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# Suppression of Nitric Oxide Production in Lipopolysaccharide-stimulated Macrophage Cells by ω3 Polyunsaturated Fatty Acids

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**Although nitric oxide (NO) is an important biological mediator, its excessive production in inflammation is thought to be a causative factor for cellular injury and, over the long term, cancer. In the present study, the effects of several fatty acids on NO production in murine macrophage cell line RAW264 cells stimulated with lipopolysaccharide were examined. Suppression of NO production was observed with the ω3 polyunsaturated fatty acids (PUFAs), docosahexaenoic acid, eicosapentaenoic acid and α-linolenic acid, in a dose-dependent fashion. In contrast, no inhibition was observed with ω6 PUFA (linoleic acid), ω9 PUFA (oleic acid) or a saturated fatty acid (stearic acid). Western and northern blot analyses suggested that suppression of the induction of inducible NO synthase gene expression is responsible for the inhibition of NO production by ω3 PUFAs. The inhibitory effect of ω3 PUFA on NO production in activated macrophages could contribute to their cancer chemopreventive influence.**

Key words: Polyunsaturated fatty acid — Docosahexaenoic acid — Eicosapentaenoic acid — Nitric oxide — Inducible nitric oxide synthase

Excessive nitric oxide (NO) production in inflammation is reported to be associated with cellular injury and cancer development,[1–7] although NO normally acts as an important mediator in various biological systems.[8,9] NO causes deterioration of cellular functions by inhibiting cell respiration[10] and DNA synthesis,[11] deaminating DNA[12] and contributing to formation of carcinogenic *N*-nitroso compounds.[5] Moreover, NO and superoxide anions, both formed in inflamed tissue, rapidly react to produce the peroxynitrite anion ($ONOO^-$), which is a strong oxidant causing DNA and tissue damage.[13] Since chronic elevation of NO synthesis, occurring in various persistent inflammatory diseases, is thought to increase the risk of carcinogenesis, inhibition of NO production by activated macrophages could be beneficial for the prevention of neoplasia as well as damage to tissue components.

Intake of fish containing ω3 polyunsaturated fatty acids (PUFAs), mainly docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), is epidemiologically associated with a reduced risk of colon cancer in man.[14] Furthermore, administration of EPA in the diet has been shown to reduce azoxymethane-induced rat colon carcinogenesis.[15] In the case of DHA, suppression of formation and growth of aberrant crypt foci,[16] thought to be preneoplastic lesions, was noted in the colon of rats given 1,2-dimethylhydrazine. Moreover, DHA was recently demonstrated to suppress intestinal polyp development in *Apc*$^{\Delta 716}$ knockout mice.[17] ω3 PUFAs interfere with metabolic pathways of arachidonic acid and thereby decrease prostaglandin $E_2$ levels.[18,19] This may partly be responsible for the cancer prevention by ω3 PUFAs. However, the molecular mechanisms by which ω3 PUFAs exhibit their anti-carcinogenic effects have yet to be fully understood. To cast light on this question, we examined the effects of PUFAs on the production of NO in a lipopolysaccharide (LPS)-stimulated murine macrophage cell line *in vitro*, and found that ω3 PUFAs such as DHA, EPA and α-linolenic acid can inhibit NO production.

The murine macrophage cell line, RAW264, obtained from Riken Cell Bank (Tsukuba), was grown in phenol red-free RPMI1640 medium supplemented with 5% fetal bovine serum (Hyclone Laboratories, Inc., Logan, UT) and antibiotics, at 37°C in a humidified atmosphere of 5% $CO_2$/95% air. RAW264 cells were plated at a density of $3\times10^5$/ml and after preincubation for 12 h were stimulated to induce NO production with 100 ng/ml LPS (*Escherichia coli* 0111:B4, Sigma, St. Louis, MO). All the fatty acids examined in the present study were ethylester forms. The ω3 PUFAs DHA, EPA and α-linolenic acid were obtained from Sagami Chemical Research Center (Sagamihara). Linoleic acid (ω6 PUFA) and oleic acid (ω9 PUFA) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka), and stearic acid (saturated fatty acid) was from Tokyo Chemical Industry (Tokyo).

RAW264 cells were pulse-stimulated with 100 ng/ml LPS for 10 min to produce NO, then washed, and the

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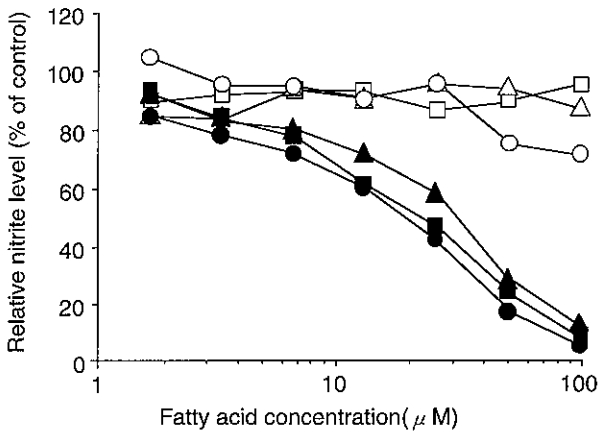


Fig. 1. Effects of fatty acids on NO production in LPS-stimulated macrophages. RAW264 cells were incubated with 100 ng/ml LPS for 10 min, washed and further incubated in fresh medium containing various concentrations of fatty acids. The extracellular accumulation of nitrite was determined by means of the Griess assay[20] 24 h after LPS stimulation. The data are percentages of control values, with LPS-stimulated cells incubated in the absence of fatty acids. Each point represents the mean for 3 wells. Similar results were also obtained in three other separate experiments. □ stearic acid, △ oleic acid, ○ linoleic acid, ■ α-linolenic acid, ▲ EPA, ● DHA.

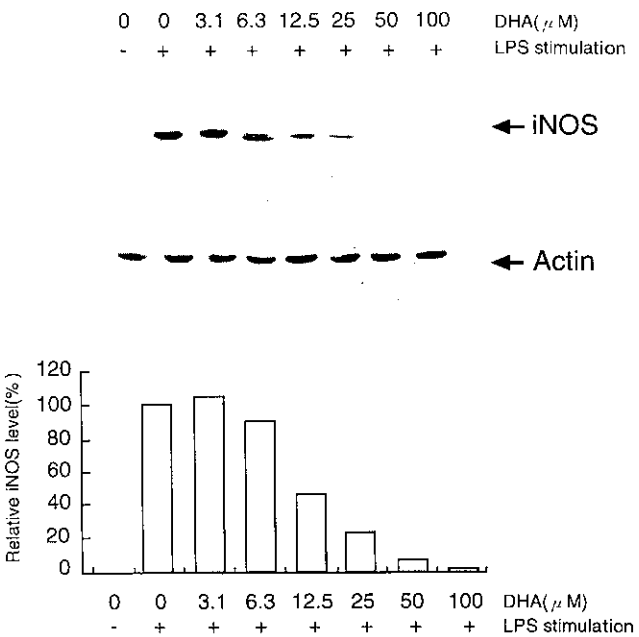


Fig. 2. Suppression of iNOS protein levels in LPS-stimulated macrophages by DHA. RAW264 cells were cultured in the absence or presence of 100 ng/ml LPS for 10 min, washed and subsequently cultured in fresh medium containing DHA at a final concentration of 3.1, 6.3, 12.5, 25, 50 or 100 $\mu M$. After 12 h of incubation, the cells were lysed in buffer solution (0.0625 $M$ Tris-HCl at pH 6.8, 2% SDS, 5% 2-mercaptoethanol, and 10% glycerol), and the lysates were briefly sonicated and boiled. Proteins in lysates of $1\times10^5$ cells were separated on 8% SDS-PAGE gels and electrophoretically transferred onto Immobilon-P membranes (Nihon Millipore, Tokyo). After blocking with skim milk, membranes were probed with anti-iNOS rabbit polyclonal antibody (Affinity Bioreagents, Inc., Golden, CO), followed by a horseradish peroxidase-linked second antibody (Amersham Life Science, Buckinghamshire, UK). The blots were then developed using an ECL detection kit (Amersham Life Science) and Hyperfilm (Amersham Life Science). To correct for variation in protein amounts, antibodies were stripped and the blots were reprobed with anti-actin rabbit antibody (Biomedical Technologies, Stoughton, MA). Each band was quantified using a densitometer (Image Master, Pharmacia Biotech, Tokyo) and total protein amounts loaded into each lane were normalized based on the actin levels. Relative protein levels were calculated with reference to LPS-stimulated control culture.

stimulated cells were incubated in fresh medium containing various concentrations of each fatty acid. Levels of inducible NO synthase (iNOS) mRNA, iNOS protein and NO were measured 6, 12 and 24 h after LPS stimulation, respectively.

NO was estimated as nitrite accumulated in the conditioned medium by means of the Griess assay.[20] As shown in Fig. 1, the nitrite levels were suppressed by ω3 PUFAs (DHA, EPA and α-linolenic acid) in a dose-dependent manner. DHA, at 50 $\mu M$, reduced NO production to less than 15% of that by LPS-stimulated control culture cells. EPA and α-linolenic acid caused similar inhibition. However, ω6 PUFA (linoleic acid), ω9 PUFA (oleic acid) and the saturated fatty acid (stearic acid) showed no appreciable effects on NO production at doses of up to 100 $\mu M$. No decrease of cell viability, measured by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay, was observed with any of the fatty acids at any dosage tested here.

To assess the effects of DHA on iNOS expression, protein levels of iNOS were analyzed by immunoblot analysis (Fig. 2). iNOS protein was hardly detectable in unstimulated RAW264 cells, but its level was markedly increased 12 h after treatment with LPS. Addition of DHA caused a dose-dependent decrease. The other two ω3 fatty acids, EPA and α-linolenic acid, exhibited similar although somewhat weaker inhibition of iNOS protein expression, while no significant change was observed with linoleic acid, oleic acid or stearic acid (data not shown).

To clarify the effects of DHA on transcription of the iNOS gene, expression of iNOS mRNA was examined by northern blot analysis. As shown in Fig. 3, iNOS mRNA levels in RAW264 cells were markedly increased at 6 h

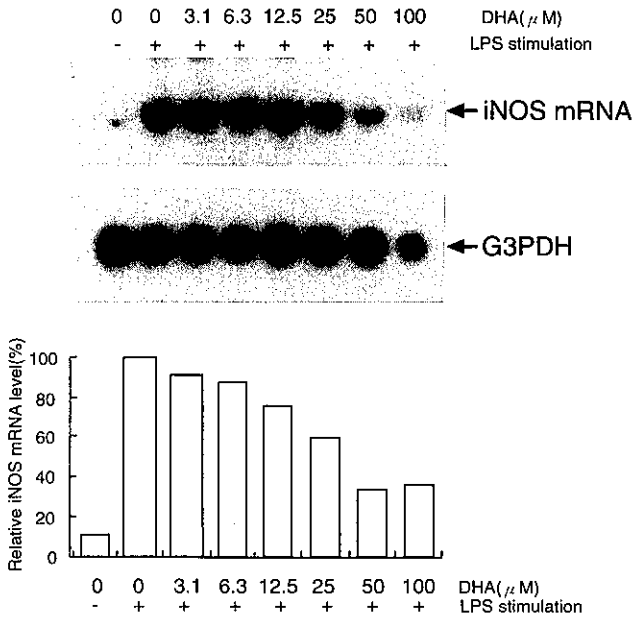


Fig. 3. Northern blot analysis confirming the inhibitory effect of DHA on iNOS mRNA expression in LPS-stimulated macrophage cells. RAW264 cells were treated with LPS and DHA as described for Fig. 2. After 6 h of incubation, total RNA was extracted from cultured cells by a modified guanidinium thiocyanate method using ISOGEN (Nippon Gene, Tokyo). Ten microgram aliquots of total RNA were briefly denatured and subjected to electrophoresis on 1.2% agarose-formaldehyde gels. RNAs were transferred to Hybond-N membranes (Amersham Life Science), fixed by UV irradiation and hybridized with an iNOS cDNA probe, obtained from the Cayman Chemical Company (Ann Arbor, MI), labeled with [$\alpha$-$^{32}$P]dCTP by random priming. After hybridization, blots were autoradiographed using Kodak XAR-5 film with an intensifying screen at $-70$°C. To correct for variations in RNA amounts, the probes were stripped and the blots were rehybridized with a $^{32}$P-labeled G3PDH cDNA probe (Clontech, Palo Alto, CA). Each band was quantified using an imaging analyzer (BAS2000, Fuji Photo Film Co., Ltd., Tokyo) and total RNA amounts loaded into each lane were normalized based on the G3PDH mRNA levels. Relative mRNA levels were calculated with reference to LPS-stimulated control cultures.

after treatment with LPS in contrast to the unstimulated case. DHA suppressed the transcription of the iNOS gene in a dose-dependent manner.

Inhibition of expression of iNOS protein and mRNA was even observed with addition of DHA 2 h after stimulation with LPS. When RAW264 cells were incubated with LPS throughout the culture, the amounts of nitrite which accumulated were more than several fold that after pulse-stimulation for 10 min. Even under those conditions, continuous DHA treatment inhibited production of NO, expression of iNOS protein and iNOS mRNA (data not shown). However, DHA was not able to suppress NO production when DHA was added in the medium 24 h after LPS stimulation, when macrophages were already fully activated and contained abundant iNOS protein. This suggests that DHA has no inhibitory effect on the enzymatic activity of iNOS.

The present study thus suggested that $\omega$3 PUFAs such as DHA, EPA and $\alpha$-linolenic acid suppress NO production in macrophages activated with LPS by inhibiting induction of iNOS gene expression. This was not the case with $\omega$6 PUFA, $\omega$9 PUFA and a saturated fatty acid. Consistent with our data, inhibition of transcription of the murine iNOS gene by DHA has recently been reported.[21] Macrophages can be stimulated to produce NO by several cytokines, such as interferon (IFN)-$\gamma$, tumor necrosis factor (TNF)-$\alpha$ and interleukin (IL)-1$\beta$. We have observed that $\omega$3 PUFAs suppressed NO production from RAW264 cells stimulated by these cytokines (data not shown). Thus, $\omega$3 PUFAs are able to suppress NO production in macrophage cells treated with not only LPS, but also several cytokines. However, the molecular mechanisms responsible for the suppression of induction of iNOS gene expression by $\omega$3 PUFAs have still to be elucidated. Moreover, the effect of $\omega$3 PUFAs on other functions of macrophages also remain to be elucidated. Macrophages produce many kinds of cytokines and some of these factors, such as TNF-$\alpha$ and IFN-$\gamma$, are indispensable to the host for defense against malignant cells. Endres *et al.* reported that $\omega$3 PUFAs suppress production of cytokines such as TNF-$\alpha$ and IL-1 from stimulated mononuclear cells.[22] Although antiinflammatory effects may contribute to the prevention of carcinogenesis, the immunosuppressive properties of these fatty acids may cause other effects which are not beneficial to host defence systems. Further studies are therefore necessary to clarify the usefulness of $\omega$3 PUFAs as cancer preventive agents.

It is widely accepted that production of NO in large amounts is a causative factor in disease processes, including cancer development.[1-7] Evidence is now accumulating that effective inhibition of NO production in inflammatory states may contribute to prevention of carcinogenesis, as well as controlling the inflammation itself. Our results obtained in the present study suggest that not only the modulation of arachidonic acid metabolism shown previously,[18,19] but also the suppression of increased production of NO from activated macrophages by $\omega$3 PUFAs, including DHA, EPA and $\alpha$-linolenic acid, are causally related to the chemopreventive effects.

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