The effects of four natural tocopherols on the proliferation and signaling pathways were examined in the human mastocytoma cell line (HMC-1). The four tocopherols inhibited HMC-1 cell proliferation with different potency ($\beta > \alpha > \gamma > \beta$). Growth inhibition correlated with the reduction of PKB (protein kinase B) phosphorylation by the different tocopherols. The reduction of PKB phosphorylation led to a decrease of its activity, as judged from a parallel reduction of GSKa/β phosphorylation. The translocation of PKB to the membrane, as a response to receptor stimulation by NGFβ, is also prevented by treatment with tocopherols. In the presence of PKC or P2A inhibitors, the reduction of PKB phosphorylation by tocopherols was still observed, thus excluding the direct involvement of these enzymes. Other pathways, such as the Ras-stimulated ERK1/2 (extracellular signal responsive kinase) pathway, were not affected by tocopherol treatment. The tocopherols did not significantly change oxidative stress in HMC-1 cells, suggesting that the observed effects are not the result of a general reduction of oxidative stress. Thus, the tocopherols interfere with PKB phosphorylation and reduce proliferation of HMC-1 cells, possibly by modulating either phosphatidylinositol 3-kinase, a kinase phosphorylating PKB (PDK1/2), or a phosphatase that dephosphorylates it. Inhibition of proliferation and PKB signaling in HMC-1 cells by vitamin E suggests a role in preventing diseases with mast cell involvement, such as allergies, atherosclerosis, and tumorigenesis.

The term vitamin E covers a group of eight lipid-soluble compounds, the four tocopherols and the four tocotrienols. α-Tocopherol is considered to be the most biologically active form, because it is specifically retained in the body by the liver through a specific transfer protein, the α-tocopherol transfer protein (α-TTP) (1). Tocopherols are considered as the main cellular, lipid-soluble, antioxidants with potential beneficial effects, such as protection against atherosclerosis, neurodegenerative diseases, and cancer. Tocopherols influence the activity of several enzymes and modulate the transcription of certain genes (2). Whereas some of the cellular effects may be the result of free radical scavenging, many effects of tocopherol are independent, such as the modulation of expression of certain genes (2, 7).

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Inhibition of HMC-1 Mast Cell Proliferation by Vitamin E

INVolVEMENT OF THE PROTEIN KINASE B PATHWAY

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The term vitamin E covers a group of eight lipid-soluble compounds, the four tocopherols and the four tocotrienols. α-Tocopherol is considered to be the most biologically active form, because it is specifically retained in the body by the liver through a specific transfer protein, the α-tocopherol transfer protein (α-TTP) (1). Tocopherols are considered as the main cellular, lipid-soluble, antioxidants with potential beneficial effects, such as protection against atherosclerosis, neurodegenerative diseases, and cancer. Tocopherols influence the activity of several enzymes and modulate the transcription of certain genes (2). Whereas some of the cellular effects may be the result of free radical scavenging, many effects of tocopherol are unrelated to its antioxidant properties. Protein kinase C (PKC) has been recognized as the main cellular target of α-tocopherol (3). Inhibition of smooth muscle cell proliferation correlated with inhibition of PKC (4). The activity of PKC was not influenced directly, but through modulation of its phosphorylation status by activation of PP2A (5). Recently, the activation of diacylglycerol kinase by tocopherol was also shown to influence PKC activity (6). However, certain cellular effects of the tocopherols were described that seemed to be PKC independent, such as the modulation of expression of certain genes (2, 7).

HMC-1 is a mastocytoma cell line with a gain of function mutation in the c-kit receptor (stem cell factor (SCF) receptor), which leads to constitutive tyrosine kinase activity independent of the c-kit ligand, SCF (8). The c-kit receptor is present in the majority of hematopoietic cells, playing indispensable functions in their proliferation and differentiation (9, 10). Mutations of c-kit are found not only in mastocytoma and leukemia, but also in other cancers, such as gastrointestinal stromal tumors or small cell lung carcinoma (11, 12). The activity of the c-kit receptor can be completely inhibited by STI571/Gleevec®, an inhibitor designed against constitutively active Abelson kinase used for the treatment of chronic myeloid leukemia (13). As for most of the receptors with tyrosine kinase activity, stimulation of c-kit leads to the activation of several signaling pathways, including the Ras-MAPK-ERK and the PI3K-PKB pathways (8, 14). A growing amount of data supports the importance of PI3K-PKB signaling in many processes, including proliferation of cancer cells, cellular migration, apoptosis, and secretion (15). PKB or Akt, a serine/threonine kinase, is a key enzyme in the phosphatidylinositol 3-kinase (PI3K)–activated pathways. PKB has a wide range of cellular targets, and its increased activity can be found in many tumors. Activation of PKB involves a membrane translocation step, followed by phosphorylation of two key regulatory sites, Ser473 and Thr308. The pleckstrin homology domain (PH), present in the PKB molecule, binds phosphatidylinositol triphosphate, produced by activated PI3K at the plasma membrane. By the same mechanisms PDK-1, a kinase phosphorylating Thr308 in PKB, becomes active. Phosphorylation of Thr308 leads, however, only to partial activation of PKB. Only after phosphorylation at the second site (Ser473) by a yet unidentified kinase, does the enzyme become fully active (16, 17). Once active, PKB can be inactivated by protein phosphatase PP2A or by PTEN, a lipid phosphatase, which hydrolyzes the products of PI3K (18).
Because of the correlation between enhanced activity of PI3K, PKB, and tumor progression, these enzymes became attractive targets for pharmacological intervention by specific inhibitors. Interestingly, several natural compounds, such as certain flavonoids, caffeine, and theophylline, were shown to be potent inhibitors of PI3K activity (19, 20). The effects of tocopherols on these enzymes are as yet unclear.

Mast cells have been implicated in two contrasting types of immune responses, the immediate hypersensitivity reactions associated with allergic phenomena and their acute activation by bacterial products during infection (21). Besides their well known central involvement in anaphylaxis, they contribute to the pathogenesis of rheumatoid arthritis, interstitial cystitis, scleroderma, and Crohn’s disease. Increased numbers of mast cells were found also in atherosclerotic lesions, in particular in fatty streaks and the shoulder regions of atheromas (22). Activated mast cells contribute to foam cell and fatty streak formation by stimulating low-density lipoprotein modification and uptake by macrophages (23). In some of these diseases it can be assumed that mast cell signaling and proliferation are deregulated, and normalization could play a beneficial role. In a model for canine atopic dermatitis, vitamin E inhibited histamine, prostaglandin D2, and chymase release both in unstimulated and mastoparan-stimulated canine mastocytoma cells (C2), but the signaling pathways involved have not been resolved in detail (24).

We chose HMC-1 cells for the study of the effects of different tocopherols on mast cell proliferation and signaling. HMC-1 tumorigenicity is the result of an activated c-kit receptor, which is located at the beginning of a signaling cascade. Specific inhibition of c-kit by STI571 allows assessing and excluding the effect of tocopherols on other signaling cascades involved in mast cell proliferation.

**EXPERIMENTAL PROCEDURES**

**Materials**—The antibodies (PKB, phospho-PKB/Ser473, phospho-PKB/Thr389, phospho-GSK3α/β, phospho-ERK1/2, ERK1/2) were obtained from Cell Signaling Technology (Beverly, MA). The inhibitors wortmannin and okadaic acid (OA) were purchased from Sigma, and stored as stock solutions in ethanol, for OA at 50 mM, for wortmannin at 0.1%. Cells were counted using the hemocytometer with 0.1% formaldehyde/PBS for 10 min, and then examined by fluorescent-activated cell sorting.

**Inhibition of PKB Phosphorylation by Tocopherol**

Inhibition by tocopherol of PKB phosphorylation was measured using the phospho-specific antibodies (Biosource, Camarillo, CA) and Western blot analysis. HMC-1 cells were cultured in medium with or without tocopherol for 24 h, washed, and then treated with or without serum for 24 h. After treatment, cells were harvested and subjected to Western blot analysis using phospho-specific antibodies. PKB phosphorylation was quantified using the phospho-specific antibodies and Western blot analysis. PKB phosphorylation was decreased in a dose-dependent manner by tocopherol treatment. PKB phosphorylation was quantified using the phospho-specific antibodies and Western blot analysis. PKB phosphorylation was decreased in a dose-dependent manner by tocopherol treatment.

**Isolation of Membrane Fractions**—Isolation of membranes and supernatants was performed according to the protocol provided by the manufacturer (Cell Signaling Technology). Membrane fractions were visualized using the ECL method (Amersham Biosciences). Phosphorylation of GSK3α/β and ERK1/2 were analyzed the same way. Results were scanned by a Lumimager (Roche Applied Science), and the intensity of the bands was quantified.

**RESULTS**

**Inhibitory Effect of Tocopherols on the Proliferation of HMC-1 Cells**—Tocopherols and tocotrienols are able to inhibit proliferation or induce apoptotic cell death in various cell lines; mast cells have so far not been tested (26). To this end the inhibitory effect of tocopherols on proliferation of the human mastocytoma cell line HMC-1 was analyzed. Proliferation of mast cells is critically dependent on SCF acting through the receptor SCFRc-kit; however, the cell line HMC-1 contains point mutations in the c-kit receptor (Val660 → Gly, Asp816 → Val), that keeps the receptor permanently active, independent of its ligand (8, 27).

Two conditions were used for the growth inhibition assay: the presence of 10% FCS, thus providing growth factors, and in the absence of FCS, without any external growth factors. Cells were treated with different tocopherols for 24 or 48 h, and cell proliferation was quantified using a hemocytometer with trypan blue exclusion. Tocopherols were used at a concentration of 50 μM, which at least for α-tocopherol represents a physiological plasma concentration that can be reached by supplementation.

After 24 h of incubation, α-tocopherol significantly inhibited growth of HMC-1 cells in the presence of serum. Under conditions without serum only slight growth of untreated cells was observed, and α-tocopherol could inhibit growth completely after the first 24 h (Fig. 1, A and B). β-Tocopherol was less potent in inhibition of cell proliferation, whereas γ-tocopherol displayed a similar effect to α-tocopherol (data not shown).

For treatments with δ-tocopherol, maximal inhibition did not occur within the first 24 h of treatment; however cells stopped growing and detached at the later time points (48 or 72 h). At these times, δ-tocopherol was more potent than α-tocopherol, either with or without serum. This inhibitory effect of δ-tocopherol occurred also at lower (10–25 μM) concentrations (not...
Inhibition of PKB Phosphorylation by Tocopherol

Effect of α-Tocopherol Treatment on PKB Phosphorylation—A generally accepted model for the non-antioxidant molecular mechanism of α-tocopherol action is through inhibition of PKCa activity that can be directly connected with the inhibition of cell growth. However, the PI3K pathway also plays an important role in cell proliferation in many cell types through activation of the PDK1/2-PKB-GSK3β pathway. In the case of mast cells that are critically dependent on the c-kit receptor, PI3K activity, especially its catalytic isoform p110α and regulatory subunit p85, was shown to be necessary for cell growth and survival (8, 28). Experiments shown in the following were designed to verify whether this pathway is involved in growth inhibition by tocopherols in the HMC-1 cell line. We chose to analyze phosphorylation of PKB as marker for PI3K activation. PKB is phosphorylated at Ser473 by a yet unidentified kinase and at Thr307 by PDK1 (29, 30).

Cells were grown and treated with 50 μM α-tocopherol either in medium containing 10% FCS or in medium without FCS for the indicated times and phosphorylation of PKB was analyzed by Western blotting. In both cases, a clear decrease in the phosphorylation of PKB at Ser473 after treatment with tocopherols was found (Fig. 2A and B). In the presence of FCS, ethanol (at a final concentration of 0.1%), as a solvent for tocopherol, caused partial inhibition of PKB phosphorylation, although the effect of α-tocopherol, especially at the later time points, was stronger (Fig. 2B). Phosphorylation at Thr307 was also inhibited, but to a lesser extent than Ser473 (data not shown).

It was of interest to determine whether the inhibition of PKB by α-tocopherol was sufficient to decrease its in vivo activity. GS3K is one of the direct targets of PKB; the activity of PKB can thus be measured by assaying GS3K-3 phosphorylation (31). Indeed, treatment with α-tocopherol leads to a decrease in phosphorylation of both GS3K-3 isoforms (GS3K-3α is phosphorylated at Ser9, GS3K-3β at Ser361), in parallel with the decrease in PKB phosphorylation. It appears that GS3K-3β (lower band, Fig. 2A and B) is more sensitive to α-tocopherol treatment than the GS3K-3α isoform (upper band).

To assess whether the four tocopherols affect PKB phosphorylation with different potency, HMC-1 cells were incubated for 24 h with increasing concentrations of tocopherols in the presence of 10% FCS. All four tocopherols reduced PKB phosphorylation in HMC-1 cells, albeit with different efficiency (Fig. 2C). The antioxidant probucol (70 μM) was less efficient in reducing PKB phosphorylation (∼27% ± 22%) when compared with the tocopherols (50 μM) (Fig. 2C). At low concentrations, δ-tocopherol showed weaker inhibition of PKB phosphorylation, which correlated with less potent growth inhibition for early time points (Fig. 1, A and B).

Inhibition of PKB Ser473 phosphorylation by the four tocopherols (50 μM) was also observed in other cell lines. MCF-7 breast cancer cells (Fig. 2D) showed a generally stronger reduction when compared with U937 myeloid leukemia cells (Fig. 2E) and Saos-2 osteosarcoma cells (Fig. 2F). The low response of Saos-2 cells correlates with the previously described absence of inhibition by tocopherols in this cell line (32, 33). Thus, inhibition of PKB phosphorylation by tocopherols appears to be a general phenomenon, although the amount of reduction is dependent on the cell type and tocopherol analogue.

Inhibition of PKB Phosphorylation by α-Tocopherol Leads to Inhibition of GS3K-3 Phosphorylation—We decided to assess the detailed mechanisms involved in inhibition of PKB phosphorylation mainly with α-tocopherol, since it is the only one which can reach plasma levels of 50 μM after supplementation and therefore reflects the most physiological situation. To assess whether other signaling pathways are involved, we examined the Ras-ERK pathway that is also activated by c-kit. Analysis of phosphorylation of ERK1/2 showed no significant inhibition by α-tocopherol, confirming that this pathway was not affected (Fig. 3A).

Inhibition of PKB Phosphorylation by α-Tocopherol Involves the c-kit-PI3K Pathway—To better characterize the mechanism by which α-tocopherol acts on the phosphorylation of PKB, different inhibitors of the enzymes involved in the c-kit-PI3K pathway were used.

STI571, a known inhibitor of c-kit and similar tyrosine ki-
nases such as the platelet-derived growth factor receptor and Abelson kinase completely inhibited PKB phosphorylation within a few minutes, at very low concentrations (50–1000 nM) in medium without serum (Fig. 3, A and B). This shows that the main pathway leading to PKB phosphorylation under these conditions is initiated by the c-kit receptor. In the presence of serum, STI571 inhibits PKB phosphorylation and serum-activated cell proliferation as well (data not shown), in agreement with results described by Heinrich et al. (13). Treatment with STI571 also reduced phosphorylation of ERK1/2. On the other hand, treatment with 1 μM wortmannin, an inhibitor of PI3Ks, leads to inhibition of PKB phosphorylation, but does not influence phosphorylation of ERK1/2 (Fig. 3A).

Increase of PKB Phosphorylation by Specific PKC Inhibitors, but Not in the Presence of α-Tocopherol—PKB can be phosphorylated at two sites, Ser\(^{473}\) and Thr\(^{307}\). PDK-1 is responsible for the phosphorylation at Thr\(^{307}\), whereas the kinase for Ser\(^{473}\) is still unknown. Since it is known that α-tocopherol is able to inhibit PKC activity by stimulation of PP2A activity, leading to dephosphorylation and consequently to deactivation of PKC, it was possible that PKB is somehow regulated by PKC or PP2A.

To assess the influence of PKC on PKB phosphorylation, two PKC inhibitors, PKC412 and Go6976, were used. PKC412, a novel inhibitor of the classical PKCs, affected PKB phosphoryl-
Inhibition of PKB phosphorylation by α-tocopherol is mediated by the PI3K-PDK pathway, and not by PKC or PP2A. Cells were treated with STI571 (c-kit tyrosine kinase inhibitor), wortmannin, (W, a PI3K-inhibitor), or δ-tocopherol (δT) at the indicated concentrations and times, and the effects on the phosphorylation of PKB or ERK1/2 assessed by Western blotting. (A and B) Cells were grown in medium with 10% FCS, treated as indicated, and the phosphorylation status in adherent and detached cells was analyzed. C, effect of PKC412 and Go6976, inhibitors of PKC, at different concentrations. D, cells were grown in medium without FCS and treated with OA (an inhibitor of PP2A) in presence or absence of α-tocopherol. Experiments were repeated three times with similar results. The phosphorylation of PKB was calculated as ratio between phospho-PKB and total PKB and expressed in percent control (0.1% ethanol).

In summary, the effect of α-tocopherol was also preserved when PKC was either inhibited by PKC inhibitors (PKC412; Go6976) or stimulated by PP2A inhibitor (OA), suggesting that the affected pathway did not directly include PKC.

δ-Tocopherol Inhibits Phosphorylation of PKB Stronger than α-Tocopherol—We decided to compare the effects of the tocopherols in more detail: α-tocopherol as the most physiological vitamin E form with equal growth inhibitory activity as γ-tocopherol; β-tocopherol as having the lowest growth inhibitory effect; and δ-tocopherol as the most potent. Interestingly, when comparing the effects of δ-tocopherol and α-tocopherol, δ-tocopherol showed a stronger inhibition of PKB phosphorylation but at a later time point after treatment. After 6 h of treatment with δ-tocopherol, no effect was observed. 24 h of treatment led to strong inhibition of phosphorylation of PKB, without any effect on phosphorylation of ERK1/2 (Fig. 3A, last two lanes).

Treatment with δ-tocopherol for 24 or 48 h resulted also in partial cell detachment indicative of cell death (Fig. 1C), similar to the effects of STI571 and wortmannin, whereas with α-tocopherol only a few cells detached. Analysis of ERK1/2 and PKB phosphorylation status in the detached cells showed that wortmannin and δ-tocopherol gave similar results. Both inhibitors did not change phosphorylation of ERK1/2 despite complete inhibition of PKB phosphorylation (Fig. 3B, detached cells). The correlation between the loss of cell adherence and loss of phosphorylation of PKB at Ser473 may suggest the involvement of integrin-linked kinase (ILK-1) in the effect of δ-tocopherol.

Differential Effects of α-Tocopherol and Other Tocopherol Species on PKB Phosphorylation—α-Tocopherol can act as an antioxidant, and the observed inhibition of PKB might be related to the putative role of reactive oxygen species (ROS) in activation of the signaling pathway. To exclude that the effect seen by α-tocopherol is because of its antioxidant properties, we compared the effects of other tocopherol species with essentially equal radical scavenging potential, in the combination with PKC412 treatment (Fig. 4, A and B). β-Tocopherol inhibited PKB phosphorylation to a lesser extent than α-tocopherol or δ-tocopherol and also only partially prevented the increased phosphorylation in the presence of 500 nM PKC412. Interestingly, δ-tocopherol inhibited PKB phosphorylation strongly after 24 h treatment, but not after 6 h treatment. Thus, inhibi-
Inhibition of PKB Phosphorylation by Tocopherol

**Fig. 4.** Effect of PKC412-inhibitor of PKC on the phosphorylation of PKB in the presence or absence of different tocopherol analogues. A and B, cells were pretreated with different tocopherols (50 μM) for the indicated times and then treated with PKC412 (500 nM). PKC412 alone at 500 nM causes increased phosphorylation of PKB in cells growing in 0% FCS medium. C, comparison of the effect of compounds that affect the amounts of reactive oxygen species on phosphorylation of PKB. Cells were treated with the indicated compounds for 6 (upper blot) and 24 h (lower blot) in 10% FCS and phosphorylation of PKB was analyzed by Western blotting. DPI, an inhibitor of NADPH oxidase was used at 10 μM, pro (probucol), an antioxidant, was used at 50 μM. The experiments were repeated three times with similar results. The phosphorylation of PKB was calculated as the ratio between phospho-PKB and total PKB and expressed as percentage of control (0.1% ethanol).

a-Tocopherol Treatment Inhibits PKB Translocation to the Membrane and Influences the PI3K-dependent Signaling Pathway—Membrane translocation is necessary for the activation of PKB and PDKs, the kinases responsible for its phosphorylation. Translocation of PKB and PDK-1 occurs because of the PH domain, in response to the formation of PI(3,4,5)P3, the product of PI3Ks activated by receptor stimulation.

The membrane association of PKB was analyzed in cells grown in 10% FCS after treatment with α- or δ-tocopherol. STI571, as inhibitor of the c-kit pathway or wortmannin, as inhibitor of PI3K, was used as controls for complete inhibition (Fig. 5A). In control cells, a small amount of PKB could be detected in the membrane fraction. Treatment with tocopherols for 24 h or with STI571 or wortmannin for 30 min led to a reduction or even complete inhibition of PKB membrane association.

Because in non-stimulated cells the amount of PKB constantly localized in the membrane fraction is small, we pretreated cells with α-tocopherol, then stimulated with NGFβ, and analyzed the effect of α-tocopherol on the signal-induced PKB membrane translocation. The treatment with α-tocopherol reduced membrane association of PKB in response to NGFβ by 40–50% (Fig. 5B).

Stimulation of cells grown in the absence of FCS for 24 h with NGFβ or lysophosphatidic acid (LPA) did not lead to increased phosphorylation of PKB. In these cells the c-kit pathway is constitutively active, and already high basal PKB phosphorylation cannot be significantly activated by additional stimuli, although the inhibitory effect of α-tocopherol remained (Fig. 5C).
The complete absence of PKB phosphorylation after treatment with STI571 suggested that under these conditions (0% FCS), c-kit-Pi3K is the only pathway leading to PKB phosphorylation. This finding offered a unique opportunity to analyze the influence of α-tocopherol on signaling pathways stimulated by specific growth factors. To evaluate the activation of PKB in response to NGFβ, we used short time pretreatment of the HMC-1 cells grown in medium without FCS with a low concentration of STI571 (50 nm). Already, this small amount of inhibitor completely blocked the c-kit-induced Pi3K pathway, resulting in the loss of phosphorylation of PKB. Additional treatment of the HMC-1 cells with NGFβ stimulated the Pi3K pathway via the tyrosine kinase receptor TrkA (36) and restored PKB activation. In cells pretreated with α-tocopherol the response to the NGFβ stimulus appeared to be significantly weaker (Fig. 5D), correlating with the less efficient translocation of PKB to the membrane fraction. Thus, tocopherol inhibits not only basal PKB phosphorylation, but also weakens its activation as response to stimulation by NGFβ.

**DISCUSSION**

HMC-1, a cell line established from peripheral blood cells of a patient with mast cell leukemia, expresses a juxtamembrane mutant c-kit, which has constitutive kinase activity (8). This leads to the activation of the Pi3K signaling pathway, resulting in the phosphorylation of downstream effectors, such as PKB, independently on the presence of growth factors in the medium. As a consequence, this cell system offers the unique possibility of analyzing the Pi3K cascade initiated by c-kit, independent of other signals. On the other hand, the signaling cascade initiated by specific growth factors can be studied after inhibition of c-kit by the potent inhibitor, STI571. HMC-1 cells grow rapidly in the presence of 10% FCS and maintain significant growth after switching to 0% FCS.

Vitamin E is known mainly as antioxidant. However, in recent years, several cellular effects, such as inhibition of growth, were demonstrated to be independent of its antioxidant activity, but rather the results of specific interactions with proteins. Different tocopherol analogues and derivatives can differently inhibit cell proliferation or induce cell death in cancer cell lines (32, 37–39). These events are not related to their radical scavenging abilities and are the object of intensive investigation. One of the models for non-antioxidant inhibition of cell growth by α-tocopherol is through inhibition of PKCα activity (5). Among enzymes whose activity can be modulated by vitamin E are 5-lipoxygenase, phospholipase A2 (40, 41), COX-2 (42), or recently, also diacylglycerol kinase (6). Some enzymes or receptors are modulated by tocopherols at the transcriptional level (reviewed in Ref. 2), such as HMG-CoA reductase as well as the LDL receptor by α-tocopherol (43), scavenger receptor CD36 by α-tocopherol (44, 45), or cytochrome P450 enzymes (CYP3A4 and CYP3A5) by δ-tocopherol and tocotrienols (46).

In this study, we have examined the effect of vitamin E on HMC-1 cell proliferation. Both α- and γ-tocopherols inhibit HMC-1 cell growth with equal potency, δ-tocopherol was more potent, particularly at later time points, and the effect of β-tocopherol was weaker. Thus, the effect of δ-tocopherol seen in HMC-1 cells appears to be similar as described in mouse mammary epithelial cells (37). Interestingly, inhibition of growth was seen in the presence and also in absence of FCS; this suggests the involvement of the c-kit-PKB-GSK3 pathway in the inhibition of cell growth in these cells, since this pathway remains active in the absence of growth factor stimulation. The c-kit receptor initiates signals leading mainly to the activation of two pathways, the Pi3K-PDK-PKB and Ras-MAPK-ERK1/2 pathway (summarized in Fig. 6). Treatment with STI571, a specific inhibitor of c-kit, inhibits both mentioned pathways.

The first signaling pathway initiated by c-kit (Pi3K-PKB-GSK-3) can be analyzed by assessing the phosphorylation state...
Inhibition of PKB Phosphorylation by Tocopherol

The inhibition of PKB phosphorylation by tocopherol is PKC-dependent (52), PKC activity is required for the proliferation of mast cells containing the activated c-kit mutant, and treatment with wortmannin leads to complete inhibition of cell growth, toward apoptosis. PK3K is also essential for Kit-ligand-mediated survival in myeloid progenitor cell lines, when no other factors are present (57). The same authors describe that activation of the PK3K-PDK-PKB pathway (Fig. 6). As reported by Shivakrupa et al. (56), PI3K activity is required for the proliferation of mast cells containing the activated c-kit mutant, and treatment with wortmannin leads to complete inhibition of cell growth, toward apoptosis. PK3K is also essential for Kit-ligand-mediated survival in myeloid progenitor cell lines, when no other factors are present (57). The same authors describe that activation of the PK3K-PDK-PKB pathway (Fig. 6).

The inhibition of PKB phosphorylation by tocopherol is sufficient to produce the inhibition of HMC-1 cell proliferation, without affecting other signaling pathways. PK3K activity is stimulated by ROS (34, 58), suggesting that tocopherols may modulate PKB phosphorylation by scavenging ROS or by inhibiting the assembly of NADPH oxidase. The assembly and activation of this multisubunit enzyme is under control of PKC, and it is weakened in the presence of a-tocopherol (35, 59). To ensure that enzymatic production of ROS is not involved in attenuation of PKB activation, we used DPI, which is a specific inhibitor of the NADPH oxidase. However, no change of phosphorylation status of PKB was observed after treatment with DPI, excluding involvement of ROS or NADPH oxidase as a target of a-tocopherol. A direct scavenging of ROS can also be excluded by comparing the effects of different tocopherols and other antioxidants on PKB phosphorylation in HMC-1 cells. In HMC-1 cells, all four tocopherol analogues had essentially similar antioxidative properties, yet \( \delta \)-tocopherol at early time points inhibited PKB phosphorylation more weakly, and at later time points, stronger, when compared with other tocopherols. Another well-known antioxidant, probucol, only modestly affected phosphorylation of PKB.

Inhibition of cell proliferation by tocopherols

PKB (53), confirming the existence of negative crosstalk between PKC, and PKB activation. Inhibition of PKB phosphorylation with higher concentration of PKC412 can be explained by a direct and nonspecific inhibition of c-kit tyrosine kinase activity by this compound (54). However, pretreatment of cells with a-tocopherol still abolishes the stimulatory effect of PKC412 on PKB phosphorylation, suggesting that the observed effects are independent of PKC inhibition.

The inhibition of PKC activity was reported to be the result of activation of the PP2A protein phosphatase and subsequent dephosphorylation of PKC, suggesting that PP2A could also be directly involved in the inhibition of PKB phosphorylation (55). The involvement of PP2A was tested by treatment with OA, an inhibitor of the protein phosphatases PP2A and PP1A. The slight stimulatory effect of OA is most likely caused by the prevention of dephosphorylation of PKB by PP2A, as described for okadaic acid or sodium orthovanadate (18). However, the inhibition of PKB phosphorylation by tocopherols also occurs after OA treatment, excluding PP2A as the primary target of the tocopherols in these cells.

Taken together, the effect of tocopherols in HMC-1 is independent of PKC or PP2A and can be most likely localized to the PI3K-PDK-PKB pathway (Fig. 6). As reported by Shivakrupa et al. (56), PI3K activity is required for the proliferation of mast cells containing the activated c-kit mutant, and treatment with wortmannin leads to complete inhibition of cell growth, toward apoptosis. PI3K is also essential for Kit-ligand-mediated survival in myeloid progenitor cell lines, when no other factors are present (57). The same authors describe that activation of the PK3K-PDK-PKB pathway (Fig. 6).

The main pathways activated by the c-kit receptor and known connections between involved enzymes are shown. Inhibitors used in this study are indicated in bold and italics. Tocopherols do not inhibit PKB by affecting PKC or the Ras-MAPK-ERK1/2 pathway, therefore a direct effect on c-kit receptor can be excluded. The tocopherols modulate most likely the activity of one of the enzymes (PI3K, PDK1/2) or their regulators (p70S6K, ILK) transmitting signals between c-kit and PKB.

Inhibition of PKB phosphorylation by tocopherols is sufficient to produce the inhibition of HMC-1 cell proliferation, without affecting other signaling pathways.

PK3K activity is stimulated by ROS (34, 58), suggesting that tocopherols may modulate PKB phosphorylation by scavenging ROS or by inhibiting the assembly of NADPH oxidase. The assembly and activation of this multisubunit enzyme is under control of PKC, and it is weakened in the presence of a-tocopherol (35, 59). To ensure that enzymatic production of ROS is not involved in attenuation of PKB activation, we used DPI, which is a specific inhibitor of the NADPH oxidase. However, no change of phosphorylation status of PKB was observed after treatment with DPI, excluding involvement of ROS or NADPH oxidase as a target of a-tocopherol. A direct scavenging of ROS can also be excluded by comparing the effects of different tocopherols and other antioxidants on PKB phosphorylation in HMC-1 cells. In HMC-1 cells, all four tocopherol analogues had essentially similar antioxidative properties, yet \( \delta \)-tocopherol at early time points inhibited PKB phosphorylation more weakly, and at later time points, stronger, when compared with other tocopherols. Another well-known antioxidant, probucol, only modestly affected phosphorylation of PKB.
Interestingly, δ-tocopherol was more potent than α-tocopherol, although its action was delayed. The late inhibition of PKB phosphorylation (after 24 h, but not after 6 h) correlates with the inhibition of cell proliferation and cell detachment at later time points. No change in phosphorylation of ERK1/2 was detected with both tocopherols. These observations suggest that both α- and δ-tocopherol may act on the same enzyme in the signaling pathway; however with a different potency. While α-tocopherol only partially inhibits cell growth and attenuates the response to stimuli, such as NGFβ, δ-tocopherol can strongly inhibit the PI3K pathway. Similar to that, in mouse mammary epithelial cells the strong effect of tocopherols is responsible for phosphorylation of PKB at Ser473 (Fig. 6). Interestingly, similar to our results with α- or δ-tocopherol, these compounds inhibit insulin-stimulated PKB activation in vivo, but do not influence ERK1/2 stimulation (20). In MCF-7 breast cancer cells, the natural phytoalexin, resveratrol, inhibits PKB phosphorylation via modulation of the PI3K pathway, possibly by increasing the degradation of the non-nuclear estrogen receptor α (ERα) by the proteasome (61). In other breast cancer cells, PKB phosphorylation is inhibited by toctrienols after stimulation by epidermal growth factor (62) and also by the two tocopherol derivatives, α-tocopherylsuccinate and α-tocopherylxylobutyric acid (63). Similar to that resveratrol reduced angiotensin II-induced PKB phosphorylation in rat aortic smooth muscle cells (64). It is possible that the combination of such natural and synthetic inhibitors may increase the inhibition of PKB phosphorylation and thus the efficiency of treatment of different cancers and of atherosclerosis prevention. In particular, the assessment of natural compounds with higher potency, such as δ-tocopherol could be indicated. δ-Tocopherol is not efficiently retained in the human body and thus reaches much lower plasma levels (possibly originating as consequence of its stronger effects) when used as supplement. However, δ-tocopherol could behave beneficially in topical treatments or may be used intramuscularly.

Taken together, in this study we examined effects of the tocopherols on the proliferation and signaling in HMC-1 cell line. All tocopherols are able to inhibit cell proliferation, albeit with different timing and potency. Repression of growth is mediated through inhibition of the PI3K pathway, as judged by analysis of PKB phosphorylation, without the involvement of PKC and ERK pathways and independent of the radical scavenging activities of tocopherols. The inhibitory effect can be localized in the events before PKB activation, but after the bifurcation of c-kit signal transduction to the Ras-MAPK-ERK1/2 and the PI3K-PKB-GSK-3 pathway; either there is a direct inhibition of PI3K or of the putative PDK2 kinase responsible for phosphorylation of PKB at Ser473 (Fig. 6). Interestingly, RRR-α-tocopherol was recently shown to inhibit Tyk2 tyrosine kinase activity in oxLDL-stimulated macrophages (65), and it remains to be shown whether a similar mechanism applies to c-kit. Other possibilities, such as inhibition of proteins involved in membrane translocation of PKB, or activation of lipid-kinases like pTEN and SH2IP/12, have also to be considered. Moreover, the mechanism of how different tocopherols can inhibit PKB phosphorylation with different efficiencies remains to be solved. The role of proteins with the ability to recognize and transport specific tocopherol analogues, such as the tocopherol-associated proteins (TAPs), is currently investigated in our laboratory (66). The ability of these proteins to bind phosphatidylinositol as well as the tocopherols with different affinities and to modulate PI3K activity in vitro may suggest these proteins confer specificity to different tocopherol species.

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Inhibition of PKB Phosphorylation by Tocopherol

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**Inhibition of HMC-1 Mast Cell Proliferation by Vitamin E: INVOLVEMENT OF THE PROTEIN KINASE B PATHWAY**

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