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Effects of Omega-3-Rich Harp Seal Oil on the Production of Pro-Inflammatory Cytokines in Mouse Peritoneal Macrophages

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ABSTRACT: Omega-3, a polyunsaturated fatty acid, is an essential fatty acid necessary for human health, and it protects against cardiovascular disease, inflammation, autoimmune diseases, and cancer. In the present study, we investigated the effects of omega-3-rich harp seal oil (HSO) on the production of nitric oxide (NO) and cytokines, such as tumor necrosis factor (TNF)-α, interleukin-(IL)-1β, IL-6, and IL-12/IL-23 (p40) in peritoneal macrophages of mice. The culture supernatants of murine macrophages exposed to lipopolysaccharide (LPS), HSO, or HSO+LPS were harvested to assay IL-1β, TNF-α, IL-6, and IL-12/IL-23 (p40) cytokines and NO. TNF-α, IL-1 β, and IL-12/IL-23 (p40) levels, except IL-6, were lower in the culture supernatants of mouse peritoneal macrophages exposed to LPS plus HSO than those of the groups exposed to LPS alone. These observations demonstrate that omega-3-rich harp seal oil downregulates the production of the pro-inflammatory cytokines such as IL-1β, TNF-α, and IL-12/IL-23 (p40). These results suggest that HSO could be potentially used as a preventive agent or as an adjunct in anti-inflammatory therapy, if more research results were accumulated.

Keywords: harp seal oil, tumor necrosis factor (TNF)-α, interleukin (IL)-1β, IL-6, IL-12/IL-23 (p40), nitric oxide

INTRODUCTION

Pathological investigation by Dyerberg et al. (1) in the 1970s revealed that Eskimos who lived on fish and seals had significantly low rates of occurrence of cardiovascular diseases, such as atherosclerosis, brain stroke, and cardiac myoinfarction. Their follow-up research proved that omega-3 (ω-3), which is known to be abundantly supplied by fish and seals, had positive effects on the development of brain cells, anti-dementia properties, reduction of cardiovascular diseases, anti-inflammatory action, and anti-cancer properties (2). ω-3 is an essential fatty acid that cannot be synthesized in the human body, but it is a useful polyunsaturated fatty acid that helps to improve fluidity in cellular membranes, helps to transduce intracellular signals, and helps to manage inflammation and immune diseases.

Coronary heart disease, cancer, rheumatic arthritis, Crohn's disease, ulcerative colitis, and lupus erythematosus are some of the immune diseases that boost inflammatory cytokines, whereas diseases, including psoriasis, asthma, type-1 diabetes, and multiple sclerosis, result from destruction of host tissue and eventually from inappropriate activation of T-lymphocytes (3). Atherosclerosis has a mechanism similar to inflammation in that both, adhesion of leucocytes and clearance of proteins in the endothelium, are promoted by tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6 derived from monocytes and macrophages (4). Since ω-3 fatty acids reduce Th1 cytokines from monocytes and macrophages, and suppress the activation of immune cells such as T-lymphocytes, NK cells, and monocytes (5-7), they play important roles in inflammation-related diseases.

Cytokines such as TNF-α, IL-1β, and IL-6 are secreted by monocytes and macrophages during an inflammatory response, and they are involved in acute protein synthesis (8). TNF-α activates T and B lymphocytes and epithelial cells, and it regulates transcription of IL-6. IL-6 activates phospholipase A2 and cyclo-oxygenase pathways of arachidonic acid (AA) metabolism in platelets, and IL-12 plays an important role in the development of Th1 cells and initiation of cell-mediated immune reactions (9-11). Nitric oxide (NO) improves blood circu-

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lation in the vascular endothelium and/or suppresses cellular toxicity, which induces necrosis of hepatic cells driven by TNF-α and reactive oxygen intermediates (ROI) (12).

Harp seal oil (HSO) is a poly-unsaturated fatty acid that is abundant in ω-3 but low in ω-6, and it is isolated from marine mammals living in the arctic area of Canada. In this research, we investigated the changes in NO concentration and inflammation-related cytokines, including IL-1β, TNF-α, IL-6, and IL-12/IL-23 (p40), in murine macrophages post peritoneal injection.

MATERIALS AND METHODS

Experimental animals and reagents

Female Balb/c mice, 6∼8-weeks old, weighing approximately 25 g, were purchased from Daehan Biolink (Eumseong, Korea). The mice were housed in temperature and humidity-controlled rooms, kept on a 12-h light/dark cycle and provided with unrestricted amount of rodent chow and drinkable water. HSO (Newfoundland Health Food, St. Josephs, NL, Canada) was used as the source of ω-3 Plus as per the formula provided by the Korean Baptist Theological University. It contained a total of 23% of ω-3, 0.5% of α-linoleic acid, 6.7% of eicosapentaenoic acid, 10.3% of docosahexaenoic acid, and 4.7% of docosapentaenoic acid. HSO was initially dissolved into dimethyl sulfoxide (DMSO) and diluted into RPMI 1640 (Gibco BRL., Grand Island, NY, USA), and no toxicity was observed within the practical range of dosage. Lipopolysaccharide (LPS), separated and purified from *Escherichia coli* (serotype O26:B6), was purchased from Sigma-Aldrich (St. Louis, MO, USA). Mouse ELISA kits to measure the secretion of TNF-α, IL-1β, IL-6, IL-10, and IL-12/IL-23 (p40) were purchased from Biolegend (San Diego, CA, USA) and Griess reagent system (Promega Corp., Fitchburg, WI, USA) was used to measure nitric oxide.

Supernatant from macrophage incubation by murine peritoneal injection

The animals were killed by exposure to carbon dioxide and the peritoneal cavity was washed with 5 mL cold PBS. The cells were harvested by Pasture pipette. Peritoneal macrophages were inoculated from the plastic dish after 30 min incubation at 37°C. Peritoneal macrophages were diluted with RPMI 1640 media supplemented with 10% fetal bovine serum so that the macrophage count was 2×10^5 cells/mL. Macrophages were seeded into 24-well tissue culture plates (Costar, Cambridge, MA, USA) at 1 mL/well and incubated with HSO at a concentration of 10 μg/mL for 6, 24, and 48 h at 37°C and 5% CO_2. Effects of a combination of LPS and HSO were also observed. DMSO (0.01%) was used as a control. The culture supernatants underwent initial centrifugation at 300 *g* for 10 min and secondary centrifugation at 1,000 *g* for 30 min. The centrifuged supernatant were aliquoted and stored at −70°C until analyses of TNF-α, IL-1β, IL-6, IL-10, IL-12/IL-23 (p40), and NO.

Cytokine measurement

Each antibody against TNF-α, IL-1β, IL-6, IL-10, and IL-12/IL-23 (p40) was diluted with coating buffer, aliquoted into a 96-well microplate, and incubated at 4°C overnight. The next day, each plate was flushed with washing buffer 4 times. The assay diluent was aliquoted at 200 μL per well, and the plate was incubated at room temperature for 1 h. The plate was rinsed with washing buffer 4 times, 100 μL of sample was added to each well, and the plate was incubated at room temperature for 2 h. The plate was flushed with washing buffer 4 times, 100 μL of detection antibody was added to each well, followed by incubation at room temperature for 1 h. The plate was again rinsed with washing buffer 4 times, 100 μL of avidin-horseradish peroxidase was added to each well, followed by incubation at room temperature for 30 min. The plate was rinsed with washing buffer 5 times, 100 μL of the substrate reagent containing tetramethylbenzidine was added to each well, followed by incubation at room temperature for 20 min, and 100 μL/well of stop solution was added afterward to stop the reaction. Optical density was measured using a microplate reader (Model 550 microplate reader, Bio-Rad, Hercules, CA, USA) at the wavelength of 450 nm (12).

Measurement of nitric oxide

We measured NO_2^- dissolved in the medium by the following method. Fifty microliters of murine peritoneal macrophage culture supernatant and an equal volume of Griess reagent [*N*-(1-naphthyl) ethylenediamine 0.1% in H_2O, sulfanilamide 1% in 5% H_3PO_4] were added to an ELISA Titer Tek plate and incubated at room temperature for 10 min. Overall NO_2^- formation was measured by the optical density at a wavelength of 540 nm.

Statistical analyses

All results are expressed as the average±standard deviation. The statistical evaluation of each experimental group was performed using Student's *t*-test, and a *P*-value lower than 0.05 was regarded as significant.

RESULTS

In this study, we observed changes in IL-1β, TNF-α, IL-6, and IL-12/IL-23 (p40) cytokines and NO formation upon treatment with LPS or HSO, and further changes

in secretion of those cytokines upon concomitant treatment with LPS and HSO.

Determination of IL-1β

Treatment of a suspension containing isolated mouse peritoneal macrophages with HSO resulted in a constant level of IL-1β, which was maintained over time (48 h, 4.69 pg/mL). The use of the bacterial toxin LPS as a stimulant, promoted formation of IL-1β up to 15.46 pg/mL (48 h) as shown in Fig. 1. A concomitant dose of LPS (2 μg/mL) and HSO (10 μg/mL) reduced the formation of IL-1β, and eventually a significant reduction in its formation to 10.78 pg/mL was observed after 48 h.

Determination of TNF-α

Formation of TNF-α was significantly diminished by treatment with HSO alone as compared to controls after 48 h, and treatment with LPS promoted formation of TNF-α. A concomitant dose of LPS and HSO, however, as shown in Fig. 2, led to a significant reduction in formation of TNF-α at 6, 24, and 48 h.

Determination of IL-6 and IL-12/IL-23 (p40)

Formation of IL-6 was significantly diminished by treatment with HSO alone for 24 h, and a concomitant treatment of LPS with HSO led to a reduction of IL-6 formation, even if the differences were not significant (Fig. 3). The treatment of a suspension containing isolated mouse peritoneal macrophages with HSO also led to a significant reduction in the formation of IL-12/IL-23 (p40) at 48 h compared to controls, and a concomitant treatment with LPS (2 μg/mL) and HSO (10 μg/mL) also led to a reduction of IL-12/IL-23 (p40) at 24 and 48 h (Fig. 4).

Determination of nitric oxide (NO)

Treatment with HSO adversely led to high levels of NO at 6 h, but a concomitant dose of LPS and HSO suppressed the level of NO in comparison with LPS alone, even though the differences were not significant (Fig. 5).

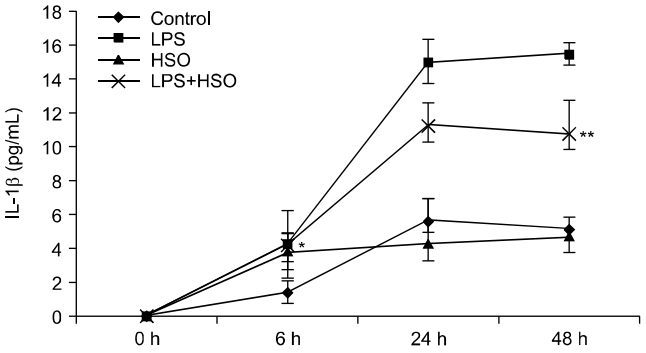

Fig. 1. Production of IL-1β cytokine by mouse peritoneal macrophages exposed to 2 μg/mL LPS, 10 μg/mL HSO, or LPS+HSO for 6, 24, or 48 h. Triplicate culture supernatants were pooled and assayed in duplicate. Data shown are the mean values± standard deviations. $^{*}P<0.05$ and $^{**}P<0.01$ compared to corresponding controls (cultured peritoneal macrophages).

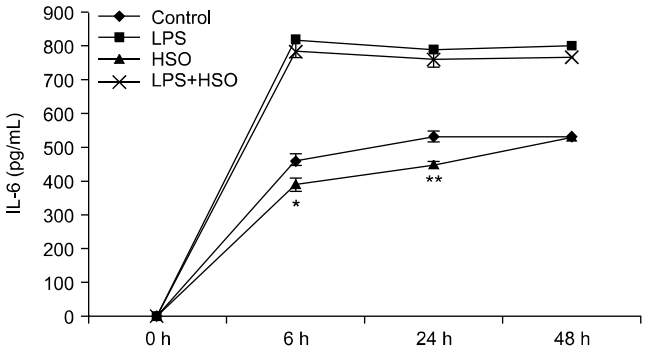

Fig. 3. Production of IL-6 cytokine by mouse peritoneal macrophages exposed to 2 μg/mL LPS, 10 μg/mL HSO, or LPS+HSO for 6, 24, or 48 h. Triplicate culture supernatants were pooled and assayed in duplicate. Data shown are the mean values± standard deviations. $^{*}P<0.05$ and $^{**}P<0.01$ compared to the corresponding controls (cultured peritoneal macrophages).

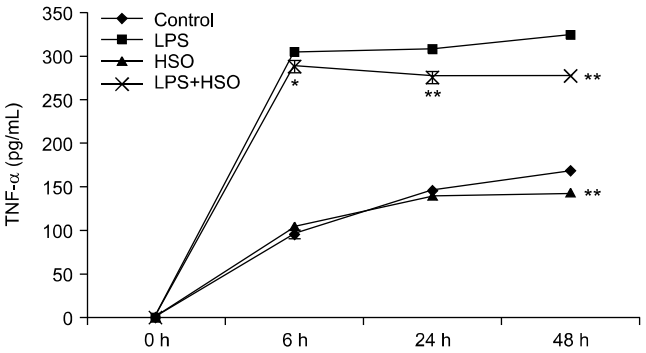

Fig. 2. Production of TNF-α cytokine by mouse peritoneal macrophages exposed to 2 μg/mL LPS, 10 μg/mL HSO, or LPS+HSO for 6, 24, or 48 h. Triplicate culture supernatants were pooled and assayed in duplicate. Data shown are the mean values± standard deviations. $^{*}P<0.05$ and $^{**}P<0.01$ compared to corresponding controls (cultured peritoneal macrophages).

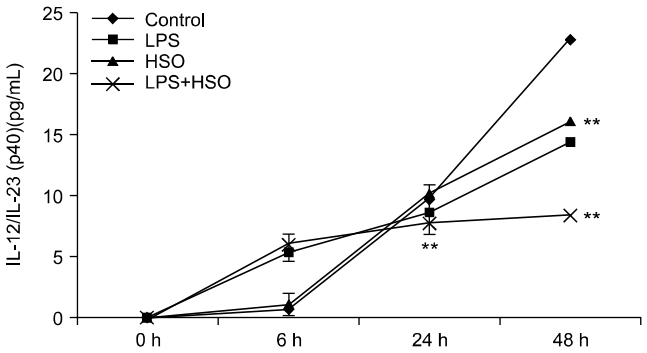

Fig. 4. Production of IL-12/IL-23 (p40) cytokine by mouse peritoneal macrophages exposed to 2 μg/mL LPS and 10 μg/mL Harp Seal Oil (HSO). Peritoneal macrophages were cultured in the presence of LPS+HSO for 6, 24, or 48 h. Triplicate culture supernatants were pooled and assayed in duplicate. Data shown are the mean values±standard deviations. $^{**}P<0.01$ compared to corresponding controls (cultured peritoneal macrophages).

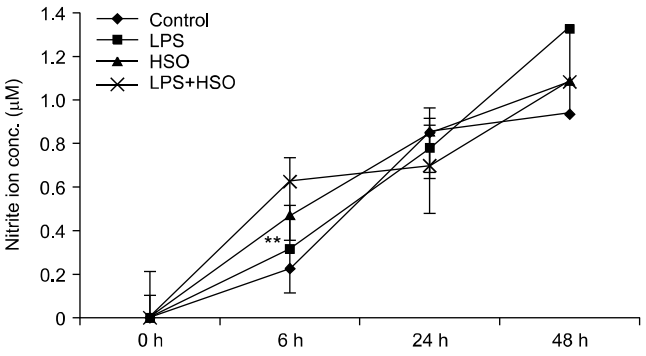

Fig. 5. Production of NO by mouse peritoneal macrophages exposed to 2 μg/mL LPS, 10 μg/mL HSO, or LPS+HSO for 6, 24, or 48 h. Triplicate culture supernatants were pooled and assayed in duplicate. Data shown are the mean values±standard deviations. $^{**}P<0.01$ compared to corresponding controls (cultured peritoneal macrophages).

DISCUSSION

We monitored changes in the levels of TNF-α, IL-1β, IL-6, IL-12/23A (p40), and NO to observe the effect of ω-3-abundant HSO on an endotoxic-LPS-based inflammatory reaction.

LPS, a compound found in the cell wall of gram-negative bacteria, is released during bacterial infection, and it is known to stimulate immune cells and potentially activate monocytes and macrophages. It also plays an important role in inflammatory and immune reactions by initiating the formation of NO and pro-inflammatory cytokines (13). This study showed significant increases in the formation of TNF-α, IL-1β, IL-6, IL-12/23A (p40), and NO upon stimulating murine peritoneal macrophages with LPS.

TNF-α, IL-1β, and IL-6 are pre-inflammatory cytokines known to cause fever, inflammation, tissue damage, and even shock; therefore, suppression of TNF-α and/or IL-1β is found to be useful in pathologies such as rheumatic arthritis, inflammatory bowel diseases, and graft rejection (14). An increase in adipose tissue also induces an increase in the levels of cytokines such as TNF-α and IL-6, and such increases reduce insulin resistance (15). Administration of ω-3 to healthy humans suppressed the efficacy of monocytes to synthesize TNF-α and IL-1β and to reduce inflammation by suppressing leukotriene B4 (LTB4). It suppressed IL-1 mRNA in patients with inflammatory and autoimmune diseases (16,17). Woods et al. (18) stated that increase in IL-6 is associated with cardiovascular diseases and inflammation, but that ω-3 fatty acid supplements suppressed both IL-6 and inflammatory reactions. Kato et al. (19) reported suppression of cancer cells as well as formation of IL-6 precursors when human colorectal cancer cells and ω-3 fatty acid were concomitantly administered to nude mice. Experimental groups of mice administered with ω-3 and ω6 showed significantly lower levels of IL-6 and IL-12 (p40) mRNA expression than mice that were administered with low levels of polyunsaturated fatty acids. Mice in an ω-3-fortified group showed lower expression levels of IL-12 p40 mRNA than those in an ω6-fortified group (16,20). NO is involved in specific- and/or non-specific immune reactions, and expression of inducible NO synthase (iNOS) can be modulated by a mixed reaction of activating compounds such as TNF-α, IFN-γ, IL-1β, and LPS. Rockey and Chung (21) reported that concomitant administration of IFN-γ and LPS synergistically boosts NO formation in proportion to LPS concentration. Our results also indicate that concomitant administration of LPS and HSO to murine peritoneal macrophages significantly reduced the production of IL-1β, TNF-α, and IL-12/IL-23 (p40) with an increase in incubation time, and reduced the formation of IL-6 and NO.

Block et al. (22) reported that ω-3 fatty acids might be more helpful than aspirin due to a better reduction of inflammation and angiogenesis. ω-6 fatty acids are known to promote the formation of pro-inflammatory leukotriene LTB (16), and therefore, an improvement in the ratio of ω-6/ω-3 essential fatty acids may be expected to suppress IL-1β-induced, IL-1β mRNA, and reduce the formation of macrophage-derived pro-inflammatory cytokines. This improvement may be helpful in reducing the use of corticosteroids (23) and cyclosporine, which are known to reduce the synthesis of IL-1β and TNF-α.

Our results therefore indicate a significant reduction in pro-inflammatory cytokines upon concomitant administration of LPS and HSO, which is abundant in ω-3 fatty acids.

In conclusion, we observed changes in the levels of expression of inflammatory cytokines, such as TNF-α, IL-1β, IL-6, IL-12/23A (p40), and NO using endotoxic LPS stimulation to observe the effects of HSO, which is known to be abundant in ω-3-polyunsaturated fatty acids. Administration of LPS to murine peritoneal macrophages led to a significant increase in TNF-α, IL-1β, IL-6, IL-12/23A (p40), and NO, and co-administration of HSO with LPS significantly reduced the formation of IL-1β, TNF-α, and IL-12/IL-23 (p40) with an increase in incubation time. The formation of NO was also reduced. Therefore, administration of ω-3-abundant HSO seemingly down-regulates the formation of pro-inflammatory cytokines. This is expected to be useful in the reduction of inflammation as well as in the management of immune diseases. We think that the molecular mechanism of the anti-inflammatory function of HSO needs to be investigated further. Also, additional experiments on the changes in IL-6 and NO levels by treatment with HSO alone may be required.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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