ANTAGONISTIC EFFECTS OF OXLDL AND α-TOCOPHEROL ON CD36 SCAVENGER RECEPTOR EXPRESSION IN MONOCYTES; INVOLVEMENT OF PKB AND PPARγ

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Running title: Involvement of PKB in CD36 inhibition by α-tocopherol

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Vitamin E deficiency increases the expression of the CD36 scavenger receptor, suggesting specific molecular mechanisms and signalling pathways modulated by α-tocopherol. We show here that α-tocopherol down-regulates CD36 expression (mRNA and protein) in oxLDL-stimulated THP-1 monocytes but not in un-stimulated cells. Furthermore, α-tocopherol treatment of monocytes leads to a reduction of fluorescent oxLDL-DiO binding and uptake. Protein kinase C (PKC) appears not to be involved since neither activation of PKC by PMA nor inhibition by PKC412 was affected by α-tocopherol. However, α-tocopherol could partially prevent CD36 induction after stimulation with a specific agonist of PPARγ (troglitazone), indicating that this pathway is susceptible to α-tocopherol action. Phosphorylation of protein kinase B (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, co-expression of PPARγ and PKB led to additive induction of CD36 expression. Altogether, our results support the existence of signalling pathways leading from PKB to PPARγ mediating 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, oxLDL 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 signalling making them more susceptible to the following pro-atherogenic stimuli. The migration of monocytes into the intima and the conversion of monocytes/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 pro- or anti-oxidant on 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 cellular signalling (reviewed in (7,9,11,12)).

For vitamin E, non-antioxidant activities have been described, such as the inhibition of vascular smooth muscle cells (VSMC) proliferation via inhibition of the PKC pathway, the modulation of phospholipase A2, cyclooxygenase-2, 5-lipoxygenase and 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 CTGF 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 cells formation is mediated by the scavenger receptors (SR-A, SR-B1 and CD36) (18). Expression of some of the 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 HDL in brain capillary endothelial cells and type II pneumocytes (23,24).

In addition to oxLDL, CD36 binds to a large variety of ligands: thrombospondin, collagens type I and IV, β-amyloid, fatty acids, anionic phospholipids and high density lipoproteins (HDL) (25). In various tissues, the uptake of long chain fatty acids is mediated by CD36/FAT (fatty acid translocase), and transgenic mice over-expressing CD36 have reduced blood lipids (26-30). The central involvement of CD36 in atherosclerosis was proven by generating CD36 knockout mice that showed reduced uptake of modified LDL and reduced atherosclerosis (28). Similarly, human monocytes/macrophages from CD36 deficient patients showed a low capacity to bind and internalize oxLDL (28,31); these monocytes have also decreased NF-κB activation after oxLDL stimulation, leading to a lower expression of inflammatory cytokines expression (32).

At the transcriptional level, CD36 expression is induced by oxLDL via the peroxisome proliferator receptor gamma (PPARγ) and the NF-E2-related factor (Nrf2) (21,33-35). Activation of CD36 by interleukin-4, 15-deoxyDelta(12,14) prostaglandin J(2), and oxLDL in murine macrophages is dependent on protein kinase C (PKC) and PPARγ (36), whereas in THP-1 cells induction by retinoic acid is independent of PPARγ and PKC (37). Cholesterol and cholesterol acetate increase CD36 expression possibly via activation of sterol regulatory binding proteins (SREBP-1/2) and subsequent activation of PPARγ (38,39). Another protein kinase that was previously shown to be activated by oxLDL is protein kinase B (PKB); in VSMC, activation of PKB by oxLDL induced proliferation (40) and in mouse bone marrow derived macrophages, it increased survival by preventing apoptosis (41). PKB has a wide range of cellular targets and its increased activity can be found during atherosclerosis and tumorigenesis (42). Activation of PKB involves a membrane translocation step, followed by phosphorylation of two key regulatory sites, Ser473 and Thr308. After phosphorylation at PKB (Ser473) by a yet unidentified kinase (“PDK-2”), the enzyme becomes fully active (43,44).

Induction of CD36 expression by oxLDL (45,46) and oxLDL uptake (46,47) was previously studied in THP-1-derived macrophages. However, THP-1 monocytes bind significant amounts of oxLDL (48) and monocytes isolated from CD36 deficient people have a decreased capacity to take up oxLDL (31,49).

We show here that vitamin E deficient rats have increased expression of CD36 mRNA, indicating specific molecular mechanisms and signalling pathways modulated in vivo by α-tocopherol. In THP-1 monocytes, the expression of the CD36 scavenger receptor is increased by oxLDL, and α-tocopherol treatment normalizes it both at the protein and mRNA level. Protein kinase C (PKC) is not involved in the observed effects but over-expression of protein kinase B (PKB) and PPARγ leads to additive induction of
CD36 promoter activity. The phosphorylation of protein kinase B is increased by oxLDL and α-tocopherol can prevent it. Hence, our results suggest the existence of signalling pathways involving PPARγ and PKB, which regulate CD36 expression in response to oxLDL and α-tocopherol in THP-1 monocytes.

MATERIALS AND METHODS

Cell culture and treatments - RRR-α-Tocopherol (Cognis) was dissolved in ethanol and the concentration of the stock solutions confirmed spectrophotometrically. Human THP-1 acute monocytic leukemia cell line (THP-1) (ATCC-TIB-202) were cultured in RPMI/10% FCS, 2 mmol/L L-glutamine, 1.0 mmol/L sodium pyruvate, 4.5 g/L glucose and 100 μmol/L ascorbic acid (Sigma). HEK293 human embryonic kidney cells were grown in DMEM/10% FCS, 2 mmol/L L-glutamine. THP-1 monocytes (10^6 per plate) were plated 24 hours before the treatment with oxLDL (20 μg/ml), α-tocopherol (50 μmol/L), or ethanol (0.1%) as solvent control. Treatments with PMA (Sigma), PKC412 (Novartis) and troglitazone (Alexis) were done as described in the text. PKC412 was kindly obtained from Dr. B. Willi (Novartis Pharma AG, Basel, Switzerland). LDL (Intracel Corp.) was oxidized by reaction with 200 μmol/L CuSO_4 for 18 hours, a procedure that leads to heavily oxidized LDL, and oxidation checked by agarose gel electrophoresis (15,50,51). Aggregated LDL were prepared by vortexing LDL (Intracel Corp.) for 1 minute.

CD36 determination in vitamin E deficient rats - Male Sprague-Dawley rats were housed five in a cage in accordance with institutional guidelines. The temperature was maintained between 21-22°C and with dark-light cycle of 12/12 (h), the lights turned on from 6.00 to 18.00. Three month-old rats were randomly divided into two dietary groups: control rats fed ad libitum with a standard pellet diet (Teklad, Harlan, Italy), and rats fed a vitamin E deficient diet. Fresh water was available ad libitum. During the experiment, the body weight of the vitamin E deficient rats was unchanged; however, they had generally increased levels of urinary aldehydes (malondialdehydes, acetaldehyde, formic acid, propionaldehydes, acetone) when compared to the control animals (Tamburini et al, data not shown). Tissue samples were taken in the morning under anaesthesia with nembutal (50 mg in 1 ml saline/kg body weight), following a 16 h (overnight) fasting period. Vitamin E levels (nmol/wet tissue) in the liver of sacrificed rats were: control group at start of experiment, 3-months old: 32.4±2.92; control group, 21-24-months old: 71.0±6.76; vitamin E deficient group, 21-months old: 1.6±0.25. The samples were stored in RNAlater (Qiagen) reagent at -80°C and later homogenized using a polytron. Total tissue RNA was isolated using a RNA extraction kit from Qiagen. Determination of rat CD36 mRNA expression by RT-PCR was done as described and quantified after normalization to rat GAPDH mRNA expression (52).

Determination of CD36 expression by FACS - THP-1 monocytes were treated as indicated and CD36 expression was analyzed by fluorescence-activated cell sorting (FACS) as previously described (15,53,54). The CD36 expression was measured using a monoclonal anti-human CD36-FITC antibody (Ancell) diluted (1:50) in PBS/1% BSA. A minimum of 10000 cells per sample was assessed; data were acquired and analyzed using the Cellquest software (FACSscan, Becton & Dickinson).

Determination of CD36 mRNA by RT-PCR - Total RNA was isolated using a RNA extraction kit from Qiagen. Semi-quantitative assays for CD36 mRNA expression were performed as previously described (15,53).

Determination of total CD36 expression by Western blots - Protein extracts and western blots were done according to standard methods with monoclonal mouse anti-human CD36 primary antibody (Ancell) and sheep anti-mouse IgG secondary antibody coupled to horseradish peroxidase (AmershamPharmaciaBiotech) (55). An anti-β-actin antibody (Sigma) was used as internal control. Western blots for PKB were done according to the protocol provided by the manufacturer (Cell Signalling Technology). Proteins were visualized using an ECL detection kit according to the manufacturer’s description (Amersham-Pharmacia-Biotech). Chemiluminescence was monitored by exposure to film (Hyperfilm ECL, Amersham-Pharmacia-
Labelling and uptake of oxLDL - Labeling of oxLDL (Intracel Corp.) with DiO (Molecular Probes) and blocking of oxLDL-DiO uptake with anti-CD36 antibodies were done as previously described (15). Uptake and binding of oxLDL-DiO was studied by FACS. The cells were pre-treated for 16 hours with oxLDL (20 μg/ml), with 50 μmol/L α-tocopherol or 0.1% ethanol solvent (control), and then incubated with oxLDL-DiO (5 μg/ml medium). For the uptake experiments the incubation was done at 37°C for 6 hours and for the binding experiment the incubation was done for 30 min at 4°C. Thereafter the cells were washed 3 times with 3% BSA/PBS, once with PBS, and fixed with 4% paraformaldehyde in PBS. FACS was performed with a FACSscan, and data were analyzed using the Cellquest software (FACSscan, Becton & Dickinson).

Plasmids, transfections and reporter assays - The luciferase reporter plasmids were: pCD36 (15), pDR1 (56), pNFkB (BD Biosciences Clonetech); the renilla internal control plasmid was pRL-TK (Promega); the PKB expression vectors pPKBwt, pPKBR25C, and pPKBK179M were kindly obtained from Dr. J. Downward (Imperial Cancer Research Fund, London, UK) and correspond to pGFP-Akt, pGFP-AktR25C and pGFP-AktK179M (57). The PPARγ expression vector used contains the PPARγ cDNA under the control of the CMV promoter (56). THP-1 monocytes were transfected for 3 hours with the indicated reporter and expression plasmids using Transfectin (Biorad) and after that treated with ethanol solvent control (0.1%) or α-tocopherol (50 μmol/L) for further 21 hours. HEK293 cells were transfected for 3 hours using Superfect (Qiagen), then the medium was changed, the cells treated with ethanol solvent control (0.1%) or α-tocopherol (50 μmol/L) and further incubated for 21 hours. Extracts were prepared and promoter activities were measured using the Dual-Luciferase Assay Kit (Promega) with a TD-20/20 luminometer (Turner Designs). The promoter-firefly-luciferase activities were normalized to the TK 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 hours, and then stained with Hoechst 33342 (5 μg/ml) for 1 hour 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 - The values are expressed as the mean ± standard deviation as indicated in the figure legends. For FACS results, the median fluorescence intensity was determined and the mean ± standard deviation calculated as mentioned in the figure legend. Student’s t-test was used to analyze the significant differences between two conditions. A P value of less than 0.05 was taken as significant and indicated by * in the graphs.

RESULTS

Vitamin E deficiency in rats leads to CD36 over-expression

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 from α-tocopherol intake for 21 months, and the expression level of CD36 mRNA measured by RT-PCR. Deficiency of α-tocopherol led to a significantly higher level of liver CD36 expression (238% ± 41, n = 4, P < 0.028) when compared to 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 surface CD36 over-expression 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 was described 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 it was checked whether α-tocopherol could interfere with this pro-atherogenic stimulus. By using oxLDL in this model system, a reduction of CD36 over-expression by α-tocopherol would be the consequence of inhibition of oxLDL-induced CD36 transcription, rather than being the result of inhibiting the oxidation of LDL.

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

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

**Apoptosis induction occurs only at high oxLDL concentrations**

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

**α-Tocopherol inhibits CD36 over-expression at the protein and mRNA level in THP-1 monocytes stimulated by oxLDL**

The results obtained by FACS could be explained by increased gene and protein expression of CD36. As previously described, western blots with anti-CD36 monoclonal antibodies showed that the CD36 protein is expressed in THP-1 monocytes mainly as a 74 kDa protein (59). Treatment of THP-1 monocytes with oxLDL (20 µg/ml) stimulated CD36 total protein expression and co-treatment with α-tocopherol (50 µmol/L) prevented completely the CD36 over-expression in western blots analyses (71 ± 38% reduction) (Figure 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/L) or of solvent control ethanol (0.1%), and CD36 mRNA expression analyzed by RT-PCR. Treatment with oxLDL led to increased expression of CD36 mRNA whereas co-treatment with α-tocopherol could normalize CD36 mRNA levels (39 ± 28% reduction) (Figure 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 CD36 scavenger receptor is involved in the uptake of oxLDL, allowing the accumulation of lipids and cholesterol that ultimately lead to foam cell formation. Consequently, inhibition of CD36 expression should reduce oxLDL uptake. To check this hypothesis, THP-1 monocytes were incubated with α-tocopherol (50 µmol/L) or solvent control ethanol (0.1%), and uptake or binding of fluorescent labeled oxLDL-DiO was analyzed by FACS. In non-stimulated THP-1 monocytes, the treatment with α-tocopherol resulted in a small and non-significant decrease of oxLDL-DiO binding and uptake (Figure 5A). In oxLDL-stimulated cells, α-tocopherol treatment led to
stronger and statistically significant inhibition of oxLDL binding and uptake (Figure 5B).

In order to check whether CD36 is the major scavenger receptor responsible for the uptake of oxLDL, THP-1 monocytes were pre-incubated for 30 minutes with an anti-CD36 monoclonal antibody before adding oxLDL-DiO for 6 hours. The uptake of oxLDL-DiO was analyzed by FACS. As described already with other cell types (15,49,60,61), an anti-CD36 antibody interfered with the uptake of oxLDL-DiO, whereas an isotype matched control antibody (mouse anti-IgM antibodies) did not, suggesting that CD36 is responsible for the oxLDL uptake in THP-1 monocytes (data not shown).

Modulation of CD36 expression in THP-1 monocytes by oxLDL and \( \alpha \)-tocopherol does not involve protein kinase C

In VSMC \( \alpha \)-tocopherol inhibits protein kinase C (PKC) by activation of protein phosphatase 2A (PP2A), leading to inhibition of proliferation (62). Since in THP-1 macrophages oxLDL are known to activate PKC (63), it was possible that the above described inhibitory effect on CD36 expression mediated by \( \alpha \)-tocopherol is 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/L, for 24 hours), CD36 was induced at the mRNA level as analyzed by RT-PCR, and at the protein level as analyzed by FACS. Co-treatment with \( \alpha \)-tocopherol (50 \( \mu \)mol/L) had no effect (Figure 6A and B).

When THP-1 monocytes stimulated with oxLDL (20 \( \mu \)g/ml, for 24 hours) were pre-incubated with a specific PKC inhibitor (PKC412, 1 \( \mu \)mol/L), \( \alpha \)-tocopherol co-treatment still reduced the expression of CD36 (Figure 6C). These results suggest that in THP-1 monocytes treated with oxLDL the inhibition of CD36 expression by \( \alpha \)-tocopherol is not directly dependent on PKC.

Modulation of CD36 expression in THP-1 monocytes by oxLDL and \( \alpha \)-tocopherol involves PPAR\( \gamma \)

The CD36 scavenger receptor has been described to be strongly activated by PPAR\( \gamma \), 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 PPAR\( \gamma \) antagonist (37), and troglitazone, a specific PPAR\( \gamma \) agonist, induces CD36 expression in mouse peritoneal macrophages (64).

To assess whether \( \alpha \)-tocopherol acts via the PPAR\( \gamma \) signalling pathway, THP-1 monocytes were treated with troglitazone (50 \( \mu \)mol/L), and with ethanol solvent control (0.1%) or \( \alpha \)-tocopherol (50 \( \mu \)mol/L). Indeed, co-treatment with \( \alpha \)-tocopherol was partially able to decrease the troglitazone-induced CD36 over-expression, as measured by FACS (reduction of 18 ± 4%) (Figure 7A) and western blot (reduction of 35 ± 19%) (Figure 7B, C).

To elucidate if 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 \( \mu \)g/ml) or troglitazone (50 \( \mu \)mol/L). Furthermore, to assess whether the observed modulation by oxLDL or troglitazone occurs via PPAR\( \gamma \) activation, a reporter plasmid containing a PPAR\( \gamma \) responsive element that controls a thymidine kinase basic promoter (pDR1) was transfected into THP-1 cells and treated as 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 PPAR\( \gamma \) (Figure 8A and B). In line with the above described results obtained with FACS (Fig. 1), western blots (Fig. 3), and RT-PCR (Fig. 4), co-treatment with \( \alpha \)-tocopherol (50 \( \mu \)mol/L) could partially reduce CD36 over-expression induced by oxLDL as well as by troglitazone (Figure 8C).

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

**oxLDL induced PKB phosphorylation is prevented by α-tocopherol**

Another protein kinase that was previously shown to be activated by oxLDL is protein kinase B (PKB). Interestingly, in human mastocytoma HMC-1 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/L), and western blots with antibodies detecting phosphorylation of PKB (Ser473) were performed. Treatment with oxLDL significantly induced PKB (Ser473) phosphorylation, and α-tocopherol reduced it (reduction of 63 ± 23%) (Figure 9).

**PKB activates CD36 expression, and stimulates PPARγ and NF-κB activity**

It was furthermore shown that activation of the PKB pathway by platelet derived growth factor (PDGF) led to induction of PPARγ gene expression in VSMC (67,68), suggesting that activation of PKB by oxLDL may be involved in the activation of PPARγ/CD36 expression. In line with this, oxLDL-induced expression of scavenger receptors SR-BI, SR-AI and CD36 is prevented by treatment with specific phosphatidylinositol-3-kinase (PI3K) inhibitors that reduce PKB activation (41,69).

To assess whether PKB can induce CD36 expression, wildtype PKB (pPKBwt), and mutant inactive PKB (pPKBR25C, mutated in the PH (Pleckstrin Homology) domain; or pPKBK179M, mutated in the kinase domain (57)), were co-transfected with a CD36-promoter-luciferase construct (pCD36, (15)) into THP-1 monocytes. Over-expression of PKBwt, but not mutant inactive PKB, induced CD36 promoter activity (Figure 10A).

The ability of PKB to activate CD36 via the PPARγ element in its promoter was investigated by co-transfection of the PKB expression vectors with a luciferase reporter plasmid carrying a binding site for this transcription factor (pDR1). In addition to that, as control, the effect of PKB expression was also checked with a reporter vector carrying a site for NF-κB (pNFκB), known to be activated by PKB. Over-expression of PKBwt, but not mutant inactive PKB, activated pDR1 and pNFκB, suggesting that both PPARγ and NFκB elements are upregulated by PKB in THP-1 cells (Figure 10A).

**PKB acts on PPARγ to induce CD36 expression**

Since co-transfection experiments with multiple vectors were difficult to perform in THP-1 cells, the effects of PKB and PPARγ expression was further assessed in HEK293 cells, which were better transfectable. Similar to THP-1 cells, PKBwt but not the PKB mutants induced CD36 expression (pCD36), in parallel with the activation of the PPARγ (pDR1) and NFκB (pNFκB) elements in HEK293 cells (Figure 10B).

To check whether PKB can activate PPARγ, pPKBwt or mutated pPKB were co-transfected with a PPARγ expression vector (pPPARγ) into HEK293 cells, and pCD36, pDR1 and pNFκB activity measured. The over-expression of PPARγ induced CD36 expression and stimulated the PPARγ element, but not the NFκB element which was only activated by PKBwt (Figure 10C). Interestingly, the effect of combined PKB and PPARγ expression was additive on CD36 expression as well as on the PPARγ element (Figure 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 acts at the earliest events of the cascade of atherosclerosis progression, the ones of oxLDL binding and uptake by monocytes. We show that α-tocopherol can prevent oxLDL-induced CD36 over-expression and reduces the binding and uptake of oxLDL. Activation by oxLDL was always required for the inhibitory
action of \( \alpha \)-tocopherol. Thus, oxLDL may stimulate THP-1 monocytes by binding to membrane receptors such as CD36, which may trigger alterations in cellular signalling.

PKC is known to be activated by oxLDL and inhibited by \( \alpha \)-tocopherol, but our results with PMA and PKC412 do not support the involvement of PKC in the inhibition of CD36 expression by \( \alpha \)-tocopherol.

Several transcription factors have been described which are modulated by \( \alpha \)-tocopherol by direct binding (e.g. PXR and possibly other nuclear receptors (70)) or by changing their activity (such as of AP-1 or NF-\( \kappa \)B) (reviewed in (12)). We found that similar to oxLDL, troglitazone activates PPAR\( \gamma \) (71,72) with consequent increased CD36 expression that can be partially normalized by \( \alpha \)-tocopherol. Thus, the reduction of CD36 expression by \( \alpha \)-tocopherol occurs via the PPAR\( \gamma \) signalling pathway and is not mediated by the PKC pathway.

\( \alpha \)-Tocopherol may modulate PPAR\( \gamma \) activity by affecting its redox state (73), its phosphorylation by MAP kinase (74), the rate of its synthesis (75)) or proteolytic degradation (76). Moreover, \( \alpha \)-tocopherol could also interfere with the action of lipid peroxidation products that were found to be increased \textit{in vivo} in vitamin E deficient rats, and that may, after being internalized with oxLDL, increase CD36 expression via PPAR\( \gamma \) activation. However, recent studies indicate that albeit aldehydes could induce CD36 expression in THP-1 monocytes, \( \alpha \)-tocopherol only partially reduced it, suggesting additional signalling pathways (77).

Our results show that oxLDL stimulates PKB phosphorylation, an event that can be inhibited by \( \alpha \)-tocopherol. Since PKB stimulates PPAR\( \gamma \) activity with consequent CD36 promoter activation, it is likely that \( \alpha \)-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 in adipocytes activates PPAR\( \gamma \) 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 (Ser473) phosphorylation, 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 EGF (80), and also by the two tocopherol derivatives, \( \alpha \)-tocopheryl succinate and \( \alpha \)-tocopheryl oxybutyric acid (81). Further studies showed that \( \gamma \)-tocotrienol induced a large decrease in the relative intracellular levels of the phosphorylated forms of PDK-1, PKB, and glycogen synthase kinase 3 (GSK-\( \alpha/\beta \)) (82,83).

The tocopherols and tocotrienols may inhibit PKB (Ser473) phosphorylation either directly, or by acting on enzymes upstream of PKB, such as receptor tyrosine kinases (Tyk2 (17)), phosphatidylinositol 3-kinase (PI3K), or a kinase phosphorylating PKB (PDK1/2) (Figure 11). Alternatively, \( \alpha \)-tocopherol may stimulate a phosphatase dephosphorylating phosphoPKB, such as protein phosphatase 2A (PP2A), or a lipid phosphatase (pTEN), which hydrolyses the products of PI3K (84,85). In addition to that, the tocopherol associated proteins (TAP), which modulate PI3K and PKB \textit{in vitro} and \textit{in vivo}, may also play a role in the observed effects of \( \alpha \)-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 and STAT3 is reduced by \( \alpha \)-tocopherol (88). In VSMC, angiotensin II-induced tyrosine phosphorylation of two major proteins (p120, p70) and ERK activation are markedly reduced by \( \alpha \)-tocopherol, whereas ERK activation by epidermal growth factor is unaffected (89). Tyrosine phosphorylation is also decreased by \( \alpha \)-tocopheryl succinate in human neutrophils via activation of a tyrosine phosphatase (90). Since class I and II PI3K 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 the uptake of fatty acids. Activation of PKB also occurs in response to insulin, which stimulates glucose uptake by increasing glucose transporters (Glut-4 and Glut-1) and by stimulating glucokinase expression (92,93). Energy from glucose is used for the biosynthesis of fatty acids and cholesterol. SREBP-1 and PPARγ are the major transcription factors regulating these processes and both are activated by PKB (39,79,94,95). Thus, the finding that PKB increases CD36 expression suggests that increased fatty acid synthesis by activating SREBP-1 is assisted by increased fatty acid uptake by activating PPARγ and subsequent activation of CD36 expression (96).

In the presence of insulin (e.g. postprandial), increased CD36 expression may be involved in removal of fatty acids from plasma and also in the regulation of the insulin secretion by fatty acids in pancreatic ß-cells (97). However, in the absence of insulin (e.g. diabetes) or during impaired insulin signalling (e.g. insulin resistance), a lower activation of CD36 expression may reduce plasma lipid removal with consequent hyperlipidemia (29). In this situation where cells are exposed to increased lipid concentrations, oxLDL and possibly glucose-oxidized LDL may further accelerate lipid uptake via CD36 over-expression consequent to activation of the PKB/PPARγ 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 PPARγ 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 adhesion of THP-1 monocytes, is inhibited by α-/γ-tocopherol and α-/γ-tocotrienol in TNF-α-activated HUVEC cells (99). It remains to be shown whether α-tocopherol influences further early atherosclerotic events induced by oxLDL, such as rolling on the endothelium, migration into the intima and the subsequent differentiation to macrophages (100).

REFERENCES


FOOTNOTES

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1The abbreviations are: EGF: epidermal growth factor; FACS: fluorescence activated cell sorting; HDL: high density lipoproteins; LDL: low density lipoproteins; oxLDL: oxidized low density lipoproteins; PI3K: phosphatidylinositol-3-kinase; PKB: protein kinase B; PKC: protein kinase C; PMA. Phorbolmyristateacetate; PP2A: protein phosphatase 2A; PPARγ: peroxisome proliferator receptor gamma; PDGF: platelet derived growth factor; VSMC: vascular smooth muscle cells.

FIGURE LEGEND

**Fig. 1.** Induction of CD36 protein expression by oxLDL is inhibited by α-tocopherol in THP-1 monocytes. Quantification by FACS of CD36 surface expression. The median fluorescence intensity was increased by oxLDL treatment to 139 ± 8% (n = 4, P < 0.005, vs control set to 100%), and α-tocopherol could reduce it to 120 ± 8 % (n = 4, P < 0.05, vs oxLDL treated).

**Fig. 2.** Induction of apoptosis by oxLDL in THP-1 monocytes only with high oxLDL concentrations. THP-1 monocytes were incubated for 24 or 48 hours with increasing concentrations of oxLDL as indicated, and the percentage of apoptotic cells with condensed nuclei was quantified (n = 2, *P < 0.05, vs control).

**Fig. 3.** Induction of CD36 protein by oxLDL is inhibited by α-tocopherol in THP-1 monocytes A) Western blot of total protein isolated from THP-1 monocytes. The blot was probed with anti-CD36 antibody and anti-β-actin antibody as control. B) A graphical display of the CD36 signal of the western blots normalized to β-actin (n = 3, *P < 0.05, vs control set to 100%; **P < 0.05 vs oxLDL alone). E: ethanol solvent control; T: α-tocopherol.

**Fig. 4.** Expression of CD36 mRNA induced by oxLDL is inhibited by α-tocopherol. A) After treatment of THP-1 monocytes as indicated, total RNA was isolated and CD36 mRNA expression quantified by RT-PCR. B) A graphical display of the CD36 signal normalized to GAPDH. Each bar represents the mean ± SD of 2 independent experiments (*P < 0.05, vs control set to 100%). E: ethanol solvent control; T: α-tocopherol.

**Fig. 5.** Binding and uptake of oxLDL in THP-1 monocytes is inhibited by α-tocopherol. THP-1 monocytes were either un-stimulated or stimulated with oxLDL (20 µg/ml) for 24 hours. A) In non-stimulated THP-1 monocytes, the treatment with α-tocopherol resulted in a small and non-significant decrease of oxLDL-DiO binding by 12 % ± 4 (n = 5, P < 0.096, vs control set to 100%) and uptake (by 11 % ± 11, n = 5, P < 0.076, vs control set to 100%). B) In oxLDL-stimulated cells, α-tocopherol treatment
led to stronger and statistically significant inhibition of oxLDL binding by 31 ± 8 % (n = 5, P < 0.048, vs control set to 100%) and uptake by 18 ± 8 % (n = 5, P < 0.009, vs control set to 100%). E: ethanol solvent control; T: α-tocopherol.

**Fig. 6.** PKC is not involved in CD36 modulation by oxLDL and α-tocopherol. PKC was stimulated by PMA for 24 hours what leads to adhesion of THP-1 monocytes and differentiation. A) Induction of CD36 mRNA expression to 205 ± 65 % (n = 3, P < 0.048, vs control set to 100%) as analyzed by RT-PCR. B) Induction of CD36 protein expression to 527 ± 17% (n = 4, *P < 0.005, vs control set to 100%) as analyzed by FACS; in both cases α-tocopherol co-treatment does 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 hours. Additional treatment with PKC412 (1 μmol/L) did not lead to further inhibition of CD36 expression (n = 3). E: ethanol solvent control; T: α-tocopherol.

**Fig. 7.** Involvement of PPARγ in the prevention of oxLDL-induced CD36 over-expression by α-tocopherol. A) Surface expression of CD36 (FACS). The mean fluorescence was increased by troglitazone treatment to 130 ± 18 % (n = 3, P < 0.02, vs control set to 100%), and α-tocopherol could reduce it to 111 ± 16 % (n = 3, P < 0.01, vs troglitazone treated). B) Western blot of total protein isolated from THP-1 monocytes, treated with troglitazone, α-tocopherol or solvent control ethanol (0.1%) for 24 hours; the blot was probed with anti-CD36 antibody and anti-β-actin antibody as control. C) A graphical display of the CD36 signal of the western blots normalized to β-actin. Each bar represents the mean ± SD of 2 independent experiments made in duplicate (n = 4, *P < 0.05, vs control set to 100%; **P < 0.01, vs troglitazone alone). E: ethanol solvent control; T: α-tocopherol.

**Fig. 8.** CD36 promoter activity stimulated by oxLDL and troglitazone is inhibited by α-tocopherol. THP-1 monocytes were transfected with luciferase reporter plasmids pCD36 or pDR1 and treated with either oxLDL (20 μg/ml) (A) or troglitazone (50 μmol/L) (B) for 24 hours. Additional treatment with α-tocopherol (50 μmol/L) (C) could reduce CD36 promoter activity. Each bar represents the mean ± SD of 2 independent experiments made in duplicate (n = 4, *P < 0.05, vs control set to 100%; **P < 0.04, vs troglitazone alone). E: ethanol solvent control; T: α-tocopherol.

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

**Fig. 10.** CD36 promoter activity is stimulated by PKB and PPARγ expression. A) THP-1 monocytes were transfected with luciferase reporter plasmids pCD36, pDR1 or pNFκB, together with PKB expression vectors, pPKBwt or pPKBR25C for 24 hours. B) HEK293 cells were transfected with luciferase reporter plasmids pCD36, pDR1 or pNFκB, together with PKB expression vectors, pPKBwt, pPKBR25C or pPKBK179M for 24 hours. C) HEK293 cells were transfected with luciferase reporter plasmids pCD36, pDR1 or pNFκB, together with PKB expression vectors, pPKBwt or pPKBR25C, with or without pPPARγ for 24 hours. Each bar represents the mean ± SD of 2 independent experiments made in duplicate (n = 4, *P < 0.05, vs control set to 100%).
**Fig. 11.** Antagonistic effects of α-tocopherol and oxLDL on signalling pathways inducing CD36 expression. OxLDL activates the PI3K/PKB pathway leading to increased CD36 expression via stimulation of PPARγ activity. Activation of PKC by oxLDL in THP-1 monocytes does not lead to induction of CD36 expression, since the PKC inhibitor has no effect (stippled arrows); however, activation of PKC by PMA can activate CD36 expression possibly by induction of adhesion and differentiation. In THP-1 monocytes, α-tocopherol reduces CD36 expression by inhibition of signal transduction initiated by oxLDL that leads to PKB phosphorylation. PPRE: Peroxisome Proliferator Response Element, Tro: troglitazone.
Figure 4
Figure 5
Figure 10
Antagonistic effects of oxLDL and α-tocopherol on CD36 scavenger receptor expression in monocytes; involvement of PKB and PPAR γ

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