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d-α-Tocopherol inhibits low density lipoprotein induced proliferation and protein kinase C activity in vascular smooth muscle cells

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Native and malondialdehyde modified low density lipoproteins have been shown to stimulate smooth muscle cell proliferation (A7r5) in vitro. The stimulation is associated with an increase of protein kinase C activity. d-α-Tocopherol, at physiological concentrations, has been found to inhibit both protein kinase C activity and cell proliferation.

Smooth muscle cell proliferation; d-α-Tocopherol; Arteriosclerosis; Low density lipoprotein

1. INTRODUCTION

One of the major risk factors for cardiovascular disease is hyperlipidemia, with elevated levels of cholesterol-rich low density lipoprotein (LDL). In combination with other factors, high plasma concentrations of LDL promote the development of atherosclerosis [1]. Arterial smooth muscle cell migration, proliferation and metabolic changes contribute to the development of atherosclerotic lesions [2,3]. LDL stimulates cell proliferation in cultured human vascular smooth muscle cells [4,5] by phospholipase C-mediated phosphoinositide formation [6]. High density lipoprotein antagonise LDL-mediated stimulation of cell proliferation [5]. Oxidatively modified LDL has been proposed to play a role in atherogenesis by inducing macrophage release of interleukin 1β, a mitogen for vascular smooth muscle cells, by inducing endothelial cell damage and by promoting foam cell formation [7,8]. LDL-lipid peroxidation may be the cause of the structural and biological changes of human oxidised LDL [9]. Receptors for normal and modified LDL exist on the macrophage cell membrane and other cells. Accumulation of modified LDL leads to the formation of foam cells, an integral part of the atherosclerotic picture [3]. An autocrine or paracrine platelet derived growth factor AA (PDGF AA) release and an induction of PDGF receptors are considered to be one of the mechanisms by which smooth muscle cells proliferate under the effect of LDL [10]. d-α-Tocopherol is able to inhibit proliferation in a number of cell lines, including rat aorta smooth muscle (A7r5 and A10), human aorta smooth muscle (HAI), mouse neuroblastoma (NB2A) and mouse fibroblast (Balb/3T3) but not in Chinese hamster ovary (LR73), human osteosarcoma (Saos-2) and mouse monocyte-macrophage (P388 Dl) [11]. d-α-Tocopherol inhibition depends on the mitogen employed to induce cell proliferation being progressively bigger on going from lysophosphatidic acid to bombesin, fetal calf serum (FCS), PDGF-BB and endothelin [11]. It has been shown that the effect of d-α-tocopherol is not related to its radical scavenging properties, but with its interaction with cell components, ultimately leading to inhibition of protein kinase C activity [12–15]. In the present study, we have investigated the role of d-α-tocopherol in the proliferation and protein kinase C activity of smooth muscle cells promoted by native and malondialdehyde modified LDL. The data show that d-α-tocopherol inhibits vascular smooth muscle cell proliferation and protein kinase C activation produced by both types of LDL. The implications of this phenomenon, with respect to the protective effect of d-α-tocopherol against the onset of atherosclerosis as studied at an epidemiological level [16], will be discussed.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EGTA, ethylene glycol-bis(β-aminoethyl ether) tetraacetic acid; FCS, fetal calf serum; HEPES, *N*-[hydroxyethyl]piperazine-*N*-*N*'-[2-ethanesulfonic acid]; LDL, low density lipoprotein; LDLmod, low density lipoprotein modified by malondialdehyde; PBS, phosphate-buffered saline; PDGF, platelet derived growth factor; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; SMC, A7r5, rat vascular smooth muscle cells.

2. MATERIALS AND METHODS

2.1. *Materials*

Tissue culture materials were purchased from Falcon Labware (Becton Dickinson & Co) and growth media and serum for cell culture were obtained from Gibco Laboratories (Grand Island, NY, USA.). [γ-^{32}P]ATP (30 Ci/mmol) was from Amersham International. d-α-Tocopherol and d-β-tocopherol were generous gifts from Hoffmann-La Roche & Co. (Basel, Switzerland). The peptide FKKSFKL (custom synthesised by Dr. Rolli, Institut für Klinische Immunologie, Inselspital Bern, Switzerland) was used as a substrate for the PKC assay. Streptolysin O (25,000 units) and phorbol 12-myristate 13-acetate were from Sigma Chemical Company, St.Louis, Mo. All other chemicals used were of the purest grade commercially available.

2.2. *Cell culture*

The cell line used, A7r5 rat aortic smooth muscle cells (SMC), was obtained from American Type Culture Collection. Cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 25 mM sodium bicarbonate, 60 U/ml penicillin, 60 mg/ml streptomycin, and 10% fetal calf serum (FCS). Cells were usually seeded into 100-mm plastic culture dishes and grown to confluence at 37°C in a humidified atmosphere of 5% CO_2 (medium pH 7.4). Culture media were changed every 3 days. In all experiments, media and sera used were from the same batch number and source.

2.3. *Effect of d-α- and d-β-tocopherol on cell growth*

Tocopherols were added to the cell cultures according to the following procedure. Following 48 h serum-deprivation, cells were restimulated to growth by addition of LDL or 10% FCS in the presence or absence of the indicated tocopherols suspended in ethanolic solution. Cells were detached by trypsinization and counted in a hemocytometer in triplicate. Viability of the cells was assessed by the Trypan blue dye exclusion method.

2.4. *Preparation and modification of human plasma low density lipoproteins*

LDL was prepared according to the method of Chung et al. [17]. Modified LDL was prepared by incubating 0.25 ml of 0.2 M malondialdehyde, generated by acid hydrolysis of malondialdehyde (bis(dimethyl acetal)) Aldrich, with 0.5 ml of LDL (10 mg/ml) at 37°C and pH 6.5 for 3 h [18]. Both native and modified LDL after 25 days at 0–4°C became unable to stimulate SMC proliferation (Özer et al., unpublished) indicating that ageing of the LDL (presumably their oxidation) induced structural changes resulting in loss of their cell growth stimulation property. Protein concentration was determined by using the kit by Pierce Co.

2.5. *Protein kinase C assay in permeabilized cells*

Measurements of protein kinase C activity in permeabilised smooth muscle cells were performed according to the procedure of Alexander et al.[19] with minor modifications. A7r5 cells after 48 h serum deprivation (G_0 phase) were preincubated for 7 h with LDL in the presence or absence of 50 μM d-α-tocopherol, and during the last 2 h in the presence (when specified) of 100 nM phorbol 12-myristate 13-acetate (PMA). Afterwards they were washed in phosphate-buffered saline (PBS), resuspended in intracellular buffer (5.2 mM $MgCl_2$, 94 mM KCl, 12.5 mM HEPES, 12.5 mM EGTA, 8.2 mM $CaCl_2$, pH 7.4) and aliquoted in 220 μl portions (1.5×10^6 cells/ml). Assays were started by adding [γ-^{32}P]ATP (40 cpm/pmol, final concentration 250 μM), peptide substrate (final concentration 100 μM) and streptolysin-O (0.6 I.U.). The reaction mixtures were incubated at 37°C for 5 min and the reaction was stopped by adding 100 μl of 25% (w/v) trichloroacetic acid in 2 M acetic acid. After being kept on ice for 10 min, samples were centrifuged for 10 min and spotted on P81 ion-exchange chromatography paper (Whatman International) which were then washed several times with 30% (v/v) acetic acid containing 1% (v/v) H_3PO_4 and once with ethanol. The P81 papers were dried, and the bound radioactivity was counted in a liquid scintillation analyser. To estimate the background phosphorylation, assays were performed in the absence of substrate. The value of ^{32}P incorporated obtained in the latter condition has been subtracted from the experimental data to account for the specific activity.

3. RESULTS AND DISCUSSION

Incubation of smooth muscle cells in the presence of 5 μg/ml LDL resulted in stimulation of cell growth (Fig. 1A). No stimulation was observed when, together with LDL, also d-α-tocopherol (50 μM) was present in the

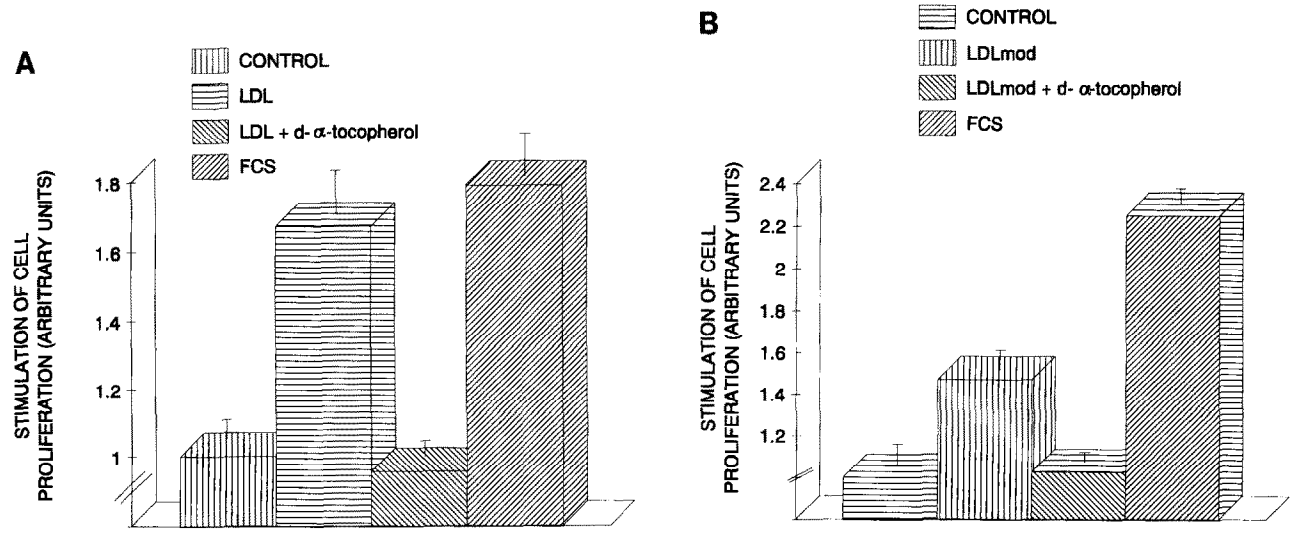

Fig. 1. Low density lipoprotein induced stimulation of smooth muscle cell proliferation. Cells (A7r5, rat aortic smooth muscle cells) were grown in DMEM containing 25 mM sodium bicarbonate, 60 U/ml penicillin, 60 mg/ml streptomycin, and 10% fetal calf serum (FCS) or 5 μg/ml LDL. d-α-Tocopherol (50 μM) was added to the cell cultures after 48h serum-deprivation at the time when cells were restimulated to growth by addition of LDL or 10% FCS. Cells were detached by trypsinization and counted in a hemocytometer. Viability of the cells was assessed by the trypan blue dye exclusion method. Results are the mean ± S.D. of three different experiments done in triplicate.

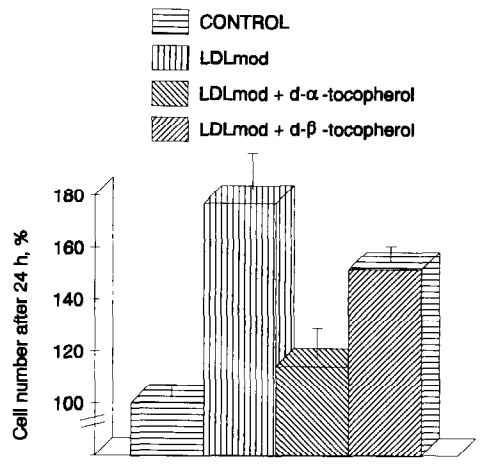

Fig. 2. Effect of d-α- and d-β-tocopherol on smooth muscle cell proliferation. The experimental conditions were similar to those described in the legend to Fig. 1. The concentration of d-β-tocopherol was 50 μM.

cell incubation medium, but no cytotoxic effects were detected. For comparison the growth promoting effect of FCS is also shown. In Fig. 1B is shown that also malondialdehyde-modified LDL induced cell growth, although to a lesser extent, but also in this case d-α-tocopherol (50 μM) was inhibitory.

Fig. 2 compares the effect of d-α- and d-β-tocopherols. The proliferation of SMC produced by modified LDL was more sensitive to d-α-tocopherol than to d-β-tocopherol. Previously published data [13] have shown that d-α- and d-β-tocopherol are taken up by smooth muscle cells in equal amounts, within experimental error. Thus a transport difference could not be responsible for the differential effects of d-α- and d-β-tocopherol. The distinct effects on cell proliferation of the 2 tocopherols could only be associated with their differences in molecular interactions since they possess very similar antioxidant and radical scavenger properties. The fact that both normal and modified LDL were able to stimulate cell proliferation under the experimental conditions employed in this study suggests that the lipoprotein receptor as well as the scavenger receptor can initiate a proliferative event. d-α-Tocopherol appears to act at some point in the cascade of events further leading to activation of cell proliferation. Inhibition of protein kinase C activity has been indicated to be a central event in the mechanism of cell growth inhibition by d-α-tocopherol [14,15]. Fig. 3 shows the effect of native and modified LDL on protein kinase C activity. Native and modified LDL stimulation of growth was linked to an increase of total protein kinase C activity of more than six-fold. With native LDL protein kinase C activity was present already in the absence of phorbol myristate acetate while with modified LDL the enzyme was in a state which required phorbol myristate acetate to show activity.

Fig. 3 shows also the effect of d-α-tocopherol on protein kinase C activity. Both protein kinase C activity induced by native and modified LDL was d-α-tocopherol sensitive. The concentration which gave full inhibition of smooth muscle cell protein kinase C (50 μM) was close to the human plasma (40 μM) d-α-tocopherol physiological concentration [16].

4. CONCLUSIONS

In the multifactorial pathogenesis of atherosclerosis, antioxidants, such as d-α-tocopherol, are supposed to play their role in preventing the oxidative modification of lipoproteins, their uptake and the consequent deposition of fat in macrophages and smooth muscle cells. The picture appears to be more complex, due to the increased expression of PDGF-AA and of PDGF receptors produced in smooth muscle cells by LDL with consequent stimulation of cell proliferation [10]. The inhibition of protein kinase C and of SMC proliferation (induced by LDL) observed in this study suggests a new and additional role for d-α-tocopherol. d-α-Tocopherol would not only prevent the oxidation of lipoproteins, but it would also counterbalance the proliferative stimulus of LDL. Such a growth effect, possibly mediated by autocrine PDGF liberation, could be prevented by inhibiting protein kinase C, a central element in the signal transduction pathway of PDGF, by d-α-tocopherol.

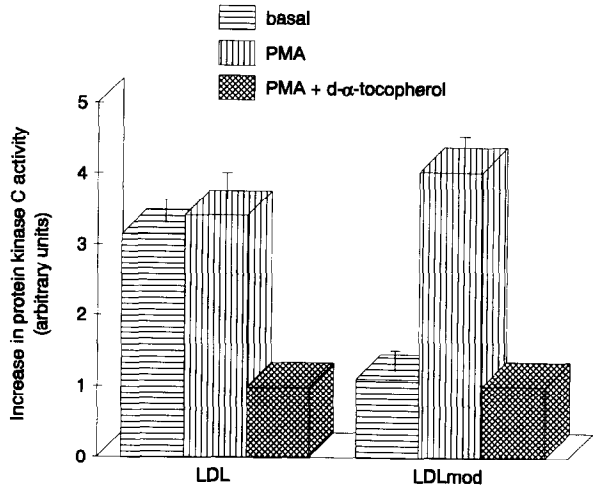

Fig. 3. Effect of d-α-tocopherol on native and modified low density lipoprotein stimulated protein kinase C activity. A7r5 cells in the late G_0 phase of the cycle, preincubated for 7 h in the presence of the indicated concentrations of LDL (5 μg/ml) and tocopherol (50 μM) (and when applicable for the last 2 h with 100 nM PMA), were washed and assayed for protein kinase C activity by adding [γ-^{32}P]ATP (40 cpm/pmol, final concentration 250 μM), peptide substrate (final concentration 100 μM) and streptolysin-O (0.6 I.U.). After 10 min incubation at 37°C the reaction was stopped by 100 μl of 25% (w/v) trichloroacetic acid and the samples centrifuged for 5 min and spotted on P81 ion-exchange chromatography paper. The background phosphorylation, measured in the absence of substrate, was subtracted.

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