Inhibition of Inflammatory Endothelial Responses by a Pathway Involving Caspase Activation and p65 Cleavage

Jiri Neuzil,* Andreas Schröder, Phillip von Hundelshausen, Alma Zernecke, Tobias Weber, Nina Gellert, and Christian Weber

Institute for Prevention of Cardiovascular Diseases, Ludwig Maximilians University, Munich, Germany

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ABSTRACT: Suppression of NFκB activation has been involved in the elimination of survival programs during endothelial cell (EC) apoptosis. We used α-tocopheryl succinate (α-TOS) to trigger apoptosome formation and the subsequent activation of executioner caspases. The level of bcl-2 was reduced by α-TOS, and its downregulation potentiated and its overexpression suppressed pro-apoptotic effects of α-TOS, indicating a mitochondrial role in α-TOS-induced apoptosis in EC. α-TOS treatment was associated with induction of TUNEL-positive apoptosis in EC with a high but not with a low proliferation index. The use of the pan-caspase inhibitor z-VAD.fmk suggested the involvement of caspases in cleavage of p65, and in inhibition of nuclear translocation of p65 and NFκB-dependent transactivation of a gene construct encoding the green fluorescence protein elicited by TNFα in contact-arrested EC. The suppression by α-TOS of inflammatory EC responses induced by TNFα such as VCAM-1 mRNA and surface protein expression and shear-resistant arrest of monocytic cells were also reversed by z-VAD.fmk. NFκB-dependent transactivation was preserved in α-TOS-treated EC stably transfected with a caspase-noncleavable p65 mutant but not with its truncated form, thus establishing a direct link between α-TOS-induced effects and p65 cleavage. Our data infer a pathway by which caspase activation in EC inhibits NFκB-dependent inflammatory activation and monocyte recruitment, and provide evidence for a relationship between pro-apoptotic and anti-inflammatory pathways.

Nuclear factor κB (NFκB)[1] is crucially involved in the expression of a variety of genes associated with "stress" situations, such as in inflammation and vascular injury (*1*, *2*). Activation of NFκB following stimulation with inflammatory cytokines requires specific phosphorylation of the inhibitory protein IκB, its ubiquitination, and degradation in the proteasome (*3*, *4*). Only then can "free" NFκB translocate to the nucleus, often as a p65−p50 heterodimer or a p65−p65 homodimer, where it binds to its recognition sequence and triggers transcription of different genes (*5*). With respect to its role in blood vessel biology and remodeling, activation of NFκB and its inhibition in vascular endothelial and smooth muscle cells have been extensively studied (*6*). It has been shown that endothelial NFκB is highly responsive to stimulation with cytokines (*6*−*8*) and regulates transcription of many genes, including those of adhesion molecules, such as VCAM-1 (*6*), and chemokines, such as IL-8 (*7*). These molecules may play a crucial role at the onset and progression of various inflammatory disorders such as atherosclerosis, primarily due to their involvement in adhesion of monocytes and lymphocytes to the endothelium and their subsequent transmigration (*9*), hallmarks of the recruitment and extravasation of circulating cells into the subendothelium. Moreover, a direct relationship between protection against pro-apoptotic pathways and NFκB activity has been documented in various models and vascular cell types, e.g., due to NFκB-dependent expression of inhibitors of apoptosis (*10*−*15*).

As some steps of the NFκB activation pathway have been postulated to be under redox control, considerable focus has been given to the role of antioxidants in NFκB activation, e.g., pyrrolidine dithiocarbamate or vitamin E (*16*, *17*). However, it has more recently been reported that α-tocopherol (α-TOH), a free, redox-active form of vitamin E (*18*, *19*), is rather inactive in modulating NFκB activity, and that the inhibitory activity of vitamin E on NFκB mobilization can be ascribed to α-tocopheryl succinate (α-TOS), a redox-silent analogue of vitamin E (*10*, *20*, *21*). In addition, α-TOS but not α-TOH has been observed to induce apoptosis in various malignant cells (*22*−*25*). Notably, this was associated with activation of caspase-3 in cells triggered to undergo apoptosis with α-TOS (*22*). Hence, recent reports that apoptosis induced by Fas or serum starvation can result in direct caspase-mediated proteolysis of NFκB, in particular cleavage of p65, suppressing NFκB activation and overriding subsequent survival programs (*12*, *13*, *15*), provide culminating evidence for an intricate link between NFκB and apoptosis.

* To whom correspondence should be addressed: Institute for Prevention of Cardiovascular Diseases, Ludwig Maximilians University, Pettenkoferstrasse 9, 80336 Munich, Germany. Fax: +49-89-5160-4352. Phone: +49-89-5160-4370. E-mail: jneuzil@klp.med.uni-muenchen.de or cweber@klp.med.uni-muenchen.de.

[1] Abbreviations: cyt *c*, cytochrome *c*; EC, endothelial cells; FACS, fluorescence-assisted cell sorting; GFP, green fluorescent protein; IFM, immunofluorescence microscopy; NFκB, nuclear factor κB; ODN, oligodeoxynucleotide; TNFα, tumor necrosis factor-α; α-TOH, α-tocopherol; α-TOS, α-tocopheryl succinate.

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Prompted by previous reports revealing that α-TOS inhibits NFκB activation (*10*, *20*, *21*) but can also trigger caspase activation and apoptosis in multiple cell types (*22*, *23*, *26*), we studied whether a link between these two phenomena exists in EC, and whether under certain conditions caspase activation can serve as a mechanism contributing to inhibition of NFκB activation. We found that α-TOS induced activation of multiple caspases but negligible cell death in confluent human EC, and that this was associated with cleavage of p65. This resulted in inhibition of NFκB activation, VCAM-1 expression, and subsequent monocyte adhesion in shear flow induced by TNFα in EC. The multiple checkpoints involved in α-TOS-mediated inhibition of NFκB activation are consistent with the emerging picture of a relationship between pro-apoptotic and anti-inflammatory pathways, and imply that caspase activation may serve as a physiological signaling pathway under sub-apoptotic or inflammatory conditions.

MATERIALS AND METHODS

Cell Culture. Human umbilical vein endothelial cells (EC) were prepared from umbilical cords, maintained in endothelial cell medium (ECM, PromoCell), and used in passages 2−5. For experiments, cells were plated on collagen-coated dishes or cover slips, and treated when >95% confluent unless specified otherwise. The human EC line ECV304 and monocytic U937 cells were maintained in RPMI-1640 medium with 10% FCS. EC were treated with DMSO solutions of α-TOS or α-TOH (Sigma) or additives as indicated.

Assessment of Apoptosis and Caspase Activity. Apoptotic parameters were evaluated by TUNEL (Boehringer Mannheim) or annexin V (Pharmingen) staining according to protocols provided by the manufacturers. Analysis was performed by scoring positive cells in situ using fluorescence microscopy or by fluorescence-activated cell sorting (FACS) (Becton Dickinson) combining EC detached during treatment with those removed by trypsinization (TUNEL) or scraping (annexin V). Caspase activity was assessed as described previously (*22*), using Ac-DEVD-pNA and Ac-VEID-pNA as substrates for caspase-3 and -6, respectively. The extent of activation of caspase-3 was estimated using anti-caspase-3 IgG (Pharmingen) recognizing its active 17 kDa form.

Adhesion Assays. Monocytic cell adhesion to endothelium under conditions of laminar flow was performed as described previously (*27*). EC grown to confluence were treated with α-TOS, stimulated with TNFα (100 units/mL; Pharmingen) for 4 h, and perfused at 1.5 dyn/cm^2 with U937 cells (10^6 cells/mL) in Hank's balanced salt solution (10 mM Hepes, 1 mM Ca^{2+}/Mg^{2+}, and 0.5% HSA) at 37 °C in a flow chamber. The number of firmly adherent monocytes as determined by the primary interaction with EC after 5 min was quantified in multiple fields recorded by video microscopy.

Transfections and Antisense Oligodeoxynucleotide (ODN) Treatments. The plasmids that were used were as follows: pcDNA3.1-MycHis vector with or without wild-type bcl-2 (*28*, *29*), pRC/CMV vector with wild-type p65 ($p65_{WT}$), caspase-noncleavable p65 ($p65_{DM}$), or a p65 mutant truncated at position 471 ($p65_{TR}$) (*12*) and pNFκB-EGFP (Clontech) containing a tandem of four κB sites.

Transient transfections of EC were performed as described previously (*12*, *30*). Briefly, 3−6 μg of plasmid DNA was added to 5×10^5 EC after preincubation in 150 μL of ECM supplemented with 25 μL of Superfect (Qiagen) and incubated for 24−36 h before treatment with α-TOS. Transfection efficacy was estimated by cotransfection with pGreen Lantern-1 (Gibco) and assessment of green fluorescent protein (GFP) expression by FACS. For stable transfections, 10^7 ECV304 cells were electroporated with 25 μg of plasmid DNA (240 V, 750 μF), selected, and maintained in RPMI medium containing 10% FCS and G418 (1 mg/mL, Calbiochem). For experiments, transfectants were grown in complete RPMI medium.

Bcl-2 was downregulated by treatment with bcl-2 phosphorothioate-modified antisense ODNs. Reverse antisense and sense bcl-2 ODNs were used as negative controls. ODNs were synthesized (MWG-Biotech) according to published sequences (*30*). Briefly, 1.5 μg of ODNs in ECM (100 μL) containing Lipofectamine (5 μL; Gibco) was added to near-confuent EC.

Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was isolated by phenol/choroform/isoamyl alcohol extraction, cDNA reverse transcribed from 2 μg of RNA, and RT-PCR performed, and RT-PCR products were analyzed with an agarose gel as described previously (*31*).

Proliferation Assays. The EC proliferation rate was evaluated by the BrdU method (Boehringer Mannheim) according to the manufacturer's protocol. EC plated at different densities were incubated with BrdU, stained with anti-BrdU-FITC, and analyzed by FACS. The percentage of BrdU-positive EC of the highest density (4.45%) was set to 1, and the proliferation index of lower-density samples was related to it. Cell cycle analysis was performed by FACS after staining nuclei with propidium iodide using the CycleTEST PLUS kit (Becton-Dickinson). Proliferation was also evaluated in situ by staining for proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, Santa Cruz, CA).

Western Blotting and Immunocytochemistry. Western blotting and immunofluorescence microscopy (IFM; staining of saponin-permeabilized cells) were performed according to standard protocols using primary antibodies to the following antigens: p65, poly(ADP-ribose) polymerase (PARP), apaf-1, VCAM-1 (Santa Cruz Biotechnology), lamin B, bcl-2 (Calbiochem), cytochrome *c* (cyt *c*), caspase-3, and caspase-9 (Pharmingen).

Statistics. Unless stated otherwise, data are given as means ± the standard deviation of at least three independent experiments, and the images that are shown are representative pictures of three to six independent experiments.

RESULTS

α-TOS Induces Caspase Activation in EC. We have recently reported that the vitamin E analogue α-TOS induced apoptosis in Jurkat cells, and that this effect involved mitochondrial destabilization and caspase activation (*22*, *23*). Here, we used α-TOS to study the effects of caspase activation in EC. Treatment of confluent as well as subconfluent EC with α-TOS but not with α-TOH for 6 h induced activation of caspase-3 and caspase-6, which was prevented by the pan-caspase inhibitor z-VAD.fmk (Figure 1A, not

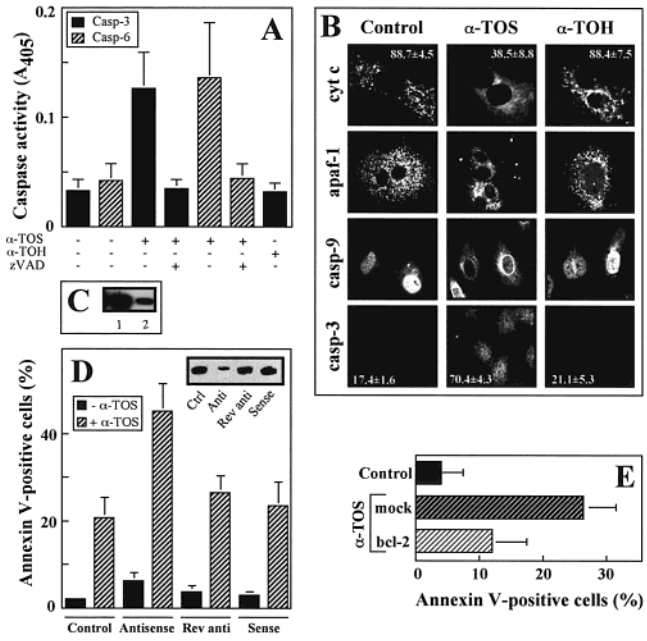


FIGURE 1: α-TOS activates caspases and mitochondrial apoptotic pathways in EC. Confluent EC were treated with α-TOS or α-TOH at 25 μM, in the absence or presence of 25 μM z-VAD.fmk. After 6 h, EC were assessed for caspase-3 and -6 activity (A). In situ staining for cyt *c*, apaf-1, caspase-9, and active caspase-3 following exposure for 6 h to 25 μM α-TOS is shown (B). The numbers indicate the percentage of cells with granular cyt *c* or active caspase-3, as obtained by visual evaluation of 100 cells (B). Immunoblotting (C) shows reduction of bcl-2 by α-TOS (2) vs control (1). Downregulation of bcl-2 by antisense but not reverse antisense or sense oligonucleotides (inset of panel C) potentiates while bcl-2 overexpression inhibits (E) phosphatidylserine externalization induced by α-TOS, as assessed by annexin V staining (D and E).

shown). IFM confirmed activation of caspase-3 and demonstrated that this was associated with a relocalization of cyt *c* or (pro)caspase-9, as was made evident by more diffuse or perinuclear staining, respectively (Figure 1C). While the granular staining for cyt *c* was consistent with a mitochondrial localization, caspase-9 appeared in a predominantly nuclear or cytosolic granular localization in untreated EC (Figure 1B). A similar caspase-9 localization was seen in the endothelial-like ECV304 cells (not shown), which is in contrast to findings which show that caspase-9 is primarily mitochondrial, e.g., in T cells (*32*). IFM in EC also showed expression of apaf-1 and its perinuclear relocalization during α-TOS treatment (Figure 1B). Similar results were also achieved in subconfluent endothelial cells (not shown). Together, our results suggest that α-TOS triggers relocalization of compartmentalized cyt *c* and caspase-9 and formation of the apoptosome, a cytosolic complex of apaf-1, caspase-9, and cyt *c*, as a prerequisite for activation of caspase-9 (*33*), followed by activation of executioner caspase-3 and -6 in EC. This extends our observations that α-TOS can destabilize lysosomes and mitochondria, likely due to its charged succinyl moiety (*23*).

To provide further evidence for an involvement of mitochondria in early pro-apoptotic events induced by α-TOS, we studied the role of the mitochondial anti-apoptotic protein bcl-2 which controls apoptosis in various cell types, including EC, and can be depleted during apoptosis (*28*, *29*). We observed a reduction in bcl-2 levels after treating confluent EC with α-TOS (Figure 1C). Downregulation of bcl-2 levels in EC by antisense ODN potentiated the externalization of phosphatidylserine (PS) induced by α-TOS, as was made evident by annexin V staining (Figure 1D). In contrast, overexpression of bcl-2 rendered EC more resistant to α-TOS (Figure 1E). Thus, bcl-2 appears to be a proximal and crucial element in α-TOS-induced effector pathways which may be consistent with an initiation of α-TOS signaling at the mitochondrial level.

The Extent of EC Apoptosis Depends on the Proliferation Status. Despite caspase activation induced by α-TOS in confluent EC, the monolayer remained intact during treatment (not shown). Caspase activation and an increased level of PS externalization induced by α-TOS were observed in both confluent and subconfluent EC (Figure 2, not shown). In contrast, a significant increase in the number of TUNEL-positive cells (Figure 2A) and substantial cleavage of PARP or lamin B (not shown), i.e., recognized measures of cell death, were only found in subconfluent but not confluent cells. EC can react with different susceptibilities to pro-apoptotic signals depending on their proliferative status, a phenomenon that is linked to expression and/or activation of cell cycle-associated proteins (*34*, *35*). We therefore studied the effects of α-TOS on EC at different proliferation rates, using EC plated at various densities before treatment with α-TOS. Subconfluent EC showed a high proliferative index which was dependent on their density, as assessed by incorporation of BrdU (not shown), while confluent EC were more quiescent than subconfluent cells, as was made apparent by cell cycle analysis (Figure 2B) or PCNA staining (not shown). The susceptibility of EC to α-TOS-induced apoptosis, as assessed by TUNEL staining, was directly proportional to the cellular proliferation status (Figure 2C). This indicates that α-TOS treatment of confluent EC represents a tool for achieving caspase activation without cell death, and such sub-apoptotic changes are insufficient to disrupt the endothelial integrity.

Inhibition of NFκB Activation by α-TOS in EC Involves Caspases. α-TOS has been shown to inhibit cytokine-induced NFκB activation, e.g., in EC, and to trigger caspase activation (*10*, *22*). Since caspases have been implicated in NFκB inhibition by cleavage of p65 (*14*), we attempted to establish a link between these mechanisms by studying the effects of α-TOS on NFκB activation by TNFα in EC. Pretreatment with α-TOS prevented nuclear translocation of NFκB in stimulated EC, as was made evident by IFM of p65 (Figure 3A). Moreover, pretreatment with α-TOS resulted in cleavage of p65 into a 54 kDa fragment (Figure 3B). As the cleavage product of p65 is still recognized by the mAb used for IFM, this suggests that the p65 fragments are retained in the cytosol without undergoing nuclear translocation (Figure 3A). Inhibition of NFκB activation was observed with α-TOS but not α-TOH, and was suppressed by cotreatment with z-VAD.fmk (Figure 3A,B), indicating an involvement of both the succinyl moiety and caspase activation.

We next studied the effects of α-TOS on NFκB-dependent transactivation after transient transfection of EC with an expression vector harboring EGFP with κB sites in the promoter region. GFP expression was induced by stimulation with TNFα; this was inhibited by pretreatment with α-TOS but not α-TOH, and the inhibitory effect of α-TOS was suppressed by cotreatment with z-VAD.fmk (Figure 3C). These results suggest that the inhibition of cytokine-

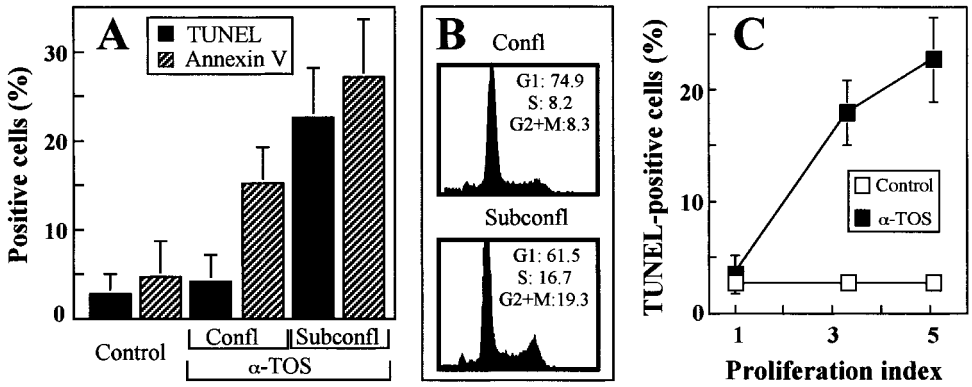


FIGURE 2: Susceptibility to apoptosis depends on to the proliferation status of EC. EC seeded at 10^6 (confluent) or 10^6 cells/well (subconfluent) treated with 25 μM α-TOS for 6 h were assessed for annexin V and TUNEL binding by FACS quantification (A). Untreated confluent and subconfluent EC were also analyzed for cell cycle pattern by FACS (B). To evaluate the relationship of proliferation status and susceptibility to apoptosis, EC were seeded at a density of 5×10^4, 2×10^5, or 10^6 cells/well, proliferation was assessed by the BrdU method, expressed as the proliferation index relative to the BrdU-positive percentage in highest-density EC, and TUNEL staining was performed (C).

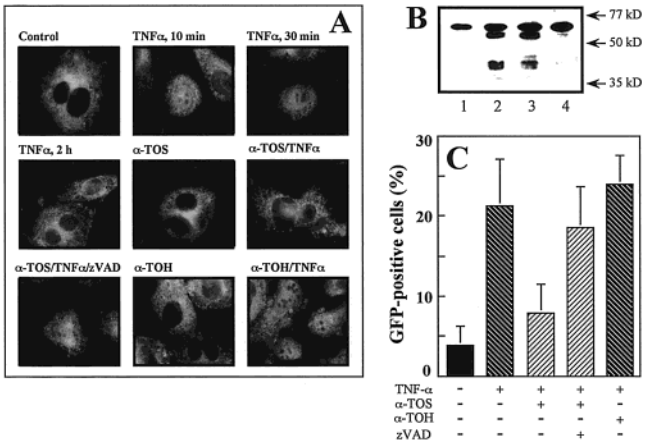


FIGURE 3: Inhibition of NFκB activation by α-TOS involves caspase activation. Translocation of p65 in EC stimulated with or without TNFα (100 units/mL) for 30 min, unless indicated otherwise, and pretreated with α-TOS, α-TOH, or z-VAD.fmk (all at 25 μM, 6 h) is shown by IFM (A). Immunoblotting of p65 in control EC (1), EC incubated for 6 h with 25 μM (2) or 50 μM α-TOS (3), or EC incubated with 25 μM α-TOS and z-VAD.fmk (4) is shown (B). κB-dependent GFP expression by transactivation in EC transiently transfected with pNFκB-EGFP treated as indicated (preincubation with α-TOS with or without zVAD.fmk or α-TOH, 6 h; stimulation with TNFα, 4 h) was assessed by FACS (C).

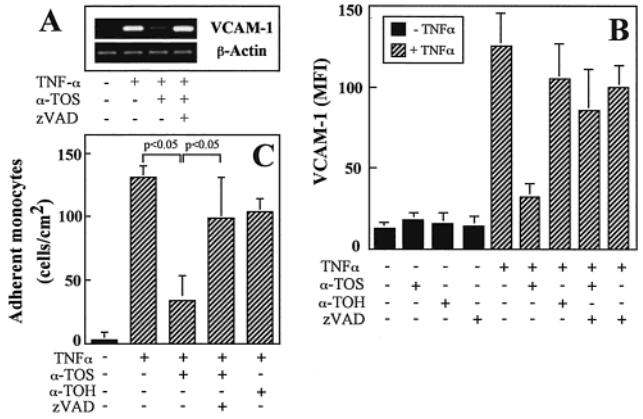


FIGURE 4: α-TOS inhibits VCAM-1 expression and monocyte arrest on activated EC. Confluent EC were pretreated with α-TOS, α-TOH, or z-VAD.fmk (25 μM each) for 6 h as indicated, stimulated with TNFα (100 units/mL, 4 h), and analyzed for expression of VCAM-1 mRNA by RT-PCR (A) or protein by FACS (B). Shear-resistant arrest of U937 monocytic cells on confluent EC pretreated and stimulated as indicated in flow is shown in C.

stimulated NFκB activation by α-TOS involves p65 cleavage mediated by caspase activation.

Collectively, these findings are consistent with the notion that α-TOS is a more potent anatagonist of NFκB transactivation than α-TOH (*10*, *20*, *21*) and suggest a link between the α-TOS-dependent induction of apoptotic programs and its inhibitory effect on NFκB activation.

α-*TOS Inhibits VCAM-1 Expression and Monocyte Arrest on Activated Endothelium*. NFκB controls the transcription of various genes expressed under inflammatory or atherogenic conditions, including VCAM-1 (*6*), which mediates monocyte arrest in flow (*36*, *37*). Since α-TOS prevented the NFκB-dependent transactivation of GFP in EC, we tested whether α-TOS inhibits endothelial expression of VCAM-1 induced by TNFα. Pretreatment of confluent EC with α-TOS but not α-TOH suppressed κB-dependent induction of VCAM-1 mRNA and surface protein expression by TNFα, as analyzed by RT-PCR, and FACS (Figure 4A−C). The inhibitory effect of α-TOS was reversed by z-VAD.fmk, suggesting an involvement of caspases (Figure 4A,B). As shown by gene transfer of IκBα, the NFκB-dependent expression of adhesion molecules, such as VCAM-1, and chemokines, such as GROα, is crucially involved in the spontaneous arrest of monocytes on TNFα-activated EC under flow conditions (*37*). In this system, we observed an enhancement in the shear-resistant arrest of U937 monocytic cells on confluent EC stimulated with TNFα (Figure 4C). These cells were used as a monocyte model, since their firm adhesion to TNFα-activated EC is mainly mediated by binding of the β1 integrin VLA-4 to VCAM-1 (*31*). As confirmed by mAb inhibition, arrest in shear flow was also largely mediated by interactions of VLA-4 and β2 integrins with VCAM-1 and ICAM-1, respectively (*31*). In agreement with the marked reduction in the level of VCAM-1 expression, pretreatment of TNFα-activated EC monolayers with α-TOS but not α-TOH resulted in a significant inhibition of monocytic cell arrest under flow conditions, and this was prevented by z-VAD.fmk, again implicating caspases (Figure 4C). Thus, inhibition of NFκB activation by a pathway involving caspases may have profound effects on EC adhesion molecule expression and monocyte recruitment under inflammatory conditions.

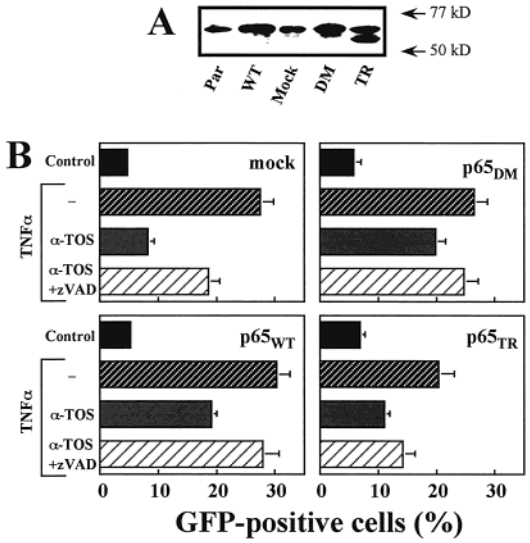


FIGURE 5: Role of p65 cleavage in inhibiting κB-dependent transactivation by α-TOS in EC. Expression of p65 in endothelial EC304 cells stably transfected with the vector (pcDNA, mock), the wild type (p65 WT), caspase-noncleavable p65 (p65 DM), or truncated p65 (p65 TR) is shown by immunoblotting in panel A. Cells were transiently cotransfected with pNFκB-EGFP, and GFP expression was analyzed after pretreatment with α-TOS, α-TOH, or z-VAD.fmk (25 μM each) for 24 h as indicated, and stimulated with TNFα (100 units/mL, 2 h) by FACS (B).

Role of p65 Cleavage in Inhibition of κB-Dependent Transactivation by α-TOS in EC. To establish a direct link between caspase activation, caspase-mediated cleavage of p65, and functional consequences, such as NFκB-dependent gene expression, we used endothelial-like ECV304 cells stably transfected with caspase-noncleavable or truncated p65 (*12*). Western blots revealed that stable transfection of ECV304 cells with $p65_{WT}$ or $p65_{DM}$ caused a 3-fold increase in the intensity of the 65 kDa band as compared to the intrinsic intensity, reflecting p65 overexpression (Figure 5A). In contrast, transafection with $p65_{TR}$ led to the appearance of an additional band at 54 kDa, corresponding to the product of the p65-truncated construct (*12*).

ECV304 is a cell line derived from HUVEC (*38*), which like their progenitor cells, show activation of NFκB in response to TNFα stimulation (*39*). To study κB-dependent transactivation under the influence of α-TOS-induced caspase activation, the ECV304 p65 transfectants characterized above were transiently cotransfected with pNFκB-EGFP, pretreated with α-TOS, stimulated with TNFα, and assessed by FACS for GFP expression. As illustrated in Figure 5B, all transfectants showed κB-dependent transactivation after stimulation with TNFα. As suggested by the percentage of GFP-positive cells, the extent of this transactivation appeared to be somewhat lower in the $p65_{TR}$ transfectants, indicative of a dominant-negative effect of the truncated p65, as shown previously (*12*), although the difference does not appear to be significant. This could be because of the fact that the efficacy of the transient transfection with pNFkB-EGFP was 25−30%. Notably, however, the inhibition of TNFα-stimulated transactivation by αTOS was less pronounced in transfectants expressing $p65_{DM}$ resistant to cleavage or in transfectants overexpressing the $p65_{WT}$ substrate than in mock or $p65_{TR}$ transfectants, indicating efficient p65 cleavage is required for inhibitory effects of α-TOS. In all cells, the inhibition of GFP transactivation by αTOS was attenuated by caspase inhibition with z-VAD.fmk (Figure 5B). Thus, our results demonstrate a link between α-TOS-induced caspase activation and inhibition of inflammatory NFκB activation due to caspase-dependent cleavage of p65, exemplifying a novel anti-inflammatory signaling pathway.

DISCUSSION

In this report, we show that activation of executioner caspases by the vitamin E analogue α-TOS in EC does not necessarily lead to cell death but under certain conditions may serve as a signaling pathway resulting in p65 cleavage and suppression of NFκB activation. Consistent with our recent observations with Jurkat cells (*22*, *23*), we found destabilization of mitochondria in EC after treatment with α-TOS. This impairment of mitochondrial integrity and/or function was characterized by formation of ROS and relocalization of cyt *c* and caspase-9. Together with the fact that α-TOS induced externalization of PS and activation of caspase-3 and -6, these findings imply the induction of early pro-apoptotic events dependent on mitochondrial destabilization, consistent with the current model of drug-induced apoptosis (*33*). According to this, the mitochondrial release of pro-apoptotic factors leads to formation of the apoptosome, a cytosolic complex consisting of cyt *c*, (pro)caspase-9, and apaf-1. As a part of this complex, caspase-9 is activated to process and thereby activate the executioner caspases, including caspase-3 and -6. An additional line of evidence for a mitochondrial role in α-TOS-induced apoptosis in EC is provided by our findings that bcl-2 overexpression is anti-apoptotic while bcl-2 downregulation renders EC more susceptible to α-TOS. This is in agreement with the known anti-apoptotic function of bcl-2 that stabilizes the mitochondrial membrane and subsequently prevents the cyt *c* release and the ensuing downstream events (*28*, *40*, *41*). Together with the observation that α-TOS treatment results in bcl-2 loss, these findings are consistent with the notion that during apotposis of EC, bcl-2 undergoes proteasome-dependent degradation (*28*, *30*).

In contrast to incubation with α-TOS, incubation of EC with the free form of vitamin E, α-TOH, did not result in any of the pro-apoptotic activation observed with α-TOS. This finding underlines the requirement for the charged succinyl moiety for induction of apoptosis, and may provide an explanation why α-TOS but not α-TOH has been found to inhibit NFκB activation in some systems (*10*, *20*, *21*). The possible function of the succinyl moiety could be destabilization of intracellular membraneous structures, including mitochondria, with the ensuing release of apoptogenic factors, and we have observed that it is required for efficient induction of apoptosis by vitamin E analogues in a variety of cell lines (*23*).

An intriguing finding in this report is the difference in response to pro-apoptotic stimulation of EC depending on their proliferation status. Thus, in confluent cells, α-TOS treatment leads to activation of executioner caspases, while only a low number of cells were TUNEL-positive, i.e., showing signs of advanced apoptosis (cf. Figures 1 and 2). In our experiments, we have regularly observed <5% detached cells when confluent EC were exposed to α-TOS, while cell detachment was a prominent feature under

subconfluent conditions. The extent of apoptotic cell death in EC proved to be directly proportional to their proliferation index (cf. Figure 2). The notion that contact-inhibited EC are resistant to execution of apoptosis induced by α-TOS is strengthened by a previous report showing that exposure of confluent endothelium to 200 μM α-TOS led to only marginal cell detachment (*10*). Moreover, we observed that contact-arrested but not subconfluent EC, when exposed to α-TOS followed by trypsinization and replating, were largely viable (not shown). This implies that the control of downstream events in the apoptotic machinery may be dependent on cell cycle-related factors.

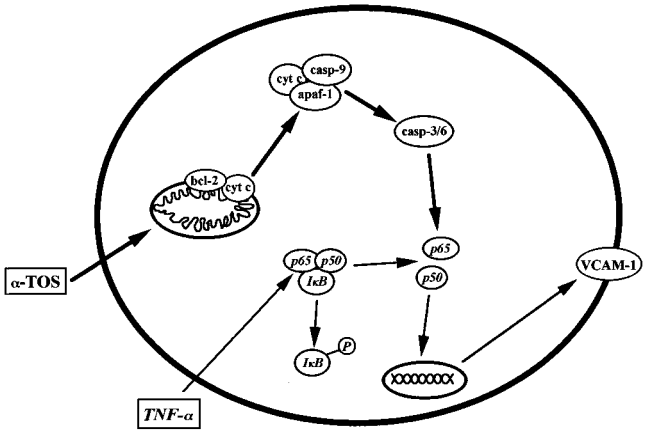
It has been reported that kinase activities of cyclin-dependent kinase-2 (cdk2) and cyclin A and expression of $p27^{Kip1}$ in EC depend on their proliferation status (*35*). While confluent cells exhibited G1 arrest, inhibition of cdk2/cyclin A kinase activities, and upregulation of p27, the opposite pattern was observed in proliferating cells. Thus, recent reports suggesting a relationship between caspase activation, cell cycle regulation, and apoptotic cell death (*42*, *43*) appear to be of paramount importance. The authors challenge the notion of executioner caspase activation as a "point of no return" and clearly document that activation of caspases with "DEVDase" activity per se is a prerequisite for cell death but does not suffice. This is based on experiments in which transfection of cells with dominant-negative cdk2 suppressed cell death following induction of apoptosis, while allowing for caspase activation, PS externalization, and mitochondrial dysfunction (*43*). These notions are further supported by a recent report showing caspase-3-dependent cleavage of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ with ensuing apoptosis in EC, and partial suppression of apoptosis upon transfection of the cells with dominant-negative cdk2 (*44*). These findings may provide an explanation for our observation that caspases can be activated in confluent endothelium without bringing forth cell death, while caspase activation appears to lead to cell death in proliferating EC.

Several anti-apoptotic proteins acting downstream from caspase have been recently characterized. These include members of the inhibitor of apoptosis protein (IAP) family. At least some of the IAPs have been suggested to be expressed following NFκB activity; they appear to antagonize the activity of caspases, and have been shown to provide resistance to apoptosis in certain cell types, e.g., in smooth muscle cells with constitutive NFκB activity (*45*−*47*). Moreover, it has been shown that the expression of some of the IAP family proteins is associated with cell cycle progression (*48*) or depends on the proliferative status of the cells (*46*). Consistent with the latter, we have observed higher levels of c-IAP1 expression in confluent EC than in subconfluent EC (J. Neuzil, unpublished). Notably, a recent report showed an interaction of IAP with cdk and other cell cycle-associated proteins in rescuing cells from apoptosis (*49*, *50*), further supporting the notion that the proliferative status may determine the fate of a cell following pro-apoptotic stimulation.

Our finding that activation of caspases by α-TOS in EC leads to p65 cleavage is consistent with a report showing caspase-dependent inhibition of NFκB activation in serum-starved EC (*12*). Whereas the authors observed cell death in serum-starved EC, caspase activation and inhibition of NFκB activation in our system using confluent EC did not lead to cell death. This difference may be due to the cell culture conditions that were used, i.e., presence of additional serum-derived survival factors, and indicates that NFκB may not be an absolute prerequisite for protection from cell death but may rather be a common link between pro-apoptotic and pro-inflammatory pathways. As recent reports show a requirement for NFkB activation for hypoxia-induced (*51*) or p53-dependent cell death (*52*), the cross-talk between these pathways seems to be bidirectional (*53*). Our data further challenge the generally accepted notion that inactivation of NFκB and cell survival are not necessarily incompatible after a given apoptogenic stimulus (*12*−*15*). Nevertheless, boosting the levels of cellular NFκB by tranfection of its essential subunit, p65, either in its natural form or mutated so that it cannot be cleaved by caspases, provides cells with additional resistance to apoptosis (*12*; J. Neuzil, unpublished), underscoring the importance of the κB-dependent prosurvival factors.

As activation of NFκB by a variety of stimuli results in transactivation of pro-inflammatory genes (*1*, *2*, *5*), our finding that treatment of EC with α-TOS inhibits transcription of the NFκB-dependent VCAM-1 gene could be deemed physiologically important. The fact that expression of VCAM-1 in confluent EC elicited with TNFα is inhibited by pretreatment with α-TOS and that adhesion of monocytic cells to such intact endothelium is markedly suppressed suggests that this vitamin E analogue is capable of reducing a prominent feature of inflamed endothelium without causing damage to it. This could be important because in atherogenesis, NFκB-dependent expression of VCAM-1 and chemokines, such as MCP-1, perpetuates a recruitment of inflammatory cells from the blood stream. Hence, our data revealing that this crucial pro-inflammatory signaling can be suppressed by α-TOS via its inhibitory effect on NFκB activation suggest that this pharmacological analogue of vitamin E could suppress inflammatory events in and around

Scheme 1: Possible Mechanism of Signaling Leading to Caspase-mediated Inhibition of NFκB Activation[a]



[a] According to the scheme, a pro-apoptotic agent such as α-TOS destabilizes membranous structures, which leads to cyt *c* release, a step which can be blocked by bcl-2. Cyt *c* forms a complex (apoptosome) with procaspase-9 and apaf-1, which is followed by release of active caspase-9. This initiator caspase then activates the executioner caspase-3 and -6, which can cleave the p65/RelA subunit of NFκB, thus preventing activation of the transcription factor by pro-inflammatory cytokines, such as TNFα. By this mechanism, a pro-apoptotic stimulus like α-TOS activates a mechanism that overrides signals otherwise inducing transcription of (pro-inflammatory) genes.

endothelium associated with a variety of immune-dependent diseases, while not causing EC death. Notwithstanding, in vivo verification of such an intriguing scenario is missing.

In conclusion, we provide data suggesting a paradigm for a relationship between NFκB activation and induction of pro-apoptotic pathways (Scheme 1) within intact endothelium, a phenomenon that is of potential physiological and/or pharmacological relevance.

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