약학석사 학위논문

Docosahexaenoic Acid Inhibits *Helicobacter pylori*-induced STAT3 Phosphorylation through Activation of PPARγ.

Docosahexaenoic Acid 가 *Helicobacter pylori* 에 의해 유도된 STAT3 인산화를 PPARγ 활성화를 통해 억제한다.

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지 현 근

Abstract

Docosahexaenoic aicd (DHA), one of the ω-3 polyunsaturated fatty acids, has received considerable attention due to its diverse health beneficial effects. It has been reported that DHA inhibits growth of *Helicobacter pylori* (*H. pylori*) *in vitro* and decreases the colony number in *H. pylori*-infected mouse stomach *in vivo*. However, the molecular mechanism underlying the gastroprotective effect of DHA hasn't been clarified. Signal transducer and activator of transcription 3 (STAT3) is a major transcription factor in cancer and activated by *H. pylori* infection *in vitro* and *in vivo*. Therefore, the aim of this study was to investigate the effect of DHA on *H. pylori*-induced activation of STAT3 signaling in human gastric cancer AGS cells. Pretreatment of AGS cells with DHA significantly reduced the expression, phosphorylation and nuclear translocation of STAT3 induced by *H. pylori* infection. Notably, DHA activated peroxisome proliferator-activated receptor γ (PPARγ) and induced expression of suppressor of cytokine signaling 3 (SOCS3). siRNA knockdown of PPARγ and SOCS3 abolished the DHA-mediated inhibition of *H. pylori*-induced STAT3 phosphorylation. DHA also markedly decreased anchorage-independent growth of AGS cells co-cultured with *H. pylori*. In conclusion, DHA prevents *H. pylori*-induced STAT3 phosphorylation in a PPARγ/SOCS3-dependent manner.

Keywords : DHA; *H. pylori*; STAT3; PPARγ; SOCS3; n-3 PUFA

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Introduction

Gastric cancer is the fourth most common cancer, and the second cause of cancer mortality in the world[1]. Despite the gastric cancer survival rate has been increasing since the development and prevalence of endoscopy, the stomach cancer is still increasing in certain regions of the world, especially in East Asia and Eastern Europe[1, 2]. Human gastric carcinogenesis is a multistep and multifactorial process. During human gastric cancer development, many hosts, bacterial, and environmental factors acting in combination contribute to gastric carcinogenesis[3]. *Helicobacter pylori* infection has been considered one of the etiologic factors of gastric cancer and over the half of population in the world are *H. pylori* positive. Accumulating evidence supports that persistent infection with *H. pylori* significantly increased the risk of gastric disease[4, 5].

H. pylori is gram-negative, microaerophilic bacterium that colonizes the stomach and induces a chronic gastritis. Eradiation of *H. pylori* by triple therapy dramatically reduced the *H. pylori*-associated gastric ulcer but the antibiotics in the triple therapy could give raise to antibiotic resistance. Therefore, we need develop a new potential agent targeting the *H. pylori*-induced STAT3 pathway to prevent gastric cancer.

Epidemiologic research have shown that the cancer incidence and the mortality are low in some region where n-3 FUFA diets are abundant[6]. Moreover, administration of the n-3 PUFA docosahexaenoic acid (DHA) can decrease pro-inflammatory mediator secretion in cancer patients[7]. DHA is

known to be one of the peroxisome proliferator-activated receptor γ (PPARγ) ligands and it has been reported that DHA can activate transcription factor PPARγ to prevent the pro-inflammatory cytokine release[8]. STAT3 has received considerable attention in gastric cancer development[9]. STAT3 is a member of transcription factors and plays an important role in inflammation, cell growth, and differentiation. In addition, *H. pylori* pathogenicity protein Cag A activates STAT3 *in vitro* and *in vivo*[10]. Suppressor of cytokine signaling (SOCS) is a negative regulator of STAT3. Interestingly, a recent study has indicated that DHA activated-PPARγ binds to SOCS3 promoter to prevent IL-17-mediated cancer growth[11].

Although there was a lot of research that supports the anti-cancer and anti-inflammatory effects of DHA, its effects on *H pylori*-induced inflammation remains elusive. Here, we report that DHA significantly inhibits *H. pylori*-induced activation of STAT3 signaling via the PPARγ-SOCS axis.

Materials and methods

Reagents

DHA (purity > 98%) was purchased from Cayman Chemical Co. (Ann Arbor, MI, USA). RPMI-1640 medium, fetal bovine serum, penicillin, streptomycin were products of GIBCO BRL (Grand Island, NY, USA). Sheep blood agar, Gaspak™ and anaerobic jars were provided by BD Biosciences (Sparks, MD, USA). Primary antibody against actin was products of Sigma-Aldrich Co. (St Louis, MO, USA). Primary antibodies for PPARγ, lamin B were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against phospho-STAT3, total STAT3, c-Myc were purchased from Cell Signaling Technology (Beverly, MA, USA). Primary antibody for α-Tubulin was a product from Biogenex (Fremont, CA, USA). Horseradish peroxidise-conjugated secondary antibodies were obtained from Pierce Biotechnology (Rockford, IL, USA). Alexa 488 conjugated-IgG, TRIzol®, propidium iodide (PI), SYBR® were provided by Invitrogen (Carlsbad, CA, USA). Polyvinylidene difluoride membranes were supplied from Gelman laboratory (Ann Arbor, MI, USA). Protease inhibitor cocktail tablets were provided from Boehringer Mannheimm (Mannheimm, Germany). The ECL chemiluminescent detection kits were purchased from ELPIS-BIOTECH (Daejon, Korea) and GH Healthcare (Piscataway, NJ, USA), respectively. A protein assay dye (Bradford) Reagent was supplied by Bio-Rad Laboratories (Hercules, CA, USA). The bicinchonic acid (BCA) protein assay reagent was obtained from Pierce Biotechnology (Rockford, IL, USA). Oligonucleotide

probe containing the PPARγ consensus sequence (5'-GGAACTAGG TCAAAGGTCATCCCCT-3') was purchased from Bioneer (Seoul, Korea). All other chemicals used were in the purest form available commercially.

Cell culture

AGS cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). AGS cells were cultured in RPMI-1640 medium supplemented with 10% v/v FBS and 100 units/mL penicillin and 100 μg/mL streptomycin at 37℃ in an incubator with humidified atmosphere of 95% O_2/5% CO_2.

Bacteria strain and growth condition

H. pylori (ATCC 43504, *cag* PAI, and *vac* A positive) was grown on 5% sheep blood agar plates and antibiotic supplements (Dents supplement) (Oxoid, Basingstoke, UK) at 37℃ under microaerophilic conditions generated by CampyPack plus in an atmosphere of 5% O_2, 10% CO_2, and 85% N_2 for 48 h.

In vitro H. pylori *infection model and drug treatment*

The cells were seeded in tissue culture plates for 24 h before infection. 12 h before infection, the medium was replaced by fresh RPMI 1640 without either antibiotic or serum. For the infection, bacteria were harvested in Brucella broth (Difco) containing 10% FBS and added to the host cells at a

multiplicity of infection (MOI) of 100. DHA was added to the cells 1 h before infection.

Western blot analysis

The cell lysates were prepared and the protein concentration was measured by using the BCA protein assay (Thermo) Reagent. The equivalent amounts of proteins (15-30 μg) were subjected to electrophoresis on 8% or 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membrane. The transferred proteins were incubated with primary antibodies in 3% BSA in PBS overnight and incubated with Amersham ECL Prime Western Blotting Detection Reagent (GH Healthcare, Piscataway, NJ, USA) according to manufacturer's instructions and visualized with the imagequant^{TM} LAS 4000 (Fujifilm Life Science, Stamford, USA).

Preparation of cytosolic and nuclear extracts

After *H. pylori* infection, cells were washed twice with ice-cold 1 x PBS, then scraped in 1 ml 1 x PBS, followed by centrifugation at 4000 rpm for 5 min at 4℃. Pellets were resuspended in hypotonic buffer A [10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM PMSF] for 15 min on ice. 10% Nonidet P-40 was then added to final concentration of 0.1% for less than 5 min. The mixture was then centrifuged at 7500 rpm for 5 min at 4℃. Supernatant was collected as the cytosolic extract and stored at -70℃. The pellets were washed twice with hypotonic buffer A and resuspended again in hypertonic buffer C [20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (pH 7.9), 20%

glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT and 0.2 mM PMSF] for 1 h on ice and centrifuged at 13 000 rpm for 15 min at 4℃. The supernatant containing nuclear proteins was collected and stored at -70℃. The protein concentrations of cytosolic and nuclear extracts were determined using the BCA protein assay reagent.

Reverse transcriptase-PCR (RT-PCR)

Total RNA was isolated from AGS cells using TRIzol® and then used for the complementary DNA synthesis using random primers. Reverse transcriptase-PCR was performed following standard procedures. PCR conditions for *SOCS3* were 30 cycles of 95℃ for 30 sec; 60℃ for 30 sec and 72℃ for 30 sec, *PPAR*γ were 30 cycles of 95℃ for 30 sec; 46℃ for 30 sec and 72℃ for 30 sec and the house keeping gene *GAPDH* were as follows: 24 cycles of 95℃ for 30 sec; 56℃ for 30 sec and 72℃ for 30 sec. The primer pairs of the expected products were as follows (forward and reverse, respectively): SOCS3, 5'-TCACCCACAGCAAGTTTCCCGC-3' and 5'-GTTGACGGTCTTCCGACAGAGATGC-3' and PPARγ, 5'-TCTCTCCGTAATGGAAGACC-3' and 5'-GCATTATGAGACATCCCCAC-3' and GAPDH, 5'-ACCCAGAAGACTGTGGATGG-3' and 5'-TCTAGACGGCAGGTCAGGTC-3'. Amplified products were resolved by 2% agarose gel electrophoresis, stained with SYBR® and visualized with the imagequant™ LAS 4000.

siRNA transient transfection

AGS cells were transfected with SOCS3-specific siRNA or PPARγ-specific siRNA or scrambled siRNA using the transfection reagent Lipofectamine for 24 h according to manufacturer's instructions. Transfected cells were then treated with *H. pylori* for the indicated times and then harvested for the next experiments. Lipofectamine® RNAiMAX are provided by Invitrogen (Carlsbad, CA, USA). Scrambled siRNA, Human PPAR gamma-specific siRNA (5'-GCUUAUCUAUGACAGAUGUGAUCUU-3') and SOCS3 specific siRNA (5'-CCAAGAACCUGCGCAUCCAUU-3') were purchased from Genolution Pharmaceuticals, Inc. (Seoul, Korea).

Luciferase assay

AGS cells were transfected with mock or PPARγ luc-vector with FuGENE (Promega, USA) according to the manufacturer's instructions. After 12 h transfection, cells were treated with DHA. Then harvest by reporter lysis buffer (Promega) and cell extract mix with a luciferase substrate, the luciferase activity was measured by the luminometer.

Electrophoretic mobility shift assay (EMSA)

The EMSA for measuring the DNA binding of PPARγ was performed using PPARγ oligonucleotide labeled with [γ-^{32}P]ATP by use of T4 polynucleotide kinase (Takar Bio Inc., Shiga, Japan). Labeled oligonucleotide was purified on a Nick column (Amersham Pharmacia Biotech, USA). The binding reaction was carried out in 20 μl of mixture containing 4 μl of

incubation buffer [10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM DTT, 1 mM EDTA, 4% glycerol, and 0.1 mg/ml sonicated salmon sperm DNA], 8 μg of nuclear extracts, and 100000 cpm of [γ-^{32}P]ATP-end labeled oligonucleotide. After 50 min incubation at room temperatre, 2 μl of 0.1% bromophenol blue was added, and samples were electrophoresed through 6% nondenaturing polyacrylamide gel at 150 V in a cold room for 2 h. To ensure the sepcificity of the binding, a competition assaay was carried out with the excess unlabeled oligonucleotide. Finally, the gel was dried and exposed to an X-ray film.

Immunocytochemical analysis of STAT3^{Tyr705} phosphorylation

AGS cells were infected with *H. pylori* for the indicated intervals. After fixation with cold 95% MeOH/5% Acetic acid for 10 min at 4℃, samples were permeabilized with 0.2% Triton X-100 5 min at room temperature and then blocked with 5% bovine serum albumin in PBST (PBS containing 0.1% Tween-20) for 1 h at room temperature. Samples were then incubated with primary antibody specific for phospho-STAT3^{Tyr705} overnight at 4℃, followed by incubation with fluorescein isothiocyanate-goat anti-rabbit IgG secondary antibody for 1 h at room temperature. Nuclear-staining was performed with propidium iodide (PI) for 5 min at room temperature. Images were assessed under a fluorescent microscopy (Nicon, Japan).

ChIP assay

ChIP assay was carried out accorindg to the EZ-ChIP™ kit protocol (17-371, Upstate, Millipore Corporation, Billerica, MA) Chromatin was sonicated to 200-1000bp fragment and incubated at 4℃ with antibody. The following antibodies were used: PPARγ (santacruz, sc-7196 X), or mouse isotype IgG (1 ug/ml; Millipore) Primers were: 5'-FGGTACTCGCTCTTGGAGCTGA-3' and 5'-AGATCCACGCTGGCTCCGT-3' for SOCS3.

Statistical analysis

Data from three independent experiments at least were expressed as the mean ± S.E.M. The statistical significance of differences between two groups was evaluated using Student's *t* test. Analysis was performed using Sigmaplot (Version 10). Statistical significance was accepted at $P \leq 0.05$, unless otherwise indicated.

Results

DHA inhibits STAT3 phosphorylation induced by *H. pylori*.

H. pylori infection is well known to increase the risk of gastric cancer[12]. In the present study, AGS cells were co-cultured with *H. pylori* (100 MOI) and harvested at different time points. The *H. pylori* infection induced phosphorylation of STAT3 which peaked at 3h (data not shown). DHA significantly decreased STAT3 phosphorylation induced by *H. pylori* infection (Figure 1A). It is well known that phosphorylated STAT3 translocates to the nucleus to exert its transcriptional activity[13, 14]. To measure the localization of phospho-STAT3, we separated cytosol and nucleus of *H. pylori*-treated AGS cells. Phospho-STAT3 existed predominantly in nucleus of the *H. pylori*-treated cells and this was inhibited by DHA (20 μM) treatment (Figure 1B). Nuclear translocation of phospho-STAT3 was also verified by immunocytochemistry (Figure 1C).

SOCS3 decreased *H. pylori*-induced STAT3 phosphorylation by DHA pre-treatment.

Although DHA has been reported to inhibit STAT3 phosphorylation induced by IL-6[15], but underlying mechanism has not been elucidated. In order to explore further how DHA decreases phosphorylation of STAT3

induced by *H. pylori* in AGS cells, we investigated negative regulator proteins participating in STAT3 signaling. SOCS3 is one of the STAT3 negative regulator proteins through competition with phosphorylated receptor[16]. To determine whether SOCS3 is induced by DHA, we conducted RT-PCR and Western blot analysis after AGS cells were treated with DHA. DHA increased the mRNA level of the SOCS3 (Figure 2A upper panel). Similarly, the protein expression of SOCS3 was markedly increased at 1h (Figure 2A lower panel). To identify whether DHA-induced SOCS3 regulates *H. pylori*-induced STAT3 phosphorylation, we utilized the siRNA targeting SOCS3 to knock down SOCS3 expression. The *H. pylori*-induced STAT3 phosphorylation was inhibited by DHA pre-treatment in AGS cells transfected with scrambled siRNA, whereas SOCS3-specific siRNA significantly abolished the inhibitory effect of DHA in *H. pylori*-infected AGS cells (Figure 2B). To confirm the effect of SOCS3-specific siRNA in AGS cells, the mRNA level of SOCS3 was detected by RT-PCR (Figure 2C). The mRNA expression of SOCS3 was markedly reduced by transfection with SOCS3-sepcific siRNA. Thus, it is likely that DHA-upregulated SOCS3 decreases *H. pylori*-induced STAT3 phosphorylation.

DHA exerts the inhibitory effect on *H. pylori*-induced STAT3 phosphorylation through SOCS3 produced via PPARγ.

DHA is a ligand of PPARγ that has been reported to play essencial role in anti-inflammation and emerged new therapeutic durg for various cancers[17, 18]. Berger et al. have reported that SOCS3 was transcribed by PPARγ in the mouse immnune cells[11]. To test the possibility that DHA could induce SOCS3 through PPARγ, AGS cells were treated with DHA (20 μM). The DHA treatment induced nuclear translocation of PPARγ which peaked at 0.25h by immunoblot analysis (Figure 3A). We also confirmed the nuclear translocation of PPARγ by immunocytochemistry (Figure 3B). To assess the activation of PPARγ, the nuclear extract was subjected to EMSA using the oligonucleotide containing the PPARγ consunsus sequence. Consistent with nuclear translocation data (Fiuger 3A), the DNA binding with PPARγ was enhanced by DHA (Figure 3C). To determine whether DHA could controll the expression of downstream target genes of PPARγ, we trasnsfected AGS cells with a PPARγ-dependent reporter construct. DHA significantly activated transcription of the PPAR reporter activity in a time-dependent manner (Figure 3D). To determine whether DHA can induce binding of PPARγ to the SOCS3 promoter region, we conducted the ChIP assay using a primer harbouring the SOCS3 promoter region. When AGS cells were treated with DHA, the biding of PPARγ with SOCS3 promoter region was increased whereas this effect was not observed in the absence of DHA (Fiugure 4A).

To convince PPARγ is the SOCS3 transcription factor, we utilized the siRNA PPARγ construct to knock down the PPARγ gene expression. We found that DHA-induced upregulation of SOCS3 was abolished by siPPARγ (Figure 4B), suggesting that mRNA expression of SOCS3 is regulated by PPARγ. To further verify whether DHA inhibit STAT3 phosphorylation via

PPARγ, siRNA which specifically targeting PPARγ was used. AGS cells were transfected with scrambled siRNA or PPARγ specific-siRNA for 24 h followed by *H. pylori* infection with or without DHA pre-treatment for 1 h. DHA reduced *H. pylori*-induced STAT3 phosphorylation in scrambled siRNA treatment AGS cells, whereas such effects were blunted in siPPARγ transfected cells (Figure 4C). A similar result was achieved with BADGE, a PPARγ antagonist (Figure 4D). Therefore, we suggest that DHA exerts an inhibitory effect on *H. pylori*-induced STAT3 phosphorylation by inducing PPARγ activation and subsequently transcription of SOCS3 mRNA.

DHA inhibits downstream signaling of the STAT3 pathway activated by *H. pylori*.

It has been reported that the PPARγ ligands including DHA have inhibitory effects on the proliferation of gastric cancer cells[19, 20]. c-Myc which is one of the target proteins of the STAT3 pathway, is implicated in cell proliferation[21]. *H. pylori* substantially increased expression of c-Myc after *H. pylori* infection and this was decreased by DHA (Figure 5A). To further support our hypothesis, we utilized the siRNA targeting expression of SOCS3. As illustrated in Figure 5B SOCS3-specific siRNA significantly abolished the inhibitory effect of DHA on c-Myc expression in *H. pylori*-infected AGS cells.

The anchorage-independent colony forming assay is widely used to assess malignant transformation of cells. To determine the oncogenic effect of *H. pylori* and anti-cancer effect of DHA, we conducted. AGS cells co-cultured

with *H. pylori* for 2 weeks developed the significantly increased number of anchorage-independent colonies (Figure 6). DHA treatment markedly decreased the anchorage-independent growth of AGS cells induced by *H. pylori* (Figure 6).

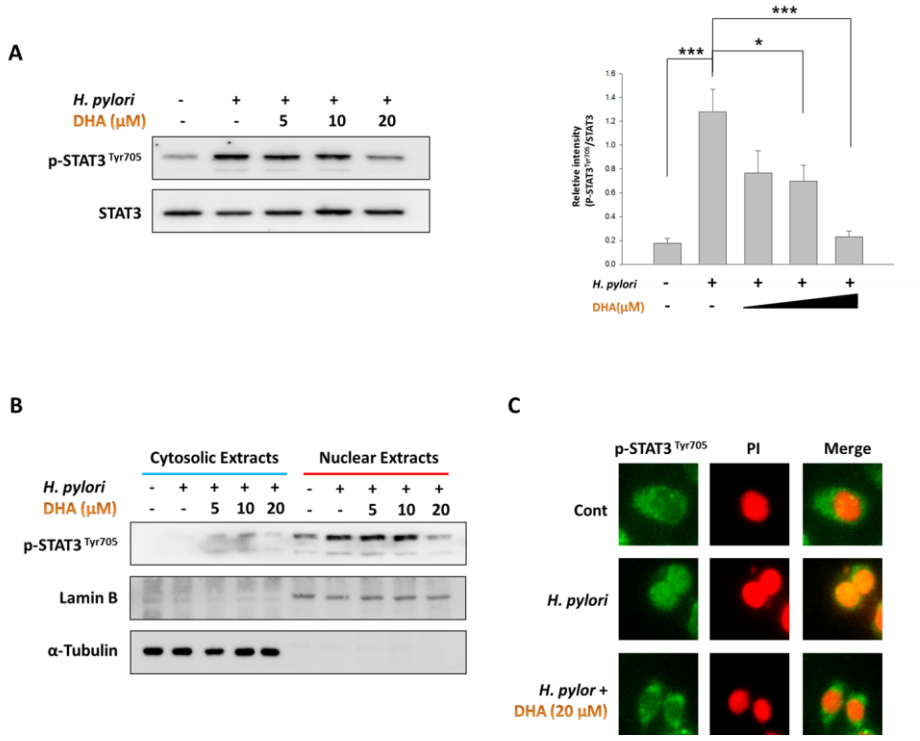


Figure 1. DHA decreases *H. pylori*-induced STAT3 phosphorylation. (A) After 1 h pre-treatment with DHA, AGS cells were infected by *H. pylori* for 3 h. Phosphorylation of STAT3 was assessed by Western blot analysis. (B) Cytosolic and nuclear extracts were immunoblotted for the measurement of phospho-STAT3 translocation. Lamin B was measured to ensure equal amounts of nuclear proteins loaded. α-Tubulin was also measured to ensure equal amounts of cytosolic proteins loaded. (C) Immunocytochemistry analysis was performed using antibodies against phospho-STAT3 after DHA pre-treatment followed by *H. pylori* infection for 3 h. Results are the means ± S.E. (n=5) *p<0.05, **p<0.005, ***p<0.001

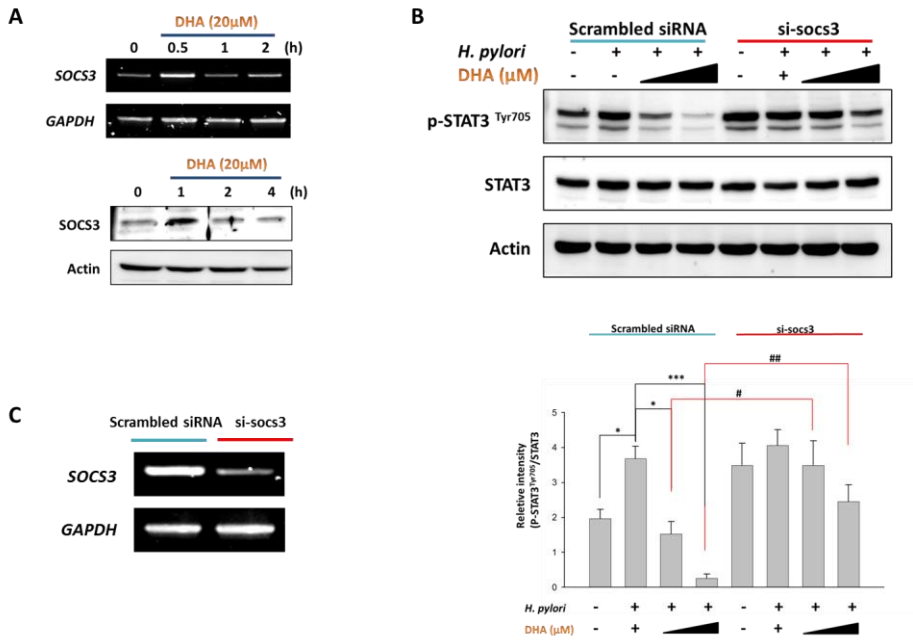


Figure 2. DHA inhibits *H. pylori*-induced STAT3 phosphorylation through SOCS3 upregulation. (A) AGS cells were incubated with DHA (20 μM) or vehicle for indicated time periods. The expression of SOCS3 was determined by semi-quantitative RT-PCR and GAPDH was measured to ensure equal amounts of cDNA loaded. The protein level of SOCS3 was measured by Western blot analysis and actin was measured to ensure equal amounts of protein loaded. (B) AGS cells were transfected with scrambled siRNA or SCOS3-specific siRNA before DHA treatment. Blockage of *H. pylori*-dependent upregulation of phospho-STAT3 expression was detected by Western blot analysis. Immunoblots were subjected to statistical analysis. (C) The expression of SOCS3 mRNA was determined by semi-quantitative RT-

PCR in the AGS cells transfected with scrambled siRNA or SOCS3 siRNA. Results are the means ± S.E. (n=4) *$p<0.05$, **$p<0.005$, ***$p<0.001$

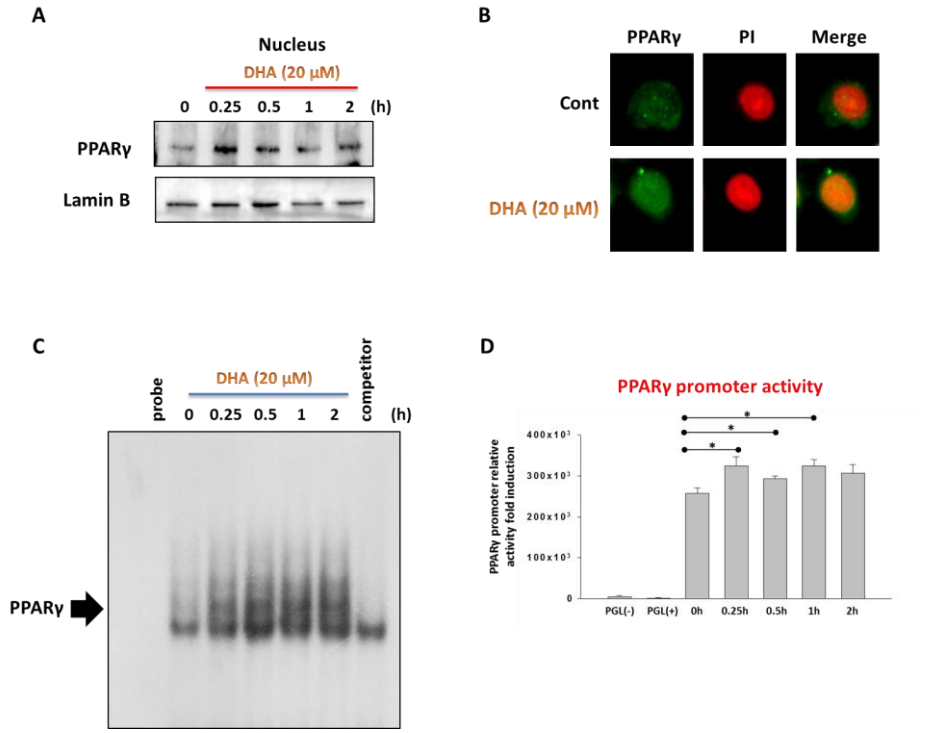
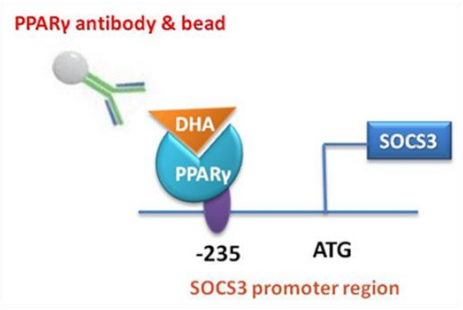


Figure 3. DHA induces PPARγ activation. (A) Nuclear extracts were prepared at the indicated intervals after incubation with DHA (20 μM). The nuclear translocation of PPARγ was determined by Western blot analysis. Lamin B was used for loading control. (B) Immunocytochemical analysis of PPARγ translocated into the nucleus was detected with a fluorescent microscopy after treatment with DHA (20 μM) for 0.5 h. (C) Nuclear extracts were incubated with the [γ-^{32}P]-labeled oligonucleotides containing the PPARγ consensus sequence. Protein-DNA complexes were separated from the free probe by electrophoresis. (D) AGS cells were treated with DHA (20 μM) after transfection with a PPARγ luciferae construct and analyzed for the

PPARγ transcriptional activity. Results are the means ± S.E. (n=5) *p<0.05, **p<0.005, ***p<0.001



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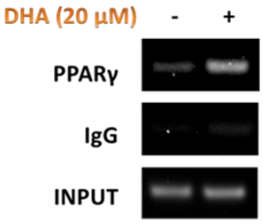

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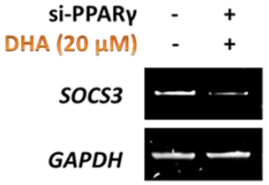

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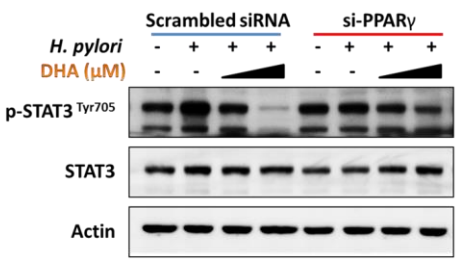

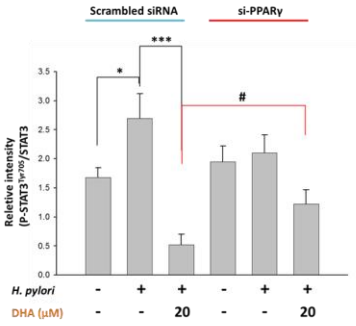

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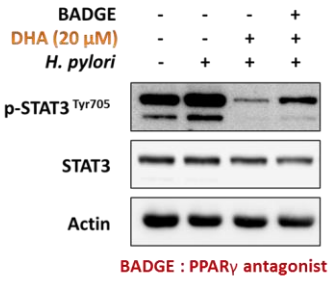

BADGE : PPARγ antagonist

Figure 4. DHA regulates SOCS3 expression via PPARγ. (A) AGS cells were treated with DHA (20 μM) for 0.5 h and harvested to determine PPRE binding activity by the ChIP assay. (B) The expression of SOCS3 mRNA was assessed by semi-quantitative RT-PCR after DHA treatment for 0.5 h in the AGS cells transfected with scrambled siRNA or PPARγ-specific siRNA. (C) AGS cells were transfected with scrambled siRNA or PPARγ-specific siRNA for 24 h. The cells were then infected with *H. pylori* after DHA (20 μM) pre-treatment for 1 h. Blockage of *H. pylori*-dependent upregulation of phospho-STAT3 expression was detected by Western blot analysis. Immunoblots were subjected to statistical analysis. (D) Inhibitory effects of BADGE (PPARγ antagonist) was determined by Western blot analysis. Results are the means ± S.E. (n=6) *$p<0.05$, **$p<0.005$, ***$p<0.001$.

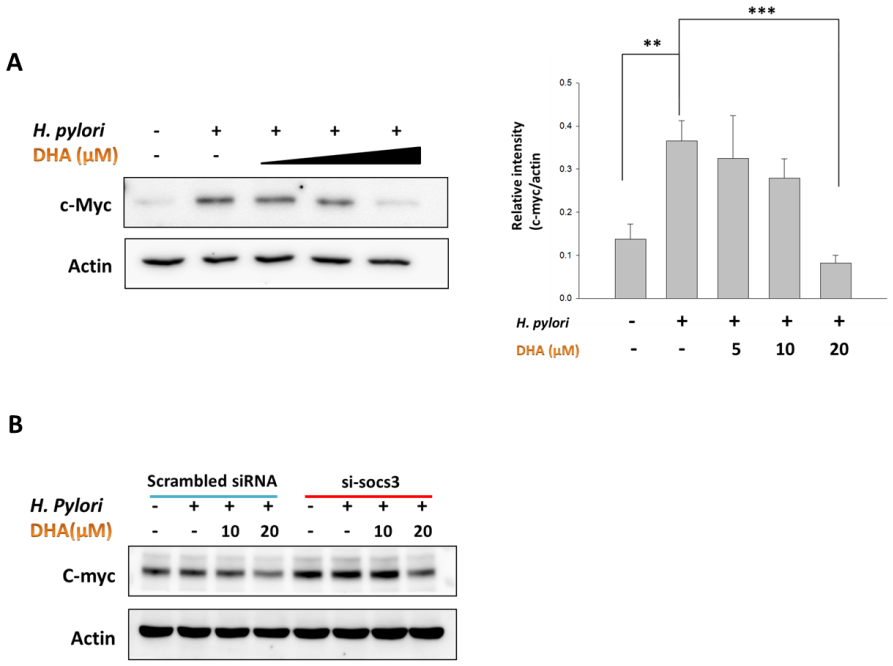

Figure 5. DHA inhibits the *H. pylori*-induced c-Myc protein expression. (A) After 1 h pre-treatment with DHA, AGS cells were infected by *H. pylori* for 3 h. c-Myc expression was assessed by Western blot analysis. (B) AGS cells were transfected with scrambled siRNA or SCOS3-specific siRNA before DHA treatment. Blockage of *H. pylori*-dependent upregulation of c-Myc was detected by Western blot analysis. Immunoblots were subjected to statistical analysis. Results are the means ± S.E. (n=5) *p<0.05, **p<0.005, ***p<0.001

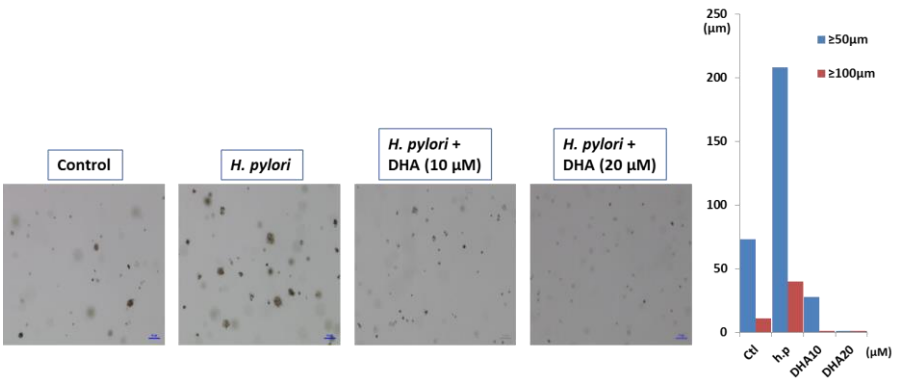

Figure 6. DHA decreased the *H. pylori*-induced anchorage-independent colony formation. AGS cells were co-cultured with *H. pylori* for 2 weeks, and medium including DHA was changed every other day.

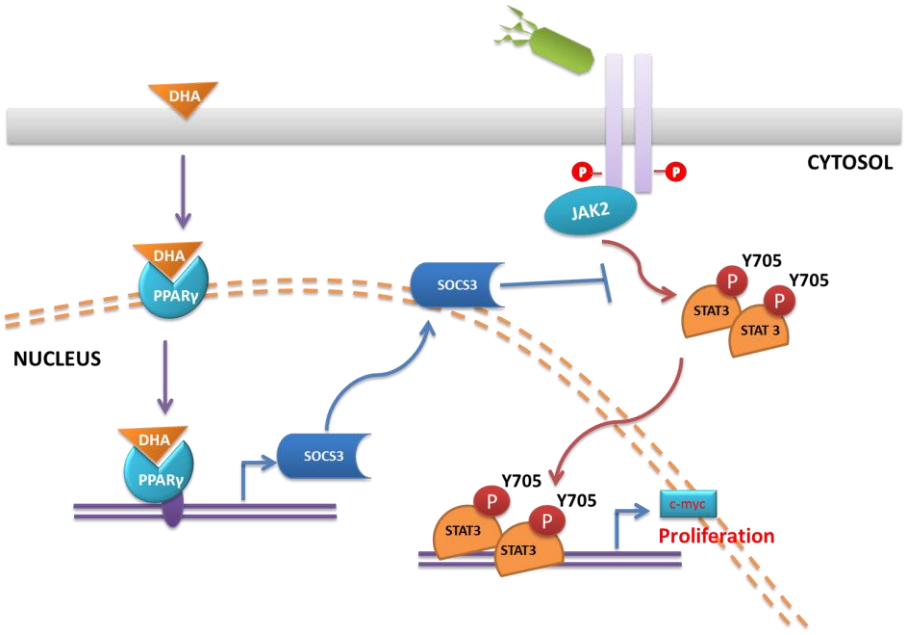

Figure 7. A proposed mechanism underlying the inhibitory effect of DHA on STAT3 phosphorylation induced by *H. pylori*. The study proposes a mechanism responsible for inhibition of STAT3 phosphorylation by DHA in AGS cells treated with *H. pylori*. The crucial role of PPARγ and SOCS3 as a feasible link between DHA and down-regulation of phospho-STAT3 is suggested.

Discussion

Helicobacter pylori infection is known for a major risk factor for the chronic gastritis and strongly associated with the development of gastric adenocarcinoma[22, 23]. Recently, some PPARγ agonists have been emerged as potential therapeutic drugs for the treatment of chronic inflammatory diseases like Alzheimer's disease, systemic lupus erythematosus, and ulcerative colitis[24-26]. Furthermore, anti-inflammatory and anti-cancer effects of PPARγ ligands have been observed in various *in vivo* and *in vitro* experiments[11, 27-29] and clinical trials have demonstrated that DHA is a potential adjuvant in the cancer chemotherapy [27, 30]. Although, several lines of evidence support DHA, one of the polyunsaturated fatty acids (PUFAs), inhibits growth and proliferation of human gastric cancer cells, the underlying mechanisms remain insufficiently clarified[31]. In this study, we investigated a series of intracellular events affected by DHA in the *H. pylori*-infected AGS cells. Our results suggest that DHA inhibits proliferation of gastric cancer cells infected by *H. pylori* in a PPARγ-SOCS3 dependent manner.

A recent study has shown that DHA 100 μM inhibits *H. pylori* growth directly and consequently attenuates the host inflammatory response in mice[32]. However, DHA (20 μM) failed to inhibit the growth of *H. pylori* directly in our experiments, and there was also no change in the pathogenicity of *H. pylori* treated with 20 μM DHA (data not shown). This concentration of DHA may not be high enough to hinder the growth of *H. pylori*. Therefore, we focused on cellular mechanism induced by DHA. We found that DHA treatment activated PPARγ nuclear translocation and binding to the SOCS3

promoter to inhibit *H. pylori*-induced STAT3 phosphorylation. To provide further evidence for this mechanism, we silenced PPARγ and SOCS3 with a siRNA knock down system. Although inhibitory effect of DHA on *H. pylori*–induced STAT3 phosphorylation was abolished by silencing of PPARγ and SOCS3, both siRNA knock down systems could not completely recovered the DHA-mediated inhibition of *H. pylori*-induced STAT3 phosphorylation. It might be suggested that the strong inhibitory effect of DHA on STAT3 phosphorylation induced by *H. pylori* could involve other signal pathways, such as NF-κB signaling. In addition, it was reported that the NF-κB singnaling pathway was suppressed by DHA and EPA[33-35]. Still another possibility was proposed by other researchers[36-39]. Park et al. (2003) have reported that even though 15-Deoxy-Delta12,14-Prostaglandin J_2 is one of the PPARγ ligands, its anti-inflammatory effects are dependent not only on PPARγ but also phosphorylated SHP2[39].

SOCS3 as a negative feedback-loop regulator of JAK/STAT signaling inhibits JAK protein's tyrosine kinase activity or competing with other molecules for phosphorylated receptor binding[40]. It is well known that SOCS3 is encoded by STAT family transcription factors to regulate overexpressed STAT3 phosphorylation. According to our experiments, expression of SOCS3 was increased after 6 h in *H. pylori* infected AGS cells (data not shown). Therefore, it is likely that SOCS3 elevated by DHA is involved in prevention of *H. pylori*-induced phosphorylation of STAT3 as schematically proposed in Figure 7. Interestingly, recent research showed that PPARγ could also serve as a transcription factor regulating SOCS3 expression to prevent cancer promotion[11, 41]. In line with this notion, we demonstrated

that DHA treatment induced PPARγ nuclear translocation and interaction with SOCS3 promoter region in AGS cells. Notably, SOCS3 mRNA was transcribed by PPARγ at early time (Figure 2A). Therefore, we suggest that DHA exerts a protective effect through SOCS3 induction to prevent *H. pylori*-activated STAT3 signaling in the human gastric cancer cells.

c-Myc is one of the STAT3 target proteins, constitutively expressed in various cancers[42]. Knock down of c-Myc protein was found to inhibit breast and colon cancer *in vitro and in vivo*[43, 44]. These studies also support the effect of DHA on gastric cancer development by reducing c-Myc protein. Similarly, our study showed that DHA significantly decreased c-Myc protein level stimulated by *H. pylori*. As shown in Figure 5, DHA significantly decreased expression of c-Myc and anchorage-independent colony formation. In the clinical study with gastric cancer patients, combination chemotherapy treatment with DHA improved survival of patients[45].

Although recent studies have shown phenomenally beneficial effects of DHA on human gastric cancer, the underlying mechanism of DHA was not fully elucidated. Thus, our study suggest DHA inhibits STAT3 phosphorylation induced by *H. pylori* through activating PPARγ, which produce SOCS3 as a transcription factor, in the human gastric cancer cells. Taken together, our results provide mechanistic basis for the chemopreventive effect of DHA on gastric cancer.

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초 록

대표적인 오메가 3 다중불포화지방산 (ω-3 polyunsaturated fatty acids) 중 하나인 Docosahexaenoic acid (DHA)는 항염증과 항암작용으로 큰 주목을 받고 있다. 최근 연구에 의하면 DHA 는 *Helicobacter pylori* (*H. pylori*)의 성장을 억제할 뿐만 아니라 *H. pylori* 에 감염된 마우스 위 내 *H. pylori* 의 콜로니 형성을 감소시켰다. 또한, 지속적인 *H. pylori* 감염은 위암 risk 를 증가시키며 염증 및 암화 과정의 주요 전사인자인 signal transducer and activator of transcription 3 (STAT3)의 활성화를 유도한다. 이러한 연구결과를 바탕으로 본 연구에서는 DHA 의 항암 효과를 *H. pylori* 에 의해 유도된 STAT3 인산화 억제를 통해 그 기전을 규명하고자 하였다.

DHA 를 사람위암상피 세포인 AGS 세포에 전 처리한 결과 *H. pylori* 에 의해 유도된 STAT3 의 인산화와 핵내 이동이 효과적으로 억제되었다. 본 연구에서는 이러한 DHA 억제 효과의 원인을 밝히고자 DHA 에 의해 활성화된 PPARγ 가 STAT3 의 음성조절인자로 알려진 suppressor of cytokine signaling 3 (SOCS3)를 유도한다는 가설을 세우고 연구를 수행하였다. AGS 세포에 DHA 를 처리하여 peroxisome proliferator-activated receptor γ (PPARγ)의 활성이 증가됨을 확인하였고 ChIP assay 를 통하여 활성화된 PPARγ 가 SOCS3 의 promoter 에 결합함을 확인하였다. 이렇게 PPARγ 에 의해 유도된 SOCS3 가 *H. pylori* 에 의해 증가된 STAT3 인산화를 억제하는지 확인하기 위하여 PPARγ siRNA 와 SOCS3

siRNA 를 이용해 두 단백질을 각각 Knockdown 시킨 후 DHA 와 *H. pylori* 를 처리하였다. 그 결과 DHA 에 의한 STAT3 의 인산화가 억제되지 않음을 확인할 수 있었다. DHA 는 또한 anchorage-independent growth assay (AIG assay)에서 *H. pylori* 를 co-culture 한 AGS 세포의 콜로니 형성을 효과적으로 감소시켰다. 결론적으로 DHA 는 *H. pylori* 에 의해 감염된 사람위암세포에서 PPARγ 와 SOCS3 단백질의 활성화를 통해 STAT3 인산화를 효과적으로 억제하여 항암 효과를 나타낸다.

주요어 : DHA; *H. pylori*; STAT3; PPARγ; SOCS3; n-3 PUFA

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