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Dualism of Oxidized Lipoproteins in Provoking and Attenuating the Oxidative Burst in Macrophages: Role of Peroxisome Proliferator-Activated Receptor-γ[1]

Barbara Fischer, Andreas von Knethen, and Bernhard Brüne[2]

Activation and deactivation of macrophages are of considerable importance during the development of various disease states, atherosclerosis among others. Macrophage activation is achieved by oxidized lipoproteins (oxLDL) and is determined by oxygen radical (ROS) formation. The oxidative burst was measured by flow cytometry and quantitated by oxidation of the redox-sensitive dye dichlorodihydrofluorescein diacetate. Short-time stimulation dose-dependently elicited ROS formation. Diphenylene iodonium prevented ROS formation, thus pointing to the involvement of a NAD(P)H oxidase in producing reduced oxygen species. In contrast, preincubation of macrophages with oxLDL for 16 h showed an attenuated oxidative burst upon a second contact with oxLDL. Taking into account that oxLDL is an established peroxisome proliferator-activated receptor-γ (PPARγ) agonist and considering the anti-inflammatory properties of PPARγ, we went on and showed that a PPARγ agonist such as ciglitazone attenuated ROS formation. Along that line, major lipid peroxidation products of oxLDL, such as 9- and 13-hydroxyoctadecadienoic acid, shared that performance. Supporting evidence that PPARγ activation accounted for reduced ROS generation came from studies in which proliferator-activated receptor response element decoy oligonucleotides, but not a mutated oligonucleotide, supplied in front of oxLDL delivery regained a complete oxidative burst upon cell activation. We conclude that oxLDL not only elicits an oxidative burst upon first contact, but also promotes desensitization of macrophages via activation of PPARγ. Desensitization of macrophages may have important consequences for the behavior of macrophages/foam cells in atherosclerotic lesions. ***The Journal of Immunology,*** **2002, 168: 2828–2834.**

Low density lipoproteins acquire a number of important pathophysiological activities as a result of oxidative modification. Oxidized low density lipoproteins (oxLDL)[3] are generated in vitro by auto-oxidation in the presence of transition metals (1, 2) or in vivo via cell-mediated mechanisms (3, 4). OxLDL has been shown to be a powerful regulator of cell signaling in provoking various responses, among others, expression of adhesion molecules on endothelial cells (5), production of proinflammatory cytokines and growth factors by vascular cells (6), or proliferation and migration of vascular cells (7). In addition, oxLDL is both a potent chemoattractant for circulating monocytes (8) and a differentiating agent that promotes transition of macrophages to lipid-loaded foam cells (9). In close association, receptor-mediated endocytosis of oxLDL by several scavenger receptor family members including macrophage class A scavenger receptor (10, 11), CD36 (12), scavenger receptor that binds phosphatidylserine and oxidized lipoprotein (13), or lectin-like oxidized low density lipoprotein receptor-1 (14) is implicated in the process of atherogenesis. Atherosclerosis is now considered a problem of wound healing and chronic inflammation and can be viewed as a response to injury, with lipoproteins or other risk factors as the injurious agents, keeping in mind the important idea that accumulation of lipid-loaded foam cells in fatty streaks are a primary event in disease progression (15).

Peroxisome proliferator-activated receptors (PPARs) are a group of lipid-activated nuclear receptors that heterodimerize with the 9-*cis*-retinoic acid receptor to form functional transcription factors that regulate genes involved in lipid and glucose metabolism (16–19). Examples are adipocyte fatty acid binding protein aP2 (20), phosphoenolpyruvate carboxykinase (21), lipoprotein lipase (22), or the brown fat uncoupling protein UCP1 (23). Activation of PPARγ is achieved by naturally occurring ligands that comprise derivatives of linoleic or arachidonic acid or synthetic anti-diabetic drugs known as thiazolidinediones (23–26).

Exposure of monocytes/macrophages to oxLDL provokes activation and expression of PPARγ, presumably via the action of 9- and 13-hydroxyoctadecadienoic acid (9- and 13-HODE), 15-hydroxyeicosatetraenoic acid, or 15-deoxy-$\Delta^{12,14}$-PGJ_2 (15dPGJ_2) (27). 9-HODE and 13-HODE are major lipid peroxidation products of oxLDL that account for nearly 67% of all lipid peroxidation products found in oxLDL (28). Along that line, PPARγ is highly expressed in foam cells of atherosclerotic lesions (16, 29). Following PPARγ activation in monocytes/macrophages, CD36 is up-regulated, which promotes a self-amplification feed-forward loop to further internalize oxLDL (27). Despite evidence for a role of PPARγ in atherogenesis, more recent studies support an anti-atherogenic role of PPARγ (18, 30, 31). Apparently, the vicious cycle of lipid accumulation in foam cells and PPARγ activation is broken by the opposing effect of PPARγ ligands on

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[3] Abbreviations used in this paper: oxLDL, oxidized lipoproteins; DCF, dichlorodihydrofluorescein diacetate; DPI, diphenylene iodonium; 15dPGJ_2, 15-deoxy-$\Delta^{12,14}$-PGJ_2; HODE, hydroxyoctadecadienoic acid; LDL, low density lipoprotein; PPARγ, peroxisome proliferator-activated receptor-γ; PPRE, peroxisome proliferator-activated receptor response element; ROS, reactive oxygen species; MnSOD, manganese superoxide dismutase; EU, endotoxin U.

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class A scavenger receptor expression, which is thereby downregulated, thus countering potentially atherosclerotic effects of CD36 induction (32).

Activation of macrophages may elicit an oxidative burst in response to agonists such as oxLDL (33). We speculated whether an acute response to oxLDL may provoke an oxidative response in macrophages, whereas a late answer may attenuate reactive oxygen radical (ROS) production. Indeed, acute ROS formation evoked by oxLDL is contrasted by its inhibition with oxLDL after being pre-exposed for 16 h. Taking the anti-inflammatory properties of PPARγ into consideration, it appears rational to propose an attenuated oxidative response via activation of PPARγ in macrophages after oxLDL preincubation. A role of PPARγ in downregulating ROS production was established when peroxisome proliferator-activated receptor response element (PPRE) decoy oligonucleotides were allowed to regain a full oxidative burst after oxLDL preincubation. We conclude that oxLDL not only generates ROS upon first contact, but also promotes PPARγ activation, which, in turn, desensitizes macrophages, i.e., reduces ROS production. Activation of PPARγ by oxLDL appears as an important determinant of the activation/deactivation balance in macrophages.

Materials and Methods

Materials

Cytochrome *c* and trolox were purchased from Sigma (Deisenhofen, Germany). Dichlorodihydrofluorescein diacetate (DCF) was obtained from Molecular Probes (Leiden, The Netherlands). Ciglitazone came from Biomol (Hamburg, Germany), and oligonucleotides were delivered by Eurogentec (Seraing, Belgium). 9(*S*)-HODE and 13(*S*)-HODE were purchased from Caymen (Ann Arbor, MI). Culture supplements and FCS were obtained from Biochrom (Berlin, Germany). The NAD(P)H inhibitor diphenylene iodonium (DPI) was obtained from Fluka (Deisenhofen, Germany). All other chemicals were of the highest grade of purity and were commercially available.

Low density lipoprotein (LDL): isolation and oxidation

Human plasma was obtained from Department of Transfusion Medicine, Faculty of Medicine, University of Erlangen-Nurnberg (Erlangen-Nurnberg, Germany). LDL was isolated by sequential ultracentrifugation as previously described (34), followed by dialysis against PBS and 200 μM EDTA (pH 8.0) at 4°C. Protein content was measured by the Lowry method (35). LDL (1 mg/ml) was oxidized by the addition of 3 μM $CuSO_4$ for 30 h in PBS at room temperature. Oxidation was terminated by adding EDTA (pH 8.0) to a final concentration of 200 μM, followed by dialysis against PBS/100 μM EDTA (pH 8.0) at 4°C. The degree of oxidation was quantified by an increased relative mobility on agarose gels (Lipidophor all in 12, Technoclone, Heidelberg, Germany), indicating an enhanced negative charge of oxidized lipoprotein. The relative mobility of oxLDL on agarose gels as an index for lipoprotein oxidation was 2.5–3.0 compared with that of native LDL.

Endotoxin test

A potential contamination of representative charges of LDL and oxLDL with endotoxin was tested at the Laboratory of Environmental Biotechnology (Munster, Germany). Results were <3.28 endotoxin U (EU)/ml for LDL, and values ranged between 27.71 and 92.13 EU/ml for oxLDL. To rule out unspecific effects of endotoxin under our experimental conditions, macrophages were exposed, according to the experimental design, to 90 EU/ml LPS. This was without any effect on the parameters under investigation.

Cell culture

The human premonocytic cell line U937 and the mouse macrophage cell line RAW 264.7 were maintained in RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated FCS (complete medium). All experiments were performed using complete RPMI.

Culture of human primary monocyte-derived macrophages

Human monocytes were isolated from buffy coats of healthy donors as previously described (36). After Ficoll gradient centrifugation, monocytes were cultured under adherent conditions in six-well plates in RPMI 1640 supplemented with 10% human serum AB, 1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 100 U/ml penicillin, and 100

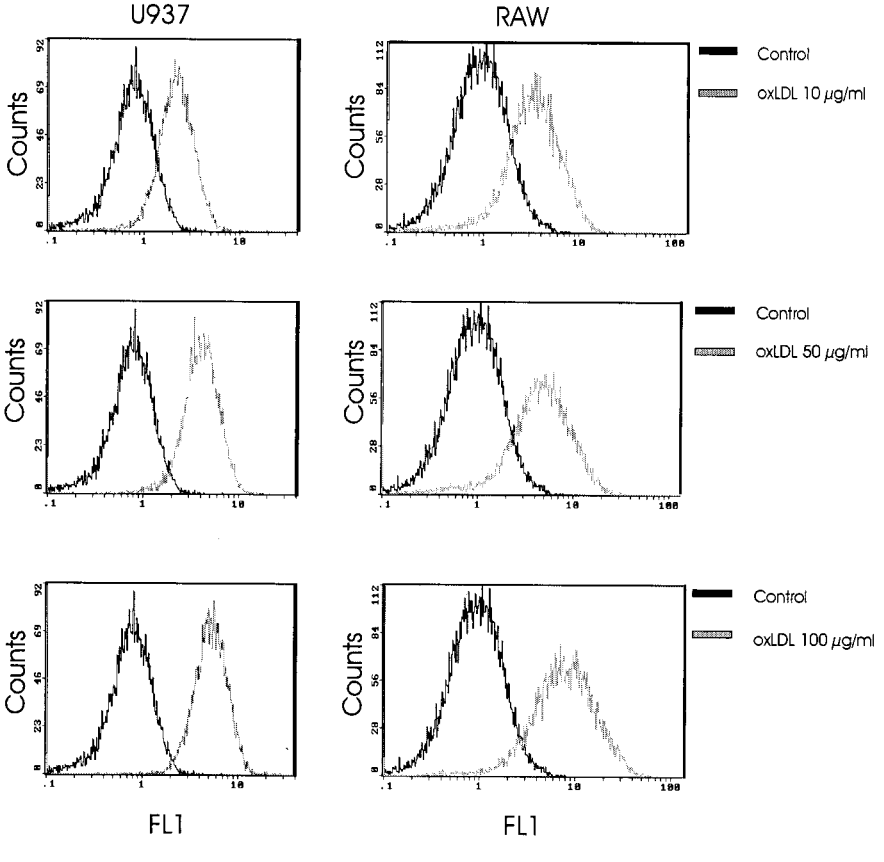

FIGURE 1. Oxygen radical production in U937 cells and RAW 264.7 macrophages in response to oxLDL. ROS production in response to 1-h stimulation with oxLDL (10–100 μg/ml) was analyzed by following oxidation of DCF using a flow cytometer. Data are representative of three similar experiments. For details see *Materials and Methods*. FL1, Fluorescence.

μg/ml streptomycin for 8 days to induce differentiation to macrophages (37). Thereafter, macrophages were preincubated with oxLDL or remained as controls.

Flow cytometry: oxygen-radical production (DCF assay)

Cells were cultured under nonadherent conditions in six-well plates at a density of 4×10^5 cells/well. Following prestimulation or addition of vehicle, cells were incubated for 30 min with 50 μM DCF. Thereafter, oxLDL at the indicated concentration was added, and incubations were continued for 1 h. Cells were harvested and resuspended in 500 μl PBS. Flow cytometry was performed using a Coulter EPICS XL flow cytometer (Beckman Coulter, Krefeld, Germany), and DCF was measured through a 530-nm long-pass filter (fluorescence 1). Data from 10,000 cells were collected to obtain significance.

Decoy approach

Cells were exposed to an oligonucleotide containing a PPRE consensus site: 5′-GGT AAA GGT CAA AGG TCA AT-3′ and 3′-A TTT CCA GTT TCC AGT TAG CCG-5′. Cells were seeded at a density of 4×10^5 cells/well into six-well plates. Oligonucleotides (3 μM) were added 24 h before cell stimulation. Cell stimulation was performed as indicated. For control reasons oligonucleotides with a mutated PPRE site (boldface) were used: 5′-GGT AAA GAA CAA AGA ACA AT-3′ and 3′-A TTT C**TT** GTT TC**T** **T**GT TAG CCG-5′.

Cytochrome c *reduction*

Superoxide-evoked reduction of ferricytochrome *c* to ferrocytochrome *c* was followed at 550 nm using a spectrophotometer (Perkin-Elmer Instruments, Rodgau-Jugesheim, Germany). Cells ($2–4 \times 10^5$) were seeded 15 h before the experiment. Medium was changed to phenol red-free medium, and cells were further incubated for 1 h without addition (control) or with different concentrations of oxLDL in the presence of 50 μM cytochrome *c*. Phenol red-free medium cytochrome *c* served as a control. The extinction coefficient used for the calculation of superoxide production is 21.5 $mM^{-1}cm^{-1}$. Superoxide production was calculated by multiplying extension $\times$ extinction coefficient^{-1} $\times$ path-length^{-1}.

Statistical analysis

Each experiment was performed at least three times, and statistical analysis was performed using ANOVA, followed by Fisher's post-hoc test. Otherwise, representative data are shown.

Results

An oxLDL-evoked oxidative burst in U937 cells, RAW 264.7 macrophages, and human primary monocyte-derived macrophages

Initially, we set up a test system to follow oxygen radical production by macrophages in response to stimulation with oxLDL. We chose human premonocytic U937 cells, the murine macrophage-like cell line RAW 264.7, and human primary monocyte-derived macrophages to avoid cell-specific or artificial cell culture responses. The oxygen burst was primarily determined by flow cytometry, looking for the conversion of the nonfluorescent DCF into a fluorescent molecule through oxidation. Activation of U937 or RAW 264.7 cells with oxLDL evoked ROS formation (Fig. 1). ROS formation was dose dependent, clearly visible at 10 μg/ml oxLDL and maximal at doses of 50–100 μg/ml oxLDL.

ROS formation became evident by following the rightward shift of fluorescence 1 (excitation, 488 nm; emission, 530 nm) with the idea that U937 and RAW 264.7 showed comparable behaviors. To quantitate ROS we determined cytochrome *c* reduction. U937 cells were subsequently treated with 50 μg/ml oxLDL for 1 h or were left unstimulated. Cytochrome *c* reduction was followed spectrophotometrically and amounted to 0.14 ± 0.01 μM $O_2^-/4 \times 10^5$ cells/h for controls vs 0.56 ± 0.074 μM $O_2^-/4 \times 10^5$ cells/h for oxLDL treatment. Data represent the mean $\pm$ SE ($n \geq 5$) and reached a statistical significant difference ($p < 0.01$). In addition, ROS formation was dose-dependently elicited in human primary monocyte-derived macrophages. ROS formation was initiated with 50 μg/ml oxLDL and reached maximal values with concentrations of 100–200 μg/ml oxLDL. Cytochrome *c* reduction indicated the generation of 0.12 ± 0.04 μM $O_2^-/2 \times 10^5$ cells/h for controls vs 0.51 ± 0.07 μM $O_2^-/2 \times 10^5$ cells/h following the addition of 100 μg/ml oxLDL or 0.91 ± 0.07 μM $O_2^-/2 \times 10^5$ cells/h in response to 200 μg/ml oxLDL.

We delineated the source of ROS production by applying the NAD(P)H oxidase inhibitor DPI at a concentration of 50 μM (Fig. 2). ROS production elicited by 10 μg/ml oxLDL was completely attenuated in the presence of DPI.

Evidently, DPI blocked a rightward shift of fluorescence 1 under the impact of oxLDL. The antioxidant trolox largely suppressed ROS formation in response to oxLDL. Incubations of RAW 264.7 macrophages with DPI, followed by the addition of the redox cycler 10 μM 2,3-dimethoxy-1,4-naphthoquinone to provoke ROS formation, showed no interference by DPI, thus ruling out that DPI functions by quenching the fluorescence (data not shown).

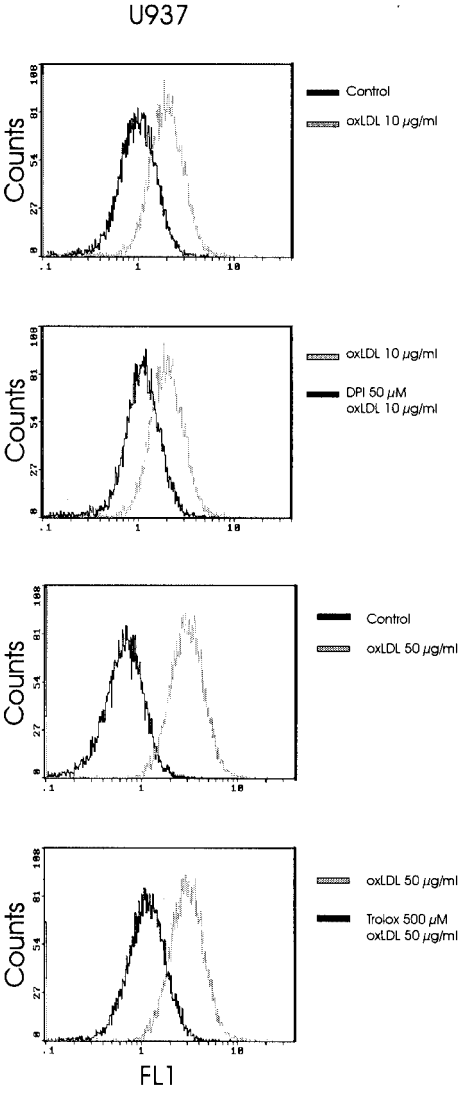

FIGURE 2. DPI and trolox blocked oxygen radical production. U937 cells were preincubated for 1 h with 50 μM DPI or 500 μM trolox or remained as controls. ROS production was initiated with 10 or 50 μg/ml oxLDL for 1 h as indicated. For details see Fig. 1 and/or *Materials and Methods*. FL1, Fluorescence.

Desensitization of macrophages by oxLDL, HODEs, or ciglitazone pretreatment

In extending experiments we investigated ROS formation of macrophages with oxLDL preincubation. During these experiments, performed using flow cytometry, we gated on viable cells to exclude any interference that may have occurred by apoptosis/necrosis. As depicted in Fig. 3, the oxidative burst in U937 or RAW 264.7 cells was initiated by the addition of 10 μg/ml oxLDL.

In the following set of experiments we pre-exposed macrophages for 16 h to 50 μg/ml oxLDL, followed by the addition of 10 μg/ml oxLDL to provoke ROS formation. Following prestimulation with oxLDL, ROS formation as a result of a second oxLDL exposure was attenuated by roughly 50–70%. This applied to both U937 and RAW 264.7 cells. Apparently, oxLDL-evoked signals provoked desensitization of macrophages when assayed for ROS formation.

Exposure of monocytes/macrophages to oxLDL elicits activation and expression of PPARγ, presumably via the action of 9- and 13-HODE. 9-HODE and 13-HODE are major lipid peroxidation products of oxLDL and account for nearly 67% of all lipid peroxidation products found in oxLDL (28). Therefore, pre-exposure of macrophages for 16 h to 0.5 μg/ml 9(*S*)-HODE or 13(*S*)-HODE, followed by the addition of 10 μg/ml oxLDL to provoke radical formation, attenuated ROS formation in RAW 264.7 macrophages under these experimental conditions (Fig. 4).

Taking into account that oxLDL is an established PPARγ activator (16, 27), we were interested in studying a potential correlation between PPARγ activation and inhibition of oxLDL-evoked ROS production. As shown in Fig. 5, activation of PPARγ by the specific agonist ciglitazone largely attenuated the oxidative burst in U937 cells.

During these examinations U937 cells were preactivated with 3 μM ciglitazone for 16 h before stimulation with 50 μg/ml oxLDL. We noticed a roughly 50% reduced ROS signal with ciglitazone present. A short prestimulation (1 h) of RAW 264.7 macrophages with the specific PPARγ agonist ciglitazone did not interfere with ROS formation. A further control experiment with the redox cycler 10 μM 2,3-dimethoxy-1,4-naphthoquinone showed that ciglitazone did not quench the fluorescence signal (data not shown). Furthermore, we noticed that preincubation of cells with oxLDL or ciglitazone for 16 h did not attenuate expression of the CD36 scavenger receptor (data not shown).

Desensitization of monocytes/macrophages by oxLDL demanded PPARγ activation

It was our further intention to demonstrate activation of PPARγ under conditions of oxLDL prestimulation to be associated with diminished ROS generation. Experimentally, we used decoy oligonucleotides to scavenge and thereby inactivate relevant transcription factors. OxLDL attenuated ROS formation via PPARγ activation. As shown in Fig. 6, oxidation of DCF was elicited in response to 10 μg/ml oxLDL in RAW 264.7 macrophages, while prestimulation with 50 μg/ml oxLDL largely attenuated ROS formation.

The presence of PPRE decoy oligonucleotides attenuated the down-modulatory behavior of oxLDL and regained DCF oxidation in response to low concentrations of oxLDL (10 μg/ml). Obviously, PPRE decoy oligonucleotides allowed a full recovery of the oxidative burst that otherwise had been suppressed by prestimulation with oxLDL. Along that line, prestimulation of RAW 264.7 macrophages with oxLDL (50 μg/ml) that had been exposed to oligonucleotides containing a mutant PPRE site beforehand were unable to regain the oxidative response after the addition of 10 μg/ml oxLDL. To exclude the involvement of PPARα in attenuating ROS formation elicited by oxLDL we preincubated RAW 264.7 with 10 μM of the PPARα agonist WY 14643 (4-chloro-6-(2,3-xylidino)-2-pyrimidinylthioacetic acid) for 16 h, followed by the addition of 50 μg/ml oxLDL to induce ROS generation for 1 h. WY 14643 slightly enhanced, rather than attenuated, ROS formation (data not shown). Therefore, we conclude attenuated ROS formation to be associated with the activation of PPARγ.

Discussion

Here we investigated ROS formation in primary monocyte-derived macrophages and macrophage cell lines in response to oxLDL. Short term (1 h) stimulation with oxLDL dose-dependently elicited ROS formation. In contrast, we provide evidence that preincubation of monocytes/macrophages with oxLDL or major lipid components of oxLDL such as HODE for 16 h attenuated the oxidative burst. Down-regulation of ROS formation was linked to PPARγ

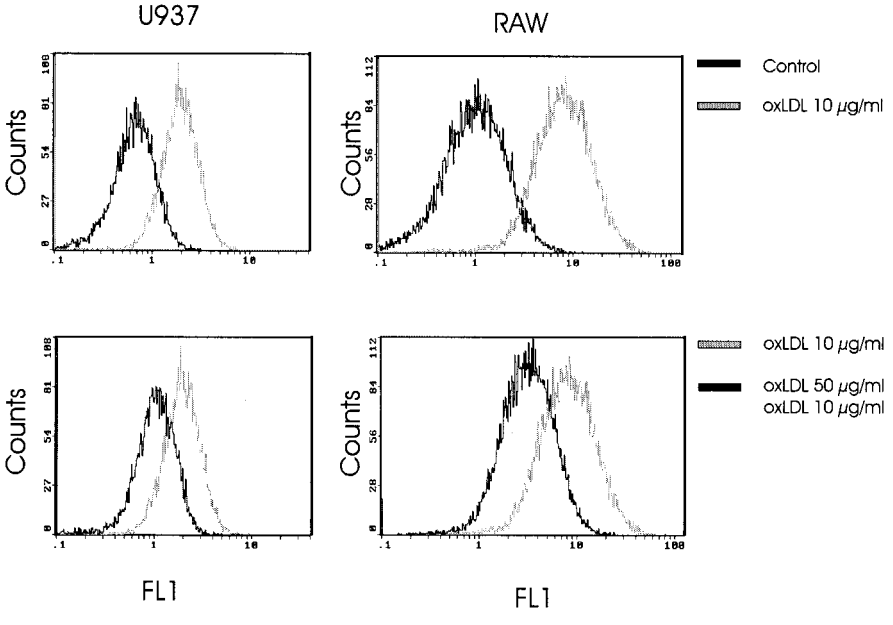

FIGURE 3. Oxygen radical production is attenuated by oxLDL pretreatment. U937 cells and RAW 264.7 macrophages were prestimulated for 16 h with 50 μg/ml oxLDL or vehicle. ROS production in response to 1-h stimulation with 10 μg/ml oxLDL was analyzed by flow cytometry, using 50 μM DCF as the redox-sensitive dye. Data are representative of three similar experiments. For details see *Materials and Methods*. FL1, Fluorescence.

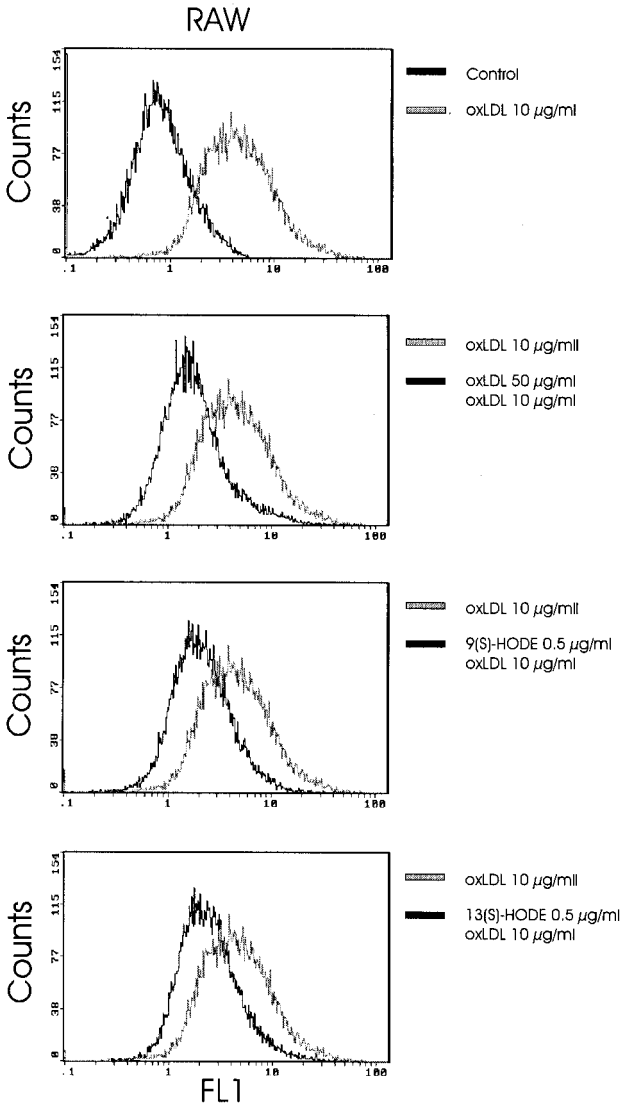

FIGURE 4. Oxygen radical formation is attenuated by pretreatment with HODEs. RAW 264.7 macrophages were prestimulated for 16 h with 0.5 μg/ml 9(*S*)-HODE, 0.5 μg/ml 13(*S*)-HODE, 50 μg/ml oxLDL, or vehicle. ROS production in response to 1-h stimulation with 10 μg/ml oxLDL was analyzed by flow cytometry, using 50 μM DCF as the redox-sensitive dye. Data are representative of three similar experiments. For details see *Materials and Methods*. FL1, Fluorescence.

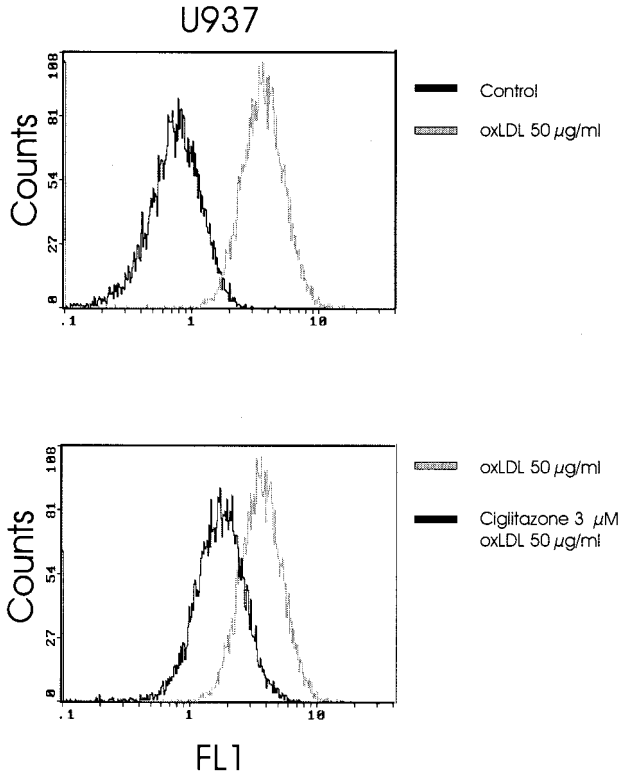

FIGURE 5. Attenuated oxygen radical production in U937 cells under the influence of the PPARγ agonist ciglitazone. U937 cells were prestimulated for 16 h with 3 μM ciglitazone or remained as controls. ROS production was elicited with 50 μg/ml oxLDL for 1 h and was analyzed by flow cytometry using 50 μM DCF as the redox-sensitive dye. Data are representative of three similar experiments. For details see *Materials and Methods*. FL1, Fluorescence.

activation by using a PPRE decoy oligonucleotide approach and was further substantiated by the use of ciglitazone, an established PPARγ agonist (schematically shown in Fig. 7). We conclude that oxLDL not only elicited an oxidative burst upon first contact, but also promoted PPARγ activation, which, in turn, attenuated ROS formation, thus contributing to macrophage desensitization.

Accumulation of oxLDL is a characteristic feature of atherosclerotic lesions and plays a fundamental role in the pathogenesis and progression of disease (15). Considering atherosclerosis as a problem of chronic inflammation the involvement of macrophages is not surprising, and macrophage transformation to lipid-loaded foam cells is a primary event in the disease program (15). Previous studies demonstrated that oxLDL evoked an oxidative burst in endothelial cells (39–41) and/or monocyte/macrophage cell lines (33). Antisense oligonucleotides directed against $p22^{phox}$, a subunit of the membrane-bound NAD(P)H oxidase system, attenuated oxLDL-evoked ROS formation in endothelial cells, suggesting a role for membrane-bound NAD(P)H oxidase as a major source of ROS (34, 42). We corroborated these findings by eliminating ROS generation in macrophages with the use of DPI, an inhibitor for O_2^--producing NAD(P)H-like oxidase systems. Furthermore, DCF oxidation in macrophages was attenuated by the antioxidant trolox, which is indicative of an oxidative response elicited by oxLDL.

An established response of monocytes/macrophages to oxLDL is activation and expression of PPARγ, presumably via formation of 13-HODE, 15-hydroxyeicosatetraenoic acid, or $15dPGJ_2$ (27). As a consequence, enforced CD36 expression after long term stimulation (≥4 days) starts an amplification loop that culminates in massive oxLDL uptake and thus foam cell formation. Along that line, high expression of PPARγ in foam cells of atherosclerotic lesions (16, 27) is established, and the action of synthetic antidiabetic drugs known as thiazolidinediones (24–26) as efficient PPARγ activators is seen.

Preincubation of monocytes/macrophages with oxLDL showed attenuated ROS formation in response to oxLDL in RAW 264.7 and U937 macrophages. Cellular desensitization may be mediated via activation of PPARγ, because oxLDL is a known PPARγ agonist. To provide further evidence of PPARγ activation in attenuating ROS formation we sought to reduce O_2^- formation in monocytes/macrophages with preincubation of specific PPARγ agonists. Indeed, ciglitazone, a classical PPARγ activator, attenuated ROS formation in response to oxLDL in RAW 264.7 and U937 macrophages, implying a role of PPARγ in negatively affecting the oxidative burst. These results are in line with previous studies showing an attenuated oxidative burst in macrophages not

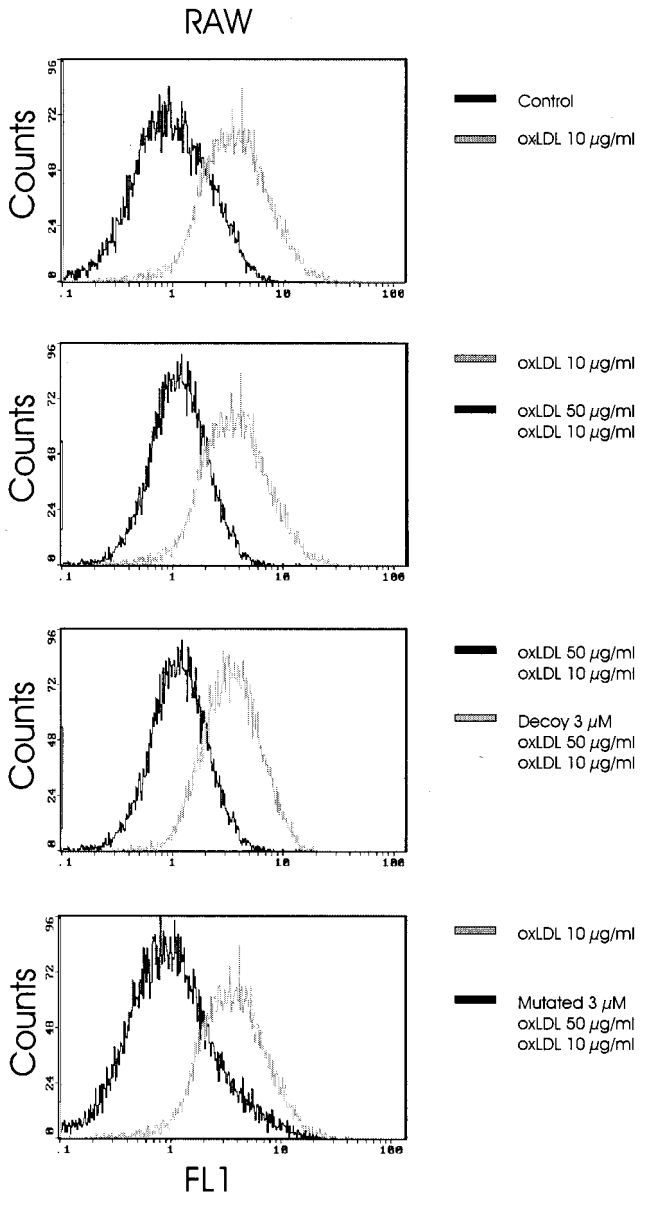

FIGURE 6. PPRE decoy oligonucleotides allowed to recover a full oxidative burst upon oxLDL addition. RAW 264.7 macrophages were incubated with PPRE decoy or a mutated oligonucleotide for 24 h or remained as controls. After changing the medium, cells were prestimulated with 50 μg/ml oxLDL or vehicle for 16 h. In turn, ROS production was initiated with 10 μg/ml oxLDL for 1 h and was analyzed by flow cytometry using 50 μM DCF as the redox-sensitive dye. Data are representative of three similar experiments. For details see *Materials and Methods*. FL1, Fluorescence.

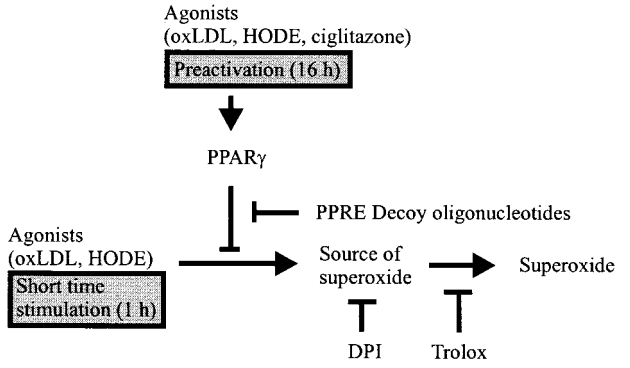

FIGURE 7. Dualism of oxLDL in provoking or attenuating the oxidative burst in macrophages. Short term stimulation with oxLDL elicited ROS formation. DPI interfered via blocking the source of ROS, while the antioxidant trolox probably scavenged ROS. Preincubation of monocytes/macrophages for 16 h with PPARγ agonists (oxLDL, HODE, ciglitazone) attenuated the oxidative burst. PPRE decoy oligonucleotides antagonized this down-modulatory behavior and allowed oxygen radical formation to be regained upon agonist challenge. See *Discussion* for details.

only in response to ciglitazone or 15dPGJ$_2$ but also as a result of LPS/IFN-γ preactivation, which demanded PPARγ activation (38). To provide unequivocal evidence that oxLDL-elicited PPARγ activation down-regulated ROS formation we employed a PPRE decoy oligonucleotide approach to scavenge and thereby inactivate a relevant transcription factor. The presence of PPRE decoy oligonucleotides antagonized the down-modulatory behavior of oxLDL and allowed a full recovery of oxLDL-mediated ROS formation that otherwise had been suppressed by PPARγ activation. The combination of decoy experiments with the use of established PPARγ activators strongly suggests that PPARγ activation reduces ROS formation and that oxLDL uses this pathways to down-regulate ROS formation in activated macrophages. Based on our finding that the action of oxLDL is shared by a so-called specific PPARγ agonist, HODE, and is attenuated by a PPRE decoy approach, we do not favor a direct scavenging effect of, i.e., glutathione, although oxLDL can increase the level of reduced glutathione in macrophages (43). In addition, according to a previous observation (44) oxLDL may up-regulate manganese superoxide dismutase (MnSOD) that, in turn, may account for decreased ROS species. Expression of MnSOD was analyzed by Western blot analysis, which excluded regulation of MnSOD under our experimental conditions in macrophages. DCF is reported to detect H_2O_2 in addition to superoxide. Therefore, enforced expression of SOD may cause faster disproportionation of superoxide, but does not affect detection of the end product of this reaction. Attenuation of ROS formation as a result of PPARγ activation appears in close association with established anti-inflammatory actions of PPARγ in macrophages, such as inhibition of NO formation or proinflammatory cytokine production (45). Macrophage stimulation by oxLDL not only caused ROS generation upon first contact, but also promoted PPARγ activation, which later plays a anti-inflammatory and cell-desensitizing role. The mechanisms by which PPARγ activation attenuated ROS formation will be the subject of further investigations. Interestingly, Inoue and co-workers (46) reported that active PPARγ reduced the expression of central NAD(P)H-oxidase components such as p22^{phox} mRNA as well as p47^{phox} protein levels in human endothelial cells. Future experiments will elaborate whether this applies to macrophages as well and whether oxLDL achieves reduced protein expression of NAD(P)H oxidase components via PPARγ that may explain reduced O_2^- formation. Moreover, we need to determine how oxLDL achieves activation of PPARγ and to elucidate the temporal sequence of events that determines the balance between cell activation and deactivation under the impact of oxLDL.

Despite the pathological role of foam cell formation under conditions of severe hyperlipidemia, it remains plausible that uptake of oxLDL by macrophages is beneficial in other circumstances, i.e., milder hyperlipidemia. Macrophage desensitization may then be considered advantageous, which goes along with the more recent support for an anti-atherogenic role of PPARγ (32). The degree of macrophage activation as a result of PPARγ signaling may have important consequences for the development of atherosclerotic lesions.

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