Modulation of α-tropomyosin expression by α-tocopherol in rat vascular smooth muscle cells

Elisabetta Aratri, Stefan E. Spycher, Isabel Breyer, Angelo Azzi*

Institut für Biochemie und Molekularbiologie, Universität Bern, Bühlstrasse 28, 3012 Bern, Switzerland

Received 10 February 1999; accepted 13 February 1999

Abstract The effect of α-tocopherol (vitamin E) on gene expression in rat vascular smooth muscle cells was studied by the differential display technique. One gene out of about 1000 genes analyzed, identified as α-tropomyosin, showed an increased transcription level caused by α-tocopherol treatment. Northern and Western blot analysis revealed a time-dependent transient up-regulation of the amount of mRNA (peak between 2 and 3 h) and protein (peak at 5 h) in α-tocopherol-treated cells. No effect was observed in cells treated with β-tocopherol.

Key words: α-Tropomyosin; α-Tocopherol; β-Tocopherol; Vitamin E; Differential display; Smooth muscle cell

1. Introduction

Atherosclerosis is the major cause of age-related disease and death in the world [1]. An early event in the onset of the disease is the migration of smooth muscle cells from the media to the intima of the arterial wall where they proliferate followed by their release of matrix components that leads to the formation of lesions protruding into the lumen [2]. α-Tocopherol, an antioxidant vitamin, has been found to reduce the risk of disease development [3–5]. In recent years, other modes of action of α-tocopherol have been demonstrated that cannot be ascribed to its antioxidant property.

α-Tocopherol has been shown to inhibit vascular smooth muscle cell proliferation through a non-antioxidant effect via protein kinase C [6,7]. Tocopherol binding proteins have been found and identified in various tissues [8–10]. A function has so far only been ascribed to the α-tocopherol transfer protein found in liver cells, where it specifically transfers α-tocopherol to lipoprotein, thus regulating tocopherol levels in the body [11]. The role of the other proteins is obscure, but it is hypothesized that they might be responsible for a tissue specific cellular regulation of tocopherol transport. The only report showing an effect of α-tocopherol on the mRNA modulation is that of an increased transcription of the gene of the α-tocopherol binding protein in rat liver [12]. No systematic study has been carried out, however, especially in view of the inhibition by α-tocopherol of protein kinase C activity, to establish if such an effect could be influenced by transcription factors with consequent transcription modulation.

In order to address the question, whether the expression of some genes is modulated by α-tocopherol in rat vascular smooth muscle cells, we chose the method of mRNA differential display [13]. In this report we show that α-tocopherol treatment of serum stimulated cells leads to a transient increase in α-tropomyosin mRNA and protein expression.

2. Material and methods

2.1. Materials

Tissue culture materials were purchased from Falcon Labware (Becton Dickinson AG, Basel, Switzerland) and Gibco Laboratories (Grand Island, NY, USA). Growth media and serum for cell cultures were obtained from Gibco Laboratories. Rat vascular smooth muscle cells (A7r5) were purchased from the American Type Culture Collection (Rockville, MD, USA). RRR-α- and RRR-β-tocopherol were generous gifts from Henkel Corporation (La Grange, USA). Actinomycin D was purchased from Sigma (St. Louis, MO, USA). [^{35}S]methionine, [α-^{32}P]dATP, [α-^{35}S]dATP and the enhanced chemiluminescence Western blotting analysis system were purchased from Amersham International.

2.2. Cell culture

A7r5 cells were grown to confluence in DMEM 10% fetal calf serum (FCS). They were made quiescent for 48 h by incubation in low serum media (DMEM/0.2% FCS). During the last 24 h, 50 μM α-/β-tocopherol was added in ethanol solution (final ethanol concentration 0.1%) or ethanol alone was added to control cells.

Cells were then stimulated with DMEM/10% FCS in the presence of 50 μM α- or β-tocopherol and harvested at the indicated times. Cells were washed with cold PBS and used for RNA (using RNeasy total RNA kit from Qiagen, Germany) or protein extraction. Actinomycin D was added at the beginning of the stimulation period, at a concentration of 5 μg/ml medium.

2.3. Differential display

Differential display [13] of total RNA was performed using two kits from GenHunter Corporation (Nashville, TN, USA) according to the manufacturers procedure.

Samples were analyzed on 6% polyacrylamide sequencing gels. Gels were briefly washed with distilled water, dried and exposed in a Bio-Rad GS-250 Molecular Imager for 18 h.

2.4. Cloning and sequencing of cDNA fragments

Differentially expressed cDNA fragments were re-amplified as described by [13]. Following this, they were cloned into the pGEM-T vector (Promega, Madison, WI, USA). Plasmid DNA sequencing was carried out at the Department of Clinical Research of the University of Bern. Sequence analysis was done using the Wisconsin Sequence Analysis Package (Version 8) from GCG (Madison, WI, USA).

2.5. Northern blot analysis

Equal amounts of total RNA (10 μg) were size-fractionated on a 1% (w/v) agarose gel in 20 mM MOPS, 5 mM Na-acetate, 0.5 mM EDTA, pH 7.0, containing 2 M (6%) formaldehyde. RNA was transferred to a Zeta-Probe membrane (Bio-Rad Laboratories) using 10×SSC (SSC: 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) and cross-linked to the membrane using UV irradiation. The cDNA fragments recovered from the differential display analyses by re-amplification with the originally used primer pair and a fragment of the encoding region of rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH, loading control) were labelled with [α-^{32}P]dATP using

*Corresponding author. Fax: (41) (31) 6313737.
E-mail: angelo.azzi@mci.unibe.ch

PII: S0014-5793(99)00277-X

the random prime labelling kit (Boehringer, Mannheim). Unincorporated label was removed using NICK spin columns (Pharmacia Biotech). Pre-hybridization (10 min) and hybridization (overnight) was performed at 42°C in 50% formamide, 0.25 M NaCl, 0.12 M NaH_2PO_4, pH 7.2, 1 mM EDTA, 7% SDS and 1×10^6 cpm/ml of heat-denatured [α-^{32}P]dATP-labelled cDNA probe. Blots were washed in 1×SSC/0.1% SDS at 42°C, then in 0.25×SSC/0.1% SDS at 42°C, 48°C, 54°C and 60°C for 20 min. Blots were exposed in a Bio-Rad GS-250 Molecular Imager for 48 h, signals quantified with the Molecular Analyst software from Bio-Rad and normalized to the GAPDH signals.

2.6. Western blot analysis

Cells were harvested by scraping and resuspended in a small volume of ice cold disruption buffer (Tris-Cl 20 mM, EDTA 5 mM, EGTA 5 mM, pH 7.4) containing protease inhibitors and 1% Triton X-100. Following disruption using a syringe G25, samples were centrifuged (10 000×*g*, 15 min, 4°C) and supernatants stored at −70°C until analysis. The protein content was determined using the method of Bradford [14]. Proteins (5–10 μg per lane) were separated using a 10% SDS-PAGE separating gel ([20]) and electro-blotted onto a Immobilon-P (Millipore, Bedford, MA, USA) membrane. α-Tropomyosin protein was detected with a polyclonal rabbit anti-chicken gizzard α-tropomyosin primary antibody (dilution 1:1000) from Sigma (St. Louis, MO, USA) and visualized by chemiluminescence (ECL, Amersham, Zürich, Switzerland) following labelling with horseradish peroxidase-labelled anti-rabbit IgG (dilution 1:10 000) (Amersham, Zürich, Switzerland). Rabbit anti-mouse actin antibody was used as primary antibody to control protein loading in addition to the protein quantitation determination. Signals were quantified densitometrically on a Bio-Rad GS-700 imaging densitometer.

2.7. Pulse-chase experiment

The culture medium was changed 75 min before harvesting of the cells with methionine-free medium. 15 min before harvesting, the cells were pulsed with [^{35}S]methionine. The pulse time was during the last 15 min before harvesting.

Proteins were extracted, quantified and immunoprecipitated using polyclonal α-tropomyosin antibodies according to the method described by Eldering et al. [15]. The same volumes of samples were loaded on a 10% SDS polyacrylamide gel, treated with Na-salicylate (15%), dried and exposed to Fuji RX X-ray film at −70°C for 20 days. Signals were quantified using a Bio-Rad GS-700 imaging densitometer.

3. Results and discussion

About 1000 transcripts were analyzed by differential display using 20 different arbitrary primer combinations. Only one band using the primer pair H-AP4 and oligo-dT primer H-T_{11}A of the GenHunter kit was reproducibly, differentially displayed (shown in Fig. 1A). DNA sequence analysis and comparison with the gene databank showed that the fragment was identical to a portion of the α-tropomyosin cDNA. Northern blot analysis (Fig. 1B) showed a significant increase for the α-tropomyosin mRNA expression of 1.6-fold for the tocopherol-treated cells compared to control after 3 h stimulation. In the presence of Actinomycin D, α-tocopherol-mediated mRNA induction was completely prevented and the mRNA level was identical to the tocopherol non-treated cells (Fig. 1B). This indicates that tocopherol treatment leads to de novo synthesis of tropomyosin mRNA and is not the result of its increased degradation rate relative to control cells.

The time course of mRNA and protein expression is shown in Fig. 2. mRNA levels peaked around 2 h after stimulation for α-tocopherol-treated cells when normalized to control cells reaching control levels after 7 h (Fig. 2A). Considering the result of Fig. 1B, the maximum mRNA synthesis rate was in a window between 2 and 3 h.

We asked the question whether the change in RNA expression would correspond to a change in the protein level. In fact, it has been shown that heterozygous knockout mice for the α-tropomyosin gene, although having a 50% reduction in the mRNA level, exhibit a normal protein amount [16]. The α-tropomyosin protein level showed a transient maximum 5 h after stimulation in the presence of α-tocopherol returning to the control levels after 7 h. This indicates that the smooth muscle cells, as found in the knockout mice, tend to maintain a constant steady state concentration of tropomyosin protein (Fig. 2A). No significant changes for mRNA and protein levels were observed when β-tocopherol (a related derivative of α-tocopherol) was used (Fig. 2B) indicating a different mode of action for the two homologues.

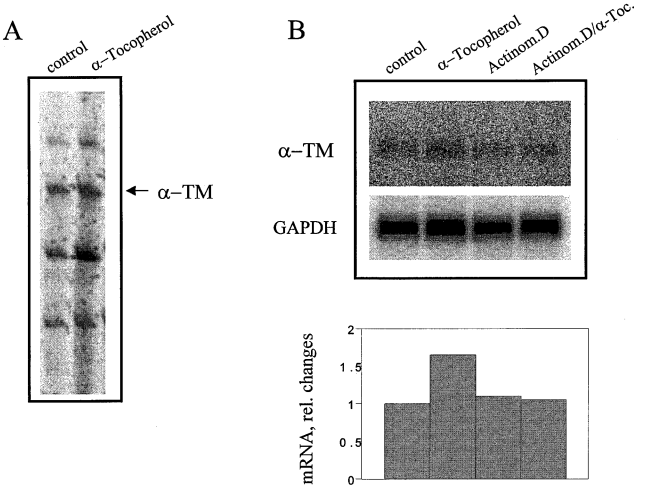

Fig. 1. Differential display and Northern blot analysis of α-tropomyosin gene expression in α-tocopherol-treated A7r5 smooth muscle cells. (A) Autoradiography obtained from mRNA differential display of synchronized cells following re-stimulation with 10% FCS in the presence of 50 μM α-tocopherol for 3 h at 37°C/5% CO_2. The arrow indicates the mRNA present in an increased amount in tocopherol-treated cells compared to controls. (B) Northern blot analysis of tropomyosin mRNA expression after 3 h treatment. 15 μg of total RNA was loaded for each lane and hybridized with the re-amplified and sub-cloned ^{32}P-labelled tropomyosin fragment recovered from the differential display gel marked with the arrow in (A). GAPDH was used as loading control.

In a next step, we analyzed the time course of tropomyosin synthesis rate by pulse-chase labelling experiments. The highest protein synthesis rate was observed in the 5 h window where also the highest accumulated protein levels (see Fig. 3A) were observed. Since the maximum protein accumulation and highest protein synthesis rate coincide (between 4 and 5 h after stimulation), it has to be assumed that an increased protein degradation occurs in the window between 5 and 7 h in order to reach the level of control cells after 7 h.

There is general agreement in support of the mechanism by which tropomyosin sterically prevents actin-myosin interactions [17]. Both in vitro and in vivo evidence suggest that multiple isoforms of tropomyosin in non-muscle cells may be required for regulating the actin filament stability, intracellular granule movement, cell shape determination and cytokinesis. Tropomyosin binding proteins such as caldesmon, tropomodulin and other unidentified proteins may be required for some of these functions [18]. The role of tropomyosin is especially important in vascular smooth muscle cells, where the force generated between thin and thick filaments provides the mechanism for cell shortening and influences the arterial

blood pressure [19]. On the other side, results have been reported suggesting that hypertensive patients may have an increased lipid peroxidation and reduced protection from vitamins C and E [20]. It has also been shown that short term oral high dose combination antioxidant therapy reduces the blood pressure [21]. An involvement of tropomyosin in the progression of atherosclerosis has also been suggested. Early after balloon injury, smooth muscle cells of the media and those that have migrated into the intima contain decreased amounts of tropomyosin and late after balloon injury tropomyosin returns toward normal values [22]. The overexpression of tropomyosin in smooth muscle cells, induced by α-tocopherol described in this study, may thus be relevant in understanding, at a molecular basis, the diminution of the blood pressure induced by antioxidants such as vitamin E. Such an event may be the consequence of a lesser contractility of smooth muscle cells containing more tropomyosin.

Furthermore, changes of tropomyosin isoforms have previously been found accompanying morphologic alterations such as those associated with neoplastic transformations in mammalian cells [23]. TGF-alpha initiates the disassembly of cytoskeletal stress fibers and suppresses the synthesis of tropomyosin isoforms and stimulates their degradation [24]. The induced expression of tropomyosin by α-tocopherol may be in part at the basis of an anti-neoplastic effect of α-tocopherol

It has been shown that PKCs are involved in remodelling of the actin cytoskeleton, partly by phosphorylating specific PKC substrates such as the MARCKS protein and pleckstrin [25,26]. A direct connection between the PKC activity and cytoskeleton modelling therefore exists. Since α-tocopherol is known to have an inhibitory effect on smooth muscle cell proliferation via inhibition of PKC activity, it might be possible that the observed tropomyosin expression is one of the signals indirectly elicited on the cytoskeleton via a reduced PKC activity. On the other hand, it might be possible that signalling pathways independent of PKC activity exist, which are triggered by α-tocopherol, leading to the observed transient modulation of tropomyosin expression. The molecular events involved in this response are the object of current investigation.

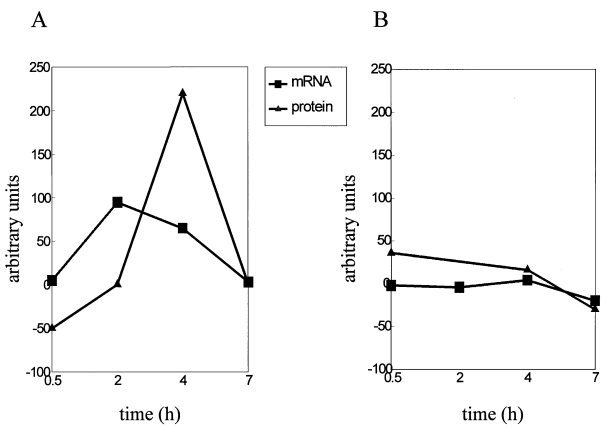

Fig. 2. The time course of mRNA and protein expression after treatment with α- (Fig. 2A) and β-tocopherol (Fig. 2B). Cells were harvested at the indicated times after treatment with tocopherol. RNA and protein were isolated as described in Section 2. Each time point was normalized to its corresponding control. The figure represents the result of one out of three independent experiments.

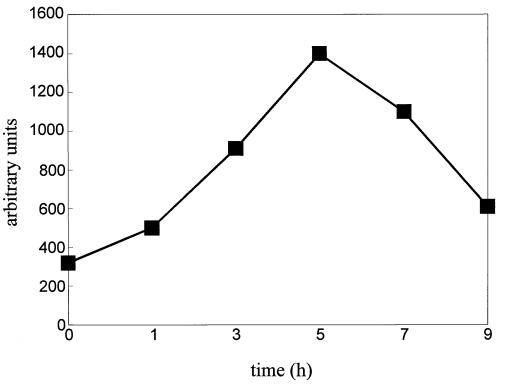

Fig. 3. Pulse-chase experiment of α-tropomyosin protein synthesis in the presence of α-tocopherol. Cells were treated as described in Section 2 and harvested at the indicated times after a 15 min [^{35}S]methionine pulse. Equal amounts of protein were loaded and signals obtained for the tocopherol treated cells were normalized to control cells.

Acknowledgements: The present study has been supported by the Swiss National Science Foundation and by F. Hoffmann-La Roche, AG. The financial support of the Stiftung für die Ernährungsforschung in der Schweiz is gratefully acknowledged. EA is a recipient of a Consortium CARSO fellowship funded by the European Community and Regione Puglia.

References

[1] Ross, R. (1993) Nature 362, 801–809.
[2] Raines, E.W. and Ross, R. (1993) Br. Heart. J. 69, S30.
[3] Gey, K.F., Moser, U.K., Jordan, P., Stahelin, H.B., Eichholzer, M. and Ludin, E. (1993) Am. J. Clin. Nutr. 57, 787S–797S.
[4] Rimm, E.B., Stampfer, M.J., Ascherio, A., Giovannucci, E., Colditz, G.A. and Willett, W.C. (1993) New Engl. J. Med. 328, 1450–1456.
[5] Stampfer, M.J., Hennekens, C.H., Manson, J.E., Colditz, G.A., Rosner, B. and Willett, W.C. (1993) New Engl. J. Med. 328, 1444–1449.
[6] Boscoboinik, D., Szewczyk, A. and Azzi, A. (1991) Arch. Biochem. Biophys. 286, 264–269.
[7] Boscoboinik, D., Szewczyk, A., Hensey, C. and Azzi, A. (1991) J. Biol. Chem. 266, 6188–6194.
[8] Arita, M., Sato, Y., Miyata, A., Tanabe, T., Takahashi, E., Kayden, H.J., Arai, H. and Inoue, K. (1995) Biochem. J. 306, 437.
[9] Gordon, M.J., Campbell, F.M., Duthie, G.G. and Dutta-Roy, A.K. (1995) Arch. Biochem. Biophys. 318, 140–146.
[10] Dutta-Roy, A.K., Gordon, M.J., Leishman, D.J., Paterson, B.J., Duthie, G.G. and James, W.P. (1993) Mol. Cell. Biochem. 123, 139–144.
[11] Arita, M., Nomura, K., Arai, H. and Inoue, K. (1997) Proc. Natl. Acad. Sci. USA 94, 12437–12441.
[12] Fechner, H., Schlame, M., Guthmann, F., Stevens, P.A. and Rustow, B. (1998) Biochem. J. 331, 577–581.
[13] Liang, P. and Pardee, A.B. (1992) Science 257, 967–971.
[14] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[15] Eldering, J.A., Grunberg, J., Hahn, D., Croes, H.J., Fransen, J.A. and Sterchi, E.E. (1997) Eur. J. Biochem. 247, 920–932.
[16] Blanchard, E.M. et al. (1997) Circ. Res. 81, 1005–1010.
[17] Reedy, M.K., Reedy, M.C. and Schachat, F. (1994) Curr. Biol. 4, 624–626.
[18] Lin, J.J., Warren, K.S., Wamboldt, D.D., Wang, T. and Lin, J.L. (1997) Int. Rev. Cytol. 170, 1–38.
[19] Bohr, D.F. and Webb, R.C. (1984) Am. J. Med. 77, 3–16.

[20] Wen, Y., Killalea, S., McGettigan, P. and Feely, J. (1996) Ir. J. Med. Sci. 165, 210–212.
[21] Galley, H.F., Thornton, J., Howdle, P.D., Walker, B.E. and Webster, N.R. (1997) Clin. Sci. 92, 361–365.
[22] Kocher, O., Gabbiani, F., Gabbiani, G., Reidy, M.A., Cokay, M.S., Peters, H. and Huttner, I. (1991) Lab. Invest. 65, 459–470.
[23] Galloway, P.G., Likavec, M.J. and Perry, G. (1990) Lab. Invest. 62, 163–170.
[24] Warren, R.H. (1997) Exp. Cell. Res. 236, 294–303.
[25] Hartwig, J.H., Thelen, M., Rosen, A., Janmey, P.A., Nairn, A.C. and Aderem, A. (1992) Nature 356, 618–622.
[26] Brumell, J.H., Craig, K.L., Ferguson, D., Tyers, M. and Grinstein, S. (1997) J. Immunol. 158, 4862–4871.