

Role of Protein Kinases on NF- κ B Activation and Cell Death in Bovine Cerebral Endothelial Cells

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Nuclear factor κ B (NF- κ B) activation is modulated by various protein kinases. Activation of NF- κ B is known to be important in the regulation of cell viability. The present study investigated the effect of inhibitors of protein tyrosine kinase (PTK), protein kinase C (PKC) and protein kinase A (PKA) on NF- κ B activity and the viability of bovine cerebral endothelial cells (BCECs). In serum-deprivation-induced BCEC death, low doses of TNF α showed a protective effect. TNF α induced NF- κ B activation within 4 h in serum-deprivation. PTK inhibitors (herbimycin A and genistein) and PKC inhibitor (calphostin C) prevented NF- κ B activation stimulated by TNF α . Likewise, these inhibitors prevented the protective effect of TNF α . In contrast to TNF α -stimulated NF- κ B activity, basal NF- κ B activity of BCECs in media containing serum was suppressed only by calphostin C, but not by herbimycin A. As well BCEC death was also induced only by calphostin C in serum-condition. H 89, a PKA inhibitor, did not affect the basal and TNF α -stimulated NF- κ B activities and the protective effect of TNF α on cell death. These data suggest that modulation of NF- κ B activation could be a possible mechanism for regulating cell viability by protein kinases in BCECs.

Key Words: NF- κ B, Cerebral endothelial cell death, Protein kinase

INTRODUCTION

Protein tyrosine kinase (PTK), protein kinase C (PKC) and protein kinase A (PKA) are involved in many cell-death and cell-protective pathways. Because protein kinases are key signaling molecules in cells and can phosphorylate various proteins imperative for cellular defense or apoptosis, their roles in cell viability are expected to be critical (Lee et al, 1993). Various stimuli have been suggested to modulate cell viability by changing the activities of various protein kinases.

PTK is implicated in both the positive and negative regulation of apoptosis. PTK was involved in apoptosis induced by ionizing radiation or CD95 receptor triggering (Szabo et al, 1998). However, inhibition of PTK in receptor-mediated signaling induced apoptosis (Otani et al, 1993; Kinoshita et al, 1995). These

phenomena were also applicable to PKA and PKC. Agents which activate PKC inhibited apoptosis of thymocyte (McConkey et al, 1989). Basal PKC activity has been reported to be important for the maintenance of cell viability (Drew et al, 1998; Villaba, 1998). However, in some paradigms, PKC participated in the apoptosis pathway (Garzotto et al, 1998). PKA also appears to be involved in proapoptotic and antiapoptotic cell programs (Ottonello et al, 1998; Srivastava et al, 1998). However, in most cases, downstream elements that would mediate the cytotoxic or cytoprotective actions of protein kinases have not been clearly identified.

NF- κ B is a transcription factor that is closely involved in inflammation, immune response, differentiation, development and cell death/survival. Recently, NF- κ B has been suggested as a cell survival molecule (Beg & Baltimore, 1996; Van Antwerp et al, 1996; Wang et al, 1996; Wu et al, 1996). An essential step in the NF- κ B activation process is phosphorylation of I κ B, an inhibitory molecule of NF- κ B, which is then dissociated from NF- κ B (Thanos & Maniatis, 1995; Baeuerle & Baltimore, 1996). Thus,

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several protein kinases have been reported to be involved in the NF- κ B activation pathway. Although I κ B kinase was discovered as a protein kinase that directly phosphorylates I κ B (Mercurio et al, 1997), it is reasonable to believe that several protein kinases constitute the complex signaling cascade resulting in I κ B phosphorylation. It is very probable that modulation of the activities of protein kinases which would participate in NF- κ B activation pathway can affect cell viability by regulating NF- κ B activity. In this study, we investigated the role of protein kinases using various protein kinase inhibitors on the NF- κ B activation and cell survival in bovine cerebral endothelial cells (BCECs) stimulated by TNF α or serum. Here, we have shown that cell death induced by protein kinase inhibitors were well correlated with the suppression of survival molecule NF- κ B.

METHODS

Materials

Tumor necrosis factor α (TNF α), genistein, herbimycin A and calphostin C were purchased from Sigma (St. Louis, MO). H 89 was obtained from Calbiochem (La Jolla, CA). Double-stranded oligonucleotides containing consensus NF- κ B binding sequence and its mutant form were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Poly (dIdC) was purchased from Pharmacia (Uppsala, Sweden). [γ - 32 P]ATP was purchased from DuPont NEN (Boston, MA). Specific antisera to p50 and p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, Utah). Cell culture products were purchased from Gibco BRL (Life Technologies, NY).

Cell cultures

Bovine cerebral endothelial cells (BCEC) were prepared and characterized as previously described (DeBault et al, 1981; Abbott et al, 1991) with modifications (Xu et al, 1998). BCECs were maintained in Dulbecco's modified eagle medium (DME) with 10% fetal bovine serum (FBS) and endothelial growth supplements. Purity of BCEC was determined by positive immunocytochemical detection of Factor VIII and vimentin, but absent any expression of fibronectin, α -actinin and glial fibrillary acidic protein. BCECs of passage 4-12, which still expressed functional bradykinin receptors based on agonist-induced calcium

influx and phosphoinositide turnover, were plated on 10 cm² culture dishes maintained in DME with 10% FBS.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared according to the method described by Lee et al (1988) with modifications (Xu et al, 1997). For EMSA, the following oligonucleotide with the NF- κ B consensus binding sequence was used: (5'-AGTTGAGGGGACTTTCCCAGGC-3'). A mutant motif with G to C substitution (5'-AGTTGAGGCGACTTTCCCAGGC-3') served as a control. Labeling of the oligonucleotide with γ - 32 P-ATP and the EMSA method have been previously detailed (Xu et al, 1997). Nuclear fractions of equal protein content (4~6 μ g) were used in each assay. The reaction mixture in a final volume of 20 μ l contained 2 μ g poly (dIdC), 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol and 0.0175 pmole 32 P-labeled DNA probe. Reactions were started by the addition of nuclear extracts and carried out for 30 min at room temperature. Samples were loaded on 4% polyacrylamide non-denaturing gel and electrophoresed for 2 h at 180 V. The dried gel was exposed to Kodak XR5 film on intensifying screen for 10~20 h at 70°C. For competition assay, an unlabeled NF- κ B oligonucleotide was added in 100-fold excess. For supershift assay, 1 μ l of anti-p65 or anti-p50 antibody was incubated with nuclear extract for 30 min at room temperature prior to binding reaction.

Cell death assessment

Quantitative assessment of cell death was based on the extent of lactate dehydrogenase (LDH) released into the medium as a consequence of cell membrane damage. LDH release is widely accepted as a reliable quantitative measure of the extent of death in a number of cell types (Koh & Choi, 1997) including BCECs (Xu et al, 1998; Kim et al, 1999). Cell death was expressed as a percentage of "total kill" with the extent of LDH release caused by treating cells with triton X-100 as 100% (Xu et al, 1998; Kim et al, 1999). For the assay, LDH released from cells was reacted with pyruvic acid and β -NADH. Oxidation of β -NADH was then followed in a SOFTmax PRO multiplate reader (Molecular Devices, Sunnyvale, CA) at 340 nm in kinetic mode.

Statistical analysis of data

All results were expressed as means \pm standard error (SE). Analysis of variance was used to assess the differences between multiple groups. If the *F* statistics were significant, the mean values obtained from each group were then compared using Fisher's least significant difference method. A *P* value < 0.05 was used as the criterion for a statistically significant difference.

RESULTS

Changes of NF- κ B activation and cell viability in serum-depleted and TNF α -stimulated BCECs

Basal NF- κ B activity was present in BCECs which

were maintained in DMEM containing 10% FBS (Fig. 1A). Basal NF- κ B activation was decreased after serum withdrawal, which was observed after 4 h of deprivation (Fig. 1A). Low doses of TNF α (3 ng/ml) activated NF- κ B of serum-depleted BCECs within 4 h (Fig. 1A). To ensure that the mobility shift bands were the NF- κ B/oligonucleotide complex, we used a NF- κ B mutant oligonucleotide with a "G" \rightarrow "C" substitution in the NF- κ B binding motif as a control. Addition of this cold NF- κ B mutant oligonucleotide (100-fold in excess) did not affect the bands (p50/p65 and p50/p50) which were associated with the labeled NF- κ B oligonucleotide probe; whereas the cold consensus NF- κ B oligonucleotide (100-fold in excess) completely abolished these bands (Fig. 1B). AP-1 or Oct-1 consensus oligonucleotide also failed to displace the labeled NF- κ B oligonucleotide probe (Fig. 1B). Incubation of nuclear extracts with antisera

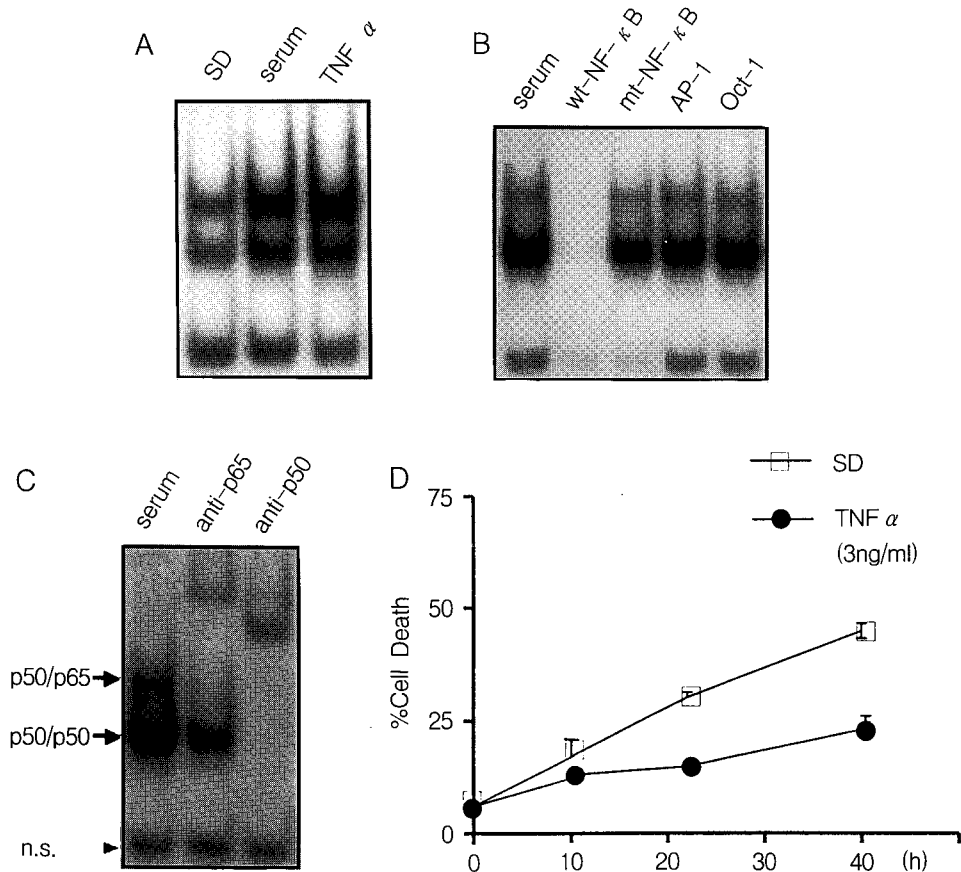


Fig. 1. Effect of TNF α on serum-deprivation induced BCEC death and NF- κ B activation. (A) Decrease of NF- κ B activity at 4 h after deprivation of serum from 10% FBS-containing media. Activation of NF- κ B in BCECs by treatment of 4 h of 3 ng/ml TNF α in serum-deprivation. (B) Competition for NF- κ B binding with cold nucleotides [wild type (wt-) or mutant- (mt-) NF- κ B, AP-1 or Oct-1] which were in excess of 100-fold. (C) Supershift with anti-p65 or anti-p50 IgG. Arrowhead denotes the nonspecific band (n.s.). (D) BCECs were treated with 3 ng/ml TNF α for 48 h. Cell death assessment was done at the indicated time.

against NF- κ B p50 or the p65 subunit, respectively, caused a supershift of the corresponding NF- κ B/oligonucleotide band (Fig. 1C). These results established the specificity of the NF- κ B binding activity assay. In cell death assessment, the cell death ratio of BCECs increased up to about 50% 40 h after serum-deprivation (Fig. 1D). TNF α reduced the cell death ratio of BCEC that was increased by serum withdrawal (Fig. 1D).

Effect of PKC-, PTK-, PKA-inhibitor on the viability of BCECs rescued by TNF α in serum deprivation

To determine the protein kinases involved in TNF α -provoked survival signaling, BCECs were pretreated for 12 h with several protein kinase inhibitors before administration of 3 ng/ml TNF α . Cell death assessment was done at 40 h of TNF α treatment. PTK inhibitors, herbimycin A and genistein, partially prevented the protective effect of TNF α on BCECs (Fig. 2A, 2B). Herbimycin A and genistein themselves did

not alter the cell death ratio of BCECs incubated in serum-deprivation media (Fig. 2A, 2B). Calphostin C, a specific PKC inhibitor, inhibited the effect of TNF α on BCECs in a dose-dependent manner. Calphostin C almost completely blocked the protective action of TNF α at 1 mM (Fig. 2C). Calphostin C itself did not affect the viability of BCECs in serum-deprivation (Fig. 2C). However, the PKA inhibitor H 89 did not change the cell death ratio that was determined by serum-deprivation or TNF α stimulation (Fig. 2D).

Effect of PKC-, PTK-, PKA-inhibitor on NF- κ B activation by TNF α in serum-deprivation

To determine the protein kinases that are implicated in NF- κ B activation signaling provoked by TNF α , nuclear extracts were obtained for EMSA at 4 h of TNF α treatment. Inhibitors of PKC, PTK, and PKA were administered 12 h before TNF α treatment. Genistein (100 μ M) and Herbimycin A (2 μ M) suppressed NF- κ B activity to the level of activity in

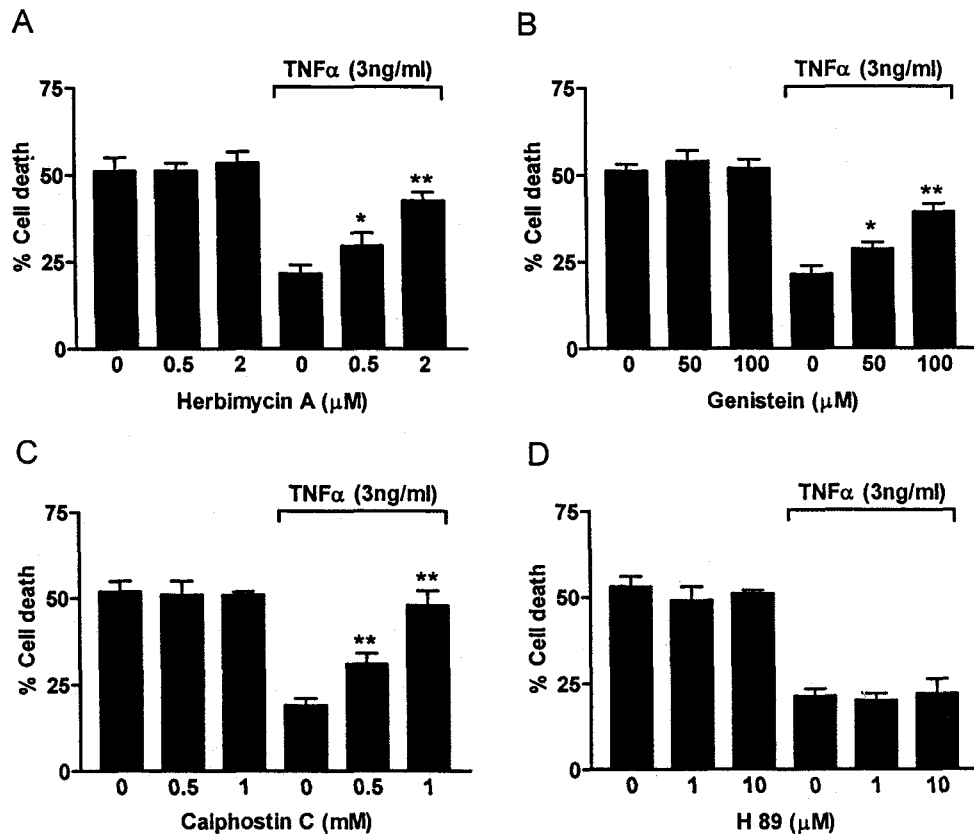


Fig. 2. Effects of PKC-, PKA-, PTA-kinase inhibitors in viability of BCEC stimulated by TNF α . BCECs were pretreated with herbimycin A (A), genistein (B), calphostin C (C) or H 89 (D) for 12 h in serum-deprivation and then TNF α (3 ng/ml) was administered. After 40 h of TNF α treatment, cell death assessment was done using LDH assay. SD indicates serum-deprivation control. Data are expressed as mean \pm SE of four independent experiments, each in quadruplicate, *P < 0.05, **P < 0.01.

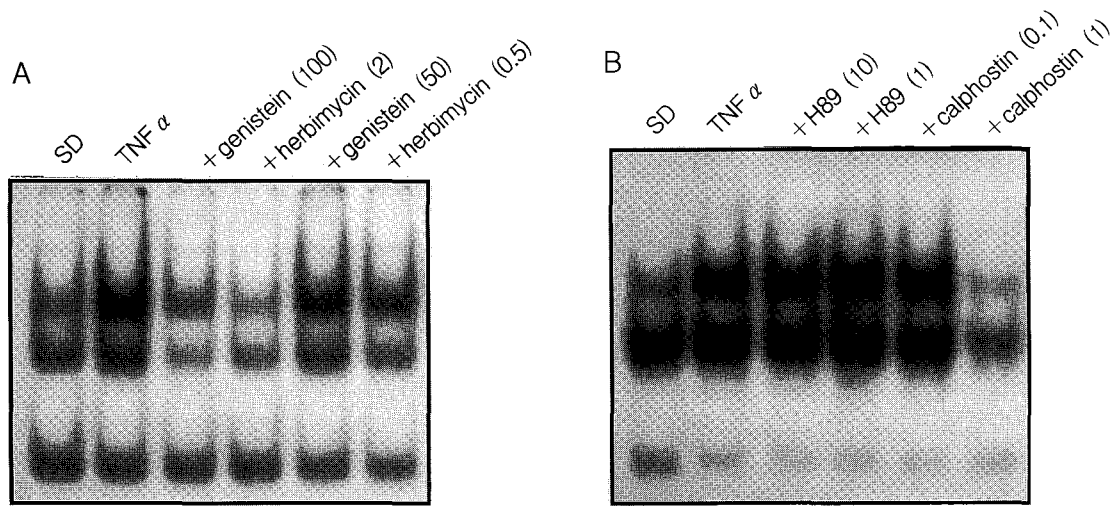


Fig. 3. Effects of PKC-, PKA-, PTA-kinase inhibitors on NF- κ B activation stimulated by TNF α . TNF α (3 ng/ml) was administered to BCECs which were pretreated with specified concentrations of herbimycin A (A), genistein (A), calphostin C (B) or H 89 (B) for 12 h in serum-deprivation. At 4 h of TNF α treatment, nuclear extracts were prepared for EMSA. SD denotes serum-deprivation control.

serum-deprivation state (Fig. 3A). Calphostin C (1 μ M) also almost completely inhibited the stimulated NF- κ B activity (Fig. 3B). However, H 89 did not inhibit NF- κ B activation up to 10 μ M (Fig. 3B). Inhibitors of the protein kinases which were used in this experiment did not affect the NF- κ B activity of serum-deprivation (data not shown).

Effect of PKC-, PTK-, PKA-inhibitor on BCEC death and NF- κ B activation in serum condition

As shown above, BCECs maintained in serum-contained media have basal NF- κ B activity. To determine whether protein kinase activities contribute to basal NF- κ B activity and cell survival, we tested the effect of protein kinase inhibitors on NF- κ B activity and cell viability in BCEC which had been growing in serum-contained media. In LDH assay, about 10% of cell death was detected in serum control due to the innate LDH present in FBS. Herbimycin A did not alter the cell death index of BCECs in serum condition for up to 40 h (Fig. 4A). H 89 also had no effect on the viability of BCECs during the same time period (Fig. 4B). However, 1 mM calphostin C induced BCEC death up to about 60 % (Fig. 4C). NF- κ B activities were measured at 8 h after treatment of protein kinase inhibitors. In accordance with results for the cell death index, herbimycin and H 89 did not inhibit basal NF- κ B activation (Fig. 4D, 4E), while calphostin C strongly inhibited basal NF- κ B activation (Fig. 4F).

DISCUSSION

Although TNF α exerts a cytotoxic role in some experimental conditions, the fate of cell death was, in most cases, determined by the balance of apoptotic and anti-apoptotic signaling triggered simultaneously by TNF α stimulation (Beg & Baltimore, 1996). The most probable anti-apoptotic molecule that is activated by TNF α is NF- κ B (Beg & Baltimore, 1996; Van Antwerp et al, 1996; Wang et al, 1996). Previously, we found that NF- κ B inhibition by pyrrolidine dithiocarbamate led to BCEC death (Kim et al, 1998). Therefore, we hypothesized that basal NF- κ B activity in BCECs was also crucial for the maintenance of normal viability, which is also the case in B cells (Wu et al, 1996). This could be supported by the fact that NF- κ B activity decreased during the serum-deprivation-induced cell death process. TNF α is a potent activator of NF- κ B (Osborn et al, 1989). To test our hypothesis, we observed the effects of TNF α on cell death and NF- κ B activation in a serum-deprivation paradigm. In this experiment, TNF α increased NF- κ B activity in a serum-deprived condition. At the same time, TNF α rescued BCECs from serum-deprivation-induced death. These results indicate that NF- κ B activation by TNF α could have a protective role in cell death induced by serum-deprivation.

To test the role of protein kinases on cell death and NF- κ B activation, we used several inhibitors of protein kinases in this experiment. PTK inhibitors (herbimycin A and genistein) and PKC inhibitor (calphostin

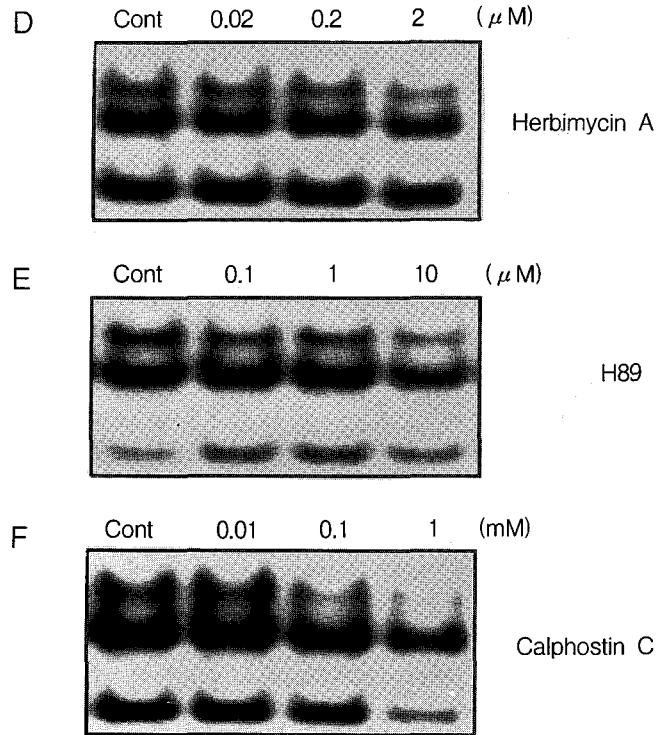
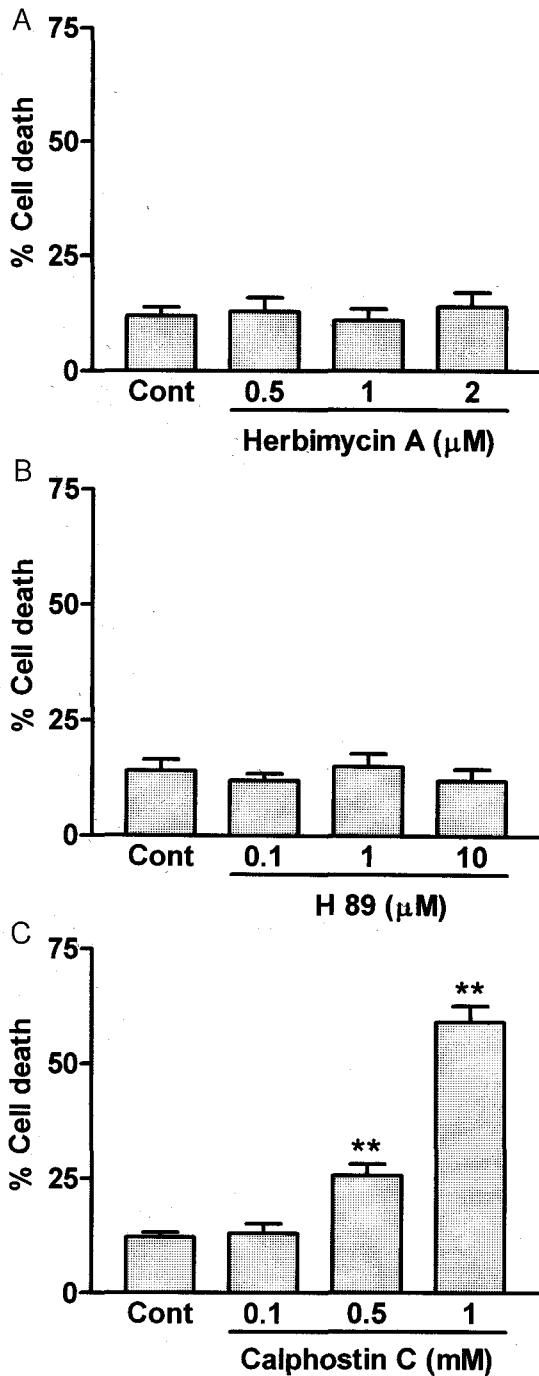


Fig. 4. Effects of PKC-, PKA-, PTA-kinase inhibitors on BCEC viability and basal NF- κ B activity. BCECs were incubated for 40 h with herbimycin A (A), H 89 (B) or calphostin C (C), and the cell death assessment was done at 40 h. BCECs were treated for 8 h with each protein kinase inhibitor, herbimycin A (D), H 89 (E) or calphostin C (F), and the nuclear extracts were obtained at 8 h. Cont denotes serum control. SD indicates serum-deprivation control. Data are expressed as mean \pm SE of four independent experiments, each in quadruplicate, ** $P < 0.01$.

C) blocked the protective effect of TNF α against cell death induced by serum-deprivation. The inhibitors of PTK and PKC not only inhibited the cytoprotective action of TNF α , but also suppressed the NF- κ B activity stimulated by TNF α . Thus, PTK and PKC could be implicated in protective signaling provoked by TNF α . However, PKA seemed not to be involved in this process because H 89 did not alter the effect of TNF α . Also, H 89 did not prevent NF- κ B activation by TNF α . Therefore, the role of protein kinases in the TNF α -evoked survival signaling pathway seems

to be related to the modulation of NF- κ B activation.

Next, we tested whether these kinases are involved in normal viability and basal NF- κ B activity in BCECs cultured in normal media containing serum. When various protein kinase inhibitors were used in this paradigm, only calphostin C, a PKC inhibitor, induced cell death. At the same time, only calphostin C inhibited basal NF- κ B activity. It seems that basal PKC activity is crucial for the maintenance of NF- κ B activity and cell survival. Similar findings were observed in Jurkat T cells and cerebellar granule cells

(Drew et al, 1998; Villaba, 1998). PTK inhibitors neither induced cell death, nor suppressed basal NF- κ B activation. Considering the fact that the dose of PTK inhibitor for inhibition of basal NF- κ B activation is much higher than those for inhibition of stimulated NF- κ B, it would be difficult to exclude the possibility that basal PTK activity also contributes to basal NF- κ B activation. In fact, basal PTK activity has been closely linked to the suppression of apoptosis (Otani et al, 1993). However, it is obvious that protein-kinase dependent modulation of BCEC death showed the best correlation with changes of NF- κ B activation.

In our study, we observed that NF- κ B inhibition was involved in BCEC death induced by the inhibition of protein kinases in TNF α -stimulated or serum-activated pathways. These results encourage further studies which could prove the intriguing hypothesis that NF- κ B is one of the active molecules which mediates the effect of PTK or PKC in antideath signaling.

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