Oral delivery of oligomeric procyanidins in Apple Poly® enhances type I IFN responses in vivo

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ABSTRACT

Type I IFN signaling is a central pathway that provides critical innate protection from viral and bacterial infection and can have regulatory outcomes in inflammatory settings. We determined previously that OPCs contained in the dietary supplement APP enhanced responses to type I IFN in vitro. Here, we confirm that OPCs from two different sources significantly increased pSTAT1, whereas a monomeric form of procyanidin did not. We hypothesized that similar responses could be induced in vivo following ingestion of APP. Ingestion of APP before injection of poly(I:C) enhanced in vivo responses to type I IFNs in mice. When human subjects ingested APP, enhanced responses to type I IFN and enhanced pSTAT1 ex vivo were detected, whereas ingestion of RES, a monomeric polyphenol, induced minimal such changes. Polyphenols are best known for induction of anti-inflammatory and antioxidant responses; however, our findings suggest a unique, nonantioxidant aspect of OPCs that is broadly applicable to many disease settings. The capacity of oral OPCs to enhance type I IFN signaling in vivo can augment innate protection and may, in part, contribute to the noted anti-inflammatory outcome of ingestion of OPCs from many sources. J. Leukoc. Biol. 95: 000–000; 2014.

Introduction

Type I IFN signaling is a central cellular process with a broad range of downstream effects. Type I IFNs are induced immediately upon sensing viral molecular patterns and signal to protect infected and uninfected cells, thus are a major component of innate antiviral immunity [1]. As such, type I IFNs are used to treat chronic viral infections [2] and have been shown to be effective therapies for a broad range of viral threats [3–6]. Type I IFN can also result in anti-inflammatory signaling and has long been used as a therapy for MS patients [7], but only recently have specific mechanisms of protection been discovered [8, 9]. Type I IFNs also have a critical role in early anti-tumor immunity by stimulating DCs [10] and have been used as cancer therapy [11]. Additionally, type I IFN signaling contributes to optimum type II IFN (IFN-γ) responses [12] and promotes hematopoiesis [13]. Type I IFN signaling is clearly important in multiple disease settings and is a clinical target. An orally delivered, novel immunotherapy that enhances this signaling pathway may have broad therapeutic application as a combination therapy or could potentially obviate IFN injections.

Polyphenols are chemical compounds found in varying concentrations and types in many dietary sources, such as pomegranates, grapes, and tea. Polyphenols have widely been shown to have antioxidant and anti-inflammatory capacity [14, 15]. APP is an extract from unripe apple peel that contains high concentrations of OPCs [16]. This supplement has notable anti-inflammatory effects in a mouse colitis model that are dependent on αβ T cells [14]. OPCs are composed of two to 10 repeats of hydroxylated flavan-3-ol building blocks, known as catechin or epicatechin (Fig. 1). OPCs from apple peels and other sources, such as grape seed and marine pine bark (Pycnogenol), are already known to be safe to ingest [17, 18]. We have defined recently the innate-immune enhancing capacity of OPCs from APP that is clearly distinct from antioxidant activity [16, 18–21]. Using dengue virus-infected human cells in vitro, we discovered that treatment with OPCs derived from APP decreased viral titers and exhibited a unique antiviral mechanism: enhanced responses to type I IFN [21]. Here, these results were confirmed by demonstrating that two robust sources of OPCs enhanced the phosphorylation of STAT1 which occurs following type I IFNR interaction, whereas a monomeric procyanidin did not. The primary goal of this investigation was to demonstrate that enhanced type I IFN signaling...
could also be measured in vivo following ingestion of APP. Ingestion of APP enhanced type I IFN signaling in mice injected with the dsRNA mimetic polyI:C, and optimal responses were detected 1 week after APP ingestion. Underscoring the medical relevance of these data, APP ingestion by human subjects induced enhanced responses to type I IFN in blood cells cultured ex vivo. These data indicate that minimal oral doses of APP enhance type I IFN responses in vivo. The capacity for a simple, safe oral supplement to enhance responses to type I IFN may have broad potential for enhancing innate protection from viral infection or for use as a combination therapy for patients receiving type I rIFN for chronic viral infection, autoimmune conditions, or cancer.

**MATERIALS AND METHODS**

**Human cells in vitro**

To assess the effects of OPCs and catechin on pSTAT1, total PBMCs were isolated using Histopaque (Sigma, St. Louis, MO, USA), as described previously [21], and cultured for 24 h with OPCs derived from APP [21], water extract of APP, a newly identified source of OPCs (methanol extract AS), catechin, or medium alone. The cells were then stimulated for 15 min with rIFN-α (100 U/ml; Human Interferon-Alpha A/D; PBL Interferon Source, Piscataway, NJ, USA), fixed, permeabilized, stained with Phosflow pSTAT1 PE antibody (BD Biosciences, San Jose, CA, USA) using the manufacturer’s protocols, and analyzed using a FACScaliber cytometer (BD Biosciences) and FlowJo Software (Ashland, OR, USA). In additional experiments, cells were treated for 3 or 24 h with APP, then washed, and incubated an additional 48 h in culture, followed by the addition of rIFN-α and similar analyses.

**Induction of type I IFN responses by polyI:C injection**

All animal studies were carried out in compliance with the Montana State University Institutional Animal Care and Use Committee. Six-week-old BALB/c mice were fed water or APP extract doses (5 mg/100 μl; based on approximate equivalence to 1-g dose in humans) [22] at varying intervals, and then 1 μg polyI:C was delivered by i.p. injection. Mice were euthanized after 4 h, and livers (one lobe) and whole spleens were homogenized, red blood cells were water-lysed, and cells were lysed for RNA extraction using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA).

**Human subjects studies**

Studies involving human subjects were carried out in compliance with the Montana State University Institutional Review Board, and as such, each subject signed informed consent documentation. In the first study, blood was collected from volunteers having ingested nothing or from the same subjects (n=11), 2 weeks later, 90 min to 2 h after ingestion of 3 g APP (six capsules; Apple Poly®, Morrill, NE, USA). This alternating pattern was repeated four times (Rounds 1–4) and continued in the absence of APP ingestion for three additional cycles (Rounds 5–7). In the second study, ingestion of APP was compared with ingestion of RES in a randomized and double-blind setting. Starting with 10 subjects/group, groups ingested 3 g APP or 3 g RES (Biotivia Transmax), and blood was collected 2 h later (Round 1). Round 2 was a repeat performed 3 weeks later. Round 3, in which subjects did not ingest supplements, was performed 4 weeks later, and the same parameters were compared. In both studies, subject groups were alternated and mixed such that blood from a maximum of six subjects was analyzed per day to diminish the possible effects of day-to-day variation biasing the results. Diets and activities of volunteers were not otherwise restricted. Total PBMCs were isolated, as described previously [21] and stimulated ex vivo with medium or 100 U/ml rIFN-α. Cells in separate wells were stimulated for 4 h and then lysed for RNA extraction and qPCR or cultured for 24 h for analyses by flow cytometry. Fold induction was calculated by dividing the IFN-treated value by the medium-only value from cells from the same donor.

**Flow cytometry with human cells**

Human PBMCs were stained with antibodies specific for CD11b (to identify mononuclear cells; Clone 1C8; Pharmingen, San Diego, CA, USA) and MHC I (Clone W6/32; BioLegend, San Diego, CA, USA) and CD86 (Clone IT2.2; BioLegend), which increase in expression in response to type I IFNs [23, 24]. Cells were stained using standard protocols for flow cytometry and analyzed using a FACScaliber cytometer. The data was analyzed using FlowJo software, MS Excel (Microsoft, Redmond, WA, USA), and Prism (GraphPad Software, La Jolla, CA, USA).
RNA extraction and RT-qPCR
RNA from mouse tissues and human cells was extracted, reverse-transcribed, and transcript levels assessed and analyzed, as described previously [21].

Statistical analyses
Statistical significance was determined using parametric and nonparametric tests. Data presented were analyzed using the unpaired, one-tailed t-test unless noted otherwise.

RESULTS AND DISCUSSION
To confirm earlier in vitro data and investigate whether the effect of APP on the type I IFN pathway was specific to compounds containing OPCs, an in vitro assay measuring pSTAT1 was performed. Human PBMCs were incubated for 24 h with a water extract of APP, a water extract of another recently identified, robust source of OPCs (AS), OPCs derived from APP, as described previously [21], catechin, or medium only. Figure 1B demonstrates that the lower concentrations of APP and AS augmented pSTAT1 on CD11b+ cells in response to rIFN-α. After 24 h incubation, AS at 1 μg/ml and all OPC sources at 10 μg/ml, including OPCs isolated from APP (Fig. 1B, inset), also induced pSTAT1 in the absence of rIFN-α. Incubations for 1, 3, or 6 h with 10 μg/ml APP did not induce any such changes (data not shown). Catechin, a monomeric procyanidin, induced responses similar to medium-only controls. These data suggest that OPCs and sources of OPCs, but not monomeric procyanidins, enhanced pSTAT1 in vitro and that monomeric polyphenols might be used as negative controls. Additional experiments addressed the necessary interval for pre-treatment with APP. To investigate a longer-term response, cells were treated with APP for 3 or 24 h, then washed, and incubated for an additional 48 h before treatment with rIFN-α. In this case, direct pSTAT1 in the absence of rIFN-α was not affected, but in response to rIFN-α, pSTAT1 was greatly increased by APP treatment over medium-only controls. These data suggest that APP treatment primes cells to better respond to type I IFN, and maximal priming response takes days to fully develop. Anti-inflammatory outcomes are the most commonly described effect of polyphenol ingestion [14, 15], which may be generally related to their antioxidant capacity. OPCs, but not monomeric forms, reduce dengue virus titers in vitro [21] and enhanced pSTAT1, even though these polyphenols have similar antioxidant capacity. Collectively, our results show that OPCs have a novel and unique effect on responses to type I IFN signaling that is distinct from the well-characterized antioxidant effects.

The effects of ingestion of APP extracts on responses to type I IFN in vivo were assessed by measuring expression of ISG transcripts following injection of mice with poly(I:C). To determine the optimal oral dose of APP, mice were divided into four treatment groups and received, by oral gavage, one, 5-mg APP dose, 36 h before injection; four, 5-mg APP doses; or water only at 36, 24, 12, and 0 h before injection or the same four doses every 12 h, given 1 week before injection. Mice were injected with poly(I:C), and tissues were collected 4 h later and processed for RT-qPCR analyses. Four hours were empirically determined to be the optimal point for ISG transcript expression following poly(I:C) injection. ISG transcripts were not detected following ingestion of APP alone nor were they detected 1 h after poly(I:C) injection (data not shown). ISG transcripts SOCS1, OAS1, IFIT1, and IFIT2 were induced more robustly in the spleen, whereas expression of CXCL10 was greater in the liver (Fig. 2A). Consistent with in vitro findings suggesting enhanced activity following multiple days (48 h) of incubation, delivery of APP 1 week before poly(I:C) injection was the most effective dosing interval for enhanced type I IFN responses, as this dose significantly increased expression of SOCS1, IFIT1, IFIT2, and CXCL10 compared with water-fed controls. Only the four doses within 36 h of injection significantly altered OAS1 expression in the liver and feeding APP did not alter OAS1 expression in the spleen. The four doses of APP within 36 h of injection also significantly affected IFIT1 and IFIT2 expression in the livers, as did one dose, 36 h before, affect IFIT2 expression. Delivery of APP 1 week before injection induced fold increases of 4.7 and 3.9 in CXCL10 expression in spleens and livers, respectively, relative to water-treated mice. This increase was much greater than the induction of other ISGs, which was between 1.8- and 2.8-fold. These data indicate that oral APP affected ISG expression in vivo, and the dosing interval that most affected gene expression was that delivered 1 week before injection of poly(I:C). The relatively long-term effect of minimal OPC ingestion was consistent with in vitro findings and may indicate that OPCs prime cells for enhanced responses to type I IFN in vivo, and optimal detection of the primed state in a large fraction of cells requires days to fully develop.

Poly(I:C) injection induces type I IFN expression; however, a potential cause of enhanced ISG expression in the APP-fed group may be further increased expression of type I IFNs themselves, so this possibility was investigated. Transcripts that encode type I IFNs were measured in spleens and livers of mice that received water or APP at the most-effective dosing interval, 1 week before poly(I:C) injection (Fig. 2B). Type I IFN transcripts were detectible following poly(I:C) injection, and levels were similar between the two groups. Relative to the livers, the type I IFN transcripts were only detectable at very low levels in spleens. The only significant difference between the water and APP-fed groups was a 1.65-fold increase in IFN-α expression in the spleens of the group that received APP relative to the water-fed mice. No significant differences between groups were noted in the liver for IFN-α or IFN-β transcripts (Fig. 2B). Similarly and as expected, there were no differences in either tissue in expression of IFN-γ transcripts (data not shown). The slightly greater expression of IFN-α in the spleens of APP-fed mice offers a possible explanation for the increase in ISG expression observed in this tissue, but this was clearly not the case in the liver, where ISGs increased independently of type I IFN expression. These data confirm our in vitro findings and suggest that ingestion of OPCs does not change type I IFN expression significantly but enhances type I IFN signaling in vivo.

To maximize the clinical and translational potential of our findings, small-scale human-subject experiments were performed to demonstrate that ingestion of APP increased re-
sponses to type I IFN by human PBMCs cultured ex vivo. In the first study, healthy subjects consumed nothing (NEG) and 2 weeks later, consumed 3 g APP, and this pattern was repeated. Responses were compared with PBMCs from the same individual; thus, each individual served as their own negative control. After 24 h in ex vivo culture and no other treatment, there was a significant increase in CD86 mean fluorescence following the first and second doses of APP compared with when the same subjects ingested nothing initially. At the beginning of Round 3, expression of CD86 remained significantly elevated compared with levels at the beginning of Round 1 and was not increased further in subsequent rounds by ingestion of APP (Fig. 3A). Thus, despite the discontinuation of APP ingestion, expression of CD86 remained elevated for several weeks, suggesting a very long-lasting effect on CD86 expression induced by multiple doses of APP. A similar pattern was evident for MHC I induction in response to rIFN-α treatment ex vivo. The fold induction of MHC I (on CD11b+ cells; Fig. 3B) was significantly greater following the first and second APP ingestion. Induction of MHC I at the start of Rounds 3 and 4 was significantly greater than at the start of Round 1 and was not increased further in these later rounds by supplement ingestion. With the use of cells from the same subjects, parallel analyses of induced gene expression in response to rIFN-α indicated a similar trend as that seen with MHC I. We measured ISG transcripts: IFIT1, MX1, SOCS1, and RIGI after type I IFN treatment for 4 h. ISGs were not expressed in the absence of type I IFN treatment. RIGI was induced consistently by type I IFN, and fold induction (over untreated cells from the same donor) was increased following APP ingestion (Fig. 3C). As with MHC I and CD86, subsequent rounds did not induce RIGI expression further, and its fold induction at the start of Round 3 and 4 without APP ingestion (NEG) was similarly elevated compared with the start of Round 1. Induction of IFIT1, SOCS, and MX1 was not as consistent as that of RIGI (data not shown). These data indicate that ingestion of minimal doses of a rich source of OPCs can enhance ex vivo expression of proteins and transcripts induced by low levels of type I IFN and that the effect of multiple doses may be long-lasting.

In the second study, ingestion of APP was compared directly with ingestion of the same dose of RES, and following two doses of each, with 3 weeks between the doses, there were significant differences between the groups. There was a significant increase in fold induction of MHC I in the cells derived from those who ingested two doses of APP compared with those who ingested the same amount of RES (Fig. 4A). However, in this study, there was no difference in CD86 expression between subjects that ingested APP or those that ingested RES; rather, CD86 mean fluorescence increased in both groups. These data suggest that CD86 expression may be increased by ingestion of polyphenols in general or is increased by the placebo effect. Similar to the findings for MHC I, fold induction of RIGI expression was significantly higher following APP ingestion compared with ingestion of RES (Fig. 4B). We expanded this study to include transcripts encoding IL-10 and IL-27, as these anti-inflammatory cytokines are likely to be involved in the benefit of type I IFN treatment in experimental autoimmune encephalomyelitis, a mouse model of MS [8, 9]. Both of these transcripts were also induced to a significantly greater level in subjects that ingested APP compared with those that ingested RES (Fig. 4B). Anti-inflammatory responses following ingestion of some polyphenol-containing supplements may be, in part, a result of enhanced type I IFN responses through enhanced expression of anti-inflammatory cytokines IL-10 and IL-27. IL-27R signaling, in particular, has been shown recently to decrease specifically inflammatory cytokine expression, primarily IFN-γ, and trafficking by CD4+ T cells [25, 26]. These cells are a primary source of inflammation in the mouse model of colitis [27]. APP-induced expression of IL-27 through enhanced type I IFN signaling may be, in part, responsible for the noted suppression of inflammation by APP that was dependent on αβ T cells in a colitis model.
We also assessed differences in pSTAT1 ex vivo in human cells following two doses of APP or RES. PBMCs from a subset of subjects were cultured overnight and then stimulated with rIFN-α for 15 min, fixed, permeabilized, and stained with the antibody specific to pSTAT1. Similar to the in vitro findings (Fig. 1B), both the basal and rIFN-α-stimulated levels of pSTAT1 were increased slightly in subjects that ingested APP compared with those that ingested RES (Fig. 4C). When this response was compared in multiple donors, there was a statistically significant increase in IFN-α-induced pSTAT1 in subjects that ingested APP compared with subjects that ingested RES (Fig. 4D). For each of the parameters shown in Fig. 4, there were no significant differences between APP and RES groups following one dose nor were the differences retained 4 weeks after the second dose (data not shown), suggesting that multiple, occasional, small doses of OPCs are necessary to retain the effects, and two doses are not sufficient for a long-lasting change. These data indicate that ingestion of APP induced enhanced type I IFN responses in mice and human subjects. This response may be dependent on OPCs found in a limited set of dietary sources.

An orally delivered, broad-spectrum therapy that enhances type I IFN responses has not been described previously. Type I IFNs are induced immediately upon sensing viral molecular patterns and are a critical component of innate antiviral immunity. Our therapeutic defense against viruses is currently limited to a few antiviral drugs and treatment with type I rIFN [4]. Our data suggest that ingested APP has long-lasting effects on expression patterns downstream of type I IFNs. These results support a novel biological effect of OPC sources in vivo that may be a viable antiviral prophylactic approach. Type I rIFN is used in antiviral therapies for human hepatitis C virus [2, 28] and is effective in models of influenza infection [3, 5]. Thus, oral OPC sources could also be used as a combination therapy to augment the effects of type I IFN therapy for chronic viral infection [2, 28], especially considering the detected responses in the liver following APP ingestion in mice. Anyone at an increased risk of viral infection and complications, including the elderly, parents of small children, teachers, and healthcare workers, could greatly benefit from enhanced innate protection from viral infection.

Type I IFN has also long been used as a therapy for MS patients [7, 29], but only recently have specific mechanisms of protection involving enhanced expression of anti-inflammatory cytokines IL-10 and IL-27 been discovered [8, 9]. Responses to type I IFN therapy differ greatly among patients; thus, an oral treatment that globally increases responses to type I IFN may increase efficacy of this therapy. A complication of type I IFN treatment is the necessity to administer large, continuous doses, resulting in IFN-specific neutralizing antibodies that render treatment less effective [29]. Use of oral OPCs as a combination therapy with injected or ingested type I IFNs could potentially decrease the effective dose and thereby, diminish side-effects. Furthermore, enhancing responses to endogenously expressed type I IFNs could obviate the need for any additional therapy. Only palliative therapy is available for most inflammatory conditions, including MS; thus, inventive therapeutic approaches are clearly warranted.

Enhancing type I IFN expression may be an unrecognized novel mechanism responsible for at least some of the noted beneficial and immunomodulatory outcomes following ingestion of OPC-containing dietary supplements. Because of the pleiotropic effects of type I IFNs, this response may be masked in other studies by the robust antioxidant and inhibitory responses common to polyphenols, or the responses are truly a unique aspect of OPCs from limited sources.

Figure 3. Ingestion of polyphenols by human subjects enhanced ex vivo responses to type I IFN. Human subjects (n=11) ingested nothing or 2 weeks later, a single dose of 3 g APP. This was repeated for four rounds. During Rounds (R) 3–7, APP ingestion was discontinued, and blood was collected from the same subjects at 2-week intervals. (A) CD86 expression on PBMCs increased following ingestion of APP and remained elevated after multiple doses. (B) Fold induction of MHC I (on CD11b+ cells, normalized to media only), by 24-h treatment with rIFN-α, increased during alternating 2-week intervals of NEG and APP ingestion and remained elevated. (C) Induction of RIGI transcripts by 4-h incubation with IFN-α was increased in Round 1 and remained elevated after Round 2 (paired, *P<0.05; **P<0.01; ***P<0.001).
There are few aspects of disease that are not affected by type I IFN signaling. Type I IFNs are critical for immune responses to tumors [10], can impact hematopoiesis [30], and have been appreciated recently as critical for protection from some bacterial infections [31–33]. Characterization of this novel effect of ingestion of OPCs on type I IFN signaling is likely to provide insights into in vivo mechanisms and a strong rationale for its recommended application in an extensive range of disease conditions. Considering the paucity and complications of existing therapies and the far-reaching implications of the type I IFN pathway, the capacity for a simple oral supplement to enhance responses to type I IFN, expressed endogenously during infection or delivered as a therapeutic recombinant protein, could strongly impact the field.

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DISCLOSURES
The authors declare no conflict of interest.

REFERENCES


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