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Modulation of macrophage cytokine production by conjugated linoleic acids is influenced by the dietary n-6:n-3 fatty acid ratio

John J. Turek, Yong Li,* Ingrid A. Schoenlein, Ken G.D. Allen,† and Bruce A. Watkins*

*Department of Basic Medical Sciences, School of Veterinary Medicine, and *Department of Food Science, Lipid Chemistry and Metabolism Laboratory, Purdue University, W. Lafayette, IN USA; and †Department of Food Science and Human Nutrition, Colorado State University, Ft. Collins, CO USA*

Conjugated linoleic acids (CLA) are a mixture of 18:2 isomeric fatty acids that have conjugated double bonds. We undertook this study to determine if CLA altered spleen fatty acid composition, macrophage cytokine production, and liver prostaglandin E_2 (PGE_2) biosynthesis in the presence of varying ratios of (n-6) and (n-3) polyunsaturated fatty acids (PUFA). Groups of six rats were fed diets (AIN-93G) containing soybean oil (SBO) or menhaden oil and safflower oil (MSO), with CLA (10 g/kg) or without CLA. Spleens of rats consuming CLA contained the t9,c11 and t10,c12 isomers of CLA and a lower content of 18:1 fatty acids. CLA significantly reduced basal and lipopolysaccharide (LPS) induced levels of interleukin-6 (IL-6) by resident peritoneal macrophages in rats given SBO. CLA also reduced basal levels of tumor necrosis factor (TNF) production in both diets, but had no effect on LPS-induced levels of TNF. CLA had no effect on IL-1 production, but macrophage IL-1 production was increased in MSO fed rats compared with SBO fed rats. Rats given MSO had reduced PGE_2 production in spleen compared with SBO fed rats. In SBO fed rats, CLA caused some reduction in PGE_2, but the effect was not significant. The effect of CLA on PGE_2 production may be dependent on the type of dietary fat consumed and the organ being studied. (J. Nutr. Biochem. 9:258–266, 1998) *© Elsevier Science Inc. 1998*

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Introduction

Conjugated linoleic acids (CLA) are an isomeric mixture of 18:2 fatty acids that have conjugated double bonds. These isomers occur naturally and are enriched in the tissues of ruminant animals[1] and in dairy products.[2] CLA has been shown to inhibit benz(a)pyrene-induced forestomach cancer in mice,[3] to suppress mammary tumors in rats,[4] and to inhibit cancer cell proliferation in vitro.[5] These isomers also have been shown to protect against the catabolic effects of endotoxin.[6] The isomers also have some antioxidant properties,[3] but the antioxidant effect may be due to a metabolite or oxidized form of the fatty acids because CLA is just as susceptible to oxidation as linoleic acid.[7] A unique property of CLA is that it appears to function as a growth factor and some animals demonstrate improved feed efficiency and weight gain.[8] Additionally, CLA has also been shown to be antihypercholesterolemic and antiatherogenic in rabbits.[9] There have been a few studies on the effects of CLA on immune function. Rats fed a diet containing 0.5% CLA had enhanced macrophage phagocytosis and foot pad swelling in response to phytohemagglutinin.[10] Mice fed 0.3% and 0.9% CLA had increased in vitro lymphocyte proliferation in response to phytohemagglutinin but not concanavalin A or lipopolysaccharide (LPS). Interleukin-2 (IL-2) production in these mice was also was stimulated by CLA.[11]

Some of the properties of CLA are similar to those of (n-3) polyunsaturated fatty acids (PUFA). PUFA of the

Address correspondence to Dr. John J. Turek, Purdue University, School of Veterinary Medicine, Department of Basic Medical Sciences, 1246 Lynn Hall, G193C, West Lafayette, IN 47907-1246 USA.
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(n-3) family may reduce cardiovascular risk factors,[12] inhibit tumor growth,[13] and attenuate the effects of endotoxin.[14] Eicosapentaenoic acid (20:5n-3) competes with arachidonic acid for the cyclooxygenase enzyme,[15] which decreases the production of 2-series prostanoids such as prostaglandin E_2 (PGE_2). PGE_2 suppresses T-lymphocyte proliferation and cytotoxic activities of natural killer cells[16,17] and inhibits the production of tumor necrosis factor-α (TNF-α)[18] and IL-12.[19] Regulation of PGE_2 is one mechanism whereby (n-3) PUFA are able to affect cytokine production by inflammatory cells.[20,21] A recent study showed that CLA is elongated and desaturated to 20:4 isomers.[22] These isomers might compete with arachidonic acid for cyclooxygenase enzyme and lower PGE_2 in a manner similar to (n-3) PUFA. To determine if CLA altered cytokine and eicosanoid production similar to (n-3) PUFA, the extent of CLA incorporation into a lymphoid organ (spleen) was determined by fatty acid analysis, and peritoneal macrophage TNF, IL-1, and IL-6, and liver homogenate PGE_2 biosynthesis was measured from rats fed varying ratios of (n-6) and (n-3) PUFA with and without CLA.

Materials and methods

Reagents

All reagents used in the experiments were from Sigma Chemical Co. (St. Louis, MO) unless specified otherwise.

Rats and diets

Groups of six male weanling Sprague-Dawley rats were fed an AIN-93G diet containing 70 g/kg of added fat for 42 d. The dietary treatments followed a factorial design consisting of soybean oil [SBO; (n-6):(n-3) ratio of 7.3:1] or menhaden oil plus safflower oil [MSO; (n-6):(n-3) ratio of 1.8:1] and two levels of CLA [0 and 1% (10g/kg diet)]. The CLA was added at the expense of the SBO or MSO treatments. The CLA was donated by Kraft Foods, Inc. (Glenview, IL) and was obtained from Nu-Chek Prep (Elysian, MN). The fatty acid composition of the diets is shown in *Table 1*.

Table 1 Fatty acid (mol/100 mol) and ingredient composition of the diets given to rats[1]

Fatty acid	Dietary lipid treatments[2]			
	SBO	SBO + CLA	MSO	MSO + CLA
12:0 (lauric)	TR	TR	0.08	0.03
14:0 (myristic)	0.20	0.19	3.64	3.07
15:0 (pentadecylic)	ND	ND	0.34	0.29
16:0 (palmitic)	10.32	8.80	12.19	10.23
16:1n-7 (palmitoleic)	0.09	TR	4.83	4.05
17:0 (margaric)	0.07	TR	0.41	0.35
18:0 (stearic)	4.63	3.96	3.11	2.60
18:1 (octadecenoic)	23.18	19.75	12.25	10.47
18:2n-6 (linoleic)	52.84	45.33	36.67	32.38
18:3n-3 (α-linolenic)	7.26	6.24	1.03	0.85
18:4n-3 (stearidonic)	ND	ND	1.89	1.64
18:2(*c*9,*t*11/*t*9,*c*11)	ND	6.52	ND	7.11
18:2(*t*10,*c*12)	ND	6.84	ND	7.34
18:2(*c*9,*c*11/*c*10,*c*12)	ND	0.55	ND	TR
18:2(*t*9,*t*11/*t*10,*t*12)	ND	0.42	ND	0.43
20:0 (arachidic)	0.35	0.34	0.27	TR
20:1n-9 (gondoic)	0.16	ND	0.73	0.65
20:4n-6 (arachidonic)	ND	ND	0.42	0.35
20:5n-3	ND	ND	8.02	6.72
22:0 (behenic)	0.47	0.42	TR	TR
22:5n-3	ND	ND	1.51	1.27
22:6n-3	ND	ND	8.57	7.20
SAT	16.04	13.71	20.04	16.57
MONO	23.44	19.75	17.81	15.17
PUFA	60.09	51.57	58.10	50.41
n-6 PUFA	52.84	45.33	37.09	32.73
n-3 PUFA	7.26	6.24	21.01	17.67
(n-6):(n-3)	7.28	7.27	1.77	1.85

[1]The semipurified basal diet contained the following (g/kg): casein, 200; corn starch, 397.486; DYETROSE, 132; sucrose, 100; cellulose, 50; L-lysine, 3; choline bitartrate, 2.5; salt mix, 35; vitamin mix, 10. Salt mix provided (mg/kg diet): $CaCO_3$, 12495; K_2HPO_4, 6860; $C_6H_5O_7K_3 \cdot H_2O$, 2477; NaCl, 2590; K_2SO_4, 1631; MgO, 840; $C_6H_5O_7Fe$, U.S.P., 212.1; $ZnCO_3$, 57.75; $MnCO_3$, 22.05; $CuCO_3$, 10.5; KIO_3, 0.35; Na_2SeO_4, 0.359; $(NH_4)_2MoO_4 \cdot H_2O$, 0.278; $Na_2O_3Si \cdot 9H_2O$, 50.75; $CrK(SO_4)_2 \cdot 12H_2O$, 9.625; LiCl, 0.609; H_3BO_3, 2.853; NaF, 2.223; $NiCO_3$, 1.113; NH_4VO_3, 0.231. Vitamin mix provided (mg/kg diet): thiamine HCl, 6; riboflavin, 6; pyridoxine HCl, 7; niacin, 30; calcium pantothenate, 16; folic acid, 2; biotin, 0.2; cyanocobalamin (B-12) (0.1%), 25; vitamin A palmitate (500,000 IU/g), 4000; vitamin E acetate (500 IU/g), 75; vitamin D_3, 1000; vitamin K_1, 0.75.
[2]Dietary treatments included soybean oil (SBO) or menhaden oil + safflower oil (MSO), with (+) or without (−) conjugated linoleic acids (CLA). Total fat content in each diet was 70 g/kg of diet and CLA was added (10 g/kg of diet) at the expense of SBO or MSO.
TR, trace amount; ND, not detected; SAT, total saturated fatty acids; MONO, total monounsaturated fatty acids; PUFA, total polyunsaturated fatty acids not including CLA.

Collection and culturing of macrophages

Rats were sacrificed via carbon dioxide (CO_2) inhalation. The skin covering the abdominal cavity was removed, an incision made, and the peritoneal cavity lavaged with sterile calcium and magnesium free Hank's balances salt solution (HBSS). The peritoneal cells were pelleted by centrifugation, the supernatant fluid discarded, and the cells resuspended in a control medium (CM) of RPMI 1640 containing 5 mg/ml bovine serum albumin (BSA), 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% L-glutamine. Cells were counted in a Coulter counter (Coulter Corporation, Hialeah, FL), and 1×10^6 cells in 7 ml of CM plated in 60 mm well plates and cultured for 90 min in a humidified CO_2 incubator at 37°C. The nonadherent cells were washed off with CM, and the remaining adherent macrophages cultured for 24 h in CM, which contained 0.01 g/L of O55:B5 *Escherichia coli* lipopolysaccharide (LPS), CM + 10 μM PGE_2, CM + 5nM leukotriene B_4 (LTB_4), CM + LPS + PGE_2, and CM + LPS + LTB_4. After 24 h the cell free supernatant fluid was collected and the adherent cells washed in HBSS and digested with 1 mol/L ammonium hydroxide and 0.2% Triton X-100 for total cellular DNA quantitation.[23]

Fatty acid analysis

Since the spleen is a major source of macrophages, the fatty acid composition of this organ was determined to ascertain the extent of CLA incorporation into a lymphoid organ. The fatty acid analysis of the spleen followed a method described previously.[24] Briefly, total lipids were extracted with chloroform/methanol (2:1, vol/vol) and saponified, and fatty acid methyl esters (FAME) were prepared using sodium methoxide. FAME were analyzed by a gas-liquid chromatograph equipped with a DB 23 column (J & W Scientific Co., Folsom, CA). Fatty acids were identified by comparison of retention times with authentic standard mixtures of FAME (Matreya Inc., Pleasant Gap, PA) and Nu-Chek Prep.

PGE_2 analysis

The liver is a large organ with a heterogeneous population of macrophages,[25] and was chosen as a reference organ to determine the effects of CLA on PGE_2 production. Liver homogenates for PGE_2 production were prepared as previously described and assayed by radioimmunoassay.[26] Liver samples were homogenized in 10 vol of 50 mmol/L potassium phosphate buffer (pH 7.4 at 4°C), and homogenates incubated with shaking at 37°C. After 10 min, the incubation was terminated with aspirin (42 mmol/L) and samples stored at −80°C until assayed.

TNF bioassay

Macrophage culture supernatant fluids were assayed in octuplicate for TNF-like activity as described previously.[27] Briefly, dilutions of macrophage culture supernatant fluid (1:10 to 1:100) were placed in wells containing L929 murine fibroblasts with growth medium containing actinomycin D. After incubation for 20 h at 37°C, the fluid in the wells was discarded, and MTT in HBSS added to each well. After 3 h, 100 μl of a solution of sodium dodecyl sulfate (SDS) in N,N dimethylformamide (pH 4.7) was added to each well and incubated at 37°C overnight. The absorbency at 600 nm was then read in a microplate reader. A standard curve using recombinant human TNF-α (Genzyme, Boston, MA) was used to determine the amount of TNF-like bioactivity.

IL-6 bioassay

IL-6 bioassay was performed using B9 cells.[28] The cells were grown in Iscove's modified Dulbecco's medium supplemented with 10% fetal calf serum (FCS), 4 pg/100 ml of mouse recombinant IL-6, and 0.05 mol/L 2-mercaptoethanol (2-ME). Macrophage culture supernatant fluid was diluted (1:100 or 1:200) with growth medium and 200 μl/well added in octuplicate to a 96 well culture plate containing B9 cells. After 72 h of incubation, 20 μl of 7.24×10^{-7} mol/L MTT in HBSS was added to each well and then 6 h later 100 μl of SDS-DMF. The absorbancy at 550 nm was recorded after an overnight incubation at 37°C. A standard curve using recombinant mouse IL-6 (Genzyme) was used to determine the amount of IL-6–like bioactivity.

IL-1 bioassay

A mouse plasmacytoma cell line (T1165.17) that proliferates in response to IL-1 was used to assay for IL-1 activity. The IL-1 receptor on the T1165.17 cell line was blocked using a monoclonal antibody (LA 15.6) to the IL-1 receptor.[29] The cell line and antibody were generously supplied by Dr. Andrew Glasebrook, Eli Lilly and Co. Indianapolis, IN. The cells were grown in minimal essential medium supplemented with 10% FCS, 0.05 mol/L 2-ME, and 200 pg/L of IL-1β. Cells were seeded in 96 well plates (5×10^5/ml), and samples assayed in quadruplicate. Four wells were blocked with antibody (1 μg/ml) 30 min before addition of samples and four wells were unblocked. Macrophage culture supernatant fluid was diluted (1:10 to 1:200) with growth medium and 200 μl/well added in quadruplicate to each well and incubated for 18 h. After 18 h MTT and SDS-DMF was added and the plates incubated and absorbancy measured as described for the IL-6 assay. The difference in absorbancy between blocked and unblocked wells, corrected for control levels, determined the amount of IL-1 activity in the macrophage culture medium. A standard curve using recombinant mouse IL-1β (Genzyme) was used to determine the amount of IL-1 bioactivity.

Statistical analysis

Data were analyzed using SAS (SAS Institute, Cary, NC) by two-way analysis of variance, following a factorial design of treatments, and significant differences between treatment means were tested using Tukey's test.[30] The factorial design of dietary treatments allowed for an n = 12 in the statistical analysis of main effects (FAT, CLA, and CLA × FAT). Variations within treatments were expressed as the standard error of the means (SEM) or pooled SEM.

Results

Composition of conjugated linoleic acids

Upon analysis, it was determined that the CLA mixture consisted of 40% 18:2 *cis* 9, *trans* 11, and 40% 18:2 *cis* 12, *trans* 10 isomers.

Spleen fatty acid composition

The results of the fatty acid analysis are found in *Table 2*. The data show that CLA incorporated into spleen lipids at equal concentrations independent of the dietary (n-6) and (n-3) PUFA levels. The concentration of the CLA t10,c12 isomer was higher than that of the 9,11 isomers. CLA significantly lowered the concentrations of 16:1(n-7), 18:1, and 20:3(n-6) fatty acids in rats fed either diet. CLA increased the incorporation of 22:6(n-3) into spleen lipids of rats given either diet and there was a significant interaction of the PUFA type with CLA that increased the concentration of 22:5(n-3) fatty acids in MSO fed rats. The net effect of CLA on spleen lipids with either diet was to lower the total concentration of monounsaturated fatty acids (18:1) but increase the concentration of (n-3) PUFA. Rats given the (n-3) PUFA diet (MSO) had significantly higher concentrations of 16:1(n-7), 18:2(n-6), 20:5(n-3), 22:5(n-3), and total (n-3) PUFA but lower 18:0, 20:1(n-9), 20:2(n-6), 20:4(n-6), total n-6 PUFA, and (n-6):(n-3) fatty acid ratio.

Liver PGE_2 production

The MSO diet significantly reduced ($P = 0.0002$) liver PGE_2 production compared with the levels from SBO fed rats (*Figure 1*). Feeding CLA caused a reduction in mean liver PGE_2 production, but the effect was not significant ($P = 0.09$).

TNF bioactivity

Feeding CLA significantly reduced basal macrophage TNF production in control medium from rats fed either SBO (42% decrease; $P = 0.01$) or MSO (54% decrease; $P = 0.01$) (*Figure 2*). The CLA-induced reduction in basal TNF bioactivity was maintained in the presence of exogenous PGE_2 ($P = 0.05$) and LTB_4 ($P = 0.03$) for the SBO and MSO diets. Exogenous PGE_2 caused a significant reduction ($P < 0.05$) in mean levels of basal TNF production for all diets except MSO. LTB_4 caused a significant increase ($P < 0.05$) of basal TNF production in macrophage control medium from SBO fed rats, but not from the MSO fed rats.

There was not a CLA-induced reduction in TNF production from LPS-stimulated macrophages. Exogenous PGE_2 significantly reduced ($P < 0.05$) TNF production for all

Table 2 Fatty acid composition of spleen from rats fed different lipid treatments[1]

Fatty acid (mol/100 mol)	Dietary group[2]				Pooled SEM	ANOVA P-value		
	SBO		MSO					
	CLA+	CLA−	CLA+	CLA−		CLA	FAT	CLA × FAT
14:0	0.55−m	0.52−m	0.97−n	0.92−n	0.06	NS	0.0001	NS
16:0	25.90	25.55	26.86	25.84	0.36	NS	NS	NS
16:1(n-7)	0.93−mx	1.62−my	1.74−nx	2.29−ny	0.16	0.0008	0.0001	NS
17:0	0.29−B	0.27−B	0.43−A	0.48−A	0.01	NS	0.0001	0.04
18:0	15.47−m	15.13−m	14.24−n	14.52−n	0.22	NS	0.0005	NS
18:1	10.71−x	14.06−y	11.05−x	13.09−y	0.38	0.0001	NS	NS
18:2(n-6)	13.37−m	13.81−m	14.60−n	15.24−n	0.26	NS	0.0001	NS
18:3(n-3)	0.44−m	0.51−m	ND−*n*	ND−*n*	0.02	NS	0.0001	NS
20:1(n-9)	0.49−m	0.49−m	0.39−n	0.45−n	0.02	NS	0.0003	NS
20:2(n-6)	1.51−m	1.42−m	0.93−n	0.99−n	0.05	NS	0.0001	NS
20:3(n-6)	1.11−mx	1.29−my	1.29−nx	1.49−ny	0.04	0.0001	0.0001	NS
20:4(n-6)	19.99−A	19.06−A	9.82−B	10.71−B	0.40	NS	0.0001	0.03
20:5(n-3)	ND−*m*	ND−*m*	3.71−n	3.55−n	0.10	NS	0.0001	NS
22:4(n-6)	3.10−A	2.81−B	0.53−C	0.58−C	0.06	NS	0.0001	0.01
22:5(n-3)	2.16−C	1.75−C	6.15−A	5.07−B	0.14	0.0001	0.0001	0.02
22:6(n-3)	1.43−mx	1.19−my	4.84−nx	4.38−ny	0.10	0.002	0.0001	NS
18:2(*c*9,*t*11/*t*9,*c*11)	0.67−x	ND−*y*	0.76−x	ND−*y*	0.04	0.0001	NS	NS
18:2(*t*10,*c*12)	1.30−x	ND−*y*	1.24−x	ND−*y*	0.03	0.0001	NS	NS
SAT	42.21−x	41.48−y	42.50−y	41.76−y	0.33	0.04	NS	NS
MONO	12.13−x	16.17−y	13.18−x	15.82−y	0.47	0.0001	NS	NS
PUFA	43.11	41.84	41.87	42.00	0.62	NS	NS	NS
n-6 PUFA	39.07−A	38.39−A	27.17−B	29.00−B	0.50	NS	0.0001	0.02
n-3 PUFA	4.03−mx	3.45−my	14.71−nx	13.00−ny	0.30	0.001	0.0001	NS
[n-6]:[n-3]	9.69−B	11.14−A	1.86−C	2.24−C	0.15	0.0001	0.0001	0.002

[1]Mean values for liver fatty acid composition (n = 6) within a row having different letters (*m* or *n* for PUFA effect; *x* or *y* for CLA effect; *A*, *B*, or *C* for interaction effect) are significantly different by two-way analysis of variance (ANOVA) and Tukey's studentized range test ($P < 0.05$). The factorial design of dietary treatments allowed for an n = 12 in the statistical analysis of main effects.
[2]Dietary treatments included soybean oil (SBO) or menhaden oil + safflower oil (MSO) and with (+) or without (−) conjugated linoleic acid (CLA). Total fat content in each diet was 70 g/kg of diet, and CLA was added at 10 g/kg diet.
SAT, total saturated fatty acids; MONO, total unsaturated fatty acids; PUFA, total polyunsaturated fatty acids; NS, not significant; ND, not detected.

diets. LTB_4 caused a significant decrease ($P < 0.05$) in LPS-induced levels of TNF from macrophages of MSO fed rats, but had no effect on macrophages from rats fed the other diets.

IL-6 bioactivity

Feeding CLA significantly reduced mean basal IL-6 levels in macrophage control medium from SBO fed rats (79.6% decrease; $P = 0.02$) (*Figure 3*). The P-values for the CLA × FAT interaction indicated that this CLA-induced reduction was dependent on the type of fat (SBO). The MSO diet significantly reduced basal macrophage IL-6 production in control medium (88.8% decrease; $P = 0.003$) compared with levels produced from rats fed SBO. CLA had no effect on macrophage IL-6 production by MSO fed rats. PGE_2 and LTB_4 did not affect IL-6 production in nonstimulated macrophages for any diet.

There was also a CLA-induced reduction in IL-6 bioactivity in the control medium of LPS-stimulated macrophages from rats fed SBO (57.4% decrease; $P = 0.04$). This effect was abolished in the presence of exogenous PGE_2 and LTB_4 for LPS-stimulated macrophages from SBO fed rats. The levels of IL-6 from LPS-stimulated macrophages were also lower in MSO fed rats compared with SBO fed rats ($P = 0.003$). This relationship was maintained in the presence of exogenous PGE_2 ($P = 0.006$) and LTB_4 ($P = 0.004$).

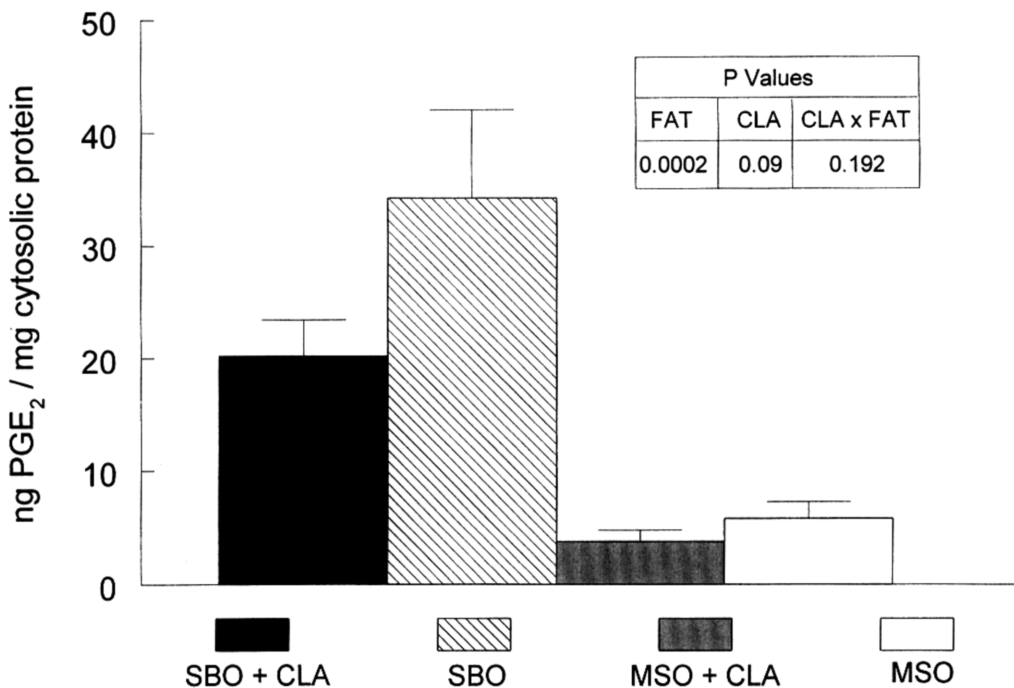
IL-1 bioactivity

Macrophage basal IL-1 production in control medium was significantly lower (53.1% decrease; $P = 0.04$) in SBO fed rats compared with MSO fed rats (*Figure 4*). This same relationship existed in the presence of LTB_4 (70.2% decrease; $P = 0.04$) and was almost significant in the presence of PGE_2 (64.9% decrease; $P = 0.06$).

The mean LPS-stimulated IL-1 levels in control medium from macrophages of SBO fed rats were lower compared with rats fed MSO, but not significantly (65% decrease; $P = 0.08$). The mean levels for IL-1 production were lower overall for rats fed diets enriched with CLA. This reduction in levels was greatest for rats fed MSO, but the effect was not significant. PGE_2 and LTB_4 had no significant effect on IL-1 production by nonstimulated and LPS-stimulated macrophages from rats fed either diet.

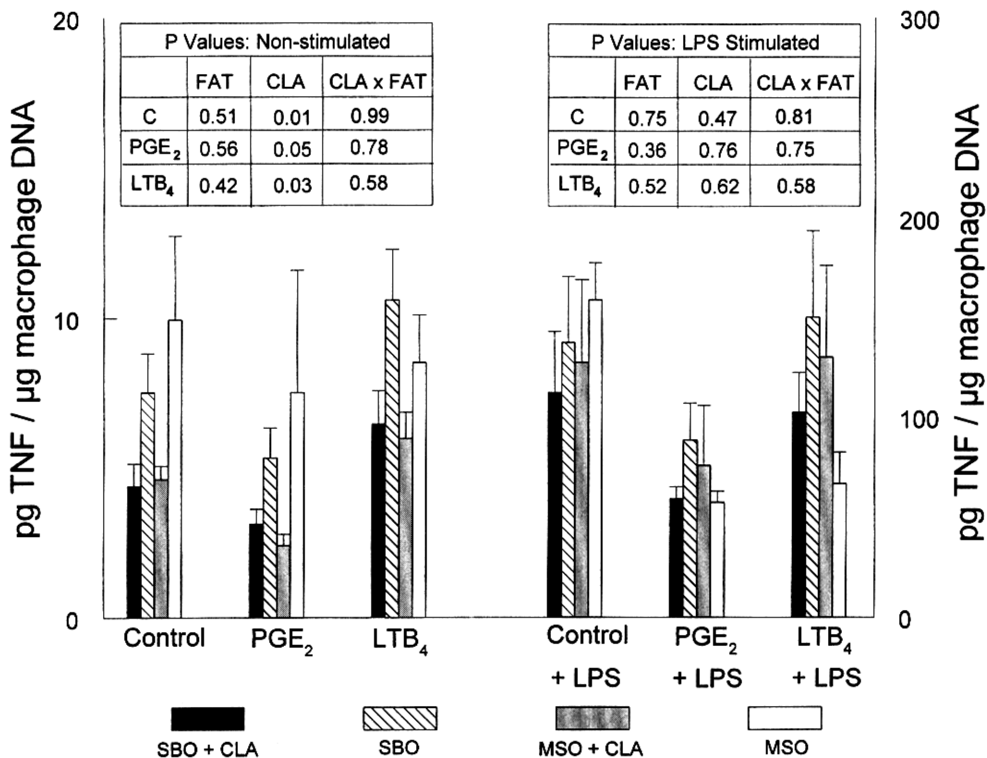
Discussion

In the present study CLA isomers were incorporated into spleen lipids at equal concentrations independent of the (n-6) and (n-3) dietary PUFA. The depression of 18:1 fatty acid incorporation into spleen lipids by CLA may be due to inhibition of delta-9 desaturase because this enzyme is required to convert stearic acid to oleic acid. The increased concentration of 22:6(n-3) may be due to CLA stimulation



P Values		
FAT	CLA	CLA x FAT
0.0002	0.09	0.192

Figure 1 Prostaglandin E_2 (PGE_2) production in liver homogenates. Dietary treatments included soybean oil (SBO) or menhaden oil + safflower oil (MSO), with (+CLA) or without conjugated linoleic acids. The P-values represent the effects of the dietary fat (SBO or MSO), the effect of the CLA, or an interaction of the type of fat with CLA (FAT × CLA). P-values ≤ 0.05 were considered statistically significant.



P Values: Non-stimulated			
	FAT	CLA	CLA x FAT
C	0.51	0.01	0.99
PGE_2	0.56	0.05	0.78
LTB_4	0.42	0.03	0.58

P Values: LPS Stimulated			
	FAT	CLA	CLA x FAT
C	0.75	0.47	0.81
PGE_2	0.36	0.76	0.75
LTB_4	0.52	0.62	0.58

Figure 2 Tumor necrosis factor (TNF)-like bioactivity from nonstimulated (left axis) and lipopolysaccharide (LPS) stimulated (right axis) rat peritoneal macrophages (mean ± SEM, n = 12 for main effects). Dietary treatments included soybean oil (SBO) or menhaden oil + safflower oil (MSO), with (+CLA) or without conjugated linoleic acids. Macrophages were cultured in control medium or control medium plus 10 μmol/L prostaglandin E_2 (PGE_2) or 5 nmol/L LTB_4 with and without 10 μmol/L LPS for 24 h. The P-values represent the effects of the dietary fat (SBO or MSO), the effect of the CLA, or an interaction of the type of fat with CLA (FAT × CLA). P-values ≤ 0.05 were considered statistically significant.

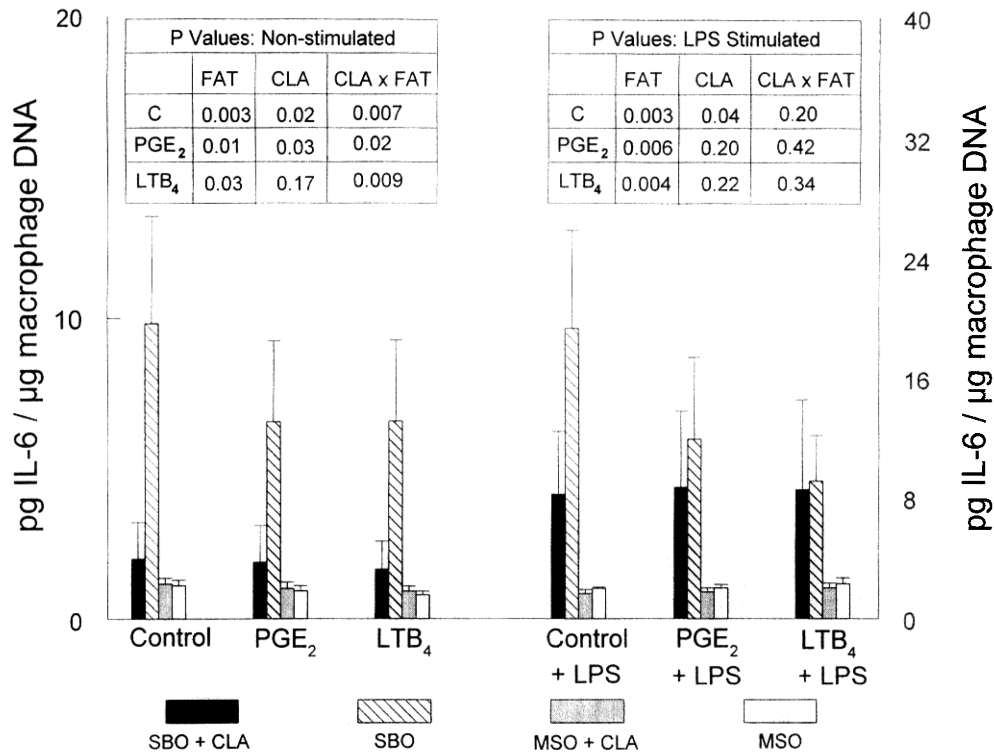


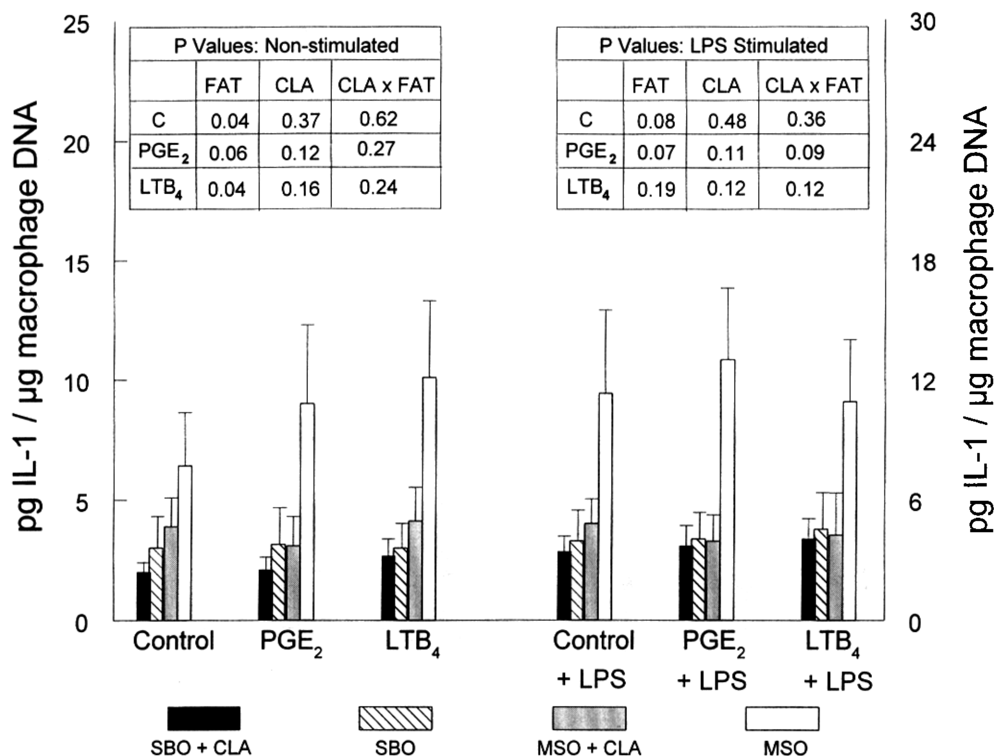
Figure 3 Interleukin-6 (IL-6) bioactivity from nonstimulated (left axis) and lipopolysaccharide (LPS) stimulated (right axis) rat peritoneal macrophages (mean ± SEM, n = 12 for factorial treatments). Dietary treatments included soybean oil (SBO) or menhaden oil + safflower oil (MSO), with (+CLA) or without conjugated linoleic acids. Macrophages were cultured in control medium or control medium plus 10 µmol/L prostaglandin E_2 (PGE_2) or 5 nmol/L LTB_4 with and without 10 µmol/L LPS for 24 h. The P-values represent the effects of the dietary fat (SBO or MSO), the effect of the CLA, or an interaction of the type of fat with CLA (FAT × CLA). P-values ≤ 0.05 were considered statistically significant.

of the beta-oxidation pathway in peroxisomes.[31] CLA did not alter the ratio of (n-6):(n-3) PUFA in spleen lipids of SBO fed rats and the concentration of 20:4(n-6) did not change with the addition of CLA for either diet. However, our analysis did not distinguish between arachidonic acid and isomers of CLA that may be elongated and desaturated.[22] The increased concentration of (n-3) PUFA in combination with C20:4(n-6) metabolites of CLA may be sufficient to alter eicosanoid formation via modulation of arachidonic acid metabolism.

The rats fed the (n-3) enriched diet (MSO) had significantly reduced PGE_2 production in liver homogenates. This reduction was expected based on other research that demonstrated the property of (n-3) PUFA to reduce PGE_2.[26,32] CLA also caused a reduction in the mean levels of PGE_2 production. Although the effect was not statistically significant, the effect was greater for rats fed SBO. This may indicate that as the dietary ratio of (n-6):(n-3) PUFA increases, CLA may have a modulating effect on PGE_2 production. The tibias and femurs of SBO fed rats in a related study demonstrated reduced ex vivo PGE_2 production.[24] This could indicate that CLA modulation of eicosanoids may also depend on the cell type or organ examined. In a study of rats fed an AIN-93G diet supplemented with either 1% CLA or linoleic acid, CLA reduced serum PGE_2 concentrations significantly, but the reduction in spleen was not significant,[33] similar to our results for liver. Recent research has demonstrated that CLA may be elongated and desaturated to 20:3 and 20:4 isomers which are incorporated into liver lipids of rats.[22] At the present time, it is not known if 20:4 isomers derived from CLA are a substrate for cyclooxygenase and lipoxygenase enzymes. However, the 20:4 isomers conceivably could compete with arachidonic acid for cyclooxygenase and thus lower PGE_2 levels and other eicosanoids of the 2-series.

Our results indicate that CLA is able to modulate cytokine production. CLA reduced basal levels of TNF-like activity independent of the type of PUFA in the diet. There have been numerous studies demonstrating (n-3) PUFA modulation of TNF production. Peritoneal macrophages of mice fed menhaden oil have enhanced TNF and IL-1 production.[34] A similar effect was observed in rats fed a diet enriched in α-linolenic acid (18:3n-3).[35] Thus, the effect of CLA on basal TNF production in rats appears to be opposite from that of (n-3) PUFA in rodents. In humans, supplementation of the diet with (n-3) PUFA resulted in lower levels of production of TNF, IL-1,[20] and IL-2[36] from peripheral blood mononuclear cells. Because of these contrasting effects of (n-3) PUFA in rodents and humans, the effects of CLA should be investigated to determine if the response in the human immune system is different from that in the rat.

In our experiments, the CLA-induced reduction in basal TNF bioactivity was maintained in the presence of exogenous PGE_2 and LTB_4 for the SBO and MSO diets. However, in MSO fed rats, exogenous LTB_4 did not increase basal TNF as observed for the other diets and it reduced



P Values: Non-stimulated			
	FAT	CLA	CLA x FAT
C	0.04	0.37	0.62
PGE_2	0.06	0.12	0.27
LTB_4	0.04	0.16	0.24

P Values: LPS Stimulated			
	FAT	CLA	CLA x FAT
C	0.08	0.48	0.36
PGE_2	0.07	0.11	0.09
LTB_4	0.19	0.12	0.12

Figure 4 Interleukin-1 (IL-1) bioactivity from nonstimulated (left axis) and lipopolysaccharide (LPS) stimulated (right axis) rat peritoneal macrophages (mean ± SEM, n = 12 for main effects). Dietary treatments included soybean oil (SBO) or menhaden oil + safflower oil (MSO), with (+CLA) or without conjugated linoleic acids. Macrophages were cultured in control medium, or control medium plus 10 µmol/L prostaglandin E_2 (PGE_2) or 5 nmol/L LTB_4 with and without 10 µmol/L LPS for 24 h. The P-values represent the effects of the dietary fat (SBO or MSO), the effect of the CLA, or an interaction of the type of fat with CLA (FAT × CLA). P-values ≤ 0.05 were considered statistically significant.

LPS-induced levels of TNF to the same extent as PGE_2. This effect was not present when CLA was included in the diet. Eicosanoids such as PGE_2 and LTB_4 regulate immune cell function primarily by modulating levels of cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP).[37] CLA appeared to block the reduction of TNF activity by LTB_4 in the presence of (n-3) PUFA, and further research is required to determine the nature of the interaction between CLA and (n-3) PUFA that influences regulation pathways associated with this leukotriene.

Exogenous LTB_4 caused a modest but insignificant increase in TNF activity from nonstimulated cells, except for MSO fed rats, in which there was a slight reduction in mean TNF levels. For LPS stimulated macrophages, LTB_4 had no effect on TNF except for levels from MSO fed rats in which there was a reduction. Eicosanoids such as PGE_2 and LTB_4 regulate immune cell function primarily by modulating levels of cAMP and cGMP.[37] Because CLA appeared to block the reduction of TNF activity by LTB_4 in the presence of (n-3) PUFA, additional research will be necessary to determine if CLA is able to affect signal transduction pathways associated with this leukotriene.

CLA reduced basal and LPS-induced IL-6 production, but this effect was dependent on the type of fat and was observed only with the high (n-6) PUFA diet. IL-6 is a prime mediator of the stress response involving the hypothalamic-pituitary-adrenocortical axis.[38] Stimulation of the stress response causes a catabolic physiologic reaction. The ability to suppress IL-6 may contribute to the effectiveness of CLA in promoting growth and feed efficiency.[8] In some experimental systems, PGE_2 has been shown to modulate IL-6 production.[39,40] In our experiments, addition of exogenous PGE_2 or LTB_4 resulted in a nonsignificant reduction in mean levels of basal and LPS-induced macrophage IL-6 from SBO fed rats. This relationship was not observed when CLA was present. Additional research will be necessary to determine if there is any interaction between the type of PUFA and CLA on eicosanoid regulation of IL-6, similar to what was observed for TNF and LTB_4 with the MSO diet. The interaction of CLA with (n-6) PUFA in reducing IL-6 production could involve nuclear transcription factors such as NF-κB. The nuclear transcription factor NF-κB is important in the regulation of cytokines such as IL-6.[41] Cells exposed to an NF-κB inducer such as LPS will rapidly phosphorylate and degrade the inhibitor protein IκB, which allows NF-κB to translocate to the nucleus and activate gene expression.[42] Linoleic acid has been shown to activate NF-κB in cultured endothelial cells.[43] It is unknown if dietary linoleic acid will activate macrophage NF-κB in a manner similar to endothelial cells. However, if this effect is present, the higher levels of IL-6 from macrophages of SBO fed rats could indicate increased activation of NF-κB due to the higher linoleic acid content of the SBO diet. Dietary CLA or some metabolite may inhibit this activation and reduce IL-6 production.

Although the mean levels of IL-1 production were lower in the presence of CLA for both diets, the effect was not statistically significant. However, the effect of CLA was greater in rats given MSO. This could indicate that CLA has some effect on IL-1 production when animals are fed a diet with a low (n-6):(n-3) ratio. The MSO diet resulted in increased macrophage IL-1 production compared with the levels from SBO fed rats. This effect is similar to what has been reported in mice, where (n-3) PUFA enhanced IL-1 production by peritoneal macrophages.[34]

PGE_2 has been shown to inhibit IL-1 production in monocytes.[44] However, determining the effect of PGE_2 on IL-1 production is dependent on the parameters examined and means of measurement. In mouse peritoneal macrophages, PGE_2 did not modulate levels of LPS-induced IL-1α mRNA, but increased the levels of IL-1β mRNA by more than tenfold.[45] In that study IL-1 activity by bioassay was repressed, but the levels by radioreceptor assay were elevated. The bioassay we used does not distinguish between IL-1α and IL-1β. In light of the complexity of the cellular response for IL-1 production, our results are not unexpected, but additional research will be necessary to clarify the role of PGE_2 on IL-1 production in our model.

Some research has demonstrated that the effect of CLA in mammary cancer prevention is independent of the level or type of fat.[46] The ability of CLA to inhibit dimethylbenz-[a]anthracene mammary tumors was maximized at 1% CLA in the diet, independent of the linoleic acid levels. That study used varying levels of corn oil (enriched in linoleic acid, 18:2n-6) and lard and did not investigate the effect of different ratios of (n-6):(n-3) PUFA. In contrast, our data demonstrate that the type of PUFA does not affect CLA reduction of basal TNF levels, but does influence the effect of CLA on basal and LPS-induced IL-6 production and perhaps liver PGE_2 production as well. Our data also indicate that there is some interaction between the type of PUFA and CLA on eicosanoid regulation of cytokine production. Future studies using diets with varying ratios of (n-6):(n-3) PUFA should prove useful in determining the mechanisms regulating the effects of CLA.

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