The effects of $n$-3 polyunsaturated fatty acid-rich total parenteral nutrition on neutrophil apoptosis in a rat endotoxemia

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Although recruited neutrophils function as first-line defense to remove bacteria, delayed apoptosis is implicated in persistent inflammation leading to organ injury. Leukotriene B$_4$, $n$-6 polyunsaturated fatty acids (PUFAs) product, is one of the mediators that delay neutrophil apoptosis. The mechanism of the beneficial effects of supplementation of fish oil-based long-chain $n$-3 PUFAs in parenteral nutrition for critically ill patients has not been fully understood. One possible mechanism is the less inflammatory $n$-3 PUFAs products can compete with proinflammatory $n$-6 PUFAs products for access to the enzymes. The aim of this study was to determine whether $n$-3 PUFAs rich parenteral nutrition may alter the composition of fatty acids in the neutrophil membrane and restore delay of neutrophil apoptosis during endotoxin-induced systemic inflammation in rats. The animals in group 1 were treated with 20% Hicaliq NC-N in Neoparen-2 for three days. The animals in group 2 (referred to as $n$-6 PUFAs products parenteral nutrition) were given parenteral nutrition solutions containing 10% soybean oil in Neoparen-2 ($n$-6/$n$-3 = 10). The animals in group 3 (referred to as $n$-3 PUFAs-rich parenteral nutrition) were administered parenteral nutrition consisting of 10% soybean oil and 10% fish oil emulsion ($n$-6/$n$-3 = 1). The $n$-3/$n$-6 ratio of the neutrophil membrane was significantly increased in group 3 and was associated with restored lipopolysaccharide-delayed-apoptosis of neutrophils in bone marrow cells and increased production of leukotriene B$_4$ from peritoneal neutrophils stimulated by lipopolysaccharide. Our preliminary results showed that $n$-3 PUFAs-rich parenteral nutrition regulated neutrophil apoptosis and prevented synthesis of pro-inflammatory eicosanoids, explaining the protective effects seen in the clinical setting.

Key Words: $n$-3 polyunsaturated fatty acids (PUFAs), parenteral nutrition (PN), apoptosis, neutrophils, inflammation

Systemic inflammatory response syndrome (SIRS) may not only be triggered by microbial invasion, but also in response to various kinds of tissue injury. Activated neutrophils are involved in these events as a first defense by releasing toxic reactive oxygen species and granule enzymes to protect the host from bacterial invasion. Despite these benefits, delayed apoptosis of recruited neutrophils and further release of pro-inflammatory mediators from persistent neutrophils may cause catastrophic collateral damage to host tissues. Therefore, it is critical to regulate the constitutive apoptotic pathway of neutrophils in the presence of severe local inflammation and removal of the neutrophil-derived secondary inflammatory response. Since LPS-, GM-CSF- and dexamethasone-induced prolongation of neutrophil survival is reversed by leukotriene (LT) B$_4$ receptor antagonist, LTB$_4$, $n$-6 polyunsaturated fatty acids (PUFAs) product, is considered to be one of the mediators that delay neutrophil apoptosis.

The nutritional component of parenteral nutrition (PN) to supply adequate calories for critically ill patients is important to help them recover from critical situations. PUFAs are classified into $n$-3 and $n$-6 systems based on the position of the first double bond from the methyl group end and have been used for lipid based PN for a long time. However, several clinical experimental reports indicated that long-term use of soybean oil-based lipid emulsions, which are $n$-6 PUFA-rich and have been used as the standard PN supplement, demonstrated less desirable results in terms of pathophysiological and immunologic profiles compared to newer emulsions containing $n$-3 PUFAs. Several recent clinical studies provided evidence of the beneficial effects of supplementation of fish oil-based long-chain $n$-3 PUFAs in PN for critically ill patients in intensive care units (ICUs). Although intravenous infusions of $n$-3 PUFAs, such as eicosapentanoic acid (EPA) or docosahexaenoic acid (DHA), were proposed for potential modifications of inflammatory and immune variables, the precise mechanisms have not been fully elucidated. While $n$-6 PUFAs products including leukotriene B$_4$ generally promote inflammation, $n$-3 PUFAs products including LTBI, which compete with $n$-6 PUFAs products for access to the cyclooxygenase and lipoxygenase enzymes, are less inflammatory or inactive. Accordingly, we hypothesized that $n$-3 PUFAs-rich PN may alter the cell membrane composition of neutrophils and may regulate neutrophil apoptosis during excessive systemic inflammation. In this study, we tested this hypothesis using a rat endotoxemia model and demonstrated that supplementation of PN with $n$-3 PUFAs in fish oil mitigated over-activation of neutrophils due to loss of apoptosis-regulatory mechanisms in the inflammatory sites. Furthermore, cell culture experiments using rat abdominal neutrophils revealed that treatment with $n$-3 PUFAs resulted in increase of LTBI production, which may play an important role in the restoration of delayed apoptosis of neutrophils. Although still primitive, our preliminary results obtained in this study may ultimately help explain the precise mechanisms of the anti-inflammatory properties of $n$-3 PUFA-supplemented PN.

Materials and Methods

Animals. Male five-week old Sprague-Dawley (SD) rats (Charles River, Yokohama, Japan) were used in all the experiments. All studies described herein were approved by the...
Institutional Animal Care and Use Committee at the laboratory of the Otsuka Pharmaceutical Factory and complied with the National Research Council’s Guide for the Humane Care and Use of Laboratory Animals.

**Reagents.** LPS (*escherichia coli*. O111:B4) was purchased from Sigma (St. Louis, MO). Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies (mAb) against rat granulocytes (Clone: HIS48, BD Pharmingen, Franklin Lakes, NJ) and anti-rat CD32 mAb (Fc gamma receptor, BD Pharmingen) were used. Annexine V-phycocerythrin (PE) apoptosis detection kit was purchased from BioVision (Mountain View, CA).

**Insertion of catheter and PN protocol.** The SD male rats were fed an essential fatty acid-deficient diet (CRF-1®), Oriental Yeast Co. Ltd., Tokyo) for two weeks. Under general anesthesia, induced using inhalation of ether, catheters for PN were placed into the rats’ external jugular veins, and the rats were then infused with 250 ml/kg/day PN solutions for three days. During PN, the animals fasted but were allowed free to access to water. Based on the results of our previous observation showing that n-3 PUFA content in cell membranes is reached within three days after infusion of the fat emulsion, we decided to administer three days of PN to the rats. Intrapertoneal (i.p.) injection of LPS (5 mg/kg) or saline was given to the animals at the end of three days of PN. There were no animal deaths in any of the groups as for 24 h of observation in each group.

**Experimental groups.** Three groups of animals were analyzed. The PN solutions utilized in this study were isocaloric and isonitrogenic. The animals in group 1 (control) were treated with 20% Hicajiq NC-N in Neoparen-2 for three days. The animals in group 2 (referred to as n-6 PUFA-rich PN) were given PN solutions containing 20% soybean oil (Intralipos®, Otsuka, Tokyo, Japan) in Neoparen-2-100 (n-6/n-3 = 10). The animals in group 3 (referred to as n-3 PUFA-rich PN) were administered PN consisting of 10% Intralipos and 10% fish oil emulsion (Omegaven®, Fresenius-Kabi, Bad Homburg, Germany) in Neoparen-2-100 (n-6/n-3 = 1.3).

**Gas chromatography.** Gas chromatography (GC) was used to determine the fatty acid composition of the plasma and cell membrane of the bone marrow (BM) cells. One hundred fifty microliter samples (plasma or homogenized BM) were mixed with 5 ml Folch solution and then 1 ml phosphate buffered saline (PBS) was added to the sample. Tritreptadecanoin (0.25 mg) was added to each sample as an internal standard. Following extraction and etherification of the lipids, the fatty acid methyl esters were separated by a Shimadzu GC-2010 gas chromatograph with an ULBON HR-SS-10 column (0.32 mm i.d. × 50 m, Shimawa Chemical Industries Ltd., Kyoto, Japan). The carrier gas was helium at a linear velocity of 25 cm/s, the injector temperature was 250°C, and the split ratio was 1:20. The oven temperature gradient began at 165°C and increased 2°C/min to 175°C, then increased 1°C/min to 190°C, and then increased to a final temperature of 205°C at 0.5°C/min. Fatty acid methyl esters were detected by flame ionization at 250°C, and they were identified by comparison of retention times with fatty acid methyl ester standards.

**Culture of peripheral blood and bone marrow cells.** Whole blood (50 μl) was diluted with 50 μl of RPMI 1640 (supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 U/ml streptomycin) and cultured in U-bottomed 96-well plates in the presence of cycloheximide at the dose of 30 μg/ml for 6 h. The cultured peripheral blood was centrifuged for 10 min at 300 g. The pellets were incubated with hypotonic saline solution (0.2% sodium chloride in distilled water) for 2 min at room temperature to lyse residual erythrocytes. Following addition of hypertonic saline solution (1.6% sodium chloride in distilled water) to normalize the osmotic pressure, leukocytes were harvested after washing with PBS. BM cells were isolated by flushing of femoral bones with 5 ml PBS using a 20-gauge needle and syringe into a 100-mm Petri dish. The BM core was disrupted to obtain a single-cell suspension by repeated-passage through the 20-gauge needle. Following centrifugation (5 min at 400 g), the erythrocytes were hypotonically lysed. The remaining cells were diluted with RPMI 1640 (supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 U/ml streptomycin) and cultured in U-bottomed 96-well plates in the presence of cycloheximide at the dose of 30 μg/ml for 6 h. Then, these cells were washed in PBS before cell surface antigen staining or annexin V staining.

**Cell staining and flow cytometric analysis.** To prevent nonspecific antibody binding, BM cells and peripheral neutrophils (1 × 10⁶ cells/reaction) were incubated with 0.1 μg of anti-rat CD32 antibody in PBS containing 1% bovine serum albumin (PBS-BSA buffer) for 5 min at 4°C. Following rinsing with phosphate buffered saline with bovine serum albumin (PBS-BSA) buffer, the cells were incubated for 60 min at 4°C with 25 ng of FITC-conjugated anti rat granulocyte marker antibody in PBS-BSA buffer. To detect apoptotic cells, the cells were incubated for 10 min at room temperature with PE-conjugated annexin V in 100 μl of annexin V staining buffer (0.14 M NaCl, 2.5 mM CaCl₂). Apoptosis was determined as the percent of rat granulocyte marker positive (neutrophils) or negative cells expressing annexin V by using two-color flow cytometry on FACSCaliber (Becton Dickinson, San Jose, CA).

**Culture of peritoneal exudative neutrophils.** The cells from peritoneal cavity neutrophils (>90%) were isolated from naive untreated rats and diluted with RPMI 1640 (supplemented with 10% fetal bovine serum, 50 U/ml penicillin, 50 U/ml streptomycin, 2 μg/ml bovine insulin, 1 mM oxalacetate, and 1 mM sodium pyruvate) and cultured in U-bottomed 96-well plates for 10 min at 37°C with 12.5 μM Ca-inophore (Sigma, St. Louis, MO) to detect the supernatant levels of LTBs.

**Measurement of LTs produced by peritoneal exudative neutrophils.** The rats were injected with casein via the i.p. route followed by sacrifice and collection of exudative cells from the peritoneal cavity at 24 h after injection. The cells were then cultured as described above. The suspension of cultured cells was immediately centrifuged to remove cells, and LTs in the supernatants were quantified by using the previously reported method. Described briefly, 13,14-dihydro-15-keto-PGE₂ (100 ng/sample) was added to the supernatant as an internal standard and citrate buffer to adjust the pH to 3.0. The solution was applied to a C18-silica column (GL Sciences Inc., Tokyo), and LTs were eluted with ethyl acetate : methanol (9 : 1, vol/vol). The eluent was concentrated under nitrogen, and an aliquot of the residual mixture was used for the quantitation of LTs by high-performance liquid chromatography (HPLC).

**Statistical analysis.** The data were expressed as mean ± standard error (SE). The differences were considered to be significant at p<0.05, as determined by the Tukey-Kramer’s test or ANOVA.

**Results**

**Infusion of n-3 PUFA-rich PN for three days changed the composition of fatty acids in plasma.** To determine if the PN changed the composition of fatty acids in plasma, plasma n-3/n-6 ratio was determined by gas chromatography. LPS treatment did not change the n-3/n-6 ratio from the baseline levels of n-3/n-6 ratio treated with control saline. Although n-6 PUFA-rich PN did not change the composition, the n-3/n-6 ratio was significantly elevated after n-3 PUFA-rich PN (Fig. 1A). These results indicated that treatment with n-3 PUFA-rich PN resulted in a significant change in the balance of n-3 and n-6 in plasma compared with the PN of n-6 PUFAs.

**The Infusion of n-3 PUFA-rich PN for three days changed the composition of fatty acids in the BM cell membrane.** Similarly, we assessed the composition of fatty acids in the BM cell membrane. The n-3/n-6 ratio was significantly increased in
The group 3 animals compared with group 1 and 2 animals, regardless of the presence of LPS treatment. These results indicated that three days of infusion of \textit{n}-3 PUFA-rich PN, but not \textit{n}-6 PUFA-rich PN, could change the composition of the BM cell membrane (Fig. 1B).

**The infusion of \textit{n}-3 PUFA-rich PN did not affect the LPS-mediated reduction in BM cell.** The number of viable cells in each cell population was calculated as the product of the total viable cell numbers in each sample, which was determined by microscopic observation after staining with trypan blue. Since there was no significant difference in cell number of peripheral blood or BM cells for each experimental group regardless of LPS treatment (data not shown), we presented the data by percentages of the target cells. Then, we examined if PUFA-rich PN altered the number of neutrophils in the peripheral blood or BM. The frequencies of neutrophils in the peripheral blood of the saline treated animals detected by flowcytometry using anti-rat granulocyte marker positive cells were comparable among the groups (group 1, 25.9%; group 2, 37.9%; group 3, 39.6%). Although LPS injection increased the percentages of neutrophils in the peripheral blood, there was no statistical difference in the number of neutrophils (data not shown), the data were shown as the percentage of apoptosis in total neutrophils. In the peripheral blood, the percentages of apoptosis in neutrophils were 56.9% in group 1, 54.2% in group 2 and 52.4% in group 3 without LPS. Of note, LPS significantly reduced the percentages of apoptosis in neutrophils up to 6.0% in group 1, 5.6% in group 2, and 7.6% in group 3 (Fig. 2A).

Likewise, the percentages of apoptosis of the neutrophil component in BM cells were 37.8% in group 1, 35.5% in group 2, and 38.4% in group 3 without LPS. The percentages of apoptosis in the neutrophil component were 23.6% in group 1 and 22.0% in group 3.

The infusion of \textit{n}-3 PUFA rich PN for three days changed the composition of fatty acids in plasma and BM cell membrane. (A) The composition of fatty acids in plasma. LPS treatment did not change the \textit{n}-3/\textit{n}-6 ratio from the baseline levels of \textit{n}-3/\textit{n}-6 ratio treated with control saline. Although \textit{n}-6 PUFA-rich PN did not change the composition, the \textit{n}-3/\textit{n}-6 ratio was significantly elevated after \textit{n}-3 PUFA-rich PN (n = 4 for each group, *p<0.01). (B) The composition of fatty acids in the BM cell membrane. The \textit{n}-3/\textit{n}-6 ratio was significantly increased in the animals in group 3 compared with the animals in groups 1 and 2, regardless of the presence of LPS treatment (n = 1; pooled cells from 4 mice for each group, *p<0.01).
2. Interestingly, the percentage of apoptosis in group 3 was 33.3% and significantly higher than those of groups 1 and 2 (Fig. 3B). These results suggested that LPS reduced the percentage of apoptosis in neutrophils in BM and n-3 PUFA-rich PN attenuated the reduction of apoptosis.

PN infusion of n-3 PUFAs increased neutrophil LTB₄ production. To determine the production of LTB₄ and LTB₅ from PUFA-treated neutrophils, we measured the concentration of LTB₄ and LTB₅ in the supernatant of migrated-neutrophils, which were obtained from the rat peritoneal cavity with in vitro LPS stimulation. Incubation of neutrophils with LPS resulted in increased secretion of LTB₄. The neutrophils isolated from the animals treated with n-3 PUFA-rich PN significantly reduced decreased LTB₄ production (Fig. 4A). The concentrations of LTB₅ produced by neutrophils in the abdominal cavity were at almost basal levels in groups 1 and 2. However, n-3 PUFA-rich PN (group 3) significantly increased to 29.6 ± 7.9 ng/10⁷ cells (Fig. 4B). These results indicated that the PN infusion of n-3 PUFAs increased the production of LTB₄ from migrated-neutrophils and decreased production of LTB₅ from migrated-neutrophils, compared to n-6 PUFA-rich PN, which may explain the mechanism of the anti-inflammatory effects afforded by n-3 PUFA-rich PN.

Discussion

The present study showed PN infusion for 3 days containing n-3 PUFAs significantly restored delayed apoptosis of the neutrophils in bone marrow, associated with increasing LTB₄ production from peritoneal neutrophils. These results suggested that n-3 PUFA-rich PN may effectively regulate neutrophil functions and may explain its efficacies observed in clinical setting. Further studies are absolutely warranted to determine the optimal ratio of n-3/n-6 for PUFA as PN supplementation, as well as detailed molecular events involved in the changes of neutrophil functions. Our results showed LPS treatment caused an increase of neutrophils in the peripheral blood, associated with reduction of BM neutrophil population in all groups regardless of the PUFA with which PN was supplemented. These observations may be explained by the recruitment of neutrophils from BM to the peripheral blood by LPS stimuli. However, we also found that the causes of reduction of neutrophils in BM with LPS may be due to neutrophil apoptosis. (15)
Our data demonstrated that neutrophils exposed to endotoxemia lost their self-regulation for apoptosis, which was attenuated by n-3 PUFA-rich PN treatment in bone marrow neutrophils. Previous reports indicated that the membrane lipid raft composition, defined as cholesterol- and sphingolipid-enriched membrane microdomains, were markedly altered by an n-3 fatty acid-containing lipid emulsion. In the presence of LPS, the signals generated via activated extracellular signal-regulated protein kinases (ERK) may inhibit the regulation of neutrophil apoptosis by p38MAPK, resulting delayed neutrophil apoptosis. Inflammation by LPS may inhibit neutrophil apoptosis mediated by Myeloid cell leukemia-1 (Mcl-1) expression.

Our results suggest that the alteration of fatty acid composition in the plasma and cell membranes change neutrophil characteristics such as LTb4 production. Thus, induction of LTb4 production may lead to the inhibition of LTb4 production, leading to attenuation of inflammation, which may explain the potent anti-inflammatory effects of n-3 PUFA-rich PN in the clinical setting. Mickleborough et al. reported that n-3 PUFA supplementation was associated with reduced LTb4 production by neutrophils from asthmatic patients with corresponding increases in LTb4 production. This report, together with our results, may support the clinical benefit of n-3 PUFA-rich PN.

Although oral intake of fatty acids demonstrated beneficial effects, intravenous administration of fatty acids through PN may provide some advantage, since few ICU patients have normal gut absorbance function and intravenously injected fatty acids directly reach the site of events without loss during digestion. In addition, Jinseisen et al. have reported that six days of infusion of PN with n-3 fatty acid-containing lipid emulsion changed plasma fatty acids significantly in surgical patients. Accordingly, the rapid alteration of membrane composition via the PN route is important to control the nutritional conditions of postsurgical patients.
In conclusion, n-3 PUFA-rich PN could alter membrane composition after only three days and attenuate LPS-mediated BM cell reduction, restore delayed neutrophil apoptosis and induce LTBs production during endotoxemia. PN containing n-3 PUFAs may be an effective method to prevent neutrophil over-activation in systemic inflammation. The balance of n-6 and n-3 PUFAs in PN may be one of the key factors to controlling the inflammatory state during PN, and it should be seriously taken into consideration for nutritional support. We believe that our observation may have significant impact in the field of critical care medicine. Further investigation toward clinical application is absolutely warranted.

**Conflict of Interest**

No potential conflicts of interest were disclosed.

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