SUPPLEMENTAL MATERIALS

EXPANED MATERIALS AND METHODS

Materials

Arachidonic acid (AA), eicosapentaenoic acid (EPA) and palmitic acid (PA) were purchased from Nu-Chek Prep (Elysian, MN). Linoleic acid (LA), oleic acid (OA) and lipopolysaccharide (LPS) were obtained from Sigma-Aldrich (St. Louis, MO). Rosiglitazone (RG) and GW9662 were obtained from Cayman Chemical (Ann Arbor, MI).

Cell Culture

Murine macrophage-like cell line J774 (A2) was grown in Dulbecco's modified Eagle's medium containing 10% FBS (v/v), 1% glutamine (v/v) and 1% penicillin/streptomycin (v/v). Thioglycollate-elicited macrophages were obtained from C57BL/6 mice (12-14 week old) by peritoneal lavage with PBS at 4 days after the injection of 1 mL of 3.8% thioglycollate broth (Sigma-Aldrich, St. Louis, MO). Cells were suspended in RPMI 1640 supplemented with 10% FBS (v/v), 1% glutamine (v/v) and 1% penicillin/streptomycin (v/v), and incubated at 37ºC for 3 h. Thereafter,

nonadherent cells were washed away and adherent cells were cultured overnight in RPMI 1640 supplemented with 10% FBS (v/v), 1% glutamine (v/v) and 1% penicillin/streptomycin (v/v) at 37ºC in humidified CO_2 (5%). For experiments requiring PPARγ mRNA knockdown J774 cells (50-70% confluence) were transfected with optimized concentrations of either mouse PPARγ short hairpin RNA (shRNA) plasmid (sc-29456-sh, Santa Cruz Biotechnology, Inc.), or control nonsense shRNA plasmid (sc-108066) using shRNA transfection reagent (sc-29528), according to the manufacturer's instructions. 24 hours after transfection, cells were treated with specific FA and processed for real-Time PCR analyses as described below.

Incubation with fatty acids

Free FA were dissolved in 100% ethanol. All FA were used as complexes of the fatty acid bound to 1% FA-free bovine serum albumin (BSA) at a molar ratio of 1:1 (FA:albumin) in serum free medium (1). Serum free medium supplemented with 1% BSA was used as control. For all experiments, the cells were washed twice with phosphate-buffered saline (PBS) and the FA-containing media were added at different

doses for 3h, while the control cells received only BSA medium. Then, cells were incubated with LPS (1 μg/mL) or the PPARγ agonist and antagonist for 4h or 21h.

RNA extraction and quantitative real-time PCR

Cells were pretreated with EPA or PA for 3h at different doses and then continuously cultured in the presence or absence of LPS at 1μg/mL for 4h. Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. RNA was reverse transcribed using the iScript™ cDNA synthesis kit (BioRad, Hercules, CA). Quantitative real-time PCR was carried out on an iCycler real-time machine (BioRad, Hercules, CA) using the SYBR® Green PCR master kit (Applied Biosystems, Foster City, CA). The primer sequences were as follows: EL (sense, 5'-TCC TGC ATA CCT ACA CGC TGT C -3'; antisense, 5'- GTC AAT GTG ACC CAC AGG CA-3'), IL-6 (sense, 5'-GAG GAT ACC ACT CCC AAC AGA CC-3'; antisense, 5'-AAG TGC ATC ATC GTT GTT CAT ACA-3'), IL-10 (sense, 5'-ATT TGA ATT CCC TGG GTG AGA AG-3'; antisense, 5'-CAC AGG GGA GAA ATC GAT GAC A-3'), IL-12p40 (sense, 5'-GGA AGC ACG GCA GCA GAA TA-3'; antisense, 5'-AAC TTG AGG GAG AAG TAG GAA TGG-3'), MR (sense, 5'-CAT

GAG GCT TCT CCT GCT TCT G-3’; antisense, 5;-TTG CCG TCT GAA CTG AGA TGG-3’) and TLR4 (sense, 5’-AAG AGC CGG AAG GTT ATT GTG-3’; antisense, 5’-CCC ATT CCA GGT AGG TGT TTC-3’), VCAM-1 (sense, 5’-AAC CAG AAA AGT TCT GCT TGA CAA GT-3’; antisense, 5’-ATT AAG TTA CAA CAG TCA GTC CAA GCA A-3’). The reaction was performed at 95ºC for 5 min followed by 40 cycles of 95 ºC for 40 s, 56.7ºC for 40 s, and 72ºC for 40 s. Transcripts of the housekeeping genes GAPDH (sense, 5'-TGC AGT GGC AAA GTG GAG AT-3'; antisense, 5'-TTG AAT TTG CCG TGA GTG GA-3') in the same incubations were used for normalization.

Western blot analyses

Cells were pretreated with different doses of EPA or PA for 3h and then stimulated with LPS (1 μg/mL) for another 21h. After treatment, cells were scraped and pelleted at 1,000g and resuspended in RIPA buffer (BioRad, Hercules, CA). An aliquot (20 μg) was electrophoresed on a 7.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). After transfer onto polyvinylidene difluoride membranes, the membrane was blocked by 10% non-fat milk solution. The primary antibodies against EL (Cayman Chemical, Ann Arbor, MI) and actin (Sigma-Aldrich, St. Louis, MO) were used for Western blot analysis and blots were developed by

chemiluminescence using SuperSignal® West Femto Maximum Sensitivity Substrate (Thermo Scientific, Williston, VT).

Animals and diets

Eight-week-old male LDL-R KO mice were purchased from Jackson Laboratory (Bar Harbor, ME) and maintained under a 12-light/dark cycle. After 1-week of acclimatization, LDL-R KO mice were randomly divided into three groups (n=6) and fed specific diets as previously described (2); a normal chow (total 5% fat, 0.02% cholesterol, w/w) or a high-fat diet (total 19% fat, 0.2% cholesterol, w/w) enriched in either n-3 (91% menhaden fish oil and 9% corn oil; Harlan Teklad; TD. 07500) or saturated fat (SAT; 78% saturated fat from coconut oil, 13% monounsaturated fat from olive oil, and 9% polyunsaturated fat from corn oil; Harlan Teklad; TD. 08081). The n-3 and SAT diets contained 0.2% cholesterol (w/w). Mice had free access to food and water and were sacrificed at 12 weeks after feeding diet. All mice were sacrificed by exsanguination and intravascular perfusion with nuclease-free diethylpyrocarbonate (ProtectRNA™ RNAse inhibitor, Sigma) treated-PBS through a cannula inserted into the left ventricle under general anesthesia (isoflurane, Baxter, Deerfield, IL) was achieved. The aorta was dissected using a stereoscopic zoom microscope after collecting blood, and then kept

at -80ºC for EL and PPARγ mRNA analysis. All animal procedures were in compliance and approved by the Institutional Animal Care and Use Committee of Columbia University.

Statistical analyses

Results are given as mean ± S.E. Statistical analyses were performed using the SPSS program (SPSS, Inc., Chicago, IL). One-way analysis of variance (ANOVA) and 2-tailed Student's t-test were used to determine statistical significance at $p<0.05$. Pearson correlation coefficients were calculated to examine the association between the EL and PPARγ or inflammatory markers.

SUPPLEMENT RESULTS

Cell viability

Based on our MTT results (data not shown), we did not observe any significant change in cell viability with treatment of FA, LPS or PPARγ agonist or antagonist compared to the vehicle control. .

SUPPLEMENTAL REFERENCES

1. Rumsey SC, Galeano NF, Lipschitz B, Deckelbaum RJ. Oleate and other long chain fatty acids stimulate low density lipoprotein receptor activity by enhancing acyl coenzyme A:cholesterol acyltransferase activity and altering intracellular regulatory cholesterol pools in cultured cells. *J Biol Chem*. 1995;270:10008-10016.
2. Chang CL, Seo T, Matsuzaki M, Worgall TS, Deckelbaum RJ. N-3 fatty acids reduce arterial LDL-cholesterol delivery and arterial lipoprotein lipase levels and lipase distribution. *Arterioscler Thromb Vasc Biol*. 2009;29:555-561.

EXPANDED FIGURE LEGENDS

FIGURE 1. Effects of different FA on EL expression in macrophages. A: J774 macrophages cultured in the presence or absence of LPS, 1 μg/mL, for 4h after preincubation with 150 μM of unsaturated (EPA, AA, LA), monounsaturated (OA) and saturated FA (PA) for 3h. EL mRNA were analyzed by real-time quantitative RT-PCR and normalized to the GAPDH mRNA. Data are expressed as the mean ± SE from 3 independent assays. ^{abc}Means not sharing a common letter are significantly different between groups at $p<0.05$ (by one-way ANOVA). *$p<0.05$ vs non-stimulated control (by student's t-test). B, D: J774 macrophages were treated with the indicated concentrations

of EPA or PA for 7h (mRNA, B) or 24 h (protein, D). EL mRNA were analyzed by real-time quantitative RT-PCR and normalized to the GAPDH mRNA. EL protein evaluated by western blotting and the signal intensity of EL protein was normalized to that of β-actin protein. Representative western blots (top) and quantification (bottom) are shown. Data are expressed as the mean ± SE from 3 independent assays. *$p<0.05$, **$p<0.01$ vs non-stimulated control (by student's t-test). C, E: J774 macrophages were pretreated with the indicated concentrations of EPA or PA for 3h and continually cultured in the presence or absence of LPS, 1 μg/mL, for 4h (mRNA, C) or 21h (protein, E). EL mRNA was analyzed by real-time quantitative RT-PCR and EL protein evaluated by western blotting. Data are expressed as the mean ± SE from 3 independent assays. *$p<0.05$, **$p<0.01$ vs non-stimulated control or LPS-stimulated control (by student's t-test). F: Peritoneal macrophages from C57BL/6 mice were pretreated with 150 μM EPA or PA for 3h and continually cultured in the presence or absence of LPS, 1 μg/mL, for 4 h. EL mRNA was analyzed by real-time quantitative RT-PCR and normalized to the GAPDH mRNA. G: Peritoneal macrophages from C57BL/6 mice were pretreated with 150 μM EPA or PA for 3h and continually cultured in the presence or absence of LPS, 1 μg/mL, for 21 h. EL protein was evaluated by western blotting (bottom: representative of three independent blots) and densitometric analyses (top). The signal intensity of EL protein was

normalized to that of β-actin protein. Data are expressed as the mean ± SE from 3 independent assays. [ab]Means not sharing a common letter are significantly different between groups at $p<0.05$ (by one-way ANOVA). *$p<0.05$ vs non-stimulated control (by student's t-test).

FIGURE 2. Effects of different ratios of EPA and PA on EL. **A**: J774 macrophages were treated with the indicated concentrations of EPA and PA for 7h and EL mRNA were analyzed by real-time quantitative RT-PCR. B: J774 macrophages were treated with the indicated concentrations of EPA and PA for 24h and EL protein was evaluated by western blotting. Representative western blots (top) and quantification (bottom) are shown. Data are expressed as the mean ± SE from 3 independent assays. *$p<0.05$, **$p<0.01$ (by student's t-test).

FIGURE 3. Effects of FA on macrophage PPARγ mRNA expression (A, B) and correlation between EL and PPARγ mRNA (C, D). J774 (A) or peritoneal macrophages from C57BL/6 mice (B) were pretreated with 150 μM EPA or PA for 3h and continually cultured in the presence or absence of LPS, 1 μg/mL for 4 h for PPARγ mRNA analysis by real-time quantitative RT-PCR. Data are expressed as the mean ± SE

from 3 independent assays. [ab]Means not sharing a common letter are significantly different between groups at $p<0.05$ (by one-way ANOVA). C, D: correlation between EL and PPARγ mRNA was determined by Pearson correlation coefficients.

FIGURE 4. Interactions of EPA and PA with a PPARγ agonist/antagonist on EL expression in macrophages. A: J774 macrophages were treated with rosiglitazone (50 μM) and/or GW9662 (10, 25, 50 μM) for 4h (mRNA) or 21h (protein). EL mRNA was analyzed by real-time quantitative RT-PCR and EL protein evaluated by western blotting (top: representative of three independent blots) and densitometric analyses (bottom). B: J774 macrophages were pretreated with 150 μM PA for 3h and continuously cultured with GW9662 (10, 25, 50 μM) for 4h (mRNA) or 21h (protein). C: J774 macrophages were pretreated with EPA (150, 300 μM) and continuously cultured with rosiglitazone (50 μM) for 4h (mRNA) or 21h (protein). D: Peritoneal macrophages from C57BL/6 mice were pretreated with 150 μM EPA and continuously cultured with rosiglitazone (25 μM) for 4h (mRNA) or 21h (protein). E: Peritoneal macrophages from C57BL/6 mice were pretreated with 150 μM PA and continuously cultured with GW9662 (25 μM) for 4h (mRNA) or 21h (protein). Data are expressed as the mean ± SE from 3 independent assays. $^*p<0.05$, $^{**}p<0.01$ (by student's t-test).

FIGURE 5. Effects of EPA and PA on mRNA expression of pro-and anti-inflammatory markers in murine peritoneal macrophages. Peritoneal macrophages from C57BL/6 mice were pretreated with 150 μM EPA or PA for 3h and continually cultured in the presence or absence of LPS (1 μg/mL) for 4 h. A: pro-inflammatory IL-6, IL-12p40, TNF-α, VCAM-1, TLR4 and CD68 mRNA were analyzed by real-time quantitative RT-PCR B: anti-inflammatory IL-10 and MR (mannose receptor) mRNA were analyzed by real-time quantitative RT-PCR. Data are expressed as the mean ± SE from 3 independent assays. *$p<0.05$, non-stimulated control vs LPS-stimulated control (by student's t-test). [ab]Means not sharing a common letter are significantly different between groups at $p<0.05$ (by one-way ANOVA).

FIGURE 6. Effects of n-3- and saturated FA-rich diets on arterial EL, PPARγ and inflammatory markers mRNA expression in LDL-R KO mice. LDL-R KO mice were fed a normal chow or a high-fat diet enriched in either n-3 or SAT for 12 weeks for EL (A), PPARγ (B), IL-6 (C), IL-12p40 (D) and IL-10 (E) mRNA analysis by real-time quantitative RT-PCR. Data are expressed as the mean ± SE. [ab]Means not sharing a common letter are significantly different between groups at $p<0.05$ (by one-way

ANOVA).

SUPPLEMENTAL FIGURE LEGENDS

SUPPLEMENTAL FIGURE Ⅰ. Effects of LPS on EL expression in J774 macrophages. J774 macrophages were incubated with increasing concentrations of LPS for 4h (mRNA) and 21h (protein). A: EL mRNA was analyzed by quantitative RT-PCR and normalized to the GAPDH mRNA. Data are mean ± SE from three independent assays. *$p<0.05$ vs non-stimulated control (by student's t-test). B: EL protein was evaluated by western blotting. The signal intensity of EL protein was normalized to that of β-actin protein. Representative western blots (top) and quantification (bottom) are shown. Data are mean ± SE from three independent assays. **$p<0.01$ vs non-stimulated control (by student's t-test).

SUPPLEMENTAL FIGURE Ⅱ. Effects of different doses of rosiglitazone on EL expression in J774 macrophages. A: J774 macrophages were treated with rosiglitazone (ROSI, 0.1, 1, 10, 25, 50 μM) for 21h for EL protein analysis by western blotting. B: J774 macrophages were treated with rosiglitazone for 4h for EL mRNA

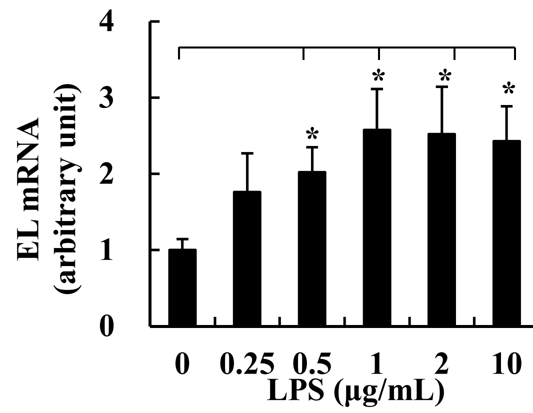
analysis by real-time PCR. Data are expressed as the mean±SE. *p<0.05, **p<0.01 (by student's t-test).

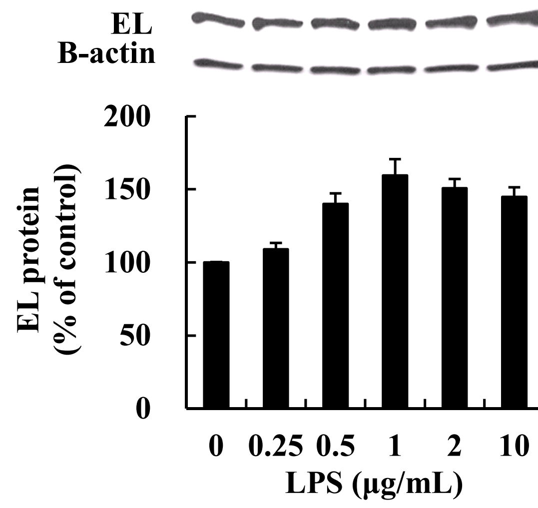
SUPPLEMENTAL FIGURE Ⅲ. Interactions of rosiglitaozone with GW9662/EPA on PPARγ mRNA expression in J774 macrophages. A: J774 macrophages were treated with rosiglitazone (ROSI, 10, 25, 50 μM) for 4h for PPARγ mRNA analysis by real-time PCR. B: J774 macrophages were treated with rosiglitazone (50 μM) and/or GW9662 (50 μM) for 4h. C: J774 macrophages were pretreated with EPA (150μM) and continuously cultured with rosiglitazone (50 μM) for 4h. Data are expressed as the mean±SE from 3 independent assays. *p<0.05, **p<0.01, ***p<0.001 (by student's t-test).

SUPPLEMENTAL FIGURE Ⅳ. Interaction of EPA with PPARγ agonist on LpL (A) and CD36 (B) mRNA expression in macrophages. J774 macrophages were pretreated with EPA (150, 300 μM) for 3h and continuously cultured with rosiglitazone (50 μM) for 4h. LpL and CD36 mRNA was analyzed by real-time PCR and normalized to the GAPDH. Data are expressed as the mean ± SE from 3 independent assays. *p<0.05, **p<0.01 (by student's t-test).

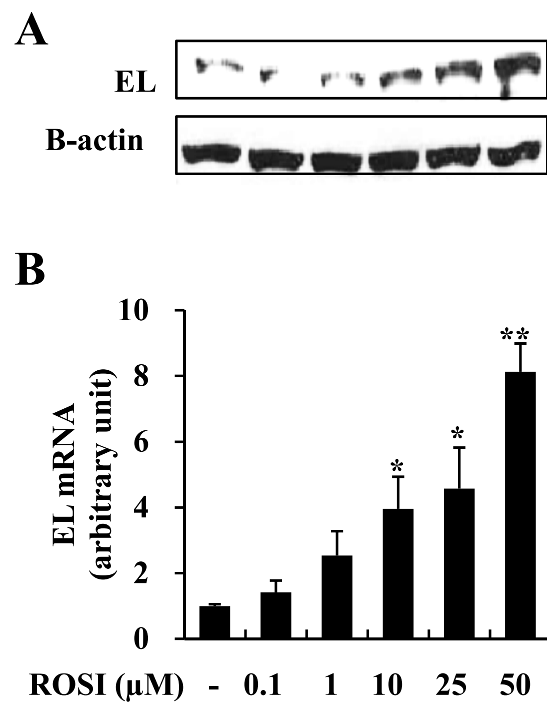
SUPPLEMENTAL FIGURE Ⅴ. Relationships between EL and PPARγ mRNA expression and changes in pro-(A, C) and anti-(B, D) inflammatory markers in murine peritoneal macrophages.

SUPPLEMENTAL FIGURE Ⅰ

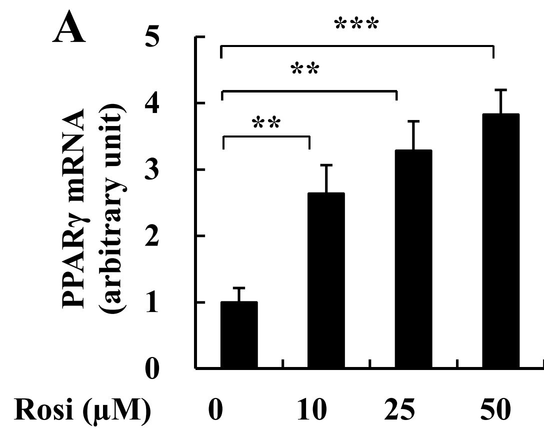


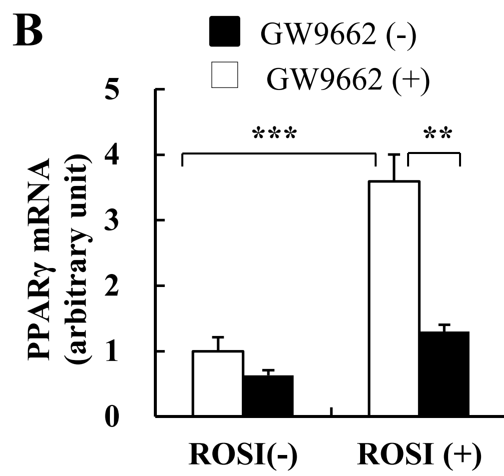


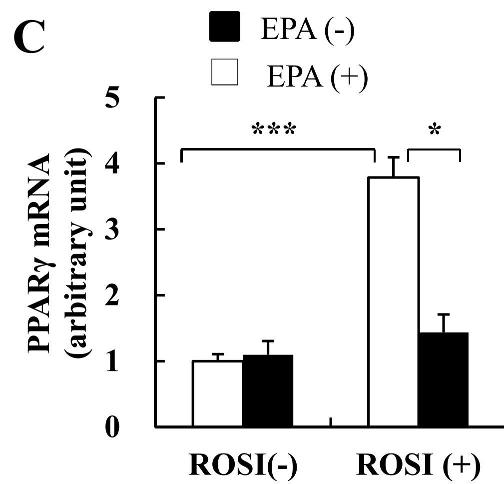
SUPPLEMENTAL FIGURE Ⅱ



SUPPLEMENTAL FIGURE Ⅲ







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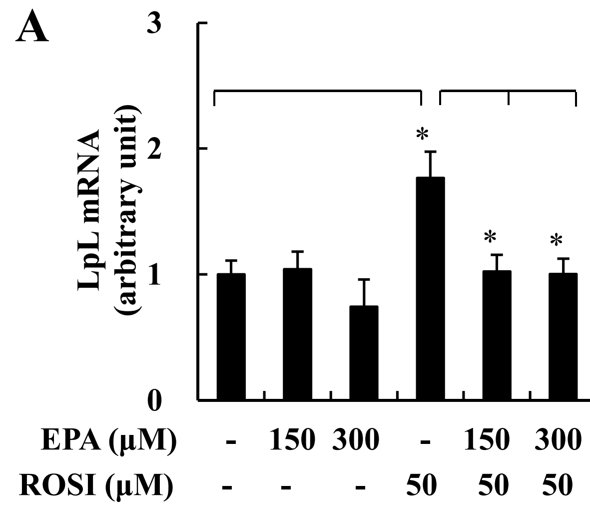

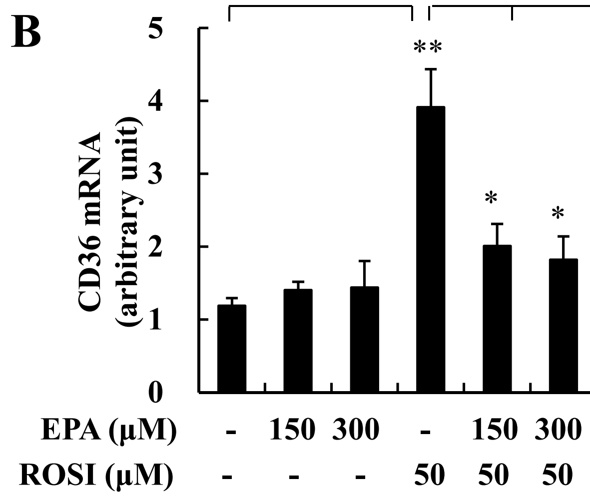

SUPPLEMENTAL FIGURE Ⅴ