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Fatty acids regulate endothelial lipase and inflammatory markers in macrophages and in mouse aorta: a role for PPARγ

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Abstract

Objectives—Macrophage endothelial lipase (EL) is associated with increased atherosclerosis and inflammation. Because of their anti-inflammatory properties we hypothesized that n-3 fatty acids (FA), in contrast to saturated FA, would lower macrophages and arterial EL and inflammatory markers.

Methods and Results—Murine J774 and peritoneal macrophages were incubated with eicosapentaenoic acid (EPA) or palmitic acid (PA) in the presence or absence of lipopolysaccaride (LPS). LPS increased EL mRNA and protein. PA alone or with LPS dose-dependently increased EL mRNA and protein. In contrast, EPA dose-dependently abrogated effects of LPS or PA on increasing EL expression. EL expression closely linked to PPARγ expression. EPA blocked rosiglitazone (a PPARγ agonist)-mediated EL activation and GW9662 (a PPARγ antagonist) blocked PA-mediated EL stimulation. EPA alone or with LPS blunted LPS-mediated stimulation of macrophage pro-inflammatory IL-6, IL-12p40, TLR4 mRNA and increased anti-inflammatory IL-10 and mannose receptor mRNA. *In vivo* studies in LDL receptor knockout mice showed that high saturated fat rich diets, but not n-3 diets, increased arterial EL, PPARγ and pro-inflammatory cytokine mRNA.

Conclusions—n-3 FA, in contrast to saturated FA, decrease EL in parallel with modulating pro- and anti-inflammatory markers, and these effects on EL link to PPARγ.

Keywords

n-3 fatty acids; endothelial lipase; inflammation; PPARγ; atherosclerosis

Inflammation adversely affects arterial wall biology.[1,2] Much evidence supports a pro-atherogenic and pro-inflammatory effect of saturated fatty acids (FA).[3,4] In contrast, protective actions with respect to the arterial wall have been attributed to n-3 FA.[5-7] n-3 FA delivered from dietary fish oil are incorporated into atherosclerotic plaques, enhancing

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stability, whereas n-6 FA do not have these effects.[5] Recent reviews indicate that increased consumption of long-chain n-3 FA, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), but not of α-linolenic acid (their n-3 essential fatty acid precursor), reduced the rates of all-cause mortality, and cardiac and sudden death.[6] n-3 FA have been shown to reduce the macrophage infiltration into the vessel wall and secretion of pro-atherogenic and pro-inflammatory growth factors and cytokines by monocytes and macrophages.[7]

Macrophages play a pivotal role in the development and progression of atherosclerosis. Endothelial lipase (EL) is one of several lipases synthesized and secreted by macrophages. High levels of EL expression have been observed in macrophages present in human atherosclerotic plaques.[8] EL deficiency is associated with a 70% decrease in atherosclerotic lesions in apoE KO mice.[9] A pro-atherogenic effect of EL in macrophages has also been ascribed to bridging functions, which plays a role in the uptake of lipoproteins[10] or recruitment of monocytes by blood vessel walls.[11] Moreover, EL expression is upregulated in macrophages by pro-inflammatory cytokines.[10] Upregulation of macrophage EL by toll-like receptor (TLR) 4 and 3 negatively modulates IL-10 and positively modulates IL-12 production, potentially influencing atherosclerosis.[12] Accordingly, EL might be considered to be an attractive pharmacological target in the prevention of atherosclerosis.

Peroxisome proliferator activated receptor (PPAR)γ also has been implicated in atherogenesis. PPARγ is highly expressed in macrophages-derived foam cells in atherosclerotic lesions[13] and activation of PPARγ has been shown to induce macrophage lipid accumulation by increasing the expression of the oxidized LDL scavenger receptor CD36 and lipoprotein lipase (LpL).[13-15] In contrast to the proposed potentially pro-atherogenic effects of PPARγ, other limited evidence suggests PPARγ may mediate anti-inflammatory effects by negatively regulating pro-inflammatory cytokine expression.[16] Also, macrophage PPARγ deficiency increases atherosclerosis in C57BL/6 and LDL receptor knockout (LDL-R KO) mice,[17] indicating an anti-atherogenic role for PPARγ. However, at present, no definitive studies support the premise that PPARγ is required for anti-inflammatory effects in macrophages.[18-22] In fact, recent clinical trials have raised concerns on increased risk of myocardial infarction and cardiovascular death in diabetic patients treated with rosiglitazone,[23, 24]a strong PPARγ agonist.

We previously demonstrated that high-saturated fat (SAT) diets increased contributions of LDL selective uptake (SU) to total arterial LDL-CE deposition and that increased SU parallels increased LpL levels and distribution in the arterial wall.[25] In contrast, n-3 rich diets decreased arterial total LDL delivery and abrogated LDL SU in parallel with changing arterial wall LpL.[26] We now questioned whether FA would regulate EL expression since different dietary FA modulated arterial LpL levels and distribution as described in our previous reports.[25-27] Specifically, we asked whether n-3 FA, EPA, in contrast to a saturated FA, palmitic acid (PA), would decrease expression of EL and, if so, would these changes correlate with changes in inflammatory markers and in PPARs which may also modulate lipid metabolism and inflammatory responses.[13,14,18]

Our results demonstrate that PA increases EL expression and decreases anti-inflammatory IL-10 expression in macrophages. In contrast, EPA decreases EL expression, in parallel with decreasing pro-inflammatory markers and increasing anti-inflammatory markers. The changes in macrophage EL by FA were strongly related to the regulation of PPARγ. Moreover, LDL-R KO mice fed SAT diets, but not n-3 diets, showed the increases in EL, PPARγ and pro-inflammatory responses in the arterial wall.

Methods

Some methods are described in more detail in online Supplemental Materials and Methods (available at http://atvb.ahajournals.org).

Cell Culture

Murine macrophage-like cells, J774 (A2), were 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 weeks old) by peritoneal lavage with PBS at 4 days after injection of 1 mL of 3.8% thioglycollate broth (Sigma-Aldrich). 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 37°C for 3 h. 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), PPARγ agonist or antagonist for 4h (for mRNA) or 21h (for protein).

For experiments requiring PPARγ mRNA knock-down, 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. Twenty four hours after transfection, cells were treated with specific FA and processed for real-time PCR analyses as described below.

Quantitative real-time PCR

Total RNA was isolated with TRIzol reagent (Invitrogen) and quantitative real-time PCR was carried out on an iCycler real-time machine (BioRad) using the SYBR® Green PCR master kit (Applied Biosystems). Values were normalized to GAPDH levels.

Western Blot

EL protein expression was analyzed by western blot normalized to β-actin protein.

Animals and Diets

Eight-weeks-old male LDL-R KO mice were purchased from Jackson Laboratory. After 1 week acclimatization, mice were fed a semipurified, normal chow (total 5% fat, 0.02% cholesterol, w/w) or a high-fat, semipurified 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) for 12 weeks similar to our previous report.[26] The aorta was dissected and measured for mRNA of EL, PPARγ, and specific inflammatory markers. All procedures were approved by the Institutional Animal Care and Use Committee of Columbia University.

Statistical Analyses

Statistical analyses were determined by one-way ANOVA (for comparing FA), 2-tailed Student's t-test, or Pearson correlation coefficients. Data are expressed as mean ± SE.

Results

LPS increases EL expression in macrophages

We first examined the effect of LPS, a potent endotoxin, on EL expression in macrophages. J774 cells were incubated with increasing concentration of LPS (0.25-10 μg/mL). LPS increased EL mRNA by a maximum of 2.5 fold at 1 μg/mL (Supplemental Figure 1A). Similarly, LPS dose-dependently increased protein levels of EL with a maximum increase also at 1 μg/mL (Supplemental Figure 1B). We thus chose concentration of LPS of 1 μg / mL for the experiments below.

EPA inhibits the increase of EL mRNA and protein induced by LPS more than other FA

To determine the effects of different FA on macrophage EL mRNA expression, J774 cells were cultured in the presence or absence of LPS, 1 μg/mL, for 4h after preincubation with 150 μM of unsaturated FA (eicosapentaenoic acid, EPA; arachidonic acid, AA; linoleic acid, LA), monounsaturated FA (oleic acid, OA) and saturated FA (palmitic acid, PA) for 3h. Exposure of cells to LA and PA alone significantly increased macrophage EL mRNA levels compared to non-FA control by 2.2 fold and 2.6 fold, respectively (Figure 1A). PA together with LPS induced 1.4 fold higher levels of EL mRNA compared to LPS alone. In contrast, EPA, AA and OA inhibited LPS-induced EL mRNA levels by 64%, 37% and 39%, respectively. Among FA tested, PA induced the strongest response on increasing EL expression, whereas EPA was most potent at inhibiting EL expression induced by LPS.

Since EPA and PA appeared to show the greatest differences on EL mRNA expression, we chose EPA and PA for dose-response experiments. J774 cells were cultured in the presence or absence of LPS, 1 μg/mL, for 4 to 21h after preincubation with 50, 150, 300 μM of EPA or PA for 3h. PA alone as well as together with LPS dose-dependently increased EL mRNA (Figure 1B and 1C). In contrast, EPA alone had no effect on EL mRNA, but completely abrogated effects of LPS on increasing EL mRNA. Consistent with mRNA expression, EPA had no significant effect on EL protein expression but inhibited LPS-mediated increase of EL protein (Figure 1D and 1E). PA alone as well as with LPS increased EL protein levels in a dose-dependent manner.

PA and LPS have similar effects on EL expression in peritoneal macrophages as in J774 cells and EPA inhibits LPS-mediated increase of EL expression

To determine whether EPA and PA also regulate EL expression in primary macrophages, we treated EPA and PA with or without LPS in peritoneal macrophages from C57BL/6 mice. Similar to J774 cells, LPS and PA alone significantly increased EL mRNA compared to control by 2.0 and 3.0 fold, respectively, whereas EPA significantly inhibited LPS-induced EL mRNA by ~30% (Figure 1F). EL protein levels were also increased by LPS and PA, but EPA significantly abrogated effects of LPS on increasing EL protein (Figure 1G). Thus, EPA can mitigate the effects of PA-mediated increases of EL in different macrophage lines.

EPA inhibits PA-mediated increases of EL

To determine effects of EPA together with PA on macrophage EL, we incubated cells with different ratios of EPA and PA (Figure 2). EL mRNA was lower in J774 cells treated with the combinations of PA plus EPA compared to the group treated only with PA; these differences became significant when EPA accounted for 1/3 or more of total FA (Figure 2A). EL protein levels showed similar responses to incubations of PA vs PA and EPA (Figure 2B).

EPA and PA have different effects on regulation of PPARγ expression

Because PPARs are linked to a number of processes important to atherogenesis,[14,28] we examined potential associations of PPARγ and PPARα with EL expression. PPARγ expression was much more affected by FA than PPARα expression. In J774 cells, but not peritoneal macrophages, EPA alone or with LPS tended to increase PPARα mRNA whereas PA had no effect (data not shown). In contrast, PA alone as well as with LPS significantly increased PPARγ mRNA expression (Figure 3A and B). In addition, PPARγ mRNA positively correlated with EL mRNA in both macrophages, respectively ($r=0.34$, $p<0.05$; $r=0.43$, $p<0.01$).

In order to determine how changes in PPARγ might affect macrophage EL expression, we treated cells with increasing concentrations of rosiglitazone and/or GW9662 in the presence or absence of EPA or PA. Rosiglitazone is a known PPARγ agonist and GW9662 is a PPARγ antagonist. Rosiglitazone (0.1~50 μM) dose-dependently increased EL protein and mRNA, whereas GW9662 markedly blocked rosiglitazone-mediated increases of EL protein and mRNA in J774 cells (Supplemental Figure 2, and Figure 4A and 4B). Moreover, GW9662 significantly inhibited PA-induced EL mRNA and protein. Of note, EPA abrogated rosiglitazone-mediated increases of EL mRNA and protein in J774 cells (Figure 4C). PPARγ mRNA expression was dose-dependently increased by rosiglitazone, whereas GW9662 and EPA abrogate effects of rosiglitazone on increasing PPARγ mRNA in J774 cells (Supplemental Figure 3). We also found that EPA inhibited the increase of mRNA levels of LpL and CD36, well known PPARγ target genes,[15,16] after incubation with rosiglitazone (Supplemental Figure 4).

To determine whether the PPARγ agonist and antagonist also regulate EL expression in primary macrophages, we treated peritoneal macrophage with rosiglitazone and GW9662 in the presence of FA. Similar to J774 cells, rosiglitazone increased EL mRNA and protein by 70% and 60%, respectively, and EPA significantly blocked rosiglitazone-induced EL mRNA expression by 86% (Figure 4D). Also, GW9662 markedly blocked the PA and rosiglitazone-mediated increases of EL mRNA by 96% and 78%, respectively, and inhibited the increase in EL protein induced by PA and rosiglitazone (Figure 4D and 4E). Thus, EL in cultured cells *in vitro* closely linked to PPARγ and was regulated by FA, in part, through modifying macrophage PPARγ expression.

To further support the above association between PPARγ and EL expression we carried out experiments where we used shRNA mediated knock-down of PPARγ expression in J774 cells. We achieved a knock-down efficiency of >80% using PPARγ shRNA as compared to control shRNA controls ($n=6$, $p<0.05$). We then compared the effects of PA on increasing EL mRNA in these PPARγ knock-down J774 cells and found a mean 63% decrease in EL mRNA expression after incubation with PA compared to control levels ($n=6$, $p=0.04$). Thus, decreases in PPARγ blunt the ability of PA to increase EL expression in macrophages, indicating that EL is modulated, in part, by PPARγ-dependent pathways.

EPA decreases pro-inflammatory markers, but increases anti-inflammatory markers in peritoneal macrophages

LPS is a bacterial endotoxin that is commonly used to stimulate inflammatory responses. Wang et al[12] reported that induction of macrophage EL by LPS can modulate macrophage inflammatory responses. To explore the relationships of EL and inflammatory markers, we compared well defined pro-and anti-inflammatory markers in peritoneal macrophages incubated with EPA and PA. LPS significantly increased pro-inflammatory cytokines, IL-6 and IL-12p40 (Figure 5A). However, EPA alone or with LPS blunted the stimulating effects of LPS on IL-6 and IL-12p40 mRNA by 62% and 60%, respectively. Also, EPA markedly

attenuated TLR4 mRNA. We also found that in J774 cells, LPS increased IL-6 and IL-12p40 mRNA by 17- and 12-fold, respectively ($p<0.001$, $p<0.001$) and that these effects were diminished by EPA (data not shown). PA and EPA effects on TNF-α expression were not similar to other pro-inflammatory markers; PA alone increased TNF-α mRNA level similar to LPS (LPS vs BSA, 56%; PA vs BSA, 55%), whereas EPA alone or with LPS had no effect. In contrast, anti-inflammatory IL-10 and mannose receptor (MR) were increased in EPA-treated cells by 2.1 and 1.5 fold, respectively (Figure 5B). PA had little effect on pro-inflammatory markers but decreased IL-10 mRNA (Figure 5A and B). Interestingly, EL mRNA showed positive correlations with increasing mRNA levels of pro-inflammatory markers such as IL-6, IL-12p40, TLR4 and vascular cell adhesion molecule-1 (VCAM-1) (Supplemental Figure 5). In contrast, there were negative correlations between EL and anti-inflammatory markers, IL-10 and MR, respectively. PPARγ mRNA was also positively correlated with pro-inflammatory IL-6, IL-12p40, TLR4 and VCAM-1 mRNA, whereas it was negatively correlated with anti-inflammatory IL-10 mRNA (Supplemental Figure 5). There were no significant correlations between PPARα and inflammatory markers (data not shown).

Dietary saturated vs n-3 diet changes arterial EL, PPARγ and inflammatory markers expression in LDL-R KO mice *in vivo*

We next investigated the potential effects of 12-week feeding of n-3-rich and SAT-rich diets on arterial EL, PPARγ and inflammatory gene expression in aorta of LDL-R KO mice which are susceptible to atherosclerosis. SAT diets led to 10-fold greater arterial EL mRNA compared to chow (Figure 6A). There was no significant difference in arterial EL mRNA levels between n-3 and chow diets. SAT diets were associated with markedly increased arterial PPARγ mRNA compared to chow diets, whereas the n-3 diets showed a 26% decrease in PPARγ mRNA (Figure 6B). Similar to *in vitro* data in macrophages, SAT diets increased arterial IL-6 and IL-12p40 mRNA 2.6-fold and 5.8-fold compared to chow, respectively, whereas arterial IL-10 mRNA was lowered in SAT-fed mice compared to chow-fed mice by 22% (Figure 6C-6E). In contrast, n-3 diets reduced both pro-inflammatory cytokine mRNA levels in aorta of LDL-R KO mice compared to chow by 74% and 50%, respectively, but increased IL-10 mRNA compared to SAT diet by 69%. Thus, *in vivo* effects of diets rich in SAT vs n-3 FA on arterial expression of EL and inflammatory markers paralleled effects observed in cultured macrophages *in vitro*.

Discussion

Macrophage-derived EL in the arterial wall is associated with increased atherosclerosis and arterial inflammatory markers in mice.[9,10,12] Because of their anti-inflammatory properties we hypothesized that n-3 FA, in contrast to saturated FA, would lower expression of EL *in vitro* and *in vivo*. Our results show that a saturated FA, PA, increases macrophage EL expression and decreases anti-inflammatory IL-10 expression. In contrast, an n-3 FA, EPA, inhibits the increase of EL expression induced by LPS and PA, and this is accompanied by decreases in pro-inflammatory markers and increases in anti-inflammatory markers in cultured macrophages. Moreover, regulation of macrophage EL in response to FA is closely linked to changes in PPARγ activation. *In vivo* studies also show that SAT diets, but not n-3 diets, increase EL, PPARγ and pro-inflammatory cytokine expression, but decrease anti-inflammatory cytokine mRNA in aorta of LDL-R KO mice, suggesting that changes in EL by FA have important regulatory roles on atherosclerosis and inflammation in *vivo* as well as in *vitro*.

LPS, a major inflammatory stimulus, can play an important role in lipoprotein metabolism and atherosclerosis.[29] Yasuda et al[10] reported that EL expression was increased by LPS. In our current study, LPS also markedly increased EL protein as well as mRNA expression in

J774 and peritoneal macrophages. Interestingly, saturated PA alone and/or with LPS increased EL mRNA and protein levels in both macrophages. In contrast, n-3 EPA had little effects on EL mRNA and protein, but markedly inhibited the increase of macrophage EL expression induced by LPS. Furthermore, EL expression was significantly lower in cells treated with a combination of PA plus EPA compared to PA alone. Based on these results, EPA suppresses the increase of macrophage EL expression induced by EL activators, PA and LPS.

PPARγ is a nuclear transcription factor that regulates numerous genes involved in lipoprotein metabolism and is highly expressed in macrophages, including foam cells of atherosclerotic lesions.[14] PPARγ activation increased macrophage LpL mRNA and protein expression,[15] and promoted uptake of oxidized LDL through induction of macrophage CD36 expression,[13,14] which suggest the potential role of PPARγ in the pathogenesis of atherosclerosis. Furthermore, the PPARγ agonists, rosiglitazone and pioglitazone, enhance macrophage apoptosis via a PPARγ-independent mechanism, and pioglitazone promotes advanced plaque progression in LDL-R KO mice through enhancement of advanced lesion macrophage apoptosis.[30] Our data indicate that EL might also contribute to atherogenesis, and that PPARγ activation is associated with this regulation of EL.

In the current study, PPARγ activation using rosiglitazone as well as PA was related to increased EL mRNA and protein, and GW9662, a PPARγ antagonist, EPA and PPARγ-knock-down cells abrogated stimulation by rosiglitazone or PA. Rosiglitazone significantly increased PPARγ mRNA in a dose-dependent manner, whereas pioglitazone, a ~10 times less potent an activator of PPARγ than rosiglitazone,[31] had little effects on PPARγ as well as on EL, in our experiments in macrophages (data not shown). Also, GW9662 and EPA blocked rosiglitazone-induced PPARγ mRNA expression. Similar to our results with EPA, n-3 FA-DHA, suppressed CD36 expression induced by PPARγ agonist through the inhibition of transcriptional activity of PPARγ in human monocytes and colon tumor cells, [32] and EPA and DHA reduced the PPARγ response element (called PPRE) reporter activity in colon cancer cells. Edwards et al[33] proposed that n-3 FA may directly, or after being metabolized, activates ERK or other pathways that counteracts PPARγ signaling. On the other hand, PA significantly increased PPARγ mRNA or protein expression in several cell types such as cardiomyocytes,[34,35] and a high fat diet enriched in PA enhanced PPARγ expression in macrophages.[36] PA also stimulated the activity of the PPRE in primary human adipocytes, suggesting activation of this nuclear signaling cascade.[37] Taken together, our findings suggest that macrophage EL expression is partially mediated by the upregulation of PPARγ, and that saturated vs n-3 FA affect the expression of EL, at least in part, by regulating PPARγ. It is possible that the transcriptional induction of EL gene might be mediated through binding PPAR-RXR heterodimer, since it is reported that CD36 and the LpL promoter is a direct target of PPAR-RXR heterodimer.[14,38] Others have also shown different effects of saturated vs n-3 FA on PPARγ signaling and these differences are also related to the specific tissue or cell analyzed.[32-37] To clearly understand whether regulation of macrophage EL is most likely mediated through PPARγ-dependent mechanism, further experiments using ligand binding assays are needed to be performed.

A PPARγ-related mechanism for regulation of arterial wall EL by FA is supported by our *in vivo* findings. SAT diets, but not n-3 diets, increased arterial EL and PPARγ mRNA in LDL-R KO mice, and EL was positively correlated with PPARγ ($p<0.01$). Ishida et al[9] reported that EL protein was increased in aorta from apoE KO mice and this was accentuated by a high fat diet (0.15% cholesterol, 21% milk fat)[10]. Also, there was a decrease in atherosclerotic lesions in animals lacking both EL and apoE compared with apoE KO alone.[10] Moreover, EL protein[39] and PPARγ mRNA[40] were increased in the

aorta, especially the atherosclerotic lesions in high-cholesterol diet fed animals. Herein we show that n-3 diets do not share the stimulatory effects of SAT diets *in vivo*.

We also found that, in parallel with the changes in macrophage EL and PPARγ, EPA alone or plus LPS reduced IL-6 and IL-12p40 mRNA in macrophages but increased the mRNA of IL-10 and MR which stimulates anti-inflammatory cytokines production including IL-10.[41] Pro-inflammatory cytokines such as IL-6 and IL-12 promote the development of atherosclerotic lesions,[42,43] whereas anti-inflammatory IL-10 have anti-atherogenic effects.[44] EL as well as PPARγ positively correlated with pro-inflammatory markers but negatively correlated with anti-inflammatory markers. Moreover, similar changes in arterial inflammatory markers were found in LDL-R KO mice, suggesting that FA-regulated inflammatory responses could also occur in *vivo*. In contrast to other inflammatory cytokines mRNA expression, there was no significant effect of EPA on TNF-α mRNA expression in macrophages. Preliminary data (not shown) on LDL-R KO mice also show that feeding of n-3 diets for 12 weeks did not affect TNF-α mRNA expression in aorta compared to chow or SAT diets. Renier et al.[45] reported that macrophages derived from mice fed n-3 diets showed a significant decrease in TNF-α mRNA after 15 weeks, but not 6 weeks, suggesting that the maximum effect of n-3 FA might require a relatively longer observation period than that used in our study.

Several studies have consistently reported the pro-inflammatory effect of EL in macrophages.[12,46] On the other hand, there have been contradictory reports on the potency of PPARγ activation on macrophage inflammatory responses. PPARγ agonists reduced pro-inflammatory cytokine production, including TNF-α and IL-6, in human monocytes,[16] whereas Thieringer et al[19] failed to obtain an inhibitory effect of PPARγ agonists on TNF-α and IL-6 production in human monocytes or macrophages. Furthermore, PPARγ activation appeared to increase plasma cytokine levels in mice after LPS administration.[19] Consistent with this finding, rosiglitazone increased LPS-induced TNF-α production in rat peritoneal macrophages.[20] In fact, a PPARγ activator exerted anti-inflammatory effects in a PPARγ-independent mechanism via inhibition of NF-κB-dependent transcription,[21] and PPAR agonists inhibited cytokine production in PPARγ-deficient macrophages,[22] indicating that anti-inflammatory effects may also be mediated by other biological pathways.

Another possible mechanism by which FA affect macrophage-derived EL may be due to regulation of TLR4 and NF-κB, a transcription factor involved in TLR activation. EL expression was induced by inflammatory cytokines and LPS in endothelial cells and macrophages through NF-κB activation.[39,47] *In vitro* treatment with n-3 FA such as EPA diminished TLR4 and NF-κB signaling, while saturated FA enhanced these.[44,46,48-50] Consistent with these findings, we found that EPA attenuated TLR4 mRNA, and this correlated with lower EL expression. Thus, it seems possible that FA modulation of EL expression may also occur through interference with TLR4 pathways in macrophages. Indeed, Rader's group has shown that EL is increased with increased TLR4 expression.[12] Experiments in TLR4 knock-down cells would be informative in determining if TLR4 is also directly linked to effects of FA on EL.

Our findings describe mechanisms whereby decreases in EL associated with dietary n-3 FA might be associated not only with higher plasma HDL levels, as described by others[51] but also decreases in inflammatory pathways contributory to atherosclerosis. EL was associated with PPARγ expression and the macrophage-derived lipase could be modified by specific FA, in part, through regulating macrophage PPARγ expression. We suggest that similar changes of the EL and inflammation could occur *in vivo*. The ability of EL to anchor LDL in macrophages could also contribute to atherogenesis.[10] We hypothesize that diets rich in n-3 FA (e.g. EPA), in contrast to saturated FA (e.g., PA) decrease progression of

atherosclerosis in humans, in part, by down-regulating inflammatory markers and PPARγ, together with decreasing macrophage EL in the arterial wall.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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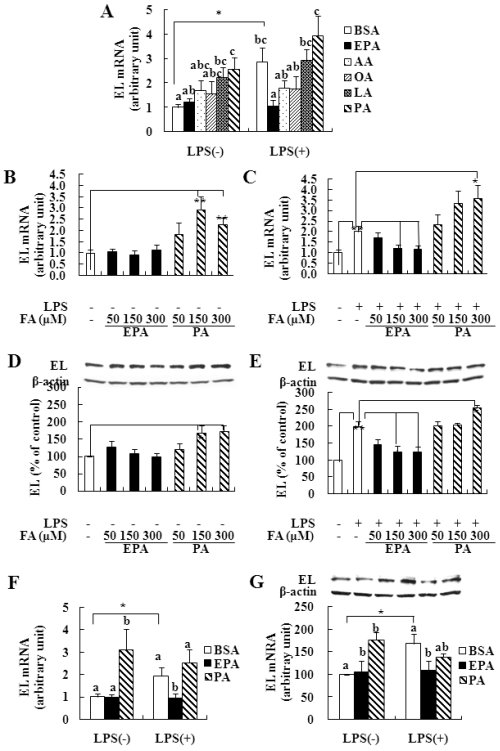

FIGURE 1. Effects of different FA on EL expression in macrophages

J774 (A-E) or peritoneal macrophages (F, G) were cultured in the presence or absence of LPS, 1 μg/mL, for 4h (mRNA, A-C, F) or 21h (protein, D, E, G) after preincubation with 150 μM of FA for 3h. abcMeans with unlike letters are significantly different at $p<0.05$ (one-way ANOVA). *$p<0.05$, **$p<0.01$ (student's t-test).

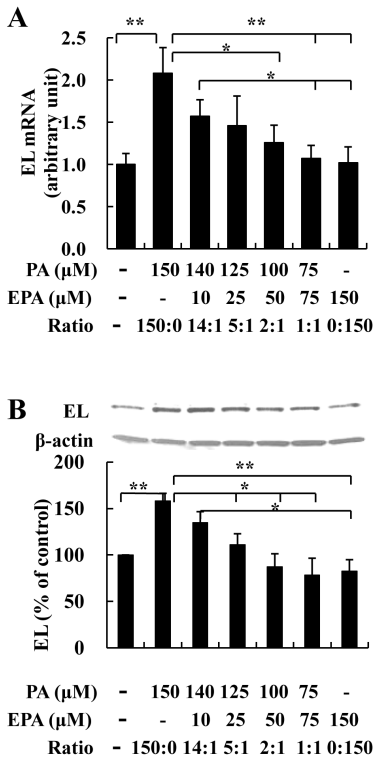

FIGURE 2. Effects of different ratios of EPA and PA on EL

J774 macrophages were incubated with the indicated concentrations of EPA and PA for 7h (mRNA, A) or 24h (protein, B). *p<0.05, **p<0.01 (student's t-test).

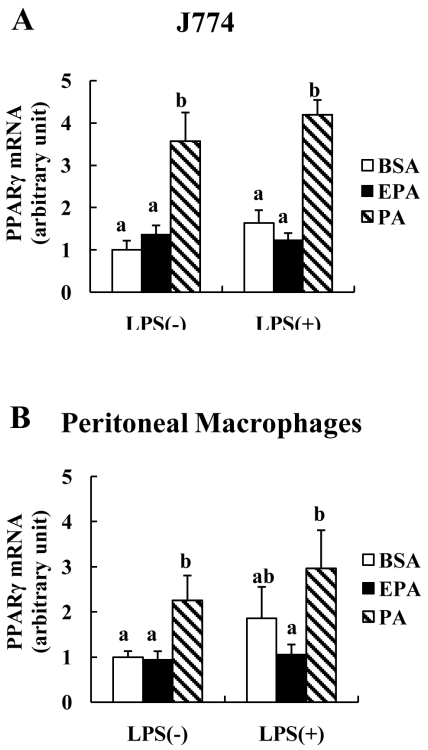

FIGURE 3. Effects of FA on macrophage PPARγ mRNA expression

J774 (A) or peritoneal macrophages (B) were incubated with 150 μM of EPA or PA as previously described in Figure 1. [ab]Means with unlike letters are significantly different at $p<0.05$ (one-way ANOVA).

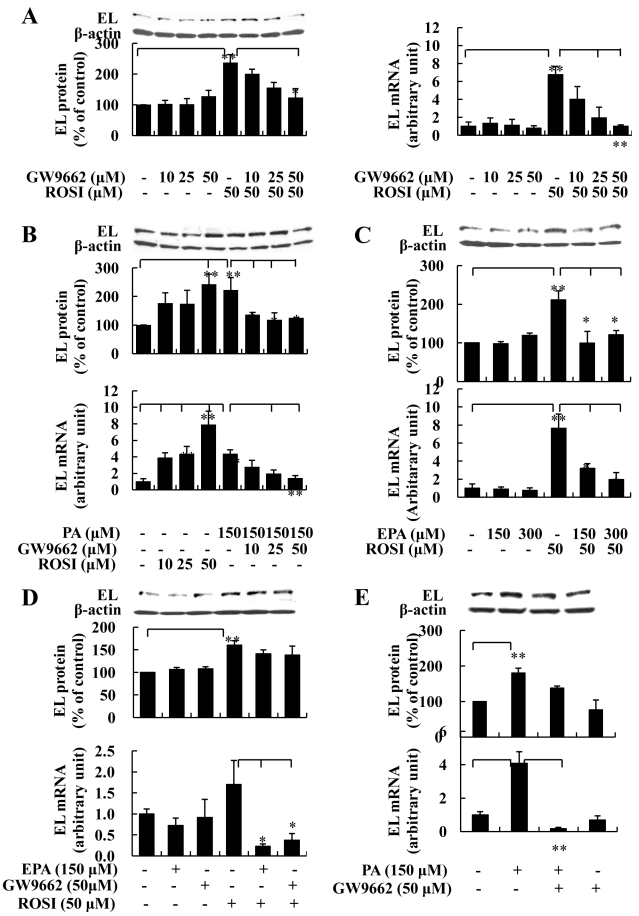

FIGURE 4. Interactions of EPA and PA with a PPARγ agonist/antagonist on EL expression in macrophages

J774 (A-C) or peritoneal macrophages (D-E) were pretreated with or without FA for 3h and then continuously cultured with PPARγ agonist rosiglitazone (ROSI) and/or PPARγ antagonist GW9662 for 4h (mRNA) or 21h (protein). *p<0.05, **p<0.01 (student's t-test).

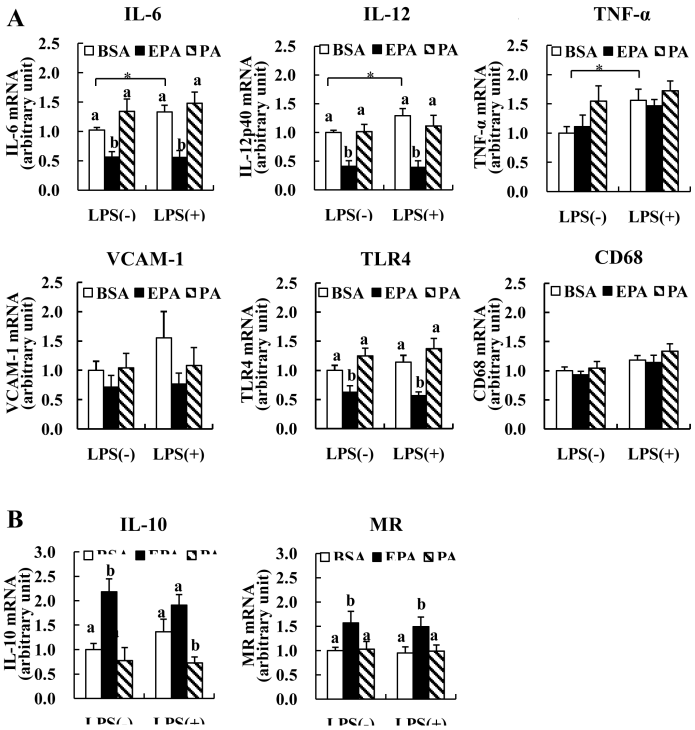

FIGURE 5. Effects of FA on pro- (A) and anti-inflammatory markers (B) mRNA expression in murine peritoneal macrophages

Cells were incubated with 150 μM of EPA or PA as previously described in Figure 1. [ab]Means with unlike letters are significantly different ($p<0.05$). *$p<0.05$ (student's t-test). MR; mannose receptor.

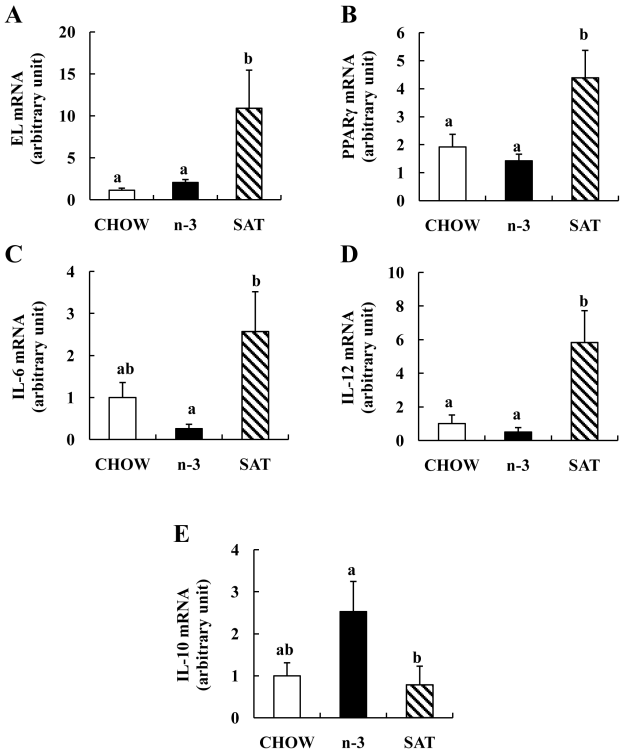

FIGURE 6. Effects of n-3- and saturated FA-rich diets on arterial EL (A), PPARγ (B) and inflammatory markers (C-E) mRNA expression in LDL-R KO mice

[ab]Means with unlike letters are significantly different at $p<0.05$ (one-way ANOVA).