Regular Article

Naturally Oxidized Olive Oil Promotes Active Cutaneous Anaphylaxis and Th2 Cytokine Production

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Received January 21, 2021; accepted March 29, 2021

The excessive ingestion of oxidized dietary oils may exacerbate some allergic diseases. We previously reported that oxidized olive oil exacerbates active cutaneous anaphylaxis (ACA), one of the immediate allergic reactions. This study was conducted to clarify the effects of oxidized olive oil on the T cell response during ACA. BALB/c female mice were orally administered naturally oxidized olive oil once every 2 d for 2 weeks after ovalbumin (OVA)/aluminum hydroxide gel sensitization, after which ACA was elicited by intracutaneous administration of OVA into the ear auricles. Compared with fresh olive oil, oxidized olive oil administration increased the antigen-specific immunoglobulin E (IgE) antibody titer 2 weeks after OVA-sensitization and vascular hyperpermeability increased due to ACA. In the oxidized olive oil-administered mice, the mRNA expression levels of T-helper 2 (Th2) cytokines, interleukin (IL)-4, -5, -6, and -10, in the lymph nodes increased, as did the proportion of cluster designation (CD)3+CD4+ cells in the spleen and lymph nodes. In CD3+CD4+ cells, the mRNA expression levels of IL-4 and GATA-binding protein 3 (GATA3), the master regulator of Th2, were higher in the oxidized olive oil-group. Antigen-stimulated specific IL-4 production was promoted in CD3+CD4+ cells of oxidized olive oil-administered mice. This suggests that oxidized olive oil exacerbates ACA by promoting Th2 dominance in immediate allergic diseases.

Key words oxidized oil; lipid peroxide; oxidative stress; allergic disease; active cutaneous anaphylaxis

INTRODUCTION

The prevalence of allergic diseases is increasing worldwide, especially in developed countries. Many studies have been conducted to clarify the cause of this problem, focusing on changes in lifestyles such as living and food environment. The hygiene hypothesis, an increase in the prevalence of allergies as a result of improved hygiene levels due to improved living environments, is well known. Infection and unhygienic environments during infancy were found to suppress the development of allergic diseases. This is thought to be based on promoting T-helper 1 (Th1) differentiation and adjusting the Th1/Th2 balance through contact with antigens in the environment. Excessively hygienic environments were suggested to inhibit Th1 differentiation, leading to a Th2-dominant state, thereby increasing the susceptibility to asthma and allergic rhinitis.

In developed countries, consumption of frying oil and processed fat is increasing due to simplification of dietary habits such as an increase in fast food intake. An increased intake of oxidized cooking oil induces oxidative stress by oxidizing unsaturated fatty acids and generating free radicals. Oxidative stress is an imbalance between oxidation and antioxidation, and is caused by overexposure to oxidants. It has been reported that thermally-oxidized cooking oils promote lipid peroxidation and causes oxidative damage of various tissues such as liver and pancreas. Oxidized soybean oil intake has been reported to enhance the production of antibodies and inflammatory mediators and to promote lymphocytes proliferation, but little information is available on the relationship between oxidized oil intake and the increased incidence or exacerbation of allergic diseases. As oxidative stress is associated with a variety of allergic diseases, such as asthma, bronchitis, and dermatitis, increased oxidative stress due to the ingestion of oxidized dietary oils may increase allergic symptoms.

The increase in oxidative stress and tissue damage caused by the ingestion of oxidized cooking oils was clarified in animal experiments. We previously demonstrated that ingestion of naturally oxidized olive oil exacerbated various allergic reactions, including the immediate type. However, the mechanisms of aggravation of the immediate allergic reaction due to the oxidized oil intake are unclear. In this study, we investigated the effects of oxidized olive oil ingestion on active cutaneous anaphylaxis (ACA), one of the immediate-type allergic reactions, using ovalbumin (OVA) to clarify the effects of oxidized oil on T cells during allergic reactions. ACA is caused by the humoral immune response related to Th2 cells and immunoglobulin E (IgE) antibody; therefore, we measured the allergic symptoms and Th2 cytokine expression levels in ACA. Furthermore, the spleen of ACA model mice was harvested to examine the Th1/Th2 balance by measuring cluster designation (CD)3+CD4+ cell distribution and Th2 cytokine production in peripheral lymphocytes.

MATERIALS AND METHODS

Preparation of Oxidized Olive Oil Oxidized olive oil was prepared by natural oxidation of olive oil for more than 6 months at room temperature. Naturally oxidized olive oil and fresh olive oil were mixed and adjusted to approximately 50 mEq/kg. The thiobarbituric acid reactive substances

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(TBARS) and acid value (AV) of oxidized olive oil were similar to those of fresh olive oil.

**Chemical Analyses** The peroxide value (POV) was measured by the American Oil Chemists’ Society official method Ja 8-87(17) using 0.01 mol/L sodium thiosulfate after dissolving oils in an acetic acid–chloroform (3:2) solution. The POV (mEq/kg) was calculated from the titration value of sodium thiosulfate.

**Animals** Animal protocols conformed to the Animal Experiment Guidelines of Setsunan University that were established by revising the guidelines of the Japanese Society for Pharmacology. This study was approved by the Committee for the Ethical Use of Experimental Animals at Setsunan University. All efforts were made to minimize animal suffering, reduce the number of animals used, and use alternatives to in vivo techniques. Female BALB/c mice (4–5 weeks old) were purchased from Japan SLC, Inc., Shizuoka, Japan, and acclimated in a specific pathogen-free room at 23 ± 1 °C and 47–67% humidity under a 12-h light–dark cycle (lights on at 7:30 a.m.) for at least one week before the start of experiments.

**ACA Sensitization** Female mice (6-weeks-old) were immunized twice at weekly intervals by intraperitoneally administering a mixture of 1 µg of OVA (Sigma-Aldrich Inc., St. Louis, MO, U.S.A.) and 1 mg of aluminum hydroxide gel as the adjuvant. Mice were orally administered 100 µL of the oxidized olive oil from the first immunization once every 2 d for 2 weeks. Thereafter, mice were intravenously injected with 250 µL of 0.5% Evans blue-saline solution, followed by the elicitation of ACA in the right ear by injecting 10 µL of 0.1 µg/µL OVA-saline solution. The left ear was sham-challenged by injecting saline solution. Thirty minutes after the challenge, mice were sacrificed under anesthesia and both ears were removed to measure extravasated dye. Non-immunized mice were challenged in the same manner as immunized mice in order to assess nonspecific dye leakage. The extraction and quantification of dye leakage were performed as described by Inagaki et al.(18).

Blood samples were collected 1 and 2 weeks after OVA-immunization. OVA-specific IgE plasma levels were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (SHIBAYAGI Co., Ltd., Gunma, Japan).

**Preparation of Splenocytes** Spleens were harvested under sterile conditions and single-cell suspensions were prepared by passage through a nylon mesh filter. After destroying red blood cells using ammonium–chloride–potassium buffer, splenocytes were suspended in growth medium or 2% fetal bovine serum (FBS)-containing phosphate-buffered saline (PBS).

**Cell Proliferation Assay** Splenocytes (200000 cells/well) were cultured in 96-well flat plates and stimulated by 100 µg/mL of OVA for 72h. After incubation with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) for 2h, the generated formazan crystals were dissolved in dimethyl sulfoxide (DMSO) and the absorbance at 570 nm was measured.

**Flow Cytometry and Cell Sorting** Splenocytes were FcR blocked with TrueStain fcX (BioLegend, Inc., San Diego, CA, U.S.A.) and then stained using combinations of fluorescently labeled anti-mouse antibodies: antigen-presenting cell (APC)-CD3, PE/Cy7-CD4, and fluorescein isothiocyanate (FITC)-CD8 (BioLegend, Inc.). Multi-stained cells were analyzed by BD FACSAria Fusion (BD Biosciences, San Diego, CA, U.S.A.), and CD3+CD4+ and CD3+CD8+ cells (each >98% purity) were isolated based on the results.

**APC Preparation** Mouse peritoneal macrophages and other hemolytic cells were disrupted by osmotic shock using one-third isotonic saline. Remaining cells were seeded onto cell culture dishes in the culture medium and incubated at 37 °C for 1 h. After washing away non-adherent cells, adherent cells were obtained as macrophages. More than 98% of the cells were macrophages according to the zymosan phagocytosis test.

**T Cell and APC Co-culture** Isolated CD3+CD4+ cells were cultured in flat 96-well plates with APC. These cells were co-cultured at a 5:1 T cell/APC ratio (500000 to 1000000). Cells were stimulated by 20 ng/mL of 12-O-tetradecanoylphorbol 13-acetate (PMA) + 1 µg/mL of ionomycin (Io) or 100 µg/mL OVA. After culturing for 48h, the amounts of interleukin (IL)-4 and interferon (IFN)-γ in the culture supernatant were measured using the Mouse Uncoated ELISA Kit (Thermo Fisher Scientific, Inc.).

**mRNA Expression Analysis** mRNA expression levels were measured by RT-PCR. Lymph nodes and isolated T cells were immersed in Sepasol RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan). Total RNA extracted according to the manufacturer’s protocol was reverse transcribed using a reverse transcription kit (Thermo Fisher Scientific, Inc.). One microliter of the cDNA solution was used for PCR using SYBR Green I Master (Roche Diagnostics, Mannheim, Germany) and LightCycler 480 System II (Roche Diagnostics, GmbH). Primer sets for IFN-γ, IL-4, -5, -6, -10, T-box expressed in T cells (T-bet), GATA-binding protein 3 (GATA3), and ribosomal protein S18 (Rps18, an internal control) were purchased (Eurolin Genomics, K.K., Tokyo, Japan). The relative mRNA expression levels were calculated as ratios based on Rps18 mRNA levels.

**Statistical Analysis** Results were statistically analyzed by the Student’s t-test or two-way ANOVA followed by Bonferroni’s multiple comparison test using LightStone Origin Pro (Tokyo, Japan). p-Values <0.05 were considered significant. The data represent the mean ± standard deviation (S.D.).

**RESULTS**

**Auricular Allergic Reactions and Cytokine mRNA Expression by ACA** To investigate the effects of oxidized olive oil on ACA, mice that were sensitized with OVA were orally administered oxidized or fresh olive oil for 2 weeks. ACA was elicited on the next day by intracutaneous administration of OVA to the right ear and vascular permeability due to the allergic reaction 30 min after elicitation was evaluated. Almost no dye leakage was observed in the non-immunized mice regardless of the oxidation degree of the administered oil. However, dye leakage due to the allergic reaction was observed in the oxidized olive oil-administered group was greater than that in the fresh olive oil-administered group (Fig. 1).

The titer of OVA-specific IgE antibody in the plasma was measured 1 and 2 weeks after sensitization. OVA-IgE was not
detected in the non-immunized mice, but was detected one week after sensitization in immunized mice (Fig. 2). The titer of OVA-IgE was significantly higher in the oxidized olive oil-administered group two weeks after sensitization. The mRNA expression levels of Th2 cytokines, IL-4, IL-5, IL-6, and IL-10, in infra-auricular parotid lymph nodes were measured two weeks after sensitization. All these cytokines mRNA levels increased approximately two-fold in the oxidized olive oil group compared with those in the fresh olive oil group (Fig. 3). Th1 cytokines, IL-12 p40 and IFN-γ, were not significantly affected by oxidized olive oil (data not shown).

Lymphocytes and T Cell Responses of Splenocytes from OVA-Sensitized Mice

To investigate whether the administration of oxidized olive oil affects peripheral T cell responses in OVA-sensitized mice, we first measured the effects of OVA treatment on the splenocyte proliferation rate. No significant increase in splenocytes by OVA treatment was observed in non-immunized mice regardless of oxidized olive oil administration (Fig. 4). Splenocytes of the immunized mice demonstrated OVA-stimulated proliferation and significantly increased in the oxidized olive oil group. The relative proportion of the T cell subsets in splenocytes and lymphocytes isolated from OVA-sensitized mice was defined by CD3 and CD4 fluorescent antibody staining and analyzed by flow cytometry. The percentage of CD3+CD4+ double-positive cells was gated and quantified, as shown in Fig. 5. CD3+CD8+ cells were not significantly different between two groups, while the percentage of CD3+CD4+ cells in the oxidized olive oil group was significantly higher than that in the fresh olive oil group.
Mice were orally administered fresh olive oil [POV = 4.98 ± 0.68 mEq/kg] or oxidized olive oil [POV = 49.5 ± 1.01 mEq/kg] 14 d after OVA sensitization. Fourteen days after sensitization, splenocytes were analyzed by flow cytometry. Shown are representative dot plots of CD3 vs. CD4 staining.

**DISCUSSION**

Sensitization with aluminum hydroxide gel as an adjuvant induces Th2 and Th2 cytokines, such as IL-4,[20] and in the induction phase of immediate allergic reactions, including ACA, symptoms appear via IgE antibody. IgE bound to effector cells, such as basophils and mast cells, binds to antigens and is activated by cross-linking to release histamine and other inflammatory mediators, thereby increasing vascular permeability.[21] A relationship between the intake of oxidized dietary oil and immune function has been reported. Lin et al. reported that intake of deep-fried oxidized soybean oil influences Th2-related antibodies and inflammatory mediators.[10] This study suggested that the intake of oxidized dietary oils increases Th2 immunity and allergic reactions.

We previously demonstrated that the consumption of oxidized olive oil exacerbates contact hypersensitivity, a delayed-type allergy, and that this exacerbation was caused by the increased production of IL-18 during the sensitization phase, resulting in the increased production of IFN-γ and Th1.[22,23] In this study, administration of an ingestible amount of oxidized olive oil increased Th2 immunity and exacerbated the immediate allergic reaction. Oxidized olive oil increased serum IgE levels and mRNA expression of Th2 cytokines in regional lymph nodes, and exacerbated ACA. As IL-4 plays an important role in the differentiation of naïve T cells into Th2, increased IL-4 production by oxidized olive oil-intake may amplify Th2. Promotion of Th2 differentiation resulted in increased IgE production in the sensitization phase, and the activation of effector cells, such as basophils and mast cells, was promoted by eliciting ACA, resulting in increased vascular permeability. Oarada reported that mitogen-stimulated cell proliferation increased in splenic lymphocytes of mice fed an oxidized soybean oil-containing diet.[11] This report supports our finding that oxidized olive oil increases splenocyte proliferation due to OVA stimulation. T follicular helper (TFH), a subset of helper T cells present in lymphoid follicles, is considered to be more involved in the control of antibody responses.

**Table 1.** Proportion of the T Cell Subpopulation

<table>
<thead>
<tr>
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<th>CD3⁺CD4⁺ (%)</th>
<th>CD3⁺CD8⁺ (%)</th>
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<tbody>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh oil</td>
<td>25.6 ± 3.06</td>
<td>13.5 ± 3.53</td>
</tr>
<tr>
<td>Oxidized oil</td>
<td>32.5 ± 1.40**</td>
<td>17.7 ± 5.15</td>
</tr>
<tr>
<td>Mesenteric LN</td>
<td></td>
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</tr>
<tr>
<td>Fresh oil</td>
<td>52.7 ± 1.12</td>
<td>20.7 ± 1.64</td>
</tr>
<tr>
<td>Oxidized oil</td>
<td>57.4 ± 1.93*</td>
<td>22.4 ± 1.25</td>
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<tr>
<td>Parotid LN</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fresh oil</td>
<td>47.0 ± 5.28</td>
<td>25.0 ± 1.83</td>
</tr>
<tr>
<td>Oxidized oil</td>
<td>54.5 ± 2.60*</td>
<td>26.7 ± 1.38</td>
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*p < 0.05, **p < 0.01 vs. the fresh olive oil group. Statistical test used was t test.

**Fig. 5. Effects of Oxidized Olive Oil on the CD3⁺CD4⁺ Cell Subpopulation**

**Fig. 6. Effects of Oxidized Olive Oil on mRNA Expression in CD3⁺CD4⁺ Cells**

CD3⁺CD4⁺ cells were sorted from splenocytes by FACS. The POV of fresh and oxidized olive oil was 4.98 ± 0.68 and 49.50 ± 1.01 mEq/kg, respectively. The mRNA expression levels of target genes were normalized by Rps18. The relative expression levels in the fresh olive oil group were designated as 100%. Fresh olive oil (□) and oxidized olive oil (■). The values are the mean ± S.D. (n = 4).

*p < 0.05, **p < 0.01 vs. the fresh olive oil group. Statistical test used was t test.
production than Th2,\textsuperscript{24} so detailed studies focusing on TFH are required on the effects of oxidized oils.

Th1 produce IFN-\(\gamma\) and promote cell-mediated immunity, whereas Th2 produce IL-4 and promote humoral immunity by antibodies.\textsuperscript{25,26} IFN-\(\gamma\) suppresses Th2 and IL-4 suppresses Th1 to reciprocally adjust their function and maintain balance.\textsuperscript{27,28} In the present study, oxidized olive oil reduced IFN-\(\gamma\), and increased IL-4 and GATA3 expression in CD3\(^+\)CD4\(^+\) cells, suggesting that it promoted Th2 dominance. Although a decrease in IFN-\(\gamma\) mRNA was observed in the oxidized olive oil-administered group, t-bet mRNA did not affect. Further examination on protein expression of the master regulators and fluctuation of Th1/Th2 by oxidized oil is needed. IL-4 is essential for Th2 differentiation,\textsuperscript{29,30} and the binding of IL-4 to IL-4R activates Janus kinase/signal transducer and activator of transcription (JAK/STAT) signaling.\textsuperscript{31,32} Expression of GATA3, the master regulator of Th2, is deeply involved in the activation of nuclear factor-kappa B (NF-\(\kappa\)B)\textsuperscript{33} induced via CD28, a co-stimulatory receptor,\textsuperscript{34} in addition to induction by IL-4/STAT6 signaling.\textsuperscript{35,36} The promotion of NF-\(\kappa\)B activation by the intake of oxidized rapeseed oil has been reported.\textsuperscript{37} These reports suggest that NF-\(\kappa\)B activity may be involved in the promotion of Th2 induction by the oxidized olive oil. Neither IFN-\(\gamma\) nor IL-4 was affected by non-specific PMA/Io stimulation, but in the presence of APC, IL-4 increased and IFN-\(\gamma\) decreased. This suggests that oxidized olive oil increased antigen-specific Th2 and cytokine production during antigen stimulation.

In conclusion, we found that oxidized olive oil increased antigen-specific IgE production during the sensitization phase, resulting in exacerbated allergic reactions, such as vascular hyperpermeability, during the elicitation phase. Oxidized olive oil also increased the proportion of CD3\(^+\)CD4\(^+\) cells in lymphatic organs and their production of antigen-specific IL-4. This suggests that the excessive intake of oxidize olive oil promotes Th2 differentiation and proliferation via an increase in IL-4 production during the sensitization phase, thereby exacerbating immediate-type allergic diseases. However, the mechanisms of Th2 response promotion and effects of oxidized olive oil on other effector cells, such as TFH, basophils, and mast cells, were unclear. Further studies are needed to clarify the exacerbation mechanisms of immediate-type allergic reactions due to the ingestion of oxidized dietary oil.

Fig. 7. Effects of Oxidized Olive Oil on IFN-\(\gamma\) and IL-4 Production by Splenocytes and Sorted T Cells
Acknowledgments We thank Eri Koike, Shingo Yamada, Masashi Okada, and Karin Mizuno at Setusunan University for their technical assistance.

Conflict of Interest The authors declare no conflict of interest.

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