Molecular and Cellular Pharmacology

Caffeic acid phenethyl ester-mediated Nrf2 activation and IκB kinase inhibition are involved in NFκB inhibitory effect: Structural analysis for NFκB inhibition

Youna Lee a,1, Dong-ha Shin b,1, Ji-Hye Kim a, Sungchae Hong a, Daekyu Choi a, Yung-Jin Kim c, Mi-Kyoung Kwak b,*, Yunjin Jung a,*

a College of Pharmacy, Pusan National University, Busan 609-735, Republic of Korea
b College of Pharmacy, Yeungnam University, Gyeongsan, Gyeongsangbuk-do 712-749, Republic of Korea
c Department of Molecular Biology, College of Nature Sciences, Pusan National University, Busan 609-735, Republic of Korea

A R T I C L E   I N F O
Article history:
Received 5 February 2010
Received in revised form 28 May 2010
Accepted 10 June 2010
Available online 20 June 2010

Keywords:
Caffeic acid phenethyl ester
NFκB
Nrf2
Inhibitory protein kappaB kinase Structural analysis

A B S T R A C T
Caffeic acid phenethyl ester (CAPE) is an active component of propolis from honeybee. We investigated potential molecular mechanisms underlying CAPE-mediated nuclear factor kappa beta (NFκB) inhibition and analyzed structure of CAPE for its biological effect. CAPE attenuated expression of NFκB-dependent luciferase stimulated with TNF-α or LPS and suppressed LPS-mediated induction of iNOS, a target gene product of NFκB. In HCT116 cells, CAPE interfered with TNF-α dependent IκBα degradation and subsequent nuclear accumulation of p65, which occurred by direct inhibition of inhibitory protein kappaB kinase (IKK). CAPE increased the expression of Nrf2-dependent luciferase and heme oxygenase-1, a target gene of Nrf2, and elevated the nuclear level of Nrf2 protein, indicating that CAPE activated the Nrf2 pathway. In HCT116 cells with stable expression of Nrf2 shRNA, CAPE elicited a reduced inhibitory effect on TNF-α-activated NFκB compared to scramble RNA expressing control cells. On the other hand, the NFκB inhibitory effect of CAPE was diminished by removal or modification of the Michael reaction acceptor, catechol or phenethyl moiety in CAPE. These data suggest that CAPE inhibits TNF-α-dependent NFκB activation via direct inhibition of IKK as well as activation of Nrf2 pathway, in which the functional groups in CAPE may be involved.

© 2010 Elsevier B.V. All rights reserved.

1. Introduction

Caffeic acid phenethyl ester (CAPE), which is structurally related to flavonoids, is a biologically active component of propolis from honeybee hives. The polyphenolic natural product has been widely used as an antioxidant against various oxidative processes in animal models and elicited numerous biological activities including antiviral, anti-inflammatory and immunomodulatory properties (Borrelli et al., 2002; Ho et al., 2005; Park et al., 2004; Son and Lewis, 2002).

Nuclear factor kappa beta (NFκB) is an important transcription factor that regulates genes involved in immunity, inflammation and cell proliferation (Li and Verma, 2002). The functional NFκB protein is a heterodimer composed of two subunits, p65 and p50 (Urban et al., 1991). Under normal conditions, NFκB is present in the cytoplasm in an inactive state, bound to IκB protein. Stimulation with proinflammatory cytokines such as TNF-α initiates an intracellular signaling cascade, resulting in the phosphorylation and subsequent degradation of IκBα by the 26S-proteasome (Tanaka et al., 2001). IκB phosphorylation is mediated by the high molecular weight inhibitory protein kappaB kinase (IKK) complex that contains two catalytic subunits named IKKα (IKK1) and IKKβ (IKK2) and a non-catalytic regulatory subunit named NEMO or IKKγ (Tang et al., 2003) The degradation of IκBα releases NFκB, allowing it to translocate into the nucleus, and activates cytokines, chemokines, antiapoptotic factors and cell growth factors that are pivotal mediators of the immune and inflammatory responses and tumorigenesis (Li and Verma, 2002). For this reason, inhibition of NFκB activation has attracted increasing attention as a therapeutic approach for chemoprevention of cancer development and intervention in immune and inflammatory reactions (Calzado et al., 2007; Yu et al., 2009).

Nuclear factor-erythroid 2 p45 (NF-Ε2)-related factor 2 (Nrf2), an important cytoprotective transcription factor, is sequestered in the cytoplasm as an inactive complex with its cytosolic repressor Kelch-like ECH associated protein 1 (Keap1), which in turn mediates protoxenmal degradation of Nrf2 by functioning as an adapter protein of an E3 ubiquitin ligase complex (Tong et al., 2006). When challenged by oxidants or electrophiles, Nrf2 is released from Keap1 repression, translocates to the nucleus, forms a heterodimer with small musculoaponeurotic fibrosarcoma proteins, recognizes and binds to a cis-acting enhancer antioxidant response element (ARE) or electrophile response element (EpRE),

1* Corresponding authors. Jung is to be contacted at Tel.: +82 51 510 2527; fax: +82 51 513 6754. Kwak, Tel.: +82 53 810 2823; fax: +82 53 810 4654.
E-mail addresses: mkwak@ynu.ac.kr (M.-K. Kwak), jungy@pusan.ac.kr (Y. Jung).
1 These authors contributed equally to this work.
0014-2999/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2010.06.016
consequently transactivating diverse antioxidant enzymes, phase II detoxification enzymes, and phase III efflux transporters.

CAPE is a well-documented inhibitor of NF-κB (Abdel-Latif et al., 2005; Natarajan et al., 1996), which may be an action mechanism for CAPE-mediated anti-inflammatory and anti-tumorigenic effects (Carrasco-Legleu et al., 2004; Fitzpatrick et al., 2001). Previous reports demonstrate that CAPE inhibits NF-κB activation by suppressing nuclear translocation and DNA binding of p65 or phosphorylation of p65, which is dependent or independent on IkBα protein degradation in the cytosol (Ang et al., 2009; Fitzpatrick et al., 2001; Onori et al., 2009; Toyoda et al., 2009). However, molecular targets and structural analysis of CAPE for inhibition of NF-κB activation still remains to be further investigated. In this study, potential molecular targets involved in CAPE inhibition of NF-κB were scrutinized along with structural analysis for the effect.

2. Materials and methods

2.1. Reagents

Recombinant human TNF-α was obtained from R & D systems (MN, USA). LPS was purchased from Sigma-Aldrich. (St. Louis, MO), CAPE was from Alexis (San Diego, CA). Catechol was purchased from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Reaction solvents were obtained from Junsei chemical Co. (Tokyo, Japan). All other chemicals were reagent-grade, commercially available products. Caffeic acid analogues used in this report were prepared in our laboratory (Choi et al., 2009). CP-NBD (YCGrARRRARTALDWSLQTE), a cell permeable specific NFκB peptide inhibitor, was purchased from Peptron (Daejeon, South Korea).

2.2. Cell culture

Human colon carcinoma HCT116 cells (ATCC, MD, USA), and murine macrophage RAW264.7 cells (ATCC, MD, USA) were grown in DMEM medium supplemented with 10% Fetal Bovine Serum, penicillin/streptomycin (Hyclone, Utah, USA).

2.3. Establishment of Nrf2 knockdown HCT116 cells

Lentiviral particles with shRNA were produced in HEK 293T cells as described previously (Shim et al., 2009). Briefly, HEK 293T cells were transfected with 1.5 μg pLKO.1-NRF2 shRNA plasmid, which contains human NRF2-specific shRNA (5′-CCGCGGTTCAATGGTTGTAATCAGTCAGGATTTACATCAGTCAGTAGGA-3′), or pLKO.1-scramble RNA plasmid, and the Packaging Mix (Sigma Co.) by using Lipofectamine™2000 (Invitrogen). Media containing lentiviral particles were harvested and collected for a further transduction. HCT116 cells were seeded on 6-well plates at a density of 2×10⁵ cells per well and were transduced with lentiviral particles containing either scramble non-specific RNA expression plasmid or NRF2 shRNA expression plasmid in the presence of 8 μg/ml of hexadimethrine bromide (Sigma Co.) for 24 h. For the selection of cells with target plasmid, cells were grown in a medium with 1 μg/ml puromycin (Sigma Co.) for up to 3 weeks. Inhibition of NRF2 was verified by using RT-PCR analysis of levels for NRF2 in obtained cell lines.

2.4. Luciferase assay

Cells were plated in 6 well plates to be 50–60% confluent on the day of transfection with either NFκB dependent luciferase plasmid (0.5 μg, a gift from Dr. M. Birrer, NCI) or ARE-luciferase plasmid (0.5 μg) and CMV Renilla luciferase plasmid (4 ng, Promega, WI, USA). Fugene (Roche, CA, USA) or Lipofectamine™2000 (Invitrogen) was used as a transfection reagent. Twenty four hours post-transfection, cells were treated with TNF-α (10 ng/ml) in the presence of various concentrations of caffeic acid phenethyl ester (CAPE) for 8 h and luciferase activity was measured and normalized to CMV Renilla luciferase activity. The data are means ± S.E.M. (n = 4). *P<0.05 vs. group treated with TNF-α only. **P<0.01 vs. group treated with TNF-α only. B. RAW264.7 cells were transfected with an NFκB responsive reporter gene plasmid and subsequently were treated with LPS (1 μg/ml) in the presence of various concentrations of caffeic acid phenethyl ester (CAPE) for 6 h and luciferase activity was measured and normalized to CMV Renilla luciferase activity. The data are means ± S.E.M. (n = 4). *P<0.05 vs. group treated with LPS only. **P<0.01 vs. group treated with LPS only B. RAW264.7 cells were 6 h-treated with LPS (1 μg/ml) in the presence of 5 μM CP-NBD (100 μM), a cell permeable specific NFκB peptide inhibitor, and were lysed to obtain whole cell lysates. iNOS protein levels were examined by Western blot. D. RAW264.7 cells were 6 h-treated with LPS (1 μg/ml) in the presence of various concentrations of CAPE and were lysed to obtain whole cell lysates. iNOS protein levels were examined by Western blot.

later and luciferase activities were measured and normalized to CMV Renilla luciferase activities using a Dual Luciferase reporter assay system (Promega).
2.5. Immunoblot analysis

Cells were lysed and nuclear and cytosolic extracts prepared as described (Andrews and Faller, 1991). Cell lysates were electrophoretically separated using 7.5% or 10% gels. Proteins were transferred to nitrocellulose membranes (Protran, Schleicher & Schuell, Keene, NH). IkBα protein was detected in the cytosolic extracts using polyclonal anti-IkBα antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and α-tubulin antibody (for cytosolic proteins) followed by addition of protein G-Agarose beads. The beads were subjected to in vitro kinase assay in the presence of various concentrations of CAPE.

2.6. In vitro kinase assay

To determine the effect of caffeic acid phenethyl ester (CAPE) on TNF-α-induced IKK activation, we performed an immunocomplex kinase assay. In brief, the IKK complex from whole-cell extracts (700 μg) was precipitated with an antibody against IKKα/β (20 μL, Santa Cruz) followed by treatment with protein G-Agarose beads (Santa Cruz). After 2 h incubation, the beads were washed and assayed in a kinase assay mixture containing 20 mM HEPES, pH 7.4, 2 mM MgCl2, 2 mM MnCl2, 10 mM NaF, 0.5 mM Na3VO4, 0.5 mM PMSF, 25 μg/ml aprotinin, 2 μg/ml pepstatin, 1 mM ATP and 1 μg of substrate glutathione S-transferase-IκBα (GST-IκBα, Santa Cruz). Drugs were pretreated 15 min before starting the kinase reaction. After incubation at 30 °C for 10 min, the reaction mixture was centrifuged and immediately boiled with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Phosphorylated GST-IκBα protein was detected by phospho-specific anti-IκBα antibodies from Cell Signaling Technology. The blots were reprobed with anti-IKKα/β antibody (Santa Cruz) to confirm equivalent amount of IKK in each kinase reaction.

2.7. RT-PCR

Total RNA was isolated using a Trizol reagent (Invitrogen, Carlsbad, CA, USA). The reverse transcriptase reaction was performed using 200 ng of total RNA in reaction mixture of nuclease free water, 10× PCR buffer (200 mM Tris–HCl (pH 8.4), 500 mM KCl), 50 mM MgCl2, 2.6. In vitro kinase assay

To determine the effect of caffeic acid phenethyl ester (CAPE) on TNF-α-induced IKK activation, we performed an immunocomplex kinase assay. In brief, the IKK complex from whole-cell extracts (700 μg) was precipitated with an antibody against IKKα/β (20 μL, Santa Cruz) followed by treatment with protein G-Agarose beads (Santa Cruz). After 2 h incubation, the beads were washed and assayed in a kinase assay mixture containing 20 mM HEPES, pH 7.4, 2 mM MgCl2, 2 mM MnCl2, 10 mM NaF, 0.5 mM Na3VO4, 0.5 mM PMSF, 25 μg/ml aprotinin, 2 μg/ml pepstatin, 1 mM ATP and 1 μg of substrate glutathione S-transferase-IκBα (GST-IκBα, Santa Cruz). Drugs were pretreated 15 min before starting the kinase reaction. After incubation at 30 °C for 10 min, the reaction mixture was centrifuged and immediately boiled with SDS sample buffer for 5 min. Finally, the protein was resolved on 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. Phosphorylated GST-IκBα protein was detected by phospho-specific anti-IκBα antibodies from Cell Signaling Technology. The blots were reprobed with anti-IKKα/β antibody (Santa Cruz) to confirm equivalent amount of IKK in each kinase reaction.

2.7. RT-PCR

Total RNA was isolated using a Trizol reagent (Invitrogen, Carlsbad, CA, USA). The reverse transcriptase reaction was performed using 200 ng of total RNA in reaction mixture of nuclease free water, 10× PCR buffer (200 mM Tris–HCl (pH 8.4), 500 mM KCl), 50 mM MgCl2,
3. Results

3.1. CAPE inhibits NF_{\kappa}B activation by TNF_{\alpha} and LPS

To examine whether CAPE effectively inhibits NF_{\kappa}B activation in our experimental conditions, HCT116 and RAW264.7 cells were transfected with an NF_{\kappa}B-dependent luciferase (NFDL) reporter plasmid in combination with an internal standard CMV Renilla luciferase plasmid. Cells were then treated with TNF_{\alpha} or LPS in the presence of CAPE (10 \mu M–50 \mu M). TNF_{\alpha} or LPS increased NFDL expression, which represents stimulants-mediated NF_{\kappa}B activation, in these cell lines. As shown in Fig. 1A and B, CAPE inhibited NFDL expression stimulated with TNF_{\alpha} or LPS in a dose-dependent manner. To confirm CAPE inhibition of the NF_{\kappa}B pathway, we examined whether CAPE can interfere with expression of an NF_{\kappa}B target gene product, inducible nitric oxide synthetase (iNOS), in RAW264.7 cells. Cells were treated with LPS in the presence or absence of CAPE at varying concentrations. NF_{\kappa}B dependency on the expression of the gene product was validated by treatment with a cell permeable NEMO binding protein, a specific NF_{\kappa}B inhibitor CP-NBD (May et al., 2000). As shown in Fig. 1C and D, induction of iNOS protein was significantly inhibited not only by the NF_{\kappa}B specific peptide inhibitor but also by CAPE in a dose-dependent manner, which is in parallel with the above luciferase result.

3.2. CAPE directly inhibits IKK in HCT116 cells

A number of NF_{\kappa}B activators including LPS and TNF_{\alpha} utilize IKK-\kappaB-p65 pathway to achieve activation of the transcription factor (Shembade et al., 2007). To examine whether CAPE inhibition of NF_{\kappa}B involved intervention in the pathway, HCT116 cells were incubated with TNF_{\alpha} in the presence of CAPE at varying concentrations and lysed to obtain the cytosolic and nuclear extracts. Western blotting was performed to analyze I\kappaB levels in the cytosol and p65 levels in the nucleus. As shown in Fig. 2A, stimulation of cells with the cytokine resulted in complete degradation of I\kappaB in the cytosol and accumulation of p65 in the nucleus. CAPE partly prevented the cytosolic I\kappaB degradation with a concomitant attenuation of the nuclear p65 accumulation. Since TNF-dependent IKK activation leads to I\kappaB degradation, it was examined whether CAPE affected TNF-dependent IKK activation. HCT116 cells were stimulated with TNF_{\alpha} in the presence of 50 \mu M of CAPE and the levels of phosphorylated IKK\alpha and IKK\beta, which represent activity of the kinases, were monitored using a phosphospecific anti-IKK antibody. As shown in Fig. 2B, TNF_{\alpha} induced phosphorylation of IKK\alpha and IKK\beta was not altered by CAPE. We next examined whether CAPE inhibited IKK\alpha/\beta activity directly. IKK\alpha/\beta were immunoprecipitated following stimulation of cells with TNF_{\alpha} and the immunocomplexes were subjected to an in vitro kinase assay, which was done in the presence of escalating dose of CAPE. As shown in Fig. 2C, CAPE attenuated phosphorylation of an IKK substrate (GST–l\kappaBz) in a dose dependent manner. These results indicate that CAPE can attenuate l\kappaB degradation and subsequent nuclear accumulation of p65 in HCT116 cells through the direct inhibition of IKK activity.

3.3. CAPE-mediated Nrf2 activation is associated with the inhibition of the NF_{\kappa}B pathway

Our data demonstrated that the inhibitory effect of CAPE at 50 \mu M on the IKK-I\kappaB-p65 pathway was not complete. However, a 50 \mu M concentration of CAPE was sufficient for complete suppression of the induction of NF_{\kappa}B-dependent luciferase and the target gene product, which suggests that there exists an additional mechanism in CAPE-mediated NF_{\kappa}B inhibition. Since it has been reported that CAPE can activate Nrf2 signaling, which may interfere with NF_{\kappa}B activation (Balogun et al., 2003; Li et al., 2008), the possibility that CAPE activation
of Nrf2 is associated with NfκB inhibitory effect was examined. To establish Nrf2 knockdown cell line, HCT116 cells were transduced with lentiviral particles containing either scramble nonspecific RNA expression plasmid or Nrf2 shRNA expression plasmid. RT-PCR analysis was done to measure the mRNA levels of Nrf2 in shHCT116 cells, which stably express Nrf2 shRNA, and sHCT116 cells, which express nonspecific scrambled RNA. As shown in Fig. 3A, the mRNA level of Nrf2 was diminished significantly in shHCT116 cells while no change in the mRNA level was observed in sHCT116 cells indicating successful knockdown of Nrf2. To ensure that CAPE can activate Nrf2 in HCT116 cells, sHCT116 cells were transfected with the ARE-luciferase plasmid followed by CAPE treatment. As shown in Fig. 3B, CAPE increased ARE-driven luciferase activity in a dose dependent manner: luciferase activity increased up to 5-fold following 50 μM CAPE. While, in Nrf2-inhibited HCT116 cells, the increase in ARE transcription activity by CAPE was much lower than that in scRNA control cells. Next, to confirm CAPE activation of Nrf2, expression of HO-1, a target gene of Nrf2, was monitored in control and Nrf2-knockdown cells using RT-PCR after treatment with CAPE. HO-1 was induced in the control cells but not in Nrf2-knockdown cells, which was elevated in a dose dependent manner (Fig. 3C). For further confirmation of CAPE activation of Nrf2, nuclear Nrf2 was detected following treatment with CAPE. As shown in Fig. 3D, CAPE elevated the level of Nrf2 in the nucleus, which represents activation of Nrf2. These results indicate that CAPE, indeed, activates Nrf2 in HCT116 cells. We next examined whether CAPE activation of Nrf2 is associated with CAPE inhibition of NfκB in HCT116 cells. HCT116 cells transfected with the NfκB-dependent luciferase plasmid were treated with TNF-α in the presence of sulforaphane, a typical Nrf2 activator. As shown in Fig. 4A, sulforaphane decreased the luciferase expression suggesting that activation of Nrf2 could inhibit TNF-α dependent NfκB activation in these cells. To examine whether CAPE-mediated Nrf2 activation is involved in CAPE inhibition of TNF-α dependent NfκB activation, control and Nrf2-knockdown cells transfected with the NfκB-dependent luciferase plasmid were treated with TNF-α in the presence of CAPE or sulforaphane. TNF-α increased the luciferase activity up to 23.2 fold and 19.4 fold in Nrf2-knockdown and control cells, respectively. As shown in Fig. 4B, sulforaphane attenuated TNF-dependent luciferase expression only in control cells. Although CAPE reduced the luciferase expression in the both cell lines, the inhibitory effect of CAPE was greater in the control cells than that in Nrf2 knockdown cells. These results suggest that CAPE-mediated Nrf2 activation participates in the inhibitory effect of CAPE on NfκB signaling in the human colon cell line.

3.4. Structural analysis of CAPE for the inhibition of NfκB activation

As shown in Fig. 5, CAPE analogues were prepared or purchased for structural analysis of CAPE for NfκB inhibition. To examine whether the functional groups, the catechol moiety and Michael reaction acceptor (MRA) in CAPE, were involved in the inhibition of NfκB activity, the NfκB luciferase assay was done in HCT116 and RAW264.7 cells. When catechol or cinamic acid phenethyl ester, which has no MRA or no catechol moiety, respectively, was incubated in cells stimulated by TNF-α or LPS, the two compounds were able to attenuate the expression of NfκB-dependent luciferase in both cell lines although the potency was lower than that of CAPE. To confirm this, LPS induction of iNOS protein in RAW264.7 cells was monitored after treatment with these compounds. Consistent with the luciferase results, the iNOS protein induction was also reduced by these chemicals (Fig. 6C). To further clarify the requirement of the functional groups, the same experiments were done using CAPE and CAPE analogues, dimethoxycinnamic acid phenethyl ester (DMC), dihydrocaffeic acid phenethyl ester (DHC), phenpropionic acid phenethyl ester (PPAPE), which do not have catechol moiety (methylated), MRA (hydrogenated) and either catechol or MRA, respectively. As shown in Fig. 6D and E, DMC and DHC elicited the inhibitory effect on TNF-α or LPS dependent NfκB activation whereas PPAPE did not affect NfκB transcription activity, suggesting that the two moieties are required for NfκB inhibition. The potency of DMC and DHC was lower than that of CAPE. Similar result was obtained in the measurement of LPS-mediated iNOS induction: DMC and DHC could attenuate iNOS induction, while no inhibitory effect was seen in PPAPE-treated cells (Fig. 6F). Next, in order to test the involvement of the phenethyl moiety in the biological function of CAPE, caffeic acid methyl (CAME), n-pentyl (CAPEN) and n-hexyl ester (CAHA) were prepared. CAME was designed to examine the structural role of the phenethyl moiety in CAPE. CAPEN and CAHA were introduced to see whether the n-alkyl moieties (similar in molecular length to the phenethyl moiety) could replace the phenethyl moiety. The potency of the derivatives for NfκB inhibition was compared with that of CAPE. As shown in Fig. 7A, B and C, substitution with methyl moiety deteriorated...
the CAPE activity for NFκB inhibition, indicating the importance of the phenethyl moiety. CAHA, which was more potent than CAPEN, was a little less potent in inhibiting NFκB than CAPE.

4. Discussion

In this study, we investigated potential molecular mechanisms underlying CAPE-mediated NFκB inhibition and identified the functional groups in CAPE involved in the inhibition of NFκB. Our data demonstrated that CAPE interfered with the activation process of NFκB, IkBα degradation and consequent p65 accumulation in the nucleus, which occurred by direct inhibition of IKK activity. In addition, CAPE activated Nrf2 pathway and the inhibitory effect of CAPE on NFκB was attenuated by knockdown of Nrf2. It was also revealed that removal or modification of the catechol moiety, Michael reaction acceptor or phenethyl ester moiety in CAPE structure impaired its ability to inhibit NFκB.

The negative effects of CAPE on expression of NFκB dependent luciferase and an NFκB target gene product, iNOS, indicate that CAPE inhibited NFκB activation induced by TNF-α or LPS, which is consistent with previous reports (Song et al., 2008) (Natarajan et al., 1996). However, intervention of CAPE in IkBα-p65 pathway resulting in NFκB inhibition seems to be dependent on cell types. It was reported that while CAPE interferes with DNA binding of p65 without prevention of degradation of IkBα protein (Natarajan et al., 1996), it delays IkBα degradation, preventing p65 translocation into the nucleus.
expression in Nrf2 knockout cells whereas the inhibitory effect of sulforaphane was significant in the control cells. In fact, this observation is not surprising since it was reported that diverse Nrf2 activators, such as phenethyl isothiocyanate, sulforaphane and curcumin, attenuate NF-κB activation (Jeong et al., 2004; Xu et al., 2005) and NF-κB signaling is enhanced in Nrf2 deficient cells (Thimmulappa et al., 2006). In particular, CAPE-mediated induction of HO-1, which produces a strong anti-inflammatory protein, was only observed in control HCT116 cells but not in Nrf2-inhibited cells. Since HO-1 induction has been implicated to the inhibition of pro-inflammatory cytokine production (Kapurczak et al., 2004; Lee et al., 2003; Li and Nel, 2006) Nrf2-dependent effect of CAPE on NF-κB signaling might be accounted for by HO-1.

Our findings that CAPE inhibition of IKK and activation of Nrf2 were involved in CAPE inhibition of NF-κB revealed potential molecular targets of CAPE. While CAPE directly inhibited IKK as shown in the result of in vitro kinase assay where CAPE prevented phosphorylation of GST-IκBα, a substrate of IKK, by immunoprecipitated IKK complex, it is not clear what is a cellular target of CAPE for activation of Nrf2. However, as discussed below, an electrophilic property of CAPE (Balogun et al., 2003) is likely to be implicated in activation of Nrf2, which suggests that Keap1, the cytosolic repressor of Nrf2, may be a cellular target for CAPE activation of Nrf2.

The structural analysis of CAPE for inhibition of NF-κB suggests that the functional groups, catechol and Michael reaction acceptor in CAPE, enabled the natural product, to interfere with NF-κB activation probably via IKK inhibition and Nrf2 activation. This argument is based on the findings that catechol and cinnamic acid phenethyl ester with MRA but no catechol was still able to inhibit NF-κB activation and PPAPE without the functional groups had no ability to inhibit NF-κB activation. Furthermore, the two functional groups seem to collaborate to inhibit NF-κB activation, which is supported by the data showing that the inhibitory potencies of DMC and DHC, which are CAPE analogues without catechol or MRA, respectively, were lower than that of CAPE. The phenethyl moiety is also likely to affect potency of CAPE in inhibiting NF-κB activity probably by means of modulating lipophilic interaction with potential molecular target(s). Substitution of the moiety with a methyl moiety deteriorated the inhibitory activity of CAPE on NF-κB but increasing lipophilicity of the alkyl substituents partially restored the activity. Nonetheless, alkyl moieties similar in molecular length to the phenethyl moiety such as pentyl and hexyl were not able to take the place of the aromatic group, which implies that the aromatic ring may be more efficient in interacting with a molecular target(s) of CAPE.

Although our data suggest that the functional groups, MRA and catechol, could be involved in the direct inhibition of IKK and/or activation of Nrf2, it remains to be investigated which functional group is responsible for modulating the activity of each target. Catechol forms semiquinones and reactive quinones that are presumed to play an important role in the generation of reactive oxygen species (ROS), which could modulate Nrf2 and NF-κB activity (Baulig et al., 2003; Christman et al., 2000). Moreover, quinones generated from catechol are able to act as an electrophile that may react with the sulfhydryl residues of Keap1, the cytosolic repressor of Nrf2, leading to activation of Nrf2 (Sato et al., 2008). The α,β-unsaturated carbonyl moiety (MRA) in the CAPE also functions as an electrophile. Indeed, 15-deoxy-Delta (12,14)-prostaglandin J(2) is suggested to activate Nrf2 by means of reaction of MRA in the endogenous compound with the sulfhydryl residues of Keap1 (Itoh et al., 2004; Kim and Surh, 2006). Our data showing that CAPE directly inhibited IKK activity suggest that IKK is a new cellular target of catechol or MRA in CAPE. It should be noted that IKK activity is impaired by cyclopentanone prostaglandins, which occurs by interaction of the thiol of cysteine 179 in IKKβ with Michael reaction acceptor in the prostaglandin (Rossi et al., 2000). It is under investigation that CAPE targets cysteine residue(s) in Keap1 and IKKβ to modulate activity of Nrf2 and IKK, which leads to the CAPE-mediated NF-κB inhibition.
5. Conclusion

Our data suggest that CAPE, a polyphenolic natural product, inhibits TNFα-dependent NF-κB activation via direct inhibition of IKK as well as activation of Nrf2 pathway, in which Michael reaction acceptor and catechol in CAPE may be involved.

Acknowledgements

This work was supported by a grant of the Korea Healthcare technology R & D Project, Ministry for Health, Welfare and Family Affairs, Republic of Korea (A080640) awarded to Y.J.

References


Li, N., Nel, A.E., 2006. Role of the NF2 mediated signaling pathway as a negative regulator of inflammation: implications for the impact of particulate pollutants on asthma. Antioxid. Redox Signal. 8, 88–98.


