INHIBITION OF THE PI3K/PKB-SIGNALING PATHWAY BY VITAMIN E: IMPLICATIONS FOR Atherosclerosis

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Introduction

Atherosclerosis is a multifactorial process with many characteristics of an inflammatory disease: major cellular participants include monocytes, macrophages, mast cells, endothelial cells, T lymphocytes, platelets, and vascular smooth muscle cells (VSMC) [1]. Activation of these cells leads to release of hydrolytic enzymes, cytokines, chemokines, and growth factors that can result in further injury.

In a recent article we describe the inhibition of the PI3K/PKB-signalling pathway by vitamin E and the consequent growth inhibition in the human mastocytoma cell line HMC-1 [2]. Besides the possible interferences of vitamin E with inflammatory, allergic, and tumorigenic processes [3], how could this finding play a role in reducing the risk of atherosclerosis development?

Mast Cells and Atherosclerosis

In human blood vessels, mast cells have been observed in the intima of carotic arteries at sites of hemodynamic stress, together with monocytes, T-lymphocytes, and dendritic cells [4]. Increased numbers of mast cells were found in atherosclerotic lesions when compared with normal intima and are often associated with macrophages and extracellular lipid [5], in particular in fatty streaks and the shoulder regions of atheromas [6]. It is still unclear to what degree a higher density of mast cells in plaques is the result of increased recruitment or the consequence of higher proliferation [7]. Activated mast cells can contribute to foam cells and fatty streak formation by stimulating LDL modification and uptake by macrophages [8], by secreting a variety of inflammatory mediators (histamine, cytokines, chemokines, leukotrienes, prostaglandins, platelet activating factor) and enzymes (tryptase, chymase, carboxypeptidase, and cathepsin G) (reviewed in [9]), possibly leading to weakening and rupture of atherosclerotic plaques [10]. It can be assumed that mast cells signaling and proliferation are deregulated during the progression of atherosclerosis and normalization by vitamin E could thus play a beneficial role.

Novel Functions of Vitamin E

The term vitamin E covers a group of eight lipid-soluble compounds - the four tocopherols (α-, β-, γ-, and δ), and the four tocotrienols. α-Tocopherol is the most biologically active form, since it is specifically retained in the body by the liver α-tocopherol transfer protein (α-TTP); the other tocopherols are metabolized and predominantly eliminated [11]. To date, several epidemiological studies and intervention trials have been performed with vitamin E, and some of them showed that it prevents atherosclerosis [12-16]. For a long time, vitamin E was assumed to act only by decreasing the oxidation of LDL, a key step in atherosclerosis initiation and foam cell formation.
However, in addition to that it was later found that vitamin E influences cellular signaling cascades and gene expression, leading at the cellular level to inhibition of VSMC proliferation, platelet aggregation, monocyte adhesion, oxLDL uptake via reduction of scavenger receptor expression, and cytokine production, all reactions implied in the progression of atherosclerosis [15,17-19]. 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 non-antioxidant cellular target of \( \alpha \)-tocopherol [20]. Inhibition of VSMC proliferation correlated with inhibition of PKC [21]. The activity of PKC was not influenced directly, but via modulation of its phosphorylation state by activation of protein phosphatase 2A (PP2A) [22]. Certain cellular effects of the tocopherol were described that are PKC independent, such as the modulation of expression of certain genes and the regulation of certain enzymes, such as 5-lipoxygenase, phospholipase A2, cyclooxygenase-2 (COX-2), and diacylglycerolkinase [19,23]. Some enzymes or receptors are modulated by tocopherols at the transcriptional level, such as the HMG-CoA reductase, the LDL receptor, the scavenger receptors CD36 and SR-BI, or the cytochrome P450 enzymes (CYP3A4 and CYP3A5) (reviewed in [19]).

In our study with HMC-1 cells, the four tocopherols inhibited cell proliferation with different potency (\( \delta > \alpha = \gamma > \beta \)), \( \delta \)-tocopherol even led to apoptosis at higher concentrations [2]. Neither PKC nor PP2A were involved in the observed effects, as judged from using inhibitors of PKC and PP2A. Other pathways, such as the Ras-stimulated ERK1/2 (extracellular signal responsive kinase) pathway, were also not affected by tocopherol treatment. However, the growth inhibition correlated with the reduction of protein kinase B (PKB) phosphorylation by the different tocopherols. The reduction of PKB phosphorylation led to a decrease of its activity, as judged from a parallel reduction of glycogen synthase kinase 3 (GSK\( \alpha/\beta \)) phosphorylation. Similar to our results with mast cells, induction of apoptosis by \( \gamma \)-and \( \delta \)-tocopherol was recently shown in related manuscripts with prostate cancer cells [24], with mouse activated macrophages [25] and with mammary epithelial cells [26]. In androgen-sensitive prostate cancer cells (LNCaP) it was suggested that the inhibition of dihydroceramide desaturase is involved in the induction of apoptosis by \( \gamma \)- and \( \delta \)-tocopherol [24].

The c-kit/PI3K/PKB Signaling Pathway in Mast Cells

HMC-1 is a mastocytoma cell line with a gain of function mutation in the c-kit receptor (stem cell factor receptor), which leads to constitutive tyrosine kinase activity and activation of phosphatidylinositol 3kinase (PI3K) independent of the c-kit ligand, stem cell factor (SCF) [27]. The c-kit receptor is present in the majority of hematopoietic cells, playing indispensable functions in their proliferation and differentiation [28,29].

A growing number of data supports the importance of PI3K/PKB signaling in many processes, including cardiovascular physiology, proliferation of cancer cells, cellular migration, apoptosis, survival, and secretion [30,31]. PKB or Akt has a wide range of cellular targets and its increased activity can be found during atherosclerosis and tumorigenesis [32]. Activation of PKB involves a membrane translocation step, followed by phosphorylation of two key regulatory sites, Ser473 and Thr308. The PH domain (pleckstrin homology domain) present in the PKB molecule binds phosphatidylinositol trisphosphate, produced by activated PI3K at the plasma membrane. By the same mechanisms PI3K-dependent kinase 1 (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 (“PDK-2”, such as ATM [33], DNA-dependent protein kinase [34], ILK [35], PKCα [36], or PKCβ [37]), the enzyme becomes fully active [38,39]. Once active, PKB can be inactivated by protein phosphatase PP2A or by PTEN, a lipid phosphatase, which hydrolys the products of PI3K [40].

In our experiments, the tocopherols interfered with PKB Ser473 phosphorylation and reduced proliferation of HMC-1 cells, possibly by modulating either c-kit tyrosine kinase directly, PI3K, a kinase phosphorylating PKB (PDK1/2), or a phosphatase dephosphorylating it. Some evidence for the involvement of tyrosine phosphorylation has been described; α-tocopherol was recently shown to inhibit Tyk2 tyrosine kinase activity in oxLDL-stimulated macrophages [41], and tyrosine phosphorylation of JAK2, STAT1, and STAT3 is decreased by α-tocopherol in oxLDL-stimulated MRC5 fibroblasts [42]. Related to that, in HT4 hippocampal neuronal cells, glutamate stimulated pp60 c-Src tyrosine kinase activity is normalized by α-tocotrienol, but not by α-tocopherol [43]. In VSMC, angiotensin II-induced tyrosine phosphorylation of two major proteins (p120, p70) and ERK activation were markedly reduced by α-tocopherol, whereas ERK activation by epidermal growth factor was unaffected [44]. Tyrosine phosphorylation is also decreased by α-tocopherylsuccinate in human neutrophils via activation of a tyrosine phosphatase [45]. Since class I and II PI3K are regulated by tyrosine phosphorylation, it can be speculated that inhibition of tyrosine kinase activity by tocopherols may ultimately lead to reduced PKB membrane translocation and phosphorylation [31].

It remains to be shown whether similar mechanisms apply to c-kit tyrosine kinase activity. 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 [27,46]. Since the ERK pathway was not affected in our study, we assume that c-kit may not be the direct target of vitamin E, although further tests need to corroborate this. Other possibilities, such as inhibition of proteins involved in membrane translocation of PKB, or activation of lipid-phosphatases, like PTEN and SHIP1/2, have also to be considered. Moreover, it remains to be solved how different tocopherols can inhibit PKB phosphorylation with different efficiencies.

The c-kit/PI3K/PKB Pathway in VSMC and Monocytes/Macrophages

Similar to mast cells, the c-kit/PKB pathway plays also an important regulatory role in VSMC and monocytes/macrophages. The VSMC in the media of adult arteries are normally quiescent or proliferate at low frequency. Proliferation of VSMC occurs during arterial injury in response to growth factors and oxLDL. Uncontrolled uptake of oxLDL by monocytes/macrophages and VSMC converts them into foam cells, a central event during the atherosclerotic process. VSMC of the media were found to express c-kit and SCF suggesting the existence of mast cell-VSMC interaction and of an autocrine loop of c-kit and its ligand on the surface of VSMC [47]. Injured vessels revealed that c-kit expression within the media and neointima was significantly increased following injury [48] and during in-stent restenosis [49]. Other stimuli that activate the PI3K/PKB pathway in VSMC are: oxLDL, which induces VSMC proliferation, migration and survival [50,51]; insulin-like growth factor, which induces VSMC proliferation and migration [52]; and angiotensin II, a hypertrophic/anti-apoptotic hormone for VSMC, which can induce VSMC polyploidization [53,54].

Activation of PI3K/PKB by platelet-derived growth factor (PDGF) furthermore led to
induction of PPARγ gene expression in VSMC [55,56], suggesting that activation of PKB by oxLDL may be involved in the activation of PPARγ/CD36 expression. In support for that, oxLDL induces the expression of scavenger receptors SR-BI, SR-AI, and CD36, whereas blocking of PI3K activity with inhibitors interferes with these events in macrophages [57,58].

**Inhibition of PKB by Natural Compounds**

Interestingly, several natural compounds were shown to be potent inhibitors of the PI3K/PKB pathway, such as certain flavonoids, caffeine, and theophylline [59,60]. The tea polyphenol, epigallocatechin-3-gallate (EGCG), inhibits ERK1/2 and PKB activity leading to growth inhibition of cervical and prostate cells [61,62]. Deguelin induces apoptosis in premalignant bronchial epithelial cells via inhibition of PKB phosphorylation [63]. Neurotoxic effects of quercetin, a natural flavonoid, analyzed in primary cortical neurons, are also connected with PI3K modulation [59]. Caffeine and theophylline are potent inhibitors of different PI3Ks with higher specificity for PI3Kδ. 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 [60]. In MCF-7 breast cancer cells, the natural phytoalexin, resveratrol, inhibits PKB phosphorylation by modulation of the PI3K pathway, possibly via increasing the degradation of the nonnuclear estrogen receptor alpha (ERα) by the proteasome [64]. Similar to that, resveratrol reduced angiotensin II-induced PKB phosphorylation in rat aortic VSMC [65]. In other breast cancer cells, PKB phosphorylation is inhibited by tocotrienols after stimulation by EGF [66], and also by the two tocopherol derivatives, α-tocopherylsuccinate and α-tocopherylxybutyric acid [67]. Further studies showed that γ-tocotrienol induced a large decrease in the relative intracellular levels of phosphorylated forms of PDK-1, PKB, and GSK3α/β [68,69]. It is possible that the combination of such natural and synthetic inhibitors may potentiate the inhibition of PKB phosphorylation. It remains to be analyzed in detail whether for some of these compounds the described atherosclerosis preventive effects are due to inhibition of PKB activity in mast cells, monocytes/macrophages, and VSMC.

**Summary**

Taken together, in this study we examined the effects of the tocopherols on the proliferation and signaling in HMC-1 cells. All four tocopherols are able to inhibit cell proliferation, albeit with different timing and potency. Repression of growth is mediated via inhibition of the c-kit/PI3K/PKB-pathway, as judged by analysis of PKB phosphorylation, without the involvement of the PKC and ERK pathways and independent of the radical scavenging activities of the 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/GSK3α/β pathway.

In our study we checked only proliferation and apoptosis of HMC-1 cells; other consequences of PKB inhibition such as secretion of inflammatory cytokines and proteases, migration, or modulation of differentiation and survival could also be affected and need to be checked. That PKB can indeed modulate secretion was shown in FcepsilonRI-stimulated mast cells, in which PKB regulates the transcriptional activation of cytokine genes via NF-κB, NF-AT, and AP-1, thus affecting the production and secretion of IL-2 and TNF-alpha [70].

The finding that the tocopherols and certain natural compounds only partially inhibit the
PKB signaling pathway could be key for their daily use as dietary chemo-preventive agents against diseases like atherosclerosis. These compounds may normalize aberrant signaling and gene expression resulting from inherent cellular heterogeneity or from environmental cues; these compounds may also interfere with activation of cell signaling resulting from inflammatory processes in the pre-pathologic state which, if not normalized would turn into overt pathology. Specific and more potent inhibitors would then be required, capable of stopping disease progression by completely blocking a de-regulated signaling pathway.

References


