

α -Tocopherol down-regulates scavenger receptor activity in macrophages

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Abstract

The aim of this study was to investigate the effect of α -tocopherol on scavenger receptor (SR) activity, SR class A (SR-A) mRNA expression and transcriptional regulation in macrophages. Scavenger receptor activity was determined quantitatively by uptake of DiI-acLDL (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate-labeled acetylated low-density lipoprotein) in rabbit peritoneal macrophages and human monocytes/macrophages in the presence and absence of different tocopherol homologues. SR-A mRNA expression was determined by Northern blotting, the activity of the transcription factor activator protein-1 (AP-1) by electrophoretic mobility shift assay. We could demonstrate that α -tocopherol down-regulates scavenger receptor activity in macrophages in a dose-dependent manner. Scavenger receptor activity was reduced by 13, 16, 18 and 24% in the presence of 1, 5, 10 and 50 μ M α -tocopherol, respectively. This effect was associated with a reduced SR-A mRNA expression and activity of AP-1 binding transcription factors in the presence of α -tocopherol. The activity of scavenger receptors in human monocyte derived macrophages incubated with 100 μ M α -tocopherol for 15 days was reduced up to 60%. Interestingly, γ -tocopherol, which is a homologue of α -tocopherol with a comparable antioxidative capacity, showed only a weak suppression of SR activity, SR-A expression and AP-1 activity. Our observations point to the conclusion that the reduction of SR-A expression and activity in presence of α -tocopherol is possibly related to its direct action on cell signaling. © 1999 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: AP-1; DiI-acLDL; Scavenger receptor; SR-A; Tocopherol; Vitamin E

1. Introduction

Several epidemiologic studies provide evidence for an association between increased intake of α -tocopherol and a reduction in the risk of coronary heart disease [1,2]. The Nurses Health study and the Health Professionals' Follow-up Study showed about 35% reduction of the incidence of major coronary heart disease among subjects in the highest quintile of vitamin E intake as compared with those in the lowest quintile [3,4]. Recently, it was shown that patients with coronary heart disease had a significant reduction in the risk of non-fatal myocardial infarction, when supplemented with high

dose vitamin E [5]. However, in the Alpha Tocopherol, Beta Carotene Cancer Prevention Study, α -tocopherol supplementation had no significant effect on the prevention of coronary heart disease in a population of Finnish smokers [6].

Several mechanisms have been suggested to contribute to the vascular effects of α -tocopherol: α -tocopherol has been shown to inhibit low-density lipoprotein (LDL) oxidation [7] and to reduce monocyte adhesion and transmigration into the intima [8]. It can prevent cytotoxic effects of oxidized lipoproteins [9], it modulates vasomotion [10] and can inhibit proliferation of smooth muscle cells [11]. These effects of α -tocopherol were suggested to be due to two major mechanisms: the antioxidative capacity of α -tocopherol or the inhibition of protein kinase C activity.

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The aim of this study was to investigate the effect of α -tocopherol on scavenger receptor (SR) activity, SR class A (SR-A) mRNA expression and transcriptional regulation in macrophages. Scavenger receptors (SRs) are cell membrane proteins which can bind chemically modified lipoproteins, such as acetylated (acLDL) and oxidized LDL (oxLDL) [12]. They are expressed in monocyte derived macrophages and mediate the endocytosis of modified lipoproteins from the extracellular space. This process can result in massive intracellular cholesterol accumulation and the transformation of macrophages into foam cells [13]. However, it is presently unknown if α -tocopherol may affect scavenger receptor expression and activity. Though a number of different classes of scavenger receptors have been identified [12,14], the present paper will focus on the regulation of class A scavenger receptors (SR-A) in macrophages in the presence of α -tocopherol.

2. Methods

2.1. Isolation and cultivation of rabbit peritoneal macrophages

Peritoneal macrophages were isolated from New Zealand White rabbits ($n = 10$). The animals were killed with an overdose of pentobarbital 3 days after intraperitoneal injection of 40 ml mineral oil. The peritoneal cavity was washed with 800 ml 0.9% NaCl. The cells were collected into 50-ml tubes containing EDTA (final concentration 0.1 g/l), pooled and washed with Hanks' balanced salt solution (0.1 g/l EDTA) and Dulbecco's modified Eagle's medium (DMEM). The cells were resuspended in DMEM containing 10% fetal calf serum (FCS), penicillin–streptomycin and diluted to a final concentration of 10^6 cells/ml. α -, β -, γ - or δ -Tocopherol (Merck, Darmstadt, Germany) was dissolved in ethanol at 0, 1, 5, 10, 50 and 100 mM and added to the cell suspension in a 1:1000 dilution with medium. The cells were plated on culture dishes and incubated in the presence of the tocopherols for up to 20 h as indicated in the legends to the figures.

2.2. Isolation and cultivation of human monocytes

Mononuclear cells were isolated from buffy coat of five healthy human blood donors by Ficoll density gradient centrifugation using standardized tubes (Vacutainer CPT cell preparation tubes; Becton Dickinson). The cells were washed with Hanks' balanced salt solution (0.1 g/l EDTA) and 10^7 mononuclear cells were plated per 35-mm dish in DMEM containing 10% FCS, penicillin–streptomycin. After 2 h of incubation (37°C, 5% CO₂), the non-adherent cells were removed. The adherent monocytes were incubated with DMEM (10%

FCS, penicillin–streptomycin) containing 0 or 100 μ M α -tocopherol. α -Tocopherol was dissolved in ethanol at 0 and 100 M and added to the cells in a 1:1000 dilution. The cells were incubated with and without α -tocopherol for 8 or 15 days.

2.3. Lipoproteins

LDL was prepared from EDTA–plasma from normolipidemic human donors by ultracentrifugation. LDL was labeled with the fluorescent probe 1,1'-diiododecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) (Molecular Probes, Eugene, OR, USA) as described by Innerarity et al. [15] and acetylated according to Basu et al. [16]. Protein concentrations were determined by the method of Lowry et al. [17]. All lipoprotein preparations were stored at 4°C in sterile containers after filtration sterilization.

2.4. Quantitative analysis of scavenger receptor activity

Following incubation in the presence and absence of tocopherols on 35-mm dishes, macrophages were incubated with 10 μ g DiI-acLDL per ml DMEM for 5 h. After incubation with DiI-acLDL, the cells were washed twice with phosphate-buffered saline (PBS) (containing 0.4% bovine serum albumin) and $3 \times$ with PBS and 2 ml of lysis reagent (0.1% sodium dodecyl sulfate (SDS) in 0.1 NaOH) were added. This reagent allowed the determination of fluorescence intensity (FI) as well as cell protein in the same sample of lysed cells. FI was measured in the lysate on microtiter plates with excitation and emission wavelengths set at 520 and 580 nm, respectively (Fluorolite 1000, Dynatech). FI of the DiI-labeled lipoprotein diluted in the lysis reagent (1:2000) was measured in order to determine the specific fluorescence intensity of the DiI-acLDL preparation used. The data were expressed as nanograms of cell associated DiI-acLDL per milligram of cell protein [18].

2.5. Northern blot

Total RNA was isolated with TRIzol™ (Gibco) from macrophages incubated with/without tocopherols before or 1, 5 or 20 h after adherence of the cells on 10-cm culture dishes. RNA was separated by electrophoresis, transferred to a Nytran membrane (turbo blotter, Schleicher & Schuell) and cross-linked by UV irradiation. A fragment of the collagenous domain of rabbit scavenger receptor corresponding to base pairs (bp) 833–1057 was amplified from rabbit cDNA by polymerase chain reaction (PCR) [19]. It was radiolabeled with ³²P by random-priming and hybridized to the membrane at 42°C in 50% formamide $5 \times$ SSPE overnight. The membranes were washed in $2 \times$ SSC, 0.1% SDS at 42°C and exposed on storage phosphor

screens. To control for variations in RNA loading, the membranes were stripped of probe and rehybridized with a random prime labeled fragment of rabbit glyceraldehyde phosphate dehydrogenase (GAPDH) corresponding to bp 557–1258 based on the sequence published by Applequist et al. [20], or rabbit β -actin corresponding to bp 1284–1718 based on the sequence published by Harris et al. [21]. Storage phosphor screens were read with a phosphorimager and quantitatively analyzed using ImageQuANT Software (Molecular Dynamics, Krefeld, Germany).

2.6. Preparation of nuclear extracts

Nuclear extracts were prepared as described by Schreiber et al. [22] from 7×10^6 rabbit peritoneal macrophages before and 3, 5 or 20 h after adhesion of the cells to 10-cm culture dishes. Briefly, the cells were washed and scraped into ice cold PBS and resuspended in 250 μ l ice-cold buffer B (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% protease inhibitor cocktail, 0.4% NP40). Protease inhibitor cocktail contained in 1 ml: 500 μ g antipain, 500 μ g aprotinin, 500 μ g leupeptin, 50 μ g pepstatin, 750 μ g bestatin, 400 μ g phosphoramidon, 500 μ g trypsin inhibitor (all obtained from Boehringer Mannheim, Germany). The cell suspension was incubated on ice for 10 min in order to allow lysis of the cells, then centrifuged at $12\,000 \times g$ for 5 min (4°C). The pellet was resuspended carefully in buffer B and centrifuged again at $12\,000 \times g$ for 5 min (4°C). The nuclear pellet was resuspended in 100 μ l buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 1% protease inhibitor cocktail) and rocked vigorously on a shaking platform for 15 min. After centrifugation at $12\,000 \times g$ for 5 min (4°C) the supernatant containing the nuclear extracts was frozen at -70°C . Protein content of the nuclear extracts was determined using the Bradford assay (Coomassie Plus, Pierce).

2.7. DNA binding assay

Double-stranded AP-1 consensus oligonucleotide was labeled with ^{32}P according to the recommendations of the supplier (Promega, Madison, USA). Nuclear protein (0.8 μ g) was incubated on ice for 30 min with 50 000 cpm of the labeled AP-1 consensus oligo. Free AP-1 was separated by electrophoresis on a 5% acrylamide–bisacrylamide gel at 4°C . The gels were dried, exposed to storage phosphor screens, read in a phosphorimager (Molecular Dynamics, Krefeld, Germany) and quantified using ImageQuANT Software.

3. Results

We found a four-fold increase in the activity of transcription factor AP-1 in freshly isolated rabbit peritoneal macrophages during the first 5 h of adhesion to culture dishes. This up-regulation of AP-1 activity was attenuated (50%) in the presence of 100 μM α -tocopherol (Fig. 1). In adherent macrophages, 24 h after isolation, we observed a marked concentration-dependent decrease of AP-1 activity in the presence of α -tocopherol. In contrast, β -, γ - and δ -tocopherol had only little or no effect on AP-1 activity (Fig. 2).

In parallel with the data obtained for AP-1, we found an increase of SR-A mRNA expression during the first 24 h of adhesion of the macrophages, which was attenuated in the presence of 100 μM α -tocopherol (data not shown). The down-regulation of SR-A expression in the presence of α -tocopherol was reproducible, as shown in Fig. 3. Interestingly, β -tocopherol also reduced SR-A expression in macrophages, although it had a much lower effect on AP-1 inhibition than α -tocopherol. In contrast, γ -tocopherol had only little effect on both SR-A expression and AP-1 activity (Fig. 3).

The α -tocopherol-mediated reduction of SR-A mRNA expression was confirmed by a comparable down-regulation of scavenger receptor activity, as measured by uptake of DiI-acLDL (Fig. 4). Scavenger receptor activity in macrophages was significantly reduced in the presence of α - and β -tocopherol, but there was only a weak and not significant effect in the presence of γ -tocopherol.

In separate experiments, we studied if the reduction of scavenger receptor activity in rabbit macrophages

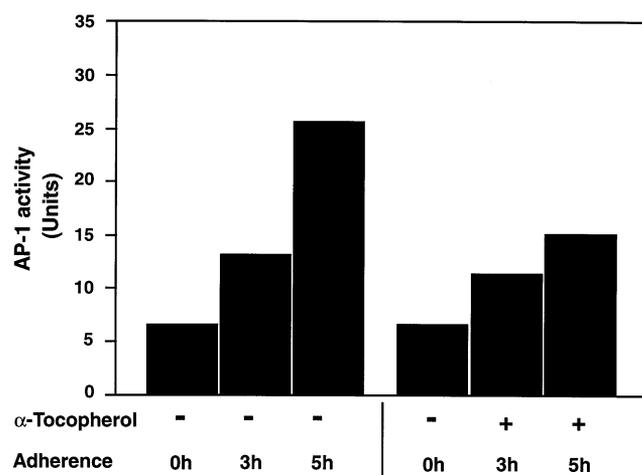


Fig. 1. α -Tocopherol reduces the time-dependent activation of AP-1 in rabbit peritoneal macrophages. AP-1 activity was analyzed by electrophoretic mobility shift assay in freshly isolated cells and 3 and 5 h after adhesion to culture dishes in the presence and absence of α -tocopherol (100 μM). The quantitation of AP-1 activity was performed by phosphorimage analysis and expressed in units of intensity.

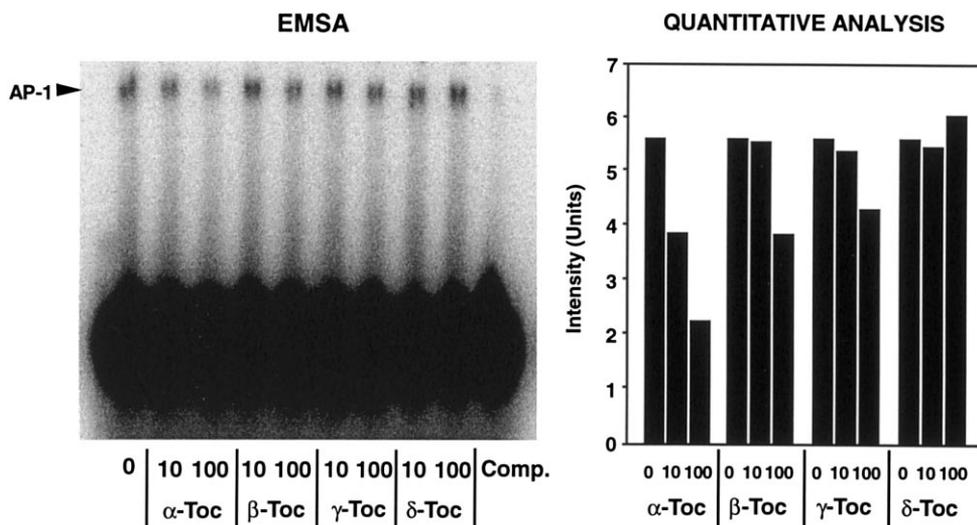


Fig. 2. Concentration-dependent effect of tocopherol homologues (10 and 100 μ M, 20 h) on AP-1 binding transcription factors in rabbit peritoneal macrophages, analyzed by electrophoretic mobility shift assay. The specificity of binding was determined in the presence of an excess of unlabeled AP-1 as a competitor (Comp.).

follows a concentration-dependent manner. As shown in Fig. 5, we found a significant, concentration-dependent decrease of DiI-acLDL uptake of -13 , -16 , -18 and -24% after preincubation with α -tocopherol (1, 5, 10, 50 μ M, respectively) for 20 h as compared to control.

In an additional set of experiments we studied, if the down-regulation of scavenger receptor activity in rabbit macrophages by α -tocopherol could also be seen in human monocytes/macrophages isolated from blood of healthy donors. As shown in Fig. 6, scavenger receptor activity was low in human monocyte derived macrophages after 8 days in culture and α -tocopherol had no significant effect at this time point. However, after 15 days in culture, there was a substantial increase in scavenger receptor activity, which was 60% lower in the α -tocopherol treated cells as compared with the controls (Fig. 6).

4. Discussion

The present study demonstrates for the first time that SR activity can be down-regulated by α -tocopherol in rabbit macrophages and human blood monocyte derived macrophages in vitro. This observation is supported by a decrease of SR-A mRNA expression in the presence of α -tocopherol.

Macrophage SR-activity is up-regulated during monocyte to macrophage differentiation [23]. Transcriptional activation of the scavenger receptor gene in macrophages was found to be mediated by AP-1 and cooperating ets-domain transcription factors [24,25]. On the basis of our observations that α -tocopherol can

down-regulate scavenger receptor activity in macrophages, we hypothesized that α -tocopherol may inhibit or delay AP-1-mediated transcriptional activation in these cells. This speculation was supported by the recent observation from Azzi et al. [26], that α -tocopherol can reduce transcriptional activation in response to phorbol 12-myristate 13-acetate in rat smooth muscle cells.

In the present paper we demonstrated that α -tocopherol reduced the activation of AP-1 during the adherence of freshly isolated rabbit macrophages. This finding points to the idea that α -tocopherol affected the activation of macrophage specific genes. The ability of α -tocopherol to inhibit gene expression was recently demonstrated for interleukin (IL) 1 mediated E-selectin expression in endothelial cells [27] and the secretion of IL-1 β from activated monocytes [28].

Chatelain et al. speculated, that the observed difference in the antiproliferative activity of tocopherol homologues in rat smooth muscle cells may be independent from their antioxidant effects and rather due to an inhibition of protein kinase C (PKC) activity [29]. However, Tasinato et al. reported recently that α -tocopherol does not act by binding to PKC directly but presumably by preventing PKC activation [11].

In order to learn if the effect of α -tocopherol is related to its antioxidative properties or to other mechanisms, we incubated macrophages with β -, γ - and δ -tocopherol, which are homologues of α -tocopherol. Chemically, the four tocopherols differ only in the presence and position of two methyl groups and have a comparable antioxidative capacity. In contrast to α -tocopherol, incubation of macrophages with γ -tocopherol did not result in a significant down-regulation of scav-

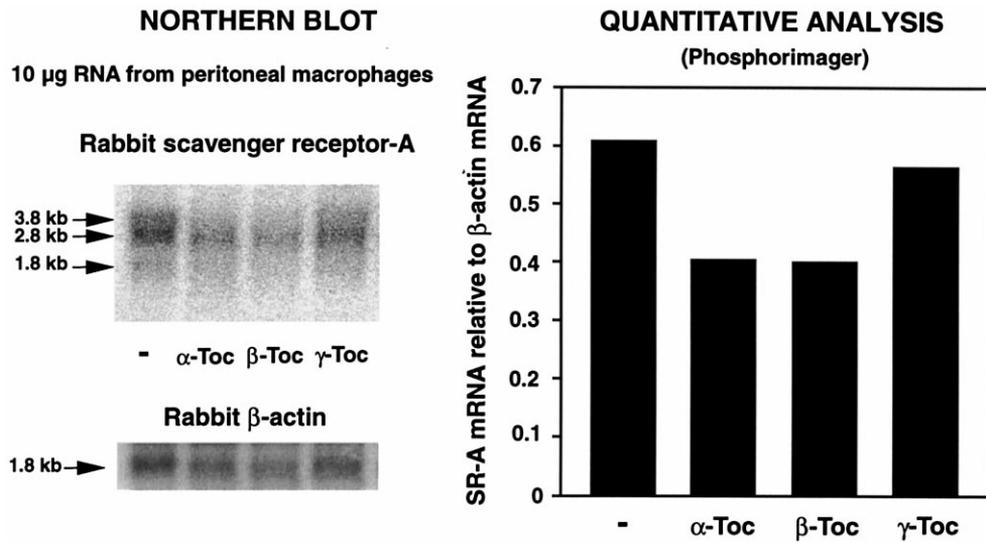


Fig. 3. Effects of α -, β - and γ -tocopherol (50 μ M, 20 h) on scavenger receptor A expression in rabbit peritoneal macrophages. Rabbit SR-A mRNA was quantified by phosphorimage analysis and normalized to β -actin mRNA.

enger receptor activity. AP-1 activity was reduced in the presence of α -tocopherol, but not by the other tocopherols. These data point to the conclusion that the down-regulation of SR activity in presence of α -tocopherol is possibly related to its direct action on cell signaling. However, β -tocopherol had a comparable effect on scavenger receptor expression as α -tocopherol but did not affect AP-1 activity. This observation indicates that, in addition to AP-1, other transcription factors may be involved in the tocopherol-mediated reduction of scavenger receptor activity.

Could the observed down-regulation of macrophage scavenger receptor activity by α -tocopherol play a role in the prevention of atherosclerosis? At present, the biological functions of class A scavenger receptors are not clear: SRs mediate cellular uptake of modified lipoproteins, which is assumed to be involved in foam cell formation [13]. The data from a recent study performed with scavenger receptor knockout mice with an apolipoprotein E (ApoE) knockout background point in this direction: SR-A and ApoE double knockout mice developed less atherosclerosis than the ApoE knockout controls [30]. On the other hand, SRs have also been implicated in the initiation of immunity and host defense because of their ability to bind a wide variety of pathogens [14].

Our data show that α -tocopherol down-regulates SR-A activity even at concentrations which are comparable to those in plasma from normal individuals [31]. However, depletion of α -tocopherol may occur locally in the vessel wall as a consequence of oxidative stress. Such a condition could be associated with an increase of scavenger receptor activity of vessel wall cells. We conclude that down-regulation of SR activity in macrophages by α -tocopherol may avoid overloading of macrophages

with oxLDL. In parallel, α -tocopherol increases the antioxidative capacity of LDL. Therefore we speculate that both the enhanced resistance of LDL against oxidation and the down-regulation of scavenger receptors by α -tocopherol may have a beneficial effect in the prevention of atherosclerosis.

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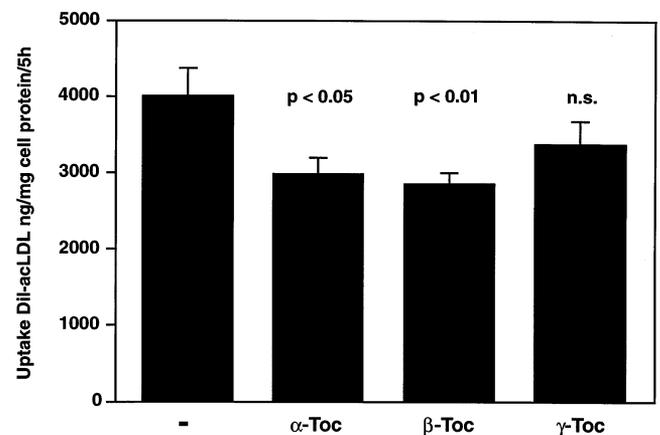


Fig. 4. Effects of α -, β - and γ -tocopherol (50 μ M, 20 h) on scavenger receptor activity in rabbit peritoneal macrophages as determined by quantitative uptake of DiI-acLDL (10 μ g/ml, 5 h). Values are mean \pm S.D. of triplicates and expressed in ng DiI-acLDL per mg cell protein, 5 h.

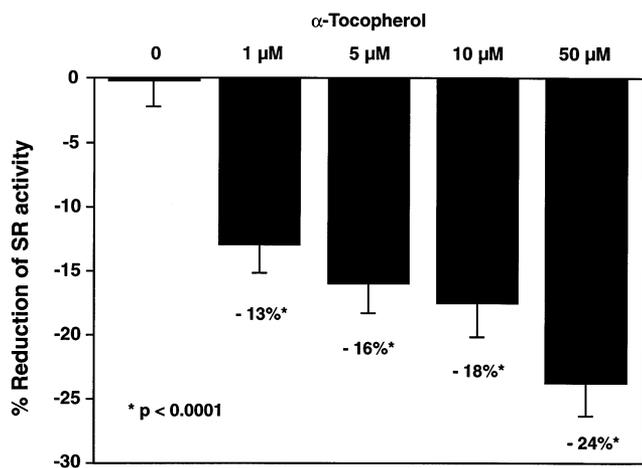


Fig. 5. Concentration dependent down-regulation of scavenger receptor activity in rabbit peritoneal macrophages after incubation with 1, 5, 10 and 50 μM α -tocopherol for 20 h. Scavenger receptor activity was determined by quantitative uptake of DiI-acLDL (10 $\mu\text{g}/\text{ml}$, 5 h) and expressed as percent reduction compared with the scavenger receptor activity in macrophages incubated without α -tocopherol. Values are mean \pm S.D. of quadruplicate dishes.

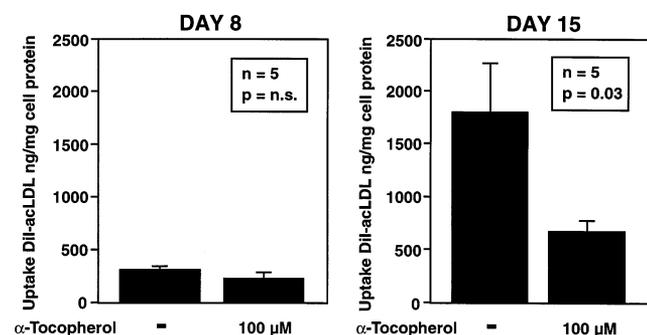


Fig. 6. Scavenger receptor activity in human monocyte derived macrophages from five normal subjects, cultivated for 8 and 15 days in the presence (100 μM) and absence of α -tocopherol (mean \pm S.D.). Scavenger receptor activity was determined by quantitative uptake of DiI-acLDL (10 $\mu\text{g}/\text{ml}$, 5 h). After 15 days of incubation, scavenger receptor activity was significantly lower ($*P < 0.05$) in cells incubated with α -tocopherol.

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