To compare NLRP3 inflammasome inhibitory function between the 2 T3 isoforms, iJ774 macrophages were pretreated with either δT3 or γT3, then stimulated with LPS/Ng. The extent to which δT3 inhibits NLRP3 inflammasome reporter activity was significantly lower than with γT3 (Figure 2E), which was also confirmed by IL-1β secretion, cleaved iGLuc protein, and cleaved caspase-1 in the medium (Figure 2F, G). Taken together, these results show that δT3 pretreatment in macrophages inhibits LPS/Ng-stimulated NLRP3 inflammasome activation, but γT3 exerts a stronger inflammasome inhibitory activity when it is normalized with 1 μM of γT3 concentration.

Discussion

Deregulation of innate immune responses and accompanied NLRP3 inflammasome activation in macrophages are key signaling events that perpetuate inflammation and expedite the onset of inflammatory disease conditions. Previously, we reported that γT3 supplementation is effective in inhibiting the NLRP3 inflammasome (8). Given the structural and functional similarities between γT3 and δT3, we tested the effectiveness of annatto δT3 in modulating the NLRP3 inflammasome in comparison with γT3. Here, we showed that annatto δT3 is a dietary source that effectively attenuates priming as well as assembly of the NLRP3 inflammasome. It is well documented that γT3 is proficient in the downregulation of MAPK and NF-κB activation (8, 15, 16). To the best of our knowledge, this is the first study to report that annatto δT3 has an immunomodulatory function to mitigate NLRP3 inflammasome activation in murine macrophages.

Recently, a pharmacokinetic study with high-dose annatto T3 showed that the maximum plasma δT3 concentrations were 1.4–1.6 μg/mL after 3–4 h of single administration of 750–1000 mg annatto δT3 in healthy men (17), which is equivalent to 3.5–4 μM. On the basis of these results, our experiment that used 1 μM annatto δT3 seems to be a reasonable and physiologically achievable concentration in humans. The acute annatto δT3 intake ≤1000 mg was reported as safe (17); however, further clinical trials are necessary to establish the safety of chronic supplementation of annatto δT3.

It is becoming more evident that targeting the NLRP3 inflammasome possesses therapeutic potential for the treatment of inflammation-mediated chronic diseases. Our results show that annatto δT3 significantly inhibits NLRP3 inflammasome activation and IL-1β production by attenuating NF-κB priming and ROS production. This suggests that annatto δT3 may constitute a cost-effective and practical approach to attenuate or delay the onset of chronic inflammatory diseases that require NLRP3 inflammasome activation for disease manifestation. Future research is warranted to confirm the effectiveness of the NLRP3 inflammasome by annatto δT3 in animal studies and in further human clinical trials.

Acknowledgments

The authors' responsibilities were as follows—SC and TB: conceived and coordinated the studies and wrote the manuscript; TB, RF, YK, and JK: performed the experiments and analyzed the data; SC: had primary responsibility for final content; and all authors: read and approved the final manuscript.

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