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 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 δT3 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

Supporting Material

Abbreviations used: iGLuc, IL-1β 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 1A) (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](https://example.com/figure1.png)
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, IkBα 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-dichlorodihydrofluorescein diacetate; HPRT, hypoxanthine-guanine phosphoribosyltransferase; iGLuc, IL-1β–Gaussia luciferase fusion construct; iJ774, J774 macrophage stably expressing iGLuc reporter construct; IkBα, 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 μM palm γT3. Pre-
treatment with 1 μM annatto δT3 significantly decreased LPS
(100 ng/mL)-mediated mRNA expression of Nlrp3, Tnfa, Il1b, and
Il18 compared with the control, but to a lesser extent than 1 μM
palm γT3 (Figure 2A). In parallel, annatto δT3 treatment signifi-
cantly reduced the following: 1) LPS-mediated MAPK phosphory-
lization of phosphorylated ERK MAPKinase (p-ERK), phosphorylated
p38 MAPKinase (p-p38), and phosphorylated JNK MAPKinase
(p-JNK); 2) degradation of inhibitor of kB (IκB), a surrogate
marker for NF-кB 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 po-
tency in attenuating ROS production, a common event required
for second signalling for NLRP3 assembly (12–14). LPS/Ng stimula-
tion 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 quan-
tify 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 pre-
treatment of either δT3 or γT3 to the nonstimulated concentrations
(Figure 2D).

To compare NLRP3 inflammasome inhibitory function be-
tween 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 con-
firmed 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 inflamma-
tory disease conditions. Previously, we reported that γT3 supple-
mentation 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 prim-
ing as well as assembly of the NLRP3 inflammasome. It is well docu-
mented 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 in-
take ≤1000 mg was reported as safe (17); however, further
clinical trials are necessary to establish the safety of chronic sup-
plementation of annatto δT3.

It is becoming more evident that targeting the NLRP3 infl-
ammosome possesses therapeutic potential for the treatment of
inflammation-mediated chronic diseases. Our results show that
annatto δT3 significantly inhibits NLRP3 inflammasome acti-
vation 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 infl-
ammasome activation for disease manifestation. Future research
is warranted to confirm the effectiveness of the NLRP3 inflam-
masome by annatto δT3 in animal studies and in further human
clinical trials.

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ceived 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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