RESEARCH ARTICLE

α-Tocopherol enhances degranulation in RBL-2H3 mast cells

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Based on the observation that 3 months α-tocopherol supplementation caused an up-regulation of the mRNA of vesicular transport proteins in livers of mice, the functional relevance was investigated in RBL-2H3 cells, a model for mast cell degranulation. In total, 24 h incubation with 100 μM α-tocopherol enhanced the basal and phorbol-12-myristyl-13-acetate/ionomycin-stimulated release of β-hexosaminidase and cathepsin D as measured by enzymatic analysis as well as Western blotting and immunocytochemistry, respectively. β-Tocopherol exerted the same effect, whereas α-tocopheryl phosphate and trolox were inactive, indicating that both the side chain and the 6-OH group at the chroman ring are essential for activation of degranulation. α-Tocopherol did not induce mRNA expression of soluble NSF-attachment protein receptor (soluble *N*-ethylmaleimide-sensitive factor-attachment protein receptor) proteins, such as *N*-ethylmaleimide sensitive fusion protein, complexin-2, SNAP23 or syntaxin-3, in the RBL-2H3 cell model. In view of the well known α-tocopherol-mediated activation of protein phosphatases, which regulate soluble NSF-attachment protein receptor activities by dephosphorylation, underlying mechanisms are discussed in terms of preventing oxidative inactivation of protein phosphatases and so far unknown functions in certain membrane domains.

Keywords:
α-Tocopherol / β-Hexosaminidase / Cathepsin D / Degranulation / Mast cells

1 Introduction

Although known for more than 80 years, the essential biological role of vitamin E is still not known. Many functions have been suggested including prevention of membranes from oxidative stress [1, 2], stabilizing membrane structures [3, 4], regulation of signal cascades [5], and controlling gene activity [6]. However, among all these functions no common denominator has been found that could explain the symptoms characteristic for vitamin E deficiency, which are disorders in neuromuscular signal transduction [7–9] and female infertility [10–12].

Recent analyses of gene expression in vitamin E-deficient and -supplemented animals identified a large number of genes influenced by the vitamin E status. Although the breakthrough has not been achieved yet, some hints are available. Gohil *et al.* found genes involved in Ca^{2+} dynamics and signaling as well as genes of the vesicular transport decreased in the brain of α-tocopherol transfer protein-deficient mice, a model of severe vitamin E deficiency [13]. A convincing number of these genes were up-regulated in the liver of vitamin E-supplemented mice [14]. In a more recent study, a cluster of genes regulating Ca^{2+}-mediated muscle contraction-relaxation cycles including sarcolipin was over-expressed in muscles of

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Abbreviations: NSF, *N*-ethylmaleimide-sensitive factor; **PI3K,** phosphatidylinositol 3-kinase; **PMA,** phorbol-12-myristyl-13-acetate; **RT,** room temperature; **SNAP,** synaptosome-associated protein; **SNARE,** soluble NSF-attachment protein receptor; **TRITC,** tetramethylrhodamin isothiocyanate; **VAMP,** vesicle-associated membrane proteins

α-tocopherol-deficient mice [15]. An uncoordinated induction was suggested to be associated with ataxia observed in vitamin E deficiency. A second cluster of up-regulated genes, including ubiquitin carboxy-terminal hydrolase-1 and cathepsins, was associated with proteolysis and inflammation response [15].

Mast cells are central effector cells in both innate and adaptive immune response. Upon stimulation, they release newly synthesized mediators, such as leukotrienes, prostaglandins, cytokines, and chemokines as well as preformed granule-associated mediators such as histamine, proteoglycans, proteases, β-hexosaminidase, or cathepsin D (for a comprehensive review see [16]). Unlike other secretory cell types like neurons or pancreatic β-cells, wherein vesicles are present at different stages ready for release, mast cells organize granules after stimulation [17]. Upon stimulation, granules fuse with the plasma membrane, and their content is released by exocytosis, in mast cells also called degranulation [18]. Membrane fusion is achieved by the concerted action of membrane-associated proteins, so-called soluble *N*-ethylmaleimide-sensitive factor (NSF)-attachment protein receptors (SNAREs), which drive membrane trafficking in most cells. One of the vesicle-associated membrane proteins (VAMPs) at the vesicular membrane and one of the syntaxins as well as two of the synaptosomal-associated proteins (SNAPs) at the target membrane form the so-called *trans* complex, the minimum requirement for membrane fusion [19, 20]. In mast cells, the neuronal SNAP25 is replaced by SNAP23, which like SNAP25 interacts with VAMP2 [21]. Together with syntaxin-4 and a second SNAP both proteins form the core complex, which brings membranes into close proximity and contributes to accurate vesicle fusion. After formation of the extremely stable core complex, SNAREs can only be disassembled under energy consumption, which is delivered by the ATPase NSF [18, 22]. Thereafter, vesicle proteins can be recycled for another round of vesicle formation.

Mast cell exocytosis can be triggered by antigen-induced cross-linking of cell-bound IgE antibodies. Subsequent aggregation of the IgE receptor FcεRI leads to the activation of several pathways, including the phosphatidylinositol 3-kinase (PI3K) pathway, calcium influx, and finally activation of functional targets such as degranulation and secretion (reviewed in [23, 24]). In addition, reactive oxygen species produced by intracellular NADPH-oxidases, lipoxygenases, or cycloxygenases have been discussed to be involved, too [25, 26]. Alternatively, mast cell degranulation can be achieved by phorbol-12-myristyl-13-acetate (PMA) and calcium ionophores to stimulate Ca^{2+} influx directly.

Vitamin E has been shown to influence a number of signaling events leading to degranulation, *e.g.* modulation of tyrosine kinases and phosphatases (reviewed in [27]). The general mechanism might be the reversal of phosphorylation since vitamin E activates protein tyrosine phosphatases and thereby inhibits enzymes, which require phosphorylation for activity. One example is the PI3K/PKB(Akt) pathway, which is inhibited by vitamin E. Underlying mechanisms apart from dephosphorylation might be interference with the recruitment of Akt to and/or its association with plasma membranes (reviewed in [27]). On the other hand, vitamin E restored the activity of PI3K and its downstream products, phosphorylated PDK and Akt, previously reduced by 7-keto cholesterol [28] and, thus, prevented apoptosis [28, 29]. This effect of vitamin E has been attributed to its capability to prevent incorporation of 7-keto cholesterol into lipid rafts, the domain where PI3K is active [29, 30]. Thus, depending on the situation, vitamin E can stimulate or inhibit signals required for degranulation. The process of degranulation itself, however, has only sporadically been investigated. We, therefore, investigated the release of β-hexosaminidase and cathepsin D from RBL-2H3 cells, a commonly used model of primary mast cells, and found it to be stimulated by vitamin E.

2 Materials and methods

RRR-α-tocopherol was a kind gift of Cognis Deutschland GmbH. all-rac-β-tocopherol was obtained from Merck (Darmstadt, Germany). α-Tocopheryl phosphate, ionomycin, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide were purchased from Sigma (Steinheim, Germany), Trolox from Fluka (Buchs, Switzerland), and PMA from Appligen (Darmstadt, Germany). All chemicals were reagent grade.

2.1 Cell culture

RBL (rat basophilic leukemia)-2H3 cells (DSMZ, ACC 312), a tumor analog of mucosal mast cells, were cultured in high-glucose DMEM containing 10% FCS, 2 mM L-alanyl-L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (all from Gibco, Karlsruhe, Germany). Ethanolic forms of vitamin E were added to FCS in concentrations ten times of that in the final experiment and incubated overnight as described previously [31]. For control, respective amounts of ethanol were applied accordingly and never exceeded 0.5% in the cell culture medium. If wanted, cells were grown in the presence of α-tocopherol for 24 or 48 h as indicated.

2.2 Stimulation of degranulation

In essential, the stimulation was performed as described previously [14] with minor modifications: RBL cells (60–80% confluent) grown in 6-well plates with or without different forms of vitamin E were washed in phenol red-free RPMI medium (Sigma). Degranulation was triggered with 10 nM PMA/1 μM ionomycin or the respective amounts of EtOH. Unspecific release was measured in cells without PMA/ionomycin treatment and subtracted from the stimulated one. After the times indicated 100 μL aliquots were taken

from the medium and stored on ice until the end of the experiment. After 40 min degranulation was stopped by placing the plate on ice. Cells were washed as described above and lysed in phenol red-free RPMI medium containing 1% Triton X-100 at room temperature (RT) and by 5 min shaking. Debris was removed by centrifugation for 10 min at 15 000 × *g* and 4°C.

2.3 β-Hexosaminidase assay

For estimation of β-hexosaminidase activity, 10 μL of undiluted medium samples or cell lysates (diluted five times in water) were incubated with 50 μL substrate solution (1 mg/mL *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide in 0.05 M citrate buffer, pH 4.5) in triplicate in 96-well plates for 45 min at 37°C. The reaction was stopped by addition of 100 μL 0.2 M NaOH, 0.2 M glycine, pH 10. The concentration of produced *p*-nitrophenol was measured at 405 nm with a microtitre plate absorbance reader (Synergy 2, Biotek Instruments GmbH, Bad Friedrichshall, Germany). The amount of produced *p*-nitrophenol was taken as measure for β-hexosaminidase activity. Released β-hexosaminidase was calculated as net degranulation in percentage of total β-hexosaminidase (summarized cell culture medium content plus content of the lysate) from which basal release has been subtracted.

2.4 Estimation of cathepsin D

For estimation of cathepsin D, protein was concentrated from 70 μL medium aliquots by acetone precipitation. Dried pellets were resuspended in 25 μL reducing Laemmli buffer and loaded onto 12.5% gels. From cell lysates 200 μL were treated identically. SDS-PAGE and Western Blot analysis were performed as described previously [32]. The primary antibody was goat-anti-human-cathepsin D (sc-6486, Santa Cruz Biotechnology, Santa Cruz, USA) used in a 1:1000 dilution in TTBS (50 mM Tris-HCl, 150 mM NaCl, 0.1% Tween-20 w/v, pH 7.5) at 4°C overnight. Peroxidase-conjugated rabbit-anti-goat antibody (1:2000) (DAKO, Hamburg, Germany) was used as secondary antibody for 1 h at RT. Proteins were detected by chemiluminescence imaging (using Supersignal West Dura, Perbio, Bonn, Germany) with a Fuji LAS3000-CCD-camera system. Bands were quantified by means of the Aida/2D densitometry 4.0 software (Raytest, Straubenhardt, Germany). Amounts of released cathepsin D is expressed as percentage of total cathepsin D (summarized cell culture media samples plus lysate content). Experiments were performed at least two times in duplicate.

2.5 Immunocytochemistry

Briefly, 100 000 cells were seeded in 12-well plates on cover slips and allowed to adhere overnight. Next day medium was replaced by 2 mL medium containing 100 μM α-tocopherol or ethanol and cells were incubated for further 48 h. After washing two times with PBS cells were fixed with 1 mL 3% formaldehyde in PBS for 30 min under gentle moving on a belly dancer at RT. After washing again two times, cells were permeabilized with 0.1% Triton X-100 in PBS for exactly 5 min at RT. Cells were washed three times with PBS and consecutively incubated for 30 min with the primary antibodies: goat-anti-human-cathepsin D (1:500) (Santa Cruz) or mouse-anti-rat-VAMP2 (1:1000) (Synaptic Systems, Göttingen, Germany). Antibodies were diluted in PBS containing 0.1 mg/mL BSA. After each incubation step, cells were washed seven times followed by 30 min incubation with fluorescent-labeled secondary antibodies in a dark room. First, donkey-anti-goat-fluorescein isothiocyanate (1:200) (Santa Cruz) was used for cathepsin D, followed by goat-anti-mouse-tetramethylrhodamin isothiocyanate (1:200) (Invitrogen, Karlsruhe, Germany) for VAMP2. In between cells were washed seven times. Cover slips were placed on slides (cell side down) in anti-fade mounting medium (DAPI Fluoromount G, Southern Biotech, Birmingham, USA) and sealed with nail polish. Confocal images were collected at excitation laser wavelengths of 561 nm (VAMP2) or 488 nm (cathepsin D) on a Leica TCS SP2 (Heidelberg, Germany) microscope.

2.6 RNA isolation

Cells were seeded into 6-well plates, grown for 4, 8, and 24 h, with and without *RRR*-α-tocopherol, respectively. After washing with PBS they were lysed in 800 μL cold Trizol (Invitrogen). RNA was isolated following the Trizol protocol according to the manufacturer's instructions. Genomic DNA was digested with 10 U RQ1 DNase (Promega, Mannheim, Germany) and RNA was cleaned up with a phenol-chloroform extraction. RNA concentrations were measured with a NanoDrop ND1000 (Peqlab Biotechnologie GmbH, Erlangen, Germany).

2.7 Quantitative real time PCR

RNA (3 μg) was reversely transcribed with 6 pmol oligo$(dT)_{15}$ primers and 180 U Moloney murine leukemia virus reverse transcriptase (Promega) in a total volume of 45 μL. Real-time PCRs (Mx3005PTM QPCR System, Stratagene, Amsterdam, Netherlands) were performed in triplicates with 1 μL of the cDNA in 25 μL reaction mixtures using SYBR Green I (Molecular Probes, Eugene, OR, USA) as fluorescent reporter. The annealing temperature was 59°C for all PCR reactions. PCR-products were quantified with a standard curve ranging from 1×10^4 to 1×10^9 copies of each amplicon. Primers (Sigma) were designed to be specific for cDNA by placing at least one primer onto an exon/intron boundary with PerlPrimer v1.1.14 [33]. *Hprt1*

Table 1. Primer sequences (5′→3), rat genes

Gene	Acc. number	Primer sequence	Product size (bp)
Cplx2	NM_053878	fwd GCAGATTCGAGATAAGTATGGGCT rev GATGCTCTCCTCTTCTTCCTCCT	157
Nsf	NM_021748	fwd ACTCTCTTGGCTCGACAGATTG rev GAGCCGTCTCTGCTCCTCTT	150
Snap23	NM_022689	fwd ATGGTCAGCCTCAGCAGACT rev CCCATATCCAGAGCCATGTT	148
Stx3	NM_031124	fwd GAAGGCACGGGATGAAACTA rev AAATGCCCAGCAACACAACT	101
Hprt1	NM_012583	fwd GTCCCAGCGTCGTGATTAGT rev GGCCTCCCATCTCCTTCAT	165

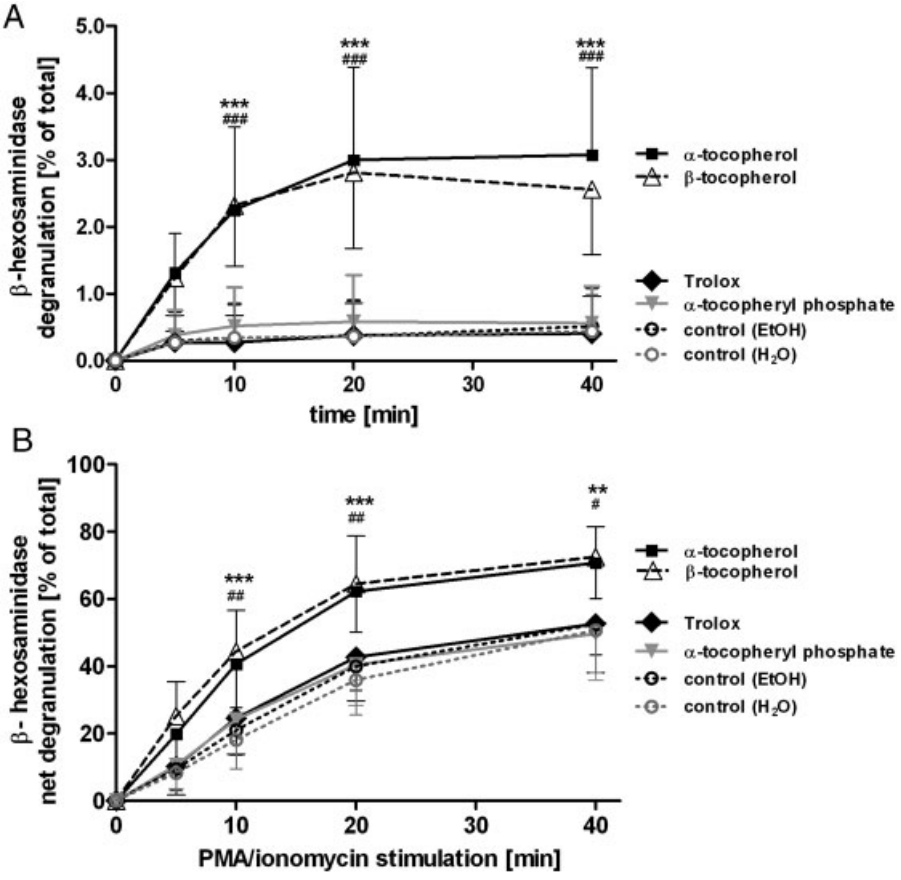

Figure 1. Influence of different forms of vitamin E on basal (A) and PMA/ionomycin-stimulated (B) degranulation of β-hexosaminidase from RBL cells. Cells were grown in 6-well plates for 24 h and further incubated for 24 h with medium containing 100 μM of the different forms of vitamin E or solvent controls. Tocopherols and Trolox were added to FCS (see Section 2) in ethanol, α-tocopheryl phosphate in water. Then cells were stimulated (B) or not (A) with 10 nM PMA/1 μM ionomycin. Released β-hexosaminidase was estimated after the times indicated and expressed as net degranulation. Amounts were calculated as percentage of total β-hexosaminidase (cellular and released) activity. Data represent means ± SD ($n = 5$, measured in duplicate). $^{**}p < 0.01$, $^{***}p < 0.001$ for α-tocopherol and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ for β-tocopherol, respectively, *versus* EtOH control at the same time point. For further details see Section 2.

was used as reference gene. Primer sequences for rat genes are summarized in Table 1.

2.8 Statistics

Results are expressed as means ± SD. Significance of difference was analyzed by two-way ANOVA (Graphpad Prism, version 5.0, San Diego, CA, USA) and Bonferroni's post-test. A value of $p < 0.05$ was considered statistically significant.

3 Results

3.1 Degranulation of β-hexosaminidase is stimulated by α- and β-tocopherol in rat mast cells

Preincubation of cells with α- or β-tocopherol stimulated the basal release of β-hexosaminidase by a low but significant degree (Fig. 1A). Both tocopherols also significantly enhanced PMA/ionomycin-mediated degranulation compared with the ethanol and water controls (Fig. 1B). The

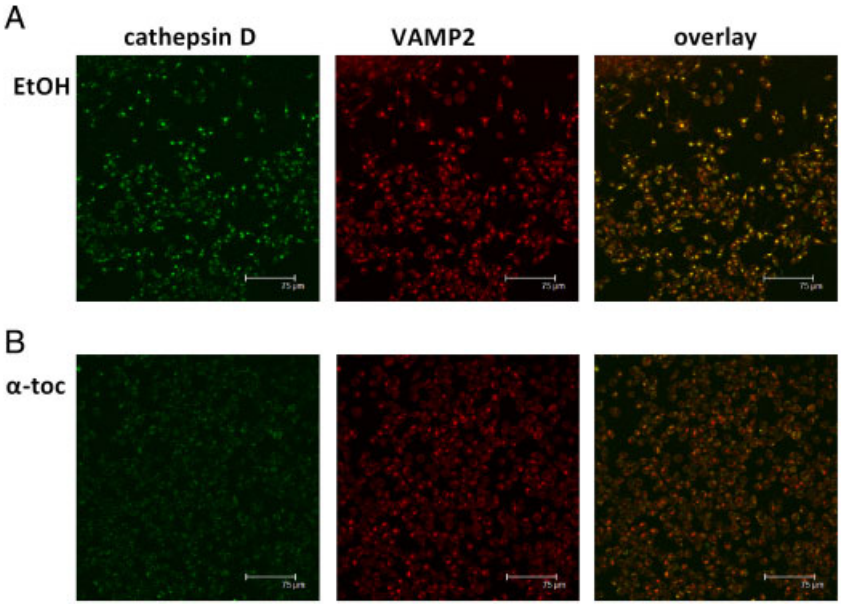

Figure 2. Loss of cathepsin D from RBL cells after incubation with α-tocopherol. Cells were grown on cover slips in the absence (EtOH) (A) or the presence (α-toc) (B) of 100 μM α-tocopherol for 48 h. Then cells fixed in paraformaldehyde were stained with the primary antibodies against cathepsin D or VAMP2 followed by visualizing with fluorescein isothiocyanate in case of cathepsin D (green) and tetramethylrhodamin isothiocyanate in case of VAMP2 (red) labeled secondary antibodies and fluorescence was imaged by confocal microscopy. The overlay shows co-localization of both proteins (yellow) in the control (EtOH) but much less in α-tocopherol-treated cells. Pictures are representative for three independent experiments. For further details see Section 2.

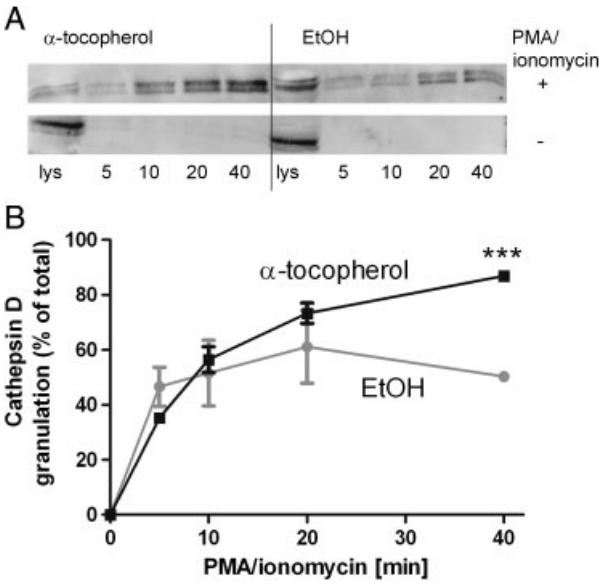

Figure 3. Stimulation of cathepsin D release from RBL cells by α-tocopherol. Cells were grown for 24 h with (α-toc) or without (EtOH) 100 μM α-tocopherol before stimulation or not with 10 nM PMA/1 μM ionomycin. After the times indicated an aliquot of the medium was removed and the cells were lysed after 40 min. Proteins in the medium and cell lysates were concentrated by acetone precipitation. (A) Western blots. Lanes 1 and 6 represent cell lysates prepared 40 min after stimulation. Lanes 2–5 and 7–10 represent medium samples taken after the time indicated. (B) Released cathepsin D quantified by densitometric evaluation of Western blots and expressed as percent of total cathepsin D. Values are means ± SD from two experiments with cells seeded and treated in duplicate. SDs at 40 min are too small to be visible. ***$p < 0.001$ *versus* the ethanol control at the same time point. For further details see Section 2.

increase in degranulation was apparent even at the earliest time points investigated indicating that, apart from increasing the absolute amount, tocopherols increased the rate of β-hexosaminidase release. In contrast, α-tocopheryl phosphate, which is discussed to be the biological active form of α-tocopherol [34], did not show any effect. Also trolox, the α-substituted chromanol with the side chain replaced by a more hydrophilic carboxyl group, did not influence degranulation.

3.2 α-Tocopherol stimulates cathepsin D release from RBL cells

A second hydrolase released from mast cells is cathepsin D. Confocal immunofluorescence microscopy revealed co-localization of cathepsin D with the vesicle marker VAMP2 (Fig. 2A). Cells cultured in the presence of 100 μM α-tocopherol for 48 h drastically decreased cathepsin D within the cells whereas VAMP2 remained nearly constant (Fig. 2B). To test whether cathepsin D is released into the cell culture medium, cells were treated with PMA/ionomycin as for the examination of β-hexosaminidase release. Cathepsin D release was evaluated by Western blotting. As obvious from Fig. 3A, faint bands of cathepsin D were detected in the medium of unstimulated, α-tocopherol pre-treated cells but not in the EtOH control. As shown for β-hexosaminidase α-tocopherol also enhanced the PMA/ionomycin-stimulated release of cathepsin D. A quantification of immunoreactivity showed an increase in cathepsin D release by α-tocopherol after stimulation with PMA/ionomycin which became significant after 40 min (Fig. 3B).

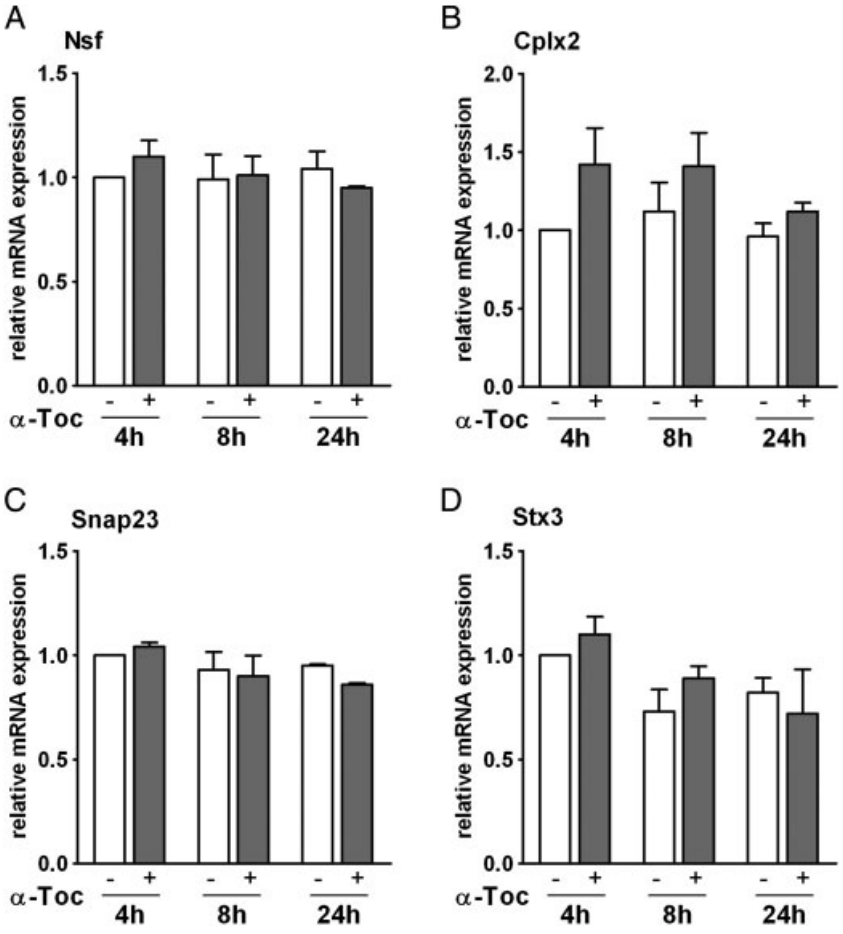

Figure 4. α-Tocopherol does not induce genes of the vesicular transport in RBL cells. Cells were seeded into 6-well plates and incubated with 100 μM *RRR*-α-tocopherol or ethanol control for the times indicated. mRNA expression was quantified by qPCR and normalized to Hprt1. Data are related to the 4 h time point of controls which was set to 1. Values are means of two individual experiments measured in triplicate ± SD. There were no significant differences. For further details see Section 2.

3.3 α-Tocopherol does not induce the expression of genes for proteins involved in the vesicular transport

A number of genes for proteins involved in the vesicular transport have been activated in α-tocopherol-supplemented mice [14]. Since some of them are also involved in the degranulation of mast cells, we tested whether they are affected by α-tocopherol and, thus, responsible for enhancement of degranulation. *N*-ethylmaleimide sensitive fusion protein (Nsf), complexin-2 (Cplx2), SNAP-23, syntaxin-3 (Stx3) are involved in the process of exocytosis in RBL cells [21, 22, 35, 36] and were, therefore, chosen for mRNA analysis. None of these genes was activated by 4, 8, and 24 h incubation with 100 μM α-tocopherol (Fig. 4).

4 Discussion

We show here that α-tocopherol enhances basal and PMA/ionomycin-stimulated degranulation from RBL mast cells. The enhancement was also observed with β-tocopherol but not with trolox, in which the long side chain is missing, or α-tocopheryl phosphate, in which the 6-OH group of the chromanol is blocked. This shows that the stimulation requires both, the side chain and the OH group. The effect of β-tocopherol was not necessarily expected. A lack of biological effects of β-tocopherol is often taken as proof that an observed function of α-tocopherol is independent from its antioxidant activity. A failure of β-tocopherol to replace α-tocopherol can also be explained by its degradation. Therefore, metabolism of both forms of tocopherol was tested by the estimation of α- and β-carboxyethyl hydroxychroman in the cell culture medium 72 h after tocopherol addition according to [37]. Neither α- nor β-carboxyethyl hydroxychroman could be detected whereas uptake of α- and β-tocopherol into the cells similarly increased with time and concentration (S. N. unpublished). Although it must not be overestimated β-tocopherol appears to have an effect in this system. Since the amount of β-tocopherol in the diet is negligible the experimental concentration was by far higher than those that can be reached *in vivo*.

The effect of α-tocopherol was not mediated by an up-regulation of genes for proteins of the vesicular transport. None of the tested genes (*Nsf*, *Cplx2*, *Snap23*, and *Stx3*) was affected, indicating that at least in the RBL cell model α-tocopherol does not directly regulate gene activities. This appears to be in contrast to previous *in vivo* findings where

feeding of α-tocopherol clearly up-regulated a cluster of cellular transport genes [14]. However, in the previous study feeding was maintained for 3 months as is usually done in most of the studies investigating vitamin E-regulated genes in microarrays. Consequently, it might be that a continuous stimulation/inhibition of processes involved in cellular trafficking led to a transcriptional activation/inhibition of genes for involved proteins as has been shown by the induction of syntaxin-1 *via* constant activation [38, 39].

A more direct effect of α-tocopherol might be the interference with protein phosphorylation, which plays a major role in the regulation of degranulation [40]. Whereas some SNAREs, like SNAP-23 appear to be active in the phosphorylated state [41], others have to be dephosphorylated for activity. One example is NSF, which regulates one of the key events in degranulation, the recycling of vesicles initiated by the disassembly of the vesicle/cell membrane fusion complex. Disassembly is stimulated by NSF, which is active in the dephosphorylated form [42]. Inhibition of NSF by tyrosine kinases induced the accumulation of SNARE complexes, lack of recycling, and decreased degranulation of RBL cells. Inhibition was reversed by the secretory vesicle-localized protein tyrosine phosphatase, PTP-MEG2 [43].

Also syntaxin-4 has to be dephosphorylated before it can assemble to SNAP23 [22]. Dephosphorylation is catalyzed by PP2A, which, like most of the proteins that are activated/inhibited by α-tocopherol, has to be recruited to the membrane to support RBL cell degranulation [44]. In accordance with the role of PPs in degranulation, okadaic acid, a potent inhibitor of protein phosphatases, inhibits degranulation of RBL cells [45], for review see [17]. Thus, the well-known activation of protein phosphatases by α-tocopherol [46] might be responsible for the stimulation of degranulation observed here.

Activity of protein tyrosine phosphatases can be regulated by the cellular hydroperoxide tone [47]. Hydroperoxides oxidize the nucleophilic cysteine in the catalytic site of the enzymes to sulfenic acid (prot-S-OH). Subsequently sulfenic acid is rapidly converted into a cyclic sulfenamide with the amide nitrogen of the neighbouring amino acid which abrogates the phosphatase activity [48]. PP2A belongs to serine/threonine phosphatases that have been less intensively investigated in relation to their inhibition by oxidation. In a more recent publication, however, it has been shown that this indeed can be the case [49]. Alternatively PP2A is potently inhibited by tyrosine phosphorylation [50] which can be reverted by PTPs.

Stimulation of mast cells results in the production of hydroperoxides. Sources are NADPH-oxidases [25] or lipoxygenases or cycloxygenases [26]. Since vitamin E inhibits all of these enzymes [51–55], it might prevent hydroperoxide production and subsequent phosphatase inhibition. Underlying mechanisms have been reported to be an inhibition of the recruitment of the enzymes or subunits there from to the membrane. However, also a redox function of vitamin E can still not be completely excluded. As outlined in [56], *in vivo* vitamin E is not able to act as an antioxidant in general. However, if concentrated in certain membrane domains it can reach a concentration which enables it to prevent oxidative modulation of proteins. Such membrane domains might be lipid rafts as suggested by Lemaire-Ewing *et al.* [57] or PUFA-containing phospholipid-rich domains as suggested by Atkinson *et al.* [58].

Although at time still speculative, it appears to be worth focussing on the function of vitamin E in membrane processes in future research. Impairment of degranulation, neurotransmitter release, cell fusion, *etc.*, can explain the prominent vitamin E-deficiency symptoms, *i.e.* disturbed female reproduction and neuronal dysfunction, much better than an unspecific antioxidant capacity.

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The authors have declared no conflict of interest.

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