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reports

Capsaicin, a component of red peppers, stimulates protein kinase CKII activity

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Protein kinase CKII (CKII), a heterotetramer composed of two catalytic (α or α′) subunits and two regulatory (β) subunits, plays a critical role in cell proliferation and anti-apoptosis. Recently, capsaicin was shown to trigger apoptosis. Therefore, we examined the effect of capsaicin on CKII activity. Although capsaicin induced apoptotic death in HeLa cells, CKII activity was increased in the cytosolic fraction of HeLa cells after treatment. Capsaicin did not change the expression of the CKIIα and CKIIβ proteins. Capsaicin stimulated the catalytic activity of recombinant CKII tetramer, but not the CKIIα subunit. Moreover, capsaicin enhanced the autophosphorylation of CKIIα and CKIIβ. Taken together, our data suggest that capsaicin stimulates the phosphotransferase activity of CKII holoenzyme by interacting with the CKIIβ subunit. [BMB reports 2010; 43(5): 325-329]

INTRODUCTION

Protein kinase CKII (CKII) is a serine/threonine protein kinase found in both the nucleus and cytoplasm of all eukaryotes (1-3). CKII is a tetrameric complex of α, α′, and β subunits and exists as an α2β2, αα′β2, or α′2β2 structure. The α and α′ subunits are the catalytic subunits, whereas the β subunit is thought to be a regulatory subunit since it mediates tetramer formation, modulates catalytic activity, and influences substrate recognition (4-6). Heparin has been shown to be an inhibitor of CKII, whereas polybasic compounds such as polyamines and polylysine stimulate CKII activity via the β subunit (7, 8). CKII catalyzes the phosphorylation of a large number of both cytoplasmic and nuclear proteins including DNA binding proteins, nuclear oncoproteins, and transcription factors (1-3). The overexpression of the CKII catalytic subunit leads to tumorigenesis in mice overexpressing myc (9). Analysis of CKII gene expression using temperature-sensitive yeast mutants has shown that CKII is required for cell cycle progression in both G_1 and G_2/M phases (10). In addition, recent observations in which CKII phosphorylates procaspase-2 or caspase substrates suggest that CKII prevents apoptosis (11-13). These observations indicate that CKII plays a critical role, not only in cell growth and proliferation, but also in antiapoptosis.

Capsaicin (8-methyl-N-vanillyl-6-noneamide, Fig. 1) is the major pungent ingredient in red pepper. Recently, capsaicin was shown to induce apoptosis in several tumor cells. For example, capsaicin induced apoptosis through Bcl-2 down-regulation and caspase-3 activation in hepatocarcinoma cells (14), and through increased intracellular reactive oxygen species (ROS) and calcium levels in glioblastoma cells (15). In addition, capsaicin induced apoptosis in pancreatic cancer cells via ROS generation and persistent disruption of mitochondrial membrane potential (16). Due to the antiapoptotic function of CKII, we chose to examine whether capsaicin would inhibit CKII activity. Unexpectedly, capsaicin actually stimulated the catalytic activity of CKII holoenzyme, but not CKIIα, suggesting that CKIIβ is necessary for this activation. In addition, capsaicin enhanced the autophosphorylation of CKIIα and CKIIβ. To our knowledge, this is the first paper reporting a non-basic CKII activator.

RESULTS AND DISCUSSION

The cleavage of poly (ADP-ribose) polymerase (PARP) is a typical feature of apoptosis (17). To determine whether capsaicin induced apoptosis in HeLa cells, cells were treated with 0.3 mM capsaicin for 9 h followed by the examination of PARP cleavage by Western blotting. The protein level of tubulin was measured as a control. The 89 kDa cleaved fragment of PARP in these cells was increased in a time-dependent manner by capsaicin treatment (Fig. 2A). The finding that capsaicin induced apoptosis suggests that it might inhibit the activity of CKII. To address this, we examined the effect of capsaicin on CKII activity. Unlike other protein kinases, CKII can utilize GTP instead of ATP as a phosphate donor (18). The synthetic peptide substrate RRREEETEEE is universally recognized as an excellent substrate by CKII (19). When the cytosolic fraction of capsaicin-treated HeLa cells was assessed using [γ-^{32}P]GTP and synthetic peptide substrate, CKII activity was found to have been stimulated in a time-dependent (Fig. 2B). To determine if

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Received 26 January 2010, Accepted 3 March 2010

Keywords: Apoptosis, Capsaicin, Phosphotransferase activity, Protein kinase CKII, Protein phosphorylation

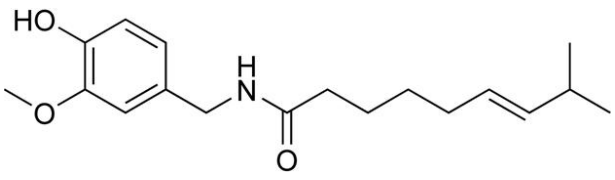

Fig. 1. Structure of capsaicin.

this stimulation of CKII activity in capsaicin-treated HeLa cells was due to increased levels of CKII protein, total protein was extracted from HeLa cells either untreated or treated with capsaicin. We then analyzed the protein levels of CKIIα and CKIIβ by immunoblot using anti-CKIIα and anti-CKIIβ antibodies. As shown in Fig. 2A, the protein levels of CKIIα and CKIIβ were not changed in capsaicin-treated cells. Taken together, these data suggest that capsaicin stimulates CKII activity and induces apoptosis through a CKII-independent pathway.

The possibility that capsaicin stimulates CKII activity indirectly in HeLa cells cannot be excluded. Thus, we next investigated the effect of capsaicin on the catalytic activity of recombinant CKII holoenzyme in the presence of [γ-^{32}P]GTP and synthetic peptide substrate. As shown in Fig. 3A, capsaicin increased the catalytic activity of CKII holoenzyme in a dose-dependent manner, suggesting that capsaicin directly regulates CKII activity. Capsaicin (0.5 mM) treatment generated a 1.5-fold increase in the phosphotransferase activity of CKII holoenzyme toward the synthetic peptide substrate. The stimulatory effect of capsaicin on CKII holoenzyme was also tested using dephosphorylated β-casein as a substrate. Quantification by densitometry revealed that capsaicin stimulated CKII activity, generating a 2.8-fold increase in β-casein phosphorylation (Fig. 3B).

CKIIβ is the regulatory subunit of the CKII holoenzyme. To determine whether CKIIβ was required for capsaicin-mediated CKII activation, we examined the effect of capsaicin on the catalytic activity of recombinant CKIIα in the presence of [γ-^{32}P] GTP and synthetic peptide substrate. Addition of capsaicin did not lead to an increase in CKIIα activity as shown in Fig. 3C, indicating that CKIIβ was necessary for CKII activation.

Since the synthetic peptide substrate (RRREEETEEE) and β-casein are artificial substrates, we examined the effect of capsaicin on the CKII-mediated phosphorylation of physiological substrates. CKII holoenzyme autocatalyzes the rapid, stoichiometric incorporation of phosphate at the CKIIα and CKIIβ subunits (20). Thus, we examined the effect of capsaicin on the autophosphorylation of CKII subunits. As shown in Fig. 4, the purified CKII autophosphorylated CKIIα and CKIIβ in the absence of capsaicin. However, capsaicin enhanced autophosphorylation of CKIIα and CKIIβ by approximately 570% and 350%, respectively.

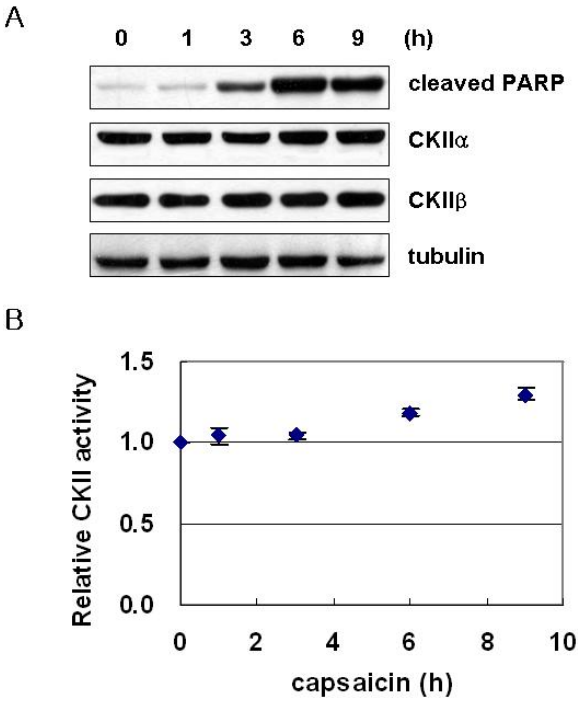

Fig. 2. Effect of capsaicin on apoptosis and CKII activity in HeLa cells. (A) HeLa cells were incubated with 0.3 mM capsaicin for 0 to 9 h, lysed, electrophoresed on a 10% (w/v) SDS-polyacrylamide gel, and visualized by Western blotting with specific antibodies against cleaved PARP, CKIIα, CKIIβ and tubulin. (B) Lysates from capsaicin-treated HeLa cells were utilized in kinase assays with [γ-^{32}P]GTP and specific CKII substrate peptide under standard assay conditions as described in Materials and Methods. ^{32}P incorporation into the substrate peptide was measured by scintillation counting. The results represent two independent experiments done in duplicate. Results are shown as the mean value ± SD.

In summary, this study demonstrates that capsaicin is able to induce the apoptosis-independent activation of CKII. It has been reported that polybasic compounds such as polyamines and polylysine stimulate CKII activity by 2 to 3-fold. The amino-terminal portion (amino acids 55 to 80) of CKIIβ contains clusters of acidic residues that are responsible for the intrinsic negative regulation of CKII activity and for interaction with polybasic compounds (21). Although capsaicin is not a basic compound, it does stimulate CKII activity in this study. It has been reported that polylysine increases the autophosphorylation of CKIIα and inhibits that of CKIIβ (22). In the present study, however, capsaicin stimulates autophosphorylation of both CKIIα and CKIIβ subunits. Therefore, these differences may suggest that the acidic domain of CKIIβ is not a candidate for the binding of capsaicin. Detailed mapping of the domain involved in the interaction will be determined by a site-directed mutagenesis study.

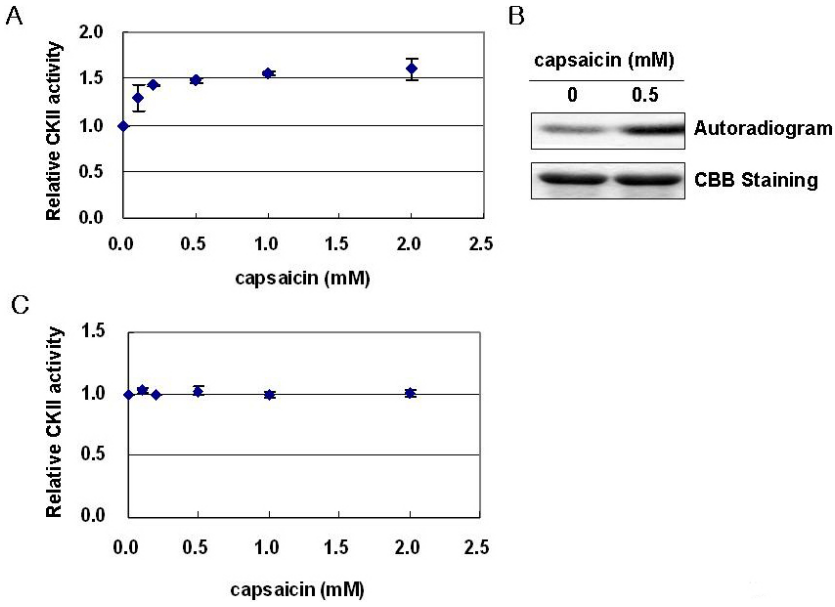

Fig. 3. Effect of capsaicin on the catalytic activity of recombinant CKII. (A) The specific CKII substrate peptide was incubated with [γ-^{32}P] GTP and purified CKII holoenzyme in the presence of various concentrations of capsaicin under standard assay conditions. ^{32}P incorporation into the substrate peptides was measured by scintillation counting. Each point represents the average $\pm$ SD of triplicate determinations. (B) Dephosphorylated β-casein was incubated with [γ-^{32}P]GTP and purified CKII holoenzyme in the absence (lane 1) or presence (lane 2) of 0.5 mM capsaicin. Reaction mixtures were boiled in sample buffer for 5 min prior to loading on a 12% (w/v) SDS-polyacrylamide gel. ^{32}P incorporation was monitored by autoradiogram. Coomassie blue (CBB) staining and autoradiography are shown. (C) The specific CKII substrate peptide was incubated with [γ-^{32}P]GTP and purified CKIIα in the presence of various concentrations of capsaicin. ^{32}P incorporation into the substrate peptides was measured by scintillation counting. Each point represents the average $\pm$ SD of triplicate determinations.

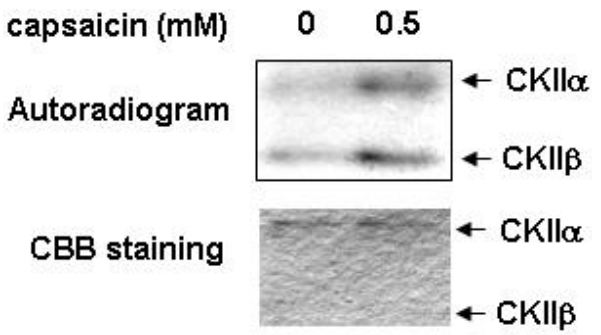

Fig. 4. Effect of capsaicin on autophosphorylation of CKII holoenzyme. Assays for CKII autophosphorylation were carried out under standard assay conditions in the absence (lane 1) or presence (lane 2) of 0.5 mM capsaicin. Without substrate, CKII holoenzyme was incubated with [γ-^{32}P]ATP and analyzed by 12% (w/v) SDS-polyacrylamide gel electrophoresis followed by Coomassie blue (CBB) staining and autoradiography.

MATERIALS AND METHODS

Cell culture and Purification of CKII

HeLa cells were grown in Dulbecco's modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. Human CKII holoenzyme and CKIIα were expressed and purified in *E. coli* as described previously (23).

Preparation of HeLa cell extract

For Western blotting, approximately 1 × 10^6 HeLa cells in 100 mm-dishes were treated with 0.3 mM capsaicin (Sigma Chemical Co.) for 0 to 9 hr, washed with ice-cold phosphate buffered saline (PBS), collected by scraping with a rubber policeman, and lysed in 100 μl of ice-cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). For the CKII activity assay, cells were lysed in lysis buffer (50 mM Tris-HCl, pH 8.0, 20 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 1% Nonidet P-40, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, and 4 mM *p*-nitrophenyl phosphate) by sonication. The particulate debris was removed by centrifugation at 12,000 × g. The volumes of the supernatants were adjusted to equalize the protein concentration.

Western blotting analysis

Capsaicin-treated or untreated cells were washed with ice-cold PBS, collected by centrifugation, and lysed in 100 μl of ice-cold RIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin). The particulate debris was removed by centrifugation at 12,000 × g. The protein concentrations of the supernatants were determined using Bradford protein dye reagent (Bio-Rad), and the volumes of the supernatants were adjusted to equalize the protein concentrations. Protein samples were separated on a 10% polyacrylamide gel in the presence of SDS, and then transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with 5% skim milk in TBST (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) for 2 h and then incubated with specific antibodies against cleaved PARP formCKIIα, CKIIβ and tubulin in 1% skim milk for 1 h.

The membrane was washed 3 times in TBST and treated with ECL system (Amersham Pharmacia Biotech).

Assay for CKII activity

The standard assay for the phosphotransferase activity of CKII was conducted in a reaction mixture containing 20 mM Tris-HCl, pH 7.5, 120 mM KCl, 10 mM $MgCl_2$, and 100 μM [γ-^{32}P] GTP in the presence of 1 mM synthetic peptide substrate (RRREEETEEE) or dephosphorylated β-casein in a total volume of 30 μl at 30°C. The reactions were initiated by the addition of purified CKII or cell lysates and incubated for 15 min. For the peptide substrate, the reaction was stopped by addition of trichloroacetic acid to a final concentration of 10% and centrifugation. Ten μl of supernatant was then applied to P-81 paper, which was washed in 100 mM phosphoric acid and measured for radioactivity by scintillation counting. For dephosphorylated β-casein, the reactions were subjected to SDS-polyacrylamide gel electrophoresis. The gels were dried and visualized by autoradiography.

Autophosphorylation of CKII

Autophosphorylation of CKII was performed in a reaction mixture containing 20 mM Tris-HCl, pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 1 mM DTT, 1 mM EGTA, 100 μM [γ-^{32}P]ATP, and 3 μg of CKII holoenzyme in a total volume of 30 μl. After incubation for 15 min at 30°C, the samples were separated on a 12% SDS-polyacrylamide gel. The gel was then stained with Coomassie blue, dried, and subjected to autoradiography.

Acknowledgements

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (KRF-2008-C00705).

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