RESEARCH ARTICLE

Effect of pistachio oil on gene expression of IFN-induced protein with tetratricopeptide repeats 2: A biomarker of inflammatory response

Jun Zhang[1,2], Penny M. Kris-Etherton[1,2], Jerry T. Thompson[3,4] and John P. Vanden Heuvel[1,2,3,4]

[1] Department of Nutritional Sciences, PA, USA
[2] Center of Excellence in Nutrigenomics, PA, USA
[3] Department of Veterinary and Biomedical Sciences, PA, USA
[4] Center for Molecular Toxicology and Carcinogenesis, The Pennsylvania State University, University Park, PA, USA

When incorporated into the diet, pistachios have a beneficial effect on lipid and lipoprotein profiles. However, little is known about potential anti-inflammatory properties. This study was conducted to determine whether pistachio oil and an organic extract from pistachio oil extract (PE) regulated expression of inflammation-related genes. A mouse macrophage cell line (RAW 264.7 cells) was treated with pistachio oil and gene expression microarray analyses were performed. Pistachio oil significantly affected genes involved in immune response, defense response to bacteria, and gene silencing, of which INF-induced protein with tetratricopeptide repeats 2 (Ifit-2) was the most dramatically reduced. PE reduced the LPS-induced Ifit-2 by 78% and the bioactive molecules contained in PE, linoleic acid, and β-sitosterol recapitulated this inhibition. Promoter analysis identified two adjacent IFN-stimulated response elements, which lie between −110 and −85bp of the 5′-flanking region of the Ifit-2 promoter, as being responsive to LPS activation and inhibition by PE. Our results indicate that pistachio oil and bioactive molecules present therein decrease Ifit-2 expressions, and due to the sensitivity of this effect, this gene is a potential biomarker for monitoring diet-induced changes in inflammation.

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1 Introduction

Inflammation is a complex biological response to harmful stimuli, pathogens, damaged cells, or irritants, and underlies the pathophysiology of many chronic diseases such as atherosclerosis, obesity, and cancer. Persistent local and/or systematic inflammation increases macrophage and lymphocyte migration and activity, leading to the release of hydrolytic enzymes, cytokines, chemokines, and growth factors, which can induce further damage and eventually result in necrosis. Inflammatory markers, such as C-reactive protein, tumor necrosis factor-α (TNF-α), ILs (IL-6, IL-1β), and many others are used to monitor the severity of inflammatory diseases.

Correspondence: Dr. John P. Vanden Heuvel, Department of Veterinary and Biomedical Sciences, 325 Life Sciences Building, The Pennsylvania State University, University Park, PA 16802, USA
E-mail: jpv2@psu.edu
Fax: +1-814-863-1696

Abbreviations: CVD, cardiovascular disease; **GAPDH,** glyceraldehyde 3-phosphate dehydrogenase; **GPX-1,** glutathione peroxidase-1; **IC,** inhibitory concentration; **Ifit,** IFN-induced protein with tetratricopeptide repeats; **ISRE,** IFN stimulated response elements; **LA,** linoleic acid; **OA,** oleic acid; **PA,** palmitic acid; **PE,** pistachio oil extract; **PO,** pistachio oil; **STAT,** signal transducer and activator of transcription; **TGF-β1,** transforming growth factor-β1

Nuts are a nutrient-dense plant food that are a rich source of protein, fiber, micronutrients, plant sterols, and other phytochemical compounds including ellagic acid, flavonoids, phenolic compounds, luteolin, and tocotrienols [1]. In addition, they are low in saturated fat, and high in unsaturated fat. Because of their favorable nutrient profile, nuts can be included in a diet recommended to decrease risk of cardiovascular disease (CVD) http://www.health.gov/dietaryguidelines/dga2005/document/. Epidemiologic studies have demonstrated reduced risk of CVD with frequent nut consumption [2–5]. The clinical studies with nuts have shown total cholesterol and LDL-cholesterol lowering effects [5, 6]. In addition, several studies have shown a reduction in markers of inflammation following nut consumption. Zhao *et al.* [7] found that a cholesterol-lowering diet was high in walnuts, compared with an average American diet, significantly decreased serum C-reactive protein and cellular adhesion molecules by 75% and 15–19%, respectively. IL-6, IL-1β, TNF-α released from isolated primary peripheral blood mononuclear cell were significantly lowered by 22, 18, and 22%, respectively, following the walnut diet compared with those released from cells isolated from individuals on an average American diet [8]. However, little is known about the effects and mechanisms of pistachios or pistachio oil (PO) on inflammation biomarkers related with CVD risk.

One of the frequent problems with assessing the effects of diet on inflammation is that the test population does not exhibit elevated levels of CVD risk factors; hence, studies aimed at identifying repressors of inflammation suffer from a lack of sensitivity. The approach depicted herein uses cell culture systems and gene expression microarrays to determine sensitive biomarkers for future use in clinical, dietary intervention studies. The inflammatory marker INF-induced protein with tetratricopeptide repeats 2 (Ifit-2) was identified by this approach to be sensitive to pistachio and bioactive molecules present in this nut. Further studies are needed to validate the Ifit-2 as a nutritionally sensitive marker of inflammation and CVD risk.

2 Materials and methods

2.1 Chemicals

Virgin PO was purchased from Jean-Marc Montegottero, France. High glucose-DMEM, LPS, palmitic acid (PA), oleic acid (OA), linoleic acid (LA), α-linolenic acid, γ-tocopherol, β-carotene, and β-sitosterol were purchased from Sigma-Aldrich, St. Louis, MO. Fatty acids were conjugated to fatty acid-free BSA based on the methods described by Calder *et al.* [9]. Fetal bovine serum was purchased from HyClone (Logan, UT). Non-essential amino acids, penicillin, and streptomycin were purchased from Invitrogen (Grand Island, NY). Rabbit polyclonal anti-Ifit2 antibody was purchased from Abcam, Cambridge, MA. Mouse polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was purchased from Fitzgerald Industries, Concord, MA.

2.2 PO preparation and analysis

The weighed oil was dissolved in DMSO with a ratio of 65:35 (oil:DMSO, v/v) at a stock concentration of 1.1 mg/μL, sealed in argon and stored at −20°C. Procedures of lipid extraction were modified from Folch method as described by Folch *et al.* [10]. Briefly, 1 g of oil was extracted twice with 20 mL chloroform/methanol 1:1 and 3:1, v/v and centrifuged at 2800 rpm for 10 min separately. Pooled supernatant was mixed with 1 mL 0.9% NaCl and centrifuged as above. Lower phase, which contains lipid soluble components, was evaporated under argon and stored at −20°C. Oil extract was dissolved in DMSO with a ratio of 10:40 (oil:DMSO, v/v) at a stock concentration of 0.2 mg/μL, sealed in argon and stored at −20°C. To minimize DMSO effect on gene expression and to maximize solubility of oil and its extract in the medium, we did a 1:200 dilution of PO and pistachio oil extract (PE) stock in the medium to achieve the highest PO and PE treatment concentration as 5.5 and 1.0 mg/mL, respectively. These concentrations did not cause toxicity as tested by CellTiter Cell Proliferation Assay (Promega, Madison, MI). Fatty acid profile analysis was performed at Venture Laboratories, Lexington, KY. FAMEs from PO having 8–24 carbon atoms were separated on a silica capillary column and determined by gas chromatography. The FAMEs were identified by comparing the retention time of peaks from samples with those of FAME standard mixtures. Quantification of FAMEs was based on the internal standard technique and on the conversion of relative peak areas into weight percentages, using the corrected response factor of each fatty acid. Fatty acids were expressed as percentage of the sum of identified fatty acids (wt%).

2.3 Cell culture

The RAW 264.7 cell line (*Mus musculus* macrophage) was obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were cultured in high glucose-DMEM containing 10% heat inactivated fetal bovine serum, 1 mM sodium pyruvate, 100 μM non-essential amino acid, and 100 U/mL penicillin and 100 μg/mL streptomycin.

2.4 RNA isolation, reverse transcription, real-time PCR

Cells were lysed and harvested using TriReagent according to the manufacturer's instructions (Sigma-Aldrich). High-capacity cDNA Archive kit (Applied Biosystems, Foster City, CA) was used for reverse transcription. Twenty nanogram *per* microliter of cDNA was amplified by SYBR

Table 1. Oligonucleotides used in real-time PCR and mutagenesis

	Forward	Reverse
Jagged-2	CATCGTGGCTGCTATCACTCA	TGTGGAAGAGCCACCCATAAC
TGF-β1	TGGATTCTGCGCTCTCTTCTC	TTTCTGGCCTCTCCGAGTTCT
GPX-1	GCAACGACATTGCCTGGAA	CGATGTCGATGGTACGAAAGC
EGR-1	TGATGTCTCCGCTGCAGATCT	GCATCATCTCCTCCAGTTTGG
Ifit-2	CCACCTTCGGTATGGCAACTT	GCCTTGTCTTGACGCTTCATT
TNF-α	AAAGCATGATCCGCGACGT	TGCCACAAGCAGGAATGAGA
IL-6	CCCAATTTCCAATGCTCTCC	TGAATTGGATGGTCTTGGTCC
IL-1β	TCCTTAGTCCTCGGCCAAGAC	GTGCCATGGTTTCTTGTGACC
wt ISRE	TGACATCAGTGTTACTTTCTGGTTTCAATTTCTCTTCTTT	GATCAAAGAAGAGAAATTGAAACCAGAAAGTAACACTGATGTCAGTAC
mutISRE1	TGACATCAGTGTTACTTTCTGATTGAAATGTCGCTTCTTT	GATCAAAGAAGAGAAATTGAAACCCGACAGTCGCTGTGATGTCAGTAC
mutISRE2	TGACATCACAGCGACTGTCGGGTTTCAATTTCTCTTCTTT	GATCAAAGAAGAGAAATTGAAACCCGACAGTCGCTGTGATGTCAGTAC
mutISRE12	TGACATCACAGCGACTGTCGGATAGAAATGTCGCTTCTTT	GATCAAAGAAGAGCGACATTTCTATCCGACAGTCGCTGTGATGTCAGTAC

EGR-1, early growth response-1; mutISRE1, mutant INF-stimulated response element 1; wt ISRE, wild-type INF-stimulated response element. All oligonucleotides are of mouse origins.

Green PCR Master Mix (Applied Biosystems) and detected by ABI 7000 Sequence Detection System (Applied Biosystems). Primer sequences used in real-time PCR are listed in Table 1.

2.5 Microarray

Details of conducting gene expression microarray have been described elsewhere [11–13]. Briefly, the Mouse Genome Oligo Set Version 1 was purchased from Operon Technologies (Alameda, CA) and was printed onto glass slides using GeneMachines Omnigrid (San Carlos, CA) at the Pennsylvania State University DNA Microarray Facility. All arrays were scanned with a GenePix 4000A scanner (Axon Instruments, Foster City, CA) and image intensity information was collected with Genepix 3.0 software (Axon Instruments). Data files from Genepix were filtered to remove bad spots and spots that were not significantly different ($p \leq 0.001$) from background. Significantly regulated genes were determined using GeneSpring 6.0 (Silicon Genetics, Redwood City, CA) with a 1.5-fold difference in expression values and $p < 0.05$. LocusLink IDs of significant genes were input into Gene Ontology Tree Machine (GOTM, http://bioinfo.vanderbilt.edu/gotm/, Vanderbilt University) for further pathway analysis.

2.6 Western blot

RAW 264.7 macrophages were seeded in 15 cm^2 plates at a density of 5×10^6/plate. Macrophages were treated with different doses of oil extract (0.125–1 mg/mL) for a total of 24 h, during which time cells were treated with LPS (100 ng/mL) for the last 6 h. After LPS treatment, cells were washed with PBS and treated with lysis buffer (0.25 M sucrose, 10 mM Tris-acetate (pH 8.1), 1 mM EDTA, and 1 mM DTT). Lysates were sequentially centrifuged at 2000 and 12 000 rpm to remove nuclear and mitochondria fractions. Supernatant was collected and protein concentration was measured by Bio-Rad DC protein assay kit (Bio-Rad Laboratories, Hercules, CA). Total soluble protein (100 μg) was separated on a 10% Tricine gel and transferred to a PVDF membrane (Immobilon P, Millipore, Bedford MA). Membrane was blocked by 5% non-fat dry milk in TBS+0.2% Tween-20 (TBS^+) at 4°C overnight. The membrane was incubated with primary antibody (anti-Ifit-2 1:800; anti-GAPDH 1:40 000) in 0.5% milk at room temperature for 2 h, washed three times with TBS^+ and incubated with horseradish peroxidase-linked secondary anti-rabbit (1:8000) and anti-mouse (1:80 000) antibodies at room temperature for 1 h. Blots were visualized by ECL plus Western blot detection kit (GE Healthcare Biosciences, Piscataway, NJ). Band intensities were determined by OptiQuant Image analysis software (Packard Instrument, Meriden, CT).

2.7 Ifit promoter constructs

Primers were designed to amplify different lengths (pGL4/−1417bp+78bp Ifit-2, pGL4/−917bp+78bp Ifit-2, pGL4/−417bp to −17bp+78bp Ifit-2) of the 5′-flanking region of the mouse Ifit2 promoter (+78bp is the start of translation). The first PCR amplification was done using Mouse Genomic DNA (Promega) as template. Amplicons were cloned into pGEM-T Easy Vector System (Promega). Fragments were digested by restrictive enzymes (Nco I; New England BioLabs, Ipswich, MA; Hind III; Promega), isolated by standard techniques and sub-cloned into pGL4 Luciferase Reporter Vector (Promega). Ifit-2 DNA sequences were confirmed by sequence analysis at the Nucleic Acid Facility, The Pennsylvania State University.

2.8 Transient transfection and luciferase reporter assay

RAW 264.7 cells were seeded and transfected with Lipofectamine according to the manufacturer's recommendations (Invitrogen). Briefly, the day before transfection, RAW 264.7 macrophages were seeded in 24-well plates at a density of 0.1×10^6/well. pGL4 vectors with insert of different lengths of 5′-flanking region of mouse Ifit-2 promoter were transfected into RAW 264.7 cells for 6 h. After overnight recovery in growth medium, cells were co-treated with PE and LPS for 24 h. Cells were lysed by addition of passive lysis buffer (Promega) and frozen at −80°C for at least 2 h. Reporter expression was determined by Luciferase Assay System (Promega) and detected by Turner TD-20/20 Luminometer (Turner Designs; Sunnyvale, CA).

2.9 Mutagenesis

Using an online transcription factor binding site analysis tool (TFSEARCH, version 1.3), we identified two adjacent IFN-stimulated response elements (ISREs) between −110 and −85bp of 5′-flanking region of Ifit-2 promoter. Sequences of wild-type and mutant ISRE1, mutant ISRE2, and both ISRE1 and ISRE2 mutant (Table 1) were cloned into pGL3 promoter vector (Promega). Mutant DNA sequences were confirmed by sequence analysis at the Nucleic Acid Facility, The Pennsylvania State University.

2.10 Statistical analysis

A p-value < 0.05 was used to determine whether the differences among the variables were significant. Normality was checked by Anderson–Darling test. General linear model, followed by Tukey *post hoc* test, was used to test the difference between treatments. The values were expressed as mean ± SEM. All data analyses were performed by MiniTAB Ver.14 (MiniTAB, State College, PA). Data were plotted by Prism 5.01 (GraphPad Software, San Diego, CA).

3 Results

3.1 PO-regulated gene expression in various pathways, especially inflammation and immunology-related pathways

Microarray can be used to test hypotheses about treatment effects on gene expression. To identify what pathways PO may affect, microarray analyses were performed using RAW 264.7 macrophages. Results indicated a total of 43 genes were significantly regulated by PO treatment (5.5 mg/mL; Table 2). The GOTM pathway analysis indicated that PO may affect many biological processes including immune response ($p = 0.00084$), defense response to bacteria ($p = 0.00717$), and gene silencing ($p = 0.00103$). To further validate the microarray data, three transcripts that were increased (Jagged-2, transforming growth factor-β1 (TGF-β1), glutathione peroxidase-1 (GPX-1)) and three that were decreased (early growth response-1 (EGR-1), INF-induced protein with Ifit-2, TNF-α) by PO were selected. Quantitative real-time PCR analysis showed expression changes of the selected genes followed by the same trends as that of the microarray results. However, changes of GPX-1 and EGR-1 were not significantly different compared with DMSO control (Fig. 1).

3.2 PE reduced Ifit-2 expression in a dose-dependent manner in response to LPS challenge and the reduction was greater than other inflammatory markers tested

Microarray results indicated that PO had a potential anti-inflammatory effect, perhaps due to the lipid component(s) in the PO, such as fatty acids, vitamins, and phytosterols. To test this hypothesis, macrophages were treated with PE, followed by LPS challenge and the mRNA levels of traditional inflammation markers, TNF-α, IL-6, IL-1β, along with Ifit-2, were quantified (Fig. 2A). Compared with non-LPS stimulated cells, Ifit-2 mRNA was significantly increased 28-fold following LPS challenge (data not shown). Compared with the LPS treatment, 0.25–1 mg/mL of PE significantly decreased Ifit-2 mRNA expression. The calculated half maximal inhibitory concentration (IC_{50}) was 0.48 mg/mL with the highest treatment concentration (1 mg/mL) resulting in a 78% reduction of Ifit-2 expression in response to LPS stimulation. The same concentration of oil extract reduced TNF-α, IL-6, IL-1β by 55, 58, and 35%, respectively, in response to LPS stimulation. LPS challenge significantly increased Ifit-2 protein level compared with non-LPS treated cells (Fig. 2B), whereas addition of PE (0.25–1 mg/mL) significantly reduced this stimulation.

Table 2. Genes regulated by pistachio oil treatment

Transcripts increased by PO	Fold
Chromobox homolog 8	+5.11
Jagged 2	+4.22
Otoraplin	+3.34
Cytochrome P450, 2c40	+2.04
RIKEN cDNA 2900002H16	+2.01
CD24a antigen	+1.85
Glycine C-acetyltransferase	+1.81
TGF β1-induced transcript	+1.77
J domain protein 1	+1.74
RIKEN cDNA 2410004C24	+1.72
S-adenosylhomocysteine hydrolase	+1.72
Enhancer of rudimentary homolog	+1.69
Gnefr	+1.66
RAN guanine nucleotide release factor	+1.65
pEL98 protein	+1.65
Differential display and activated by p53	+1.63
Breakpoint cluster region protein 1	+1.62
RNA binding motif protein 3	+1.59
Manic fringe homolog	+1.58
Phosphatidylethanolamine binding protein	+1.58
Regulator of G-protein signaling 14	+1.56
8-Oxoguanine DNA-glycosylase 1	+1.55
Calmodulin 3	+1.52
Glutathione peroxidase 1	+1.50
VHSV induced gene 1	−5.27
Ifit 3	−3.57
Immune-responsive gene 1	−3.42
Ifit 2	−3.28
Ifit 1	−3.17
SCYB	−3.06
Myxovirus resistance 1	−2.76
INF-activated gene 202B	−2.61
Homeo box B5	−2.61
INF-activated gene 204	−2.50
STAT 2	−2.35
Schlafen 3	−2.27
cAMP-GEF II	−2.25
STAT 1	−2.16
TNF	−2.07
Nischarin	−2.05
Early growth response 1	−1.98
Apolipoprotein C-IV	−1.91

RAW 264.7 macrophages were seeded in 6-well plates at a density of 1.0 × 106/well the day before treatment. Cells were treated with either PO (5.5 mg/mL) or DMSO (vehicle control) for 24 h before harvest. To achieve the high-RNA purity required by microarray, RNA samples were obtained by using RNeasy Mini Kit (QIAGEN, Valencia, CA). Detailed array analyses are described in the Section 2. cAMP-GEF, cAMP-regulated guanine nucleotide exchange factor; VHSV, viral hemorrhagic septicemia virus; SCYB, small inducible cytokine B subfamily.

3.3 Identification of bioactive compounds in PO responsible for Ifit-2 reduction

Organic extracts of PO decreased Ifit-2 mRNA and protein levels in a dose-dependent manner. The predominant saturated, monounsaturated, and polyunsaturated fatty acids in the oil were PA, OA, and LA (Fig. 3A). These fatty acids were examined to determine if they were responsible for the Ifit-2 response from PE. Following treatment of LPS-stimulated RAW264.7 cells with the aforementioned fatty acids, only LA treatment (0–100 μM) significantly reduced Ifit-2 mRNA in a dose-dependent manner (Fig. 3B). The calculated IC_{50} of LA was about 60 μM while the other fatty acids (PAs, OAs, and α-linolenic acid), did not significantly affect Ifit-2 expression following LPS challenge (data not shown). Other lipid soluble compounds found in pistachio, γ-tocopherol, β-carotene, and β-sitosterol, were examined in an identical manner (Fig. 3B). Each of these bioactive molecules reduced LPS-stimulated Ifit-2, in particular β-carotene, albeit with IC_{50} values in the μM range (IC_{50} are 3, 2, and 1.2 μM, respectively of γ-tocopherol, β-carotene, and β-sitosterol.)

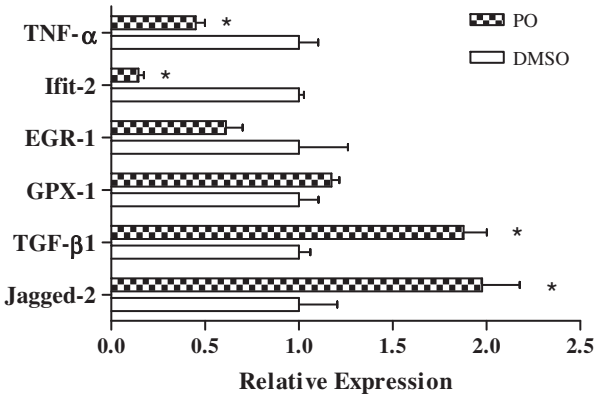

Figure 1. Effect of PO on global gene expression. RAW 264.7 macrophages were seeded in 6-well plates at a density of 1.0×10^6/well the day before treatment. Cells were treated with either PO (5.5 mg/mL) or DMSO (vehicle control) for 24 h. To validate microarray results, mRNA levels of three upregulated genes (bottom three genes in the figure) and three down-regulated genes (top three genes in the figure) were quantified by real-time PCR. All values were adjusted by housekeeping gene 18S ribosomal RNA. *indicates that the difference between control and PO treatment was significantly different ($p < 0.05$). TGF, transforming growth factor; GPX, glutathione peroxidase; EGR, early growth response; TNF, tissue necrosis factor.

3.4 Identification of Ifit-2 promoter region responsive to pistachio treatment of RAW 264.7 cells

RAW 264.7 cells were transfected with different length of Ifit-2 promoter/luciferase reporter constructs (described in section 2.8) and treated with DMSO (data not shown), LPS and co-treated with PE+LPS (Fig. 4). Luciferase activity induced by LPS was significantly decreased by PE co-treatment of pGL4/−1417bp+78bp Ifit-2 and pGL4/−417bp+78bp Ifit-2 constructs. The region between −317bp and the transcription start site (+78bp) was examined more closely. LPS induced a significant increase of luciferase activation

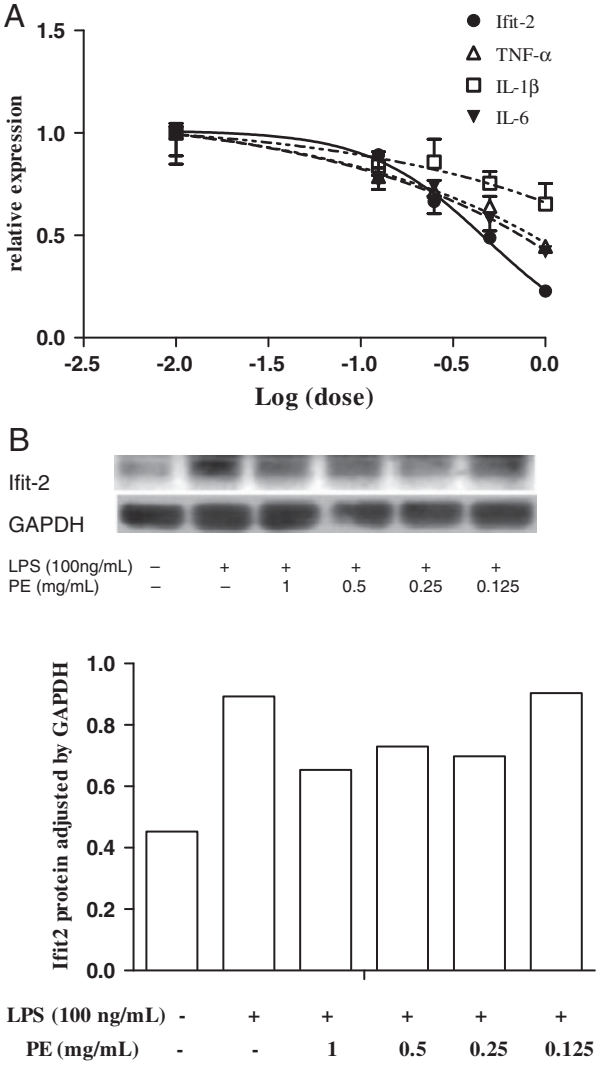

Figure 2. Expression changes of inflammation-related genes following co-treatment of PE and LPS. RAW 264.7 macrophages were treated with PE (as indicated dose) for a total of 24 h, during which time they were co-treated with LPS (100 ng/mL) for the last 6 h. mRNA levels were measured by real-time PCR. All values were adjusted by housekeeping gene 18S ribosomal RNA. (A) Significant treatment effects were observed for genes Ifit-2 ($p<0.001$), TNF-α ($p=0.001$), and IL-6 ($p<0.001$). No significant treatment effect was observed of IL-1β ($p=0.237$). (B) Western blot showed that LPS significantly increase Ifit-2 protein compared with non-LPS control. PE significantly decreased Ifit-2 protein through 0.25–1 mg/mL concentration. 0.125 mg/mL PE did not affect Ifit-2 protein level. Ifit2 and GAPDH band intensities were estimated by subtracting background intensities. Relative Ifit2 protein levels were plotted after GAPDH adjustment.

among pGL4/−317bp+78bp Ifit-2 to pGL4/−117bp+78bp Ifit-2 constructs. PE addition to LPS had a significant inhibitory effect of luciferase activation. However, LPS failed to stimulate luciferase activity of pGL4/−17bp+78bp Ifit-2 construct and PE response was not observed for this construct.

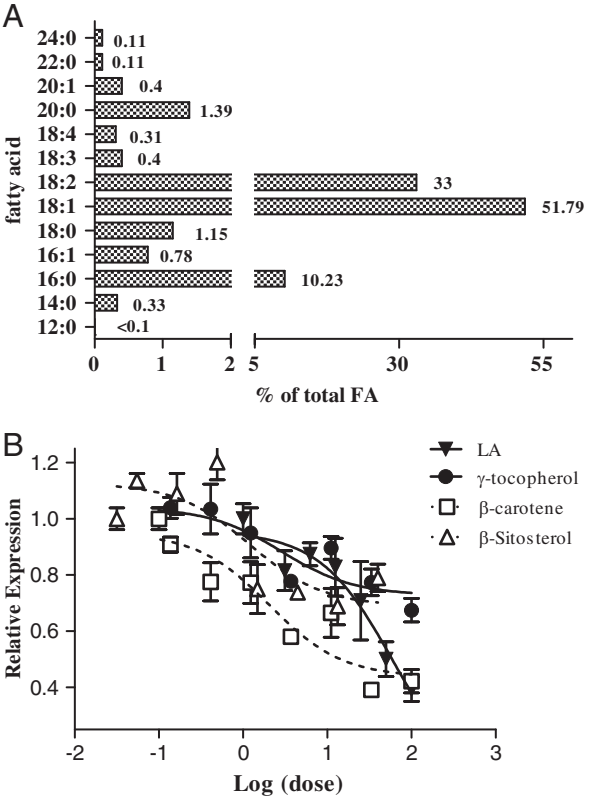

Figure 3. Identification of bioactive compounds responsible for Ifit-2 reduction. (A) Fatty acid was expressed as the number of carbon atoms: the number of double bonds within the carbon chain. Fatty acid profile analysis indicated that the predominant saturated, monounsaturated and polyunsaturated fatty acids are PA, OA and LA, respectively. Values next to the bar represent the percentage of individual fatty acid of total fatty acid. (B) RAW 264.7 macrophages were treated with different doses of fatty acids (100–25 μM; 1:2 dilution), γ-tocopherol and β-carotene (100 μM to 400 nM; 1:3 dilution), and β-sitosterol (40 μM to 160 nM; 1:3 dilution) for a total of 24 h, during which time they were co-treated with 100 ng/mL LPS for the last 6 h. LA treatment significantly reduced Ifit-2 expression in a dose dependent manner ($p<0.001$). Significant treatment effects were only observed at micro molar ranges of vitamins and phytosterol treatment. The calculated IC_{50} of LA, γ-tocopherol, β-carotene, and β-sitosterol were 60, 3, 2, and 1.2 μM, respectively.

3.5 Mutation of ISREs in Ifit-2 promoter region changed responses to pistachio treatment in RAW264.7 cells

Ifit-2 promoter analyses suggest that PE decreased Ifit expression by affecting transcription and that the region from −117 to −17bp contains the enhancer element involved. This region contains two ISRE elements (Fig. 5). After macrophages were transfected with a plasmid containing the wild-type ISRE sequence, a significant induction of luciferase activity was observed following LPS stimulation. However, this was not detected in cells transfected with three mutant ISRE luciferase constructs

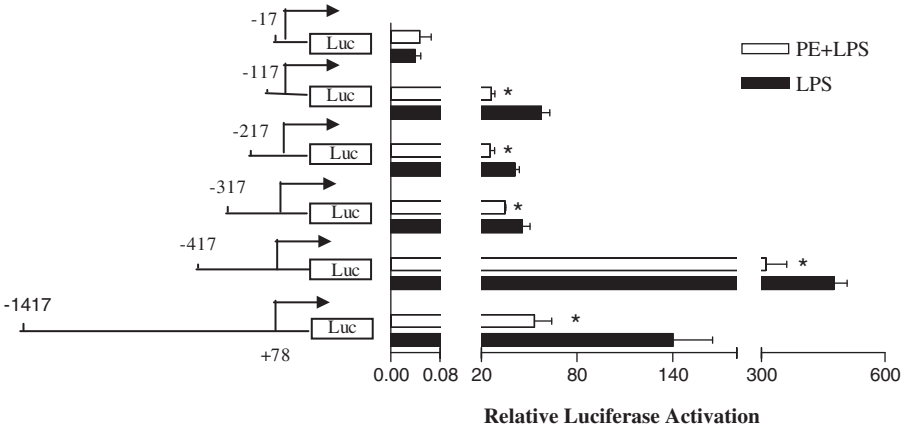

Figure 4. Mouse Ifit-2 promoter activity changes after PE treatment of RAW 264.7 cells. pGL4 vectors with insert of different lengths (−1417 to −17 bp) of 5′-flanking region of mouse Ifit-2 promoter were transfected into RAW 264.7 cells. After recovering overnight, RAW 264.7 macrophages were treated with DMSO (vehicle control; data not shown); LPS (100 ng/mL), and co-treated with LPS+PE (0.5 mg/mL) for 24 h. *indicates significantly different from LPS treatment ($p<0.05$) when compared LPS+PE co-treatment with LPS. All luciferase values were adjusted by respective protein levels.

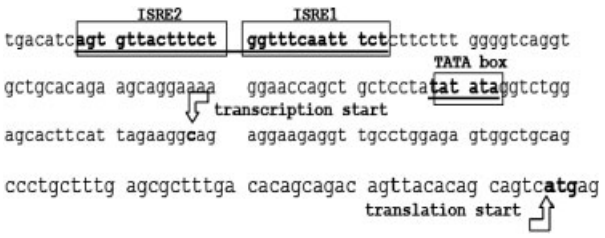

Figure 5. Prediction of response element – ISRE in Ifit promoter region. Two ISREs were found adjacent at −110 to −85 bp 5′-flanking region of Ifit-2 promoter based on research conducted by Bluyssen *et al.* [14] and online analysis tool TFSEARCH, version 1.3. ISRE, INF-stimulated response element.

(Fig. 6A). For the wild-type construct, luciferase activity was significantly decreased following the co-treatment of PE with LPS (data not shown). When both ISREs were mutated, compared with wild-type macrophages response, the inhibitory effect of PE on Ifit-2 expression was reversed (52% increase) following co-treatment with LPS (Fig. 6B). Mutation of ISRE1 and ISRE2 alone did not significantly change luciferase activity despite a trend of an increase (33 and 15%, respectively).

4 Discussion

In these *in vitro* model systems, PO and its extract significantly decreased inflammatory markers including Ifit-2, TNF-α, and IL-6. The most potent effects were observed for Ifit-2, which was more responsive to PE than other biomarkers commonly used to identify inflammation. Ifit genes are markers of inflammation that are induced by LPS or IFN stimulation and are attenuated by glucocorticoids *in vitro* [14–17]. In a gene profiling study conducted by Ovstebo *et al.* [18], Ifit-2 was increased 82-fold in response to LPS stimulation, as were Ifit-1 (51-fold), Ifit-3 (42-fold) and Ifit-5 (22-fold). Our microarray results revealed that mouse Ifit family members were reduced by PO treatment. In mice, three members of this gene family are ISG-56 (also known as Ifit-1 or GARG-16/Ifi-56), ISG-54 (also known as Ifit-2 or GARG-39/Ifi-54), and ISG-49 (also known as Ifit-3 or GARG-49/IRG-2), which are homologous to the human ISG-56, ISG-54, and ISG-60 genes, respectively [16, 19, 20]. Mutations of Ifit identified in humans lead to reduced expression in response to IFN and/or LPS activation [14, 21]. Ifit is also involved in virus infection, auto-immunologic disease, and systemic lupus erythematosus [22–27]. Taken together, these data indicate that Ifit family members are responsive to inflammatory signaling and their expression is associated with diseases in humans.

Microarray and other data presented herein identified Ifit-2, as being a biomarker of inflammation that is regulated by PO and its extract. Although significant treatment effects of PE were also observed for TNF-α and IL-6, changes in Ifit-2 mRNA were more dramatic and seen at lower doses. IL-1β mRNA is the least sensitive of the established inflammatory biomarkers and was significantly reduced only at the highest PE dose. Preliminary studies were conducted to determine if the Ifit response could be observed in response to dietary pistachio. Peripheral blood mononuclear cells were isolated from subjects participating in a clinical pistachio feeding study [28]. The two pistachio diets (3 and 1.5 ounces/day) reduced peripheral blood mononuclear cells Ifit-2 mRNA level by −40 and −30%, respectively, compared with a baseline high saturated fat average American diet (unpublished observations).

Like other tree nuts, pistachios and PO are sources of many bioactive components, including fatty acids, phytosterols, vitamins, and minerals http://www.nal.usda.gov/

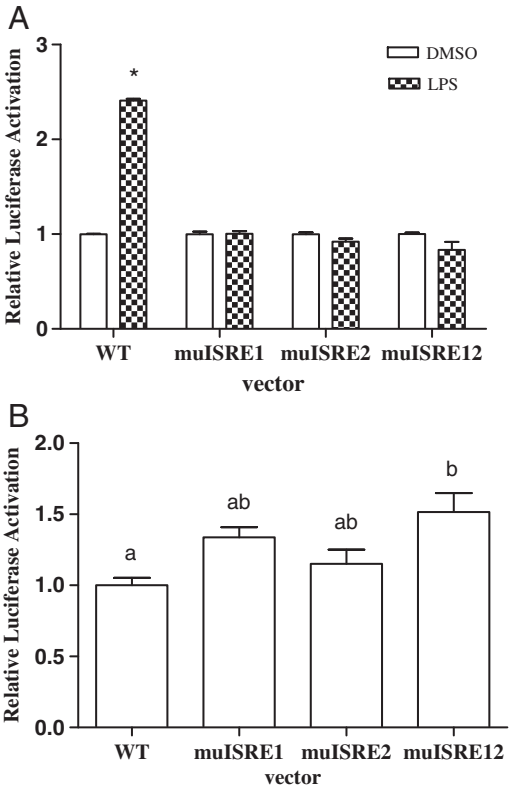

Figure 6. Wild-type and mutant ISREs in response to PE treatment. Oligonucleotides from −117 to −77 bp (including two ISREs) 5′-flanking region of Ifit-2 promoter, as well as mutations of sequences for ISRE1, ISRE2 and ISRE1 and 2, were cloned into pGL3 promoter vectors as described. RAW 264.7 macropahges were transfected with different plasmid constructs and treated with DMSO (not shown), PE (0.5 mg/mL), LPS (100 ng/mL) and co-treatment for 24 h. (A) *indicates significantly different from DMSO ($p<0.05$); (B) bars with different letters differ ($p<0.05$). All luciferase values were adjusted by respective protein levels.

fnic/foodcomp/search/. Besides inhibition of Ifit-2 by PE, we also tested the effect of PO on Ifit-2 expression. PO reduced Ifit-2 expression in a similar manner as that of PE. For example, the highest dose (5.5 mg/mL) inhibited Ifit-2 mRNA by −85% in response to LPS challenge with an IC_{50} of 0.97 mg/mL (data not shown). Pistachios are particularly a rich source of unsaturated fatty acids (predominantly as OA) and phytosterols. Although OA showed anti-inflammatory effects by reducing nuclear factor-kappaB and activating protein 1 expression in some *in vitro* studies [29–34], there was no significant effect of oleate on Ifit-2 expression in our study (25–100 μM; data not shown). Other predominant fatty acids in nuts, such as PA and α-linolenic acid, also had no effect on Ifit-2 expression at the same concentration range. However, LA showed a significant inhibitory effect on Ifit-2 expression in a dose-dependent manner. In addition, lipid soluble vitamins γ-tocopherol and β-carotene both significantly reduced Ifit-2 expression with IC_{50} of 3 and 2 μM, respectively. Other bioactive compounds in pistachios, such as phytosterols, could also have anti-inflammatory effects [35–39]. In support, β-sitosterol decreased LPS-stimulated Ifit-2 expression with an IC_{50} of 1.2 μM. Based on the fatty acid profile analysis and information present elsewhere http://www.nal.usda.gov/fnic/foodcomp/search/, the estimated availability of LA, γ-tocopherol, β-carotene and β-sitosterol in the PE ($IC_{50} \approx 0.5$ mg/mL) is approximately 175 μM, 270 nM, 3 nM, and 2.4 μM, respectively. Thus, the most likely scenario is that LA and β-sitosterol are responsible for the observed Ifit-2 reduction from the pistachio extract with minor contributions from the other fatty acids and bioactive molecules.

PO decreases Ifit expression by affecting its transcription *via* elements in the −110 to −85bp 5′-flanking promoter region, and area which includes two adjacent ISREs. Previous studies with mouse macrophages have shown that ISRE transcription activity was regulated by LPS treatment [40–44]. LPS interacts with the membrane toll-like receptor-4 and activates nuclear factor-kappaB and/or activating protein 1, followed by an increase in proinflammaory gene expression. LPS stimulation also can mediate endogenous INF expression and activate Janus kinase signal pathway and signal transducer and activator of transcription (STAT) signal pathway. The activated signal pathways would lead to INF regulatory factor binding to ISRE and/or INF-γ activated sequence. In our microarray results, STAT1 and STAT2 were decreased over twofold, indicating a possible pathway regulated by pistachio treatment. It is not clear which of these transacting factors is being affected by pistachio and extracts derived from this nut due to their complex nutrient combination as whole food package. However, based on the mutational analysis of the Ifit-2 promoter, the ISRE is the key *cis*-acting element. This response element is found in a variety of pro-inflammatory genes including TNFα and IL-1α. Presumably, the number, arrangement or sequences flanking the ISRE in Ifit-2 render this particular inflammatory gene more responsive to the effects of pistachio and its components.

In summary, PO has beneficial effects on the regulation of inflammation-related genes, notably a relatively understudied potential biomarker Ifit-2. Compared with more conventional inflammatory and CVD markers, extracellular inflammation-stimulated Ifit-2 gene expression was most affected by PE treatment. Thus, these results suggest that PO beneficially affects CVD risk, in part, by modulating the inflammatory response as measured by common inflammatory markers TNF-α and IL-6 as well as Ifit-2. The fact that LA and β-sitosterol are responsible for this effect, and these are components of other tree nuts and plant-derived foods, suggests that Ifit-2 may be a sensitive biomarker for the anti-inflammatory effects and CVD risk in dietary intervention studies.

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