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ω -6 lipids regulate PPAR turnover via reciprocal switch between PGC-1 alpha and ubiquitination

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Abstract

Objective—Dietary ω -6 lipids such as linoleic acid and its oxidized forms (13-HPODE-OxLA) interact with peroxisome proliferator-activated receptors (PPARs) and elicit pro- and anti-atherogenic effects in vascular cells. Ligand-dependent PPAR protein turnover is promoted by ubiquitination, but attenuated by binding to its co-activator, peroxisome proliferator-activated receptor gamma coactivator-1 (PGC-1 α). The objective of our study was to investigate if the dual atherogenic effects of ω -6 lipids are due to its regulation of PPAR turnover.

Methods and Results—In rat aortic smooth muscle cells (RASMCs), oxidized linoleic acid (OxLA) at 10–50 μ M induced and stabilized PPAR γ protein at earlier time points (0–4 hrs) but suppressed it at 12 hrs. Conversely, it activated PPAR γ protein turnover at a later time point (12 hours). Pre-treatment with the proteasome inhibitor (MG132) prevented OxLA mediated loss of PPAR stability and transactivity. Co-immunoprecipitation studies indicated a ligand mediated time-dependent reciprocal exchange of PPAR interaction between ubiquitination and PGC-1 α . This ω -6 lipid mediated time-dependent switch between PPAR degradation versus stability helped modulate the pro- and anti- atherogenic effects of these dietary lipids.

Conclusion—Our findings provide insights into the dual pro- and anti- atherogenic effects of dietary ω -6 lipids on vascular cells by the regulation of PPAR turnover.

Keywords

Oxidized lipids; Proteasome degradation; Vascular cells

Peroxisome proliferators-activated receptors (PPARs), PPAR α and PPAR γ are nuclear receptors that regulate the transcription of genes related to lipid metabolism and insulin sensitivity^{1, 2}. In vascular cells, activation of PPARs (α , γ) by various synthetic ligands induces anti-atherogenic responses including apoptosis of vascular smooth muscle cells and inhibition of their proliferation and migration^{2, 3}.

PPAR turnover is a highly complex phenomenon. All PPAR subtypes (α , β/δ , γ) undergo degradation through the ubiquitin-proteasome system (UPS) which involves a complex multi-step process beginning with the binding of the activated polypeptide ubiquitin to the

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lysine residues of the targeted protein followed by the step-wise action of three enzymes, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin-protein ligase (E3), resulting in a multi-ubiquitinated protein, which is then rapidly degraded by the 26S proteasome system⁴. The complexity of the PPAR isotype specific turnover arises due to its differential regulation upon ligand-binding. Synthetic ligands of PPAR modulate its turnover differently, for example Wy14,643 prevent PPAR α degradation through directly decreasing ubiquitination of PPAR α protein⁵; however, the PPAR γ agonist, rosiglitazone increases its degradation through the UPS⁶. PPAR turnover can also be regulated by its interactions with its co-activator proteins especially, PGC-1 (Peroxisome proliferator-activated receptor gamma coactivator-1)⁷ which inhibits protein degradation by inhibiting ubiquitin ligase⁸. Since the transactivity of PPARs are tightly related to its turnover through the UPS, modulation of this process results in altered PPAR mediated effects.

As one of the essential dietary lipids, linoleic acid (LA, 18:2n-6), an ω -6 polyunsaturated fatty acid (PUFA) that is abundant in the typical Western diet⁹, undergoes oxidation during processing of oils and foods, thus forming atherogenic compounds such as oxidized linoleic acid (OxLA) [13-hydroperoxyoctadecadienoic acid (13-HPODE) and 13-hydroxyoctadecadienoic acid (13-HODE)]¹⁰. These atherogenic ω -6 lipids are major components of low-density lipoproteins and are abundantly present in the atherosclerotic lesions¹¹. Unlike the PPAR synthetic ligands, these endogenous lipids have a low-affinity for both PPAR α and PPAR γ , but still exhibit vascular effects^{12, 13}. However, the physiological significance of the interactions between these endogenous lipids and PPARs remains unclear. We and others have shown that these ω -6 lipids have multiple vascular effects¹¹ which include both pro-^{14, 15} and anti-atherogenic^{16, 17} effects. We also recently observed that the ω -6 lipids (LA and OxLA) activated Egr-1/MCP-1 mediated pro-inflammatory pathway in vascular smooth muscle cells, by modulating the levels of PPARs¹⁸. This time dependent modulation of ω -6 lipids on PPAR transactivational effects might be attributed to their ability to regulate PPAR turnover. Hence the objective of this study was to investigate if these lipids regulate PPAR turnover and thereby modulate PPAR transactivity.

Materials and Methods

Materials

Linoleic acid (LA) and soybean lipoxidase were obtained from Sigma (St. Louis, MO). MG132 (proteasome inhibitor) was obtained from Fisher Scientific (South Bend, IN). Rabbit PPAR α and PPAR γ polyclonal antibody were obtained from VWR (Gilbertsville, PA). Rabbit PGC-1 α polyclonal antibody was obtained from Cell signaling (Boston, MA). Rabbit ubiquitin antibody was obtained from Santa Cruz (Santa Cruz, CA). PPRE-luciferase construct [p(AOX₃)-TKSL] was a gift (Dr. Richard Niles, Marshall University, Huntington, WV). pSV Sport-PPAR α vector, pSV Sport-PPAR γ vector and pSV Sport vector constructs were obtained from Invitrogen (Carlsbad, CA).

Cell Culture and Treatment

Primary rat aortic smooth muscle cells (RASMCs) were cultured in smooth muscle cell growth media (ATCC, Manassas, VA) and used at a passage <20. Quiescent cells were treated with either 50 μ M LA or 10, 25, 50 μ M OxLA for 0, 1, 4, and 12 hours. Oxidized linoleic acid (OxLA) was prepared by oxidizing linoleic acid (0.1mM) using soybean lipoxidase (100 U/100 μ mol, 1 hr at 37°C), as described previously to generate 13-HPODE and HODE¹⁹. Typically, >98% of LA was converted to OxLA. The control (CTRL) was defined as the cells treated with vehicle (alcohol) alone. The *acute exposure phase* was defined as time points between 0-4 hrs. The *sub-acute exposure phase* was defined as time

points between 4–12 hrs. Time points were chosen to accommodate time required for PPAR turnover. All treatments were performed in duplicates and repeated independently three times.

Quantitative Real-Time PCR (qPCR)

Total RNA was extracted from the treated RASMCs using Tri-reagent kit (Sigma, St- Louis, MO) according to the manufacturer's protocol. PPAR α , PPAR γ , Cytochrome P450 family 4 subfamily a polypeptide 1 (Cyp4a1), Lipoprotein lipase (LPL) and β -actin (house-keeping gene) mRNA levels were measured in duplicates by qPCR in myiQ Bio-Rad system. The respective primers used were: PPAR α (NM_017096) 5'-atgccagtactgccgtttc -3', 3'-ggccttgacctgtgtcatgt -5'; PPAR γ (NM_013124) 5'-cattttcaagggtgccagt -3', 3'-gaggccagcatgtgttagat -5'; Cyp4a1 (NM_021838) 5'-atgagcgtctctgcactgag -3', 3'-ttggagaaaggaggaagg -5'; LPL (NM_012598) 5'-ggaggtgccacaaataaaa -3', 3'-ctgaccagcgggaagtaggag -5'; β -actin (BC145810) 5'-tcggtgtgaacggattggccgta-3', 3'-atggactgtggtcatgagcccttc -5'. A sequence detection program calculated a threshold cycle number (Ct) at which the probe cleavage-generated fluorescence, exceeded the background signal using the Pfaffl equation²⁰.

Western Blotting Analysis

The whole cell lysates were subjected to SDS-PAGE. After transfer, blots were probed individually with a solution of anti-rat PPAR α (1:2000), PPAR γ (1:7000) or β -actin (1:1000) as housekeeping protein and then analyzed using the chemiluminescence detection method (Millipore, Billerica, MA). The protein levels were quantified by measuring the density of the bands on the autoradiograph using densitometry (Bio-Rad, Hercules, CA). The results were expressed as the ratio of protein levels in treated samples compared to CTRL.

Co-Immunoprecipitation and detection of Ubiquitinated-PPAR or PPAR-PGC-1 α complexes

RASMCs pretreated with or without the proteasome inhibitor, 40 μ M MG132 for 2 hours, were exposed to either 50 μ M LA or 10–50 μ M OxLA for an additional 4 or 12 hrs. CTRL was defined as cells that had only vehicle treatment. At the end of the respective time points, the cells were lysed on ice in NP-40 buffer (20mM Tris HCL, 137 mM NaCL, 10% glycerol, 1% Nonidet P-40 (NP-40), 2mM EDTA). Co-immunoprecipitation of PPAR α or PPAR γ protein was performed using 50 μ g of cell extracts following the kit manufacturer's protocol (Pierce, Rockford, IL). Either Ubiquitinated PPAR protein (IP:PPAR, IB: Ub) or the PPAR-PGC-1 α bound proteins (IP:PPAR, IB: PGC-1 α) were detected in the immunoprecipitated PPAR protein beads by Western Blotting using either monoclonal ubiquitin antibody (1:500) or PGC-1 α antibody (1:1000). The protein bands on the blots were detected using densitometry and analyzed following the methods discussed under Western blotting. The results were expressed as the ratio of protein bands in treated samples compared to CTRL. Each experiment was performed in duplicate and repeated independently three times.

PPAR transactivity measurement using PPRE- Luciferase Reporter Assay

The transient co-transfection of RASMCs was performed using PPRE-firefly luciferase construct [p(A-OX3)-TKSL] with the constructs of, PPAR α (pSV Sport-PPAR α vector), PPAR γ (pSV Sport-PPAR γ vector), or pSV Sport vector (control vector) in 24-well plates (50,000 RASMCs per well) using the lipofectamine-2000 transfection reagent (Promega, Madison, WI). After 24 hrs of transfection, the cells were pretreated using 40 μ M MG132 or the equivalent concentration of DMSO (vehicle) in serum free EMEM containing 1% charcoal stripped fetal bovine serum for 2 hrs prior to either 50 μ M LA or 10, 25, 50 μ M OxLA treatments for 4 or 12 hrs. After treatment, the cell lysates were assayed for firefly

(PPRE) luciferase activities using the Luciferase Reporter Assay System (Berthold, Germany). Each experiment was performed in duplicate and repeated three times. The cells with co-transfection of PPRE and pSV Sport construct were used as the baseline value. The relative luciferase units (RLU) were expressed as the ratio of the relative luciferase units in the treated samples/baseline value.

Statistical Analysis

The real-time PCR results were expressed as “mean of fold change $\pm$ SEM (Standard Error of Δ Ct mean)” in the experimental gene compared to the house keeping gene using the Pfaffl method ($2^{-(\Delta\Delta Ct)}$)²⁰. All statistics were performed at the Δ Ct stage in order to exclude potential bias due to averaging of data transformed through the equation $2^{-(\Delta\Delta Ct)}$. The PPRE relative luciferase units and Western blotting results were expressed as “mean $\pm$ SEM”. One way ANOVA was used for the comparison between two treatments. Significance was confirmed by post-hoc analysis using Fisher’s least significant difference (Fisher’s LSD) test. A $p < 0.05$ was considered statistically significant. The correlation between changes in mRNA levels of Cyp4a1 and LPL and the PPAR α and PPAR γ protein levels, respectively were calculated for the entire time course using Pearson’s correlation analysis. A two-tailed value of $p < 0.05$ indicated statistical significance.

Results

Time dependent effect of ω -6 lipids on mRNA levels of PPARs

ω -6 lipids, LA and OxLA treatments on RASMCs had a time dependent effect on mRNA levels of PPARs as determined by qPCR. As shown in Fig. 1A, 50 μ M LA treatments had no apparent effect on PPAR α mRNA levels between 1-4 hrs, but increased PPAR α mRNA levels (not significantly) at 12 hours compared to CTRL. OxLA, at all concentrations, had no apparent effect on the mRNA levels of PPAR α between 1-4 hours; however, the levels significantly increased by 2.81 fold ($p < 0.05$) and 3.49 fold ($p < 0.05$), at 25 and 50 μ M OxLA, respectively at 12 hours.

In contrast, the mRNA regulation of PPAR γ by LA and OxLA had an opposite trend to that observed on PPAR α mRNA. PPAR γ mRNA levels were increased significantly at 4 hrs, by both 50 μ M LA (11.09 fold ($p < 0.01$)) and by 25 μ M (6.58 fold ($p < 0.05$)) and 50 μ M (12.07 fold ($p < 0.01$)) OxLA treatments, but decreased to near baseline levels at 12 hrs, compared to the CTRL, as shown in Fig. 1B.

Differential effects of ω -6 lipids on PPAR α turnover

PPAR turnover through the UPS is ligand-dependent. Alternately, the PPAR co-activator PGC-1 α , can attenuate ubiquitin ligase of the UPS pathway⁸. Therefore, the stability of PPAR protein may be dependent on its reciprocal interaction between PGC-1 α and ubiquitin. As shown in Fig. 2A, RASMCs treated with LA (50 μ M) or OxLA at all concentrations, contrary to their effect on mRNA levels, increased PPAR α protein levels at the acute phase (4 hrs). At similar time points, co-immunoprecipitation studies (IP:PPAR, IB:Ub) showed that the levels of ubiquitinated-PPAR α proteins were attenuated by both LA and OxLA in a concentration-dependent manner. In contrast, although PGC-1 α protein levels at 4 hrs were unchanged, co-immunoprecipitation (IP:PPAR, IB:PGC-1 α) showed that the PPAR α -PGC-1 α complex was significantly enhanced by LA and OxLA at this time point. Administration of the proteasome inhibitor, MG132, did not alter the levels of ubiquitinated-PPAR α or PPAR α -PGC-1 α at 4 hrs.

At 12 hours, (Fig. 2B), LA and OxLA treatment significantly ($p < 0.05$) decreased PPAR α protein levels. The baseline levels of PGC-1 α protein were still unchanged at this time

point; however, LA and OxLA significantly attenuated the levels of PPAR α -PGC-1 α complex and enhanced the ubiquitination of PPAR α in a concentration dependent manner. Likewise, the addition of MG132 accumulated more PPAR α -ubiquitin complex than PPAR α -PGC-1 α complex.

These findings suggest that LA and OxLA probably recruit PGC-1 α to stabilize the PPAR α protein and attenuate PPAR α ubiquitination at an earlier time point (*1-4 hours*). However, at a later time point, since there was a lower PPAR α -PGC-1 complex formation it resulted in increased PPAR degradation, *i.e.* appearance of higher levels of ubiquitinated-PPAR α protein.

Differential effects of ω -6 lipids on PPAR γ turnover

As shown in Fig. 3A, both LA and OxLA decreased PPAR γ protein at the *acute phase* (*1-4 hrs*). This process was accompanied with an increased accumulation of ubiquitinated-PPAR γ protein and decreased levels of PPAR γ -PGC-1 α complex. Inhibition of the proteasome by the addition of MG132 still showed enhanced ubiquitinated-PPAR γ protein in the presence of 50 μ M LA and 10-25 μ M OxLA treatments, but not in OxLA 50 μ M. In contrast, addition of MG132 only slightly altered the levels of PPAR γ -PGC-1 α complex. Fig. 3B showed that at the *sub-acute phase* (*12 hours*), LA and OxLA stabilized PPAR γ protein levels at all concentrations and hence no apparent changes in PPAR γ protein levels were seen, but a significantly lower ubiquitinated-PPAR γ levels was observed. The addition of MG132 exhibited an accumulation of ubiquitinated-PPAR γ in the lipid treated cells. Neither the levels of PGC-1 α or PPAR γ -PGC-1 α complex changed at this time point with or without MG132 treatment. These findings indicated that the OxLA interactions with PPAR γ are different from that of PPAR α .

Modulation of PPAR turnover by ω -6 lipids altered PPAR transactivity

Results in Fig. 4A, showed that LA and OxLA activated PPAR transactivity in a time and concentration-dependent manner in RASMCs. After *4 hrs* of treatment, there was an increase in PPAR α transactivity as measured by induction of PPRE-luciferase activity. There was a significant increase in RLU by 10, 25 and 50 μ M ($p < 0.05$) OxLA treatments. In the presence of the proteasome inhibitor MG132 at *4 hrs*, there was no significant modification of the transactivity of PPAR α compared to the baseline, though still significantly higher than CTRL levels. However, at *12 hrs* treatment, there was very little PPAR α transactivity, as seen by a decrease in RLU levels by LA and even further decrease by 10, 25 ($p < 0.05$) and 50 μ M ($p < 0.05$) OxLA. However, MG132 pretreatment significantly induced both the basal and ligand mediated PPAR α transactivity compared to *12 hours* baseline levels, although the lipid treatment had significantly lower RLU than its baseline levels, Fig. 4B.

Ligand mediated activation of PPAR γ response genes are short-lived⁶. Therefore, PPAR γ transactivity was measured only at *4 hrs* (Fig. 4C). There was no induction of PPAR γ transactivity by OxLA at *4 hours*. However in the presence of proteasome inhibitor MG132, there was an induction of transactivity both in CTRL ($p < 0.01$) and lipid treated cells, compared to treatments without the proteasome inhibitor. Lipid treatment however, inhibited PPRE transactivity compared to CTRL.

Modulation of PPAR turnover by ω -6 lipids altered the expression of PPAR response genes

Ligand mediated PPAR turnover through the UPS modulates PPAR transactivity^{5, 6}. Since OxLA treatment modulated the turnover of PPAR in a time dependent manner by

reciprocally switching its interactions with the UPS versus PGC-1 α complex, it should also have an altered effect on the downstream PPAR response genes at similar time points.

The gene expression of Cyp4a1 and LPL as representative downstream genes regulated by either PPAR α or PPAR γ ^{21, 22}, was determined. Regulation of PPAR α target genes is dependent upon the stability and availability of the PPAR α protein⁵. In our studies, as shown in Fig. 5A, similar to what was observed with PPAR α protein stability, treatment of RASMCs with LA or OxLA up-regulated Cyp4a1 gene expression around 4 hrs, after which there was a down-regulation of Cyp4a1 mRNA levels by 12 hrs. Pearson's correlation showed a significant positive correlation between Cyp4a1 mRNA levels at various time points with PPAR α protein levels (insert Fig. 5A). Pre-treatment with MG132 prior to the lipid treatment showed an earlier increase in Cyp4a1 mRNA in the acute phase (1-4 hrs) and stabilization at 12 hrs (Fig. 5B). This suggested a time-dependent stability of PPAR α protein by the ω -6 lipids.

Lipoprotein lipase (LPL) is a PPAR γ response gene^{22, 23}. Similar to the weaker effects of OxLA on PPAR γ protein turnover, the mRNA levels of LPL was lower around 1-4 hrs after LA or OxLA treatment. LPL mRNA levels returned to baseline levels at 12 hrs after both LA and OxLA treatments, as shown in Fig. 5C. There was also a weak positive correlation between LPL mRNA levels and PPAR γ protein levels at similar time points (insert Fig. 5C). Conversely, MG132 addition attenuated the decrease in LPL mRNA seen in the presence of LA and OxLA, although a significant increase in LPL mRNA was seen only at 50 μ M OxLA ($p < 0.05$) treatment, Fig. 5D.

Discussion

Ligand dependent and independent regulation of PPARs is an area of intense research. Nuclear receptors such as PPARs are regulated by small-molecule ligands by inducing conformational changes and co-repressor or co-activator interactions. Other than the synthetic ligands, TZDs or fibrates, PPARs are also activated by several small molecule ligands that exhibit diverse chemical structures including oxidized and nitrated fatty acids^{12, 13}. Though the cellular fate of PPARs are related to the UPS⁴, in that these are short-lived proteins that undergo ubiquitination and proteasomal degradation, the various PPAR isotypes differ in respect to their ligand-dependent turnover. PPAR α expression undergoes diurnal variations and has been shown to exhibit a short half-life (1–3 hours)⁵. Ligand activation seemed to stabilize the PPAR α protein and prevent degradation through the proteasome pathway. This ligand mediated stabilization, though rapid, is only transient, which can be attributed to either rapid metabolism of the ligand or the recruitment of PPAR co-activators or co-repressors. In contrast, ligand binding to PPAR γ resulted in a negative auto-regulation of the protein, *i.e.* ligand binding increased the degradation of the PPAR γ protein by the UPS pathway^{6, 24}.

PGC-1 α , initially identified as the protein that interacts with PPAR γ ⁷, was mostly known for its role as a transcriptional co-activator in the regulation of adaptive thermogenesis and numerous pathways that controls metabolism and energy homeostasis²⁵. PGC-1 α is also an important PPAR co-activator, which interacts differently with the two PPAR isotypes. For example, it uses its nuclear hormone receptor interacting motif, LXXLL (Leu-Xaa-Xaa-Leu-Leu) present in its N-terminal region to interact with the AF-2 (activation function-2) region present in the C-terminal domain of the nuclear receptors such as PPAR α ²⁶. However, the interaction between PGC-1 α and PPAR γ is through its hinge region of PPAR γ instead of the LXXLL/AF-2 binding site. Also, there is an absolute requirement of the LXXLL motif in the PGC-1 α for its ligand dependent interaction with PPAR α , however it can use non-LXXLL motifs to interact with PPAR γ ²⁷. Interestingly, the AF-2 region of the PPARs that

interacts with the N-terminal motif of PGC-1 α also contains the sequence responsible for ubiquitination. This reveals the importance of the AF-2 region in the ubiquitination mediated degradation of PPARs²⁸.

Our studies indicate that ω -6 lipids, such as LA and OxLA, differently regulated the turnover of the two PPAR isotypes in a time and concentration-dependent manner. Analysis of the PPAR mRNA and protein levels showed that these lipids, in a concentration-dependent manner, stabilized and activated PPAR α protein in the acute phase (*1-4 hrs*) and decreased it at a later time point (*12 hours*), but had the opposite effect on PPAR γ turnover. Co-immunoprecipitation studies showed that LA and OxLA induced a time-dependent competition between PGC-1 α and ubiquitin binding to PPARs. This data also suggested that the interaction of PGC-1 α with PPARs might enhance PPAR protein stability. In contrast, a loss of PGC-1 α and PPAR interaction, when replaced with ubiquitin binding, contributed to PPAR degradation. The common binding site AF-2, present in PPARs that interacts with both PGC-1 α and ubiquitination pathway may be responsible for the reciprocal competition between the two regulating proteins, resulting in either stabilization or degradation of the PPAR protein. Our findings also implicated that ω -6 lipids modulated the switch between the interaction of PGC-1 α or ubiquitin to PPAR α and PPAR γ in a time-dependent manner. This might suggest that the reciprocal switch between PGC-1 α and ubiquitination actually determines the half-life of the two PPAR isotypes.

In our studies both unoxidized and oxidized LA modulated PPAR turnover in vascular cells. It is well known that both unoxidized LA and its oxidized forms (OxLA) are natural ligands for PPARs and modulate PPAR activity; however, structure-function studies reveal differences in their extent of interactions with PPARs^{13, 29} which might be attributed to the presence or absence of additional polar groups in the lipid. In addition, we have shown earlier that although the majority (<90%) of the unoxidized lipids entered vascular cells unchanged, possibly through passive diffusion, in contrast, only around 3-10% of OxLA entered these cells unchanged³⁰, thus speculating that there either is a receptor-mediated uptake for oxidized lipids or these lipids interact with macromolecules in the membrane, triggering a vascular response. Therefore, although we observed both LA and OxLA to have an effect on PPAR turnover, due to differences in their intra-cellular availability and their ability to covalently interact with PPAR proteins, OxLA might be more efficient than LA at similar concentrations, to modulate PPAR turnover and transactivity. Interestingly, our results did indicate that the effect of these endogenous lipids were more regulatory of PPAR protein levels compared to PPAR mRNA levels. The lipids also expressed a higher regulatory effect on PPAR α compared to PPAR γ turnover, which might partially be attributed to the levels of these isotypes in the vascular smooth muscle cells. Since PPARs are present in several tissues, the tissue specificity of these endogenous lipids on PPAR turnover will be important to investigate in the future.

Ligand binding also stabilizes PPAR's transactivity. Thus, we found that PPRE-luciferase transactivity and the levels of the PPAR response genes - Cyp4A1 and LPL, also responded to the OxLA treatment in a time and concentration dependent manner. Therefore, the dual vascular effects of ω -6 lipids can be attributed to the balance between the relative levels and activity of PGC-1 α and UPS interaction with PPAR isotypes, which, in turn, provides the regulatory convergence point for the induction of PPAR response gene targets. Our results also suggested that the extent of exposure time of ω -6 lipids may be the key factor in modulating PPAR turnover, which may also explain our earlier findings on the time-mediated modulation of the Egr-1/MCP-1 pathway by OxLA in vascular cells¹⁸. Though our studies do provide insights into the possible mechanism involved in the dual behavior (pro- and anti-atherogenic effects) of ω -6 lipids on vascular cells; more studies are needed to conclude if oxidized lipids are beneficial or harmful.

Conclusion

In conclusion, we have demonstrated that the endogenous lipid mediators, such as LA and OxLA, which are available in excess through dietary sources or generated at lower levels *in vivo*, but still can reach significant concentrations during pathological conditions, in a time and concentration dependent manner, can modulate PPAR turnover, thereby, regulating vascular effects.

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HIGHLIGHTS

- Dietary ω -6 lipids such as linoleic acid and their oxidized forms exhibit both pro- and anti-atherogenic effects on vascular cells.
- ω -6 lipids are biological ligands for the transcription factor peroxisome proliferators-activated receptors (PPAR).
- Our study reports that ω -6 lipids by modulating PPAR turnover and transactivity in a ligand and time dependent mechanism by reciprocal interaction of PPARs between PGC-1 and ubiquitination, are able to exhibit dual atherogenic effects on vascular cells.

Figure 1. Time-dependent induction of PPAR mRNA by ω -6 lipids
mRNA levels of PPAR α and PPAR γ from RASMCs treated with 50 μ M LA or 10-50 μ M OxLA for 0-12 hrs were determined using qPCR. Control (CTRL)-vehicle only. Data expressed as relative expression of fold change $\pm$ SEM (Standard error of Δ CT mean). **A.** PPAR α mRNA; **B.** PPAR γ mRNA. *:p< 0.05, **: p< 0.01.

Figure 2. ω -6 lipids modulate PPAR α turnover via a reciprocal switch between PGC-1 α and UPS

Western blot of cell lysates from RASMCs exposed to 50 μ M LA or 10-50 μ M OxLA for 4 or 12 hrs (with or without pre-treatment with proteasome inhibitor, 40 μ M MG132) to detect PPAR α or PGC-1 α protein. Co-immunoprecipitation followed by Western blot of cell lysates to detect ubiquitinated PPAR α (IP: PPAR α , IB:Ub) or PPAR α -PGC-1 α (IP:PPAR α , IB: PGC-1 α) proteins. **A-B.** PPAR α turnover at 4 hrs and 12 hrs. Control (CTRL): vehicle only. The results expressed as mean $\pm$ SEM, defined by the ratio of protein levels in treated samples compared to CTRL. *: $p < 0.05$.

Figure 3. ω -6 lipids modulates PPAR γ turnover via a reciprocal switch between PGC-1 α and UPS

Western blot of lysates of RASMCs exposed to 50 μ M LA or 10–50 μ M OxLA for 4 or 12 hrs (with or without pre-treatment with 40 μ M MG132) to detect PPAR γ or PGC-1 α protein. Co-immunoprecipitation followed by Western blot of cell lysates to detect ubiquitinated PPAR γ (IP: PPAR γ , IB: Ub) or PPAR γ -PGC-1 α (IP: PPAR γ , IB: PGC-1 α) proteins. **A-B**. PPAR γ turnover at 4 hrs and 12 hrs. Control (CTRL): vehicle only. The results expressed as mean $\pm$ SEM defined by the ratio of protein levels in treated samples compared to CTRL. *: $p < 0.05$.

Figure 4. Modulation of PPAR turnover by ω -6 lipids altered PPAR transactivity RASMC co-transfected with PPRE-luciferase construct along with either PPAR α , PPAR γ or pSV Sport vector (control vector) followed by pretreatment with or without MG132 prior to exposure to ω -6 lipids for 4 or 12 hrs for PPAR α or 4 hrs only for PPAR γ transactivity. Relative Luciferase Units (RLU), the ratio of luciferase activity in the treated samples to the CTRL. The data expressed as mean $\pm$ SEM. **A-B.** PPAR α transactivity at 4 hrs and 12 hrs. **C.** PPAR γ transactivity at 4 hrs. *: comparison within each group, $p < 0.05$. \$: comparison between groups.

Figure 5. Modulation of PPAR turnover by ω -6 lipids altered PPAR response genes mRNA levels of Cyp4a1 and LPL, the respective target genes of PPAR α and PPAR γ were determined using RT-qPCR. **A-B:** Cyp4a1 mRNA expression in the presence or absence of MG132 (insert- correlation between Cyp4a1 and PPAR α protein); **C-D:** LPL mRNA expression in the presence or absence of MG132 (insert-correlation between LPL and PPAR γ protein); *:p < 0.05.