ARTICLE
Journal of Cellular Biochemistry 116:398–407 (2015)

Journal of Cellular Biochemistry

Induction of VEGF Expression by Alpha-Tocopherol and Alpha-Tocopheryl Phosphate via PI3Kγ/PKB and hTAP1/SEC14L2-Mediated Lipid Exchange

Jean-Marc Zingg,* Angelo Azzi, and Mohsen Meydani

Vascular Biology Laboratory, JM USDA-Human Nutr. Res. Ctr. On Aging, Tufts University, Boston, MA 02111, USA

ABSTRACT

In several studies, vitamin E has been observed to influence angiogenesis and vasculogenesis. We recently showed that the phosphorylated form of α-tocopherol (αT), α-tocopheryl phosphate (αTP), increases the expression of the vascular endothelial growth factor (VEGF). Thus, αTP may act as an active lipid mediator increasing VEGF expression, angiogenesis, and vasculogenesis. Here, we investigated the molecular signaling mechanisms by which αTP induces VEGF expression using cultured HEK293 cells as model system. αT and more so αTP increased VEGF-promoter activity in a phosphatidylinositol-3-kinase gamma (PI3Kγ)-dependent manner. In contrast, after overexpression of PI3Kγ and/or protein kinase B (PKB), VEGF promoter activity was inhibited by αT and more so by αTP. Inhibition by αT and αTP was dependent on the lipid kinase activity of PI3Kγ, whereas an induction was seen with the protein kinase activity, consistent with a model in which PKB inhibition by αT or αTP occurs only when activated at the plasma membrane and possibly involves a phosphatase such as PHLPP1. PI3Kγ-induced VEGF expression was reduced when the human tocopherol-associated protein 1 (hTAP1/SEC14L2) was overexpressed suggesting formation of an inactive PI3Kγ/hTAP1 heterodimer, that could be reactivated by αT and more so by αTP. We suggest a novel signaling mechanism by which αTP stimulates PI3Kγ activity by stimulating hTAP-mediated phosphatidylinositol exchange and presentation to the enzyme and/or dissociation of an inactive heterodimer. At cellular level, hTAP may act as sensor for intracellular lipid information (location, type, and amount of lipid) and translate it into responses of PI3K-mediated signaling and gene expression. J. Cell. Biochem. 116: 398–407, 2015.

KEY WORDS: VEGF; SEC14-LIKE PROTEINS; TOCOPHEROL; PHOSPHOLIPIDS; VITAMIN E; KINASE; TOCOPHERYL PHOSPHATE; hTAP; SUPERNATANT PROTEIN FACTOR; SPF; PKB; PI3K; PI3Kγ

Vitamin E was discovered as a dietary factor essential for reproduction in rats [Evans and Bishop, 1922]. Since then, vitamin E has revealed many important molecular properties such as the scavenging of reactive oxygen and nitrogen species, and the modulation signal transduction and gene expression (reviewed in [Zingg, 2007]). A congenital disease, ataxia with vitamin E deficiency (AVED), which is characterized by low levels of α-tocopherol (αT) in plasma due to mutations in the α-tocopherol transfer protein gene (α-TTP), has been described [Ben Hamida et al., 1993]. An effect of vitamin E on angiogenesis and vasculogenesis has been the subject of several studies but the molecular mechanisms involved are not clear (reviewed in [Zingg et al., 2012]). Using THP-1 monocytes, we recently observed that the phosphorylated form of α-tocopherol (αT), α-tocopheryl phosphate (αTP), induced VEGF expression leading to increased angiogenesis in human umbilical vascular endothelial cell (HUVEC) in culture, whereas αT was not effective [Zingg et al., 2010a].

VEGF is an endothelial cell-specific mitogen that promotes angiogenesis and mediates, among other actions, a successful pregnancy to the final stage by inducing angiogenesis and vasculogenesis during placenta and embryonic development [Zygmunt et al., 2003; Haigh, 2008]. We proposed that fetal resorption in the vitamin E-deficient state may be the consequence of decreased expression of VEGF as a result of insufficient production of VEGF in the absence of vitamin E, followed by impaired formation of an extensive vascular net in the placenta leading to placental ischemia, and inadequate nutrient supply to the fetus [Zingg et al., 2012]. In support of this concept, increased placental angiogenesis and vascular

Grant sponsor: US Department of Agriculture; Grant number: 58-1950-0-014; Grant sponsor: National Institute of Food and Agriculture (NIFA/USDA); Grant number: 2010-65200-20395.

*Correspondence to: Jean-Marc Zingg, Vascular Biology Laboratory, Office 621, JM USDA-Human Nutr. Res. Ctr. On Aging, Tufts University, 711 Washington St Boston, MA 02111, USA. E-mail: Jean-Marc.Zingg@tufts.edu

Manuscript Received: 7 September 2014; Manuscript Accepted: 26 September 2014

Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 7 October 2014

DOI 10.1002/jcb.24988 • © 2014 Wiley Periodicals, Inc.

network formation was recently detected in pregnant ewes supplemented with vitamin E, possibly resulting from stimulation of VEGF expression [Kasimanickam et al., 2010]. Accordingly, in mice deficient in vitamin E as result of a knockout of the α-TTP gene (α-TTP$^{-/-}$), the absence of embryonic blood vessels in the trophoblast was observed, in addition to a failure of trophoblasts to survive and an abnormally forming small labyrinth [Jishage et al., 2001]. A lower level of VEGF in VEGF knockout mice (homozygous and even heterozygous) leads to abnormal embryonic blood vessel formation and impairs the production of a viable offspring [Carmeliet et al., 1996; Ferrara et al., 1996]. Likewise, successful blastocyst implantation for pregnancy establishment is critically dependent on the presence of sufficient expression of VEGF [Sengupta et al., 2007]. In the brain, VEGF in the Purkinje cell layer of cerebellum is necessary for proper granule cell migration [Ruiz de Almodovar et al., 2010], and mice not able to upregulate VEGF during hypoxia develop motor neuron degenerations that are symptoms related to vitamin E-deficient mice [Oosthuyse et al., 2001; Ulatowski et al., 2014].

Regulation of VEGF expression by vitamin E has been reported in several in vitro and in vivo experimental systems, in which either activation [Zhang et al., 2004; Daghini et al., 2007] or inhibition of VEGF [Tang and Meydani, 2001; Nespereira et al., 2003; Schindler and Mentlein, 2006] has been observed, but the molecular mechanisms of modulation of VEGF by vitamin E are yet to be understood (reviewed in [Zingg et al., 2012]). We reported that the induction of VEGF by αTP was due to upregulation of the phosphatidylinositol-3-kinase (PI3K) and/or protein kinase B (PKB/Akt) signaling pathway, a pathway that is downregulated by αT [Munteanu et al., 2006]. We suggested that αTP acts as an active "lipid mediator" and activates the PI3K/PKB pathway and that the un-phosphorylated αT inhibits its activation in THP-1 cells [Kempna et al., 2004; Munteanu et al., 2006; Numakawa et al., 2006]. Thus, the cellular response to αT may depend on the ratio of αT/αTP and thus on degree of their interconversion in different tissues or cell types. Based on these results we proposed that in certain tissues or conditions, αT is converted locally to αTP, which activates the PI3K/PKB signaling pathway and ultimately stimulates VEGF production leading to increased angiogenesis and vasculogenesis (reviewed in [Zingg et al., 2010b]).

In a recent study, we have shown that αT and more so αTP activate in vitro phosphatidylinositol-3-kinase gamma (PI3Kγ) activity by inducing tocopherol-associated protein 1 (hTAP1/SEC14L2)-mediated lipid exchange [Zingg et al., 2014]. By using HEK293 cells as a model system, we propose here novel molecular signaling mechanisms by which αT and more so αTP can both stimulate and inhibit the VEGF promoter depending on the activation state of the PI3Kγ/PKB pathway.

MATERIALS AND METHODS

MATERIALS

RRR-α-tocopherol (αT) (from Cognis, Cincinnati, OH) was dissolved in ethanol as 50 mM stock solutions and the concentrations confirmed spectrophotometrically. Stock solutions (50 mM) of α-tocopheryl phosphate (αTP), D-α-[5-methyl-14C]-tocopheryl phosphate (14C-αTP) (0.13 μCi/mg) (provided by Phosphagenics Ltd (Melbourne, Australia)) and D-α-[5-methyl-14C]-tocopherol (14C-αT) (57 mCi/mmole) (Amersham Pharmacia Biotech) were prepared in ethanol or water [Munteanu et al., 2004; Negis et al., 2007]. The specific inhibitor of PI3Kγ, AS-605240 (5-(Quinoxalin-6-ylmethylene)thiazolidine-2,4-dione) (Alexis Biochemicals, San Diego, CA) was dissolved in DMSO.

CELL CULTURE

HEK293 cells (ATCC, CRL-1573) were grown in Dulbecco's modified Eagle's medium, 10% fetal calf serum, and 2 mmol/L L-glutamine containing 100 μg/mL streptomycin and 100 U penicillin.

TRANSFECTION

HEK293 cells (70% confluent) were transfected with pCGCG-luc, a reporter plasmid containing 3169bp of the human VEGF promoter in front of the *firefly* luciferase gene (kindly provided by S. J. Prior, University of Maryland, Baltimore, MD [Prior et al., 2006]), with expression vectors for phTAP1, phTAP2, phTAP3, or phTTP [Kempna et al., 2003; Zingg et al., 2008], PI3Kγ-wt, PI3Kγ-mut, PI3Kγ-L, PI3Kγ-P, PI3Kγ-LP (all kindly provided by Dr. H. A. Rockman, Duke University, Durham, NC [Ma et al., 1998; Naga Prasad et al., 2005]), pPKBwt, pPKB(R25C), and pPKB(K179M) (kindly provided by Dr. J. Downward, Imperial Cancer Research Fund, London, UK [Watton and Downward, 1999; Munteanu et al., 2006]), together with the *Renilla* internal control plasmid pRL-TK (Promega, Madison, WI), for 3 h using Fugene (Promega) as transfection reagent, and then treated with αT (40 μM) or αTP (40 μM) for additional 21 h. Extracts were prepared, and promoter activities were measured using the Dual-Luciferase assay kit (Promega) by means of a GLOmax luminometer (Promega). The VEGF promoter-*firefly* luciferase activities were normalized to the thymidine kinase promoter-*Renilla* luciferase activities, and the activities of the control transfections were set to 100%.

UPTAKE OF αT AND αTP

HEK293 cells were plated in 24 wells plates at 70% confluency overnight, transfected for 24 h with expression vectors for hTAP1, hTAP2, hTAP3, or hTTP, or empty control vector pMH [Kempna et al., 2003], and after changing medium incubated with ^{14}C-αT (40 μM) or ^{14}C-αTP (40 μM) for 5 h. Cells were washed with PBS, trypsinized, removed to eppendorf tubes and washed two times with PBS. The radioactivity associated with the cell pellet was measured using a scintillation counter and the data plotted as % of total input.

STATISTICAL ANALYSIS

All values are expressed as the mean ± standard error of the mean (SEM) as explained in the figure legends. Student's *t*-test was used to determine the significant differences between two conditions. A $P < 0.05$ was considered as significant and indicated by * or # in the graphs.

RESULTS

αT AND αTP REGULATE VEGF PROMOTER ACTIVITY VIA MODULATION OF PI3Kγ AND PKB

Using gene expression arrays and THP-1 monocytes, we recently detected a number of genes, such as VEGF, that are upregulated specifically by αTP [Zingg et al., 2010a]. αTP stimulated PKB (Ser473)

phosphorylation and free radicals production in a wortmannin and AS-605240-sensitive manner, suggesting the involvement of phosphatidylinositol-3-kinases (PI3K) [Zingg et al., 2010a, 2014], whereas αT antagonized these reactions. Interestingly, VEGF [Dutra et al., 2011], angiogenesis [Madeddu et al., 2008; Siragusa et al., 2010], as well as the production of free radicals [Lehmann et al., 2009] are all regulated by PI3Kγ suggesting that αTP may indeed activate these events via PI3Kγ.

To assess further whether αTP activates VEGF expression via PI3Kγ, expression vectors for wild-type or inactive mutant PI3Kγ (pPI3Kγ-wt or pPI3Kγ-mut, respectively [Ma et al., 1998; Naga Prasad et al., 2005]) were transfected together with a human VEGF-promoter-luciferase reporter vector (pCGCG-Luc [Prior et al., 2006]) into HEK293 cells. These cells were chosen since unlike THP-1 monocytes, transfection of HEK293 is highly efficient allowing co-transfection of multiple vectors. Moreover, HEK293 cells may represent a suitable model since they express low levels of VEGF [Liang et al., 2002] and PI3Kγ [Hirsch et al., 2000], yet VEGF can be activated [Kurig et al., 2009; Zingg et al., 2012].

VEGF-promoter activity was increased with αTP (Fig. 1A), and in contrast to THP-1 cells, an intermediate activation was also observed by αT possibly resulting from its conversion to αTP in these cells [Zingg et al., 2010a, 2012, 2014]. Overexpression of pPI3Kγ-wt strongly activated VEGF-promoter activity, but in this case αT and αTP effects were inhibitory (Fig. 1A). These results suggest that αT and more so αTP can increase the endogenous PI3Kγ activity, which is similar to the previously described in vitro results [Zingg et al., 2014]. However, since after PI3Kγ overexpression, the effects of αT and αTP on PI3Kγ/PKB/VEGF signaling were inhibitory (Fig. 1A), it is plausible that additional regulation may occur when the levels of PI3Kγ are high in cells. In these situations, PKB is most likely membrane-attached and fully activated by phosphorylation as in HMC-1 mastocytoma cells or other cancer cells [Munteanu et al., 2006], and αT and αTP may either interfere with PKB membrane translocation and/or activate phosphatases able to de-phosphorylate and inactivate PKB, such as PP2A [Ricciarelli et al., 1998; Wei and Xia, 2006] or PH domain and leucine-rich repeat protein phosphatase 1 (PHLPP1) [Huang et al., 2013] (reviewed in [Zingg, 2007]). In fact, when pPKBwt was overexpressed, αT and more so αTP-inhibited VEGF-promoter activity, but not when the mutants pPKB(R25C) (mutated in the PH-domain required for membrane translocation) and pPKB(K179M) (mutated in the catalytic domain) were overexpressed, both without (Fig. 1B) and with (Fig. 1C) additional overexpression of PI3Kγ-wt. These results are in line with a model in which the inhibitory effect occurs only with membrane-associated activated PKB which becomes de-phosphorylated as result of activation of PHLPP1 by αT, αTP or PI3P [Huang et al., 2013].

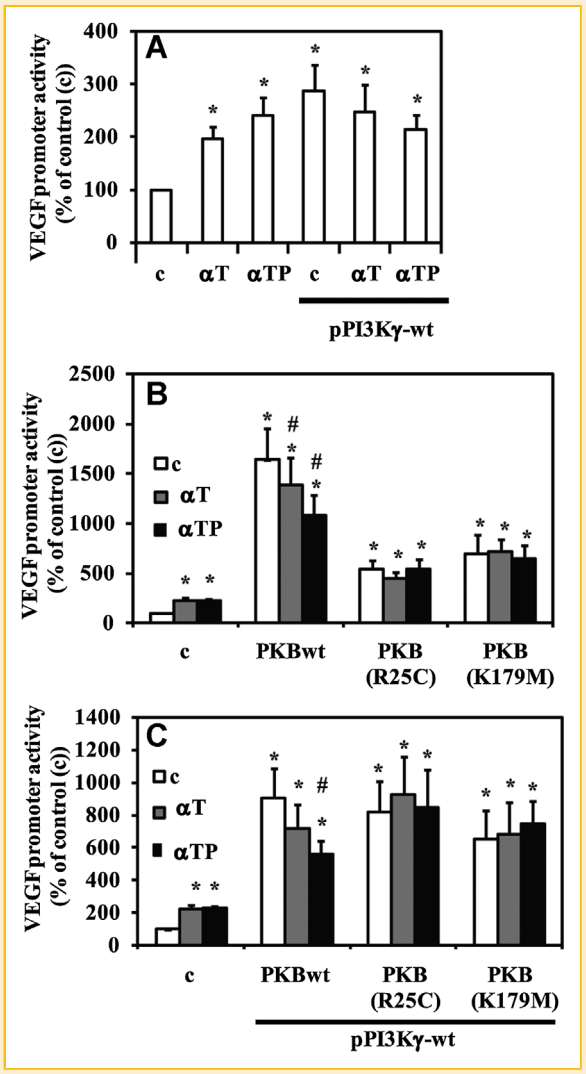

Fig. 1. αT and αTP increase VEGF expression in HEK293 cells, but inhibit it when activated by PI3Kγ or PKB. (A) The treatment with αTP, and less with αT, induces VEGF promoter activity in human embryonic kidney 293 (HEK293) cells; when PI3Kγ is overexpressed, αT and more so αTP reduce PI3Kγ-induced VEGF promoter activity (±SEM, n = 4, *$P < 0.05$ relative to untreated control (c)). (B) αT and more so αTP inhibit VEGF-promoter activity after overexpression of pPKBwt, but not when the mutants pPKB(R25C) (mutated in the PH-domain) and pPKB(K179M) (mutated in the catalytic domain) are overexpressed (n = 4, *$P < 0.05$ relative to untreated control (c), both in the absence (B) and presence (C) of PI3Kγ overexpression (n = 4, *$P < 0.05$ relative to untreated control (c), #$P < 0.05$ relative to untreated pPKBwt control).

αT AND αTP INCREASE VEGF PROMOTER ACTIVITY IN AN hTAP1-DEPENDENT MANNER VIA MODULATION OF PI3Kγ

In previous studies, the recombinant human tocopherol-associated protein 1 (hTAP1/SEC14L2) reduced the in vitro activity of the PI3Kγ possibly by sequestering the substrate phosphatidylinositol (PI) and/or forming an inactive PI3Kγ/hTAP1 heterodimer; addition αT or αTP stimulated PI3Kγ, possibly by facilitating egress of PI from hTAP1 to the enzyme [Kempna et al., 2004; Zingg et al., 2014]. In view of these in vitro results it appeared that in HEK293 cells PI3Kγ is only activated by αTP when hTAP1 is overexpressed. Therefore, it was interesting to investigate, whether overexpression of hTAP would alter the response of PI3Kγ/PKB/VEGF to αT or αTP. To assess whether the ability of αT or αTP to activate PI3Kγ and the VEGF-promoter is regulated by hTAP1, the above experiments (Fig. 1A) were performed in the presence of an expression vector for hTAP1 (phTAP1) or of an empty control vector (pMH), both in the presence of wild-type or inactive mutant PI3Kγ (pPI3Kγ-wt or pPI3Kγ-mut,

respectively). Similar to the experiments done in vitro [Zingg et al., 2014], hTAP1 expression reduced the ability of PI3Kγ-wt to activate the VEGF-promoter, and the addition of αT and more so αTP reactivated hTAP1-inhibited PI3Kγ and VEGF-promoter activity (Fig. 2A). When PI3Kγ-mut was used, these regulatory effects of hTAP1 and αT or αTP were absent or much weaker (Fig. 2B). VEGF-promoter activity was only induced by αT and αTP after over-expression of hTAP1, but not of the related proteins hTAP2/SEC14L3, hTAP3/SEC14L4, or hTTP (Fig. 2C). Inhibition of PI3Kγ by the specific inhibitor AS-605240 (1 μM) prevented induction of VEGF promoter activity, suggesting that in these cells PI3Kγ is the isoform responsive to αT and αTP (Fig. 2D). Interestingly, activation of the VEGF promoter by αT and αTP still occurred in the presence of the src inhibitor PP2, indicating that this tyrosine kinase, which acts upstream of the PI3K, is not involved in the observed effects (Fig. 2D). These results suggest that increase of VEGF promoter activity by αT and more so αTP involves PI3Kγ and hTAP1, whereas, in the absence of hTAP1 when only PI3Kγ is overexpressed αT and αTP suppress VEGF promoter activity (Figs. 1A and 2A).

DIFFERENTIAL REGULATORY EFFECTS OF αT AND αTP ON PI3Kγ LIPID AND PROTEIN KINASE ACTIVITY

PI3Kγ can either phosphorylate PI or several proteins including tropomyosin, MAPK, Ras, 4EBP1, or PKCα [Bondeva et al., 1998; Naga Prasad et al., 2005]. Protein phosphorylation activity has been first characterized as auto-phosphorylation and has been measured only with the cytosolic form of PI3Kγ [Bondeva et al., 1998; Dolle et al., 2011]. Moreover, PI3Kγ can serve as scaffold for proteins such as β-ARK, PDE3B, and PDE4, for kinase-independent regulation of cellular processes [Dolle et al., 2011]. To evaluate whether the regulatory effects of αT and αTP on VEGF expression occur as a result of phosphorylation of protein or lipid (PI), PI3Kγ mutant forms [Naga Prasad et al., 2005], with either only lipid kinase activity (PI3Kγ-L), only protein kinase activity (PI3Kγ-P), or both reconstituted lipid and protein kinase activity (PI3Kγ-LP) were transfected into HEK293 cells with pMH (empty control vector) or with hTAP1 co-transfection, and the effects of αT and αTP treatment on VEGF expression measured. Interestingly, VEGF expression mediated by lipid kinase activity of PI3Kγ-L was inhibited by αT and αTP both in the presence and absence of hTAP1 (Fig. 3A). Whereas, VEGF expression mediated by PI3Kγ-P protein kinase activity was stimulated (Fig. 3B). In contrast, for the reconstituted PI3Kγ-LP lipid and protein kinase the two responses were apparently almost neutralized by each other (Fig. 3C). Overexpression of hTAP1 mainly increased VEGF expression when PI3Kγ protein kinase was also expressed (Fig. 3B). These results suggest that in cells the stimulatory effects of αT and more so αTP on VEGF expression may involve the cytosolic protein kinase activity of PI3Kγ, possibly requiring hTAP1-

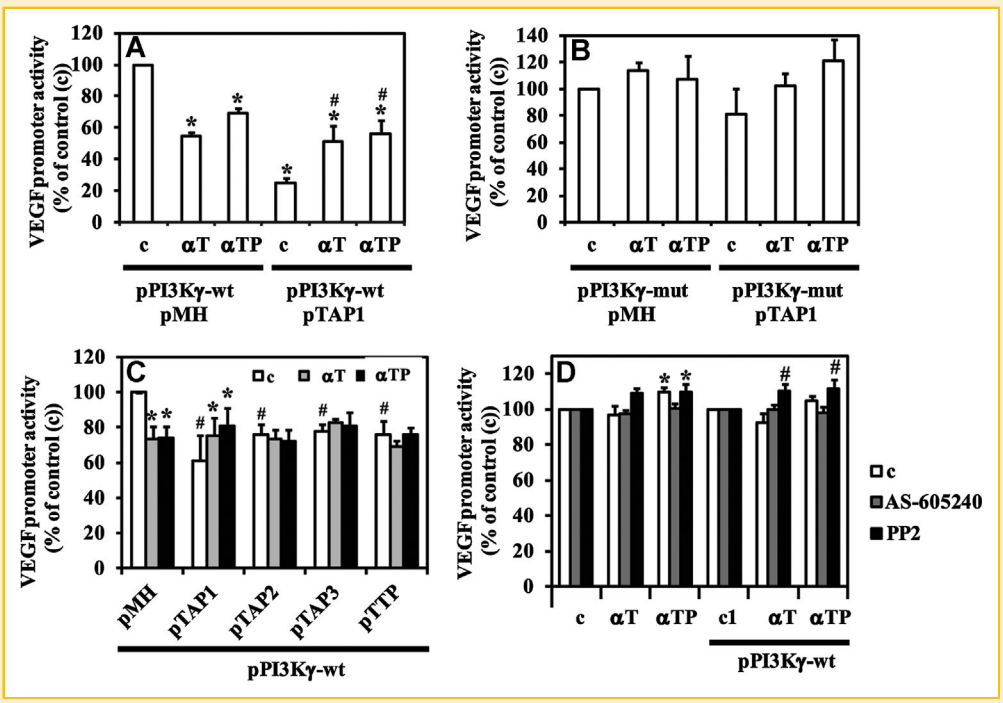

Fig. 2. Modulation of VEGF expression by hTAPs, α-TTP, αT, or αTP. (A) Overexpression of hTAP1 reduces PI3Kγ-induced VEGF promoter activity; in this case αT and more so αTP reactivate VEGF promoter activity (±SEM, n = 4, *$P < 0.05$, relative to empty control vector (pMH), #$P < 0.05$ relative to untreated control), most likely by enhancing lipid exchange and presentation of phosphatidyl inositol to PI3Kγ by means of the nanoreactor formed by hTAP1 similar to previously published in vitro results with recombinant enzymes [Zingg et al., 2014]. With empty control vector (pMH), inhibition of VEGF expression was seen. (B) VEGF promoter activity is only weakly changed by αT and αTP after overexpression of a mutant form of PI3Kγ; in this case overexpression of hTAP1 only weakly reduces VEGF promoter activity that is restored by αT and αTP (±SEM, n = 4). (C) Overexpression of hTAP2, hTAP3, or hTTP did not change the response of the VEGF-promoter to αT or αTP (±SEM, n = 4, *$P < 0.05$, relative to untreated control, #$P < 0.05$ relative to untreated empty control vector (pMH)). (D) Induction of VEGF promoter activity by αTP is inhibited by the specific PI3Kγ inhibitor AS-605240 (1 μM), but not by the c-src tyrosine kinase inhibitor PP2 (10 μM) (± SEM, n = 4, *$P < 0.05$ relative to control (c), #$P < 0.05$ relative to control (c1)).

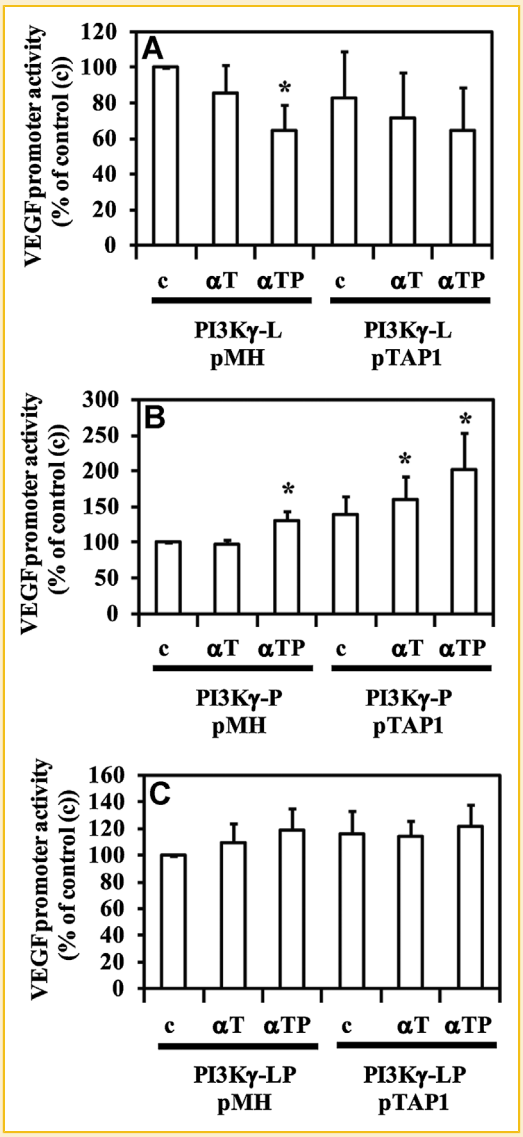

Fig. 3. Modulation of VEGF expression by PI3Kγ lipid kinase (PI3Kγ-L), PI3Kγ protein kinase (PI3Kγ-P), or reconstituted PI3Kγ lipid and protein kinase (PI3Kγ-LP) activity and hTAP1. (A) αT and more so αTP reduced VEGF promoter activity in PI3Kγ-L overexpressing HEK293 cells, both in the presence of empty control vector (pMH)) or of hTAP1 ($n = 6$, $^*P < 0.05$ relative to untreated control (c)). (B) αT and more so αTP increased VEGF promoter activity in PI3Kγ-P overexpressing HEK293 cells, both in the presence of empty control vector (pMH) or of hTAP1 ($n = 6$, $^*P < 0.05$ relative to untreated control (c)). (C) αT and αTP weakly increased VEGF promoter activity in reconstituted PI3Kγ-LP overexpressing HEK293 cells, both in the presence of empty control vector (pMH) or of hTAP1 ($n = 6$, $^*P < 0.05$ relative to untreated control (c)).

mediated transport of αT, αTP, or PI to cytosolic PI3Kγ and/or allosteric activation of its kinase activity by these lipids. Whereas the inhibitory effects of αT and αTP on VEGF expression may occur when the lipid kinase activity of PI3Kγ generates sufficient PI3P to trigger PKB translocation to the plasma membrane and then becomes inactivated by the PKB phosphatase PHLPP1 [Huang et al., 2013].

OVEREXPRESSION OF hTAPs AND α-TTP INFLUENCES CELLULAR αT AND αTP UPTAKE

It is plausible that the higher stimulation of VEGF promoter activity by αTP in the presence of hTAP1 is the result of increased transport and cellular uptake of αTP. Thus, hTAP1/2/3, and as control α-TTP, were overexpressed in HEK293 cells and the uptake of radioactive D-α-[5-methyl-14C]-tocopherol (14C-αT) or D-α-[5-methyl-14C]-tocopheryl phosphate (14C-αTP) measured. Within the time of the experiment (5 h), about half of αTP was taken up when compared to αT (Fig. 4). Thus, since αTP despite a reduced uptake was more active than αT, αTP may act as an intact and more active molecule. Cellular αT and αTP uptakes were differently regulated, in that hTAP1, hTAP3, and hTTP-inhibited αT uptake (Fig. 4A), whereas they increased it for αTP (Fig. 4B). Thus, since the three proteins hTAP1, hTAP3, and hTTP induced an increased αTP uptake, but only one, hTAP1, increased VEGF expression (Fig. 2C), the effects seen on VEGF expression cannot be solely due to increased αTP uptake. Most likely increased VEGF expression is the consequence of enhanced lipid/protein catalytic activity of PI3Kγ, e.g., as result of increased lipid presentation and exchange by hTAP1 and/or dissociation of an inactive heterodimer [Ile et al., 2006; Zingg et al., 2014]. However, it remains to be determined to what degree binding and activation of a receptor and/or transporter by αTP at the plasma membrane such as CD36 [Zingg et al., 2014] contributes to enhanced activation of endogenous PI3Kγ activity by αTP in certain cell types.

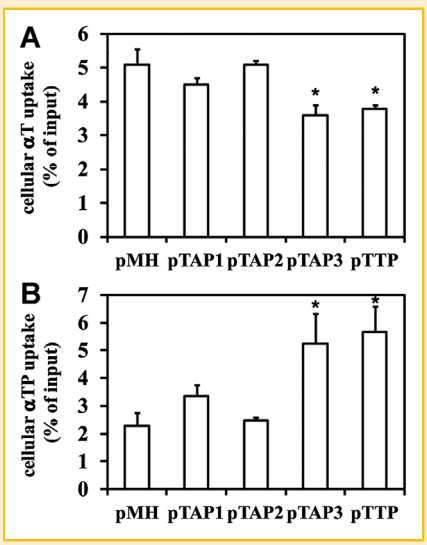

Fig. 4. Uptake of αTP into HEK293 cells by hTAPs and hTTP proteins. HEK293 were transfected with expression vectors for hTAP1, hTAP2, hTAP3, hTTP, or empty control vector pMH [Kempna et al., 2003], and then treated with (A) ^{14}C-αT (40 μM) or (B) ^{14}C-αTP (40 μM) for 5 h. Cellular uptake of αT or αTP was measured as described in materials and method. The radioactivity associated with the cell pellet was measured using a scintillation counter, and the data expressed as % of total input (±SEM, $n = 4$, $^*P < 0.05$).

DISCUSSION

In previous studies, we observed increased expression of VEGF and of angiogenic activity secreted from THP-1 monocytes after in vitro treatment with αTP [Zingg et al., 2010a, 2012, 2014]. Here, we analyzed in detail the signaling mechanisms by which αTP induces VEGF by using a human embryonic kidney cell line (HEK293) as a model system. Although the relevance of embryonic kidney cells for angiogenesis is to date unclear, supplementation of normal pigs with vitamin E and C increased VEGF expression and angiogenesis in the kidney suggesting a regulatory role of αT and/or αTP for angiogenesis in this tissue [Daghini et al., 2007].

We find that αTP and to a lesser degree also αT increases VEGF expression. Some activation by αT suggests either an intrinsic lower ability of this compound to activate PI3Kγ or some conversion to αTP by αT kinase activity in these cells [Zingg et al., 2014]. Using a specific inhibitor of PI3Kγ, AS-605240, we show that VEGF induction by αTP is mediated by PI3Kγ, which is expressed in HEK293 at low levels [Hirsch et al., 2000]. Interestingly, when PI3Kγ and/or PKB were overexpressed, αT and more so αTP inhibited VEGF expression, suggesting that after activation and translocation to the plasma membrane, these enzymes become inhibitable by αT and αTP, most likely by increasing PKB dephosphorylation by PHLPP1 [Huang et al., 2013]. Moreover, overexpression of hTAP1 with and without PI3Kγ inhibited VEGF expression, but in this situation αT and more so αTP could stimulate it. These results suggest that in the presence of hTAP1, αT and more so αTP increased PI3Kγ activity either by disrupting an inactive PI3Kγ/hTAP1 heterodimer or by facilitating lipid exchange and presentation, as supported also by our earlier in vitro data [Zingg et al., 2012].

As outlined in the reaction scheme (Fig. 5), the ratio between hTAPs, enzymes and different lipid ligands may be an important factor in determining the cellular response to αT and αTP and activation of PI3Kγ and VEGF expression. The source of αT and αTP (intracellular, extracellular), the efficiency of transport and the conversion of αT to αTP by αT kinase or vice versa of αTP to αT by a phosphatase in a given tissue and cell type [Zingg et al., 2010a] may explain that in response to αT, both induction [Numakawa et al., 2006] and inhibition [Munteanu et al., 2006] of the PI3K/PKB pathway has been observed. Additional regulatory mechanisms of PI3Kγ regulation by αTP may occur, as suggested by experiments with mutant PI3Kγ enzymes either having only lipid kinase, protein kinase, or both reconstituted lipid and protein kinase activities. In these experiments, αT and more so αTP inhibited VEGF expression when the lipid kinase was active triggering PKB presence at the plasma membrane, whereas they stimulated VEGF expression via protein kinase activation. However, when both kinase activities were present an intermediate response was obtained. Since protein kinase activity was mainly observed with cytosolic PI3Kγ [Bondeva et al., 1998; Dolle et al., 2011], hTAP1 may play a double role by transporting activating lipids to cytosolic locations, and/or by stimulating catalytic turnovers by increasing lipid exchange and presentation to enzymes.

In this study we focused only on the regulation of the VEGF promoter activity by αT and αTP in a cell culture system, without an

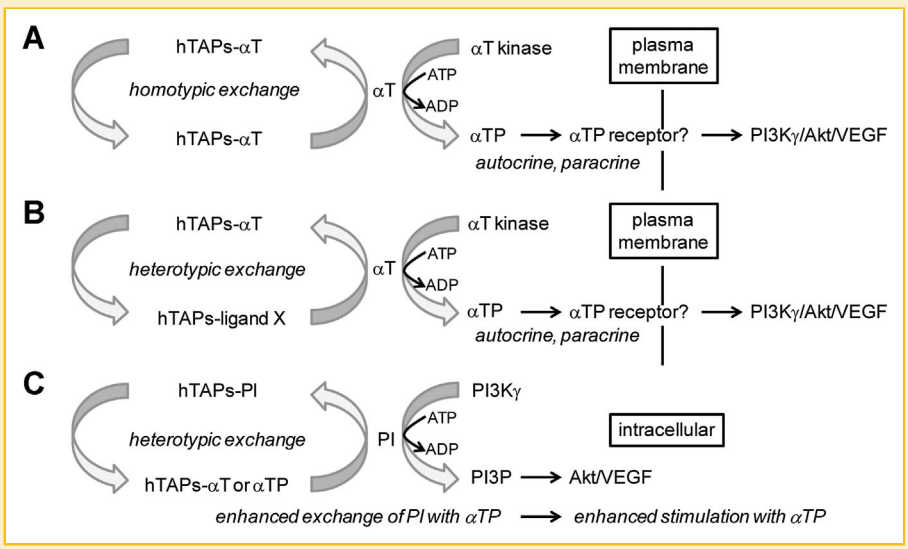

Fig. 5. Reaction scheme showing lipid exchange with hTAPs and αT, αTP, or PI and stimulation of PI3Kγ/PKB/VEGF based on a molecular model for SEC14p-like proteins [Ile et al., 2006; Bankaitis et al., 2010; Ghosh and Bankaitis, 2011]. (A) The hTAPs may stimulate phosphorylation of αT by an αT kinase by homotypic αT exchange, or (B) by heterotypic αT exchange with an unknown lipid ligand X, such as phosphatidylcholine (PC) or PI. (C) hTAPs may stimulate phosphorylation of PI by PI3Kγ by heterotypic exchange of PI with αT, αTP or other tocopherols and tocotrienols each with different efficiency. αTP produced in (A) and (B) (e.g., in VSMC, [Zingg et al., 2014]) may be secreted and when extracellular may either stimulate an αTP receptor leading to activation of PI3Kγ/PKB/VEGF (paracrine/autocrine), or when intracellular as in (C) directly stimulate PI3Kγ by heterotypic exchange with PI. Other enzymes such as other PI3K, PI4K, phospholipases or squalene epoxidase may use analogous reaction mechanisms with their respective substrate lipids.

in depth analysis of the transcription factors activated by PI3Kγ/PKB stimulation. The in vivo regulation of the VEGF expression is very complex and several transcription factors that are regulated by PI3K/PKB such as Hif1α and CREB are involved [Pages and Pouyssegur, 2005]. Moreover, additional post-transcriptional regulatory mechanisms of VEGF mRNA and protein induction by αTP may be involved that may need to be investigated further, such as alternative splicing and mRNA stability, miRNAs, and proteasome inhibition [Munteanu et al., 2007; Arcondeguy et al., 2013].

In monocytes/macrophages, the observed stimulation of VEGF production may enhance reparative angiogenesis and tissue remodeling as observed after experimental myocardial infarction occurring in a PI3Kγ/PKB-dependent manner [Siragusa et al., 2010]. In the placenta, the stimulation of VEGF expression and vasculogenesis potentially by αTP may explain the essentiality of vitamin E against fetal resorption (reviewed in [Zingg et al., 2012]). Moreover, induction of VEGF expression may explain other effects of vitamin E, such as the prevention of ischemia/reperfusion injury in the cardiovascular and nervous system (Lambrechts et al., 2003; Zhang et al., 2004; Mukherjee et al., 2008). Additionally, αT, after conversion to αTP, may possibly promote survival of muscle cells and neurons, stimulate neurite outgrowth, and prevent neurodegenerative processes [Jin et al., 2006; Sakowski et al., 2009; Ulatowski et al., 2014]. A rapid generation of a functional vascular system triggered by αTP and VEGF may be particularly required in newly formed tissues and organs such as the placenta, embryo, and possibly needed during repair after tissue injury, thus avoiding nutrient/oxygen deprivation and ischemia.

It should be emphasized that in this study a relatively high concentration of αT and αTP was used for cell treatments; such high concentrations may occur only for αT in plasma and tissues after supplementation. The tissue levels of αTP are generally low since foods contain only low amounts of αTP [Ogru et al., 2003] and since the uptake into the body as intact molecule is not efficient [Libinaki et al., 2005; Gianello et al., 2005, 2007; Mustacich et al., 2007; Zingg et al., 2010a]. A local synthesis of αTP in specific cells may thus be necessary to reach high enough concentrations for its function at precise subcellular sites relevant for signaling. In our previous experiments, no direct effect was detected when endothelial cells were exposed to αTP [Zingg et al., 2010a], suggesting that αTP may orchestrate angiogenesis in endothelial cells by triggering VEGF production and secretion from neighboring cells in the vascular system (e.g., from vascular smooth muscle cells (VSMC), monocytes/macrophages, kidney cells, or trophoblasts [Pennington et al., 2012]).

We hypothesize that in the normal physiological situation, the pro-angiogenic signal coming from αTP can be switched off by dephosphorylation to αT [Zingg et al., 2010a]. It is possible that chronic activation of PI3K/PKB/VEGF by αTP in a pathological situation (e.g., by aberrant activation of αT kinase or inactivation of αTP phosphatase in tumor tissues) may facilitate the development, growth, and migration of neoplastic cells by increasing VEGF expression, angiogenesis, and tumor growth. On the other hand, increased expression of alkaline phosphatase in certain cancer cells may prevent the stimulatory effects of αTP. These possible correlations may be at the basis of the finding that no adverse effects have been reported during dietary supplementation with αTP in animal models [Libinaki et al., 2005; Gianello et al., 2007], and high concentrations of αTP (possibly beyond the hydrolytic capacity of the phosphatases) inhibited cell proliferation and migration, and induced apoptosis in cancer cell lines [Munteanu et al., 2004; Rezk et al., 2007; Saitoh et al., 2009; Zingg et al., 2010a]. In fact, our present results suggest that αTP rather reduces VEGF expression when the PI3K/PKB signaling pathway is activated, as it often occurs in cancer cells.

So far no genetic disease has been linked to hTAPs [Nile et al., 2010], but specific polymorphisms in hTAP1 have been linked with prostate cancer risk [Wright et al., 2009; Zingg and Azzi, 2009]. Reduced expression of hTAP1 in proliferating prostate and breast cancer cells indicates that it interferes with cell proliferation [Ni et al., 2005; Wen et al., 2007; Wang et al., 2009; Johnykutty et al., 2009], possibly as shown here as a result of hTAP-mediated decreased signaling to PI3Kγ/PKB/VEGF, Ras/Erk, and Raf kinase [Johnson and Kornfeld, 2010]. The binding of the synthetic vitamin E analog α-tocopheryl succinate (αTS) to hTAPs inhibits cell proliferation and induces apoptosis by affecting the Ras, Mek/Erk and PI3K/PKB pathways, and αT or αTP may act in a similar manner [Ni et al., 2005; Donapaty et al., 2006; Neuzil et al., 2006]. Moreover, downregulation of hTAP1 in tumor tissues may remove its inhibitory effect on PI3K/PKB/VEGF and facilitate the development, proliferation, and migration of neoplastic cells by increasing angiogenesis and tumor growth (reviewed in [Zingg et al., 2012]). In this situation, excess vitamin E may stimulate tumor angiogenesis and e.g., contribute to a higher risk for prostate cancer observed in some studies with vitamin E supplementation [Lippman et al., 2009; Klein et al., 2011].

Taken together, we describe novel signaling pathways by which αTP increases VEGF expression involving PI3Kγ and hTAP1-mediated lipid exchange, whereas VEGF expression is decreased when PI3Kγ/PKB are overexpressed. The enhanced expression of VEGF induced by αTP may explain not only the essential roles of vitamin E on reproduction, but also its effects against ischemia/reperfusion injury and during wound healing. It may also serve as a survival factor for brain and muscle cells.

ACKNOWLEDGMENTS

This study was supported in part by grants from the US Department of Agriculture, under Agreement No. 58-1950-0-014 and the National Institute of Food and Agriculture (NIFA/USDA), grant No. 2010-65200-20395 (MM, JMZ). We thank Dr. S. J. Prior (University of Maryland, Baltimore, MD) for providing the human VEGF-promoter-luciferase construct, Dr. H. A. Rockman (Duke University, Durham, NC) for PI3Kγ−wt, PI3Kγ−mut PI3Kγ-L, PI3Kγ-P, and PI3Kγ-LP expression vectors, and Dr. J. Downward (Imperial Cancer Research Fund, London, UK) for pPKBwt, pPKB (R25C), and pPKB(K179M) expression vectors. We thank Stephanie Marco for her assistance in the preparation of this manuscript. None of the authors has declared any potential competing interests. Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the author(s) and do not necessarily reflect the view of the U.S. Department of Agriculture.

REFERENCES

Arcondeguy T, Lacazette E, Millevoi S, Prats H, Touriol C. 2013. VEGF-A mRNA processing, stability and translation: a paradigm for intricate regulation of gene expression at the post-transcriptional level. Nucleic Acids Res 41:7997–8010.

Bankaitis VA, Mousley CJ, Schaaf G. 2010. The Sec14 superfamily and mechanisms for crosstalk between lipid metabolism and lipid signaling. Trends Biochem Sci 35:150–160.

Ben Hamida M, Belal S, Sirugo G, Ben Hamida C, Panayides K, Ionannou P, Beckmann J, Mandel JL, Hentati F, Koenig M, et al. 1993. Friedreich's ataxia phenotype not linked to chromosome 9 and associated with selective autosomal recessive vitamin E deficiency in two inbred Tunisian families. Neurology 43:2179–2183.

Bondeva T, Pirola L, Bulgarelli-Leva G, Rubio I, Wetzker R, Wymann MP. 1998. Bifurcation of lipid and protein kinase signals of PI3Kgamma to the protein kinases PKB and MAPK. Science 282:293–296.

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A. 1996. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 380:435–439.

Daghini E, Zhu XY, Versari D, Bentley MD, Napoli C, Lerman A, Lerman LO. 2007. Antioxidant vitamins induce angiogenesis in the normal pig kidney. Am J Physiol Renal Physiol 293:F371–F381.

Dolle C, Westermann M, Schilli-Westermann M, Kirsch C. 2011. Influence of liposome composition and membrane binding on protein kinase activity of PI3Kgamma. Biochem Biophys Res Commun 404:968–973.

Donapaty S, Louis S, Horvath E, Kun J, Sebti SM, Malafa MP. 2006. RRR-alpha-tocopherol succinate down-regulates oncogenic Ras signaling. Mol Cancer Ther 5:309–316.

Dutra RC, Cola M, Leite DF, Bento AF, Claudino RF, Nascimento AF, Leal PC, Calixto JB. 2011. Inhibitor of PI3Kgamma ameliorates TNBS-induced colitis in mice by affecting the functional activity of CD4+CD25+FoxP3+ regulatory T cells. Br J Pharmacol 163:358–374.

Evans HM, Bishop KS. 1922. Fetal resorption. Science 55:650.

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. 1996. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 380:439–442.

Ghosh R, Bankaitis VA. 2011. Phosphatidylinositol transfer proteins: negotiating the regulatory interface between lipid metabolism and lipid signaling in diverse cellular processes. Biofactors 37:290–308.

Gianello R, Hall WC, Kennepohl E, Libinaki R, Ogru E. 2007. Subchronic oral toxicity study of mixed tocopheryl phosphates in rats. Int J Toxicol 26:475–490.

Gianello R, Libinaki R, Azzi A, Gavin PD, Negis Y, Zingg JM, Holt P, Keah HH, Griffey A, Smallridge A, West SM, Ogru E. 2005. Alpha-tocopheryl phosphate: a novel, natural form of vitamin E. Free Radic Biol Med 39:970–976.

Haigh JJ. 2008. Role of VEGF in organogenesis. Organogenesis 4:247–256.

Hirsch E, Katanaev VL, Garlanda C, Azzolino O, Pirola L, Silengo L, Sozzani S, Mantovani A, Altruda F, Wymann MP. 2000. Central role for G protein-coupled phosphoinositide 3-kinase gamma in inflammation. Science 287:1049–1053.

Huang PH, Chuang HC, Chou CC, Wang H, Lee SL, Yang HC, Chiu HC, Kapuriya N, Wang D, Kulp SK, Chen CS. 2013. Vitamin E Facilitates the Inactivation of the Kinase Akt by the Phosphatase PHLPP1. Sci Signal 6:ra19.

Ile KE, Schaaf G, Bankaitis VA. 2006. Phosphatidylinositol transfer proteins and cellular nanoreactors for lipid signaling. Nat Chem Biol 2:576–583.

Jin K, Mao XO, Greenberg DA. 2006. Vascular endothelial growth factor stimulates neurite outgrowth from cerebral cortical neurons via Rho kinase signaling. J Neurobiol 66:236–242.

Jishage K, Arita M, Igarashi K, Iwata T, Watanabe M, Ogawa M, Ueda O, Kamada N, Inoue K, Arai H, Suzuki H. 2001. Alpha-tocopherol transfer protein is important for the normal development of placental labyrinthine trophoblasts in mice. J Biol Chem 276:1669–1672.

Johnykutty S, Tang P, Zhao H, Hicks DG, Yeh S, Wang X. 2009. Dual expression of alpha-tocopherol-associated protein and estrogen receptor in normal/benign human breast luminal cells and the downregulation of alpha-tocopherol-associated protein in estrogen-receptor-positive breast carcinomas. Mod Pathol 22:770–775.

Johnson KG, Kornfeld K. 2010. The CRAL/TRIO and GOLD domain protein TAP-1 regulates RAF-1 activation. Dev Biol 341:464–471.

Kasimanickam RK, Kasimanickam VR, Rodriguez JS, Pelzer KD, Sponenberg PD, Thatcher CD. 2010. Tocopherol induced angiogenesis in placental vascular network in late pregnant ewes. Reprod Biol Endocrinol 8:86.

Kempna P, Reiter E, Arock M, Azzi A, Zingg JM. 2004. Inhibition of HMC-1 mast cell proliferation by vitamin E: involvement of the protein kinase B pathway. J Biol Chem 279:50700–50709.

Kempna P, Zingg JM, Ricciarelli R, Hierl M, Saxena S, Azzi A. 2003. Cloning of novel human SEC14p-like proteins: cellular localization, ligand binding and functional properties. Free Radic Biol Med 34:1458–1472.

Klein EA, Thompson IM, Jr, Tangen CM, Crowley JJ, Lucia MS, Goodman PJ, Minasian LM, Ford LG, Parnes HL, Gaziano JM, Karp DD, Lieber MM, Walther PJ, Klotz L, Parsons JK, Chin JL, Darke AK, Lippman SM, Goodman GE, Meyskens FL, Jr, Baker LH. 2011. Vitamin E and the risk of prostate cancer: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). Jama 306:1549–1556.

Kurig B, Shymanets A, Bohnacker T, Prajwal, Brock C, Ahmadian MR, Schaefer M, Gohla A, Harteneck C, Wymann MP, Jeanclos E, Nurnberg B. 2009. Ras is an indispensable coregulator of the class IB phosphoinositide 3-kinase p87/p110gamma. Proc Natl Acad Sci USA 106:20312–20317.

Lambrechts D, Storkebaum E, Morimoto M, Del-Favero J, Desmet F, Marklund SL, Wyns S, Thijs V, Andersson J, van Marion I, Al-Chalabi A, Bornes S, Musson R, Hansen V, Beckman L, Adolfsson R, Pall HS, Prats H, Vermeire S, Rutgeerts P, Katayama S, Awata T, Leigh N, Lang-Lazdunski L, Dewerchin M, Shaw C, Moons L, Vlietinck R, Morrison KE, Robberecht W, Van Broeckhoven C, Collen D, Andersen PM, Carmeliet P. 2003. VEGF is a modifier of amyotrophic lateral sclerosis in mice and humans and protects motoneurons against ischemic death. Nat Genet 34:383–394.

Lehmann K, Muller JP, Schlott B, Skroblin P, Barz D, Norgauer J, Wetzker R. 2009. PI3Kgamma controls oxidative bursts in neutrophils via interactions with PKCalpha, p47phox. Biochem J 419:603–610.

Liang Y, Li XY, Rebar EJ, Li P, Zhou Y, Chen B, Wolffe AP, Case CC. 2002. Activation of vascular endothelial growth factor A transcription in tumorigenic glioblastoma cell lines by an enhancer with cell type-specific DNase I accessibility. J Biol Chem 277:20087–20094.

Libinaki R, Ogru E, Gianello R, Bolton L, Geytenbeek S. 2005. Evaluation of the safety of mixed tocopheryl phosphates (MTP)-A formulation of alpha-tocopheryl phosphate plus alpha-di-tocopheryl phosphate. Food Chem Toxicol 44:916–932.

Lippman SM, Klein EA, Goodman PJ, Lucia MS, Thompson IM, Ford LG, Parnes HL, Minasian LM, Gaziano JM, Hartline JA, Parsons JK, Bearden JD, 3rd, Crawford ED, Goodman GE, Claudio J, Winquist E, Cook ED, Karp DD, Walther P, Lieber MM, Kristal AR, Darke AK, Arnold KB, Ganz PA, Santella RM, Albanes D, Taylor PR, Probstfield JL, Jagpal TJ, Crowley JJ, Meyskens FL, Jr, Baker LH, Coltman CA, Jr. 2009. Effect of selenium and vitamin E on risk of prostate cancer and other cancers: the Selenium and Vitamin E Cancer Prevention Trial (SELECT). Jama 301:39–51.

Ma AD, Metjian A, Bagrodia S, Taylor S, Abrams CS. 1998. Cytoskeletal reorganization by G protein-coupled receptors is dependent on phosphoinositide 3-kinase gamma, a Rac guanosine exchange factor, and Rac. Mol Cell Biol 18:4744–4751.

Madeddu P, Kraenkel N, Barcelos LS, Siragusa M, Campagnolo P, Oikawa A, Caporali A, Herman A, Azzolino O, Barberis L, Perino A, Damilano F, Emanueli C, Hirsch E. 2008. Phosphoinositide 3-kinase gamma gene

knockout impairs postischemic neovascularization and endothelial progenitor cell functions. Arterioscler Thromb Vasc Biol 28:68–76.

Mukherjee S, Lekli I, Das M, Azzi A, Das DK. 2008. Cardioprotection with alpha-tocopheryl phosphate: Amelioration of myocardial ischemia reperfusion injury is linked with its ability to generate a survival signal through Akt activation. Biochim Biophys Acta 1782:498–503.

Munteanu A, Ricciarelli R, Massone S, Zingg JM. 2007. Modulation of proteasome activity by vitamin E in THP-1 monocytes. IUBMB Life 59:771–780.

Munteanu A, Taddei M, Tamburini I, Bergamini E, Azzi A, Zingg JM. 2006. Antagonistic effects of oxidized low density lipoprotein and alpha-tocopherol on CD36 scavenger receptor expression in monocytes: involvement of protein kinase B and peroxisome proliferator-activated receptor-gamma. J Biol Chem 281:6489–6497.

Munteanu A, Zingg JM, Ogru E, Libinaki R, Gianello R, West S, Negis Y, Azzi A. 2004. Modulation of cell proliferation and gene expression by alpha-tocopheryl phosphates: relevance to atherosclerosis and inflammation. Biochem Biophys Res Commun 318:311–316.

Mustacich DJ, Vo AT, Elias VD, Payne K, Sullivan L, Leonard SW, Traber MG. 2007. Regulatory mechanisms to control tissue alpha-tocopherol. Free Radic Biol Med 43:610–618.

Naga Prasad SV, Jayatilleke A, Madamanchi A, Rockman HA. 2005. Protein kinase activity of phosphoinositide 3-kinase regulates beta-adrenergic receptor endocytosis. Nat Cell Biol 7:785–796.

Negis Y, Meydani M, Zingg JM, Azzi A. 2007. Molecular mechanism of alpha-tocopheryl-phosphate transport across the cell membrane. Biochem Biophys Res Commun 359:348–353.

Nespereira B, Perez-Ilzarbe M, Fernandez P, Fuentes AM, Paramo JA, Rodriguez JA. 2003. Vitamins C and E downregulate vascular VEGF and VEGFR-2 expression in apolipoprotein-E-deficient mice. Atherosclerosis 171:67–73.

Neuzil J, Dong LF, Wang XF, Zingg JM. 2006. Tocopherol-associated protein-1 accelerates apoptosis induced by alpha-tocopheryl succinate in mesothelioma cells. Biochem Biophys Res Commun 343:1113–1117.

Ni J, Wen X, Yao J, Chang HC, Yin Y, Zhang M, Xie S, Chen M, Simons B, Chang P, di Sant′agnese A, Messing EM, Yeh S. 2005. Tocopherol-associated protein suppresses prostate cancer cell growth by inhibition of the phosphoinositide 3-kinase pathway. Cancer Res 65:9807–9816.

Nile AH, Bankaitis VA, Grabon A. 2010. Mammalian diseases of phosphatidylinositol transfer proteins and their homologs. Clin Lipidol 5:867–897.

Numakawa Y, Numakawa T, Matsumoto T, Yagasaki Y, Kumamaru E, Kunugi H, Taguchi T, Niki E. 2006. Vitamin E protected cultured cortical neurons from oxidative stress-induced cell death through the activation of mitogen-activated protein kinase and phosphatidylinositol 3-kinase. J Neurochem 97:1191–1202.

Ogru E, Gianello R, Libinaki R, Smallridge A, Bak R, Geytenbeck S, Kannar D, West S. 2003. Vitamin E phosphate: An endogenous form of vitamin E. Medimond Srl 127–132.

Oosthuyse B, Moons L, Storkebaum E, Beck H, Nuyens D, Brusselmans K, Van Dorpe J, Hellings P, Gorselink M, Heymans S, Theilmeier G, Dewerchin M, Laudenbach V, Vermylen P, Raat H, Acker T, Vleminckx V, Van Den Bosch L, Cashman N, Fujisawa H, Drost MR, Sciot R, Bruyninckx F, Hicklin DJ, Ince C, Gressens P, Lupu F, Plate KH, Robberecht W, Herbert JM, Collen D, Carmeliet P. 2001. Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. Nat Genet 28:131–138.

Pages G, Pouyssegur J. 2005. Transcriptional regulation of the vascular endothelial growth factor gene–a concert of activating factors. Cardiovasc Res 65:564–573.

Pennington KD, Schlitt JM, Schulz LC. 2012. Isolation of primary mouse trophoblast cells and trophoblast invasion assay. J Vis Exp 1–6.

Prior SJ, Hagberg JM, Paton CM, Douglass LW, Brown MD, McLenithan JC, Roth SM. 2006. DNA sequence variation in the promoter region of the VEGF gene impacts VEGF gene expression and maximal oxygen consumption. Am J Physiol Heart Circ Physiol 290:H1848–H1855.

Rezk BM, van der Vijgh WJ, Bast A, Haenen GR. 2007. Alpha-tocopheryl phosphate is a novel apoptotic agent. Front Biosci 12:2013–2019.

Ricciarelli R, Tasinato A, Clement S, Ozer NK, Boscoboinik D, Azzi A. 1998. Alpha-Tocopherol specifically inactivates cellular protein kinase C alpha by changing its phosphorylation state. Biochem J. 334:243–249.

Ruiz de Almodovar C, Coulon C, Salin PA, Knevels E, Chounlamountri N, Poesen K, Hermans K, Lambrechts D, Van Geyte K, Dhondt J, Dresselaers T, Renaud J, Aragones J, Zacchigna S, Geudens I, Gall D, Stroobants S, Mutin M, Dassonville K, Storkebaum E, Jordan BF, Eriksson U, Moons L, D′Hooge R, Haigh JJ, Belin MF, Schiffmann S, Van Hecke P, Gallez B, Vinckier S, Chedotal A, Honnorat J, Thomasset N, Carmeliet P, Meissirel C. 2010. Matrix-binding vascular endothelial growth factor (VEGF) isoforms guide granule cell migration in the cerebellum via VEGF receptor Flk1. J Neurosci. 30:15052–15066.

Saitoh Y, Yumoto A, Miwa N. 2009. Alpha-tocopheryl phosphate suppresses tumor invasion concurrently with dynamic morphological changes and delocalization of cortactin from invadopodia. Int J Oncol 35:1277–1288.

Sakowski SA, Heavener SB, Lunn JS, Fung K, Oh SS, Spratt SK, Hogikyan ND, Feldman EL. 2009. Neuroprotection using gene therapy to induce vascular endothelial growth factor-A expression. Gene Ther 16:1292–1299.

Schindler R, Mentlein R. 2006. Flavonoids and vitamin E reduce the release of the angiogenic peptide vascular endothelial growth factor from human tumor cells. J Nutr 136:1477–1482.

Sengupta J, Lalitkumar PG, Najwa AR, Charnock-Jones DS, Evans AL, Sharkey AM, Smith SK, Ghosh D. 2007. Immunoneutralization of vascular endothelial growth factor inhibits pregnancy establishment in the rhesus monkey (*Macaca mulatta*). Reproduction 133:1199–1211.

Siragusa M, Katare R, Meloni M, Damilano F, Hirsch E, Emanueli C, Madeddu P. 2010. Involvement of phosphoinositide 3-kinase gamma in angiogenesis and healing of experimental myocardial infarction in mice. Circ Res 106:757–768.

Tang FY, Meydani M. 2001. Green tea catechins and vitamin E inhibit angiogenesis of human microvascular endothelial cells through suppression of IL-8 production. Nutr Cancer 41:119–125.

Ulatowski L, Parker R, Warrier G, Sultana R, Butterfield DA, Manor D. 2014. Vitamin E is essential for Purkinje neuron integrity. Neuroscience 260:120–129.

Wang X, Ni J, Hsu CL, Johnykutty S, Tang P, Ho YS, Lee CH, Yeh S. 2009. Reduced expression of tocopherol-associated protein (TAP/Sec14L2) in human breast cancer. Cancer Invest 27:971–977.

Watton SJ, Downward J. 1999. Akt/PKB localisation and 3′ phosphoinositide generation at sites of epithelial cell-matrix and cell-cell interaction. Curr Biol 9:433–436.

Wei Q, Xia Y. 2006. Proteasome inhibition down-regulates endothelial nitric-oxide synthase phosphorylation and function. J Biol Chem. 281:21652–21659.

Wen XQ, Li XJ, Su ZL, Liu Y, Zhou XF, Cai YB, Huang WT, Gao X. 2007. Reduced expression of alpha-tocopherol-associated protein is associated with tumor cell proliferation and the increased risk of prostate cancer recurrence. Asian J Androl 9:206–212.

Wright ME, Peters U, Gunter MJ, Moore SC, Lawson KA, Yeager M, Weinstein SJ, Snyder K, Virtamo J, Albanes D. 2009. Association of variants in two vitamin E transport genes with circulating vitamin E concentrations and prostate cancer risk. Cancer Res 69:1429–1438.

Zhang B, Tanaka J, Yang L, Sakanaka M, Hata R, Maeda N, Mitsuda N. 2004. Protective effect of vitamin E against focal brain ischemia and neuronal death through induction of target genes of hypoxia-inducible factor-1. Neuroscience 126:433–440.

Zingg JM. 2007. Modulation of signal transduction by vitamin E. Mol Aspects Med 28:481–506.

Zingg JM, Azzi A. 2009. Comment re: vitamin E transport gene variants and prostate cancer. Cancer Res 69:6756.

Zingg JM, Kempna P, Paris M, Reiter E, Villacorta L, Cipollone R, Munteanu A, De Pascale C, Menini S, Cueff A, Arock M, Azzi A, Ricciarelli R. 2008. Characterization of three human sec14p-like proteins: alpha-Tocopherol transport activity and expression pattern in tissues. Biochimie 90:1703–1715.

Zingg JM, Libinaki R, Lai CQ, Meydani M, Gianello R, Ogru E, Azzi A. 2010. Modulation of gene expression by alpha-tocopherol, alpha-tocopheryl phosphate in THP-1 monocytes. FRBM 49:1989–2000.

Zingg JM, Libinaki R, Meydani M, Azzi A. 2014. Modulation of phosphorylation of tocopherol and phosphatidylinositol by hTAP1/SEC14L2-mediated lipid exchange. PLoS One 9:e101550.

Zingg JM, Meydani M, Azzi A. 2010. Alpha-tocopheryl phosphate – an active lipid mediator? Mol Nutr Food Res 54:1–14.

Zingg JM, Meydani M, Azzi A. 2012. Alpha-tocopheryl phosphate – an activated form of vitamin E important for angiogenesis and vasculogenesis? Biofactors 38:24–33.

Zygmunt M, Herr F, Munstedt K, Lang U, Liang OD. 2003. Angiogenesis and vasculogenesis in pregnancy. Eur J Obstet Gynecol Reprod Biol 110(Suppl1): S10–S18.