Caffeic Acid Phenethyl Ester Inhibits Endothelial Tissue Factor Expression

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Caffeic acid phenethyl ester (CAPE) is a component of honeybee hives with various beneficial properties. Tissue factor (TF), the key trigger of thrombosis, is expressed in human endothelial cells. This study was designed to investigate whether CAPE modulates TF expression in human aortic endothelial cells (HAECs). Western blots and real-time polymerase chain reactions were performed. CAPE (10−7–10−5 m) inhibited tumor necrosis factor (TNF)-α induced endothelial TF protein expression by 2.1-fold at 10−5 m (p<0.0001). Similarly, TF surface activity was reduced (p<0.02). In contrast, TF mRNA expression, TF promoter activity, and mitogen-activated protein (MAP) kinase activation remained unaltered. In conclusion, CAPE inhibits TF protein expression and activity at the posttranscriptional level thereby exhibiting anti-thrombotic potential.

Key words coagulation; thrombosis; endothelial function

Cardiovascular disease and acute coronary syndromes (ACS) in particular are a major cause of death and disability.1) Rupture of atherosclerotic plaques exposes a highly procoagulant plaque content to the circulating blood thereby promoting thrombus formation and ultimately vessel occlusion.1) Tissue factor (TF) is the key initiator of coagulation and thereby involved in the pathogenesis of ACS.2) Increased levels of TF have indeed been observed under these circumstances.2) Hence, inhibition of TF seems to be a promising target for reducing cardiovascular events.

Caffeic acid phenethyl ester (CAPE) is an active component of propolis from honeybee hives.3) It has various beneficial anti-inflammatory and immunomodulatory properties. It inhibits platelet aggregation, and exerts anti-oxidant and anti-angiogenic effects.1,5,6) CAPE reduced restenosis rates after balloon angioplasty of rat carotid arteries and diminished atherosclerotic plaque formation in the aorta of apoE−/− mice.6,7) However, the effect of CAPE on coagulation and thrombosis has not been assessed yet.

This study investigates whether CAPE modulates TF expression in human aortic endothelial cells (HAECs).

MATERIALS AND METHODS

HAECs (Clonetics) were stimulated with tumor necrosis factor (TNF)-α (R&D Systems, Minneapolis, MN, U.S.A.) for 5 h.8) CAPE (10−7–10−5 m; Cayman Chemicals, Ann Arbor, MI, U.S.A.) was added 1 h prior to stimulation. Cytotoxicity was assessed by a colorimetric assay for detection of lactate dehydrogenase (LDH; Roche, Basel, Switzerland).

Western blot analysis was performed as described.9) Antibodies against human TF (American Diagnostica, Stamford, CT, U.S.A.) and vascular cell adhesion molecule-1 (VCAM-1; R&D Systems) were used at 1:2000 and 1:1000 dilution, respectively. Antibodies against phosphorylated p38 mitogen-activated protein (MAP) kinase (p38), phosphorylated p44/42 MAP kinase (extracellular regulated kinase (ERK)), and phosphorylated c-Jun terminal NH₂ kinase (JNK; Cell Signaling, Danvers, MA, U.S.A.) were used at 1:1000, 1:5000, and 1:1000 dilution, respectively. Antibodies against total p38, total ERK, and total JNK (Cell Signaling) were used at 1:2000, 1:5000, and 1:1000 dilution, respectively. Antibodies against phosphorylated p70S6 kinase (p70S6K) and total p70S6K (Cell Signaling) were each applied at 1:2000 dilution. Antibody against inhibitor kappa B (1xB)-α (Santa Cruz, Dallas, TX, U.S.A.) was used at 1:2000 dilution. Blots were normalized to alpha-tubulin (αT, 1:20000; Sigma, Buchs, Switzerland) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Chemicon, Temecula, CA, U.S.A.) expression.

TF promoter activity was determined by a colorimetric assay (American Diagnostica).

TF and VCAM-1 mRNA expression were determined by real-time polymerase chain reaction (RT-PCR) on an MX3000P PCR cycler (Stratagene, Amsterdam, the Netherlands) using the SYBR Green JumpStart Taq Ready Mix kit (Sigma) as previously described.10) To verify the size of the bands, cDNA was loaded on a 1.5% agarose gel. The TF promoter (−227 bp to +121 bp) was cloned upstream of the firefly luciferase reporter gene, and a recombinant adenoviral vector was constructed as previously described.11) HAEcs were transduced with the vector Ad5/hTF/Luc (100 pfu/cell) for 1 h. An adenoviral vector without reporter gene (VQAd/Empty) was used as a negative control. After transduction, HAEcs were grown for 24 h and then serum-starved for 24 h before TNF-α stimulation with or without CAPE (1×10−5 M, 1 h) pretreatment. Firefly luciferase activity was determined in cell lysates using a lumino-meter (Berthold Technologies, Bad Wildbad, Germany) and normalized to the protein concentration in the lysates.

Statistics Data are given as mean±S.E.M. Unpaired Student’s t-test was performed for statistical analysis. A p-value <0.05 denoted a significant difference.

The authors declare no conflict of interest. These authors contributed equally to this work.

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RESULTS

CAPE Inhibits TNF-α Induced TF Protein Expression and Surface Activity CAPE (10^{-7}–10^{-5} m) concentration-dependently impaired TNF-α induced TF protein expression with a 2.1-fold decrease at 10^{-5} m (n=5; p<0.0001 versus TNF-α alone; Fig. 1A). A significant decrease in TF expression was observed between 3 and 7 h with maximal inhibition occurring after 7 h (n=3; p<0.001 versus 3 h TNF-α; Fig. 1B). Similarly, CAPE (10^{-5} m) inhibited TF surface activity (n=5; p<0.02 versus TNF-α alone; Fig. 1C). CAPE dose-dependently reduced VCAM-1 expression with a 1.9-fold decrease at 10^{-5} m (n=5; p<0.0001 versus TNF-α alone; Fig. 1D). CAPE did not alter basal TF protein expression and surface activity nor basal VCAM-1 protein expression (n=5; p=n.s. for all three parameters).

CAPE Does Not Affect MAP Kinase Activation, Nuclear Factor-kappa-B (NF-κB) Activation, TF Promoter Activity, and TF mRNA Expression CAPE (10^{-5} m) did not alter TNF-α induced activation of the MAP kinases JNK, p38, and ERK nor did it elicit a significant effect on p70S6 kinase activation (p=n.s. for all kinases; Fig. 2A). To assess NF-κB activation, expression of the inhibitory protein iκB was analyzed. CAPE did not alter the TNF-α induced iκB-α degradation pattern (n=4; p=n.s.; Fig. 2B), but prolonged its resynthesis at 60 min following incubation (n=4; p=0.045; Fig. 2B). TNF-α increased TF promoter activity by 2.2-fold (n=7; p<0.001), while CAPE alone or with TNF-α did not alter activity of the promoter (n=7; p=n.s. versus unstimulated control and TNF-α, respectively; Fig. 2C). Expression of TF mRNA (n=5; p=n.s.; Fig. 2D) and VCAM-1 mRNA (data not shown; n=5; p=n.s.) remained unaltered in the presence of CAPE.

DISCUSSION

This study demonstrates that CAPE inhibits TNF-α induced TF protein expression and surface activity in HAECs. Biologically active TF is located at the cell surface, while the remainder of cellular TF forms an intracellular and an encrypted pool. The effect of CAPE on cellular TF expression was paralleled by a decreased TF surface activity. The latter effect was less pronounced which can be explained by the dis-
tribution of TF in different cellular compartments as well as by the presence of encrypted TF.

The time course of the experiments was based on a previous study demonstrating that CAPE inhibited NF-κB activation after 1 h of preincubation. In line with that observation, CAPE was added 1 h prior to stimulation at maximal concentrations of $10^{-5}$ m. Similar CAPE concentrations have previously been applied without any cytotoxic effects, and cytotoxicity was not observed in our experiments.

Cytokines such as TNF-α are well known as inducers of endothelial TF expression and detectable at high concentrations at sites of vascular inflammation and particularly in atherosclerotic plaques. Since CAPE inhibited TNF-α induced TF expression, it is conceivable that it may exert this effect under the inflammatory conditions usually encountered in ACS. Furthermore, the inhibitory effect of CAPE on VCAM-1 expression is consistent with reduced formation of atherosclerotic plaques in the presence of the flavonoid.

TF protein expression is mainly regulated at a transcriptional level. However, CAPE did not alter activation of MAP kinases and p70S6K nor did it affect degradation pattern of IκB-α. A small delay in IκB-α resynthesis was observed with CAPE, a phenomenon that has been described previously. However, since TF promoter activity and TF mRNA expression remained unaffected by the flavonoid, the changes in the kinetic profile of IκB-α degradation seem not to be mediating the effect of CAPE in the present study, which is in line with earlier observations.

Hence, CAPE modulates TF expression at a posttranscriptional level in endothelial cells. This kind of regulation has previously been described for TF expression in this cell type.

Taken together, CAPE inhibits TF protein expression at a posttranscriptional level. Hence, CAPE, a naturally occurring mediator, exerts anti-thrombotic potential and represents a promising approach to reduce the risk of ACS.

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