Induction of CD36 expression by oxidized LDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-γ

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Abstract **CD36, a class B scavenger receptor, is a macrophage receptor for oxidized low density lipoprotein (OxLDL) and may play a critical role in atherosclerotic foam cell formation. We have previously demonstrated that OxLDL, macrophage-colony stimulating factor (M-CSF), and interleukin-4 (IL-4) enhanced expression of CD36. The effect of OxLDL on CD36 is due, in part, to its ability to activate the transcription factor, PPAR-γ (peroxisome proliferator activated receptor-γ). Other PPAR-γ ligands (15-deoxy$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2) and the thiazolidinedione class of antidiabetic drugs) also increase CD36 expression. We have now evaluated signaling pathways involved in the induction of CD36. Treatment of RAW264.7 cells (a murine macrophage cell line) with protein kinase C (PKC) activators (diacylglycerol and ingenol) up-regulated CD36 mRNA expression. Specific inhibitors of PKC reduced CD36 expression in a time-dependent manner, while protein kinase A (PKA) and cyclic AMP agonists had no effect on CD36 mRNA expression. PKC inhibitors reduced basal expression of CD36 and blocked induction of CD36 mRNA by 15d-PGJ_2, OxLDL and IL-4. In addition, PKC inhibitors decreased both PPAR-γ mRNA and protein expression and blocked induction of CD36 protein surface expression by OxLDL and 15d-PGJ_2 in human monocytes, as determined by FACS. 15d-PGJ_2 had no effect on translocation of PKC-α from the cytosol to the plasma membrane. These results demonstrate that two divergent physiological or pathophysiological agonists utilize a common pathway to up-regulate of CD36 gene expression. This pathway involves initial activation of PKC with subsequent PPAR-γ activation. Defining these signaling pathways is critical for understanding and modulating expression of this scavenger receptor pathway.**—Feng, J., J. Han, S. F. A. Pearce, R. L. Silverstein, A. M. Gotto, Jr., D. P. Hajjar, and A. C. Nicholson. **Induction of CD36 expression by oxidized LDL and IL-4 by a common signaling pathway dependent on protein kinase C and PPAR-γ. *J. Lipid Res.* 2000.** 41: **688–696.**

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Macrophage scavenger receptors are thought to play a significant role in atherosclerotic foam cell development because of their ability to bind and internalize OxLDL (1–4). Two major classes of macrophage scavenger receptors, designated type A and type B, have been identified (class C scavenger receptors are macrophage specific scavenger receptors from *Drosophila* (5)). In addition, two other macrophage receptors, MARCO (macrophage receptor with a collagenous structure) and CD68 (macrosialin), may also contribute to the uptake of modified lipoproteins (6, 7).

CD36 is a class B scavenger receptor, a class that also includes SR-BI, an HDL receptor (8). CD36 is expressed by monocyte/macrophages (9), platelets (10), microvascular endothelial cells (11), retinal pigment epithelium (12), and adipose tissue (13). Like the type A scavenger receptors (14), CD36 recognizes a broad variety of ligands including OxLDL (15, 16), anionic phospholipids (17), apoptotic cells (18), thrombospondin (TSP) (19), collagen (20), effete photoreceptors (12), and *Plasmodium falciparum*-infected erythrocytes (21). Unlike the class A receptors, which recognize the oxidized apoprotein portion of the lipoprotein particle (22), CD36 binds to the lipid moiety of OxLDL (16). Binding of OxLDL to CD36-transfected cells is inhibited by anionic phospholipid vesicles (17). CD36 may also bind HDL (23). However, SR-BI mediates uptake of HDL-CE with much greater efficiency than CD36 (24).

We have shown that phorbol myristate acetate (PMA), IL-4 and M-CSF induce monocyte expression of CD36 mRNA (25). We have also demonstrated that OxLDL can stimulate its own uptake by induction of CD36 gene expression (26). The mechanism(s) by which OxLDL up-regulates CD36 involves activation of the transcription factor, PPAR-γ (27, 28). PPAR-γ is a member of a nuclear

Abbreviations: PPAR-γ, peroxisome proliferator-activated receptor-γ; M-CSF, macrophage-colony stimulating factor; IL-4, interleukin-4; OxLDL, oxidized low density lipoprotein; 15d-PGJ_2, 15-deoxy$\Delta^{12,14}$ prostaglandin J_2.

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hormone superfamily that can heterodimerize with the retinoid X receptor (RXR) and acts as a transcriptional regulator of genes encoding proteins involved in lipid regulation (29). Oxidized LDL, and two oxidized linoleic acid metabolites, 9-HODE and 13-HODE, induce both PPAR-γ activation and CD36 expression in monocyte/macrophages (28). Other PPAR-γ ligands (15-deoxy$\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ_2) and the thiazolidinedione class of antidiabetic drugs) also increase CD36 expression (28).

OxLDL has multiple effects on signaling pathways and gene expression patterns in macrophages and vascular cells. One of the earliest events that occurs in macrophages after exposure to OxLDL is enhancement of protein kinase C (PKC) activity (30). PMA, a PKC agonist, is a potent inducer of CD36 (25). In studies reported herein, we set out to determine the temporal pattern of signaling events and the relationship of PKC and PPAR-γ activation involved in inducing CD36 expression.

EXPERIMENTAL PROCEDURES

Materials

Ingenol, 1,2-diacyl-*sn*-glycerols, Calphostin C, Gö 6983, and myristolated protein kinase C inhibitor (19–27) (mPKCI) were purchased from Calbiochem (La Jolla, CA). 15-Deoxy$\Delta^{12,14}$ prostaglandin J_2 was obtained from Caymen Chemical (Ann Arbor, MI). IL-4 was obtained from R&D Systems (Minneapolis, MN).

Cells and cell lines

RAW 264.7 cells (ATCC, Rockville, MD), a murine macrophage cell line, were cultured in 60-mm petri dishes with RPMI 1640 medium containing 10% fetal calf serum, 50 μg/ml of penicillin/streptomycin, and 2 mM glutamine. Cells were used to conduct experiments when they were about 90% confluent. All treatments were performed in serum-free media.

Isolation of LDL and preparation of OxLDL

Low density lipoprotein (LDL, d 1.019–1.063 μm/ml) was isolated from normal human plasma by sequential ultracentrifugation, dialyzed against phosphate-buffered saline (PBS) containing 0.3 mM EDTA, sterilized by filtration through a 0.22 μm filter, and stored under N_2 at 4°C. Protein content was determined by the method of Lowry et al. (31).

OxLDL was prepared by dialysis of LDL (500 μg/ml) in PBS containing 5 μM $CuSO_4$ for 10 h at 37°C, followed by dialysis in PBS containing 0.3 mM EDTA for 2 × 12 hr. The purity and charge of both LDL and OxLDL were evaluated by examining electrophoretic migration in agarose gel. The degree of oxidation of LDL and OxLDL was determined by measuring the amount of thiobarbituric acid reactive substances (TBARS) (32). LDL had TBARS values of <1 nmol/mg while OxLDL had TBARS values of >10 and <30 nmol/mg. All lipoproteins were used within 3 weeks after preparation.

Isolation of total RNA, purification of poly (A^+) RNA and Northern blotting

Cells were lysed in RNAzol™ B (Tel-Test, Inc., Friendswood, TX), extracted with chloroform, and total cellular RNA was precipitated in isopropanol. The dried pellet of total RNA was dissolved with distilled water after washing with 80% and 100% ethanol. The poly (A^+) RNA was purified from about 100 μg of total RNA using PolyATtract™ mRNA Isolation System III (Promega, Madison, WI) and dissolved in 20 μl distilled water. Total RNA (20 μg) or poly (A^+) RNA for each sample was loaded on a 1% formaldehyde agarose gel and transferred overnight to a Zeta-probe^R GT Genomic Tested Blotting Membrane (Bio-Rad Laboratories, Richmond, CA) in 10× SSC by capillary force. The blot was UV cross-linked for 2 min and pre-hybridized with Hybrisol™ (Intergen) for 15 min before the addition of ^{32}P-randomly primed labeling probe. The blot was washed once for 30 min with 2× SSC and 0.2% SDS, and twice for 20 min with 0.2× SSC and 0.2% SDS at 65°C after overnight hybridization. The blot was autoradiographed by exposure to X-ray film (X-Omat™ AR, Kodak, Rochester, NY). Semiquantitative assay of autoradiograms was assessed by densitometric scanning using NIH Image software (Bethesda, MD). The probe for CD36 is a *NsiI-BglII* digest (base pairs 193–805). The original murine CD36 cDNA was obtained from Dr. Gerda Endemann. The cDNA probe for murine PPAR-γ was generated by reverse transcription-polymerase chain reaction (RT-PCR). The sequences of 5′- and 3′- of oligonucleotides (33) used were CAGAGATGCCAT TCTGGC (bases: 10–28) and GTCTTTCCTGTCAAGATCG (bases: 716–697), respectively. The same blot was used to rehybridize with a ^{32}P-labeled probe for glyceraldehyde phosphate dehydrogenase (GAPDH) as a control for loading and integrity of RNA.

Western blotting

Whole cell lysates were prepared from macrophages which were washed with cold PBS and lysed in buffer containing 20 mM Tris (pH 7.4), 137 mM NaCl, 2 mM EDTA, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 10 μg/ml aprotinin. The extracts were cleared in an Eppendorf centrifuge (13,000 *g* for 5 min at 4°C). After separation by SDS-PAGE, proteins were transferred to a nylon/cellulose membrane. The membrane was blocked with PBS containing 0.05% Tween 20 and 3% fat-free milk for 1 h, followed by immunoblotting with antibodies for 5 h at room temperature. The blots were incubated again with appropriate secondary antibodies conjugated with horseradish peroxidase for 1 h at room temperature followed by three washes for 10 min each in washing buffer (TBS + 0.05% Tween 20). Protein visualization on each immunoblot was developed and performed with DuPont Western blot chemiluminescence reagent. Rabbit polyclonal anti-PKC-α was obtained from Santa Cruz and used at a concentration of 1:2000. Anti-mouse PPAR-γ1,2 was obtained from Calbiochem (La Jolla, CA) and used at a concentration of 1:1000.

Flow cytometric analysis of CD36 expression

Monocytes were placed in serum-free media (X-vivo, Biowhittaker) for 10 h prior to treatment with 15d-PGJ_2 (2 μM) or OxLDL (50 (μg/ml) with or without PKC inhibitors [calphostin C (0.5 μM) or Gö 6983 (2 μM)] for 16 h. The cells were then harvested, washed with PBS/1%BSA, and incubated with anti-CD36 (FA6-152, 5 μg/ml) (34) followed by a secondary antibody labeled with fluorescein isothiocyanate (FITC) for detection by fluorescence-assisted cell sorting (Coulter Epics XL flow cytometer, Coulter Inc.). Appropriate isotype matched control fluorescent antibodies were also used to measure non-specific binding.

RESULTS

Induction of CD36 mRNA by PPAR-γ agonists

The effect of PPAR-γ ligands (15d-PGJ_2 and its immediate precursor, PGJ2) on CD36 mRNA expression was evaluated in RAW cells. Cells cultured in serum-free RPMI me-

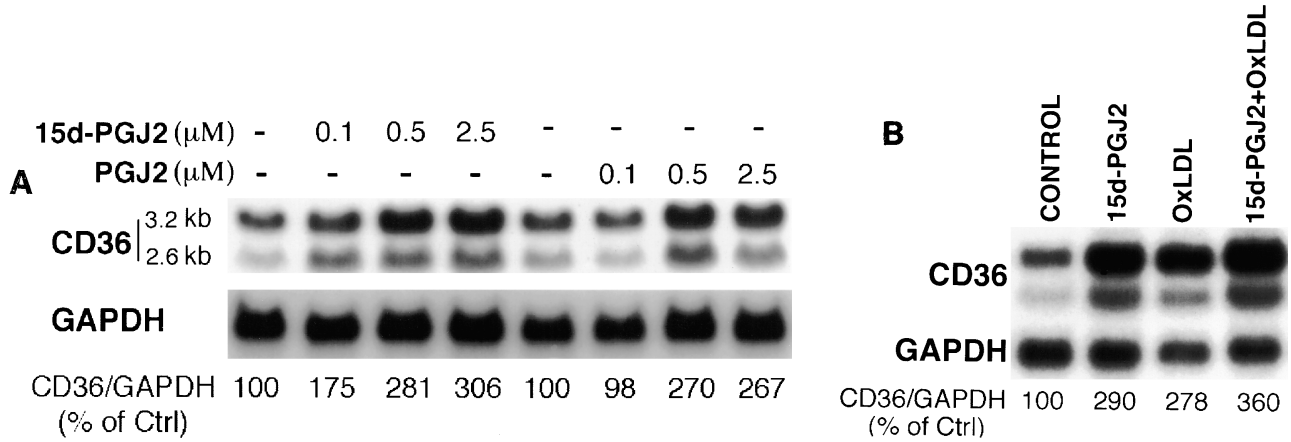

Fig. 1. Effects of OxLDL, PGJ2, and 15d-PGJ_2 on CD36 mRNA expression. A: RAW 264.7 cells were incubated with the indicated concentrations of 15d-PGJ_2 or PGJ2 for 12 h. Total RNA (20 μg per lane) was analyzed by Northern blotting using a ^{32}P-labeled cDNA probe for CD36. The blots were re-hybridized with ^{32}P-labeled GAPDH cDNA. B. RAW 264.7 cells were treated with OxLDL (50 μg/ml) or 15d-PGJ_2 (2 μM) alone or in combination for 14 h. Total RNA (20 μg per lane) was analyzed by Northern blotting using a ^{32}P-labeled cDNA probe for CD36. The blots were re-hybridized with ^{32}P-labeled GAPDH cDNA.

dium for 10 h were then treated for 12 h with 15d-PGJ_2 or PGJ2 (each at 0.1, 0.5, and 2.5 μM). Both reagents were equally effective and induced CD36 mRNA expression by 3-fold with peak expression observed at a concentration of 0.5–2.5 μM (**Fig. 1** A). CD36 is expressed as two messages of 3.2 and 2.6 kB which are produced as the result of alternative splicing (35). In a separate experiment, OxLDL and 15d-PGJ_2 each induced CD36 mRNA approximately 3-fold (Fig. 1B). There was no synergistic increase in CD36 mRNA by a combination of OxLDL and 15d-PGJ_2 (Fig. 1B), suggesting that these two agonists utilize a common signaling pathway.

Activation of PKC increases CD36 mRNA expression

OxLDL has been shown to induce PKC activity and expression of PKC-isotypes, specifically PKC-α (30). CD36 is also induced by PMA (28), a potent PKC activator. To evaluate the sequence of molecular signaling events involved in OxLDL induction of PKC activity and CD36 gene expression, RAW macrophages were incubated with PKC activators for 4 h. Agonists included cell-permeable diacylglycerol (DAG) analogs (DAG-125, 130, 135, 140, each at 20 μM) and ingenol (25 μM). All of the PKC activators tested increased expression of CD36 mRNA. Maximal expression (about 4-fold above control) was seen in response to DAG-130 (**Fig. 2** A). To evaluate the effect of inhibition of PKC activity on macrophage expression of CD36 mRNA, a selective protein kinase C (PKC) inhibitor, calphostin C (1 μM), was incubated with macrophages. Northern analysis demonstrated that calphostin C suppressed CD36 mRNA expression in a time-dependent manner, from 65% (1–3 h) to 19% (6 h) (Fig. 2B). To determine the specificity of PKC

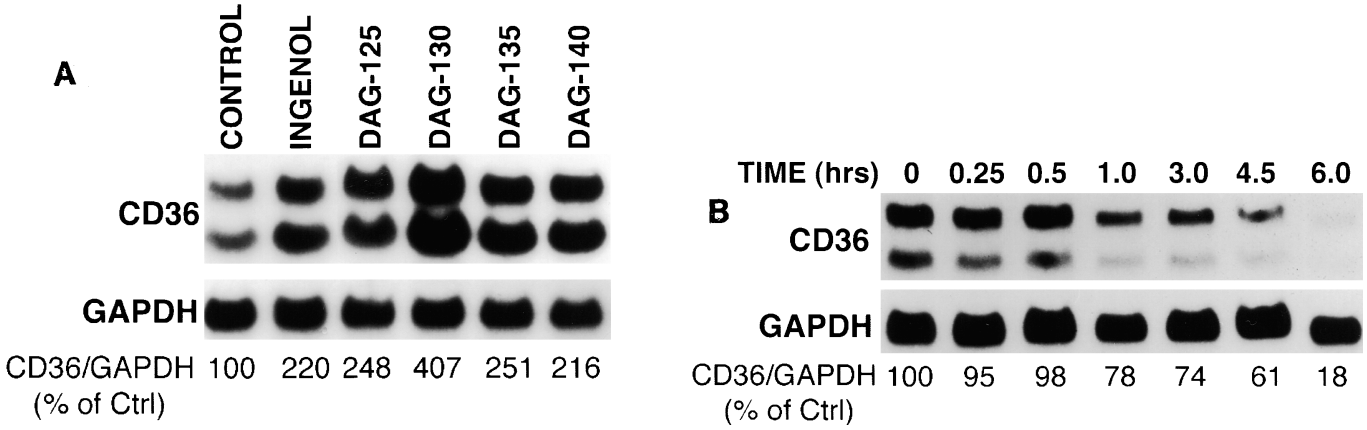

Fig. 2. A: PKC activators up-regulate CD36 expression. RAW 264.7 cells were treated with physiologic PKC activators DAG-125, 130, 135, 140 (20 μM each) and ingenol (25 μM) for 4 h. Total RNA (20 μg per lane) was analyzed by Northern blotting using ^{32}P-labeled probe for CD36 and re-hybridized with ^{32}P-labeled GAPDH cDNA. B: Time course of CD36 mRNA suppression by calphostin C. RAW 264.7 macrophages were treated with calphostin C (1 μM) for time periods as indicated. Total RNA (20 μg per lane) was analyzed by Northern blotting using a ^{32}P-labeled cDNA probe for CD36. The blots were re-hybridized with ^{32}P-labeled GAPDH cDNA.

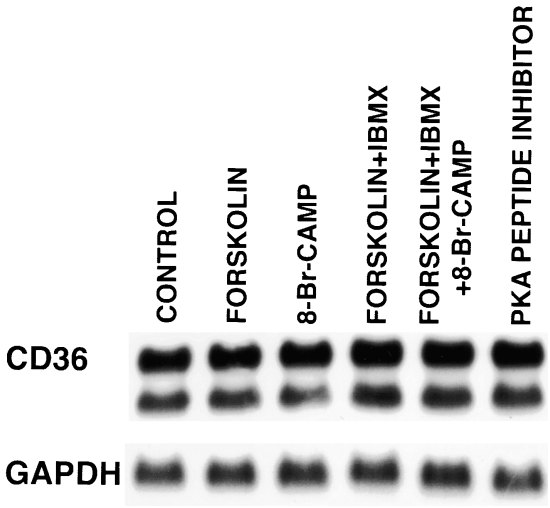

Fig. 3. Effect of PKA and cAMP agonists on CD36 mRNA expression. RAW 264.7 cells were cultured with forskolin (100 μM), 8-Br-cAMP (200 mM) [alone or in combination with IBMX (50 μM)], or PKA peptide inhibitor (1 μM) for 10 h. Total RNA (20 μg per lane) was analyzed by Northern blotting using a ^{32}P-labeled cDNA probe for CD36. The blots were re-hybridized with ^{32}P-labeled GAPDH cDNA.

activators in the induction of CD36, we evaluated CD36 expression in response to agonists of other signaling pathways. Activators of protein kinase A (PKA) and cAMP had no influence on CD36 mRNA expression (**Fig. 3**). Similarly, forskolin (100 μM) (an activator of adenylate cyclase), cAMP analog 8-Br-cAMP (200 μM) and its inhibitor IBMX (50 μM), and a PKA peptide inhibitor (1 μM) had no effect on CD36 mRNA expression (Fig. 3).

PKC inhibitors prevent 15d-PGJ$_2$ and OxLDL-induced CD36 expression

To determine whether PKC activity is required for 15d-PGJ$_2$-induced expression of CD36 mRNA, we evaluated expression of CD36 mRNA in RAW macrophages after treatment with 15d-PGJ$_2$ (2 μM) alone, or in combination with PKC inhibitors, calphostin C (0.5 μM), Gö 6983 (2 μM) or myristoylated PKC peptide (19–27) inhibitor (50 μM) (**Fig. 4** A). Macrophages were pre-incubated with the PKC inhibitors for 20 min prior to the addition of 15d-PGJ$_2$. There was a 2-fold induction of CD36 by 15d-PGJ$_2$. Induction of CD36 in response to 15d-PGJ$_2$ was variable, usually ranging from 2- to 4-fold. Each of the three PKC inhibitors completely inhibited CD36 mRNA expression induced by 15d-PGJ$_2$. In addition, calphostin C significantly diminished both basal levels of CD36 expression in RAW macrophages and expression induced in response to OxLDL (Fig. 4B).

PKC inhibitors block induction of monocyte surface expression of CD36 by OxLDL and 15d-PGJ$_2$

Human monocytes were placed in serum-depleted media and then treated with agonists [(OxLDL (50 μg/ml) or 15d-PGJ$_2$ (2 μM)], inhibitors (Gö 6983 (2 μM) or calphostin C (0.5 μM)), or agonists plus inhibitors, for 16 h. The samples were then incubated with anti-CD36 (FA6) or with an isotype matched control IgG. The log mean channel fluorescence for the control IgG was 0.157 ± 0.107. The untreated monocytes, which were analyzed for constitutive expression of CD36, gave a mean channel fluorescence of 0.649 ± 0.004 (**Fig. 5** A). In the presence of Gö 6983 or calphostin C, basal expression of CD36, as determined by the log mean channel fluorescence, was significantly reduced: Gö 6983 (0.249 ± 0.009), calphostin C (0.246 ± 0.0028), control (untreated) monocytes (0.649 ± 0.004) (Fig. 5A). Treatment with either OxLDL or 15d-PGJ$_2$ increased log mean channel fluorescence indicating increased surface expression of CD36: OxLDL (1.4 ± 0.14), 15d-PGJ$_2$ (2.105 ± 0.0919) (Fig. 5B). Co-incubation of calphostin C with OxLDL or 15d-PGJ$_2$ resulted in log mean channel fluorescence levels that were significantly less than in untreated cells: calphostin C + OxLDL (0.22 ±

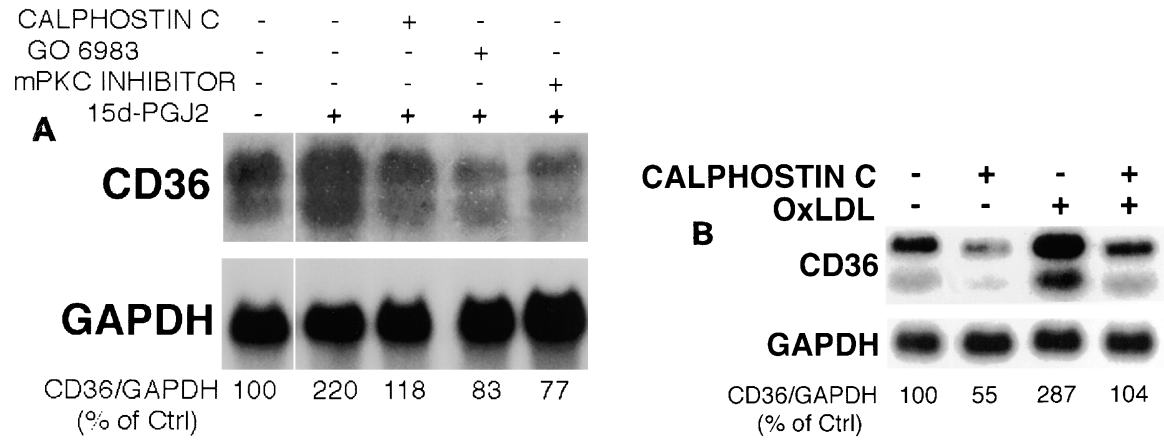

Fig. 4. PKC inhibitors prevent 15d-PGJ$_2$ and OxLDL-induced CD36 mRNA expression. A: RAW macrophages were incubated in media alone (control), with 15d-PGJ$_2$ (2 μM), or with 15d-PGJ$_2$ (2 μM) in combination with PKC inhibitors [calphostin C (0.5 μM), Gö 6983 (2 μM), and mPKCI (10 μM)], for 14 h. Total RNA (20 μg per lane) was analyzed by Northern blotting using a ^{32}P-labeled cDNA probe for CD36. The blots were re-hybridized with ^{32}P-labeled GAPDH cDNA. B: RAW macrophages were incubated in media alone (control), calphostin C (0.5 μM), OxLDL (50 μg/ml), or OxLDL and calphostin C for 14 h.

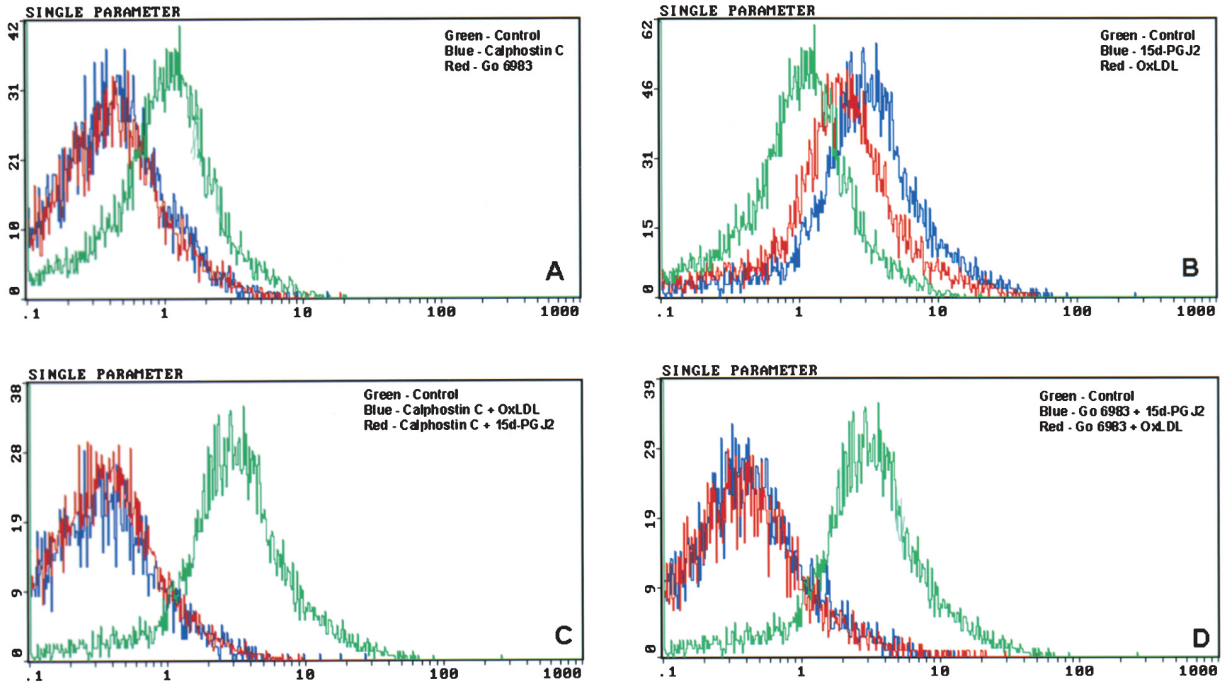

Fig. 5. Effect of PKC inhibitors on CD36 surface expression induced by OxLDL and 15d-PGJ_2. Monocytes were placed in serum-depleted media and then treated with agonists [(OxLDL (50 μg/ml) or 15d-PGJ_2 (2 μM)], PKC inhibitors (Gö 6983 (2 μM) or calphostin C (0.5 μM)), or agonists plus inhibitors, for 16 h. The samples were then incubated with anti-CD36 (FA6) or with an isotype matched control IgG. The log mean channel fluorescence for the control IgG was 0.157 ± 0.107. Panel A: Incubation of monocytes with PKC inhibitors, Gö 6983 or calphostin C. Log mean channel fluorescence: Gö 6983 (0.249 ± 0.009), calphostin C (0.246 ± 0.0028), control (untreated) monocytes (0.649 ± 0.004). Panel B: Incubation of monocytes with OxLDL (50 μg/ml) or 15d-PGJ_2 (2 μM). Log mean channel fluorescence: OxLDL (1.4± 0.14), 15d-PGJ_2 (2.105 ± 0.0919), control (untreated) monocytes (0.649 ± 0.004). Panel C: Co-incubation of PKC inhibitor calphostin C with OxLDL or 15d-PGJ_2. Log mean channel fluorescence: calphostin C + OxLDL (0.22 ± 0.003), calphostin C +15d-PGJ_2 (0.221 ± 0.022), control (untreated) monocytes (0.649 ± 0.004). Panel D: Co-incubation of PKC inhibitor Gö 6983 with OxLDL (50 μg/ml) or 15d-PGJ_2 (2 μM). Log mean channel fluorescence: Gö 6983 + OxLDL (0.241 ± 0.004) and Gö 6983 + 15d-PGJ_2 (0.229 ± 0.34), control (untreated) monocytes (0.649 ± 0.004).

0.003), calphostin C + 15d-PGJ_2 (0.221 ± 0.022), control (untreated) monocytes (0.649 ± 0.004) (Fig. 5C). Similarly, incubation with Gö 6983 resulted in log mean channel fluorescence levels that were significantly less than in untreated cells: Gö 6983 + OxLDL (0.241 ± 0.004) and Gö 6983 + 15d-PGJ_2 (0.229 ± 0.34), control (untreated) monocytes (0.649 ± 0.004) (Fig. 5D). A similar effect was also seen in tissue culture-derived macrophages which had been plated for 4 days and in which constitutive CD36 expression is up-regulated (data not shown).

PKC inhibitors antagonize CD36 expression induced by IL-4

To determine whether regulation of CD36 expression induced by another agonist known to up-regulate expression of CD36 was also mediated by the PKC pathway, we treated macrophage cell lines with a PKC inhibitor prior to CD36 induction by IL-4 (5 ng/ml or 10 ng/ml) (**Fig. 6**). As before, incubation with calphostin C (1 μM) inhibited basal CD36 expression. Co-incubation of IL-4 and calphostin C (1 μM) inhibited IL-4-induced CD36 expression by 50% (Fig. 6).

PPAR-γ mRNA and protein expression is inhibited by PKC inhibitors

To further characterize the temporal relationship of PKC and PPAR-γ activation involved in up-regulation of CD36

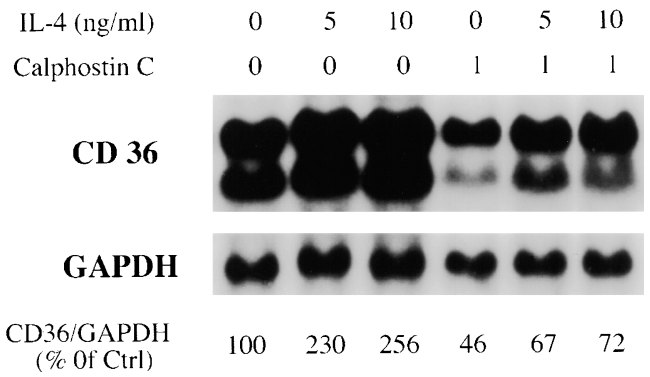

Fig. 6. Effect of PKC inhibitors on CD36 expression induced by IL-4. RAW macrophages were incubated in media alone (control), with IL-4 (5 ng/ml or 10 ng/ml), with calphostin C (1 μM), or in combination with calphostin C and IL-4, for 4 h. Total RNA (20 μg per lane) was analyzed by Northern blotting using a ^{32}P-labeled cDNA probe for CD36. The blots were re-hybridized with ^{32}P-labeled GAPDH cDNA.

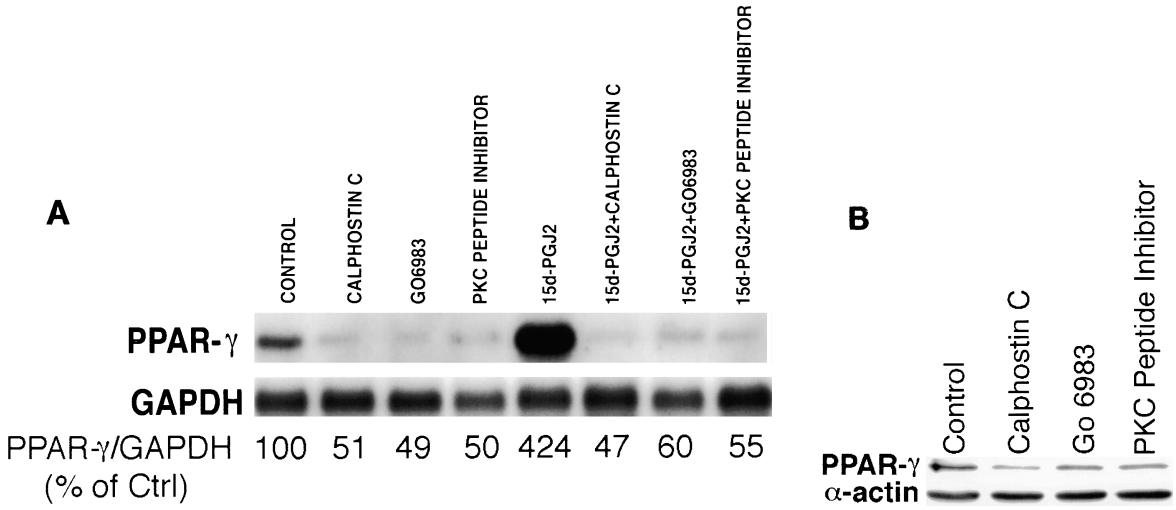

Fig. 7. Effect of PKC inhibitors on 15d-PGJ_2 induced expression of PPAR-γ mRNA and protein. A: RAW macrophages were incubated in media alone (control), with 15d-PGJ_2 (2 μM), or with 15d-PGJ_2 (2 μM) in combination with PKC inhibitors [calphostin C (0.5 μM), Gö 6983 (2 μM), and mPKCI (10 μM)], for 14 h. Total RNA was extracted and used to purify poly(A^+) RNA as described in Experimental Procedures. After electrophoresis of RNA, Northern blots were probed using a ^{32}P-labeled PPAR-γ cDNA probe. The blots were re-hybridized with ^{32}P-labeled GAPDH cDNA. B: Macrophages were treated with PKC inhibitors [calphostin C (0.5 μM), Gö 6983 (2 μM), and mPKCI (10 μM)] for 14 h. Protein was separated on 12% SDS-PAGE gel. The immunoblot was incubated with rabbit anti-PPAR-γ and anti-α-actin as described in Experimental Procedures.

expression, we evaluated the effect of PKC inhibitors on basal and 15d-PGJ_2-induced PPAR-γ mRNA levels. Treatment of RAW 264.7 macrophages with PKC inhibitors, calphostin C (0.5 μM), Gö 6983 (2 μM), or myristoylated PKC peptide (19–27) inhibitor (50 μM), reduced basal levels of PPAR-γ mRNA relative to macrophages incubated in media alone. Treatment of macrophages with 15d-PGJ_2 (2 μM) increased PPAR-γ mRNA expression by 4-fold (**Fig. 7** A). Importantly, the increase in PPAR-γ mRNA expression induced by 15d-PGJ_2 was completely abolished in the presence of PKC inhibitors (Fig. 7A). Similarly, basal levels of PPAR-γ protein, as evaluated by Western blot, were inhibited by PKC inhibitors [calphostin C (0.5 μM), Gö 6983 (2 μM) and mPKCI (10 μM)] (Fig 7B). However, PPAR-γ protein levels were not reduced to the same degree by PKC inhibitors as PPAR-γ message levels.

Western localization of PKC after treatment with 15d-PGJ_2

To clarify the sequence of signaling events leading to CD36 gene expression, we evaluated activation and translocation of PKC after addition of PPAR-γ ligands. In untreated cells, PKC-α is predominantly cytosolic in location (**Fig. 8**). RAW macrophages incubated with PMA (1 μM) rapidly (within 10 min) induced PKC-α translocation from cytosolic to membrane fractions. At 1 h after treatment, only a small fraction of PKC-α could be detected in the membrane fraction (Fig. 8). In contrast, 15d-PGJ_2 had no effect on PKC-α translocation (Fig. 8). These results suggest that activation and translocation of PKC is an upstream signaling event, and precedes (rather that follows) activation of PPAR-γ and subsequent CD36 gene expression.

DISCUSSION

Oxidation of low density lipoprotein is thought to be a critical early event in the pathogenesis of atherosclerosis. Several cellular receptors involved in binding and internalizing OxLDL have been characterized as "scavenger receptors." Unlike the LDL receptor, scavenger receptors are not subject to negative regulation by high levels of intracellular cholesterol. For this reason, they are thought to play a significant role in atherosclerotic foam cell development. The acetylated LDL receptor was the first macrophage scavenger receptor identified, isolated, and cloned (36, 37). This receptor, which is now referred to as type A or class A scavenger receptor, is expressed on macrophages but not on peripheral blood monocytes. Modification of LDL by acetylation, acetoacetylation, or malondialdehyde treatment abolishes the positive charge on lysine residues of LDL and prevents recognition by the LDL receptor, while facilitating scavenger receptor binding. As

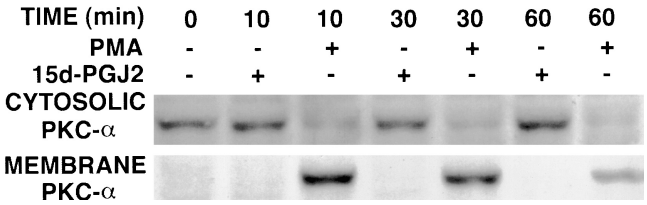

Fig. 8. Effects of 15d-PGJ_2 and PMA on cellular localization and expression of PKC-α. A: RAW macrophages were serum-depleted for 14 h, and treated with either PMA (1 μM) or 15d-PGJ_2 (2 μM) for the times indicated. The cytosolic and membrane protein was separated in 12% SDS-PAGE gel. The immunoblot was incubated with rabbit anti-PKC-α (1:2000).

these modifications do not occur under physiological conditions, the natural ligand for this receptor was unclear until it was demonstrated that OxLDL partially competes for the binding of acetylated LDL (AcLDL) to macrophages (38).

Because the type A scavenger receptor could not completely account for all OxLDL binding to macrophages, efforts were undertaken to identify other scavenger receptors. Endemann and her colleagues (15) used an expression cloning strategy to identify CD36 as a receptor on murine macrophages that recognized OxLDL but not AcLDL. We and others (16, 17, 34) subsequently showed that CD36 cDNA transfected cells bind and internalize OxLDL. Furthermore, CD36 is up-regulated by cytokines known to be present in atheromatous lesions (25), and may account for as much as 70% of OxLDL uptake by human macrophages (39). A genetic polymorphism in the CD36 gene (40) results in deficient expression of CD36 (NAK^{a-} phenotype). Significantly, monocyte-derived macrophages isolated from these patients bind 40% less OxLDL and accumulate 40% less cholesteryl ester than cells derived from normal controls (41), further implicating CD36 as a physiological OxLDL receptor. Similarly, macrophages derived from CD36 null mice bind and internalize up to 60% less OxLDL than macrophages derived from wild-type mice (42).

We had previously shown that OxLDL could stimulate its own uptake by induction of CD36 gene expression (26). The mechanism(s) by which OxLDL up-regulated CD36 was shown to involve activation of the transcription factor, PPAR-γ (27, 28). Because OxLDL has multiple effects on signaling pathways and gene expression patterns of macrophages and vascular cells, we set out to define the temporal pattern of signaling events initiated by OxLDL binding to macrophages resulting in CD36 expression. We have now shown that OxLDL and other activators of PKC increase CD36 mRNA. In addition, both basal expression and increased expression of CD36 in response to diverse agonists, OxLDL and IL-4, are blocked in response to PKC inhibitors. These data imply that signal transduction initiated by ligation of cognate receptors for these ligands converges and utilizes PKC activation to increase expression of CD36. This would not have been anticipated or predicted based on the known signaling mechanisms of these receptors. Our data also suggest that activation of PKC most likely precedes PPAR-γ activation as 15d-PGJ_2, a PPAR-γ ligand, had no effect on PKC-α translocation from the cytoplasm to the cell membrane. However, we cannot rule out the possibility that other isoforms of PKC may be activated in response to PPAR-γ ligands.

PPAR-γ plays an important role in regulating genes encoding proteins involved in the regulation of lipid (29) and glucose metabolism (43). The natural ligands that bind and modulate PPAR-γ activity in vivo remain to be established. However, PGJ2, 15d-PGJ_2 and some polyunsaturated fatty acids have been demonstrated to stimulate PPAR-γ-dependent transcription (43). In addition, synthetic ligands such as the thiazolidinedione class of antidiabetic drugs have been identified as specific PPAR-γ activators (44, 45). PPAR-γ is expressed by monocyte/macrophages (27, 46) and may negatively regulate expression of proinflammatory genes (46). It is also expressed in macrophage-derived foam cells within atherosclerotic lesions (27) where its pattern of expression is correlated with the presence of oxidation-derived epitopes (47).

PPAR-γ is induced by 12-o-tetradecanoylphorbol 13-acetate (TPA) (47), interleukin-4 (48), and M-CSF (47). IL-4-dependent transcription of the CD36 gene in macrophages is dependent on induction of 12/15-lipoxygenase and the generation of endogenous ligands for PPAR-γ (48). Our findings, that induction of CD36 expression by OxLDL is dependent on activation of PPAR-γ and PKC and is inhibited by PKC inhibitors, are consistent with these observations. In addition, incubation of macrophages with OxLDL has been demonstrated to enhance PKC activity (30), resulting in changes in both the levels and distribution of PKC isoforms. Activation of PKC by calcium and diacylglycerol or phorbol ester redistributes the enzyme from a cytosolic location in resting cells to a membrane-associated site after activation (49). However, in contrast to our findings, Ricote et al. (47) could not demonstrate that a PKC inhibitor (bisindolylmaleimide II, 1 μM) blocked induction of PPAR-γ by OxLDL in THP-1 cells. They concluded that more than one signaling pathway regulates PPAR-γ expression in macrophages (47). The reason for the discrepancy in these data with our results is unclear, but may be due to the different PKC inhibitors utilized in the two studies or to the difference in cell types.

Activation of PKC appears to be a consistent feature of scavenger receptor expression as both CD36 and the type A scavenger receptor are induced by PKC agonists. In contrast to CD36, which is markedly increased in macrophages by phorbol esters (50) and PPAR-γ ligands (27, 28), PPAR-γ ligands inhibit expression of the type A scavenger receptor induced by phorbol ester (27). The mechanism by which this occurs was elucidated by Ricote et al. (46) who showed that PPAR-γ inhibited expression of type A scavenger receptor gene in response to 15d-PGJ_2 and synthetic PPAR-γ ligands by antagonizing the activities of the transcription factors AP-1, STAT, and NF-kappa B.

In summary, our data demonstrate that macrophage basal expression of CD36 and expression in response to both OxLDL and IL-4 is blocked or reduced by PKC inhibitors. 15d-PGJ_2, a PPAR-γ ligand, has no effect on PKC-α translocation, implying that PKC activation precedes PPAR-γ activation. These signaling pathways play a critical role in regulating macrophage expression of CD36. An understanding of the signaling events initiated by binding of OxLDL to macrophages may help in designing strategies to inhibit expression of CD36 and to block the cycle by which lipids drive expression of this scavenger receptor.

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