

# 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 1 $\beta$  (IL-1 $\beta$ ) are critical determinants that precipitate disease progression. The seeds of annatto (*Bixa orellana* L.) contain tocotrienols (T3s), mostly (>90%) in the  $\delta$  form ( $\delta$ T3). 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  $\delta$ T3 significantly attenuated NLRP3 inflammasome by decreasing IL-1 $\beta$  reporter activity, IL-1 $\beta$  secretion, and caspase-1 cleavage against lipopolysaccharide (LPS) followed by nigericin stimulation. With regard to mechanism, annatto  $\delta$ T3 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  $\delta$ T3 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-1 $\beta$ . 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- $\kappa$ B signaling through pattern recognition receptors, resulting in transcriptional activation for inflammasome scaffold proteins and pro-IL-1 $\beta$ , 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-1 $\beta$  secretion (4). Accordingly, bioactive molecules that inhibit inflammasome priming or suppress inflammasome assembly signals would be effective in mitigating NLRP3 inflammasome activation and IL-1 $\beta$  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  $\delta$  isoform ( $\delta$ T3), whereas  $\delta$ T3 is a relatively minor fraction compared with  $\alpha$ T3 and  $\gamma$ T3 in other sources of T3 (e.g., palm and rice bran oil) (6). There is increasing evidence that annatto  $\delta$ T3 exerts health benefits against inflammation (7), but its immunomodulatory function is unknown. We previously showed that  $\gamma$ T3, an unsaturated



**Keywords:** annatto, delta-tocotrienol, NLRP3 inflammasome, IL-1 $\beta$ , 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-1 $\beta$ -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).  $\gamma$ T3 and  $\delta$ T3 possess similar molecular characteristics and exert strong potency in downregulating inflammation and oxidative stresses compared with  $\alpha$ T3 (7, 9). Given the high availability and easy preparation of  $\delta$ T3 from annatto plants, it is of interest to determine whether  $\delta$ T3 is capable of suppressing NLRP3 inflammation for therapeutic application. The aim of this study was to determine whether annatto  $\delta$ T3 inhibits the NLRP3 inflammasome and to compare its efficacy with palm  $\gamma$ T3. Herein, we report that annatto  $\delta$ T3 is a bioactive dietary source to suppress NLRP3 inflammasome activation.

## Methods

Annatto  $\delta$ T3 (90%  $\delta$ T3) was provided by American River Nutrition, and  $\gamma$ T3 (>90%) was provided by Carotech. The experimental details are shown in **Supplemental Methods**.

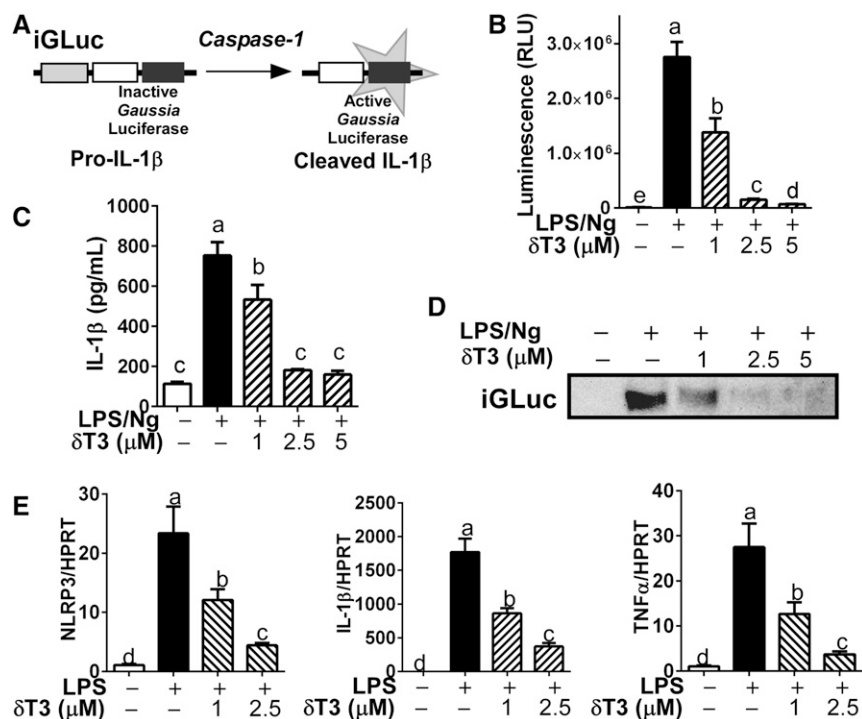
All of the data are presented as means  $\pm$  SEMs. The data were statistically analyzed by using either Student's *t* test or 1-factor

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 $\delta$ T3 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 1A**) (10) were pretreated with 1, 2.5, and 5  $\mu$ M  $\delta$ T3 or vehicle control (DMSO). The NLRP3 inflammasome was stimulated by priming with LPS followed by nigericin (LPS/Ng). Pretreatment with 1–5  $\mu$ M  $\delta$ T3 significantly decreased inflammasome reporter activity in a dose-dependent manner compared with the control (**Figure 1B**). Consistently,  $\delta$ T3 pretreatment markedly lowered IL-1 $\beta$  secretion in medium in iJ774 macrophages (**Figure 1C**). IL-1 $\beta$ -Gaussia luciferase fusion construct (iGLuc) protein is secreted from macrophages upon caspase-1 cleavage of pro-IL-1 $\beta$ . Confirming the NLRP3 inflammasome activation, treatment



**FIGURE 1**  $\delta$ T3 inhibited NLRP3 inflammasome activation. iJ774 macrophages were pretreated with  $\delta$ T3 (0, 1, 2.5, and 5  $\mu$ M), 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-1 $\beta$  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  $\mu$ M) 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  $\pm$  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-1 $\beta$ -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  $\alpha$ ;  $\delta$ T3,  $\delta$ -tocotrienol.

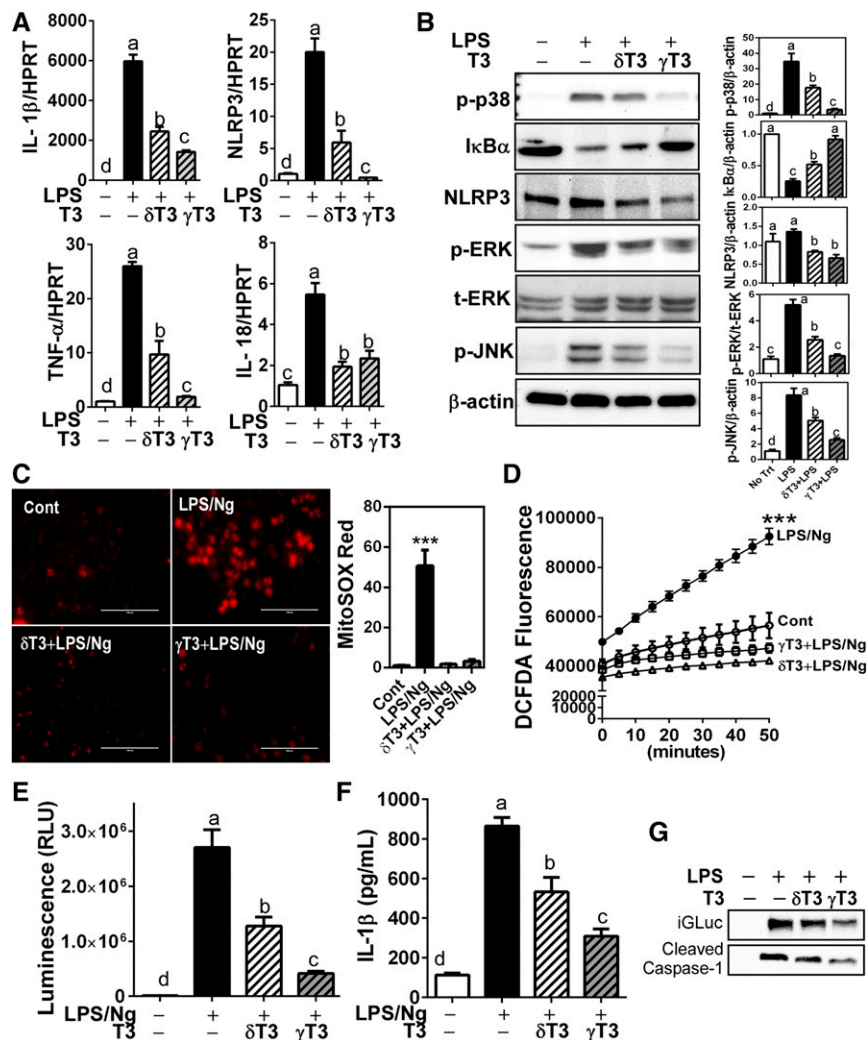
with δT3 of >1 μM abolished iGLuc and IL-1β secretion in the medium (Figure 1C, D).

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-dichlorofluorescein 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 MAPKinase; *Tnfa*, tumor necrosis factor α; Trt, treatment; T3, tocotrienol; δT3, δ-tocotrienol; γT3, γ-tocotrienol.

assembly signal, and compared its efficacy with 1  $\mu$ M palm  $\gamma$ T3. Pretreatment with 1  $\mu$ M annatto  $\delta$ T3 significantly decreased LPS (100 ng/mL)-mediated mRNA expression of *Nlrp3*, *Tnfa*, *Iil8*, and *Iilb* compared with the control, but to a lesser extent than 1  $\mu$ M palm  $\gamma$ T3 (Figure 2A). In parallel, annatto  $\delta$ T3 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  $\kappa$ B (I $\kappa$ B $\alpha$ ), a surrogate marker for NF- $\kappa$ B activation; and 3) protein concentrations of NLRP3, the scaffold of inflammasome, but a lesser degree than  $\gamma$ T3-treated cells (Figure 2B).

Next, we examined whether  $\delta$ T3 and  $\gamma$ T3 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  $\delta$ T3 and  $\gamma$ T3 (Figure 2C). To further quantify ROS quenching rate, RAW 264.7 macrophages were preloaded with 2,7-dichlorofluorescein 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  $\delta$ T3 or  $\gamma$ T3 to the nonstimulated concentrations (Figure 2D).

To compare NLRP3 inflammasome inhibitory function between the 2 T3 isoforms, iJ774 macrophages were pretreated with either  $\delta$ T3 or  $\gamma$ T3, then stimulated with LPS/Ng. The extent to which  $\delta$ T3 inhibits NLRP3 inflammasome reporter activity was significantly lower than with  $\gamma$ T3 (Figure 2E), which was also confirmed by IL-1 $\beta$  secretion, cleaved iGLuc protein, and cleaved caspase-1 in the medium (Figure 2F, G). Taken together, these results show that  $\delta$ T3 pretreatment in macrophages inhibits LPS/Ng-stimulated NLRP3 inflammasome activation, but  $\gamma$ T3 exerts a stronger inflammasome inhibitory activity when it is normalized with 1  $\mu$ M of  $\gamma$ T3 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  $\gamma$ T3 supplementation is effective in inhibiting the NLRP3 inflammasome (8). Given the structural and functional similarities between  $\gamma$ T3 and  $\delta$ T3, we tested the effectiveness of annatto  $\delta$ T3 in modulating the NLRP3 inflammasome in comparison with  $\gamma$ T3. Here, we showed that annatto  $\delta$ T3 is a dietary source that effectively attenuates priming as well as assembly of the NLRP3 inflammasome. It is well documented that  $\gamma$ T3 is proficient in the downregulation of MAPK and NF- $\kappa$ B activation (8, 15, 16). To the best of our knowledge, this is the first study to report that annatto  $\delta$ T3 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  $\delta$ T3 concentrations were

1.4–1.6  $\mu$ g/mL after 3–4 h of single administration of 750–1000 mg annatto  $\delta$ T3 in healthy men (17), which is equivalent to 3.5–4  $\mu$ M. On the basis of these results, our experiment that used 1  $\mu$ M annatto  $\delta$ T3 seems to be a reasonable and physiologically achievable concentration in humans. The acute annatto  $\delta$ T3 intake  $\leq$ 1000 mg was reported as safe (17); however, further clinical trials are necessary to establish the safety of chronic supplementation of annatto  $\delta$ T3.

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  $\delta$ T3 significantly inhibits NLRP3 inflammasome activation and IL-1 $\beta$  production by attenuating NF- $\kappa$ B priming and ROS production. This suggests that annatto  $\delta$ T3 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  $\delta$ T3 in animal studies and in further human clinical trials.

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