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.

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MA), respectively. $\alpha_i4,7,10,13,16,19$-docosahexaenoic acid (DHA), 5,8,11,14-eicosapentaenoic acid (or arachidonic acid (AA)), and $\alpha_i5,8,11,14,17$-eicosapentaenoic acid were from Cayman Chemical Co. (Ann Arbor, MI). Docosanonic acid, trans-13-docosenoic acid (brassid acid), $\alpha_i13$-docosenoic acid (or erucic acid), $\alpha_i13,16$-docosatrienic acid, $\alpha_i13,16,19$-docosatrienonic acid, and $\alpha_i7,10,13,16$-docosatetraenoic acid were from Sigma Chemical Co. (St. Louis, MO). All reagents, except the LPS itself, contained less than 0.012 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. S X 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 Griess reagent (1% sulfanilamide, 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% H$_3$PO$_4$) (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 0.10 uM palmitic acid, 0.20 uM stearic acid, 0.08 uM oleic acid, 0.02 uM linoleic acid, and 0.00 uM (undetectable amounts) of palmitoleoic, 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/acetetic acid 50/50/1 (vol/vol/vol). Oxidized samples were discarded. HSA (100 ng/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 $1 \times 10^7$ 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° with [32P]-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.** Nuclei from $10^7$ macrophages were isolated as previously described (16). pCMV plasmids containing the cDNA insert for iNOS, described above, or a 1.1-kb BamHI-HindIII fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (17) were denatured and placed onto GeneScreen Plus membranes using a slot-blot apparatus (Bio-Rad Labs., Hercules, CA). Nascent 32P-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 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 bar), or 100 μM DHA (solid bar). 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).

Hibitation. 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 iNOS gene transcription. First, DHA inhibited the increased iNOS
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-2-yl)-2,5-diphenyxtetrazolium (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 IFNγ + 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).
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 life in rodents. Maximum concentrations in the maternal serum non-esterified fatty acids to adrenalectomy and ovariectomy in developing rats. 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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