Journal of Animal and Feed Sciences, 20, 2011, 206–223

Effects of dietary fish oil on NF$_\kappa$B gene expression and related signaling in spleen of chickens stimulated with lipopolysaccharide*

X. Yang[1,2], Xi He[2], B. Zhang[2], Y. Yang[2], J. Yuan[2] and Y. Guo[2,3]

[1]*Department of Animal Nutrition and Feed Science, College of Animal Science and Technology, Northwest A&F University YangLing, 712100, P.R. China*

[2] *Department of Animal Nutrition and Feed Science, College of Animal Science and Technology, China Agricultural University Beijing, 100094, P.R. China*

(Received 27 June 2010; revised version 29 April 2011; accepted 18 June 2011)

ABSTRACT

The study was conducted to investigate the effects of fish oil and maize oil on nuclear factor kappa B (NFκB) gene expression and the downstream pathways of intracellular signaling in spleen of chickens after lipopolysaccharide (LPS) stimulation. Two hundred eighty eight chickens were assigned in a 2×2 factorial design. Factors were dietary fat type (4.5% maize oil or 4.5% fish oil) and immunological stimulation (LPS or saline). LPS increased levels of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (AA) of spleen in chickens after the second LPS stimulation on 28 d of age. Fish oil alleviated the increase of EPA and DHA in spleen of chickens after LPS stimulation at 27 d of age. Fish oil supplementation decreased prostaglandin 2 (PGE_2) production and the activity of cyclooxygenase 2 (COX_2) after LPS stimulation. LPS stimulation increased the activity of phospholipase C (PLC) in spleen of chickens. And fish oil inhibited activity of PLC in spleen of chickens stimulated by LPS. Meanwhile fish oil decreased the production of

* Supported by the National Natural Science Foundation of China (No. 31001017), the Natural Science Foundation of Shaanxi Province (No. 2010JQ3002), the Support of Opening Program State Key Laboratory of Animal Nutrition (No. 2004DA125184F0809), the Natural Science Foundation Council of China (No. 30671520), the State Outstanding Young Scientist Scholarship of China (No. 30425037) and the Central University Special Fund Basic Research and Operating Expenses (No. QN2009020)

[3] Corresponding author: e-mail: guoym9899@yahoo.com.cn

inositol triphosphate (IP_3) in spleen of chickens stimulated by LPS. Fish oil alleviated the mRNA abundance elevation of nuclear factor kappa B (NFκB) after LPS stimulation. These results showed that fish oil down-regulated the production of IP_3 and PGE_2 through inhibiting the activity of PLC and COX_2 in spleen of chickens, respectively. The results of NFκB gene expression suggested fish oil might alleviate immune stress at the level of transcription.

KEY WORDS: fish oil, immune stress, signaling, NFκB, chickens

INTRODUCTION

Polyunsaturated fatty acids (PUFA) are categorized according to the position of the last double bond near their methyl end into n-3 and n-6 series. Animals cannot introduce double bonds between carbon 9 and the methyl end of fatty acid. Therefore, animals cannot convert n-6 PUFA into n-3 PUFA or *vice versa*. Fish oil is the main dietary source of n-3 PUFA enriched with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA); and maize oil is the main dietary source of n-6 PUFA enriched with arachidonic acid (AA). Fish oil modulates the immune responses including genes expression and lymphocytes proliferation, thereby exerting beneficial effects on inflammatory processes (Liu et al., 2003; Yang et al., 2008). Cyclooxygenase (COX), also known as prostaglandin endoperoxide synthase or as prostaglandin G/H synthase, is a key membrane bound enzyme responsible for the oxidation of AA to prostaglandin E_2 and the oxidation of EPA to prostaglandin E_3. Two COX isoforms have been identified, COX_1 and COX_2. COX_1 enzyme is expressed constitutively in most tissues and cells to control synthesis of those PGs primarily involved in the regulation of homeostatic functions throughout the body and remains constant under either physiological or pathological conditions. COX_2 is an intermediate response gene that encodes a protein normally absent from most cells but highly inducible in certain cells in response to inflammatory stimuli resulting in enhanced PG release. PGs formed by COX_2 primarily mediate pain and inflammation. COX_1 was present in both normal and neoplastic tissue equally. COX_2 is also induced by a variety of factors including tumour promoters, cytokines, growth factors and hypoxia and is overexpressed in rheumatoid arthritis (Simmons et al., 2004). Its utilization of AA also perturbs the level of intracellular free AA and subsequently affects cellular functions. The anti-inflammatory activity of corticosteroids correlates with preventing the release of AA from storage phospholipids, and glucocorticoids inhibit the expression of COX_2 (Tapiero et al., 2002).

Some reviews have summarized that constituents in fish oil are anti-inflammatory, and the most likely candidates for this beneficial effect are the two n-3 fatty acids found in highest abundance in fish oil, EPA and DHA

(McMurray et al., 2000; Calder and Field, 2002; Yang et al., 2008). Spleen, an important immune organ of poultry, can transport lymphocytes to peripheral blood and reclaim lymphocytes from peripheral blood. Lymphocytes of spleen can bind and recognize antigens, which activates immune responses. However, it is unclear how fish oil regulates signaling of lymphocytes by immune stress. NF-κB is found in almost all animal spleen cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, and bacterial or viral antigens (Gilmore, 2006; Perkins, 2007). NF-κB plays a key role in regulating the immune response to infection. Conversely, incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development. In order to assess immune capabilities regulated by fish oil after immune stimulation, it is essential to understand the immune signaling. The present study was designed to determine the effects of dietary fish oil and maize oil on NFκB gene expression and the downstream pathways of intracellular signaling in spleen of chickens subjected to an immune stress, LPS.

MATERIAL AND METHODS

Animals and experimental procedures

These 288 chickens were assigned in a 2×2 factorial arrangement. The main factors were dietary fat type (4.5% maize oil or 4.5% fish oil) and immunological stimulation (LPS or saline). Birds were randomly assigned to four groups of seventy-two chicks each, with 6 replications of twelve chicks in a cage with constant lighting. Feed and water were available *ad libitum* in each dietary group. On 20 and 27 d of age, all chickens were injected intra-abdominally with either 1 mg/kg body weight (BW) of *E. coli* LPS (*Escherichia coli* serotype 055: B_5, Sigma) or sterile saline. The animal care and experimental procedures conformed to the regulations of China Agricultural University Animal Care and Use Committee. Fish oil and maize oil were supplied by Dongmiao Co. Ltd. and China Corn Oil Co. Ltd., respectively. The fatty acid profiles of fish oil and maize oil were analysed by gas chromatography as shown in Table 1.

Spleen sampling and analytical procedures

Spleen sampling. After LPS injection at 20 d of age, the spleen from a chicken every replication on 21 d of age was aseptically removed. After LPS injection at 27 d of age, the spleen from a chicken every replication on 28 d

Table 1. Fatty acids composition of fish oil and maize oils

Fatty acids	Composition, %	
	fish oil	maize oil
C14:0	8.2	0.03
C14:1	0.35	-
C16:0	15.42	12.36
C16:1	7.92	0.14
C18:0	4.21	1.84
C18:1	26.96	28.17
C18:2(n-6)	_	55.78
C18:3(n-3)	1.05	0.81
C20:1	13.88	0.33
C20;2	0.45	0.02
C20:3	0.68	-
C20:4(n-6)	0.16	-
C20:5(n-3)	10.02	-
C22:0	-	0.12
C22:6(n-3)	10.61	-
C24:0	-	0.40

of age was aseptically removed. The spleens were preserved in liquid nitrogen immediately and then transferred to appropriate tubes. All tubes were stored at -80°C until analysis.

Extraction and isolation of lipids and measurement of fatty acids composition. Lipids were extracted from spleen by modified method according to Kramer and Zhou (2001). The 0.5 g portions of tissues, stored frozen at -80°C, were quantitatively pulverized using a stainless steel mortar and pestle kept at dry ice temperature. The pulverized content was transferred into a beaker containing 5 ml chloroform/methanol (2:1), gently homogenized, and C:17 was added as an external standard. Then sample filtered through a sintered glass funnel to collect total lipids.

The methylation procedures were NaOH/methanol at 50°C for 30 min, and HCl/methanol at 80°C for 60 min, and then added 1 ml water and 6 ml heptane. Chromatographic analysis was performed on a HP6890 chromatograph equipped with a flame ionization detector system. Separations were conducted using a 100 m × 0.25 mm capillary column (CP-Sil 88) with a 0.25 μm stationary phase thickness. The carrier gas was purified helium at a flow rate of 1.7 ml/min. The gas chromatography (GC) conditions were as follows: injector temperature 250°C, detector temperature 250°C, initial oven temperature 180°C for 45 min, increased to 215°C at a rate of 10°C/min, then maintained at 215°C for 17 min. The sample and carrier gas mixture (helium) were carried at 1:40.

Measurement of phospholipase A_2 (PLA_2), COX and prostaglandin$_2$ (PGE_2). Activity of PLA_2 was measured according to the method of Reynolds et al. (1992).

The spleen was homogenized in 9 volume (W/V) of ice-cold PBS with a polytron tissue homogenizer for 2 min. The supernatant was obtained by centrifugation at 10000 rpm for 15 min, 4°C. Protein concentration of the supernatant was determined by the method of Lowry with bovine serum albumin as the standard (Stoscheck, 1990).

Activity of COX in spleen of chickens was determined according to the method of Meng et al. (2003). The 60 µl supernatant stemming from spleen of chicken was averagely divided into three tubes: A, B, C, respectively. The 60 µl reaction mixture containing 20 µl Tris-HCl buffer (pH 7.5, 100 mM), 20 µl hematin (H3281, Sigma; 20 µmol/l) and 20 µl 2',7'-dichlorodihydrofluorescein diacetate (D6883, Sigma; 50 µmol/l) were added to each tube. Then 20 µl nimsulide (N1016, Sigma) (2.5 µmol/l) were added to C tube. The tubes were incubated for 10 min at 30°C. Twenty µl substrate (9 µmol/l AA) was added to A tube and B tube without adding inhibitor (nimsulide). The reaction was performed for 20 min at 30°C. Then 2 ml of cold PBS were added to all tubes. A bilateral laser scanning fluorescence miscroscope (Cary Eclipse, Varian) with an argon ion laser beam for excitation was used for fluorescent measurement of COX activities and expression. Excitation and emission wavelengths were 450 and 550 nm, respectively. Data of COX activity indicated as fluorescence intensity (FI). FI_C minus FI_A was COX_1 activities. FI_B minus FI_C was COX_2 activities. FI_B minus FI_A was total COX activities, and total COX activities included COX_1 activities and COX_2 activities. The chicken spleen supernatant prepared as above was harvested and stored at -80°C until analysed. The PGE_2 levels of the supernatant were measured by PGE_2 kit (900-001, Assay Designs Inc.), according to the manufacturer instructions. CV of intra-assay precision determined by taking samples containing low, medium and high concentrations of PGE2 was 8.9, 5.8 and 17.5%, respectively. CV of inter-assay precision determined by measuring three samples with low, medium and high concentrations of PGE2 was 3.0, 5.1 and 3.9%, respectively. The percentage cross reactivity for PGE_2, PGE_1, PGE_3, PGA_2, $PGF_{1\alpha}$ was 100, 70, 16.3, 0.1, 1.4 and 0.1%, respectively. The percentage sample recovery was 101.3%.

Membrane fluidity of splenocytes. Splenocyte membrane was isolated by the methods of Giraud et al. (2000). The tissues (1 g) were homogenized at 4°C in a glass tube with a Teflon pestle attached to a motorized homogenizer (1000 rpm) with four 15-s bursts. The sample was filtered through cheese cloth and centrifuged at 1500 *g* for 10 min. The resulting microsomal pellet was resuspended in 1.0 ml $NaHCO_3$ (1 mM, pH 7.5), then 3 ml of 81% sucrose (w/v) were added. The microsome suspension from spleen was layered on a discontinuous sucrose gradient built up by carefully adding 1.5 ml 53.4% sucrose, 3.0 ml 48.0% sucrose and 1.5 ml 42.9%, then centrifuged at 70.000 *g* for 90 min at 4°C. The white, fluffy layer formed at the interface of the 42.9 and 48.0% sucrose layers. The pellet was harvested and washed with PBS (1 mM, pH 7.5, with 30% sucrose). The entire

preparation procedure was conducted at 4°C. Protein concentration of the plasma membranes was determined by the method of Lowry with bovine serum albumin as the standard (Stoscheck, 1990). The same volume of sample prepared was mixed with 2 ml PBS (1 mM, pH 7.5) containing 1,6-diphenyl-1,3,5-hexatriene (DPH, 2×10^{-6} mol/l prepared from a DPH stock solution of 2×10^{-3} mmol/l in tetrahydrofuran). The suspension was incubated for 30 min at room temperature. The sample was excited with vertically polarized light (362 nm) and emission (438 nm) was measured through the polarizer both parallel and perpendicular to the excitation polarizer. The parallel (I//) and perpendicular (I') fluorescence intensities were recorded at 37°C after careful temperature equilibration. The term G, which was calculated by $I_{90°,0°}/I_{90°,90°}$, was used to normalize the determinations of the parallel and perpendicular fluorescence intensities The fluorescence polarization (FP) was calculated:

$$FP= (I// – G\ I')/(I// + G\ I')$$

The fluorescence polarization refers to the rotational motion of the DPH distributed throughout the hydrophobic core of the lipid bilayers and is inversely proportional to the membrane fluidity; therefore, the lower the FP, the more fluid is the membrane.

Measurement of phospholipase C (PLC), sphingomyelinases (SMase) and IP_3. Membranous PLC and SMase activity were determined as described previously (Wu et al., 1997). After homogenizing, the spleen suspensions were centrifuged at 800 *g*, 4°C for 15 min, the supernatant was centrifuged again at 100.000 *g* at 4°C for 60 min. The pellets were resuspended in buffer (20 mmol/l Tris-HCl, pH 7.0, 10 mmol/l EGTA, 1 mmol/l DTT, 1 mmol/l PMSF, 0.5 mg/ml sodium deoxylcholate and 0.1 mol/l NaCl), treated by sonification several times for 2 min at 4°C, and used as membranous PLC and SMase. The supernatant was used for determination of membranous PLC and SMase. Protein concentration of the supernatant was determined by the method of Lowry with bovine serum albumin as the standard (Stoscheck, 1990). The IP_3 levels of the supernatant were measured by IP_3 Fluorescence Polarization kit (90-0037-01, Amersham Biosciences), according to the manufacturer instructions.

PLC assay. For Ca^{2+}-dependent PLC, the reaction mixture (500 µl) contained 100 mmol/l Tris-HCl, pH 7.0, 1.5 mmol/l $CaCl_2$ (omitted in controls), and 5 mmol/l L-α-phosphatidylcholine (P3556, Sigma). The reaction was started by adding 10 µl of the sample to the mixture. After incubation at 37°C for 15 min, the reaction was stopped by adding 2 ml chloroform/methanol/HCl (2:1:0.035, vol/vol/vol). The reaction mixture was then vigorously agitated in a vortex mixer and centrifuged at 1000 *g* for 10 min. Each supernatant (160 µl) was transferred to another mixture (0.5 ml) containing 0.21 mol/l Tris, pH 9.8, 50 mmol/l $MgCl_2$, and 4 units of alkaline phosphatase (TaKaRa). After incubation

at 37°C for 30 min, 2 ml of chromogen solution (0.31% ammonium molybdate, 0.66 mol/l sulphuric acid, 0.011% malachite green and 0.018% Tween-20) was added, and the resulting mixture was incubated at 37°C for another 20 min. The optical density was measured at 660 nm (wavelength). Enzyme activity was expressed as nmol per min per mg of protein (nmol/min · mg). For Ca^{2+} - independent PLC, the reaction mixture containing 1 mmol/l EDTA and the procedure were the same as in the assay for Ca^{2+}- dependent PLC except that $CaCl_2$ was excluded. The control tubes for Ca^{2+}- dependent PLC were used as test tubes for Ca^{2+}- independent PLC, and the tubes in which samples were added after the reaction was stopped were set as control.

SMase assay. For acid SMase, the reaction mixture (500 µl) contained 100 mmol/l NaAc-HAc, pH 4.5, and 100 mmol/l sphingomyelin (S7004, Sigma). The reaction was started by adding to the mixture 10 µl of the sample enzyme. For neutral SMase, the reaction mixture (500 µl) contained 100 mmol/l Tris-HCl, pH 7.2, and 100 mmol/l sphingomyelin (S7004, Sigma). The subsequent method was the same as in the assay for Ca^{2+}- dependent PLC.

Real-time quantitative RT-PCR. Total splenic RNA was extracted using the the TRIzol Reagent (Invitrogen, Life Technologies) according to the manufacturer's instructions. Total RNA was reverse transcribed according to the method described by Sijben et al. (2001) and Lai et al. (2005). Cycle parameters for the RT procedure were 1 cycle of 37°C, 1 h; 1 cycle of 70°C, 15 min; and 1 cycle of 4°C, 5 min. The RT products (cDNA) were stored at -20°C for relative quantification by PCR. The reverse transcribed products (cDNA) were for relative quantification by PCR. NFκB amplification primers were designed using the Primer Premier 5.0 software programme. The following sequences of PCR primer pairs were used: forward 5'-GTG TGA AGA AAC GGG AAC TG-3', reverse 5'- GGC ACG GTT GTC ATA GAT GG -3'.

Real-time PCR for quantification of NFκB mRNA levels in chicken spleens were quantified using a method based on that of Sijben et al. (2001). Briefly, quantitative analysis of PCR was carried out according to optimized PCR protocols and SYBR Green PCR master Mix Kit (Applied Biosystems). For the PCR reaction, the following experimental run protocol was used: enzyme incubation (50°C for 2 min), denaturation programme (95°C for 10 min), amplification and quantification programme repeated 38 times (94°C for 20 s, different annealing temperature for different target genes for 20 s, 72°C for 20 s with a single fluorescence measurement), melting curve programme (65-95°C with a heating rate of 0.1°C/s and a continuous fluorescence measurement) and finally 72°C for 10 min. The annealing temperature for NFκB was 53°C. Quantification was based on the increased fluorescence detected by the DNA Engine Opticon 2 fluorescence detection system (MJ Research).

Statistical analysis

Statistical analysis of the chicken data were conducted using the GLM procedure of SAS (2001) in a 2×2 factorial arrangement of treatments in a randomized complete block design, and the following model was used:

$$Y_{ijk} = \mu + a_i + b_j + (ab)_{ij} + e_{ijk}$$

where: Y_{ijk} - the digestion index in the k^{th} chicken on the a_i diet on the b_j stimulation, μ - the overall meal, a_i - the fixed effect of the i^{th} diet; b_j - the fixed effect of the j^{th} stimulation; $(ab)_{ij}$ - the interaction term and e_{ijk} - the random error connected with ijk - observation.

Correlations analysis between COX and PGE_2, PLC and IP_3 were conducted using the Pearson procedure. SAS codes for correlation coefficient test were provided in the following section.

```
proc corr data = a outp = corr; *outp with pearson correlation coefficient;
                                var x y; run
```

where: proc corr - analysis programme for correlations; outp - the product moment correlation coefficient; var - analytical parameters; a - the data file for COX, PGE_2, PLC and IP_3, x, y - the variables of COX and PGE_2, PLC and IP_3, respectively.

RESULTS

Growth performance

There were no significant effects of dietary oils on body weight gains (BW = 0.708 kg) on 21 d of age in the chickens. During the first LPS stimulation period (from 21 to 27 d of age), LPS stimulation did not reduced body weight gains of chickens. After the second LPS stimulation, chickens (BW = 1.65 kg) stimulated with LPS had lower body weight gains ($P<0.05$) than that in those (BW = 1.75 kg) treated with saline during week 4 to week 6. Feed conversion efficiency was significantly higher in chickens stimulated with LPS than in those treated with saline.

Major PUFA composition of splenocytes

In all 4 feeding groups, neither LPS stimulation nor dietary oils affected the levels of LA, EPA and DHA in spleen of chickens at 21 d of age, but the level of AA was decreased by the first LPS stimulation at 21 d of age (Table 2). Among the four groups, chickens fed fish oil diet had the highest levels of EPA and DHA in spleen after the second LPS stimulation.

Table 2. Effects of feeding different oils and lipopolysaccharide stimulation on polyunsaturated fatty acids composition of splenocytes in chickens, mol%

Item			LA C18:2	AA C20:4	EPA C20:5	DHA C22:6
21 d	Maize oil	saline	6.36	24.62	0.28	0.94
		LPS	6.13	22.48	0.28	0.95
	Fish oil	saline	6.29	25.18	0.28	0.98
		LPS	6.34	20.86	0.30	1.00
	SEM[1]		0.09	0.48	0.003	0.01
P	oils		0.7119	0.4490	0.2533	0.0807
	mitogen		0.6414	0.0002	0.1353	0.4102
	interaction		0.4520	0.1319	0.3808	0.7917
28 d	Maize oil	saline	7.01	25.70	0.31	0.98
		LPS	5.67	23.35	0.37	1.01
	Fish oil	saline	6.80	22.19	0.41	1.04
		LPS	5.42	18.80	0.51	1.25
SEM			0.19	0.57	0.02	0.03
P	oils		0.4193	<0.0001	<0.0001	<0.0001
	mitogen		<0.0001	<0.0001	<0.0001	<0.0001
	interaction		0.9365	0.3029	0.0118	0.0012

[1] SEM - pooled standard error

Membrane fluidity of splenocytes

The effects of diets supplemented with different oils on membrane fluidity in splenocytes membrane of chicken stimulated with LPS were investigated by measuring FP (Table 3). FP is inversely correlated to membrane fluidity. After the first and second LPS stimulation, FP of splenocyes membrane of chickens on 21 and 28 d of age was significantly lowered in response to LPS stimulation. No significant differences in FP of splenocytes membranes were observed between chickens consuming fish-oil diets and chickens consuming maize oil.

Table 3. Effects of different oils and lipopolysaccharide stimulation on membrane fluidity of splenocytes in chickens

Item			Fluorescence polarization (P)	
			21 d	28 d
Maize oil		saline	0.31 ± 0.01	0.33 ± 0.01
		LPS	0.23 ± 0.01	0.24 ± 0.01
Fish oil		saline	0.32 ± 0.002	0.34 ± 0.01
		LPS	0.24 ± 0.01	0.25 ± 0.01
P	oils		0.2224	0.1472
	mitogen		<0.0001	<0.0001
	interaction		0.8476	0.9090

data are expressed as means ± SE

PLA_2, COX activities and PGE_2 production

There were no significant differences in activities of PLA, COX and COX_1 in spleen of chickens on 21 d and 28 day of age between groups consuming diets supplemented with fish oil and maize oil (Table 4). Both the first and second LPS stimulation increased the activities of PLA and COX. Meanwhile chickens given fish oil compared with those given maize oil had lower activities of COX. There were significant interactions on COX_2 activities and PGE_2 production between dietary oils and LPS stimulation in chickens. Fish oils ameliorated the increasing of COX_2 activities and PGE_2 production in spleen of chickens stimulated with LPS.

Table 4. Effects of dietary oils and LPS stimulation on spleen PLA2, COX and its metabolite in chickens

Item		PLA_2, U/mg		PGE_2, pg/mg		COX, a. u.		COX_1, a. u.		COX_2, a. u.	
		21 d	28 d	21 d	28 d	21 d	28 d	21 d	28 d	21 d	28 d
Maize oil	saline	0.17	0.17	165.95	138.51	15.11	14.92	13.84	13.54	1.26	1.38
	LPS	0.21	0.24	282.75	255.33	17.93	18.50	14.19	14.50	3.74	4.00
Fish oil	saline	0.15	0.15	59.36	56.13	15.23	15.36	13.78	14.02	1.45	1.33
	LPS	0.20	0.22	103.03	114.59	15.72	18.63	13.59	14.50	2.12	3.35
SEM		0.60	0.01	0.01	17.03	0.39	0.42	0.29	0.22	0.21	0.25
P											
oils		0.1248	0.2440	<0.0001	<0.0001	0.1243	0.5528	0.5982	0.5924	<0.0001	0.0009
mitrogen		<0.0001	0.0001	0.0002	<0.0001	0.0196	<0.0001	0.8992	0.1264	<0.0001	<0.0001
interaction		0.6995	0.6444	0.0415	0.0025	0.0877	0.7560	0.6716	0.5966	<0.0001	0.0031

SEM - pooled standard error. Data of COX activity indicated as fluorescence intensity; PLA_2 - phospholipase A2; PGE_2 - prostaglandin E_2; COX - cyclooxygenase

SMase activities in spleen

The effects of dietary different types of oils on SMase activities in spleen of chickens stimulated by LPS were shown in Table 5. Dietary oils did not significantly affect activities of SMase in spleen of chickens on 21 and 28 day of age. One day after the first and second LPS stimulation, the activities of acid SMase of membrane and plasma in spleen were significantly higher in chickens stimulated with LPS than that in chickens treated with saline. However, there was no significant interaction on the acid SMase activities in spleen of chickens between LPS stimulation and dietary oils. One d after the first LPS stimulation,

Table 5. Effects of feeding different oils and lipopolysaccharide stimulation on SMase activities of spleen in chickens, nmol/min mg

Item		Membrane acid		Plasma acid		Membrane neutral		Plasma neutral	
		SMase							
		21d	28d	21d	28d	21d	28d	21d	28d
Maize oil	saline	3.36	3.89	6.05	6.78	3.41	3.76	6.56	5.82
	LPS	3.86	4.78	6.93	7.44	3.78	4.27	6.52	8.73
Fish oil	saline	3.27	3.92	6.21	6.14	3.15	3.96	6.02	6.51
	LPS	3.77	4.37	6.37	7.07	3.65	4.18	6.89	7.86
SEM		0.08	0.16	0.11	0.20	0.08	0.15	0.20	0.41
P	oils	0.4807	0.5290	0.2882	0.1969	0.2057	0.8554	0.8400	0.9045
	mitogen	0.0006	0.0394	0.0099	0.0474	0.0063	0.2597	0.3109	0.0077
	interaction	1.0000	0.4721	0.0627	0.7290	0.6510	0.6476	0.2678	0.2903

SEM - pooled standard error. SM_{ase} - sphingomyelinases

the activities of membrane neutral SMase in spleen were significantly higher in chickens stimulated with LPS than that in chickens treated with saline. However, one day after the second LPS stimulation, the activities of plasma neutral SMase in spleen were significantly increased. There was no significant interaction on the neutral SMase activities in spleen of chickens between LPS stimulation and dietary oils.

Membrane PLC activities in spleen

Membrane Ca^{2+} - independent PLC activities in spleen between chickens given maize oil and fish oil were not significantly different on 21 and 28 day of age (Table 6). LPS stimulation did not significantly affect the activities membrane Ca^{2+} - independent PLC. There were significant interactions on membrane Ca^{2+} -dependent PLC in the spleen between dietary oils and LPS stimulation in chickens. Fish oils decreased the increasing activity of membrane Ca^{2+} - dependent PLC in the spleen of chickens after the first and second LPS stimulation.

Table 6. Effects of feeding different oils and lipopolysaccharide stimulation on PLC activities of spleen in chickens

Item		Membrane Ca^{2+} - dependent PLC		Membrane Ca^{2+} - independent PLC	
		nmol/min mg			
		21 d	28 d	21 d	28 d
Maize oil	saline	11.18	12.44	24.64	23.66
	LPS	15.17	14.84	24.56	23.71
Fish oil	saline	13.61	12.64	24.76	24.18
	LPS	12.86	12.90	24.36	23.80
SEM		0.35	0.28	0.12	0.16
P	oils	0.8704	0.0562	0.8732	0.3745
	mitogen	<0.0001	0.0056	0.3320	0.5261
	interaction	0.0004	0.0216	0.5186	0.6319

data in the same column with different superscripts differ significantly ($P<0.05$); PLC - phospholipase C

IP_3 levels of splenocytes

No significant differences in splenocytes of chickens on 21 and 28 day of age were observed between groups given maize oil and fish oil (Table 7). After the first and second LPS stimulation, chickens on 21 and 28 day of age had higher levels of IP_3 in splenocytes than those of chickens injected by saline. There were significant interactions on IP_3 production between dietary oils and LPS stimulation in chickens. Fish oils attenuated the increase of IP_3 production in splenocytes of chickens stimulated with LPS.

Table 7. Effects of feeding different oils and lipopolysaccharide stimulation on IP_3 production of splenocytes in chickens, IP_3, pmol/mg

Item		21 day	28 day
Maize oil	saline	5.71 ± 0.49	5.88 ± 0.39
	LPS	9.12 ± 0.24	9.09 ± 0.13
Fish oil	saline	6.11 ± 0.39	6.17 ± 0.13
	LPS	8.07 ± 0.12	8.41 ± 0.05
P	oils	0.3574	0.3899
	mitogen	<0.0001	<0.0001
	interaction	0.0495	0.0388

data in the same column with different superscripts differ significantly ($P<0.05$); IP_3 - inositol triphosphate

NFκB gene expression of spleen

There was an interaction between LPS stimulation and dietary oil (P=0.0079 for the first stimulation; P=0.0017 for the second stimulation) for the mRNA abundance of $NF_κB$ (Table 8). Among chickens stimulated with LPS, those fed fish oil diets had lower mRNA abundance of $NF_κB$ than those fed maize oil diets.

Table 8. Effects of dietary oils and lipopolysaccharide stimulation on $NF_κB$ mRNA of spleen in chickens

Item		Nuclear factor kappa B ($NF_κB$)	
		21 day	28 day
Maize oil	saline	0.15 ± 0.01	0.16 ± 0.01
	LPS	0.26 ± 0.01	0.24 ± 0.01
Fish oil	saline	0.16 ± 0.01	0.18 ± 0.01
	LPS	0.22 ± 0.01	0.21 ± 0.01
P	oils	0.2084	0.5363
	mitogen	<0.0001	<0.0001
	interaction	0.0079	0.0017

SEM - pooled standard error

Correlation analysis

Table 9 showed the correlation between COX_2 and PGE_2, PLC and IP_3 in spleen of chickens on 21d and 28 d of age. A good correlation existed between COX_2 and PGE_2 (R^2=0.791; P<0.05, 21d of age; R^2=0.756; P<0.05, 28 d of age). And there was a good correlation between IP_3 productivity and membrane Ca^{2+} -dependent PLC activity.

Table 9. Correlation analysis among parameters

Item		21 day		Item		28 day	
		COX_2	MPLC			COX_2	MPLC
21 day	PGE_2	0.791*		28 day	PGE_2	0.756*	
	IP_3		0.753*		IP_3		0.706*

MPLC and PPLC indicated as membrane phospholipase C and plasma phospholipase C respectively. An asterisk (*) indicates a significant correlation P<0.05. PGE_2 - prostaglandin E_2; COX_2 - cyclooxygenase 2. IP_3 is the abbreviation of inositol triphosphate

DISCUSSION

In our study, there was no significant interaction in splenocytes fatty acids profile between the first LPS injection and fish oil or maize oil, but LPS stimulation had dramatic effects on splenocytes AA in chickens (Table 2). This result goes against previous studies (Selvaraj and Cherian, 2004; Wiesenfeld et al., 2005). The effects of dietary oils on changes of fatty acids composition of splenocytes was not greatly significant compared with the culturing lymphocytes in the medium supplemented with fatty acids (Yaqoob et al., 1995). It was a pity that we can not explain it commendably. Our future studies will investigate what could lead to the phenomena. The second LPS stimulation decreased the proportions of LA and AA, whereas there was significant interaction in the proportions of EPA and DHA of splenocytes in chickens on 28 d of age. And maize oil inhibited the increasing proportions of DHA and EPA in spleen of chickens stimulated with LPS. The result suggested that dietary fish oils increase the proportions of n-3 PUFA (DHA and EPA) of splenocytes in chicken stimulated by LPS. Some studies showed that the increasing availability of n-3 PUFA in the diets (fish oil, as a good source of n-3 PUFA) played an important role in ameliorating inflammatory immune responses (Korver and Klasing, 1997; Korver et al., 1998; Gaines et al., 2003; Liu et al., 2003; Yang et al., 2006).

In the present study, we found that LPS stimulation increased the membrane fluidity of splenocytes in chickens (Table 3). These effects have been observed previously in the other study (Calder et al., 1994). Altered fatty acids composition

might be expected to influence immune cell function. Changes of fatty acid composition in membrane of splenocytes led to changes of membrane fluidity. Supplementation of the culture medium with particular fatty acids (AA, DHA and EPA) led to the increasing of membrane fluidity of lymphocytes (Calder et al., 1994). However, the effects were not observed in our study (Table 3). The present study indicated that fish oil would not change the membrane fluidity in splenocytes of chickens at inflammation status. The fatty acid composition changes of splenocytes membrane resulted from diet in our study were less extreme than those changes seen in cell culture (Yaqoob et al., 1995). We cannot explain this result, and we will investigate the possible reason. No significant interactions between fish oil and LPS on membrane fluidity of splenocyte indicated that fish oil would not modulate immune inflammatory of chickens stimulated with LPS through changing the membrane fluidity of splenocytes.

Our present study showed that dietary fish oil did not significantly affect the PLA_2 activity, but effectively inhibited the COX_2 activity and PGE_2 production of spleen in chickens stimulated with LPS (Table 4). From our study, we find a good correlation between COX_2 and PGE_2 (R^2=0.791, $P<0.05$, 21 d of age; R^2=0.756, $P<0.05$, 28 d of age). In addition, the correlation between COX_2 activity and PGE_2 production of spleen on 21 and 28 d of age indicated that COX_2 played a key role in PGE_2 production (Table 9). Similar to the result, Arntzen et al. (1998) found that after supplementing with n-3 PUFA, PGE_2 were significantly reduced in decidual cell stimulated by LPS. Whereas, supplementing with n-6 PUFA, there was a significant increase in PGE_2 of decidual cell stimulated by LPS. The result is consistent with our previous findings in laying hens too (Guo et al., 2004). Dietary PUFA affects immune cellular functions not only by themselves but also by metabolites, such as PGE_2 (He et al., 2007). Usually, the highly abundant AA is liberated from membrane phospholipids by PLA_2 to be used for eicosanoid synthesis. AA metabolized through COX gives rise to PGE_2. Li and Watkins (1998) reported that dietary fish oil reduced *ex vivo* PGE_2 biosynthesis in rats fed diets rich in n-3 PUFA. n-3 PUFA showed suppression of COX_2 expression and PGE_2 production in the cultured murine monocytic cells when stimulated *in vitro* with LPS (Lee et al., 2003). Based on these studies, it is crucial for the lower PGE_2 production of spleen in chickens stimulated with LPS that fish oil inhibits COX_2 activity. It may be that one way fish oil exerts its beneficial effects on inflammatory responses of chickens stimulated with LPS is by lessening of inflammatory mediators, such as PGE_2.

In our present study, LPS stimulation increased SMase activity of spleen in chickens. However, fish oil did not ameliorate the increasing of SMase activity (Table 5). No significant interactions between fish oil and LPS on SMase of splenocyte indicated that fish oil did not modulate inflammatory response of

chickens through changing the SMase activity of splenocytes. Signaling through the sphingomyelin pathway is ubiquitous and evolutionarily conserved, ceramide may paly a role as a second messenger in intracellular signal transduction. The ceramaide generation is *via* SMase (Pena et al., 1997). The outcome of signaling through this pathway is apoptosis of lymphocytes. The signaling pathway for ceramide involves the action of SMase, sphingomyelin-specific forms of PLC, which hydrolyses the phosphodiester bond of sphingomyelin, a phospholipid preferentially found in the plasma membrane of mammalian cells yielding ceramide and phosphorylcholine (Kolesnick and Martin, 1998). Receptors as distinct as those for TNFα, IL-1 signal *via* the sphingomyelin pathway following ligand binding, which gives rise to inflammatory responses (Pushkareva et al., 1995; Spiegel et al., 1996).

In our work, we evaluated the participation of the PLC activity and IP_3 production in the activation of splenocytes in chickens stimulated by LPS (Tables 6 and 7). Phosphatidylcholine specific PLC is able to hydrolyse phosphatidylinositol-4,5-bisphosphate (PIP_2), then produce the DAG necessary for PKC activation in several signaling receptors and concomitant production of IP_3 for Ca^{2+} mobilization. Recent data have suggested that several cytokine receptors as well as other cell surface receptors can mediate DAG-dependent PKC activation without activation of phosphoinositide-PLC isoforms (PLC-γ_2), IP_3 production in female BALB/c mice (Moreno-García et al., 2005). Whereas, our data demonstrated that LPS stimulation mediated its crucial activity of spleen in chickens that leads directly to the activation of PC-PLC and IP_3 production. While it is clear that the PLC signal is absolutely involved in inflammatory responses in cultured murine lymphocytes (Kolesnick and Martin, 1998). From our study, we found that fish oil attenuated the PLC activity and IP_3 production in spleen of LPS-injected chickens. Meanwhile, there was a good correlation between IP_3 productivity and membrane Ca^{2+}-dependent PLC activity (Table 9). These events indicated that fish oil would ameliorate immune inflammatory through changing the PLC activity and IP_3 production in splenocytes of chickens stimulated with LPS. Kumar and Chakrabarti (2000) reported that Ca^{2+} mobilization initialized by IP_3 was necessary to activation of PKC, which participated the spleen T lymphocyte proliferation stimulated by Con A. Fish oil decreased IP_3 production in splenocytes of chickens, which would inhibited intracellular Ca^{2+} mobilization. And inhibition of intracellular Ca^{2+} mobilization down-regulated Ca^{2+}-dependent PLC activity that it was involved with production of IP_3 again.

Meanwhile, our data demonstrated that there were significant interactions between fish oil and LPS stimulation on NFκB gene expression in spleen of chickens at 21 and 28 d of age. And fish oil ameliorated the increase of $NF_{\kappa}B$ mRNA in chickens stimulated with LPS (Table 8). It is possible that fish oil

ameliorates inflammatory responses in chickens stimulated with LPS through attenuating $NF_{\kappa}B$ gene transcription, the similar results was seen in piglets (Liu et al., 2003). In a previous study, EPA prevented LPS-induced TNFα expression by preventing NFκB activation in cultured monocytic THP-1 cells (Zhao et al., 2004). Similarly, RAW cells grown in EPA-rich media had less TNF mRNA expression and a decreased activation of the $NF_{\kappa}B$ (Lo et al., 1999). Several proinflammatory cytokines, such as TNFα, IL-1, IL-6, are encoded by target genes of NFκB-activation pathway. PKC-dependent degradation of IκB result in translocation of NFκB into the nucleus, which give rise to the production of the pro-inflammatory cytokines (Karin and Greten, 2005). The stimuli including inflammatory cytokines and immune-related stress such as LPS lead to the activation of NFκB, which can accelerate expression of COX_2, PLA_2, proinflammatory cytokines and acute phase response protein, etc. (Garg and Aggarwal, 2002; Bonizzi and Karin, 2004).

CONCLUSIONS

The results of these experiments give insight into a potential dietary method to ameliorate immune inflammatory responses that might occur during infectious stimulation. The signaling of immunomodulatory nutrients such as fish oil in inflammation is that PLC and COX_2 activity decreased by fish oil inhibited IP_3 and PGE_2 production, respectively. These results showed that fish oil acted as an anti-inflammatory agent, which may be associated with down-regulation of production of IP_3 and PGE_2, respectively. The results of NFκB gene expression suggested fish oil might alleviate immune stress at the level of transcription.

REFERENCES

Arntzen K.J., Brekke O.L., Vatten L., Austgulen R., 1998. Reduced production of PGE2 and PGF2a from decidual cell cultures supplemented with N-3 polyunsaturated fatty acids. Prostagland. Other Lipid Mediators 56, 183-195

Bonizzi G., Karin M., 2004. The two NFκB activation pathways and their role in innate and adaptive immunity. Trends Immunol. 25, 280-288

Calder P.C., Field C.J., 2002. Fatty acids, inflammation and immunity. In: P.C. Calder, C.J. Field, H.S. Gill (Editors). Nutrition and Immune Function. Cambridge. CABI Publishing, pp. 57-92

Calder P.C., Yaqoob P., Harvey D.J., Watts A., Newsholme E.A., 1994. Incorporation of fatty acids by lymphocytes and the effect on fatty acid composition and membrane fluidity. Biochem. J. 300, 509-518

Gaines A.M., Carroll J.A., Yi G.F., Allee G.L., Zannelli M.E., 2003. Effect of menhaden fish oil supplementation and lipopolysaccharide exposure on nursery pigs: II. Effects on the immune axis when fed simple or complex diets containing no spray-dried plasma. Domest. Anim. Endocrinol. 24, 353-365

Garg A., Aggarwal B.B., 2002. Nuclear transcription factor-KB as a target for cancer drug development. Leukemia 16, 1053-1068

Gilmore T.D., 2006. Introduction to NF-κB: players, pathways, perspectives. Oncogene 25, 6680-6684

Giraud M.N., Motta C., Boucher D., Grizard G., 2000. Membrane fluidity predicts the outcome of cryopreservation of human spermatozoa. Hum. Reprod. 15, 2160-2164

Guo Y.M., Chen S.Y., Xia Z.G., Yuan J.M., 2004. Effects of different types of polyunsaturated fatty acids on immune function and PGE2 synthesis by peripheral blood leukocytes of laying hens. Anim. Feed Sci. Tech. 116, 249-257

He X., Zhang H.J., Yang X.J., Zhang S., Dai Q.Z., Xiao W.J., Ren G.P., 2007. Modulation of immune function by conjugated linoleic acid in chickens. Food Agric. Immunol. 18, 169-178

Karin M., Greten F.R., 2005. NFκB: Linking inflammation and immunity to cancer development and progrssion. Nature Rev. Immunol. 5, 749-759

Kolesnick R.N., Martin K., 1998. Regulation of ceramide production and apoptosis. Annu. Rev. Physiol. 60, 643-665

Korver D.R., Klasing K.C., 1997. Dietary fish oil alters specific and inflammatory immune responses in chicks. J. Nutr. 127, 2039-2046

Korver D.R., Roura E., Klasing K.C., 1998. Effects of dietary energy and oil source on broiler performance and response to an inflammatory challenge. Poultry Sci. 77, 1217-1227

Kramer J.K.G., Zhou J.Q., 2001. Analytical techniques for conjugated linoleic acid (CLA) analysis. Eur. J. Lipid Sci. Technol. 103, 594-632

Kumar S., Chakrabarti R., 2000. [8-(diethylamino) octyl -3,4,5-trimethoxybenzoate, HCl], the inhibitor of intracellular calcium mobilization, blocked mitogen-induced T cell proliferation by interfering with the sustained phase of protein kinase C activation. J. Cell. Biochem. 76, 539-547

Lai C.H., Yin J.D., Li D.F., Zhao L.D., Qiao S.Y., Xing J.J., 2005. Conjugated linoleic acid attenuates the production and gene expression of proinflammatory cytokines in weaned pigs challenged with lipopolysaccharide. J. Nutr. 135, 239-244

Lee J.Y., Plakidas A., Lee W.H., Heikkinen A., Chanmugam P., Bray G., Hwang D.H., 2003. Differential modulation of Toll-like receptors by fatty acids: Preferential inhibition by n-3 polyunsaturated fatty acids. J. Lipid Res. 44, 479-486

Li Y., Watkins B.A., 1998. Conjugated linoleic acids alter bone fatty acid composition and reduce ex vivo prostaglandin E-2 biosynthesis in rats fed n-6 or n-3 fatty acids. Lipids 33, 417-425

Liu Y.L., Li D.F., Gong L.M., Yi G.F., Gaines A.M., Carroll J.A., 2003. Effects of fish oil supplementation on the performance and the immunological, adrenal, and somatotropic responses of weaned pigs after an *Escherichia coli* lipopolysaccharide challenge. J. Anim. Sci. 81, 2758-2765

Lo C.J., Chiu K.C., Fu M., Lo R., Helton S., 1999. Fish oil decreases macrophage tumor necorsis factor gene transcription by altering the NF-κB activity. J. Surg. Res. 82, 216-221

McMurray D.N., Jolly C.A., Chapkin R.S., 2000. Effects of dietary n-3 fatty acids on T cell activation and T cell receptor-mediated signaling in a murine model. J. Infec. Dis. 182, S103-S107

Meng D.S., Wang F.J., Wang S.L., 2003. Effects of quercetin on metabolism of cyclooxygenase in IEC-6 cells stimulated by the erum of burned rats. Acta Acad. Med. Militaris Tertiae 25, 1345-1348

Moreno-García M.E., López-Bojórques L.N., Zentella A., Humphries L.A., Rawlings D.J., Santos-Argumedo L., 2005. CD38 signaling regulates B lymphocyte activation via a phospholipase C (PLC)-γ2-independent, protein kinase C, phosphatidylcholine-PLC, and phospholipase D-dependent signaling cascade. J. Immunol. 174, 2687-2695

Pena L.A., Fuksf Z., Kolesnick R., 1997. Stress-induced apoptosis and the sphingomyelin pathway. Biochem. Pharmacol. 5, 615-621

Perkins N.D., 2007. Integrating cell-signalling pathways with NF-κB and IKK function. Nature Rev. Mol. Cell Biol. 8, 49-62

Pushkareva M., Obeid L.M., Hannun Y.A., 1995. Ceramide: an endogenous regulator of apoptosisi and growth suppression. Immunol. Today 16, 294-297

Reynolds L.J., Hughes L.L., Dennis E.A., 1992. Analysis of human synovial fluid phospholipase A2 on short chain phosphatidylcholine-mixed micelles: Development of a spectrophotometric assay suitable for a microtiterplate reader. Anal. Biochem. 204, 190-197

SAS, 2001. SAS/STAT User´s Guide. Release 8.2. Stat. Anal. Syst. SAS Institute Inc. Cary, NC

Selvaraj R.K., Cherian G., 2004. Dietary *n*-3 fatty acids reduce the delayed hypersensitivity reaction and antibody production more than *n*-6 fatty acids in broiler birds. Eur. J. Lipid Sci. Technol. 106, 3-10

Sijben J.W., Schrama J.W., Parmentier H.K., van der Poel J.J., Klasing K.C., 2001. Effects of dietary polyunsaturated fatty acids on in vivo splenic cytokine mRNA expression in layer chicks immunized with Salmonella typhimurium lipopolysaccharide. Poultry Sci. 80, 1164-1170

Simmons D.L., Botting R.M., Hla T., 2004. Cyclooxygenase isozymes: the biology of prostaglandin synthesis and inhibition. Pharmacol. Rev. 56, 387-437

Spiegel S., Foster D., Kolesnick R., 1996. Signal transduction through lipid second messengers. Curr. Opin. Cell Biol. 8, 159-167

Stoscheck C.M., 1990. Quantitation of protein. Methods Enzymol. 182, 50-69

Tapiero H., Ba G.N., Couvreur P., Tew K.D., 2002. Polyunsaturated fatty acids (PUFA) and eicosanoids in human health and pathologies. Biomed. Pharmacotherapy 56, 215-222

Wiesenfeld P.L., Babu U.S., Raybourne R.B., Gaines D., O'Donnell M., Myers M.J., 2005. Effect of dietary fish-meal on chicken serum, liver and spleen fatty acid metabolism. Int. J. Poultry Sci. 4, 728-733

Wu X.Z., Lu H., Zhou L., Huang Y., Chen H., 1997. Changes of phosphatidylcholine-specific phospholipase C in hepatocarcinogenesis and in the proliferation of rat liver cancer cells. Cell Biol. Int. 21, 375-381

Yang X., Guo Y., He X., Yuan J., Yang Y., Wang Z., 2008. Growth performance and immune responses in chickens after challenge with lipopolysaccharide and modulation by dietary different oils. Animal 2, 216-223

Yang X., Guo Y., Wang Z., Nie W., 2006. Effects of dietary supplementation with different oils on coccidiosis in chickens. Avian Pathol. 35, 373-378

Yaqoob P., Newsholme E.A., Calder P.C., 1995. Influence of cell culture conditions on diet-induced changes in lymphocyte fatty acid composition. Biochim. Biophys. Acta 1255, 333-340

Zhao Y., Joshi-Barve S., Barve S., Chen L.H., 2004. Eicosapentaenoic acid prevents LPS-induced TNF-α expression by preventing NF-κB activation. J. Amer. Coll. Nutr. 23, 71-78