Antagonistic Effects of Oxidized Low Density Lipoprotein and α-Tocopherol on CD36 Scavenger Receptor Expression in Monocytes

IN Volvement of Protein Kinase B and Peroxisome proliferator-activated Receptor-γ*

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Vitamin E deficiency increases expression of the CD36 scavenger receptor, suggesting specific molecular mechanisms and signaling pathways modulated by α-tocopherol. We show here that α-tocopherol down-regulated CD36 expression (mRNA and protein) in oxidized low density lipoprotein (oxLDL)-stimulated THP-1 monocytes, but not in unstimulated cells. Furthermore, α-tocopherol treatment of monocytes led to reduced expression of CD36. Dioctadecylcarbocyanine perchlorate binding and uptake. Protoporphyrin IX (PPIX) and the induction of signal transduction cascades (1). Expression of PKB at Ser473 was increased by oxLDL, and α-tocopherol could prevent this event. Expression of PKB stimulated the CD36 promoter as well as a PPARγ element-driven reporter gene, whereas an inactive PKB mutant had no effect. Moreover, coexpression of PPARγ and PKB led to additive induction of CD36 expression. Altogether, our results support the existence of PKB/PPARγ signaling pathways that mediate CD36 expression in response to oxLDL. The activation of CD36 expression by PKB suggests that both lipid biosynthesis and fatty acid uptake are stimulated by PKB.

In many cell types, oxidized low density lipoproteins (oxLDL)2 modulate cellular processes such as apoptosis, adhesion, migration, gene expression, and the induction of signal transduction cascades (1). Exposure of monocytes to oxLDL may alter gene expression and signaling, making them more susceptible to the following pro-atherogenic stimuli. The migration of monocytes into the intima and the conversion of monocytes into macrophages into foam cells represent initial steps in atherosclerosis. Current strategies to prevent atherosclerosis are aimed either at lowering the cholesterol load of lipoproteins or at reducing oxidative stress.

Vitamin E is a redox-active natural compound that can act, depending on the conditions, as a pro- or antioxidant on low density lipoproteins (LDL) in vitro and in vivo (2–5). The major form of vitamin E in human plasma is α-tocopherol, and reduced plasma levels of α-tocopherol, such as in vitamin E-deficient mice, increase the incidence of atherosclerosis (6). Animal and cell culture studies strongly suggest that vitamin E can prevent atherosclerosis; however, the anti-atherogenic effects in clinical trials are still controversial (7–10). α-Tocopherol in lipoproteins (mainly LDL) and also in the subendothelial space has been assumed to play a central role in reducing atherosclerosis by preventing lipid peroxidation and consequent lesion development. Nevertheless, since many compounds exist that can interfere with the oxidation of LDL without being equally effective, alternative modes of action have been proposed for atherosclerosis prevention, such as modulation of gene expression and cell signaling (reviewed in Refs. 7, 9, 11, and 12).

For vitamin E, non-antioxidant activities have been described, such as inhibition of vascular smooth muscle cell (VSMC) proliferation via inhibition of the protein kinase C (PKC) pathway; the modulation of phospholipase A2, cyclooxygenase-2, and 5-lipoxygenase and the release of interleukin-1β; the reduction of cholesterol ester formation and uptake; the prevention of inflammation and monocyte/macrophage adhesion to the endothelium; the induction of connective tissue growth factor expression possibly involved in plaque stabilization (13); and the inhibition of scavenger receptor expression in smooth muscle cells and macrophages (14–17).

The uptake of modified LDL leading to foam cell formation is mediated by scavenger receptors (class A; class B, type I; and CD36) (18). Expression of some scavenger receptors is increased at the atherosclerotic lesion (19), possibly as a result of a positive feedback loop mediated by oxLDL and its lipid content (19–22). These receptors play also a major role in the uptake of vitamin E from high density lipoproteins in brain capillary endothelial cells and type II pneumocytes (23, 24).

In addition to oxLDL, CD36 binds to a large variety of ligands: thrombospondin, collagen types I and IV, β-amyloid, fatty acids, anionic phospholipids, and high density lipoproteins (25). In various tissues, the uptake of long chain fatty acids is mediated by CD36/fatty acid translocase, and transgenic mice overexpressing CD36 have reduced blood lipids (26–30). The central involvement of CD36 in atherosclerosis has been demonstrated by generating CD36 knockout mice, which show reduced uptake of modified LDL and reduced atherosclerosis (28). Similarly, human monocytes/macrophages from CD36-deficient patients show a low capacity to bind and internalize oxLDL (28, 31); these mono-

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2 The abbreviations used are: oxLDL, oxidized low density lipoprotein(s); LDL, low density lipoprotein(s); VSMC, vascular smooth muscle cell(s); PKC, protein kinase C; PPARγ, peroxisome proliferator receptor-γ; PKB, protein kinase B; HEK293, human embryonic kidney 293; PMA, phorbol 12-myristate 13-acetate; RT, reverse transcription; FACS, fluorescence-activated cell sorting; DIO, 3,3′-dioctadecyloxacarbocyanine perchlorate.
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cytes show also decreased NF-κB activation after oxLDL stimulation, leading to lower expression of inflammatory cytokines (32).

At the transcriptional level, CD36 expression is induced by oxLDL via the peroxisome proliferator-activated receptor-γ (PPARγ) and NF-E2-related factor-2 (Nrf2) (21, 33–35). Activation of CD36 by interleukin-4, dependent on PKC and PPAR

tained between 21 and 22 °C with a dark/light cycle of 12/12 h and with Dawley rats were housed five in a cage. The temperature was main-

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membrane translocation step, followed by phosphorylation of two pathways modulated in vivo by α-tocopherol. In THP-1 monocytes, expression of the CD36 scavenger receptor was increased by oxLDL, and α-tocopherol treatment normalized it at both the protein and mRNA levels. PKC was not involved in the observed effects, but over-

Induction of CD36 expression by oxLDL (45, 46) and oxLDL uptake (46, 47) was previously studied in THP-1-derived macrophages. How-

We show here that vitamin E-deficient rats have increased expression of CD36 mRNA, indicating specific molecular mechanisms and signal-

 MATERIALS AND METHODS

Cell Culture and Treatments—(R,R,R)-α-Tocopherol (Cognis) was dissolved in ethanol, and the concentration of the stock solutions was confirmed spectrophotometrically. The human acute monocytic leu-

ylation pathways modulated in vivo by α-tocopherol. In THP-1 monocytes, expression of the CD36 scavenger receptor was increased by oxLDL, and α-tocopherol treatment normalized it at both the protein and mRNA levels. PKC was not involved in the observed effects, but over-

proliferation (40); and in mouse bone marrow-derived macrophages, it

Determination of CD36 mRNA expression by reverse transcription (RT)-PCR was done as described and quantified after normalization to rat glyceraldehyde-3-phosphate dehydrogenase mRNA expression (52).

Determination of CD36 mRNA expression by RT-PCR—Total RNA was isolated using the RNA extraction kit (Qiagen Inc.). Determination of rat CD36 mRNA expression by reverse transcription (RT)-PCR was done as described and quantified after normalization to rat glyceraldehyde-3-phosphate dehydrogenase mRNA expression (52).

Determination of CD36 Expression by Fluorescence-activated Cell Sorting (FACS)—THP-1 monocytes were treated as indicated, and CD36 expression was analyzed by FACS as described previously (15, 53, 54). CD36 expression was measured using fluorescein isothiocyanate-conjugated anti-human CD36 monoclonal antibody (Ancell Corp.) diluted 1:50 in phosphate-buffered saline and 1% bovine serum albumin. A minimum of 10,000 cells/sample was assessed; data were acquired and analyzed using CellQuest software (FACSscan, BD Biosciences).

Determination of CD36 mRNA expression by RT-PCR—Total RNA was isolated using the RNA extraction kit. Semiquantitative assays for CD36 mRNA expression were performed as described previously (15, 53).

Determination of Total CD36 Expression by Western Blotting—Protein extraction and Western blotting were done according to standard methods with mouse anti-human CD36 monoclonal primary antibody (Ancell Corp.) and sheep anti-mouse IgG secondary antibody coupled to horseradish peroxidase (Amersham Biosciences) (55). Anti-β-actin antibody (Sigma) was used as an internal control. Western blotting for PKB was done according to the protocol provided (Cell Signaling Technology). Proteins were visualized using an ECL detection kit (Amersham Biosciences) according to the manufacturer’s recommendations. Chemiluminescence was monitored by exposure to Hyperfilm ECL film (Amersham Biosciences), and the signals were analyzed using a Lumi-

Labeling and Uptake of oxLDL—Labeling of oxLDL with 3,3’-dioctadecyloxacarbocyanine perchlorate (DiO; Molecular Probes) and blocking of oxLDL-DiO uptake with anti-CD36 antibody were done as described previously (15). The uptake and binding of oxLDL-DiO were studied by FACS. The cells were pretreated for 16 h with 20 µg/ml oxLDL, 50 µmol/liter α-tocopherol, or 0.1% ethanol (solvent control) and then incubated with oxLDL-DiO (5 µg/ml of medium). For the uptake experiments, incubation was carried out at 37 °C for 6 h; and for the binding experiment, incubation was performed at 4 °C for 30 min. Thereafter, the cells were washed three times with phosphate-buffered saline and 3% bovine serum albumin and once with phosphate-buffered saline and fixed with 4% paraformaldehyde in phosphate-buffered saline. FACS was performed with a FACSScan, and data were analyzed using CellQuest software.

Plasmids, Transfections, and Reporter Assays—The firefly luciferase reporter plasmids used were pCD36 (15), pDR1 (56), and pNF-κB (Clontech), and the Renilla internal control plasmid used was pRL-TK (Promega Corp.). The PKB expression vectors pPKBwt, pPKB(R25C),
and pPKB(K179M) were kindly provided by Dr. J. Downward (Imperial Cancer Research Fund, London, UK) and correspond to pGFP-Akt, pGFP-Akt(R25C), and pGFP-Akt(K179M), respectively (57). The PPARγ expression vector used contains the PPARγ cDNA under the control of the cytomegalovirus promoter (56). THP-1 monocytes were transfected for 3 h with the indicated reporter and expression plasmids using Transfectin (Bio-Rad) and then treated with 0.1% ethanol (solvent control) or 50 μmol/liter α-tocopherol for an additional 21 h. HEK293 cells were transfected for 3 h using Superfect (Qiagen Inc.). The medium was then changed, and the cells were treated with 0.1% ethanol (solvent control) or 50 μmol/liter α-tocopherol and incubated for an additional 21 h. Extracts were prepared, and promoter activities were measured using the Dual-Luciferase assay kit (Promega Corp.) with a TD20/20 luminometer (Turner Designs). The promoter-firefly luciferase activities were normalized to the thymidine kinase promoter-Renilla luciferase activities, and the activities of the control transfections were set to 100%.

Apoptosis Assay—THP-1 cells were incubated with increasing concentrations of oxLDL for 24 and 48 h and then stained with Hoechst 33342 (5 μg/ml) for 1 h at 37 °C (58). A total of 200 cells for each treatment were counted, and the percentage of apoptotic cells with condensed nuclei was quantified.

Statistical Analysis—Values are expressed as the mean ± S.D. as indicated in the figure legends. For FACS results, the median fluorescence intensity was determined, and the mean ± S.D. was calculated as described in the figure legends. Student’s t test was used to analyze the significant differences between two conditions. A p value <0.05 was taken as significant and is indicated by asterisks in the figures.

RESULTS

Vitamin E Deficiency in Rats Leads to CD36 Overexpression—Our previous in vitro results suggested that, in VSMC and HL-60 cells, the expression level of CD36 is inhibited by α-tocopherol, leading to reduced uptake of oxLDL (15). To assess whether a similar regulation takes place also in vivo, rats were deprived of α-tocopherol intake for 21 months, and the expression level of CD36 mRNA was measured using RT-PCR. α-Tocopherol deficiency led to a significantly higher level of liver CD36 expression (238 ± 41% (n = 4); p < 0.028) compared with rats kept on a normal diet (set to 100 ± 38% (n = 4)). The increased level of urinary aldehydes observed in vitamin E-deficient animals (data not shown) suggests increased oxidative stress; thus, α-tocopherol could inhibit CD36 expression by reducing oxidative stress or, alternatively, by interfering with signal transduction and gene expression modulated by oxidized molecules.

α-Tocopherol Inhibits CD36 Surface Overexpression Induced by oxLDL in THP-1 Monocytes—The in vivo results described above could be explained by increased generation of oxLDL in vitamin E-deficient animals, an event that possibly could be prevented by supplementation with α-tocopherol. On the other hand, it has been shown that α-tocopherol can reduce CD36 expression in VSMC and monocytes/macrophages by interfering with signal transduction and CD36 gene expression (15, 17).

To delineate these two pathways affected by α-tocopherol, CD36 expression was induced by oxLDL in THP-1 monocytes, and we checked whether α-tocopherol could interfere with this pro-atherogenic stimulus. By using oxLDL in this model system, a reduction of CD36 overexpression by α-tocopherol would be the consequence of inhibition of oxLDL-induced CD36 transcription rather than the result of inhibition of LDL oxidation.

THP-1 monocytes were treated for 24 h with oxLDL (20 μg/ml), ethanol (0.1%; solvent control), or α-tocopherol (50 μmol/liter), and CD36 surface expression was analyzed by FACS. oxLDL significantly induced CD36 surface expression in THP-1 monocytes, and α-tocopherol reduced it by 18 ± 11% (Fig. 1).

Treatment with α-tocopherol in the absence of oxLDL stimulation did not reduce CD36 expression in THP-1 monocytes as described previously (53) and was therefore specifically antagonizing only upon oxLDL stimulation. Neither LDL nor aggregated LDL increased CD36 expression in this cell line (data not shown).

Apoptosis Induction Only at High oxLDL Concentrations—Treatment with oxLDL is also known to induce apoptosis, and it seemed possible that the observed increase in CD36 expression was a consequence of cell death and cellular toxicity. However, when THP-1 cells were incubated for 24 and 48 h with increasing concentrations of oxLDL (10–80 μg/ml), significant apoptosis as measured by assessing the number of condensed nuclei was observed only with the highest concentration (80 μg/ml) and after 48 h (Fig. 2). Taken together, these results suggest that oxLDL induce CD36 expression by activating a signaling cascade and that α-tocopherol can interfere with this event.

α-Tocopherol Inhibits CD36 Overexpression at the Protein and mRNA Levels in THP-1 Monocytes Stimulated by oxLDL—The results obtained by FACS could be explained by increased gene and protein expression of CD36. Western blotting with anti-CD36 monoclonal antibody showed that CD36 protein is expressed in THP-1 monocytes mainly as a 74-kDa protein as described previously (59). Treatment of THP-1 monocytes with oxLDL (20 μg/ml) stimulated total CD36 protein expression, and co-treatment with α-tocopherol (50 μmol/liter) prevented CD36 overexpression completely (reduction of 71 ± 38%) in Western blot analyses (Fig. 3).

To assess whether modulation of CD36 protein expression by oxLDL and α-tocopherol in THP-1 monocytes is the result of changes in gene expression, THP-1 monocytes were incubated with oxLDL (20 μg/ml) in the presence of α-tocopherol (50 μmol/liter) or ethanol (0.1%; solvent control), and CD36 mRNA expression was analyzed by RT-PCR. Treatment with oxLDL led to increased expression of CD36 mRNA, whereas co-treatment with α-tocopherol normalized CD36 mRNA levels (reduction of 39 ± 28%) (Fig. 4). Thus, in THP-1 monocytes, oxLDL stimulates CD36 gene and protein expression, effects that are prevented by α-tocopherol.

Uptake and Binding of oxLDL-DiO Are Inhibited by α-Tocopherol in THP-1 Monocytes Stimulated by oxLDL—Expression of the CD36 scavenger receptor is involved in the uptake of oxLDL, allowing the accu-
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In unstimulated THP-1 monocytes, treatment with oxLDL resulted in a small and non-significant decrease in oxLDL-DiO binding (12 ± 4% (n = 5); p < 0.096 versus the control set to 100%) and uptake (11 ± 11% (n = 5); p < 0.076 versus the control set to 100%). In oxLDL-stimulated cells, α-tocopherol treatment led to stronger and statistically significant inhibition of oxLDL binding and uptake (Fig. 5B).

To check whether CD36 is the major scavenger receptor responsible for the uptake of oxLDL, THP-1 monocytes were preincubated for 30 min with anti-CD36 monoclonal antibody before addition of oxLDL-DiO for 6 h. The uptake of oxLDL-DiO was analyzed by FACS. As described previously for other cell types (15, 49, 60, 61), anti-CD36 antibody interfered with the uptake of oxLDL-DiO, whereas an isotype-matched control antibody (mouse anti-IgM antibody) did not (data not shown), suggesting that CD36 is responsible for oxLDL uptake in THP-1 monocytes.

Modulation of CD36 Expression in THP-1 Monocytes by oxLDL and α-Tocopherol Does Not Involve PKC—In VSMC, α-tocopherol inhibits PKC by activation of protein phosphatase 2A, leading to inhibition of proliferation (62). Because oxLDL are known to activate PKC in THP-1 macrophages (63), it was possible that the above-described inhibitory effect on CD36 expression mediated by α-tocopherol was the result of PKC inhibition.

The involvement of PKC in CD36 modulation was investigated by treating THP-1 monocytes with an activator of PKC (PMA) and a specific PKC inhibitor (PKC412). CD36 expression was analyzed by FACS.

When THP-1 monocytes were differentiated to macrophages by treatment with PMA (5 nmol/liter) for 24 h, CD36 was induced at the mRNA level as analyzed by RT-PCR and at the protein level as analyzed by Western Blot.
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FIGURE 6. PKC is not involved in CD36 modulation by oxLDL and α-tocopherol. PKC was stimulated by PMA for 24 h, which led to adhesion of THP-1 monocytes and differentiation. A, CD36 mRNA expression was induced to 205 ± 65% (n = 3) as analyzed by RT-PCR. * p < 0.048 versus the control set to 100%. B, CD36 protein expression was induced to 527 ± 17% (n = 4) as analyzed by FACS. * p < 0.005 versus the control set to 100%. In both cases, α-tocopherol co-treatment did not reduce CD36 expression. C, THP-1 monocytes were stimulated with oxLDL (20 μg/ml) in the presence or absence of α-tocopherol for 24 h. Additional treatment with PKC412 (1 μmol/liter) did not lead to further inhibition of CD36 expression (n = 3). E, ethanol (solvent control); T, α-tocopherol; FITC, fluorescein isothiocyanate.

by FACS. Co-treatment with α-tocopherol (50 μmol/liter) had no effect (Fig. 6, A and B).

When THP-1 monocytes stimulated with oxLDL (20 μg/ml) for 24 h were incubated with a specific PKC inhibitor (PKC412; 1 μmol/liter), α-tocopherol co-treatment still reduced expression of CD36 (Fig. 6C). These results suggest that, in THP-1 monocytes treated with oxLDL, inhibition of CD36 expression by α-tocopherol is not directly dependent on PKC.

Modulation of CD36 Expression in THP-1 Monocytes by oxLDL and α-Tocopherol Involves PPARγ—The CD36 scavenger receptor has been described to be strongly activated by PPARγ, a nuclear receptor responsive to lipid peroxidation products present in oxLDL. In PMA-stimulated THP-1 cells, CD36 is inhibited completely by GW9662, an irreversible PPARγ antagonist (37); and troglitazone, a specific PPARγ agonist, induces CD36 expression in mouse peritoneal macrophages (64).

To assess whether α-tocopherol acts via the PPARγ signaling pathway, THP-1 monocytes were treated with troglitazone (50 μmol/liter) and with ethanol (0.1%; solvent control) or α-tocopherol (50 μmol/liter). Co-treatment with α-tocopherol partially decreased troglitazone-induced CD36 overexpression as measured by FACS (reduction of 18 ± 4%) (Fig. 7A) and Western blotting (reduction of 35 ± 19%) (Fig. 7, B and C).

To determine whether the CD36 promoter is activated at the transcriptional level, THP-1 monocytes were transfected with a CD36 promoter-luciferase reporter vector containing 380 bp of the human CD36 promoter (pCD36) (15) and treated with oxLDL (20 μg/ml) or troglitazone (50 μmol/liter). Furthermore, to assess whether the observed modulation by oxLDL or troglitazone occurred via PPARγ activation, a reporter plasmid containing a PPARγ-responsive element that controls a thymidine kinase basic promoter (pDR1) was transfected into THP-1 cells and treated as described above. Activation of the pCD36 and pDR1 constructs with similar potency by oxLDL and troglitazone suggests that these compounds stimulate the CD36 promoter via activation of PPARγ (Fig. 8, A and B). In line with the above-described results obtained by FACS (Fig. 1), Western blotting (Fig. 3), and RT-PCR (Fig. 4), co-treatment with α-tocopherol (50 μmol/liter) partially reduced CD36 overexpression induced by oxLDL as well as by troglitazone (Fig. 8C).

Altogether, these results led to the conclusion that oxLDL and α-tocopherol act antagonistically on the PPARγ signaling pathway in THP-1 monocytes, without direct involvement of PKC. This is in line with previous experiments in which PPARγ activity was up-regulated by oxLDL in a PKC-independent manner (65).

oxLDL-induced PKB Phosphorylation Is Prevented by α-Tocopherol—Another protein kinase that was shown previously to be activated by oxLDL is PKB. Interestingly, in human HMC-1 mastocytoma cells, PKB phosphorylation is inhibited by tocopherols (66), suggesting that the observed effects of oxLDL and α-tocopherol on CD36 expression could be the result of modulation of PKB activity.

THP-1 cells were incubated with oxLDL (20 μg/ml) in the presence or absence of α-tocopherol (50 μmol/liter), and Western blotting with antibody detecting phosphorylation of PKB at Ser473 was performed. Treatment with oxLDL significantly induced phosphorylation of PKB at Ser473, and α-tocopherol reduced it (reduction of 63 ± 23%) (Fig. 9).

PKB Activates CD36 Expression and Stimulates PPARγ and NF-κB Activities—It has furthermore been shown that activation of the PKB pathway by platelet-derived growth factor leads to induction of PPARγ gene expression in VSMC (67, 68), suggesting that activation of PKB by oxLDL may be involved in activation of PPARγ/CD36 expression. In line with this, oxLDL-induced expression of scavenger receptors (class A, type I; class B, type I; and CD36) is prevented by treatment with specific phosphatidylinositol 3-kinase inhibitors that reduce PKB activation (41, 69).

To assess whether PKB can induce CD36 expression, wild-type PKB (pPKBwt) and mutant inactive PKB (pPKB(R25C)), mutated in the pleckstrin homology domain; or pPKB(K179M), mutated in the kinase domain (57) were cotransfected with a CD36 promoter-luciferase construct (pCD36) (15) into THP-1 monocytes. Overexpression of wild-type PKB, but not mutant inactive PKB, induced CD36 promoter activity (Fig. 10A).

The ability of PKB to activate CD36 via the PPARγ element in its promoter was investigated by cotransfection of the PKB expression vectors with a luciferase reporter plasmid carrying a binding site for this
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transcription factor (pDR1). In addition to this, as control, the effect of PKB expression was checked using a reporter vector carrying a site for NF-κB (pNF-κB), known to be activated by PKB. Overexpression of wild-type PKB, but not mutant inactive PKB, activated pDR1 and pNF-κB, suggesting that both PPARγ and NF-κB elements are up-regulated by PKB in THP-1 cells (Fig. 10A).

PKB Acts on PPARγ to Induce CD36 Expression—Because cotransfection experiments with multiple vectors were difficult to perform in THP-1 cells, the effects of PKB and PPARγ expression were further assessed in HEK293 cells, which can be transfected with higher efficiency. Similar to THP-1 cells, wild-type PKB, but not mutant PKB, induced CD36 expression (pCD36) in parallel with activation of the PPARγ (pDR1) and NF-κB (pNF-κB) elements in HEK293 cells (Fig. 10B).

To determine whether PKB can activate PPARγ, pPKBwt or mutant pPKB was cotransfected with a PPARγ expression vector (pPPARγ) into HEK293 cells, and pCD36, pDR1, and pNF-κB activities were measured. Overexpression of PPARγ induced CD36 expression and stimulated the PPARγ element, but not the NF-κB element, which was activated only by wild-type PKB (Fig. 10C). Interestingly, the effect of combined PKB and PPARγ expression on CD36 expression as well as on the PPARγ element was additive (Fig. 10C). These results suggest that CD36 expression is activated by PKB via the previously described PPARγ element in its promoter sequence (33, 55).

DISCUSSION

In this study, we investigated whether α-tocopherol (the most abundant form of vitamin E in human plasma) acts at the earliest events of the cascade of atherosclerosis progression: oxLDL binding and uptake by monocytes. We have shown that α-tocopherol prevented oxLDL-induced CD36 overexpression and reduced the binding and uptake of oxLDL. Activation by oxLDL was always required for the inhibitory action of α-tocopherol. Thus, oxLDL may stimulate THP-1 monocytes to Induce CD36 Expression on CD36 expression as well as on the PPARγ element as additive (Fig. 10C). These results suggest that CD36 expression is activated by PKB via the previously described PPARγ element in its promoter sequence (33, 55).

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Involvement of PPARγ in the prevention of oxLDL-induced CD36 overexpression by α-tocopherol. A, surface expression of CD36 as analyzed by FACS. The mean fluorescence was increased by troglitazone treatment to 130 ± 18% (n = 3; p < 0.02 versus the control set to 100%), and α-tocopherol reduced it to 111 ± 16% (n = 3; p < 0.01 versus troglitazone-treated). B, Western blot of total protein isolated from THP-1 monocytes treated with troglitazone, α-tocopherol, or ethanol (0.1%; solvent control) for 24 h. The blot was probed with anti-CD36 antibody and anti-β-actin antibody as a control. Each bar represents the mean ± S.D. of two independent experiments performed in duplicate (n = 4), *, p < 0.05 versus the control set to 100%; **, p < 0.05 versus troglitazone-treated.

FIGURE 7. Involvement of PPARγ in the prevention of oxLDL-induced CD36 overexpression by α-tocopherol. THP-1 monocytes were transfected with the luciferase reporter plasmid pCD36 or pDR1 and treated with either oxLDL (20 μg/ml) (A) or troglitazone (Tro; 50 μmol/liter) (B) for 24 h. Additional treatment with α-tocopherol (50 μmol/liter) (C) reduced CD36 promoter activity. Each bar represents the mean ± S.D. of two independent experiments performed in duplicate (n = 4). *, p < 0.05 versus the control set to 100%; **, p < 0.05 versus oxLDL-treated. C, control; E, ethanol (solvent control); T, α-tocopherol.

FIGURE 8. CD36 promoter activity stimulated by oxLDL and troglitazone is inhibited by α-tocopherol. THP-1 monocytes were transfected with the luciferase reporter plasmid pCD36 or pDR1 and treated with either oxLDL (20 μg/ml) (A) or troglitazone (Tro; 50 μmol/liter) (B) for 24 h. Additional treatment with α-tocopherol (50 μmol/liter) (C) reduced CD36 promoter activity. Each bar represents the mean ± S.D. of two independent experiments performed in duplicate (n = 4). *, p < 0.05 versus the control set to 100%; **, p < 0.05 versus oxLDL-treated. C, control; E, ethanol (solvent control); T, α-tocopherol.

FIGURE 9. α-tocopherol-stimulated PKB phosphorylation at Ser473 is inhibited by α-tocopherol. THP-1 monocytes were treated with oxLDL (20 μg/ml) and either ethanol (0.1%; solvent control) or α-tocopherol (50 μmol/liter) for 24 h. Upper panel, Western blot of total protein isolated from THP-1 monocytes. The blot was probed with anti-phospho-Ser473 PKB antibody and subsequently with anti-PKB antibody. Anti-β-actin antibody was used as a control. Lower panel, graphical display of the relative PKB phosphorylation of the Western blots. Treatment with oxLDL induced phosphorylation of PKB at Ser473 to 135 ± 15% (n = 3; p < 0.001 versus the control set to 100%), and α-tocopherol reduced it to 72 ± 12% (n = 3; **, p < 0.0003 versus oxLDL-treated). E, ethanol (solvent control); T, α-tocopherol.

PKC is known to be activated by α-tocopherol, but our results with PMA and PKC412 do not support the involve-
ment of PKC in the inhibition of CD36 expression by α-tocopherol. Several transcription factors have been described that are modulated by α-tocopherol by direct binding (e.g. pregnane X receptor (PXR)) (70) or by changing their activity (such as AP-1 or NF-κB) (reviewed in Ref. 12). We found that, similar to oxLDL, troglitazone activates PPARγ (71, 72) with consequent increased CD36 expression that can be partially normalized by α-tocopherol. Thus, the reduction of CD36 expression by α-tocopherol occurs via the PPARγ signaling pathway and is not mediated by the PKC pathway.

α-Tocopherol may modulate PPARγ activity by affecting its redox state (73), its phosphorylation by mitogen-activate protein kinase (74), the rate of its synthesis (75), or proteolytic degradation (76). Moreover, α-tocopherol could also interfere with the action of lipid peroxidation products that were found to be increased in vivo in vitamin E-deficient rats and that may, after being internalized with oxLDL, increase CD36 expression via PPARγ activation. However, a recent study indicates that, although aldehydes can induce CD36 expression in THP-1 monocytes, PPARγ only partially reduces it, suggesting additional signaling pathways (77).

Our results show that oxLDL stimulates PKB phosphorylation, an event that can be inhibited by α-tocopherol. Because PKB stimulates PPARγ activity with consequent CD36 promoter activation, it is likely that α-tocopherol affects oxLDL-stimulated CD36 expression via inhibition of PKB phosphorylation. In hepatocytes, increased PKB activity has been shown to activate SREBP-1 (78), which activates PPARγ in adipocytes by the production of endogenous ligands (79), and it remains to be shown whether a similar activation cascade is functional in THP-1 monocytes.

The tocopherols were recently described to interfere with PKB phosphorylation at Ser473, leading to reduced proliferation of HMC-1 mast cells (66). In other cell lines such as breast cancer cells, PKB phosphorylation is inhibited by tocotrienols after stimulation by epidermal growth factor (80) and also by the two tocopherol derivatives α-tocopheryl succinate and α-tocopherylxybutyric acid (81). Further studies have shown that γ-tocotrienol induces a large decrease in the relative intracellular levels of the phosphorylated forms of PDK1, PKB, and glycogen synthase kinase-3 (82, 83).

The tocopherols and tocotrienols may inhibit PKB phosphorylation at Ser473 either directly or by acting on enzymes upstream of PKB, such as receptor tyrosine kinases (Tyk2) (17), phosphatidylinositol 3-kinase, and a kinase phosphorylating PKB (PDK1/2) (Fig. 11). Alternatively, α-tocopherol may stimulate a phosphatase dephosphorylating phospho-PKB such as protein phosphatase 2A or a lipid phosphatase (pTEN) that hydrolyzes the products of phosphatidylinositol 3-kinase (84, 85). In addition, the tocopherol-associated proteins, which modulate phosphatidylinositol 3-kinase and PKB in vitro and in vivo, may also play a role in the observed effects of α-tocopherol on CD36 expression in THP-1 monocytes (86, 87).

Several studies indicate that tyrosine phosphorylation is modulated by the tocopherols. In oxLDL-stimulated MRC5 fibroblasts, tyrosine phosphorylation of JAK2 (17), phosphatidylinositol 3-kinase, and a kinase phosphorylating PKB (PDK1/2) (Fig. 11). Alternatively, α-tocopherol may stimulate a phosphatase dephosphorylating phospho-PKB such as protein phosphatase 2A or a lipid phosphatase (pTEN) that hydrolyzes the products of phosphatidylinositol 3-kinase (84, 85). In addition, the tocopherol-associated proteins, which modulate phosphatidylinositol 3-kinase and PKB in vitro and in vivo, may also play a role in the observed effects of α-tocopherol on CD36 expression in THP-1 monocytes (86, 87).

Several studies indicate that tyrosine phosphorylation is modulated by the tocopherols. In oxLDL-stimulated MRC5 fibroblasts, tyrosine phosphorylation of JAK2, STAT1 (signal transducer and activator of transcription), and STAT3 is reduced by α-tocopherol (88). In VSMC, angiotensin II-induced tyrosine phosphorylation of two major proteins...
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(p120 and p70) and ERK (extracellular signal-regulated kinase) activation are markedly reduced by α-tocopherol, whereas ERK activation by epidermal growth factor is unaffected (89). Tyrosine phosphorylation is also decreased by α-tocopherol succinate in human neutrophils via activation of a tyrosine phosphatase (90). Because class I and II phosphatidylinositol 3-kinases are regulated by tyrosine phosphorylation, it can be speculated that inhibition of tyrosine kinase activity by tocopherols may ultimately lead to reduced PKB membrane translocation and phosphorylation (91).

When extrapolated to other cell types, our results suggest that activation of PKB leads to increased CD36 expression, ultimately increasing fatty acid uptake by activating PPAR-γ (91). When PKB is activated, it increases CD36 expression, which leads to increased fatty acid uptake (91). In this situation in which cells are exposed to oxLDL, it can be speculated that inhibition of tyrosine kinase activity by tocopherols may result in reduced PKB membrane translocation and phosphorylation (91).

In the presence of insulin (e.g. postprandial), increased CD36 expression may be involved in removal of fatty acids from plasma and also in regulation of insulin secretion by fatty acids in pancreatic β-cells (97). However, in the absence of insulin (e.g. diabetes) or during impaired insulin signaling (e.g. insulin resistance), a lower activation of CD36 expression may lead to insufficient plasma lipid removal with consequent hyperlipidemia (29). In this situation in which cells are exposed to increased lipid concentrations, oxLDL and possibly glucose-oxidized LDL may further accelerate lipid uptake via CD36 overexpression consequent to activation of the PKB/PPAR-γ pathway (98).

In summary, our findings show that α-tocopherol reduces the cellular effects of oxLDL by interfering with CD36 gene and protein expression, and our data suggest that PKB and PPAR-γ are involved in this process. Thus, α-tocopherol may have a beneficial role not only in tissue macrophages, but already at earlier times such as during plasma and tissue monocyte activation. Recently, it was shown that one of the first steps in atherogenesis, the adherence of THP-1 monocytes, is inhibited by α/β-

tocopherol and α/γ-tocotrienol in tumor necrosis factor-α-activated human umbilical vein endothelial cells (99). It remains to be shown whether α-tocopherol influences further early atherosclerotic events induced by oxLDL in monocytes, such as rolling on the endothelium, migration into the intima, and subsequent differentiation to macrophages (100).

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