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Effects of caffeic acid phenethyl ester (CAPE) on membrane potential and intracellular calcium in human endothelial cells

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Abstract. – BACKGROUNG AND OBJECTIVES: Caffeic acid phenethyl ester (CAPE) is an active component of the resin propolis obtained from beehives. Propolis has a long history of medicinal use and a number of studies have already reported on some of its pharmaceutical properties. This study aimed to explore the effects of CAPE on the cytosolic Ca^{2+} concentration, cell proliferation, membrane potential and NO levels in human endothelial cells.

MATERIALS AND METHODS: Isolated human umbilical vein endothelial cells (HUVEC) were incubated with CAPE (1-100 μM) at 37°C for 48 hours. Cell proliferation was estimated by counting cell numbers with use of a Neubauer chamber. The effect of CAPE (1-100 μM) on the membrane potential was measured with the fluorescence dye DIBAC4(3) whereas its effect on the cytosolic Ca^{2+} concentration was measured by use of the fluorescence probe Fluo-3 AM (Invitrogen, Leiden, Netherlands). NO production was assayed using the flourophore DAF~AM (Invitrogen, Leiden, Netherlands). Changes in fluorescence intensity was determined with the GENios plate reader (Genios, Tecan, Austria).

RESULTS: A Dose-dependent hyperpolarization of the endothelial cell membrane was observed with CAPE stimulation. The initial increase in the intracellular Ca^{2+} concentration showed a subsequent decrease over time. CAPE stimulation also resulted in an increase in NO production; however, at higher doses a decrease in NO levels was observed. HUVEC proliferation was inhibited by CAPE.

CONCLUSIONS: Here we report on the effect of CAPE stimulation on the cytosolic Ca^{2+} concentration, cell proliferation, membrane potential and NO production in HUVEC in a dose-dependent manner. These findings provide important insights into some potential key roles that both calcium and the membrane potential play in the CAPE activation of endothelial cells in a concentration-dependent manner.

Key Words:
Caffeic acid phenthyl ester, Endothelial cells, Calcium, Mebrane potential.

Introduction

The vascular endothelium is a monolayer of cells lining the entire innerl surface of the vasculature and heart. Endothelial cells are involved in numerous physiological and pathophysiological responses including permeability, regulation of vascular smooth muscle tone, blood flow, blood pressure, blood coagulation, cell and tissue growth, contractility and rythmicity[1-3]. Many mediators initiate these diverse functions. Calcium homeostasis has a major role in maintaining endothelial cell integrity and function. In endothelial cells, Ca^{2+} mobilizing agonists typically induce membrane hyperpolarization, as well as production of vasodilator compounds such as nitric oxide (NO). The later, is correlated with the increase in the intracellular calcium concentration[4,5]. Although calcium is a universal second messenger that triggers endothelial cell contraction, it can also lead to endothelial dysfunction. Endothelial dysfunction has been implicated in the imitation and propagation of vascular diseases including atherosclerosis, hypertension, cardiac hypertrophy and congestive heart failure[3]. NO is derived by several isoforms of nitric oxide synthase (NOS): neuronal (nNOS, NOS1), inducible (iNOS, NOS2), and endothelial (eNOS, NOS3). nNOS and eNOS are constitutive enzymes that are controlled by intracellular Ca^{2+} and the consequent activation of calmodulin (CaM). iNOS is neither a constitutively expressed nor a calcium-dependent enzyme[7,8].

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Caffeic acid phenethyl ester (CAPE) is an active component of propolis produced from the hives of the honeybee. Propolis has been used in traditional medicine as an antioxidant, anti-inflammatory, anti-carcinogenic and as well as a suppressive for tumor-induced angiogenesis[9,10]. Moreover, CAPE is a specific inhibitor of NF-kB, which is an important transcriptional factor maintaining apoptosis[11]. It has been shown in several studies that CAPE has a capability to inhibit the formation of capillary-like structures in HUVECs[10,12-14]. CAPE and fluorinated derivates have a cytoprotective effect and protect HUVEC against oxidative stress induced by menadione[15,16]. In addition, CAPE was shown to reduce neointima formation in rat carotid artery with endothelial injury[17] and also act as a cardioprotective agent particularly in acute myocardial ischemia-reperfusion injury[18]. Furthermore, CAPE was shown to modulate vascular activity *in vitro* by increasing the cytosolic Ca^{2+} concentration in rat aortic smooth muscle cell[19]. However, the effects of CAPE on Ca^{2+} homeostasis in endothelial cells remain unclear.

This study was designed to explore the effects of CAPE on cytosolic Ca^{2+} concentration in HUVEC, a widely used model for studying the vascular endothelium. We investigated not only the effect of different concentrations of CAPE in the modulation of Ca^{2+} homeostasis in HUVEC, but also the effect in proliferation, membrane potential and NO levels.

Materials and Methods

Chemicals

Bis-1,3-dibutylbarbituric acid-trimethine oxonol $DiBAC_4(3)$, and fluo-3~AM and DAF~FM diacetate were obtained from Invitrogen (Leiden, Netherlands); 1,2-bis-(*o*-aminophenoxy)-ethane-N,N,N',N'-tetra-acetic acid tetra~acetoxymethyl ester (BAPTA~AM) from Merck KGaA (Darmstadt, Germany); fetal calf serum (FCS) from Biowest (Seeveta, Germany) and Hank's balanced salt solution (HBSS) from PAA Laboratories GmbH (Linz, Austria); endothelial basal growth medium and supplements from Promo Cell (Heidelberg, Germany); trypsin-EDTA (0.05%) from Sigma (Deisenhofen, Germany). Caffeic acid phenethyl ester was obtained from Calbiochem (Darmstadt, Germany). All other reagents and chemicals used in this study were of technical grade.

The HEPES-buffered physiological bath solution was made of (concentrations in mmol/L): $CaCl_2$ 1.25; *D*-glucose 5.5; HEPES 10; KCl 5; $MgCl_2$ 0.5; NaCl 140. The pH was adjusted to 7.3-7.4 with 1M NaOH.

Cell Isolation and Culture

Human umbilical vein endothelial cells (HUVEC) were isolated from umbilical cords obtained from the University Hospital in Giessen (Germany), according to the method described by Jaffe et al[19]. The umbilical cord was washed with HBSS and later filled with 0.05% collagenase solution and incubated at 37°C for 30 minutes. The collagenase solution together with cells was centrifuged at 1000 rpm for 8 minutes. The supernatant was discarded and the cell pellet was re-suspended in the endothelial cell growth-medium enriched with 10% fetal calf serum (FCS) supplemented with growth factors and 1% penicillin plus streptomycin for 24 hours at 37°C. The medium was exchanged every 2-3 days. All experiments were performed with cells from the passage 2nd and 3rd.

HUVEC Proliferation Assay

HUVEC proliferation was investigated according to the protocol described previously by Schaefer et al (21). Cells were seeded in 12-well plates (Becton Dickinson, Heidelberg, Germany), at a density of 10,000 cells/well. After 24 hours of incubation the medium was replaced by endothelial cell growth-medium with 10% FCS. CAPE (1-10 µmol/l) and/or BAPTA (10 µmol/l) were also added to the medium for the experiment. After 48 hours, cells were trypsinized and the cell number was counted with a Neubauer chamber. The mean values of two counts per well were used for the statistical analysis.

Measurement of the Membrane Potential

Changes in endothelial membrane potential were investigated with the fluorescence dye $DiBAC_4(3)$, which responds the changes in the membrane potential. HUVECs were seeded on 24-well plates and grown for 48 hours. The cells were loaded with $DiBAC_4(3)$ 0.5 µmol/l for 30 minutes at 37°C in the dark., TCell stimulation with CAPE (1-100 µmol/l) followed and changes in membrane potential were measured using a microplate-plate reader Genios (Tecan, Austria) at 37°C, capable of measuring changes in fluorescence, absorbance, and glow luminescence with a spectrum range of 340-700 nm. Changes in the

membrane potential were measured for 30 minutes in intervals of 30 seconds with a wavelength of 485 nm and emission is detected at 535 nm. Fluorescence data was analysed and presented in percentage as compared to non-stimulated control.

$[Ca^{+2}]_i$ Assay in HUVEC

Changes in $[Ca^{+2}]_i$ concentration were detected with the fluorescence probe fluo-3 ~AM with an excitation wavelength of 485 nm and emission at 535 nm. Just after the HUVECs were cultured, the cells were loaded with fluo-3 ~AM (5 µmol/l) for 60 minutes at 37°C in the dark; the medium containing fluo-3 ~AM was replaced by HEPES-buffered bath solution for de-esterification for 25-30 minutes. HUVECs were stimulated with CAPE (1-100 µmol/l). Changes in fluorescence intensity of the calcium signal were measured by microplate-plate reader at 37°C up to 60 minutes at intervals of 60 seconds. The data was normalized to parallel non-stimulated control.

NO Production Assay

NO production was assayed by using the flourophore DAF~AM, with an excitation wavelength of 485 nm and emission at 535 nm. HUVEC were cultured in 24 well plates for 48 hours. The cells were loaded with NO sensitive fluorophore DAF~AM, for 60 minutes at 37°C in darkness, later the cells were further incubated for 30 minutes at 37°C for de-esterification. The cells were stimulated with CAPE (1-100 µM). NO synthesis led to an increase in fluorescence measured with a microplate-plate reader. The data was normalized to parallel non-stimulated control.

Statistical Analysis

Non-parametric tests Kruskal-Wallis test and Mann-Whitney test were applied to the data obtained (SPSS version 18.0; SPSS Inc. Chicago, IL, USA). Data were expressed as mean ± SD. The significance level was $p < 0.05$.

Results

Effect of CAPE on Intracellular Ca^{2+}

CAPE stimulation resulted in a significant increase of the intracellular Ca^{2+}. At lower concentrations (1-10 µM) oscillations in the intracellular Ca^{2+} were observed while relatively higher concentrations (100 µM) led to a rapid increase in Ca^{2+} levels after 4 minutes and attained a lag phase after ~25 minutes. Ca^{2+} levels were monitored up to 60 minutes after stimulation with various concentrations of CAPE 1-100 µM (Figure 1A). Calcium levels were statistically analyzed at 0, 4, 20 and 32 minutes after stimulation. The 0 minute can be considered around 30 seconds after stimulation due to the time gap. At 0 minute, a significant increase in intracellular Ca^{2+} for all concentrations was observed (Figure 1B). Further, it was observed that various concentrations caused statistically highly significant Ca^{2+} oscillations at various time points. On the other, at lower concentrations, CAPE caused Ca^{2+} oscillations which sometimes were close to the control levels.

Effect of CAPE on Membrane Potential

HUVEC stimulation with various concentrations of CAPE (1-100 µM) acted in a concentration-dependent manner. At higher concentrations (100 µM) there was a rapid and prolonged decrease in the cell membrane potential (Figure 2A), during the 30 minutes of measurement. None significant changes in membrane potential were observed at CAPE 1 µM concentration. Further, CAPE 10, 100 µM induced a significant decrease in membrane potential (Figure 2B), after 20 and 30 minutes of application.

NO Synthesis

NO synthesis was studied by NO sensitive flurophore DAF (Figure 3A). HA hghly significant increase in NO levels were observed after 6 and 8 min of stimulation with CAPE (1-5 µM). Application of CAPE 100 µM also resulted in a significant increase at 6 and 8 minutes followed by a later lag phase, suggesting that lower CAPE concentrations have a prolonged effect on NO levels perhaps by affecting eNOS (Figure 3B).

HUVEC Proliferation

Stimulation of HUVECs with 24 hours of CAPE stimulation resulted in concentration dependent decrease in proliferation. CAPE 1-10 µM caused highly significant decrease in HUVEC proliferation (Figure 4). It was also observed that prolonged exposure to higher concentration (100 µM) of CAPE is lethal to HUVEC.

Discussion

In our study, CAPE was shown to induce membrane hyperpolarization as well as an increase in intracellular Ca^{2+} concentration in endothelial

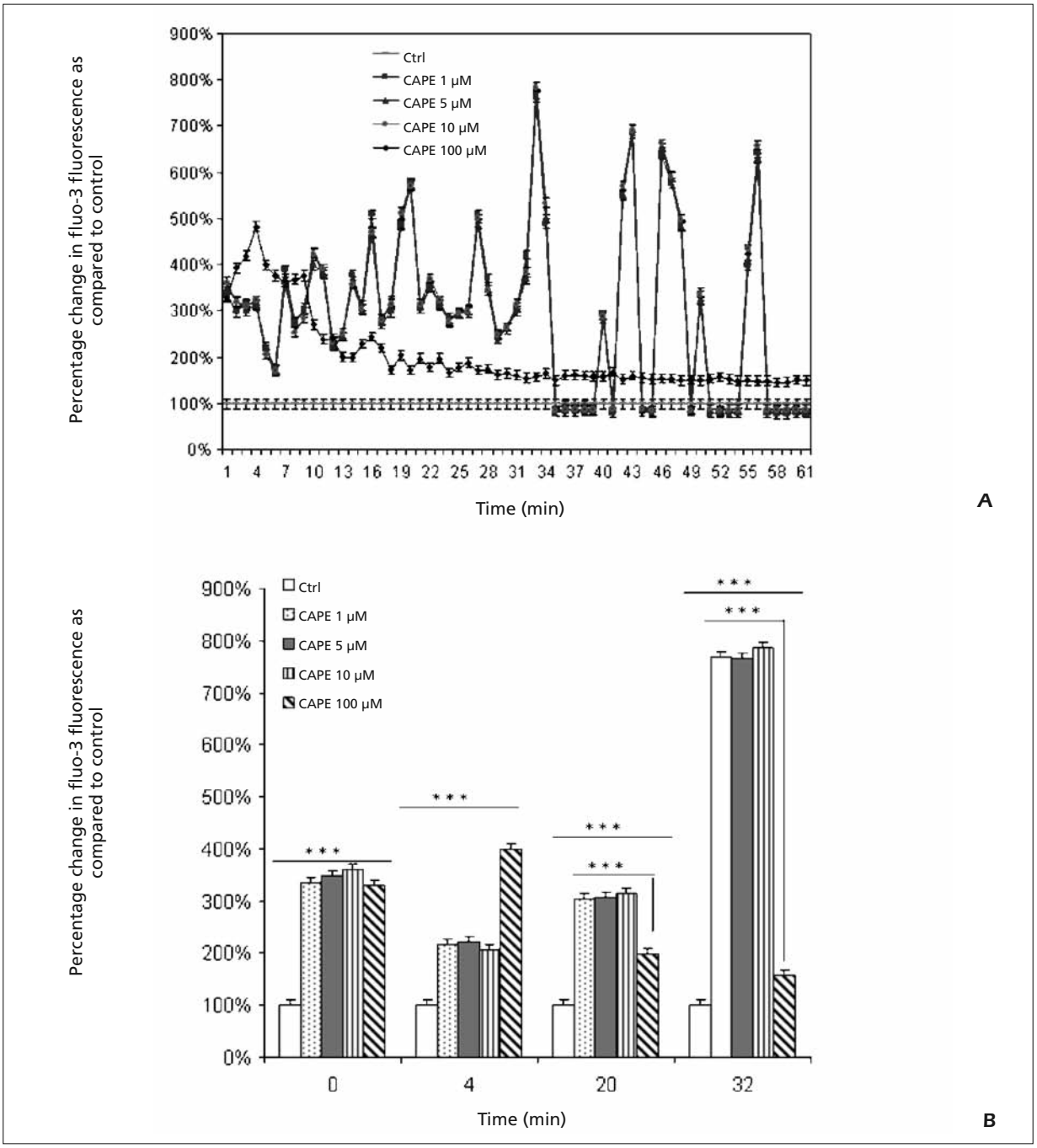

Figure 1. A, Time-course of CAPE stimulated intracellular Ca^{2+} recording. HUVEC were stimulated with CAPE 1-100 µM, and changes in fluo-3 fluorescence were measured with a plate reader for 60 min after addition of various concentrations of CAPE (n=24). ***B,*** TCAPE caused a significant change in the intracellular Ca^{2+} levels, in a concentration dependent manner. Data are shown as percentage of untreated control at 0, 4, 20 and 32 min after addition of CAPE 1-100 µM. The intracellular Ca^{2+} levels were analysed using fluo-3, a Ca^{2+} sensitive dye. The significance level was set as $p \leq 0.05$. The ***Indicate significance (n=24).

cells. Although several studies have described the effect of CAPE on endothelial cells[9-10,12,14-16], its effects on intracellular calcium, membrane potential, NO levels and their correlation with cellular proliferation are not investigated.

CAPE induced rapid changes in intracellular Ca^{2+} in HUVEC, and induced membrane hyperpolarization in a concentration-dependent manner. Furthermore, it also caused an increase in nitric oxide levels in endothelial cell, but notably with

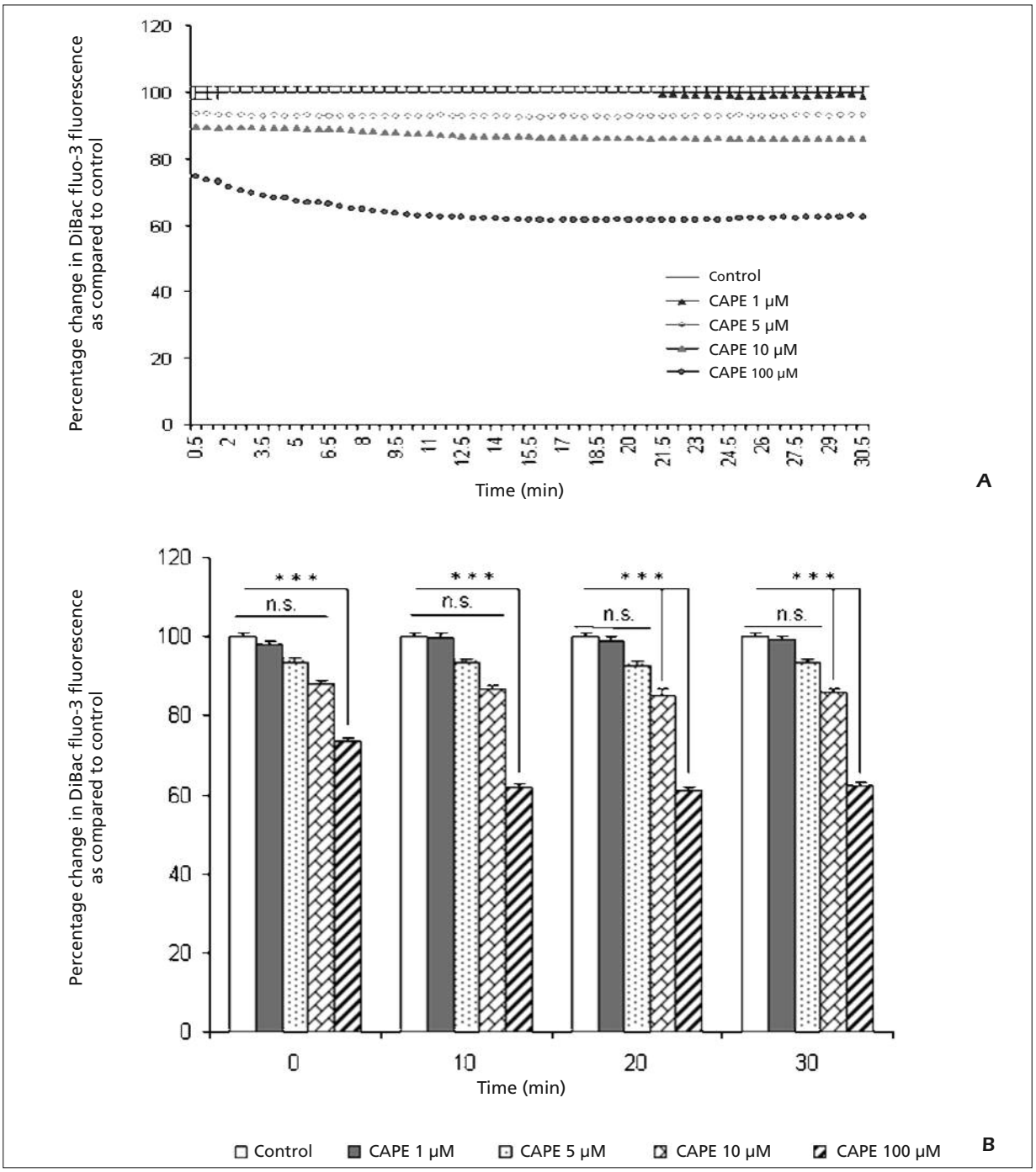

Figure 2. A, Time-course of CAPE stimulated membrane potential. HUEVC were stimulated with CAPE 1-100 µM, and changes in DiBac4 (3) fluorescence were measured with plate reader for 30 min after addition of various concentrations of CAPE. (n=24). ***B,*** CAPE caused a significant change in the endothelial cell membrane potential in a concentration dependent manner. Data are shown as percentage of untreated control at 0, 10, 20 and 30 min after addition of CAPE 1-100 µM. The membrane potential was analysed using the fluorescence probe DiBac4 (3). The significance level was set as $p \leq 0.05$. The `*´ indicate significance. (n=24).

the course of time higher concentration of CAPE decreased NO levels. Additionally; HUVEC exposed to CAPE (1-10 µM) showed a reduced proliferation but higher doses (e.g. 100 µM) resulted in cell deathThese data collectively suggested that low doses of CAPE could possibly have a protective role, but with increasing concentrations of CAPE can result in damaging effects.

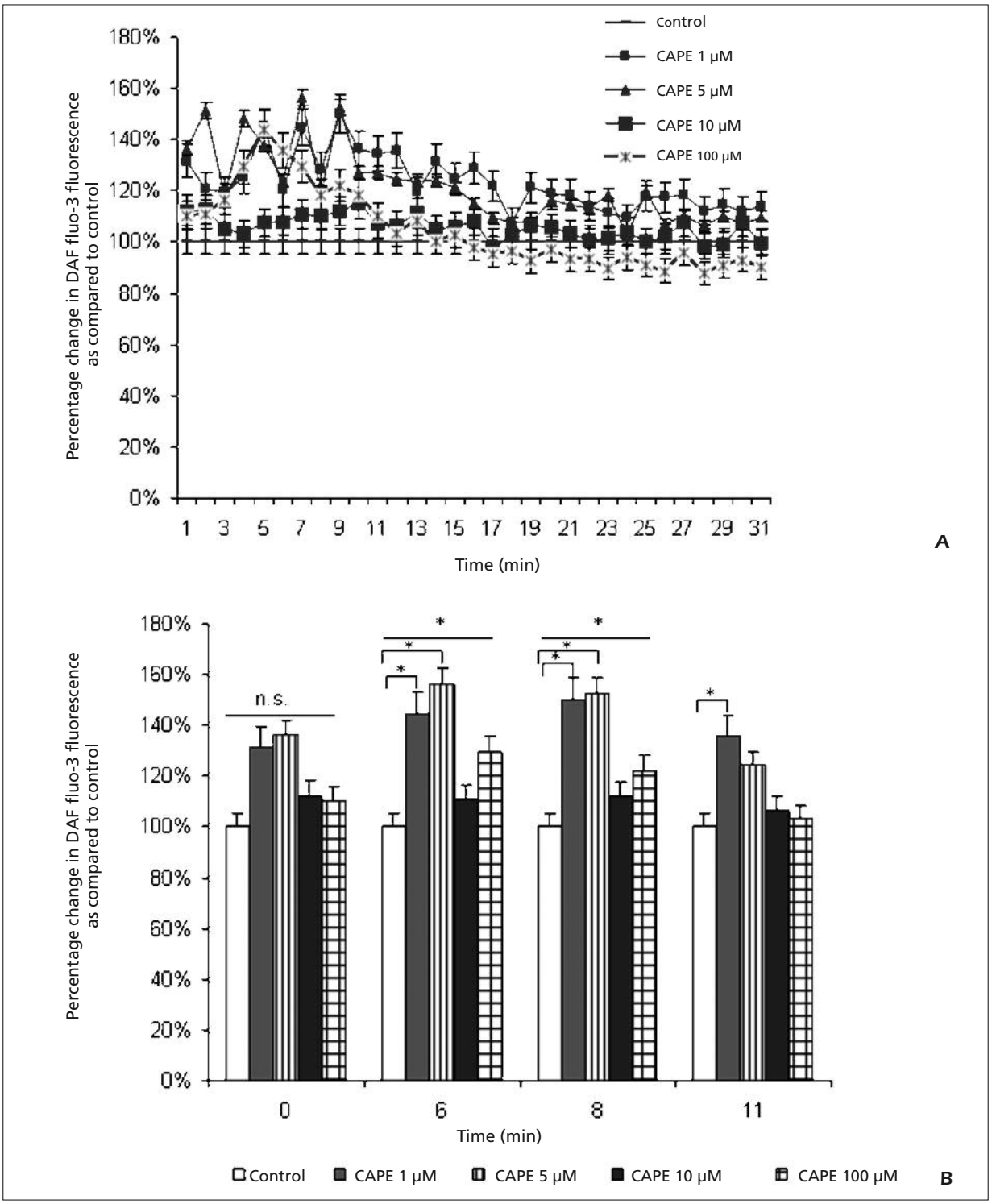

Figure 3. A, Time course of CAPE stimulated NO levels. HUEVC were stimulated with CAPE 1-100 µM, and changes in NO levels were assessed by DAF, an NO sensitive dye. NO levels were measured using a plate reader for 30 min after addition of various concentrations of CAPE. (n=12). ***B,*** CAPE caused a significant increase in NO levels in a concentration dependent manner. Data are shown as percentage of untreated control at 0, 6, 8 and 11 min after addition of CAPE 1-100 µM. The NO levels were assessed by DAF, a NO sensitive dye. The significance level was set as $p \leq 0.05$. The `*´ is for significance. (n=12).

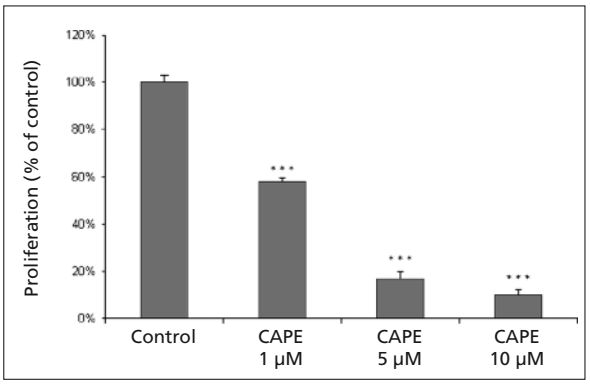

Figure 4. Effect of CAPE (1-10 μM) on HUVEC proliferation. CAPE effectively reduced HUVEC proliferation rate in a concentration dependent manner. HUVEC were cultured in 12 well plated and stimulated with CAPE (1-10 μM) and counted with the Neubauer chamber. Data are expressed as percentage of untreated control. The significance level was set as $p \leq 0.05$. The `*´ is for significance. (n=6).

Caffeic acid phenethyl ester, derived from propolis, has an antitumoral, anti-inflammatory, antioxidant, apoptotic and anti-angiogenic properties (and there are several studies reporting on its antitumor properties[10,12-14,22]. It has been reported that CAPE selectively inhibits the growth of several transformed cell lines and has selective cytotoxic and apoptotic effect on malignant cells[23,24]. Avci et al[24] reported recently that the apoptotic cell population increased with a dose-dependent manner in CCRF-CEM acute lymphoblastic leukemia cells exposed to increasing concentrations of CAPE. Although it has been reported that CAPE has not shown detrimental effects on normal cells, it is effective in suppressing both tube formation and proliferation in HUVECs in a concentration-dependent manner[12,22]. This concentration-dependent inhibition of HUVEC proliferation by CAPE is also confirmed by our results. In addition; in our study, CAPE has showed cytotoxicity at higher concentrations.

Table I. Summary of CAPE effects, all effects are significant ($p \leq 0.05$).

CAPE effects	N	Time (h)	Change %
Intracellular Ca-concentration	24	1	800
Membrane potential	24	0.5	-30
NO synthesis	12	0.5	150
Proliferation	6	24	-80

Endothelial cells modulate the intracellular Ca^{2+} concentration by calcium release from internal Ca^{2+} stores and/or influx from the extracellular space. In endothelial cells, increased intracellular Ca^{2+} concentration stimulates the calcium-activated K^+ channels and leads to hyperpolarization of the plasma membrane[25]. Calcium is the key second messenger in endothelial cells, involved in regulating a variety of cellular functions. Although Ca^{2+} is crucial for maintaining endothelial cell integrity and function, the increase in intracellular Ca^{2+} concentration in endothelial cells induces membrane dysfunction. It is well reported that there is a direct relationship between Ca^{2+} homeostasis and endothelial cell permeability[6]. Long et al[27] reported that CAPE has a capability in both inhibition of KCl concentration-dependent contractions in normal Krebs solution and reduction of the calcium concentration-dependent contraction in high K^+ depolarization medium in the porcine coronary arteries. This relaxant effect may be the result of NO released from the endothelium. In this study, we have shown that intracellular Ca^{2+} concentration in HUVEC increased in the presence of CAPE. Interestingly , the higher CAPE concentrations resulted in adecrease in the elevated intracellular calcium levels to normal. Previously, it was also reported that CAPE modulates the intracellular Ca^{2+} levels in rat aortic smooth muscle cells in aortic rings preparation[19]. In current work, we have shown that CAPE inhibits HUVEC proliferation in a concentration-dependent manner.

The observed inhibitory effect of CAPE on cell proliferation may be the result of increased intracellular calcium also in a concentration-dependent manner. In contrast to the intracellular Ca^{2+} concentration, higher CAPE concentrations increased membrane hyperpolarization. It has been reported that the rise in intracellular Ca^{2+} concentration is related to the increase in endothelial cell permeability[28].

During the 60 minutes recording experiment, after the first 30 minutes, strong Ca^{2+} oscillations were observed at CAPE (1-10 μM) which may be due to increased cell membrane permeability. Increased intracellular Ca^{2+} has been reported to play a vital role in the regulation cell death[29]. In the current study, CAPE treatment of HUVEC increases the intracellular Ca^{2+} concentration, which may lead to cell death. Sheng et al[30] reported that stimulation of vascular endothelial cells by ATP leads to increase of intracellular Ca^{2+} concentration and the subsequent activation

of small and intermediate conduction, Ca^{2+} activated K^+ channels, membrane hyperpolarization and acute NO production. Namely, it has been shown that endothelial membrane hyperpolarization, arising from calcium-activated potassium channel activity, directly determines the magnitude of stimulated NO synthesis in response to Ca^{2+}-mobilizing agonist[30]. In this study, we have demonstrated that CAPE could evoke changes in membrane hyperpolarization and cytosolic Ca^{2+} concentration leading to an increased production of NO in endothelial cells. On the other hand, at higher concentration CAPE decrease NO levels in HUVEC.

Conclusions

Collectively, our results suggest that low dose of CAPE (1 μM) has a protective effect on HUVECs, whereas high dose of CAPE might impair endothelial cell function. We have shown that CAPE alters cytosolic Ca^{2+} concentration, cell proliferation, membrane potential and NO production in HUVEC dose-dependent manner. Our findings provide interesting insights into possible key roles that calcium and the membrane potential may play in the concentration dependent CAPE activation of endothelial cells.

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