Brief Definitive Report

Transcription of the Murine iNOS Gene Is Inhibited by Docosahexaenoic Acid, a Major Constituent of Fetal and Neonatal Sera as Well as Fish Oils

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Summary

Macrophage activation is deficient in the fetus and neonate when the serum concentrations of docosahexaenoic acid (DHA) are 150 μM, or 10–50-fold higher than in the adult. We now show that DHA inhibits production of nitric oxide (NO) by macrophages stimulated in vitro by IFNγ plus LPS, or by IFNγ plus TNFα. The half-maximal inhibitory activity of DHA was ~25 μM. There were strict biochemical requirements of the fatty acid for inhibition. Polyenoic fatty acids with 22 carbons were more inhibitory than those with 20 carbons. Among 22-carbon fatty acids, those with a greater number of double bonds and a double bond in the n-3 position were more inhibitory. DHA was the most inhibitory of the polyenoic acids we tested.

Inducible nitric oxide synthase (iNOS) is the enzyme responsible for the production of NO by macrophages. NO production is initiated after new iNOS enzyme is synthesized following transcription of the iNOS gene. In macrophages stimulated by IFNγ plus LPS, DHA inhibited accumulation of iNOS mRNA, as measured by Northern blotting, and iNOS transcription, as measured by nuclear run-on assays. We transfected RAW 264.7 macrophages with a construct containing the iNOS promoter fused to the chloramphenicol acetyl transferase gene. DHA inhibited activation of this promoter by IFNγ plus LPS. By inhibiting iNOS transcription in the fetus and neonate, DHA may contribute to their increased susceptibility to infection.

Deficits in macrophage functions in the fetus, the neonate, and the parts of the placenta perfused by the fetal circulation may contribute to the increased susceptibility of the fetoplacental unit and newborn to infection (1, 2). Despite deficient functions in vivo, murine neonatal macrophages can be activated to kill P815 mastocytoma cells (3) and express Ia in vitro (4). This suggests the presence of an inhibitor of macrophage activation in the perinatal internal environment.

We have chosen to investigate docosahexaenoic acid (DHA) as one inhibitor of fetal and neonatal macrophage activation. The serum concentrations of DHA in the day-old rat are 150 μM and decline during the first weeks after birth to levels of ~5 μM (5). Similar large differences between fetal and adult concentrations of DHA have also been documented in Rhesus monkeys (6), and can be calculated in human, rat, and pig from the amounts of DHA known to be bound to its physiologic carrier protein AFP (7). DHA is a major fatty acid constituent of brain and retina, and the high fetal serum levels may be required for the rapid intrauterine growth and development of these tissues (8).

Nitric oxide (NO) produced by activated macrophages may be one effector molecule in the host defense against intracellular pathogens (9, 10). We now show that DHA, at concentrations present in the fetal and neonatal serum, inhibited NO production by macrophages stimulated by IFNγ and LPS in vitro. NO production by macrophages results when the gene for inducible nitric oxide synthase (iNOS) is activated, and new enzyme is produced. Transcription of the iNOS gene was inhibited by DHA.

Materials and Methods

Reagents. R+10 media was RPMI-1640 with 10 mM Hepes, 2 mM L-glutamine, 50 U/ml penicillin, 50 mg/ml streptomycin, and 10 % FCS. R+10 itself contained 3 μM DHA and 3.3 μM AA (7). Murine recombinant IFNγ and TNFα were from GIBCO BRL (Grand Island, NY) and Genzyme (Cambridge,

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MA), respectively. *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA), 5,8,11,14-eicosapentaenoic acid [or arachidonic acid (AA)], and *cis*-5,8,11,14,17-eicosapentaenoic acid were from Cayman Chemical Co. (Ann Arbor, MI). Docosanoic acid, *trans*-13-docosenoic acid (brassidic acid), *cis*-13-docosenoic acid (or erucic acid), *cis*-13,16-docosatrienoic acid, *cis*-13,16,19-docosatrienoic acid, and *cis* 7,10,13,16-docosatetraenoic acid were from Sigma Chemical Co. (St. Louis, MO). All reagents, except the LPS itself, contained less than .012 ng/ml endotoxin by the Limulus Amebocyte Lysate (LAL) assay.

Macrophages. Peritoneal exudate was harvested from C3H/NIH mice 4 d after 1.5 cc 3% thioglycollate ip. Mice were housed according to NIH and institutional guidelines. 5×10^4 peritoneal exudate cells were placed in each well of 96-well flat microtiter plates (Catalog no. 3596, CoStar, Cambridge, MA), incubated in R+10, 5% CO_2, 37°C, for 24–48 h, and washed twice with RPMI-1640. Over 95% of the remaining adherent cells were macrophages.

Measurement of NO. NO is rapidly converted to nitrite which was assayed using the Greiss reagent (1% sulfanilamide, 0.1% *N*-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H_3PO_4) (10, 11). 50 ml of the supernatant from each well was incubated for 10 min with 50 ml of Griess reagent. Optical densities were then obtained on a TiterTech Multiskan at 540 nm (using 690 nm as a reference). NO_2 in the samples was calculated from a standard curve using $NaNO_2$. All values shown are the mean ± SEM of triplicate wells.

Delipidated Human Serum Albumin (HSA). Albumin was delipidated using acid charcoal (7, 12). R+10 containing 8 uM of delipidated albumin had 0.10 μM palmitic acid, 0.20 μM stearic acid, 0.08 μM oleic acid, 0.02 μM linoleic acid, and 0.00 μM (undetectable amounts) of palmitooleic, arachidonic, and docosahexaenoic acids (7).

Fatty Acid-HSA Complexes. All the fatty acids were used as complexes of fatty acid bound to delipidated HSA. The fatty acids were stored under N_2, −20°C, at a concentration of 20 mg/cc in ethanol. On the day of the experiment, the fatty acids were examined by thin-layer chromatography on KG Silica Gel plates (Whatman International Ltd, Maidstone, England) using ethyl ether/petroleum ether/acetic acid 50/50/1 (vol/vol/vol). Oxidized samples were discarded. HSA (100 mg/cc in RPMI) was added directly to the fatty acid (20 mg/cc) such that the fatty acid/HSA molar ratio was 6:1. The samples were thoroughly flushed with nitrogen, sealed, covered with aluminum foil, vortexed for 10 min, and incubated at 37°C for ~2 h. The desired final dilutions were then prepared in R+10. 100 uM fatty acid contained 0.165% ethanol. We found that this concentration of ethanol did not affect NO production. The proper formation of these DHA/HSA complexes was critical. The presence of DHA as micelles, as opposed to DHA/HSA complexes, would result in toxicity to the macrophages.

Northern Blotting. Total RNA was isolated from 1×10^7 macrophages using the single-step guanidinium thiocyanate method (13). After electrophoresis on a 1% agarose gel, the mRNA was transferred to GeneScreen Plus membranes (NEN Research Products, Boston, MA), hybridized for 16–18 h at 42°C with [^{32}P]-dCTP nick-labeled DNA probes for iNOS or cyclophilin (Promega Prime-a-Gene Kit; Madison, WI). Membranes were washed twice in both 0.1%SDS/1% SSC (15 min) and 1% SDS/0.1% SSC (1 h) before autoradiography. The cDNA probes were the 664-bp AccI fragment of iNOS cDNA (14), and the 743-bp EcoRI-HindIII fragment of cyclophilin cDNA (15).

Nuclear Run-on Assay. Nuclei from 10^7 macrophages were isolated as previously described (16). pCMV plasmids containing the cDNA inserts for iNOS, described above, or a 1.1-kb BamHI-HindIII fragment of glyceraldehye-3-phosphate dehydrogenase (GAPDH) (17) were denatured and placed onto GeneScreen Plus membranes using a slot-blot apparatus (Bio-Rad Labs., Hercules, CA). Nascent ^{32}P-labeled mRNA was hybridized with the immobilized DNA for 48 h, washed, and then detected by autoradiography. The densities of the resultant bands were analyzed using a GS300 Transmittance/Reflectance scanning densitometer (Hoeffer Scientific, San Francisco, CA).

CAT Assays. The p1iNOS-CAT construct (a generous gift from Drs. Xie and Nathan [18]) contained 1,749 bp of the 5′-flanking region of the murine iNOS gene fused to the chloramphenicol acetyl transferase gene. RAW 264.7 macrophages were transiently transfected, and analyzed for CAT activity according to previously published techniques (18).

Results and Discussion

We chose to use DHA bound to carrier protein because nonesterified DHA in fetal and newborn serum is bound to hydrophobic areas of the AFP carrier protein (19). Delipidated HSA rather than AFP was chosen as the carrier protein for two reasons: (*a*) HSA is similar to AFP in structure, and has hydrophobic binding sites for nonesterified fatty acids (20, 21). (*b*) Unlike AFP, HSA is readily available, and has not been reported to regulate macrophage activation (22).

We found that DHA inhibited NO production by macrophages stimulated by IFNγ plus LPS (Fig. 1). The half-maximum inhibitory concentration of DHA was approximately 25 μM, and is comparable to the concentrations found in fetal and neonatal sera (5, 7). Fig. 2 indicates that increasing amounts of IFNγ or LPS did not reverse the in-

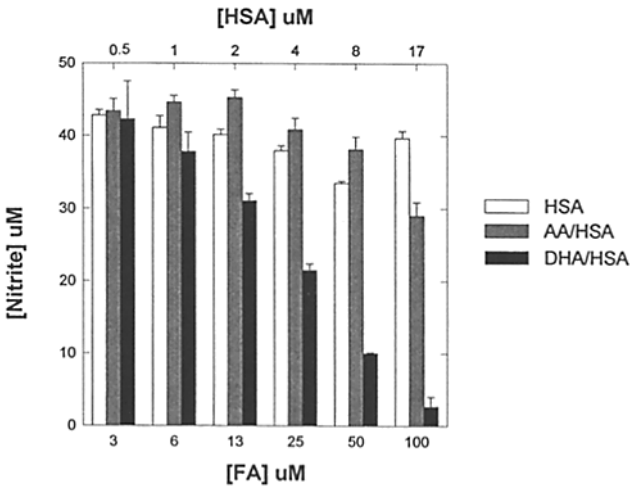

Figure 1. Inhibition of NO production by various doses of DHA or AA. Macrophages were stimulated for 24 h by IFNγ (100 U/ml) plus LPS (100 ng/ml) in the presence or absence of DHA or AA. The conditioned media was assayed for NO (as nitrite). The DHA (*solid bars*) or AA (*cross-hatched bars*) were administered as fatty acids bound to HSA. The top *x*-axis is the HSA concentration; the bottom *x*-axis, the fatty acid concentration. Macrophages stimulated by neither IFNγ nor LPS produced 1.0 ± 0.2 uM NO. The mean and standard error of triplicate determinations are shown.

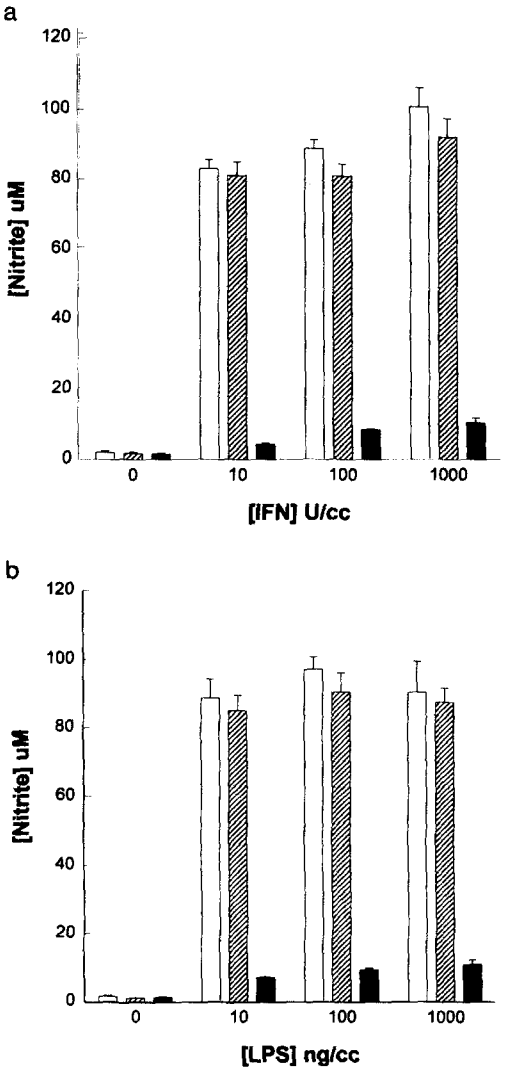

Figure 2. Effects of different doses of IFNγ and LPS on DHA inhibition. Macrophages were cultured as in Fig. 1 in the presence of media only (*open bars*), HSA (*cross-hatched bars*), or 100 uM DHA (solid bars). Macrophages stimulated by 100 ng/cc LPS plus the amounts of IFNγ shown on the *x*-axis (*a*), or 100 U/cc IFNγ plus the amounts of LPS shown on the *x*-axis (*b*).

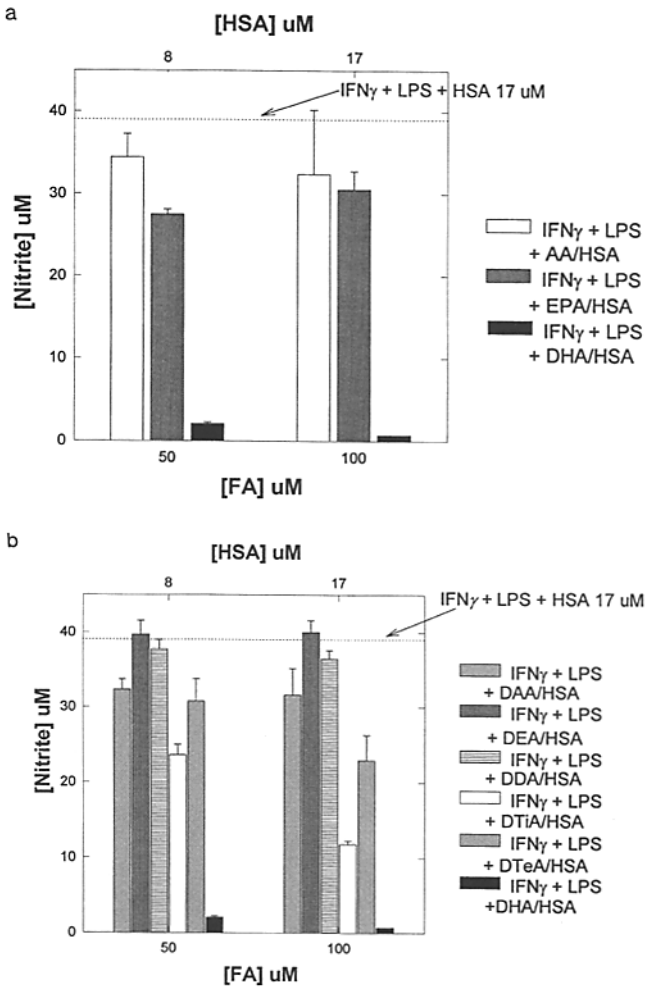

Figure 3. Inhibitory effects of DHA versus other fatty acids. Macrophages were cultured as in Fig. 1. The fine dotted line (*IFNγ* + LPS + HSA 17 μM) is the NO released by macrophages stimulated with these agents, but no fatty acid. In the absence of IFNγ or LPS, no detectable NO was produced. (*a*) DHA is all *cis*-4,7,10,13,16,19 docosahexaenoic acid (6 double bonds, 22 carbons, n-3); EPA is all *cis*-5,8,11,14,17-eicosapentaenoic acid (5 double bonds, 20 carbons, n-3); AA is arachidonic acid or 5,8,11,14-eicosatetraenoic acid (4 double bonds, 20 carbons, n-6) (*b*) DAA is docosanoic acid (22 carbons, zero double bonds); DEA is *cis*-13-docosenoic acid (22 carbons, 1 double bond, n-9); DDA is all cis-13,16 docosadienoic acid (22 carbons, 2 double bonds, n-9); DTiA is all *cis*-13,16,19-docosatrienoic acid (22 carbons, 3 double bonds, n-3); DTeA is all cis 7,10,13,16 docosatetraenoic acid, 22 carbons, 4 double bonds, n-6); and DHA is all cis 4,7,10,13,16,19 docosahexaenoic acid (6 double bonds, 22 carbons, n-3).

hibition. Furthermore, NO production was similar in the presence of media only versus delipidated HSA. This excludes the possibility that HSA was interfering with the LPS–macrophage interaction.

DHA was, by far, the most inhibitory of the nine 20- and 22-carbon fatty acids tested (Fig. 3), and was the only one of these present at high concentrations in fetal and neonatal sera (23). AA and EPA are of particular interest (Fig. 3 *a*). Both are 20-carbon polyenoic acids. AA is an n-6 fatty acid which is a major polyenoic fatty acid in the phospholipids of humans fed the usual American diet, and rodents fed standard laboratory chow. EPA and DHA are both n-3 fatty acids found in large amounts in phospholipids of animals fed fish oil–enriched diets (8). Among 22-carbon fatty acids (Fig. 3 *b*), inhibition generally increased with the number of double bonds. However, docosatetraenoic acid (DTeA), with four double bonds, was less inhibitory than docosatrienoic acid (DTiA), with three double bonds. This indicates the importance of the n-3 double bond because DTiA is an n-3 fatty acid while DTeA is n-6. Remarkably, a similar pattern of inhibition occurred when we examined IFNγ-induced Ia expression by macrophages (24).

Three sets of data indicated that DHA inhibited iNOS gene transcription. First, DHA inhibited the increased iNOS

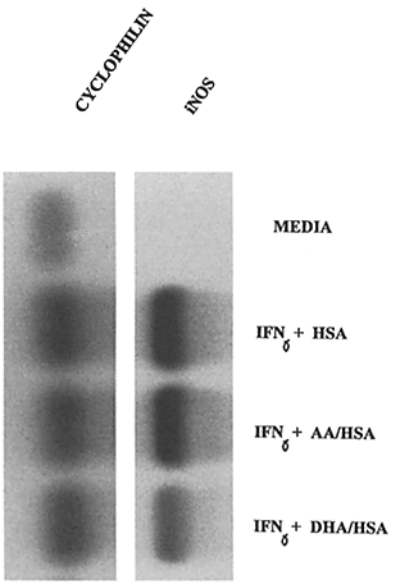

Figure 4. DHA inhibits the increased abundance of iNOS mRNA of macrophages stimulated by IFNγ plus LPS. Macrophages were stimulated with media or with IFNγ (100 U/ml) plus LPS (100 ng/ml) in the presence of 50 uM DHA, AA, or the HSA carrier protein. At the end of a 20-h culture period, the abundance of iNOS and cyclophilin mRNA was determined by Northern analysis. See text for densitometry.

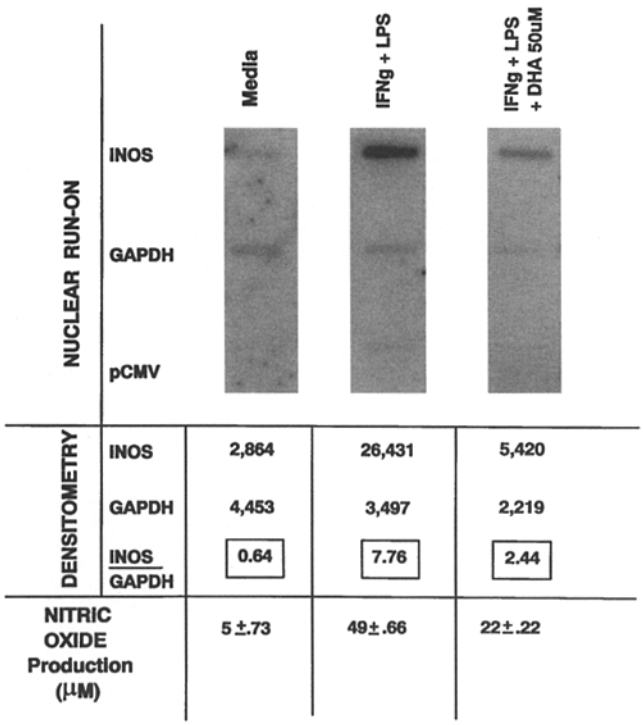

Figure 5. DHA inhibits transcription of iNOS mRNA. Macrophages were cultured either in media, IFNγ (100 U/ml) plus LPS (100 ng/ml), or IFNγ plus LPS and DHA 50 μM. (*Top*) A photomicrograph of the nuclear run-on autoradiograph. (*Middle*) Densitometry data. (*Bottom*) NO production.

mRNA abundance caused by IFNγ plus LPS (Fig. 4). By densitometry, the ratio of iNOS: cyclophilin mRNA abundance was zero in macrophages cultured in media alone, and increased to 0.97 after macrophages were stimulated with IFNγ plus LPS. The ratio was 1.07 in stimulated macrophages exposed to AA. DHA decreased this ratio to 0.55. Second, nuclear run-on assays showed that DHA inhibited iNOS mRNA transcription stimulated by IFNγ plus LPS (Fig. 5). By densitometry, the ratio of nascent iNOS mRNA to GAPDH mRNA was 0.64 in macrophages cultured in media, increased to 7.76 after stimulation with IFNγ plus LPS, and decreased to 2.44 after exposure to DHA, IFNγ and LPS. This inhibition of mRNA transcription was similar to the inhibition of NO production by DHA: IFNγ plus LPS stimulated macrophages produced 49 μM NO; DHA decreased this to 22 μM, or 44% of the stimulated amount. Third, we transfected RAW 264.7 macrophages with a construct containing the iNOS promoter fused to the CAT gene (18). As shown in Fig. 6, IFNγ plus LPS activated the promoter, and DHA prevented activation. The percent acetylated chloramphenicol, determined by phosphoimagery, was 0.82% for A (media), 3.84% for B (IFNγ + LPS), and 1.56% for C (IFNγ + LPS + DHA).

Inhibition by DHA was not a nonspecific toxic effect. DHA did not inhibit the ability of macrophages to ingest and catabolize bacteria (7), nor the conversion of 3-(4,5-dimethylthiazol-1-yl)-2,5-diphenyltetrazolium (MTT) to its formazan by the mitochondrial electron transport system (24).

The effects of DHA on several other macrophage activation pathways has been examined. We found that DHA inhibited NO production if TNFα was substituted for the LPS portion of IFNγ plus LPS stimulation. Inhibition ranged from approximately 60% at low doses of IFNγ + TNFα stimulation, to 30% at higher doses of IFNα + TNFα. We previously reported that DHA inhibited macrophage Ia-expression after stimulation by IFNγ or IL-4 (24), and lysis of the TNFα-resistant (25) P815 mastocytoma cells after stimulation by IFNγ plus LPS (7). Others have shown that production of TNFα after stimulation by LPS in vitro is not inhibited by DHA (26).

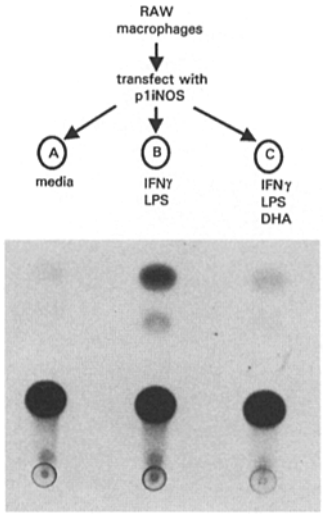

Figure 6. DHA inhibits activation of the p1iNOS promoter in a CAT assay. RAW 264.7 were transfected with p1iNOS, and then aliquoted onto three dishes. Dish A received media only. Dish B received IFNγ (100 U/ml) plus LPS (100 ng/ml). Dish C received IFNγ plus LPS plus DHA (50 μM).

Our data may help explain the increased susceptibility of the fetus, neonate, and placenta to intracellular pathogens such as Listeria monocytogenes (27). The concentrations of DHA which inhibit NO production are present in neonatal and fetal serum (5, 7) and those areas of the placenta perfused by the fetal circulation.

AFP has a special role in maintaining the high fetal concentrations of DHA. This 70,000–mol wt glycoprotein is present at concentrations of 5–10 mg/ml in fetal serum during the first trimester in humans and throughout fetal life in rodents. Maximum concentrations in the maternal serum, on the other hand, do not exceed 500 ng/ml (for review see reference 20). DHA has a much higher affinity for AFP in the fetal circulation than for albumin in the maternal circulation, and therefore DHA partitions to the fetal circulation (19). The DHA in the fetal and neonatal serum is bound to AFP. DHA bound to AFP may be the molecule actually responsible for the inhibition of macrophage activation previously attributed to AFP (22, 28).

DHA is a major fatty acid component of fish oil diets (29). Inhibition of NO production by macrophages by DHA may also contribute to the beneficial effects of such diets on some autoimmune diseases (for example see references 31–33), and preventing transplant rejection (34).

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