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Free Radical Biology & Medicine, Vol. 25, No. 2, pp. 229–241, 1998
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0891-5849/98 $19.00 + .00

PII S0891-5849(98)00062-8

Original Contribution

ANTIOXIDANT REGULATION OF PHORBOL ESTER-INDUCED ADHESION OF HUMAN JURKAT T-CELLS TO ENDOTHELIAL CELLS

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(*Received* 7 *October* 1997; *Revised* 16 *January* 1998; *Accepted* 9 *March* 1998)

Abstract—Regulation of adhesion molecule expression and function by reactive oxygen species via specific redox sensitive mechanisms have been reported. The effects of clinically safe antioxidants in the regulation of adhesion molecule expression in human endothelial cells (ECV), and adherence of human Jurkat T cells to ECV cells were investigated. The thiol antioxidant, α-lipoate, at clinically relevant doses down-regulated phorbol 12-myristate 13-acetate (PMA)-induced adhesion molecule expression and cell-cell adhesion. Inhibition of PMA-induced ICAM-1 and VCAM-1 expression as well as PMA-induced adhesion of Jurkat T-cells to ECV cells by α-lipoate was dose dependent (50–250 μM). The effect was significant for ICAM-1 ($p < .01$) and VCAM-1 ($p < .01$) expression in cells pretreated with 100 μM α-lipoate compared to PMA-activated untreated cells. Inhibition of PMA-induced adhesion molecule expression and cell-cell adhesion was more pronounced when a combination of antioxidants, α-lipoate and α-tocopherol, were used compared to the use of either of these antioxidant alone. The regulation of adhesion molecule expression and function by low concentration of antioxidants investigated does not appear to be NF-κB regulated or transcription dependent because no change in the mRNA response was observed. Protein kinase C (PKC) has been suggested to regulate PMA-induced adhesion molecule expression by post-transcriptional stabilization of adhesion molecule mRNA. α-Lipoate pretreatment did not influence the response of PKC activity to PMA. Oxidants are known to be involved in the regulation of cell adhesion processes. Treatment of ECV cells with PMA induced generation of intracellular oxidants. α-Lipoate (100 or 250 μM) treatment decreased PMA-induced generation of intracellular oxidants. The inhibitory effect of low concentration of α-lipaote alone or in combination with α-tocopherol on agonist-induced adhesion processes observed in this study may be of potential therapeutic value. © 1998 Elsevier Science Inc.

Keywords—Adhesion molecules, Gene regulation, Free radicals, Redox, Thiols, Signal transduction

INTRODUCTION

Cell-cell adhesion is critical in the generation of effective immune responses and is dependent upon the expression of a variety of cell surface receptors.[1] Intercellular adhesion molecule-1 (ICAM-1, CD54) and vascular cell adhesion molecule-1 (VCAM-1, CD 106) are inducible cell surface glycoproteins.[2] The expression of these surface proteins are known to be induced in response to activators such as cytokines (tumor necrosis factor-α, TNF-α; interleukin-1α & β, IL-1 α & β), phorbol 12-myristate 13-acetate (PMA), lipopolysaccharide and oxidants.[2] The ligands for ICAM-1 and VCAM-1 on lymphocyte are lymphocyte function-associated antigen-1 (LFA-1, CD11a/CD18) and very late antigens-4 (VLA-4), respectively.[2] The inappropriate or abnormal sequestration of leukocytes at specific sites is a central component in the development of a variety of autoimmune diseases and pathologic inflammatory disorders. Focal expression of ICAM-1 have been reported in arterial endothelium overlying early foam cell lesions in both dietary and genetic models of atherosclerosis in rabbits.[3] A role of VCAM-1 in the progression of coronary lesions has also been suggested.[4] Loss or gain of cell surface molecules is thought to determine the mobilization, emigration and invasiveness of epithelial cancer cells.[5] These studies indicated that changes in the adhesive properties of solid tumor cells, such as neo-expression of ICAM-1 or MUC18, are important determinants of the metastatic capability of individual malignant cells.[5]

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Monocytes from patients with *diabetes mellitus* are known to have increased adhesion to endothelial cells in culture.[6]

Therapeutic agents that block leukocyte-endothelial interactions have been reported to have marked effects on the progression of inflammatory responses in a number of in vivo models.[2] Regulation of both ICAM-1 and VCAM-1 gene expression has been related to oxidative stress through specific reduction-oxidation (redox) sensitive transcriptional or posttranscriptional factors.[7,8] Thus, antioxidants have been suggested to have therapeutic potential in pathologies related to changes in cellular adhesion. Thiol antioxidants such as pyrrolidine dithiocarbamate (PDTC) and *N*-acetylcysteine (NAC) have been reported to effectively inhibit expression of adhesion molecules induced by cytokines or oxidants.[7–13] The concentration of NAC used in these studies were in millimolar range, however. Such levels are much higher than that of the reported values (25 μM) that have been achieved in plasma after supplementation of NAC to humans.[14] The other thiol modulating agent studied, PDTC, to our knowledge has never been tested for safe human use.

Recent studies have highlighted considerable interest in the therapeutic value of the metabolic thiol antioxidant α-lipoic acid or thioctic acid (1,2 dithiolane-3-pentanoic acid) especially with regard to pathologies that are associated with redox imbalances.[15,16] α-Lipoic acid has been used safely for a long time in Germany to treat complications associated with diabetes.[17] Recently, α-lipoic acid has also been introduced to the United States as dietary supplement. Apart from the essential role of α-lipoic acid as a co-factor in oxidative metabolism, much of the current interest has been centered on the antioxidant and redox properties of exogenously supplied α-lipoic acid which is a free, and non protein bound compound.[15,16] Exogenously supplied α-lipoic acid is readily taken up by a variety of cells and tissues where it is rapidly reduced by NADH or NADPH dependent enzymes to a potent antioxidant and reducing agent dihydrolipoate (6,8 dithiooctanoic acid, DHLA).[18,19] Both α-lipoate and its reduced form DHLA readily scavenge a variety of reactive oxygen species including hypochlorous acid, and hydroxyl and singlet oxygen radicals and DHLA additionally scavenges superoxide and peroxyl radicals.[15] With a standard reduction potential of -0.32 V for the DHLA/α-lipoic acid couple, DHLA is a strong biological reductant that may chemically reduce glutathione disulfide (GSSG) to glutathione (GSH), the redox potential of the GSH/GSSG couple being -0.24 V.[15] Treatment of human Jurkat T cells or peripheral blood lymphocytes with α-lipoic acid has been reported to increase the intracellular glutathione levels in these cells by increasing the bioavailability of cysteine.[20,21] α-Lipoate has also been reported to suppress nuclear factor-κB (NF-κB) activation induced by oxidants, PMA or TNF-α in a number of cells.[22] The redox sensitive transcription factor NF-κB is a pleiotropic regulator of many genes involved in immune and inflammatory responses, including ICAM-1 and VCAM-1.[23]

In the present study, we investigated the regulation of and mechanisms underlying phorbol ester-induced (i) expression of ICAM-1 and VCAM-1 by antioxidants in human endothelial cell line, and (ii) adhesion of lymphocyte to endothelial cells by thiol and phenolic antioxidants *e.g.* α-lipoate and α-tocopherol. A major emphasis of the study was to explore the possible effects of these antioxidants on cell adhesion at low concentrations that are clinically achievable. Various antioxidants are known to act in co-operation such that the oxidized form of one antioxidant is recycled to the potent reduced form by another antioxidant.[24] α-Lipoate has been postulated to interact through its reduced form DHLA in an antioxidant network where it recycles the oxidized form of major antioxidants such as vitamin C (ascorbate) and E (α-tocopherol).[15] Thus, we investigated whether a combination of antioxidants, α-lipoate and α-tocopherol, is more effective in the regulation of agonist-induced cellular adherence compared to the treatment of single antioxidant.

MATERIALS AND METHODS

Cells and cell culture

Human Jurkat T cells clone E6-1 (American Type Culture Collection, ATCC, Bethesda, MD) were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin, 110 mg/L sodium pyruvate and 2 mM L-glutamine (University of California, San Francisco, CA). ECV304 (ECV), a spontaneously transformed immortal endothelial cell line established from the vein of an apparently normal human umbilical cord, were obtained from ATCC (Bethesda, MD). ECV cells were grown in medium 199 supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were maintained in a standard culture incubator with humidified air containing 5% CO_2 at 37°C.

α-Lipoate (racemate mixture, ASTA Medica, Frankfurt, Germany) stock solution for ECV pretreatment was prepared fresh in alkaline phosphate buffered saline (PBS) and pH was adjusted to 7.4. Stock solution of α-tocopherol (Sigma, St. Louis, MO) was prepared fresh in dimetyl sulfoxide (DMSO) at concentrations such that the final concentration of the solvent in cell suspension never exceeded 0.1%. Respective controls were treated with equal volume of DMSO.

Expression of adhesion molecules

ECV monolayers were pretreated with antioxidants for the time period indicated in the respective figure legends. Following the pretreatment, cells were activated using 100 nM PMA for 24 h. To study ICAM-1 expression, cells were incubated with fluorescein isothiocyanate (FITC) coupled to ICAM-1 monoclonal antibody (Immunotech, Cedex, France) for 30 min at 4°C. Cells were washed twice in dulbecco's PBS, pH 7.4 (D-PBS) and finally resuspended in fresh D-PBS. Expression of ICAM-1 was immediately assayed with a flow cytometer as described in the following section. For VCAM-1 expression, the cells were incubated with a monoclonal VCAM-1 antibody (Immunotech, Cedex, France) for 30 min at 4°C. The cells were washed twice with D-PBS and incubated with FITC-labeled goat anti-mouse IgG monoclonal antibody (Coulter, Miami, FL). The cells were washed twice with D-PBS and analyzed by flow cytometry. Appropriate isotypic controls were used for background fluorescence in ICAM-1 and VCAM-1 assay.

Flow cytometric analyses. The fluorescence and light scattering properties (forward scatter, FS; side scatter, SS) of the cells were determined by using an EPICS-Elite (Coulter, Miami, FL) flow cytometer. Cells with FITC-conjugated antibodies were excited using a 488 nm argon ion laser and emission of FITC was recorded at 525 nm. In each sample, at least 10,000 gated viable cells were examined. A logarithmic scale was used to measure both background and endothelial cell fluorescence. Background fluorescence was then subtracted from endothelial cell fluorescence, allowing linear comparisons of ICAM-1 and VCAM-1 expression between various samples.

Adhesion of Jurkat T-cells to ECV cells

Calcein labeling of cells. Calcein acetoxymethyl ester (calcein-AM, Molecular Probes, Eugene, OR) was used to fluorescently label Jurkat T-cells. Non-fluorescent calcein-AM is lipophillic and is cleaved by intracellular esterases to yield highly charged fluorescent calcein that is retained by viable cells. The excitation and emission wavelengths for calcein molecule are 480 and 530 nm, respectively. The fluorescence labeling of Jurkat T-cells was achieved by incubating cells (1×10^7 cells/ml) with 5 μM calcein-AM in RPMI 1640 for 30 min at 37°C and 5% CO_2. The calcein-AM was prepared as a 1 mM stock in DMSO and stored at −20°C. After loading of calcein-AM, cells were washed thrice with PBS to remove excess dye. Cell viability, as detected by trypan blue exclusion, was >95% up to 3 h after loading. Cells were finally resuspended in phenol red free RPMI 1640 containing 10% fetal calf serum at a density of 2×10^6 cells/ml.

Cell-cell adhesion assay. Monolayers of ECV were seeded at a density of 10^4 cells/well in 96 well tissue culture plates (Falcon 3072, Becton Dickinson, Franklin Lakes, NJ). After 24 h of seeding, the cells were activated with 100 nM PMA for 24 h. Before cell-cell adhesion assay, the ECV monolayers were washed thrice with PBS. Calcein-AM labeled Jurkat T-cells (2×10^5 cells/well) were co-cultured with ECV monolayer for 1 h in a culture incubator with humidified air containing 5% CO_2 at 37°C. Blank wells with ECV monolayer alone were maintained in final Jurkat T-cell suspension medium. After the co-culture period, the non-adherent Jurkat T-cells were removed from monolayers by washing each well four times with D-PBS using a multichannel pipette with wide open tips. The fluorescence intensity of each well was measured using a fluorescence plate reader (CS-9301, Shimadzu, Corporation, Columbia, MD). The relative contributions of ICAM-1 and VCAM-1 to the adherence of Jurkat T-cells to ECV was investigated by treating ECV with anti-human ICAM-1 (10 μg/ml) or anti-human VCAM-1 (10 μg/ml) antibodies for 30 min prior to co-culture of ECV with Jurkat T-cells.

Determination of protein kinase C activity

ECV cells were treated with 100 nM PMA for 1 h to obtain maximum activation of protein kinase C (PKC). Following such activation the cells were washed twice with D-PBS and resuspended in reaction buffer (5.2 mM $MgCl_2$, 94 mM KCl, 12.5 mM HEPES, 12.5 mM EGTA, and 8.2 mM $CaCl_2$, pH 7.4) into 200 μl portions (0.1×10^6 cells). The measurement of PKC activity was started by addition of streptolysin-O (0.3 IU, Sigma, St. Louis MO) mixed with [γ-^{32}P] ATP (300-450 cpm/pmol, final concentration 250 μM, DuPont, NEN, Boston, MA), and 100 μM peptide substrate (Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ala-Ala-Lys-Lys, Sigma, St. Louis MO). After 10 min incubation at 37°C, the reaction was stopped by addition of 100 μl of 25% trichloroacetic acid in 2M acetic acid. The samples were left on ice for 10 min and then centrifuged to remove the proteins. Samples (25 μl each) were then spotted on phosphocellulose disc papers (Gibco BRL, Gaithersburg, MD). The disc papers were washed twice with 1% phosphoric acid, and twice with deionized water. The total radioactivity on each disc was determined using liquid scintillation analyzer. The background phosphorylation in the absence of the substrate peptide was subtracted from all samples.[25,26] To determine the specificity of the obtained results for PKC,

additional measurement of the enzyme activity was done using a pseudosubstrate PKC peptide inhibitor (Arg-Phe-Ala-Arg-Lys-Gly-Ala-Leu-Arg-Gln-Lys-Asn-Val-His-Glu-Val-Lys-Asn; Sigma, St. Louis, MO) as reported previously.[25]

Nuclear extraction and electrophoretic mobility shift assay (EMSA)

ECV cells pretreated with antioxidants were activated with PMA (100 nM) for 2 h to obtain maximal NF-κB activation that ranges between 1–2 h. Translocation of NF-κB proteins to the nucleus was assayed using nuclear protein extracts. Nuclear extracts from ECV cells were prepared from $\sim 1 \times 10^6$ cells as previously described.[27,28] with slight modifications.[29] EMSAs were performed essentially as described earlier.[29,30] Binding reaction mixtures (20 μl) containing 10 μg protein of nuclear extract, 2 μg poly(dI-dC) (Pharmacia, Sweden), ^{32}P-labeled probe (NF-κB consensus oligonucleotide, Promega Inc., WI), 50 mM NaCl, 2 mM $MgCl_2$, 0.2 mM Na_2EDTA, 1 mM DTT, 10% (v/v) glycerol and 4 mM Tris-HCl (pH 7.9) were incubated for 30 min at room temperature. Proteins were separated by electrophoresis in a native 6% polyacrylamide gel using a Tris-borate-EDTA running buffer (12.5 mM Tris-borate containing 0.25 mM Na_2EDTA, pH 8.0), followed by autoradiography. The NF-κB probe (Promega Inc., WI):

5′ . . . AGT TGA GGG GAC TTT CCC AGG C
3′ . . . TCA ACT CCC CTG AAA GGG TCC G

was labeled with [γ-^{32}P]dATP (Du Pont NEN, Boston, MA) using T4 polynucleotide kinase (Promega Inc., WI). The labeled probe was purified using a NAP-5 column (Pharmacia, Sweden) equilibrated with 10 mM Tris-HCl (pH 7.5) containing 100 mM NaCl and 1 mM EDTA and stored in aliquots. For quantification, NF-κB bands on the gel was scanned using the NIH Image 1.58b29 software (NIH, MD).

mRNA determination using reverse transcription (RT)-Polymerase chain reaction (PCR)

Antioxidant pretreated ECV cells were activated with PMA (100 nM, 8 h). The level of mRNA was determined using RT-PCR as described below.

RNA extraction. Total RNA was prepared using guanidium isothiocyanate following the method of Chomczynski and Sacchi.[31] Briefly, endothelial cells were rinsed with ice-cold PBS, and lysed in acid guanidine thiocyanate. The lysate was drawn through a 26-gauge needle and extracted with acid phenol/chloroform (5:1). After 30 min incubation on ice, the mixture was centrifuged for 30 min at $12000 \times g$. The solution of RNA fraction was collected and applied to ethanol-sedimentation, then the RNA pellet was washed twice with 75% ethanol, dried, and dissolved in diethylpyrocarbonate-treated water. Quantification and purity of RNA was assayed by A260/A280 absorption, and RNA samples with ratio greater than 1.9 was used for further analysis.

RT-PCR. RT was performed using a RNA PCR Kit (Perkin Elmer, Branchburg, NJ). One μg of total RNA was reverse transcribed to cDNA following the manufacturer's recommended procedures. RT-generated cDNA encoding human ICAM-1,[32] VCAM-1[32] and GAPDH (glyceraldehyde 3-phosphate dehydrogenase, as a positive control and an internal standard) genes were amplified by PCR using specific primers as described below:

ICAM-1 (upper primer): 5′-TATGGCAACGACTCCT-TCT-3′
ICAM-1 (lower primer): 5′-CATTCAGCGTCACCT-TGG-3′
VCAM- 1 (upper primer): 5′-ATGACATGCTTGAGC-CAGG-3′
VCAM- 1 (lower primer): 5′-GTGTCTCCTTCTTTGA-CACT-3′

The primers were custom synthesized at the University of California-Berkeley, Barker Hall facility. PCR was performed using a RNA PCR-Kit. The reaction volume was 50 μl containing (final concentration): PCR buffer (1×), deoxynucleotide (0.2 mM each), $MgCl_2$ (2 mM), Taq DNA polymerase (2U), oligonucleotide primer (0.5 μM each) and RT products. After an initial denaturation for 2 min at 95°C, 35 cycles of amplification (95°C for 1 min, 58°C for 1 min and 72°C for 1.5 min) were performed followed by 7 min extension at 72°C.

Analysis of PCR products. A 10 μl aliquot from each PCR reaction was electrophoresed in a 1.7% agarose gel containing 0.2 μg/ml ethidium bromide. The gel was then photographed under ultraviolet transillumination. For quantification, PCR bands on the photograph of the gel was scanned using the NIH Image 1.58b29 software (NIH, MD). The ICAM-1 and VCAM-1 signals were normalized relative to the corresponding GAPDH signal from the same sample.

Flow cytometric assay of intracellular peroxides

Intracellular peroxides were detected using dichlorodihydrofluorescein-diacetate (DCFH-DA, Molecular

Probes, Eugene, OR) as described previously.[33] Following treatment of cells or not with α-lipoate and PMA, cells were washed three times with PBS. Cells were then detached from monolayer and resuspended in PBS and incubated with DCFH-DA (25 μM) for 30 min at 37°C. Dichlorofluorescein (DCF) fluorescence was detected using a 488 nm argon ion laser for excitation in a flow cytometer (XL, Coulter, Miami, FL) and the 530 nm emission was recorded in fluorescence channel 1 (FL1). Data were collected from at least 10,000 viable cells.

Statistical analyses

Results are presented as mean ± SD of at least three separate experiments. Difference between means of groups were determined by Student's t-test and one-way analysis of variance. The minimum level of significance was set at $p < 0.05$.

RESULTS

Down-regulation of PMA-induced expression of ICAM-1 and VCAM-1 by α-lipoate

The expression of ICAM-1 and VCAM-1 was assayed in α-lipoate pretreated ECV cells following activation with PMA (100 nM) for 24 h. Constitutive expression of ICAM-1 was very low in ECV cells. The expression was markedly induced in response to PMA treatment for 24 h (Fig. 1). Pretreatment of ECV cells with α-lipoate (100 or 250 μM) for 48 h down-regulated PMA-induced expression of ICAM-1 (Fig. 1). Such down-regulation of PMA-induced ICAM-1 expression by α-lipoate was dose dependent (50–500 μM). The effect was already significant (~50 %, $p < 0.01$) in cells pretreated with 100 μM α-lipoate compared to PMA-activated non-treated cells (Fig. 2). With increasing concentrations (250 or 500 μM) of α-lipoate pretreatment, further decrease in PMA-induced ICAM-1 expression was observed (Fig. 2). Treatment with 50 or 100 μM α-lipoate for 48 or 72 h did not influence the constitutive expression of ICAM-1.

To characterize the kinetics of the inhibitory effect of α-lipoate on PMA-induced expression of ICAM-1, ECV cells were pretreated with 50 or 100 μM α-lipoate for different time periods (24, 48, or 72 h), and then activated with PMA for 24 h. In cells that were pretreated with 50 μM lipoate, PMA-induced ICAM-1 expression remained unchanged up to 48 h (Fig. 3). A significant decrease in the PMA-induced levels of ICAM-1 was observed in cells that were pretreated with 50 μM α-lipoate for 72 h. Following treatment of cells with 100 μM lipoate for 48 or 72 h, a significant decrease in PMA-induced ICAM-1 expression was observed compared to PMA-activated untreated cells (Fig. 3).

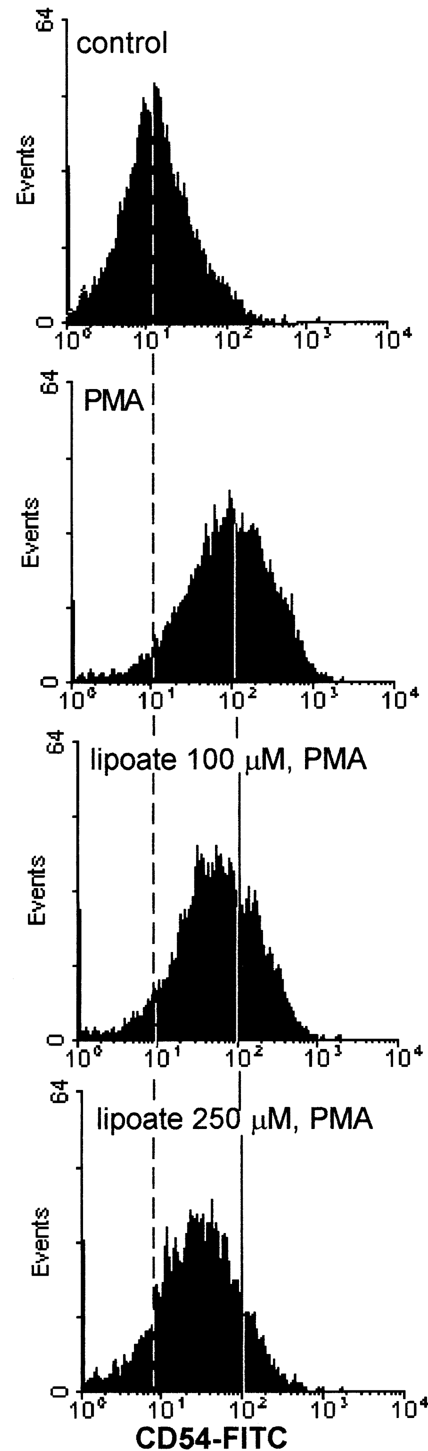


Fig. 1. Down-regulation of PMA-induced ICAM-1 expression in ECV cells by α-lipoate. Flowcytometric analysis of ECV cells pretreated with α-lipoate (100 or 250 μM, 48 h) and then activated with 100 nM PMA (24 h). Hatched line represents the median value of log ICAM-1-FITC signal of control (no PMA) cells and the solid line represents median value of log ICAM-1-FITC signal of cells activated with PMA. Data are given as histograms of cell number versus log-ICAM-1-FITC fluorescence intensity. Histogram represent one of three essentially identical experiments.

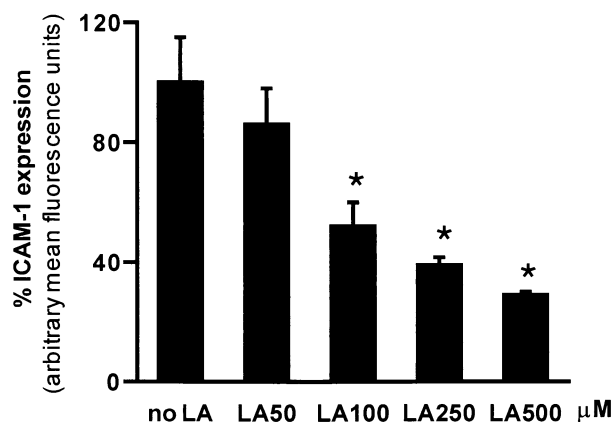


Fig. 2. Dose-relationship of α-lipoate (LA) pretreatment on PMA-induced ICAM-1 expression in human vascular endothelial (ECV) cells. ECV cells were pretreated with α-lipoate (50–500 μM) for 48 h and then activated with 100 nM PMA for 24 h. Data are mean ± SD of at least three experiments. $*p < .01$ when compared with LA non-treated (no LA) cells.

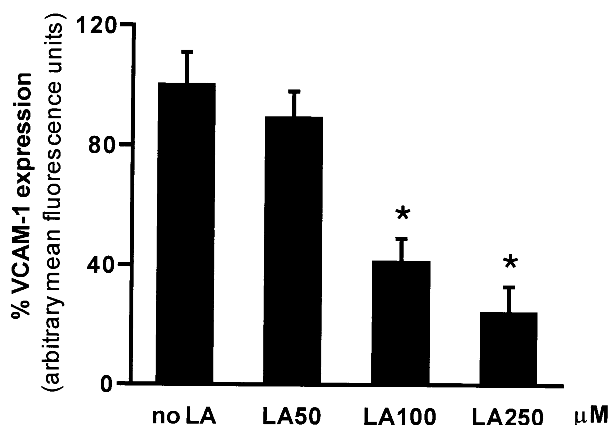


Fig. 4. Dose-relationship of α-lipoate (LA) pretreatment on PMA-induced VCAM-1 expression in human endothelial (ECV) cells. ECV cells were pretreated with α-lipoate (50–250 μM) for 48 h and then activated with 100 nM PMA for 24 h. Data are mean ± SD of at least three experiments. $*p < .01$ when compared with LA non-treated (no LA) cells.

The effect of α-lipoate pretreatment on PMA-induced expression of VCAM-1 in ECV cells was determined (Fig. 4). The constitutive expression of VCAM-1 in ECV cells was observed to be very low. PMA-induced VCAM-1 expression was down-regulated by α-lipoate treatment in a dose dependent manner. A ~60% decrease ($p < .01$) in induced VCAM-1 expression was observed in cells that were pretreated with 100 μM α-lipoate for 48 h compared to the corresponding α-lipoate untreated controls (Fig. 4). Higher concentration (250 μM) α-lipoate pretreatment for 48 h decreased (65%, $p < .01$) PMA-induced VCAM-1 expression compared to the controls (Fig. 4).

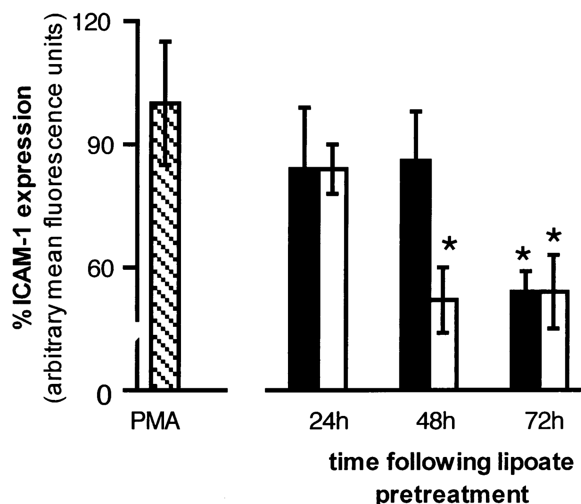


Fig. 3. Effect of α-lipoate (LA) pretreatment time on PMA-induced ICAM-1 expression in human vascular endothelial (ECV) cells. ECV cells were pretreated with α-lipoate (solid bars, 50 μM; open bars, 100 μM) for 24, 48 or 72 h and then activated with 100 nM PMA for 24 h. Data are mean ± SD of at least three experiments. $*p < .01$ when compared with LA non-treated (no LA) cells.

Synergistic inhibitory effect of α-lipoate and α-tocopherol on PMA-induced ICAM-1 expression

ECV cells were either pretreated with α-lipoate or α-tocopherol alone or in combination of these antioxidants for 48 h. The cells were then activated with PMA for 24 h and ICAM-1 expression was studied (Fig. 5a). Pretreatment of ECV with 50 μM α-lipoate or 50 μM α-tocopherol alone for 48 h did not significantly influence PMA-induced ICAM-1 expression (Fig. 5a). However, when cells were pretreated with 50 μM α-lipoate and 50 μM α-tocopherol in combination for 48 h, significant decrease in PMA-induced ICAM-1 expression was observed compared to cells that were treated with either 50 μM α-lipoate or 50 μM α-tocopherol alone (Fig. 5a). Pretreatment of cells with α-lipoate (50 or 100 μM) and 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC, 10 μM), a short phytl chain α-tocopherol derivative, for 48 h was also more effective in inhibiting PMA-induced ICAM-1 expression in ECV cells compared to the pretreatment of cells with either of these antioxidant alone (Fig. 5b).

Antioxidants inhibit PMA-stimulated adhesion of Jurkat T-lymphocytes to ECV endothelial cells

A lymphocyte adhesion assay was performed to verify (i) the functional integrity of the adhesion molecules expressed by ECV following PMA-activation; and (ii) whether the inhibitory effect of lipoate on the expression

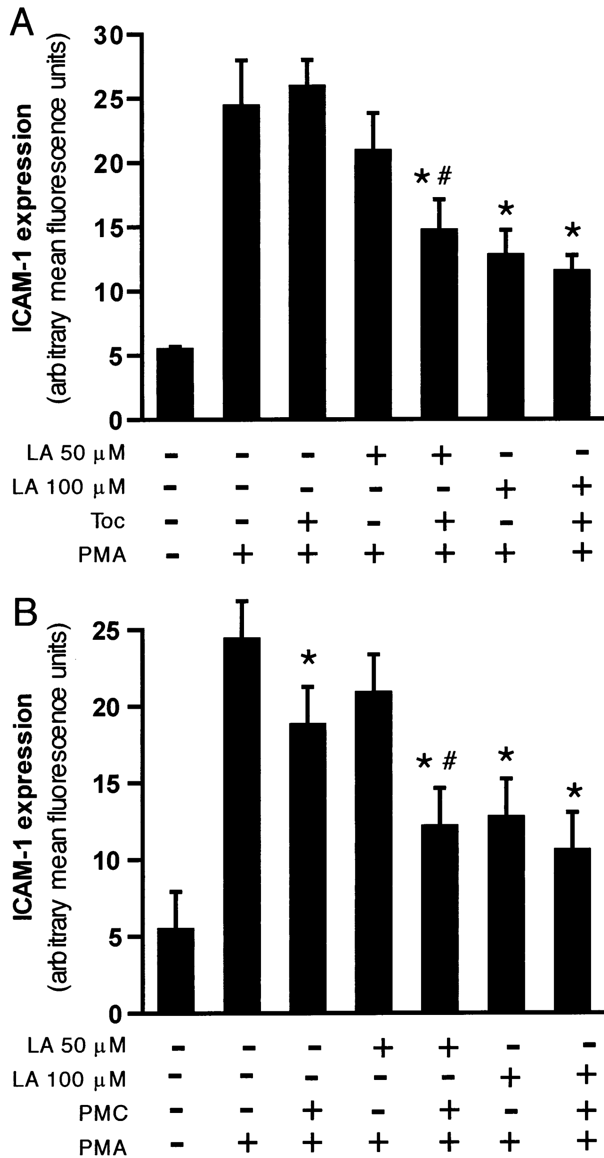


Fig. 5. Synergistic effect of α-lipoate (LA) and α-tocopherol (Toc) or 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC) pretreatment on PMA-induced ICAM-1 expression in human endothelial (ECV) cells. ECV cells were pretreated with α-lipoate (50 or 100 μM) or/and **A)** α-tocopherol (50 μM), **B)** PMC (10 μM) for 48 h and then activated with 100 nM PMA for 24 h. Data are mean $\pm$ SD of at least three experiments. $*p < .05$ when compared with PMA-induced ICAM-1 expression. $\#p < .01$ when compared with PMA-induced ICAM-1 expression in cells pretreated with α-lipoate (50 or 100 μM) alone for 48 h.

of ICAM-1 and VCAM-1 was also effective in down-regulating actual cell to cell adhesion. A significant ($p < .01$) decrease in the adherence of Jurkat T-cells to ECV cells was observed following pretreatment of ECV cells with α-lipoate (100 or 250 μM) for 48 h (Fig. 6). Anti human ICAM-1 antibody treatment of ECV cells markedly (85–90%) blocked PMA-induced Jurkat T-cell adhesion to ECV cells. Anti human VCAM-1 antibody

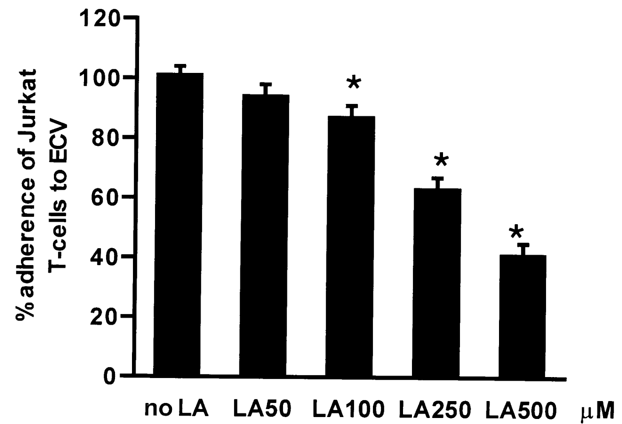
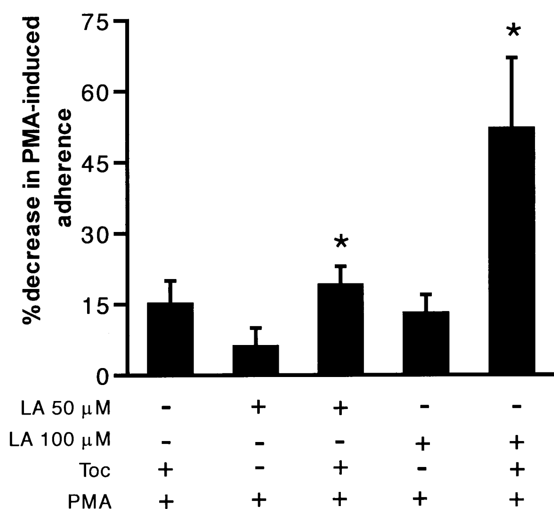


Fig. 6. Adhesion of human Jurkat T-cells to PMA-activated endothelial (ECV) cells is inhibited by α-lipoate (LA) pretreatment. ECV cells were pretreated with α-lipoate (50–500 μM) for 48 h and then activated with 100 nM PMA for 24 h. Cells were washed three times with PBS and then co-cultured with calcein-AM labelled Jurkat T-cells for 1 h. Data are mean $\pm$ SD of at least five experiments. $*p < .01$ when compared with LA non-treated (no LA) PMA-induced adhesion of Jurkat T-cells to ECV.

treatment of ECV cells had no significant effect on the adhesion of Jurkat T-cell to ECV (not shown). Pretreatment of ECV cells with α-lipoate and α-tocopherol in combination for 48 h was more effective in inhibiting PMA-stimulated adherence of Jurkat T-cells to ECV compared to the treatment of cells with lipoate or α-tocopherol alone (Fig. 7a). A marked 50% inhibition of PMA-stimulated adherence of Jurkat T-cells to ECV cells was observed following pretreatment of ECV cells with α-lipoate (100 μM) and α-tocopherol (50 μM) combination compared to the corresponding untreated cells (Fig. 7a). Pretreatment of cells with α-lipoate (50 or 100 μM) and PMC (10 μM) for 48 h was clearly more effective in inhibiting PMA-induced adherence of Jurkat T-cells to ECV compared to the pretreatment of ECV cells with α-lipoate alone (Fig. 7b). Pretreatment of ECV cells with PMC (10 μM) for 48 h was similarly effective in down-regulating PMA-induced ICAM-1 expression compared to cells pretreated with a combination of α-lipoate (50 or 100 μM) and PMC (10 μM) for 48 h (Fig. 7b). Pretreatment of ECV cells with α-lipoate and α-tocopherol in combination for 48 h was more effective in inhibiting PMA-stimulated adherence of Jurkat T-cells to ECV compared to the treatment of cells with α-lipoate and PMC in combination for 48 h (Figs. 7a and b).

Effects of antioxidant pretreatment on PKC activity

Treatment of ECV cells with PMA (100 nM, 1 h) resulted in 2-fold increase in the activity of PKC compared to cells that were not activated with PMA (Fig. 8). Presence of the indicated pseudosubstrate inhibitor pep-



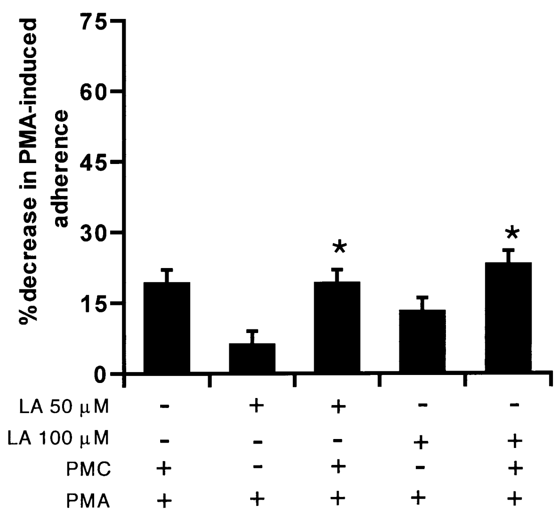


Fig. 7. Synergistic treatment of α-lipoate (LA) and α-tocopherol (Toc) or 2,2,5,7,8-pentamethyl-6-hydroxychromane (PMC) on the adherence of Jurkat T-cells to PMA-activated human endothelial (ECV) cells. ECV cells were pretreated with α-lipoate (50–100 μM) alone or/and **(A)** α-toc (50 μM), **(B)** PMC (10 μM) for 48 h and then activated with 100 nM PMA for 24 h. Cells were washed three times with PBS and then co-cultured with calcein-AM labelled Jurkat T-cells for 1 h. Data are mean $\pm$ SD of five experiments. $*p < .01$ when compared with PMA-induced adhesion of Jurkat T-cells to ECV in cells pretreated with α-lipoate alone for 48 h.

tide in the reaction mixture for the assay consistently inhibited 75% of PMA-induced PKC activity in ECV cells. Pretreatment of ECV cells with 100 μM α-lipoate for 48 h had no effect on constitutive or PMA-induced PKC activity (Fig. 8). Treatment of ECV cells with α-tocopherol alone or in combination with α-lipoate significantly down-regulated PMA-induced PKC activity. No synergistic action of α-tocopherol and α-lipoate

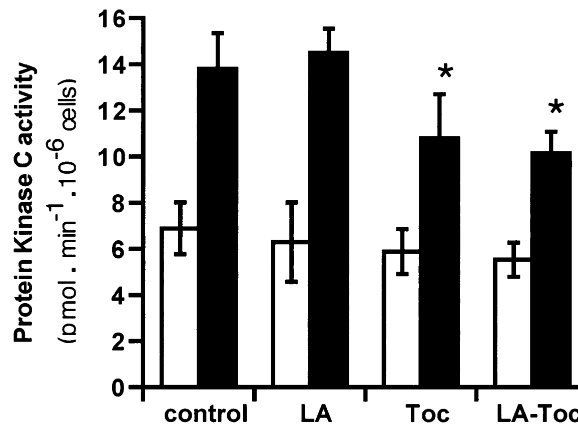


Fig. 8. Effect of α-lipoate (100 μM, 48 h, LA) pretreatment alone or in combination with α-tocopherol (50 μM, 48 h, Toc) on PKC activity in ECV cells either treated with (solid bars) or not (open bars) with PMA. ECV cells were pretreated with α-lipoate or α-tocopherol for 48 h and then activated with PMA (100 nM) for 1 h. Data are mean $\pm$ SD of at least three experiments. $*p < .001$ when compared with PMA-induced PKC activity.

was observed on the inhibition of PMA-induced PKC activity (Figure 8).

Antioxidant regulation of PMA -induced NF-κB activation

Treatment of ECV cells with PMA (100 nM, 2 h) resulted in NF-κB activation (Fig. 9). The peak response of such activation in ECV cells was observed following 1–2 h of PMA treatment (not shown). The specificity of the NF-κB band was identified in a competition assay by treating the nuclear protein of positive control extracts with a 1000 fold excess of cold consensus probe or cold mutant probe (one base pair altered, Santa Cruz Biotechnology Inc.) for 15 min before the actual binding reaction with ^{32}P labeled consensus probe was performed (not shown). Pretreatment of ECV cells with 100 or 250 μM α-lipoate for 48 h partially inhibited PMA-induced NF-κB activation (Fig. 9). A marginally potentiated inhibitory effect was observed when ECV cells were pretreated with 100 or 250 μM α-lipoate in combination with 50 μM α-tocopherol (Fig. 9).

Effect of α-lipoate treatment on PMA-induced ICAM-1 and VCAM-1 mRNA expression

To determine whether the inhibitory effect of α-lipoate pretreatment on PMA-induced ICAM-1 and VCAM-1 expression was at the messenger level of ICAM-1 and VCAM-1 expression, constitutive and PMA (100 nM, 8 h)-induced ICAM-1 and VCAM-1 mRNA expressions were evaluated using RT-PCR. The mRNA levels of cell adhesion molecules (CAM) have

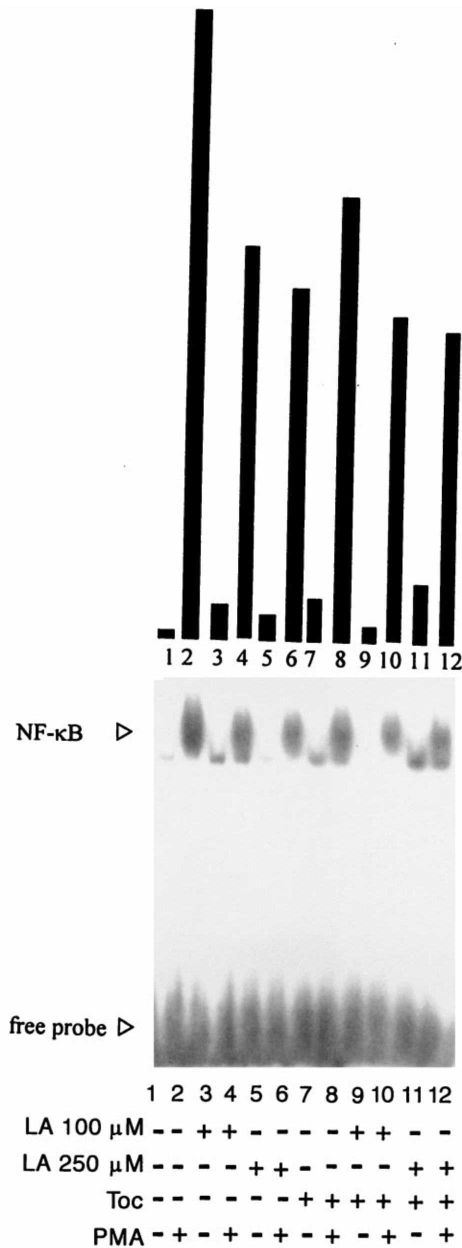


Fig. 9. Effect of antioxidant pretreatment of PMA-induced NF-κB activation in human endothelial (ECV) cells. *Lane 1* (left to right), no treatment; *lane 2*, 100 nM PMA, 2 h; *lane 3*, 100 μM α-lipoate (LA), 48 h; *lane 4*, 100 μM α-lipoate, 48 h, 100 nM PMA, 2 h; *lane 5*, 250 μM α-lipoate, 48 h; *lane 6*, 250 μM α-lipoate, 48 h, 100 nM PMA, 2 h. *lane 7*, 50 μM α-tocopherol (Toc), 48 h; *lane 8*, 50 μM α-tocopherol, 48 h, 100 nM PMA, 2 h; *lane 9*, 100 μM α-lipoate + 50 μM α-tocopherol, 48 h; *lane 10*, 100 μM α-lipoate + 50 μM α-tocopherol, 48 h, 100 nM PMA, 2 h; *lane 11*, 250 μM α-lipoate + 50 μM α-tocopherol, 48 h; *lane 12*, 250 μM α-lipoate + 50 μM α-tocopherol, 48 h, 100 nM PMA, 2 h. Activation of NF-κB was assayed from nuclear extracts of ECV cells using EMSA described in methods.

been reported to be maximum following 8–10 h of PMA treatment,[34] therefore, we studied effect of lipoate on PMA-induced CAM mRNA levels after 8 h. Pretreatment of ECV with 100 μM α-lipoate for 48 h did not influence the level of mRNA transcripts of ICAM-1 in non-stimulated or PMA-stimulated cells (Fig. 10). However, pretreatment of cells with 250 μM α-lipoate for 48 h decreased both constitutive as well as PMA-induced ICAM-1 mRNA expression. No such changes were observed in response to 100 or 250 μM α-lipoate pretreatment on the constitutive or PMA-induced VCAM-1 mRNA expression (not shown). Treatment of ECV cells with α-lipoate (100 μM) and α-tocopherol (50 μM) in combination did not influence PMA-induced ICAM-1 mRNA expression (not shown).

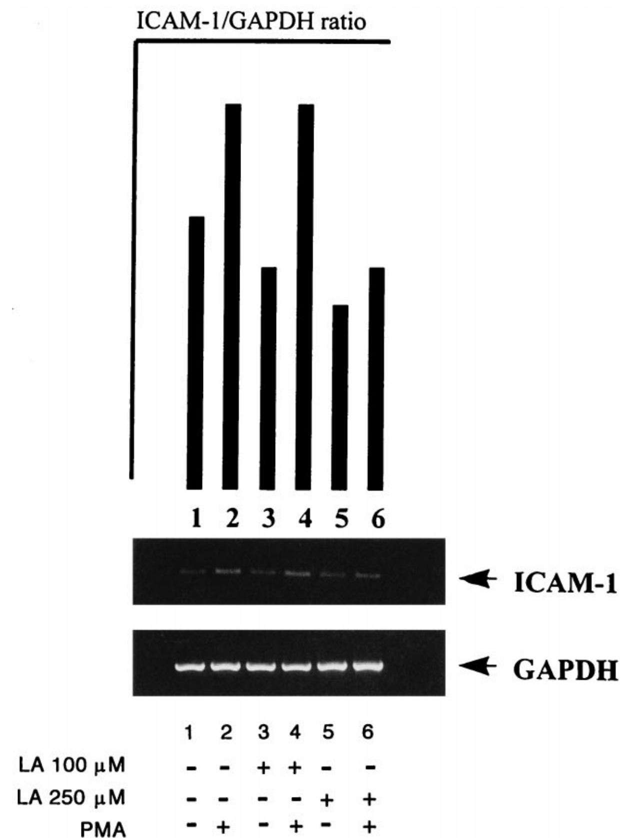


Fig. 10. Effect of α-lipoate (LA) pretreatment on ICAM-1 mRNA expression in PMA-activated human endothelial (ECV) cells following α-lipoate pretreatment. ECV cells were pretreated with α-lipoate (100 or 250 μM, 48 h) and then activated with 100 nM PMA (8 h). *Lane 1*, control (no PMA); *lane 2*, 100 nM PMA, 8 h; *lane 3*, 100 μM α-lipoate, 48 h, no PMA 8 h; *lane 4*, 100 μM α-lipoate, 48 h, 100 nM PMA, 8 h; *lane 5*, 250 μM α-lipoate, 48 h, no PMA 8 h; *lane 6*, 250 μM α-lipoate, 48 h, 100 nM PMA, 8 h. mRNA level was determined using RT-PCR described in methods.

PMA-induced accumulation of intracellular peroxides in ECV cells

PMA (100 nM) treatment increased the level of intracellular peroxides, detected by dichlorofluorescein (DCF) fluorescence, in ECV cells. A significant ($p <$

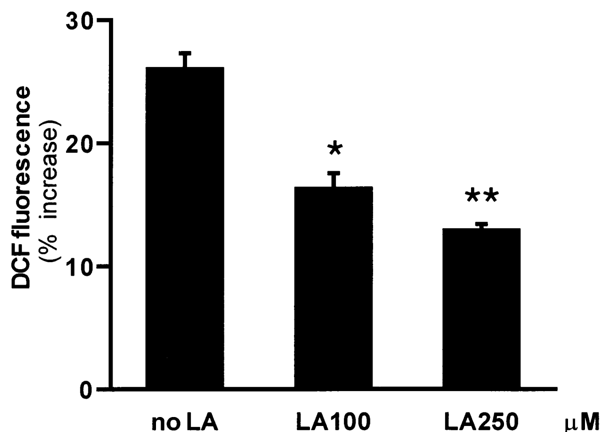


Fig. 11. Intracellular peroxides in response to PMA treatment and the effect of α-lipoate pretreatment. ECV cells were pretreated with α-lipoate (100 or 250 μM) for 48 h and then treated with 100 nM PMA for 6 h. Intracellular peroxides were detected using dichlorodihydrofluorescein (DCFH). Non fluorescent DCFH is converted to highly fluorescent dichlorofluorescein (DCF) following reaction with organic and inorganic peroxides. Following treatment and incubation with α-lipoate and PMA, cells were washed three times with PBS. Cells were then incubated with DCFH for 30 min at 37°C. Fluorescence was read using a Coulter flow cytometer. Data are presented as percentage increase in DCF fluorescence compared to PMA-non treated control cells. Data are mean ± SD of at least three experiments. $*p < .05$; $*p < .001$ when compared with LA non-treated (no LA) PMA-induced DCF fluorescence.

.001) increase in DCF fluorescence was observed in cells that were treated with PMA for 6 h compared to the PMA non-treated cells (Fig. 11). Such increase in intracellular oxidants was maximum following 6 h of PMA treatment. A significant ($p < .05$) decrease in the intracellular oxidant generation in PMA-treated cells was observed following pretreatment of ECV cells with α-lipoate (100 or 250 μM) for 48 h (Fig. 11).

DISCUSSION

Reactive oxygen species (ROS) are known to play an important role in the regulation of cell adhesion.[35,36] Previous studies have suggested that activation of agonist-induced ICAM-1 and VCAM-1 transcription in endothelial cells is dependent, at least in part, on the activation of reduction-oxidation (redox)-sensitive transcription factors NF-κB and AP-1.[8,34] The activation of these transcription factors were inhibited by thiol antioxidants such as NAC, PDTC and α-lipoate.[22] Such observations have led to a number of studies where the role of thiol antioxidants in regulation of cell adhesion has been investigated.

Multifold induction of ICAM-1 expression by PMA and other cytokines have been reported in various tissues and cell lines.[34] In ECV cells, PMA treatment resulted in intracellular oxidation which was followed by markedly increased expression of ICAM-1 and VCAM-1. In this study we report for the first time that clinically relevant at low concentrations (50 and 100 μM) the thiol antioxidant α-lipoate inhibits phorbol ester induced adhesion of Jurkat T-cells to ECV cells, and also suppresses phorbol ester induced expression of ICAM-1 and VCAM-1 in ECV cells. In this study a synergistic effect of α-lipoate and α-tocopherol was clearly evident in inhibiting agonist induced cell-cell adherence.

The thiol antioxidants NAC and PDTC have been studied for their ability to regulate the expression of adhesion molecules and cell-cell adherence. Both of these compounds are known to be able to down-regulate oxidant, cytokine or PMA-induced ICAM-1 and VCAM-1 expression.[7,8,10,11] NAC is a clinically safe drug.[37] However, in all of the studies showing the efficacy of NAC to inhibit agonist-induced ICAM-1 or VCAM-1 expression, high millimolar concentration of the drug was necessary. Pharmacokinetic studies of NAC in humans show that only up to 25 μM of NAC is available in human plasma following oral intake.[14] PDTC, the other thiol antioxidant used widely in these studies, has never been tested safe for human use. α-Lipoate has been safely used for human therapy to treat complications associated with diabetes.[17,38] Pharmacokinetic studies of α-lipoate have shown that following a single orally administered dose (10 mg/kg body wt), the plasma concentration may reach up to 60–70 μM. Higher concentrations of α-lipoate in circulation can be achieved if the drug is administered intravenously.[17] We observed that at clinically relevant concentrations, α-lipoate inhibited PMA-induced ICAM-1 and VCAM-1 expression in ECV cells. Under the treatment conditions used in this study, no cytotoxic effect of α-lipoate was observed (not shown).

Cell-cell adhesion is expected to be mediated by a large number of adhesion molecules some of which are characterized at present. Thus, although study of the expression of individual adhesion molecules are informative, prediction of actual cell-cell adhesion based on such data may not always be correct. This is clearly evident in our own data where although the combination of PMC and α-lipoate decreased PMA induced ICAM-1 expression similar to the combination of α-tocopherol and α-lipoate, the latter combination had a much more marked effect in decreasing actual cell-cell adhesion than the combination of PMC and α-lipoate. Thus, the assay of actual cell to cell adhesion is a very meaningful parameter that needs to be directly monitored. Our results show that pretreatment of ECV cells with α-lipoate and/or the other antioxidants studied clearly decreased PMA-induced adhesion of Jurkat T-cells to ECV. Thiol antioxidants such as PDTC (50–100 μM) and NAC (20 mM) have been shown to inhibit agonist-induced adhe-

sion of leukocytes to endothelial cells.[8,12,39] In addition to thiol antioxidants other antioxidants such as α-tocopherol, probucol and ascorbate have also been reported to regulate cellular adhesion processes.[39,40]

Antioxidants have been suggested to function in a network in which oxidized antioxidants are recycled by other antioxidants to their respective reduced potent state.[24] α-Lipoate has been postulated to interact through its reduced form DHLA in an antioxidant network where it recycles the oxidized forms of major antioxidants vitamin C and E.[15] Our hypothesis that combination of antioxidant may be more effective than the use of single antioxidant in the regulation of cellular adhesion was validated by the observation that combined use of α-lipoate and α-tocopherol was more effective in the down-regulation of PMA-activated ICAM-1 expression, as well as cellular adhesion compared to the use of α-lipoate or α-tocopherol alone. PMC, a short chain derivative of α-tocopherol and a potent inhibitor of TNF-α induced activation of NF-κB,[41] was also found to be effective in inhibiting PMA-induced expression of ICAM-1 when used in combination with α-lipoate compared to the use of α-lipoate or PMC alone. Although PMC acted synergistically with α-lipoate to inhibit PMA-induced ICAM-1 expression no such synergistic effect was observed in results obtained from the determination of cell-cell adhesion. A similar effect of the α-lipoate/α-tocopherol combination and α-lipoate/PMC combination on PMA-induced ICAM-1 expression, and increased efficacy of the former combination than the latter to inhibit PMA-induced cell-cell adhesion suggests that the two phenolic antioxidants α-tocopherol and PMC might act through different pathways.

The molecular mechanisms underlying the inhibitory action of antioxidants on cell adhesion processes is not well understood. Although it has been suggested that reactive oxygen species may act via increasing the expression of adhesion molecules, other pathways of action may not be ruled out.[36] In the current experimental model, PMA treatment increased the level of intracellular peroxides and antioxidant pretreatment of cells clearly decreased the level of peroxides in PMA treated cells. This direct effect of antioxidants on the state of intracellular peroxides in PMA treated cells may represent an important mechanism of antioxidant action.

NF-κB is a ubiquitously expressed multi-subunit transcription factor that is involved, together with other transcriptional activators, in the regulation of various adhesion molecules such as ICAM-1, VCAM-1 and E-selectin.[23] Thiols such as PDTC, NAC (millimolar range) and α-lipoate have been shown to inhibit NF-κB activated by diverse stimuli such as TNF-α, IL-1β, PMA or hydrogen peroxide.[22] Antioxidant treatment partially inhibited NF-κB activation by PMA in ECV cells. However, such inhibition may not be a primary mechanism by which antioxidants at low concentration used in present study inhibited PMA-induced adhesion molecule expression and cell adhesion. In experiments using RT-PCR detection of mRNA of adhesion molecules, we did not observe any significant change in PMA-activated mRNA levels by α-lipoate treatment at low concentration. Following PMA treatment, involvement of PKC to up-regulate ICAM-1 expression at a post-transcriptional level by stabilizing ICAM-1 mRNA has been suggested.[42] We examined the activity of PKC following PMA activation in ECV cells treated with antioxidants. No effect of low dose of α-lipoate pretreatment to ECV on PMA-induced PKC activity suggests that the inhibitory effect of lipoate on adhesion molecule expression is not mediated by PKC dependent post transcriptional regulation. We observed that α-tocopherol treatment of ECV cells down-regulated PMA-induced PKC activity in ECV cells. Similar effects of α-tocopherol on PMA-induced PKC activity have been previously reported in smooth muscle cells.[26,43]

Transcriptional regulation of ICAM-1 and VCAM-1 gene expression is complex and may involve the interaction of a number of transcription factors.[23] Higher concentration of α-lipoate (250 μM) pretreatment decreased ICAM-1 mRNA level in ECV cells. It is plausible that, at least at low concentrations, the site of action of α-lipoate inhibiting PMA-induced cell adhesion processes is primarily at the translation or post-translational level in ECV cells. *N*-terminal Ig-like domain of ICAM-1 and the first and fourth Ig-like unit of VCAM-1 contain two additional cysteine residues.[44] The presence of these cysteine residues suggest that the formation of extra disulfide bond might be of functional importance for these proteins. It has been demonstrated that these ICAM-1 and VCAM-1 domains bind to integrins.[44] Alteration of thiol/disulfide moities in receptor proteins in response to oxidant and antioxidant action has been suggested to result in altered binding of ligands and impaired receptor coupling.[22] The α-lipoate/DHLA redox couple has been previously shown to alter thiol/disulfide moities of protein. DHLA has been reported to reduce redox active di-cysteine structure of thioredoxin.[45] Thioredoxin is a 12 kD small protein that reduces disulfide bonds in protein.[45] Free α-lipoate/DHLA also promotes reduction of thioredoxin in cultured cells.[46] Thus, it may be hypothesized that the α-lipoate/DHLA redox couple either directly or indirectly, via thioredoxin, may alter thiol/disulfide moities in the ICAM-1 and VCAM-1 proteins that results in altered binding of ligands and impaired receptor coupling.

In summary, this work shows that clinically relevant concentrations of antioxidants that are known to be safe for human use may influence cell-cell adhesion. Thus,

results of this study suggest a remarkable therapeutic potential of antioxidants such as α-lipoic acid and α-tocopherol in pathologies where changes in cellular adhesion properties are implicated in the etiology.

Acknowledgements—Research supported by NIH DK 50430 and Asta Medica AG, Germany. SR and CKS are also affiliated to the Department of Physiology, University of Kuopio, Finland. HK is currently on leave from the Institute of Medical Science, Center for Adult Diseases, Kurashiki 710, Japan.

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ABBREVIATIONS

DCF—dichlorofluorescein
DCFH-DA—dichlorodihydrofluorescein-diacetate
DHLA—6,8 dithiooctanoic acid
ECV—ECV304, human endothelial cell line
EMSA—electrophoretic mobility shift assay
FITC—fluorescein isothiocyanate
GSH—reduced glutathione
GSSG—glutathione disulfide
HUVEC—human umbilical vein endothelial cells
ICAM-1—Intercellular adhesion molecule-1
NAC—*N*-acetyl-L-cysteine
NF-κB—nuclear factor-κB
PMA—phorbol 12-myristate 13-acetate
PMC—2,2,5,7,8-pentamethyl-6-hydroxychromane
PDTC—pyrrolidine dithiocarbamate
VCAM-1—vascular cell adhesion molecule-1