Available online at www.sciencedirect.com
SCIENCE DIRECT®
Biochemical Pharmacology 67 (2004) 841–854
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Enzymatically modified low-density lipoprotein upregulates CD36 in low-differentiated monocytic cells in a peroxisome proliferator-activated receptor-γ-dependent way

K. Jostarndt[a], T. Rubic[a], H. Kuhn[b], M.W. Anthosen[c], L. Andera[d], N. Gellert[a,e], M. Trottman[a], Christian Weber[a,f], B. Johansen[c], N. Hrboticky[a], J. Neuzil[a,e,g,*]

[a]*Institute for Prevention of Cardiovascular Diseases, Ludwig Maximilians University, 80336 Munich, Germany*
[b]*Institute of Biochemistry, University Clinics Charite, Humboldt University, 10117 Berlin, Germany*
[c]*Department of Biology, Norwegian University of Science and Technology, 7491 Trondheim, Norway*
[d]*Institute of Molecular Genetics, Czech Academy of Sciences, 14220 Prague, Czech Republic*
[e]*Heart Foundation Research Centre, School of Health Sciences, Griffith University Gold Coast Campus, Southport, 9726 Queensland, Brisbane, Australia*
[f]*Department of Cardiovascular Molecular Biology, University Hospital, 52074 Aachen, Germany*
[g]*Division of Pathology II, Faculty of Health Sciences, University Hospital, 58185 Linköping, Sweden*

Received 2 August 2003; accepted 30 September 2003

Abstract

Peroxisome proliferator-activated receptor-γ (PPARγ) has been suggested to upregulate CD36. Since free oxidized polyunsaturated fatty acids are PPARγ ligands, we studied the effects of LDL modified by the simultaneous action of sPLA2 and 15-lipoxygenase (15LO) on CD36 expression and PPARγ activation in monocytic cells. Exposure of MM6 cells, which do not express CD36 or other scavenger receptors, to such enzymatically modified LDL (enzLDL) resulted in upregulation of CD36 surface protein and mRNA expression. Similar effects were observed with free 13-hydroperoxyoctadecadienoic acid but not its esterified counterpart. Less pronounced effects were observed with LDL modified by 15LO alone. Upregulation of CD36 was inversely correlated to the state of cell differentiation, as showed by lower response to enzLDL of the scavenger receptor-expressing MM6-sr and THP1 cells. Importantly, LDL modified by sPLA2 and 15LO did not efficiently induce upregulation CD36 in PPARγ-deficient macrophage-differentiated embryonic stem cells confirming a role of PPARγ in CD36 expression in cells stimulated with enzLDL. Our data show that LDL modified with physiologically relevant enzymes stimulates CD36 expression in non-differentiated monocytes and that this process involves PPARγ activation. These effects of enzLDL can be considered pro-atherogenic in the context of early atherosclerosis.

Keywords: Modified low-density lipoprotein; Monocytic cells; Phospholipase A2; 15-Lipoxygenase; Peroxisome proliferator-activated receptor-γ; Atherosclerosis

* Corresponding author. Tel.: +61-7-5552-9109; fax: +61-7-5552-8804.
E-mail address: j.neuzil@griffith.edu.au (J. Neuzil).

Abbreviations: CE-HODE, cholesteryl ester hydroxyoctadecadienoic acid; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; dPGJ$_2$, deoxy-$\Delta^{12,14}$-prostaglandin J$_2$; enzLDL, enzymatically modified low-density lipoprotein; ES cells, embryonic stem cells; FACS, fluorescence-assisted cell sorting; HETE, hydroxyeicosatetraenoic acid; HODE, hydroxyoctadecadienoic acid; LDLR, LDL receptor; LIF, leukemia inhibitory factor; LPS, lipopolysaccharide; 15LO, 15-lipoxygenase; MM6, Mono Mac 6; NBF, neutral-buffered formalin; oxLDL, oxidized LDL; sPLA2, secretory phospholipase A2; PPARγ, peroxisome proliferator-activated receptor-γ; PPRE, PPAR-response element; PUFA, poly-unsaturated fatty acids; REM, relative electrophoretic mobility; RT–PCR, reverse transcriptase–polymerase chain reaction; TNFα, tumor necrosis factor-α.

1. Introduction

Atherosclerosis is a disease whose initiation and progression involves dysregulation of the immune system, and this process can be induced and/or exacerbated by oxidatively modified LDL [1]. Multiple reports have shown that such LDL is pro-atherogenic [2], and that this activity is associated in particular with minimally modified LDL [2–4]. Several studies have suggested that lipoproteins isolated from atherosclerotic lesions resemble mildly oxidized LDL with specific modified lipids rather than heavily oxidized LDL [5,6], and this invokes the role of certain

0006-2952/$ – see front matter © 2003 Published by Elsevier Inc.
doi:10.1016/j.bcp.2003.09.041

enzymes, such as 15-lipoxygenase (15LO). Involvement of 15LO in LDL oxidation has been stipulated based on *in vitro* [7,8] as well as *in vivo* data [6,9–11]. 15LO is capable of oxygenating a variety of substrates, with free polyunsaturated fatty acids (PUFA) being preferred over their esterified counterparts [12]. These moieties can be generated in LDL by the action of various lipolytic enzymes. We have found that both secretory phospholipase A2 (sPLA2) and lipoprotein lipase greatly enhance 15LO catalyzed oxidation of LDL lipids [13]. The role of sPLA2 in atherogenesis has also been suggested [14], and its expression can be upregulated by mildly oxidized LDL [15].

The concerted action of sPLA2 with 15LO on LDL gives rise to several specific oxidation products with hydroxyoctadecadienoic acid (HODE) and hydroxyeicosatetraenoic acid (HETE) being particularly abundant in modified lipoproteins [12]. A number of biological activities of hydroxy fatty acids have been suggested [16], including activation of peroxisome proliferator-activated receptor-γ (PPARγ) [17]. PPARγ is a member of a family of nuclear transcription factors with pleiotropic modulatory effect on expression of genes involved in lipid metabolism, with consequences for the development of various pathologies [18] including atherosclerosis [19].

Upon interacting with a ligand, PPARγ binds to PPAR-response element (PPRE) in the promoter region of target genes [20–22]. Activation of PPARγ may also involve specific phosphorylation [23]. The notion of potential role of PPARγ in atherogenesis has been supported by reports showing activation of PPARγ in monocytic cells exposed to oxidized LDL (oxLDL) [17], with ensuing upregulation of the scavenger receptor CD36 and oxLDL uptake, and differentiation of the cells into macrophages [24].

A role for PPARγ in CD36 upregulation is particularly intriguing, as this scavenger receptor appears to recognize the modified lipids rather than modified apolipoprotein B [25].

PPARγ has also been implicated in the regulation of macrophage apoptosis [20], in secretion of proinflammatory cytokines [26–28], and in oxLDL-dependent downregulation of the chemokine CCR2 expression [29]. A link between 15LO metabolism and PPARγ-dependent signaling has been suggested by experiments in which macrophages were exposed to the TH_2-derived cytokines [30], since IL4 and IL13 are potent stimulators of 15LO expression [31].

The involvement of PPARγ activation in the above-mentioned processes has been concluded largely from experiments using agonistic PPARγ ligands, such as 13HODE, deoxy-$\Delta^{12,14}$-prostaglandin J_2 ($dPGJ_2$), and the antidiabetic thiazolidinedione and non-steroid anti-inflammatory drugs. However, recent reports have suggested that PPARγ is not necessarily required in all of these processes. For example, it now appears that PPARγ activation may not be involved in inhibition of proinflammatory cytokines production by macrophages exposed to LPS [32]. The most direct evidence for a possible role of PPARγ in various cellular processes comes from recent experiments with PPARγ-deficient mbryonic stem cells (ES cells), which have been differentiated into macrophages [33,34]. These reports indicate that PPARγ is required neither for macrophage differentiation nor for secretion of pro-inflammatory cytokines. However, it appears important for upregulation of CD36 by various PPARγ agonists, such as $dPGJ_2$ or the thiazolidinedione drugs [33,34].

As 15LO in concert with sPLA2 generates PPARγ ligands in the LDL particle [13], we investigated whether LDL modified with the two enzymes (enzymatically modified low-density lipoprotein, enzLDL) regulates CD36 expression in monocytic cells and whether this process is PPARγ-dependent. Our data suggest that upregulation of CD36 expression by enzLDL in non-differentiated monocytic cells involves PPARγ, and these findings are consistent with a role of enzymatically oxidized LDL in early phases of atherogenesis.

2. Materials and methods

2.1. Cell culture and treatment

Mono Mac 6 (MM6) and MM6-sr cells were grown in RPMI 1640 medium containing 10% FCS and supplements as specified elsewhere [35]. THP1, U937 and Jurkat cells were grown in RPMI 1640 medium with 10% FCS. Endothelial cells were prepared from umbilical cords and maintained in the endothelial cell medium (Promo Cell) [36]. Human fibroblasts were prepared as described [37] and maintained in DMEM with 10% FCS. Human monocytic cells were prepared from blood of healthy volunteers by immunomagnetic sorting using anti-CD14 IgG beads (Miltenyi Biotec), adhered to plastic, and maintained in complete DMEM. The PPARγ$^{-/-}$ ES cells were prepared and differentiated into macrophages as described [33]. In brief, PPARγ$^{-/-}$ (clone AC5) and PPARγ$^{+/+}$ (clone J1) ES cells were grown in DMEM supplemented with 10 ng/mL mouse recombinant LIF (Sigma) on a confluent monolayer of STO feeder cells. When about 75–80% confluent, ES cells were separated from STO cells, and re-plated in a 6-well plate at the density of $0.1–0.3 \times 10^6$ per well in the IMDM/DMEM LIF-free medium. After 3–4 days, embryonic bodies formed. The cells were then supplemented with fresh medium containing 10% of the medium conditioned by growth of the IL3- and MCSF-overexpressing L929 cells [33], and incubated for further 4–5 days before use in experiments.

Cells were exposed to 50 μg/mL LDL modified as shown in figure legends for 1–3 days. In some experiments, cells were treated with 13HODE (Cayman), cholesteryl ester hydroxyoctadecadienoic acid (CE-HODE) prepared by 15LO-dependent oxygenation of cholesteryl linoleate [38], lysophosphatidyl choline, 7-ketosterol, 25-hydroxy-

cholesterol, indomethacin (all Sigma), $dPGJ_2$ (Cayman), or tumor necrosis factor-α (TNFα) (PharMingen). Where indicated, cells were co-treated with NH_4Cl (15 mM), or pre-treated with anti-LDLR IgG (250 μg/mL; 2 hr, 37°; clone C-7; Santa Cruz) or irrelevant mouse IgG (MOPC-1, Sigma).

Cell viability was regularly checked by the trypan blue method, which revealed minimal cell death (less then 10%) in the experiments performed during this study. Modest (not more than 25%) cell death was observed in cells exposed for prolonged periods to heavily oxidized LDL or $dPGJ_2$.

2.2. LDL preparation, modification and analysis

LDL was prepared by 2-hr ultracentrifugation from plasma obtained from healthy volunteers using the Beckman TLX table-top ultracentrifuge [39]. Protein concentration of LDL was adjusted to 0.2 mg/mL, and the lipoprotein incubated at 37° with 5 μM $CuSO_4$ for 3 (ox_3LDL) or 20 hr (ox_{20}LDL). For enzymatic modifications, LDL (3.6 mg/mL) was treated with soybean 15LO (Sigma, L6632; 10^6 units/mg protein) at 0.15 mg enzyme/mL LDL (loLDL), or porcine sPLA2 (Sigma, P6534; 1.6×10^4 units/mg protein) at 63 μg enzyme/mL LDL for 12 hr (plaLDL). LDL treated with the two enzymes together is referred to as enzLDL. In some experiments, rabbit reticulocyte 15LO [40] and human recombinant sPLA2 [41] were used for LDL modification at activities comparable with those of soybean 15LO and porcine sPLA2, respectively. Following incubation with the enzymes, LDL was reisolated using the 2-hr ultracentrifugation method [39]. In some experiments, enzLDL was incubated with fatty acid-free BSA (Sigma, A0281) for 3 hr at 37° (0.2 mg/mL LDL protein and 10 mg/mL BSA), and the lipoprotein re-isolated by ultracentrifugation as above.

Control and modified LDL were analyzed for HETE and HODE by HPLC as described elsewhere [7]. The method is based on extraction of the lipid before (free HODE/HETE) and after hydrolysis (free plus esterified HODE/HETE), followed by sequential reverse-phase, straight-phase and chiral-phase HPLC for resolution of the positional isomers and enantiomers. The extent of LDL oxidation was also assessed by estimation of relative electrophoretic mobility (REM) using agarose gel electrophoresis.

2.3. RT–PCR

Total RNA was isolated from MM6 cells (2×10^6) using the Quantum Prep® AquaPure RNA isolation kit (BioRad) according to the manufacturer's protocol. All primers were synthesized by Metabion. The sequences for actin, CD36 and LDL receptor (LDLR), and the reverse transcriptase–polymerase chain reaction (RT–PCR) conditions have been described [42,43], the sequences used for PPARγ were: CATGCTTGTGAAGGATGCAAG (forward) and TTCTGAAACCGACAGTACTGACAT (reverse). For all RT–PCR reactions the Ready-To-Go RT–PCR-Beads (Amersham Pharmacia) were used. PCR products were analyzed by agarose electrophoresis and quantified by HPLC using a DEAE ion-exchange column (Perkin-Elmer) with a solvent gradient of 0.3–0.6 M NaCl buffered at pH 9.0 [44]. Quantitative real-time PCR was carried out in some cases for CD36, LDLR and PPARγ using 18S rRNA as the house-keeping gene. The primers used were as above for RT–PCR, except for those for 18S rRNA, which were described elsewhere [45]. The analyses were carried out in the ABI PRISM 7700 Sequence Detector (Applied Biosciences) using conditions as published [45].

2.4. LDL uptake studies

Modified LDL (1.5 mg/mL) was fluorescence-labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) at 0.225 mg/mL in the presence of lipid-deficient calf serum (2 mL/mg LDL protein) and 100 μM ascorbic acid for 8 hr at 37°. Cells were washed with RPMI medium containing 0.5% BSA and adjusted to 2×10^6 mL^{-1}. Subsequently, cells were transferred to 96-well plates (0.2×10^6 per well) and incubated with labeled LDL (10 μg/mL) for 2 hr at 37° alone or after specific binding had been blocked with unlabeled LDL (500 μg/mL) for 15 min. In some cases, cells were pre-incubated (2 hr, 37°) with anti-LDLR IgG (clone C-20; Santa Cruz) at 250 μg/mL. After incubation, cells were washed with PBS containing 0.5% BSA, fixed with 2% neutral-buffered formalin (NBF), and LDL uptake assessed by fluorescence-assisted cell sorting (FACS; (FACScan).

2.5. Flow cytometry

Cell surface expression of CD36 was evaluated by FACS. Cells were treated as indicated, washed with Hank's Balanced Salt Solution containing 10 mM HEPES, 1 mM Ca^{2+}, 1 mM Mg^{2+} and 0.5% BSA, and non-specific binding blocked by incubation with 5% human serum for 30 min at 4°. Cells (0.5×10^6) were incubated with mouse monoclonal anti-human anti-CD36 IgG (clone FA6-152; Immunotech) at 2.5 μg/mL or with an irrelevant mouse IgG (MOPC-1, Sigma) for 30 min at 4°, followed by a secondary FITC-conjugated anti-mouse IgG (Sigma). Cells were then fixed with 2% NBF and CD36 expression assessed by FACS analysis. In some cases, fixed cells were permeabilized, before antibody treatment, with 0.02% saponin (Sigma) in 2% FCS (30 min, room temperature). CD14 expression was assessed as described above for CD36, using FITC-conjugated anti-CD14 IgG (My4, Coulter) or an irrelevant FITC-conjugated isotype (MCP-11, Coulter). For PPARγ staining, cells were fixed with 2% NBF and permeabilized with acetone–methanol (1:1) for 2 min on ice.

After blocking unspecific binding, cells were incubated with anti-PPARγ IgG (clone E-8, Santa Cruz) for 30 min at

room temperature, reacted with secondary FITC-conjugated IgG, and subjected to FACS analysis. The level of expression of the surface markers was expressed as mean fluorescence intensity, a value calculated by the CellQuest-Pro software (Becton Dickinson), which is based on the distribution of fluorescence within the whole cell population assessed.

2.6. Cell fractionation and Western blotting

Cytosolic and nuclear extracts were prepared as described elsewhere [46]. In brief, cells (10^7) were washed with PBS and resuspended in 100 μL hypotonic buffer (10 mM HEPES, pH 7.3, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF). After centrifugation, cells were lyzed by resuspension in 300 μL lysis buffer (10 mM HEPES, pH 7.3, 10 mM KCl, 1.5 mM $MgCl_2$, 0.4% Nonidet P-40, 1 mM DTT, 1 mM PMSF, 1 μg/mL leupeptin, 15 μg/mL aprotinin). Following a 10-min incubation at 4° on ice, nuclei were collected by centrifugation for 1 min at 8000 *g*, and the supernatant used as the cytosolic fraction. The pellet was washed in buffer composed of 20 mM KCl, 20 mM HEPES (pH 7.3), 22% glycerol, 0.2 mM EDTA, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM PMSF, 1 μg/mL leupeptin and 15 μg/mL aprotinin, and resuspended in 41 μL of the above buffer and 39 μL of the same buffer with 0.6 M KCl. After a 30-min incubation on ice, nuclear proteins were recovered in the supernatant following a 15-min centrifugation at 8000 *g*.

Western blotting was performed according to a standard protocol, following SDS–polyacrylamide gel electrophoresis and transfer of the resolved proteins onto nitrocellulose membranes. Primary antibodies to the following antigens were used: PPARγ (mouse IgG1; clone E-8), p65 (rabbit IgG; clone C-20; both Santa Cruz). In some cases, anti-PPARγ IgG was pre-incubated with a PPARγ-neutralizing peptide (Santa Cruz). Secondary antibodies (Santa Cruz) and the ECL system (Amersham) were used for visualization.

2.7. Assessment of PPARγ binding to PPRE

Two approaches were taken. First, the EMSA assays were performed as follows. The oligonucleotides for PPARγ EMSA contained the consensus binding motif AG *GT*C AAA *GG*T CA (Santa Cruz). The cells (2×10^7) were treated as indicated, and the nuclear extracts prepared by hypotonic lysis and subjected to EMSA as described [47]. The other approach was detection of PPARγ DNA binding using the TransAM kit (Active Motif). Briefly, the cell extract was incubated in wells with immobilized consensus or mutant PPRE sequence, and the bound protein detected in a spectrophotometer (450 nm) following incubation with anti-PPARγ IgG followed by a secondary, HRP-conjugated antibody and the developing solution.

2.8. Decoy approach

The approach used recently was adapted [48]. Briefly, MM6 cells were exposed to an oligonucleotide comprising the PPRE consensus sequences 5′-GGT AAA GGT CAA AGG TCA AT-3′ and 3′-A TTT CCA GTT TCC AGT TAG CCG-5′. The mutant sequences 5′-GGT AAA GAA CAA AGA ACA AT-3′ and 3′-A TTT *CTT* GT*T T*CT TGT TAG CCG-5′ were used as a negative control. The cells were incubated with the oligonuceotides (5 μM) for 24 hr before exposure to the lipoproteins, treated with enzLDL, and expression of CD36 assessed.

2.9. Immunofluorescence microscopy

Cells grown on coverslips were fixed with NBF, permeabilized on ice for 2 min with acetone–methanol (1:1), and incubated at room temperature with anti-PPARγ IgG (clone E-8) followed by anti-mouse FITC-IgG. For CD36 analysis, cells were fixed with NBF, and reacted with anti-mouse CD36 (clone H-300; Santa Cruz) followed by secondary FITC-IgG. The cells were mounted in Mowiol or Vectashield (Vector Laboratories) and observed in the Leica DMRBE microscope fitted with a computer-linked RGB camera. Images were acquired with the software SPOT32 and processed in PhotoShop. The level of CD36 expression was quantified in the immunohistochemical preparations by image analysis. This was performed using the National Institutes of Health software package (http:/rsb.info.nih.gov/nih-image/).

Corresponding sections of acquired images from control and treated cells were assessed for the level of fluorescence. Three sections were randomly chosen in each image, and three images from each treatment were processed in this way. Triplicates were averaged, and the arbitrary value of the ‘amount of green color’ of treated cells was then related to that of control cells.

2.10. Statistics

Unless specified otherwise, the data shown are mean values ± SD derived from three independent experiments. The asterisk at individual values indicates statistically using significant difference as specified in legends to individual figures, with $P < 0.05$, determined by the Student’s *t* test.

The images are representative of at least three independent experiments.

3. Results

3.1. Presence of specific oxidation products in LDL modified with sPLA2 and 15LO

Our previous findings suggested that exposure of LDL to lipases resulted in hydrolysis of ester lipids, generating free

Table 1
Formation of hydroxy fatty acids under various incubation conditions[a]

LDL treatment	HODE + HETE (μg/mg)	
	With hydrolysis	No hydrolysis
Native LDL	0.9 ± 0.9	0
LDL + Cu (3 hr)	11.9 ± 3.1	0
LDL + Cu (20 hr)	2.3 ± 0.5	0
LDL + 15LO	13.3 ± 0.7	0
LDL + sPLA2	2.1 ± 0.9	0
LDL + sPLA2 + 15LO	7.8 ± 1.8	1.9 ± 0.7

[a]LDL was treated as shown and analyzed for total HODE and HETE before and after hydrolysis of the lipoprotein as detailed in Section 2.

PUFA [12], the preferred substrate for 15LO [13]. Thus, concerted action of sPLA2 may not only accelerate 15LO-mediated LDL modification but also yield specific difusible oxidation products, which may exhibit bioactivity (Table 1, Fig. 1A). Incubation of LDL with 15LO alone led to formation of no discernible free oxygenated PUFA, while exposure of LDL to 15LO plus sPLA2 gave rise

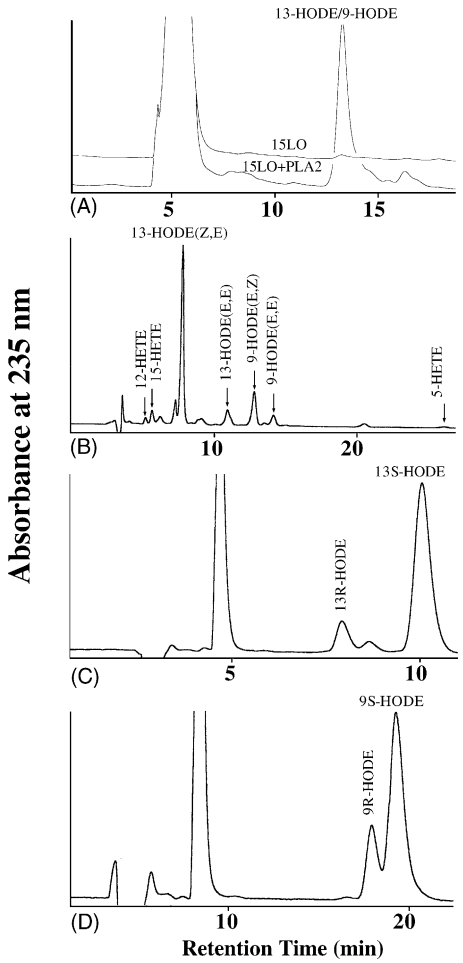


Fig. 1. Enzymatic treatment of LDL generates minimally modified lipoprotein with specific oxidation products. LDL was exposed to 15LO with or without sPLA2 for 20 hr, as described in Section 2, and analyzed by reverse-phase (A), normal-phase (B) and chiral-phase chromatography (C, D). The stereoisomers resolved as shown in panels C and D are derived from 13HODE(*Z*, *E*) and 9HODE(*E*, *Z*), respectively.

Table 2
Isomeric composition of hydroxy fatty acids formed during LDL modification induced by sPLA2 and 15LO[a]

Product	Share (%)
12HETE	1.6 ± 1.1
15HETE	3.4 ± 1.4
5HETE	0.3 ± 0.3
13HODE(*Z*, *E*)	51.0 ± 7.2 (1:9)
13HODE(*E*, *E*)	8.8 ± 3.6
9HODE(*E*, *Z*)	17.1 ± 1.1 (1:5)
9HODE(*E*, *E*)	6.9 ± 5.1

[a]LDL was treated with sPLA2 and 15LO, and resolved by sequential HPLC for the positional and enantiomeric isomers for HETE and HODE, and for chiral isomers for 13HODE(*Z*, *E*) and 9HODE(*E*, *Z*). The numbers in parenthesis show ratio of the *R* and *S* stereoisomers.

to high amounts of free HODE and HETE. More detailed analysis of HODE isomers generated in LDL exposed to sPLA2 plus 15LO revealed enzymatic origin of majority of the HODE formed, detected primarily as the 13*S*-HODE(*Z*, *E*) isomer (Fig. 1B and C, Table 2). Enzymatic origin was also documented by the presence of the minor oxidation product, 9HODE, as the 9*S*-HODE(*E*, *Z*) isomer.

Together with the low REM value (Table 3), these data suggest that concerted action of sPLA2 and 15LO on LDL generated mildly modified lipoprotein resembling that isolated from early lesions [6].

3.2. Upregulation of CD36 in low-differentiated monocytic cells by enzymatically modified LDL

We next studied whether enzLDL can upregulate expression of the scavenger receptor CD36 in monocytic cells with different basal level of CD36 expression. For these experiments, we used MM6 cells (virtually no CD36 expression), MM6-sr cells (medium CD36 expression) and THP1 cells (high basal CD36 expression) (Fig. 2A). Assessment of the kinetics of CD36 upregulation by enzLDL and oxLDL in MM6 cells (Fig. 2B) suggested the greatest effect of the former after a 3-day incubation. Exposure of MM6, MM6-sr and THP1 cells to enzLDL, ox_3LDL and ox_{20}LDL resulted in an increase in CD36 expression in all cases, with the highest increase in CD36 protein level in MM6 cells exposed to enzLDL. In contrast, THP1 cells were least responsive to the lipoprotein. The effect of LDL modified with human recombinant sPLA2 plus rabbit reticulocyte 15LO was similar to that of

Table 3
REM values of LDL treated with different agents[a]

Treatment	REM
Native LDL	1
LDL + sPLA2 + 15LO	1.4 ± 0.08
LDL + Cu (3 hr)	2.3 ± 0.3
LDL + Cu (20 hr)	4.47 ± 0.5

[a]LDL was treated and analyzed for REM by agarose electrophoresis as specified in Section 2.

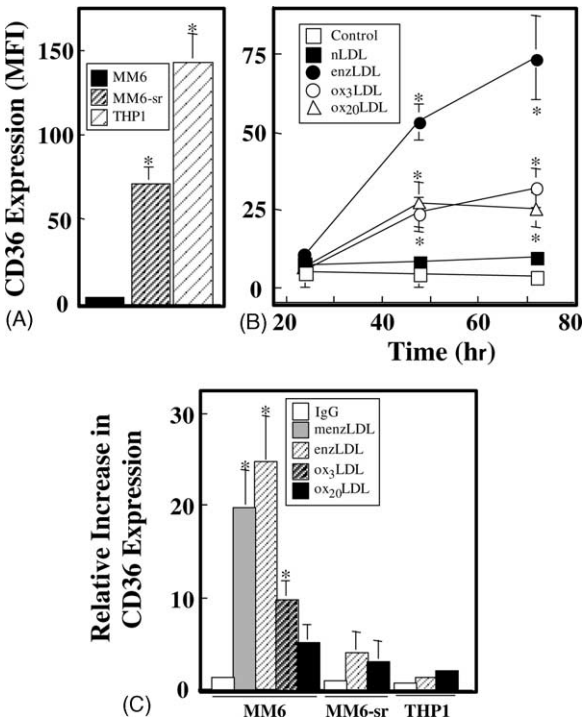


Fig. 2. Enzymatically modified LDL upregulates CD36 expression in monocytic cells. MM6, MM6-sr and THP1 cells were analyzed by FACS for basal surface expression of CD36, expressed as median fluorescence intensity (A). Panel B shows kinetics of CD36 expression in control MM6 cells or cells treated with LDL modified as shown. In panel C, MM6, MM6-sr and THP1 cells were exposed for 3 days to native LDL, enzLDL, LDL modified with mammalian sPLA2 and 15LO (menzLDL), ox_3LDL and ox_{20}LDL (50 μg/mL each), CD36 expression assessed and expressed as the increase over its basal level. The empty bars indicated background fluorescence, i.e. that of cells treated with enzLDL, but in which anti-CD36 IgG was replaced by an irrelevant IgG. Data are derived from three independent experiments and are presented as mean ± SD. The asterisk indicates significant difference from the MM6 cells (A), the control (B), and the ox_{20}LDL-treated cells (C).

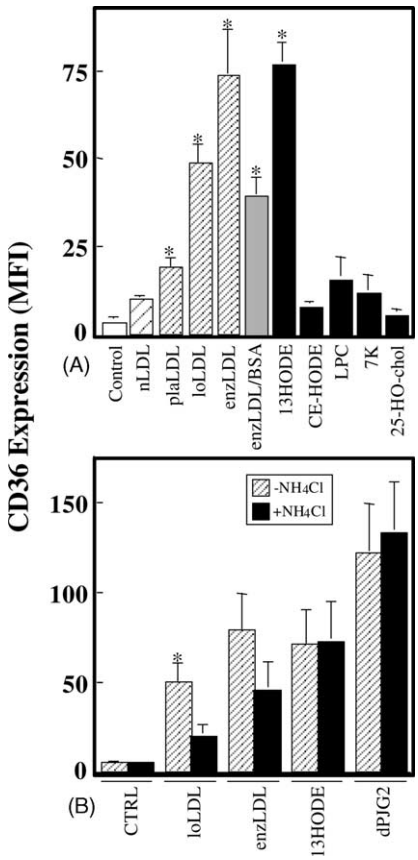


Fig. 3. 13HODE upregulates expression of CD36. (A) MM6 cells were treated for 3 days with nLDL, LDL modified with sPLA2 (plaLDL) or 15LO (loLDL), or the two enzymes together (enzLDL) (50 μg/mL each), 13HODE, CE-HODE (both 15 μM), LPC (100 μM), 7-ketocholesterol (7K), or 25-hydroxycholesterol (25-HO-chol) (both 10 μg/mL), and with enzLDL pre-incubated with BSA (lipoprotein at 50 μg/mL), and assessed by FACS for CD36 expression. MM6 cells were exposed for 3 days to loLDL, enzLDL (both 50 μg/mL), 13HODE (15 μM) or $dPGJ_2$ (3 μM) in the absence or presence of NH_4Cl (15 mM), and CD36 expression assessed by FACS (B). Data are derived from three independent experiments and are presented as mean ± SD. The asterisk indicated significant difference from the control (A), and from the cells treated in the presence of NH_4Cl (B).

enzLDL (Fig. 2C). Assessment of CD36 expression in permeabilized cells suggested that the increase in the CD36 protein was rather due to CD36 upregulation than mobilization of cytosolic CD36 stores (data not shown). Moreover, exposure of MM6 cells to enzLDL as well as to 13HODE resulted in their differentiation as documented by enhanced surface expression of the maturation marker CD14 (not shown).

3.3. 13HODE—a major bioactive constituent of enzLDL

As LDL modified with 15LO plus sPLA2 was more efficient in CD36 upregulation than LDL treated with 15LO only, while exposure to sPLA2 alone was ineffective (Fig. 3A), we investigated the effect on CD36 expression of the major products of the enzyme(s), i.e. 13HODE and CE-HODE, respectively. As shown in Fig. 3A, unlike free HODE, its esterified counterpart was inactive. This indicates that the superiority of LDL modified with sPLA2 plus 15LO over the 15LO-modified lipoprotein in CD36 upregulation can be ascribed to the presence in the lipoprotein of the free oxygenated PUFA. Moreover, stripping enzLDL of unesterified oxidized PUFAs by incubation with BSA lowered the efficacy of the lipoprotein in CD36 upregulation (Fig. 3A). As LDL modified with 15LO only caused CD36 upregulation in MM6 cells, albeit to a lesser extent than did enzLDL, we wanted to see if there was a participation of lysosomal enzymes in cellular processing of the lipoprotein. We used NH_4Cl to suppress the lysosomal hydrolytic activity. As documented in Fig. 3B, NH_4Cl inhibited CD36 upregulation by 15LO-modified LDL, while this inhibition was insignificant in case of enzLDL; NH_4Cl had no effect on CD36 upregulation in cells exposed to 13HODE or $dPGJ_2$ (a cyclopentanone prostaglandin and a strong agonist of PPARγ [21]). This suggests involvement of the lysosomal apparatus in LDL processing, resulting in a subsequent increase in CD36 expression. Other constituents of oxidized LDL tested, i.e.

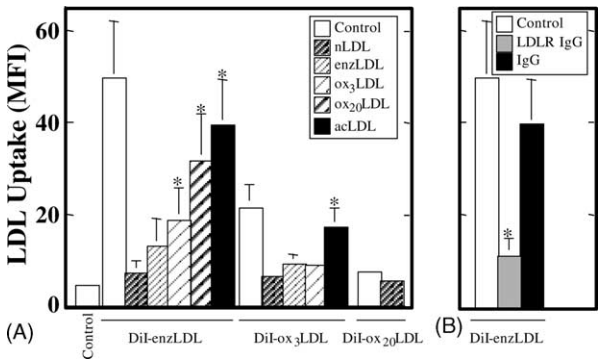


Fig. 4. Non-differentiated monocytes take up enzLDL via the LDL receptor. MM6 cells were treated for 2 hr with DiI-labeled enzLDL, ox_3LDL or ox_{20}LDL (10 μg/mL each) following pre-incubation with the vehicle, 100 μM unlabeled LDL or LDL modified as indicated (A), or pre-incubated with anti-LRL-R IgG or irrelevant IgG (B), and assessed for DiI fluorescence by FACS analysis. Data are derived from three independent experiments and are presented as mean ± SD. Asterisks show significant difference from the control.

lysophosphatidyl choline, 7-ketocholesterol, and 25-hydroxycholesterol had little effect on CD36 expression (Fig. 3A).

3.4. Uptake of enzLDL via the LDL receptor in low-differentiated monocytic cells

The fact that enzLDL triggered CD36 upregulation in the low-differentiated MM6 cells suggested that these cells, lacking the scavenger receptor, may take up enzLDL via LDLR. Incubation of the cells with DiI-enzLDL resulted in its uptake, which was blocked by pre-incubation with excess native LDL but not with heavily oxidized LDL, or by pre-incubation with anti-LDLR IgG but not the irrelevant antibody (Fig. 4). These cells were, however, very inefficient in uptake of ox_{20}LDL (Fig. 4) or acLDL (not shown), consistent with their very low expression of the scavenger receptor. However, pre-incubation with heavily oxidized LDL did not block uptake of enzLDL (Fig. 4). Endocytosis of enzLDL via the LDLR may be a route by which the enzymatically modified lipoprotein can reach the lysosomal apparatus for further processing.

3.5. Activation of CD36 transcription by enzLDL

As modified LDL has an effect on the surface expression of lipoprotein receptors, we next investigated the effect of exposure of MM6 cells to modified LDL on the mRNA levels of CD36 and LDLR. Figure 5 shows that there was a significant increase in the CD36 transcript in cells exposed to enzLDL and its bioactive constituent 13HODE, and, to a lesser degree, oxLDL, but not to native LDL, consistent with the surface levels of the CD36 protein (Cf Fig. 2). Concomitantly, there was a modest but non-significant decrease of LDLR mRNA in cells exposed to enzLDL. The differences in CD36 and LDLR mRNA, as shown in Fig. 5 were obtained using a semi-quantitative RT–PCR

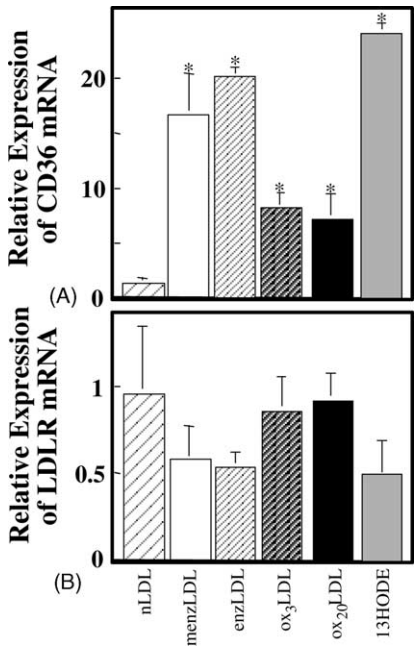


Fig. 5. Modified LDL causes different expression of CD36 and LDLR in non-differentiated monocytes. MM6 cells (2×10^6) were exposed for 3 days to the vehicle (PBS), native LDL, menzLDL, enzLDL, ox_3LDL, ox_{20}LDL (50 μg/mL each), or 13HODE (15 μM), total RNA isolated, and RT–PCR for LDLR (A) and CD36 (B) performed and evaluated, using actin as the house-keeping gene, as described in Section 2. The level of mRNA in cells treated as indicated is expressed relative to the level of the mRNA in non-treated cells. Data are derived from three independent experiments and are presented as mean ± SD. Asterisks indicate significant difference from cells treated with nLDL.

evaluated by HPLC analysis of the transcripts. To see whether this approach provided reliable results, we analyzed by real-time PCR CD36 and LDLR mRNA isolated from control cells and cells treated with 13HODE. This showed 26 ± 3.2 and 0.72 ± 0.25 fold change for CD36 and LDLR mRNA, respectively, which is in good agreement with the RT–PCR data in Fig. 5.

3.6. PPARγ plays a role in upregulation of CD36 by enzLDL

There has recently been controversy concerning the involvement of PPARγ in various processes, as often the notion for a role of the transcription factor has been based on the use of ligands/agonists of PPARγ, some of which may be pleiotropic. We thus asked if PPARγ is important for CD36 upregulation in MM6 cells exposed to enzLDL. First, we investigated if stimulation of the cells with the modified lipoprotein leads to upregulation/activation of PPARγ. Figure 6A shows that there was no significant difference in the level of PPARγ mRNA in MM6 cells stimulated with LDL regardless of the type of its modification or with 13HODE. Consistent with the result, we found no increase in the PPARγ protein level by FACS analysis of MM6 cells exposed either to differently modified LDL or to 13HODE (not shown).

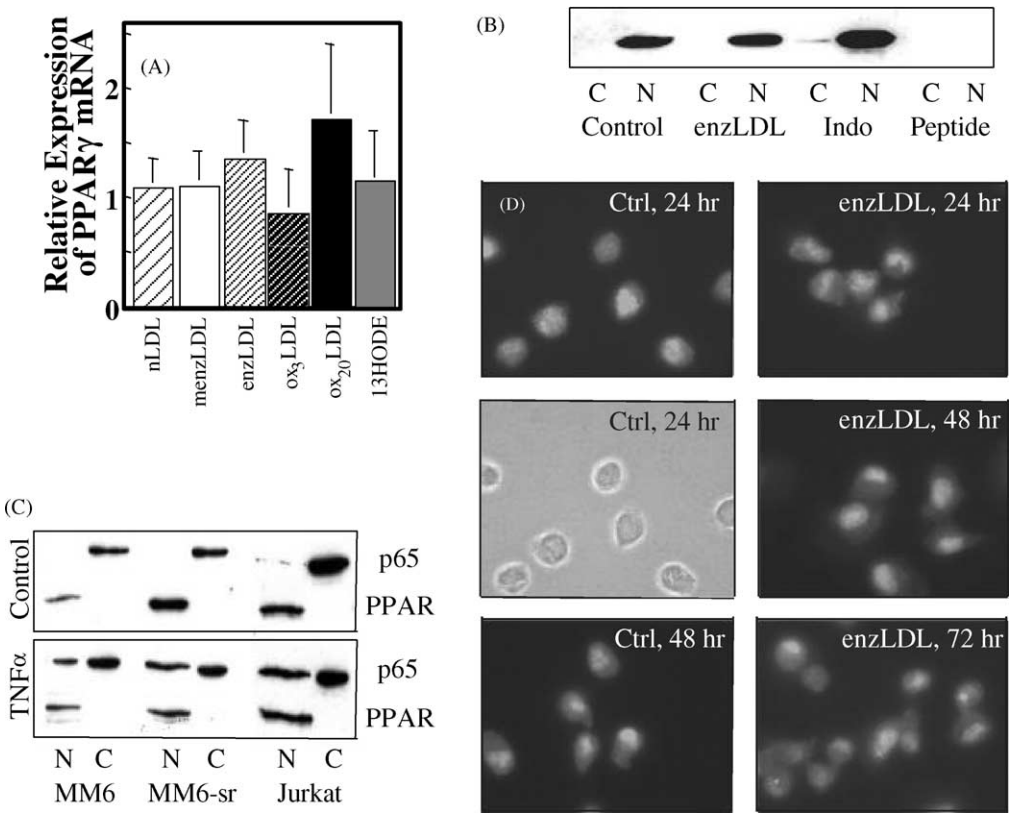


Fig. 6. PPARγ is expressed in monocytic cells. (A) MM6 cells (2×10^6) were treated for 48 hr with the vehicle, enzLDL, ox_{20}LDL (both 50 μg/mL), or 13HODE (15 μM), total RNA isolated, and RT–PCR for PPARγ mRNA performed and evaluated, using actin as the housekeeping gene. The level of mRNA in cells treated as indicated is expressed relative to the level of the mRNA in non-treated cells. (B) MM6 cells were exposed for 48 hr to the vehicle, enzLDL (50 μg/mL) or indomethacin (Indo; 100 μM), cytosolic (C) and nuclear (N) fractions prepared and assessed for PPARγ by immunoblotting. For PPARγ immunoblotting, control cells were also probed with anti-PPARγ IgG pre-incubated with a specific PPARγ neutralizing peptide. (C) Control and TNFα-stimulated (100 units, 30 min) MM6, MM6-sr and Jurkat cells were subjected to Western blotting for p65 and PPARγ in the nuclear and cytosolic fractions. (D) Human peripheral blood monocytes were prepared by anti-CD14 IgG immunomagnetic sorting, adhered to plastic, exposed for 24, 48 and 72 hr to the vehicle or enzLDL (50 μg/mL), fixed, permeabilized and immunostained for PPARγ followed by FITC-conjugated secondary IgG. Images were taken using fluorescence microscopy with blue excitation. Phase-contrast microscopy is shown for control cells. Data are derived from three independent experiments and are presented as mean ± SD.

We next studied cellular localization of PPARγ protein as it has been suggested by some researchers to reside in the cytoplasm and to translocate to the nucleus during differentiation/stimulation [21,49,50]. Western blotting analysis of nuclear and cytosolic fractions of MM6 cells revealed that PPARγ resided in the nucleus even before stimulation with enzLDL, and its level was similar in control cells and cells treated with the lipoprotein (Fig. 6B). We found that the nuclear level of the PPARγ protein was elevated when the cells were exposed to the non-steroid anti-inflammatory drug indomethacin, an activator of the transcription factor [51]. The specificity of the antibody used was verified by its pre-incubation with a PPARγ neutralizing peptide (Fig. 6B). PPARγ was exclusively nuclear in all non-stimulated cell lines tested, including MM6-sr, Jurkat (Fig. 6C), U937 and THP1 cells (not shown). To control for cell fractionation, we performed Western blotting, using the same extracts, for p65, a subunit of the nuclear factor-κB residing in non-stimulated cells in the cytoplasm and translocating into the nucleus upon exposure of cells to pro-inflammatory cytokines. As shown in Fig. 6C, p65 was found in the cytosolic fraction before and in the nuclear fraction after stimulation with TNFα, while in the same blots, PPARγ resided in the nucleus regardless of TNFα treatment.

Nuclear localization of PPARγ has been associated with more differentiated cells, and we have observed this, as expected, in terminally differentiated cells like human fibroblasts or endothelial cells (not shown). We observed that the least differentiated monocytic cells used here, the MM6 cells, featured nuclear PPARγ as did other cell types studied, before exposure to PPARγ ligands (see above). To find out more about localization of PPARγ in relation to differentiation, we prepared human peripheral blood monocytes, incubated them with enzLDL for different periods, after which we analyzed them for PPARγ by immunofluorescence microscopy. In these cells, PPARγ was localized largely in the nucleus as well (Fig. 6D). In conclusion, cytosolic-to-nuclear translocation of PPARγ does not play a role in CD36 upregulation by enzLDL in MM6 cells.

Thus, it appears that PPARγ participation in CD36 upregulation in MM6 cells by enzLDL, if at all, may be

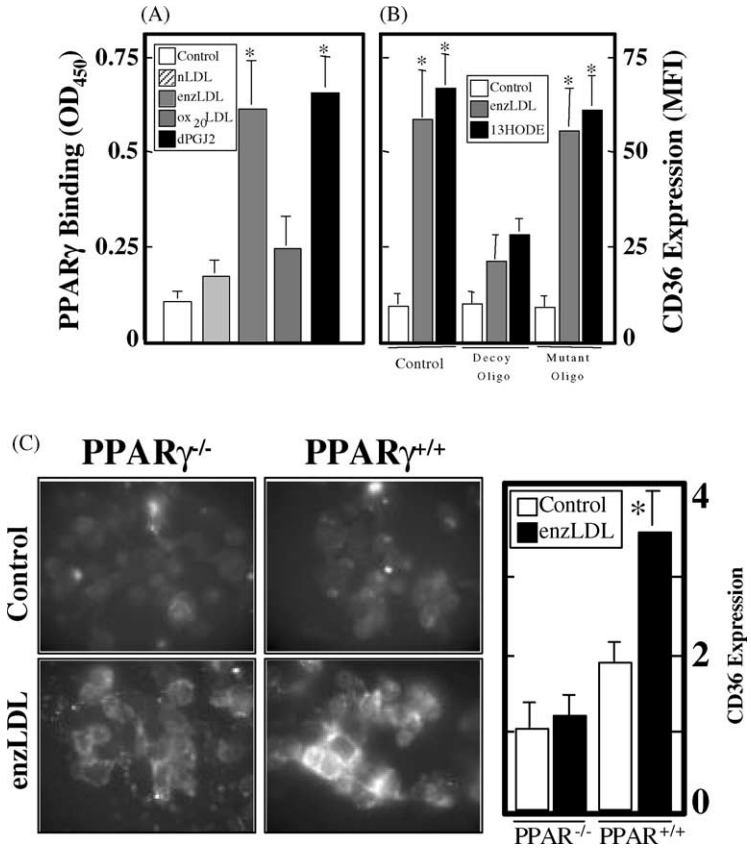


Fig. 7. PPARγ plays a role in enzLDL-induced upregulation of CD36. (A) MM6 cells (2×10^7) were incubated for 12 hr with native LDL, enzLDL, ox_{20}LDL (50 μg/mL each) or $dPGJ_2$ (3 μM), nuclear extracts prepared by hypotonic lysis, and binding of PPARγ to the PPAR-response element determined as detailed in Section 2. (B) MM6 cells were pre-treated with decoy PPRE oligonucleotides or their mutant counterparts, exposed to enzLDL, and assessed for CD36 expression by FACS analysis. (C) $PPAR\gamma^{+/+}$ and $PPAR\gamma^{-/-}$ ES cells were differentiated into macrophages, incubated for 2 days with native LDL, enzLDL or ox_{20}LDL (50 μg/mL each), and assessed for CD36 expression by immunofluorescence microscopy. The bar graph shows relative expression of CD36 derived from the immunostaining by image analysis, as is related to the expression of CD36 in the control $PPAR\gamma^{-/-}$ cells (see Section 2 for details). Data are derived from three independent experiments and are presented as mean ± SD. The images are from three independent experiments. The asterisks indicate significant difference from the control.

at the level of its binding to the PPRE in the CD36 gene promoter mediated by 13HODE, a ligand of PPARγ and a major constituent of enzLDL.

We thus performed EMSA using nuclear extracts from MM6 cells exposed to modified LDL or $dPGJ_2$. Treatment of the cells with enzLDL or ox_{20}LDL resulted in more binding to PPRE, as also found for $dPGJ_2$ (not shown). To confirm these findings, we used a novel technique that is superior to EMSA assays, i.e. ELISA-type assessment of PPARγ binding to its consensus PPRE sequence immobilized in 96-well plates. Compared to the EMSA approach, this technique is more reproducible, due to standardized preparation of nuclear extracts, and is also several fold more sensitive. Data in Fig. 7A show that MM6 cells treated with enzLDL exerted DNA binding of PPARγ, while this was not observed with cells exposed to native LDL (nLDL) or oxLDL. On the other hand, $dPGJ_2$ caused strong DNA binding. These data suggest that enzLDL may induce PPARγ binding to the PPRE.

Another piece of evidence for the role of PPARγ in CD36 upregulation by enzLDL was obtained in experiments in which the cells were pre-treated with a 'decoy' PPRE oligonucleotide. As revealed in Fig. 7B, such pre-incubation largely suppressed the effect of enzLDL as well as that of 13HODE on CD36 expression, presumably since PPARγ, following cell exposure to enzLDL could not bind to PPRE, while pre-incubation with a mutant PPRE oligonucleotide did not block CD36 upregulation by enzLDL.

To get direct evidence whether PPARγ is involved in CD36 upregulation in monocytes/macrophages after stimulation with enzLDL, we used PPARγ-deficient ES cells differentiated into macrophages. Exposure of ES macrophages to enzLDL resulted in CD36 expression in the PPARγ-proficient cells but less so in the PPARγ-deficient macrophages (Fig. 7C). Evaluation of the level of CD36 expression using image analysis of the immunohistochemical preparations showed that the expression of CD36, relative to that in the control cells ($PPAR\gamma^{-/-}$ cells, control), was 1.2 ± 0.3 for untreated $PPAR\gamma^{+/+}$ cells, 1.9 ± 0.3 for enzLDL-treated $PPAR\gamma^{-/-}$ cells, and 3.5 ± 0.4 ($P < 0.05$) for enzLDL-treated $PPAR\gamma^{+/+}$ cells.

This finding strongly suggests that PPARγ plays a role in CD36 expression in macrophages stimulated with modified

LDL, including enzLDL, and provides a link between LDL modified with sPLA2 and 15LO, and CD36 expression.

4. Discussion

The main problem we wanted to address here was whether mildly oxidized LDL has the propensity to regulate events of early phases of atherogenesis. We chose to modify LDL with two enzymes that may play a significant role in the early phases of the disease, i.e. sPLA2 [52,53] and 15LO [54–56]. This notion is based on *in vitro* experiments and on circumstantial evidence from *in vivo* settings. For example, the sPLA2 protein is overexpressed in intima in both early and advanced atherosclerotic lesions, by intimal macrophages and proliferating smooth muscle cells [14,15,57]. Due to the net positive charge of the protein, the enzyme colocalizes with pro-atherogenic LDL particles on negatively charged extracellular matrix proteoglycans, and the enzyme has been shown to be highly reactive against LDL in the proteoglycan-bound state [58,59]. The potential role of sPLA2 in atherogenesis is further illustrated by enhanced atherosclerosis and modified lipoproteins in mice overexpressing the lipase [60], and by a correlation between sPLA2 expression and the stage of atherosclerosis [57].

The strongest evidence for a role of 15LO in atherosclerosis stems from earlier observations (reviewed in [54–56]), and from more recent reports showing the presence of lipoxygenase-specific oxidation products in human atherosclerotic lesions [6,61]. Further, regulation of atherogenesis by genetic manipulation of 15LO has been documented using different mouse models of the disease, including the apolipoprotein E- and LDLR-deficient animals [10,11,62,63].

We have previously observed that concerted action of PLA2 and 15LO on LDL greatly enhances the level of oxidized PUFAs in the lipoprotein [12,13]. That is, PLA2 first liberates PUFAs esterified in surface phospholipids of LDL, and these are then preferentially oxygenated by 15LO [12]. Such modifications result in generation of specific oxidation products, majority of which are derived from linoleic and arachidonic acid. Consistent with this notion, we observed here that 12HETE, 15HETE, 9HODE and 13HODE are formed at higher levels with 13HODE as the major product. Moreover, the fact that majority of the isomers of 13HODE detected was in the form of 13HODE(*Z*, *E*), of which about 90% was the *S* stereoisomer, is direct evidence for enzymatic origin of the oxygenated free PUFA [8,13].

An important role in atherosclerosis progression is played by scavenger receptors that are crucial for uptake of oxLDL [25,64], further activation of the cells [1], and generation of the foam cell phenotype [2,65]. We were interested if enzLDL can regulate expression of the scavenger receptor CD36 that has been shown to recognize oxidized lipids rather than protein within LDL [25]. Our hypothesis was based on previous observations showing that 13HODE can regulate the expression of CD36 [17]. In agreement with this, we found that exposure of monocytic cells to enzLDL resulted in upregulation of CD36 (Fig. 2). Importantly, the increase in the level of CD36 protein was most profound in MM6 cells, which do not express CD36 unless activated, while it was lower in MM6-sr cells with modest, and lowest in THP1 cells with high basal CD36 expression. These observations may be important in the context of early phases of atherogenesis characterized by minimally modified LDL and low differentiated monocytic cells [1,65].

13HODE, being abundant in enzLDL, may be a main principle of bioactivity of the lipoprotein. We, therefore, studied whether 13HODE and several other constituents of oxidized LDL exert a regulatory effect on CD36 expression in MM6 cells. As expected, and in line with other reports, 13HODE upregulated CD36 expression, to a similar extent as did enzLDL. None of the other constituents of modified LDL tested, i.e. lysophosphatidyl choline, CE-HODE, 7-ketocholesterol and 25-hydroxycholesterol (markers of more heavily oxidized LDL [66]), showed any effect. The fact that lysophosphatidyl choline did not regulate CD36 expression is consistent with the results documenting that LDL treated with sPLA2 only showed a relatively low effect (Fig. 3), further stresses the importance of a cooperative action of sPLA2 and 15LO. Our observation that CE-HODE, a product of oxygenation of cholesteryl linoleate by 15LO, had no effect, is in agreement with earlier results with THP1 cells [17]. However, we did see upregulation of CD36 expression in MM6 cells exposed to LDL modified with 15LO alone (Fig. 3), albeit to a lower extent than was the case for enzLDL. A possible explanation of this is that, before exerting bioactivity, 15LO-modified LDL needs to be internalized and processed in the acidic compartment of the cell. In support of this theory, we observed that inhibition of lysosomal activity suppressed upregulation of CD36 expression. In this context, we cannot explain why 13HODE caused upregulation of CD36 expression while CE-HODE was completely inactive. Although not clear at present, it is possible that oxygenated free PUFAs cross plasma membrane more easily [67,68], while their esterified counterparts need to be internalized as constituents of modified LDL.

We used 13HODE at 15 μM, since preliminary experiments showed that the effect of 13HODE on CD36 was saturated at about 10 μM (not shown). Similar concentrations of the oxidatively modified PUFA (10–50 μM) were used by others (see, e.g. [29]) to mimic the effect of oxidized or minimally modified LDL on gene expression in monocytes/macrophages.

The fact that enzLDL and 15LO-modified LDL caused upregulation of CD36 expression in low-differentiated monocytic cells and that the effect of the latter could be counteracted by inhibiting the activity of the acidic apparatus, suggests internalization of the lipoproteins.

However, MM6 cells express very low levels of scavenger receptors [43,69], considered necessary for uptake of oxidatively modified LDL [25,64]. There is a report, though, showing that fibroblasts can internalize LDL modified by 15LO-overexpressing cells via LDLR [70].

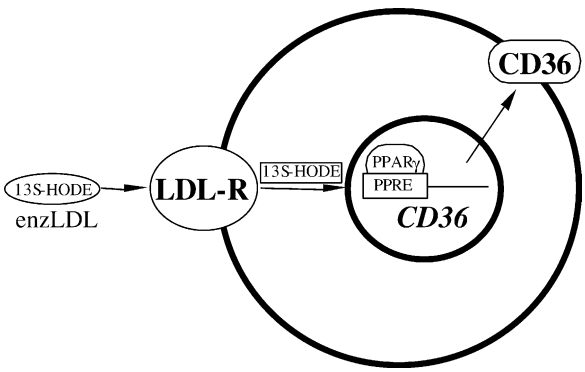
Consistent with this, we observed that MM6 cells took up enzLDL via LDLR (Fig. 4), and that blocking this uptake inhibited enzLDL-dependent upregulation of CD36 expression (not shown). In this respect, a recent report showing foam cell formation from macrophages exposed to native LDL [71] is of interest, since it suggests that the LDLR may have atherogenic functions, at least under some circumstances. On the other hand, MM6 cells internalized neither oxLDL nor acLDL (Fig. 4), a process that requires scavenger receptors [25,64]. The observation that oxLDL caused some upregulation of CD36 expression in MM6 cells may be explained by the presence of non-specific oxidation products derived from the LDL's lipidic and proteineous constituents, and their adducts [72]. It cannot be ruled out that some of these components of oxLDL may translocate into the cell.

Recent reports suggested that 13HODE, a product of concerted action of sPLA2 and 15LO on LDL, is bioactive. In this context, the report that 13HODE is a ligand for PPARγ [17,24] that promotes binding of the transcription factor to the PPAR-response element in the promoter region of a number of genes, including CD36 [18,22], is of interest. While PPARγ is an important mediator of a number of (patho)physiological processes [18,19], its role may have been overestimated as often, its involvement has been judged based on the use of agonists of the transcription factor, such as $dPGJ_2$. It has now become obvious that PGJ_2 is rather pleiotropic, so that not all of its bioactivities are mediated by PPARγ [32–34,73]. A potentially confounding factor is that no specific antagonists for PPARγ are available. For example, the synthetic compound BADGE, that has been shown to antagonise PPARγ in preadipocyte cells [74], was found agonistic in epithelial cells [75] and highly toxic towards MM6 cells (J.K. *et al.*, unpublished data). As deficiency in PPARγ is lethal during embryogenesis, the establishment of $PPAR\gamma^{-/-}$ ES cells [33,34] was important. Recent progress in differentiation of ES cell *in vitro* made it possible to show that PPARγ was crucial for adipocyte differentiation [76] but, unlike assumed earlier [24], dispensable for differentiation of ES cells into macrophages [33,34].

We thus asked if upregulation of CD36 expression in MM6 cells by enzLDL involves PPARγ. Immunoblotting and immunofluorescence microscopy analyses showed that there was a high nuclear expression of PPARγ in all cell types tested, regardless of stimulation (Figs. 6 and 7). This refers not only to the terminally differentiated cells like fibroblasts and endothelial cells, but also to monocytic cells, including MM6, MM6-sr, THP1, U937 and human peripheral blood monocytic cells, and Jurkat T lymphoma cells. These findings contradict some of the previous report that the PPARγ protein is not expressed in undifferentiated human monocytic cells [20], and that PPARγ translocates into the nucleus upon stimulation [21,49,50]. Collectively, we present data showing high level of the PPARγ protein in the nucleus regardless of the cell type and the differentiation stage studied, and little effect on its expression during stimulation of MM6 or human peripheral blood monocytes with enzLDL.

Therefore, if PPARγ is involved in upregulation of CD36 expression in MM6 cells exposed to enzLDL, it may be regulated on the level of its binding to PPRE. Analysis of MM6 cells using the TransIT or EMSA techniques suggested that enzLDL caused association of PPARγ with the response element. To get more direct evidence, we treated $PPAR\gamma^{-/-}$ and $PPAR\gamma^{+/+}$ ES cells differentiated into macrophages with enzLDL, and found an increase of CD36 expression in the PPARγ-proficient but not PPARγ-deficient cells. This strongly suggests that PPARγ is a positive regulator of CD36 expression, mediating the effect of enzLDL in monocytes/macrophages, and is consistent with earlier reports in which THP1 cells were exposed to highly oxidized LDL or to 13HODE [17,24]. Contrary to these studies, which used copper-oxidized LDL and more differentiated macrophages, we employed here LDL modified by concerted action of 15LO and sPLA2, giving rise to specific and well-characterized lipid oxidation products, and low-differentiated macrophages. Out conditions may, therefore, better mimic the initial stages of atherosclerosis.

Although the fact that modified LDL upregulated CD36 via PPAR γ is not novel *per se*, we present novel data in this report suggesting the following scenario (Scheme 1). LDL modified by concerted action of sPLA2 and 15LO, rich in bioactive oxygenated PUFAs, such as 13HODE, is internalized via LDLR. The lipoprotein is processed in the acidic compartment, and 13HODE causes binding of PPARγ to the PPRE. This results in induction of expression



Scheme 1. Possible regulatory mechanism of CD36 expression in monocytic cells by enzymatically modified LDL. LDL modified by the concerted action of sPLA2 and 15LO, rich in bioactive oxygenated PUFAs (in particular 13HODE), is internalized via LDLR. Free 13HODE then causes binding of PPARγ to the PPAR-response element. This results in induction of expression of the scavenger receptor CD36, promoting uptake of more heavily oxidised LDL. In this context, the effect of enzLDL is pro-atherogenic.

of a variety of genes, including the scavenger receptor CD36. Our results show that enzymatically modified LDL can cause differentiation of monocytic cells into a cell type that can take up more heavily oxidized LDL via the scavenger receptor. In this context, the effect of LDL modified by the concerted action of sPLA2 and 15LO can be considered pro-atherogenic.

We have recently shown that enzLDL can also induce apoptosis in monocytic cells via, at least partially, a pathway different from that involved in CD36 upregulation, and we proposed that the apoptosis-inducing activity of enzLDL may be viewed as antiatherogenic with regards to the initial stages of atherosclerosis [77]. Therefore, our findings suggest a dichotomic activity of enzLDL towards monocytic cells. In conclusion, as minimally modified LDL and low-differentiated monocytic cells are hallmarks of initial phases of atherosclerosis, these findings can deepen our understanding of the molecular mechanisms underlying early artherogenesis, and identify a causal link between LDL modified by sPLA2 and 15LO, and CD36 expression.

Acknowledgments

The authors are indebted to R. Evans and A. Chawla for providing them with PPARγ-knockout and wild-type ES cells and advise on their differentiation. Results in this report form a part of doctoral thesis of K.J. This work was supported in part by the August-Lenz Stiftung, the DFG grants We-1913/2 (C.W.) and Ku-961/7-1 (H.K.), the University of Linköping grant 83081030 (J.N.), and a grant from the National Heart Foundation of Australia G01B0262 (J.N.).

References

[1] Ross R. Atherosclerosis—an inflammatory disease. N Engl J Med 1999;340:115–26.
[2] Steinberg D. Low density lipoprotein oxidation and its pathobiological significance. J Biol Chem 1997;272:20963–6.
[3] Liao F, Berliner JA, Mehrabian M, Navab M, Demer LL, Lusis AJ, Fogelman AM. Minimally modified low density lipoprotein is biologically active *in vivo* in mice. J Clin Invest 1991;87:2253–67.
[4] Neuzil J, Kontush A, Weber C. Vitamin E in atherosclerosis: linking the chemical, biological and clinical aspects of the disease. Atherosclerosis 2001;57:257–83.
[5] Niu X, Zammit V, Upston JM, Dean RT, Stocker R. Coexistence of oxidised lipids and α-tocopherol in all lipoprotein density fractions isolated from advanced human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 1999;19:1708–18.
[6] Kühn H, Belkner J, Zaiss S, Fahrenklemper T, Wohlfeil S. Involvement of 15-lipoxygenase in early stages of atherogenesis. J Exp Med 1994;179:1903–11.
[7] Upston JM, Neuzil J, Stocker R. Oxidation of LDL by recombinant human 15-lipoxygenase: evidence for non-enzymatic, α-tocopherol-dependent oxidation of esterified core and surface lipids. J Lipid Res 1996;37:2650–61.
[8] Belkner J, Stender H, Kühn H. The rabbit 15-lipoxygenase preferentially oxygenates LDL cholesterol esters, and this reaction does not require vitamin E. J Biol Chem 1999;273:23225–32.
[9] Yla-Herttuala S, Rosenfeld ME, Parthasarathy S, Glass CK, Sigal E, Witztum JL, Steinberg D. Colocalization of 15-lipoxygenase mRNA and protein with epitopes of oxidized low density lipoprotein in macrophage-rich areas of atherosclerotic lesions. Proc Natl Acad Sci USA 1990;87:6959–63.
[10] Cyrus T, Witztum JL, Rader DJ, Tangirala R, Fazio S, Linton MF, Funk CD. Disruption of the 12/15-lipoxygenase gene diminishes atherosclerosis in apo E-deficient mice. J Clin Invest 1999;103:1597–604.
[11] Harats D, Shaish A, George J, Mulkins M, Kurihara H, Levkovitz H, Sigal E. Overexpression of 15-lipoxygenase in vascular endothelium accelerates early atherosclerosis in LDL receptor-deficient mice. Arterioscler Thromb Vasc Biol 2000;20:2100–5.
[12] Upston JM, Neuzil J, Witting PK, Alleva R, Stocker R. Oxidation of free fatty acids in low-density lipoprotein by 15-lipoxygenase stimulates non-enzymic, α-tocopherolmediated peroxidation of cholesteryl esters. J Biol Chem 1997;37:30067–74.
[13] Neuzil J, Upston JM, Witting PK, Scott K, Stocker R. Secretory phospholipase A2 and lipoprotein lipase enhance 15-lipoxygenase-induced enzymic and non-enzymic lipid peroxidation of low-density lipoproteins. Biochemistry 1998;37:9203–10.
[14] Hurt-Camejo E, Andersen S, Standal R, Rosengren B, Sartipy P, Stadberg E, Johansen B. Localization of non-pancreatic secretory phospholipase A2 in normal and atherosclerotic arteries. Activity of the isolated enzyme on low-density lipoproteins. Arterioscler Thromb Vasc Biol 1997;17:300–9.
[15] Anthonsen MW, Stengel D, Hourton D, Ninio E, Johansen B. Mildly oxidized LDL induces expression of group IIa secretory phospholipase A2 in human monocyte-derived macrophages. Arterioscler Thromb Vasc Biol 2000;20:1276–82.
[16] Kühn H. Biosynthesis, metabolization and biological importance of the primary 15-lipoxygenase metabolites 15-hydro(pero)xy-5*Z*, 8*Z*, 11*Z*, 13*E*-eicosatetraenoic acid and 13-hydro(pero)xy-9*Z*, 11*E*-octadecadienoic acid. Prog Lipid Res 1996;35:203–26.
[17] Nagy L, Tontonoz P, Alvarez JG, Chen H, Evans RM. Oxidized LDL regulates macrophage gene expression through ligand activation of PPARγ. Cell 1998;93:229–40.
[18] Kersten S, Desvergne AR, Wahli W. Roles of PPARs in health and disease. Nature 2000;405:2411–7.
[19] Neve BP, Fruchart JC, Staels B. Role of the peroxisome proliferator-activated receptors in atherosclerosis. Biochem Pharmacol 2000;60:1245–50.
[20] Chinetti G, Griglio S, Antonucci N, Torra IP, Delerive P, Majd Z, Fruchart J, Chapman J, Najib J, Staels S. Activation of proliferator-activated receptors-α and -γ induces apoptosis of human monocyte-derived macrophages. J Biol Chem 1998;273:25573–80.
[21] Bishop-Bailey D, Hla T. Endothelial cell apoptosis induced by the peroxisome proliferator-activated receptor ligand 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2. J Biol Chem 1998;274:17042–8.
[22] Fajas L, Auboeuf D, Raspe E, Schoonjans K, Lefebvre AM, Saladin R, Najib J, Laville M, Fruchart JC, Deeb S, Vidal-Puig A, Flier J, Briggs MR, Staels B, Vidal H, Auwerx J. The organization, promoter analysis, and expression of the human PPARγ gene. J Biol Chem 1997;272:18779–89.
[23] Zhang B, Berger J, Zhou G, Elbrecht A, Biswas S, White-Carrington S, Szalkowski D, Moller DE. Insulin- and mitogen-activated protein kinase-mediated phosporylation and activation of proliferator-activated receptor-γ. J Biol Chem 1996;271:31771–4.
[24] Tontonoz P, Nagy L, Alvarez JG, Thomazy VA, Evans RM. PPARγ promotes monocyte-macrophage differentiation and uptake of oxidized LDL. Cell 1998;93:241–5.
[25] Silverstein RL, Febbraio M. CD36 and atherosclerosis. Curr Opin Lipidol 2000;11:483–91.

[26] Ricote M, Li AC, Wilson TM, Kelly CJ, Glass CK. The peroxisome proliferatoractivated receptor-γ is a negative regulator of macrophage activation. Nature 1998;392:79–82.
[27] Jiang C, Ting AT, Seed B. PPARγ agonists inhibit production of monocyte inflammatory cytokines. Nature 1998;391:82–6.
[28] Chung SW, Kang BY, Kim SH, Pak YK, Cho D, Trinchieri G, Kim TS. Oxidized low density lipoprotein inhibits interleukin-12 production in lipopolysaccharide-activated mouse macrophages via direct interactions between peroxisome proliferator activated receptor-γ and nuclear factor-κB. J Biol Chem 2000;275:32681–7.
[29] Han KH, Chang MK, Boullier A, Green SR, Li A, Glass CK, Quehenberger O. Oxidized LDL reduces monocyte CCR2 expression through pathways involving peroxisome proliferator-activated receptor-γ. J Clin Invest 2000;106:793–802.
[30] Huang JT, Welch JS, Ricote M, Binder CJ, Willson TM, Kelly C, Witztum JL, Funk CD, Conrad D, Glass CK. Interleukin-4-dependent production of PPARγ ligands in macrophages by 12/15-lipoxygenase. Nature 1999;400:378–82.
[31] Heydeck D, Thomas L, Schnurr K, Trebus K, Thierfelder WE, Ihle JN, Kühn H. Interleukin-4 and -13 induce upregulation of the murine macrophage 12/15-lipoxygenase activity: evidence for the involvement of transcription factor STAT6. Blood 1998;91:2505–10.
[32] Thieringer R, Fenyl-Melody J, Le Grand CB, Shelton BA, Detmers PA, Somers EP, Carbin L, Moller DE, Wright SD, Berger J. Activation of peroxisome proliferator—activated receptor-γ does not inhibit IL-6 or TNF-α responses of macrophages to lipopolysaccharide *in vitro* or *in vivo*. J Immunol 2000;164:1046–54.
[33] Chawla A, Barak Y, Nagy L, Liao D, Tontonoz P, Evans RM. PPARγ dependent and independent effects on macrophage-gene expression in lipid metabolism and inflammation. Nat Med 2001;7:48–52.
[34] Moore KJ, Rosen ED, Fitzgerald ML, Randow F, Andersson LP, Altshuler D, Milstone DS, Mortensen RM, Spiegelman BM, Freeman MW. The role of PPARγ in macrophage differentiation and cholesterol uptake. Nat Med 2001;7:41–7.
[35] Hrboticky N, Draude G, Hapfelmeier G, Lorenz R, Weber PC. Lovastatin decreases the receptor-mediated degradation of acetylated and oxidized LDLs in human blood monocytes during the early stage of differentiation into macrophages. Arterioscler Thromb Vasc Biol 1999;19:1267–75.
[36] Neuzil J, Schröder A, von Hundelshausen P, Zernecke A, Weber T, Gellert N, Weber C. Inhibition of inflammatory endothelial responses by a pathway involving caspase activation and p65 cleavage. Biochemistry 2001;40:4686–92.
[37] Neuzil J, Weber T, Schröder A, Lu M, Ostermann G, Gellert N, Mayne GC, Olejnicka B, Negre-Salvayre A, Sticha M, Coffey RJ, Weber C. Induction of apoptosis in cancer cells by α-tocopheryl succinate: molecular pathways and structural requirements. FASEB J 2001;15:403–15.
[38] Belkner J, Wiesner R, Kühn H, Lankin VZ. The oxygenation of cholesterol esters by the reticulocyte lipoxygenase. FEBS Lett 1991;279:110–4.
[39] Sattler W, Mohr D, Stocker R. Rapid isolation of lipoproteins and assessment of their peroxidation by high-performance liquid chromatography with postcolumn chemiluminescence. Methods Enzymol 1994;233:469–89.
[40] Rapoport SM, Schewe T, Wiesner R, Halangk W, Ludwig P, Janicke-Höhne M, Tannert C, Hiebsch C, Klatt D. The lipoxygenase of reticulocytes. Purification, characterization and biological dynamics of the lipoxygenase its identity with the respiratory inhibitors of the reticulocyte. Eur J Biochem 1979;96:545–61.
[41] Johansen B, Kramer RM, Hession C, McGray P, Pepinsky RB. Expression, purification and biochemical comparison of natural and recombinant human non-pancreatic phospholipase A_2. Biochem Biophys Res Commun 1989;187:544–51.
[42] Draude G, von Hundelshausen P, Frankenberger M, Ziegler-Heitbrock HW, Weber C. Distinct scavenger receptor expression and function in the human $CD14^+/CD16^+$ monocyte subset. Am J Physiol 1999;276:H1144–9.
[43] Scheithe R, Heidenthal AK, Danesch U, Mauthner E, Hapfelmeier G, Becker A, Pietsch A, Weber PC, Hrboticky N. Acetylated LDL endocytosis by the human monocytic Mono Mac 6-sr cells is not mediated by the macrophage type I and II scavenger receptors. Arterioscler Thromb Vasc Biol 1997;17:1024–31.
[44] Draude G, Lorenz RL. TGF-β1 downregulates CD36 and scavenger receptor A but upregulates LOX-1 in human macrophages. Am J Physiol 2000;278:H1042–8.
[45] Headrick JP, Willems L, Ashton KJ, Holmgren K, Peart J, Matherne GP. Ischaemic tolerance in aged mouse myocardium: the role of adenosine and effects of A1 adenosine receptor overexpression. J Physiol 2003;549:823.
[46] Alksnis M, Barkhem T, Stromstedt PE, Ahola H, Kutoh E, Gustafsson JA, Poellinger L, Nilsson S. High level expression of functional full length and truncated glucocorticoid receptor in Chinese hamster ovary cells. J Biol Chem 1991;266:10078–85.
[47] Iwashima Y, Eto M, Horiuchi S, Sano H. Advanced glycation end product-induced peroxisome proliferator-activated receptor γ gene expression in the cultured mesangial cells. Biochem Biophys Res Commun 1999;264:441–8.
[48] Fischer B, von Knethen A, Brune B. Dualism of oxidized lipoproteins in provoking and attenuating the oxidative burst in macrophages: role of peroxisome proliferator-activated receptor-γ. J Immunol 2002;168:2828–34.
[49] Shibuya A, Wada K, Nakajima A, Saeki M, Katayama K, Mayumi T, Kadowaki T, Niwa H, Kamisaki Y. Nitration of PPAR γ inhibits ligand-dependent translocation into the nucleus in a macrophage-like cell line, RAW 264. FEBS Lett 2002;525:43–7.
[50] Han J, Hajjar DP, Zhou X, Gotto AM, Nicholson AC. Regulation of peroxisome proliferator-activated receptor—gamma-mediated gene expression. A new mechanism of action for high density lipoprotein. J Biol Chem 2002;277:23582–6.
[51] Lehmann JM, Lenhard JM, Oliver BB, Ringold GM, Kliewer SA. Peroxisome proliferator-activated receptors α and γ are activated by indomethacin and other nonsteroidal anti-inflammatory drugs. J Biol Chem 1997;272:3406–10.
[52] Sartipy P, Hurt-Camejo E. Modification of plasma lipoproteins by group IIA phospholipase A_2: possible implications for atherogenesis. Trends Cardiovasc Med 1999;98:232–8.
[53] Hurt-Camejo E, Camejo G, Peilot H, Oorni K, Kovanen P. Phospholipase A_2 in vascular disease. Circ Res 2001;89:298–304.
[54] Kühn H, Chan L. The role of 15-lipoxygenase in atherogenesis: pro- and antiatherogenic actions. Curr Opin Lipidol 1997;8:111–7.
[55] Cathcart MK, Folcik VA. Lipoxygenases and atherosclerosis: protection versus pathogenesis. Free Radic Biol Med 2000;28:1726–34.
[56] Funk CD, Cyrus T. 12/15-Lipoxygenase, oxidative modification of LDL and atherogenesis. Trends Cardiovasc Med 2001;11:116–24.
[57] Schiering A, Menschikowski M, Mueller E, Jaross W. Analysis of secretory group II phospholipase A2 expression in human aortic tissue in dependence on the degree of atherosclerosis. Atherosclerosis 1999;144:73–8.
[58] Sartipy P, Johansen B, Camejo G, Rosengren B, Bondjers G, Hurt-Camejo E. Binding of human phospholipase A_2 type II to proteoglycans. Differential effect of glycosaminoglycans on enzyme activity. J Biol Chem 1996;271:26307–14.
[59] Sartipy P, Johansen B, Gasvik K, Hurt-Camejo E. Molecular basis for the association of group IIA phospholipase A_2 and decorin in human atherosclerotic lesions. Circ Res 2000;86:707–14.
[60] Ivandic B, Castellani LW, Wang XP, Qiao JH, Mehrabian M, Navab M, Fogelman AM, Grass DS, Swanson ME, de Beer MC, de Beer F, Lusis AJ. Role of group II secretory phospholipase A2 in atherosclerosis: 1. Increased atherogenesis and altered lipoproteins in transgenic mice

expressing group IIa phospholipase A2. Arterioscler Thromb Vasc Biol 2000;19:1284–90.
[61] Folcik VA, Nivar-Aristy RA, Krajewski LP, Cathcart M. Lipoxygenase contributes to the oxidation of lipids in human atherosclerotic plaques. J Clin Invest 1996;96:504–10.
[62] Cyrus T, Pratico D, Zhao L, Witztum JL, Rader DJ, Rokach J, FitzGerald GA, Funk CD. Absence of 12/15-lipoxygenase expression decreases lipid peroxidation and atherogenesis in apolipoprotein E-deficient mice. Circulation 2001;103:2277–82.
[63] George J, Afek A, Shaish A, Levkovitz H, Bloom N, Cyrus T, Zhao L, Funk CD, Sigal E, Harats D. 12/15-Lipoxygenase gene disruption attenuates atherogenesis in LDL receptor-deficient mice. Circulation 2001;104:1646–50.
[64] Linton MF, Fazio S. Class A scavenger receptors, macrophages, and atherosclerosis. Curr Opin Lipidol 2001;12:489–95.
[65] Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. Circulation 2001;104:365–72.
[66] Brown AJ, Jessup W. Oxysterols and atherosclerosis. Atherosclerosis 1999;142:1–28.
[67] Auge N, Santanam N, Mori N, Keshava C, Parthasarathy S. Uptake of 13-hydroperoxylinoleic acid by cultured cells. Arterioscler Thromb Vasc Biol 1999;19:925–31.
[68] Ek-Von Mentzer BA, Zhang F, Hamilton JA. Binding of 13-HODE and 15-HETE to phospholipid bilayers, albumin, and intracellular fatty acid binding proteins. J Biol Chem 2001;276:15575–80.
[69] Scheithe R, Hrboticky N, Ziegler-Heitbrock HW, Weber PC. Receptor-specific uptake and degradation of acetylated, oxidized and native LDL in the human monocytic Mono Mac 6-sr cell line. Biochem Biophys Res Commun 1994;202:334–9.
[70] Sigari F, Lee C, Witztum JL, Reaven PD. Fibroblasts that overexpress 15-lipoxygenase generate bioactive and minimally modified LDL. Arterioscler Thromb Vasc Biol 1997;17:3639–45.
[71] Kruth HS, Huang W, Ishii I, Zhang WY. Macrophage foam cell formation with native low density lipoprotein. J Biol Chem 2002; 277:34573–80.
[72] Hevonoja T, Pentikainen MO, Hyvonen MT, Kovanen PT, Ala-Korpela M. Structure of low density lipoprotein (LDL) particles: basis for understanding molecular changes in modified LDL. Biochim Biophys Acta 2000;1488:189–210.
[73] Hinz B, Brune B, Pahl A. 15-Deoxy-$\Delta_{12,14}$-prostaglandin J2 inhibits the expression of proinflammatory genes in human blood monocytes via a PPAR-γ-independent mechanism. Biochem Biophys Res Commun 2003;302:415–20.
[74] Wright HM, Clish CB, Mikami T, Hauser S, Yanagi K, Hiramatsu R, Serhan CN, Spiegelman BM. A synthetic antagonist for the peroxisome proliferator-activated receptor-γ inhibits adipocyte differentiation. J Biol Chem 2000;275:1873–7.
[75] Bishop-Bailey D, Hla T, Warner TD. Bisphenol A diglycidyl ether (BADGE) is a PPARγ agonist in an ECV304 cell line. Br J Pharmacol 2000;131:651–4.
[76] Rosen ED, Sarraf P, Troy AE, Bradwin G, Moore K, Milstone DS, Spiegelman BM, Mortensen RM. PPARγ is required for the differentiation of adipose tissue *in vivo* and *in vitro*. Mol Cell 1999;4:611–7.
[77] Jostarndt K, Gellert N, Rubic T, Weber C, Kühn H, Johansen B, Hrboticky N, Neuzil J. Dissociation of apoptosis induction and CD36 upregulation by enzymatically modified low-density lipoprotein in monocytic cells. Biochem Biophys Res Commun 2002;290: 988–93.