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ScienceDirect
Journal of Nutritional Biochemistry 21 (2010) 680–686
Journal of Nutritional Biochemistry

Cocoa polyphenols attenuate hydrogen peroxide-induced inhibition of gap-junction intercellular communication by blocking phosphorylation of connexin 43 via the MEK/ERK signaling pathway☆

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Received 18 June 2008; received in revised form 20 March 2009; accepted 26 March 2009

Abstract

Cocoa, a good source of dietary antioxidative polyphenols, exhibited anticarcinogenic activity in animal models, but the molecular mechanisms of the chemopreventive potential of cocoa remain unclear. Inhibition of gap-junction intercellular communication (GJIC) is strongly related to tumorigenesis. Cocoa polyphenol extracts (CPE) dose dependently attenuated hydrogen peroxide (H_2O_2)-induced inhibition of GJIC in rat liver epithelial (RLE) cells. CPE inhibited the H_2O_2-induced phosphorylation and internalization of connexin 43, which is a regulating protein of GJIC in RLE cells. The H_2O_2-induced accumulation of reactive oxygen species and activation of extracellular signal-regulated kinase were inhibited by CPE treatment. However, CPE did not block H_2O_2-induced phosphorylation of p38 mitogen-activated protein kinase. An *ex vivo* kinase assay demonstrated that CPE inhibited the H_2O_2-induced mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK) 1 activity in RLE cell lysates. *Ex vivo* pull-down assay data revealed that CPE directly bound with MEK1 to inhibit MEK1 activity. These results indicate that CPE protects against the H_2O_2-induced inhibition of GJIC through antioxidant activity and direct inhibition of MEK activity, which may contribute to its chemopreventive potential.

Keywords: Cocoa polyphenols; Connexin 43; Gap-junction intercellular communication; Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase

1. Introduction

Naturally occurring antioxidants reportedly play a major role in ameliorating oxidative damage caused by free radicals. There are multiple lines of evidence that many antioxidative phytochemicals exhibit potent antitumor-promoting activities combined with low toxicity and very few adverse side effects [1]. There is increasing evidence that phenolic phytochemicals can be absorbed into the human body via dietary consumption in amounts that are, in principle, sufficient to exert antioxidant or other biological activities *in vivo*. The widely consumed beverages cocoa, coffee, and black and green teas are known to be rich in phenolic phytochemicals. A German study found that chocolate was a good source of dietary antioxidant catechins, second only to black tea [2]. Cocoa contains more phenolic phytochemicals and exhibits a higher antioxidant capacity than teas and red wine [3]. Cocoa extracts inhibited chemical-induced tumorigenesis in the mammary gland, pancreas [4] and lung [5] in experimental animals. However, the underlying molecular mechanisms and molecular target(s) for the potential chemopreventive effects of cocoa remain unclear.

Carcinogenesis is a multistage process consisting of initiation, promotion and progression stages, each of which is a possible target for chemopreventive agents. In particular, targeting the tumor promotion stage (a reversible and long-term process) appears to be more practical for intervening in carcinogenesis than targeting the tumor initiation stage (an irreversible and short-term process). Gap junctional intercellular communication (GJIC) is driven by the passive diffusion of small (<1000 Da) hydrophilic molecules such as glucose, glutamate, glutathione and ions or signal molecules such as cyclic adenosine monophosphate and adenosine triphosphate (ATP) which are essential cellular components for maintaining homeostasis. Normal GJIC allows cells to control their population density and,

☆ This work was supported by research grants (nos. R01-2007-000-11957-0 and M10510140004-08N1014-00410) from the Korea Science and Engineering Foundation (KOSEF) funded by the Korean government.
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doi:10.1016/j.jnutbio.2009.03.014

ultimately, modulates cell proliferation and growth in multicellular organisms [6]. There is abundant evidence that loss of GJIC is strongly involved in carcinogenesis, particularly the tumor promotion process [7]. Abnormal GJIC is attributable to phosphorylation of connexin 43 (Cx43), which is a major protein constituting gap-junction channels, and a reduction in the amount of Cx43 and modification to the Cx43 distribution due to internalization and degradation [8]. Previous studies indicated that activation of extracellular signal-regulated kinase (ERK) and p38 mitogen-activated protein kinase (MAPK) signaling pathways is strongly related to phosphorylation of Cx43 and inhibition of GJIC in rat liver epithelial (RLE) cells [9].

Reactive oxygen species (ROS) cause several human diseases — including cancer, inflammation, heart disease and cerebrovascular disease — through multiple mechanisms. Although the DNA damage induced by ROS initiates cancer, ROS are also involved in tumor promotion. The involvement of ROS, particularly hydrogen peroxide, in the tumor promotion process is supported by both *in vivo* and *in vitro* studies [10,11]. Recent reports suggest that the carcinogenicity of H_2O_2 is associated with the inhibition of GJIC [9,12]. The present study investigated the possible protective effects of cocoa polyphenol extracts (CPE) against the H_2O_2-induced inhibition of GJIC in RLE cells.

2. Materials and methods

2.1. Chemicals

D-media fetal bovine serum (FBS) and penicillin/streptomycin were purchased from GIBCO BRL (Carlsbad, CA, USA). H_2O_2, Lucifer yellow, ammonium ferrous sulfate, sodium dodecyl sulfate (SDS), acrylamide and Tris-HCl were obtained from Sigma-Aldrich (St. Louis, MO, USA). Dichlorofluorescein diacetate (DCF-DA) was purchased from Molecular Probes (Eugene, OR, USA). The antibodies against phosphorylated MAPK/ERK kinase (MEK) (Ser217/221), phosphorylated ERK (Tyr202/Tyr204), total MEK and total ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and anti-Cx43 antibody was purchased from Zymed (San Francisco, CA, USA). The MEK1 activity assay kit was obtained from Upstate Biotechnology (Lake Placid, NY, USA). CNBr-Sepharose 4B, glutathione-Sepharose 4B and [γ-^{32}P]ATP were purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA), and a protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals used were of analytical or HPLC grade.

2.2. Sample preparation

Cocoa extracts were prepared as described previously [13]. Briefly, commercial cocoa powder (50 g) was extracted with 500 ml of 50% (v/v) aqueous ethanol under reflux for 6 h. After the extraction, the solution was filtered twice to collect the extract. The collected CPE were concentrated at 50°C under reduced pressure, frozen and dried.

2.3. Cell culture

RLE cells kindly provided by Dr. J.E. Trosko at Michigan State University (USA) were cultured in D-media supplemented with 10% FBS (GIBCO BRL) and penicillin/streptomycin in a 37°C humidified incubator containing 5% CO_2 and 95% air.

2.4. Bioassay of GJIC

GJIC was measured by the scrape-loading/dye-transfer (SL/DT) technique as described previously [9]. Briefly, confluent cells were pretreated with CPE at the indicated concentrations for 30 min before being exposed to 100 μM H_2O_2 for 1 h. Following incubation, the cells were washed twice with 2 ml of PBS. Lucifer yellow was added to the washed cells, and three scrapes were made with a surgical steel-bladed scalpel at a low light intensity. These three scrapes were performed to ensure that each scrape traversed a large group of confluent cells. After 3 min of incubation, the cells were washed with 2 ml of PBS and then fixed with 2 ml of a 4% formalin solution. The number of communicating cells as indicated by the dye was counted under an inverted fluorescence microscope (Olympus Ix70, Okaya, Japan). All data are presented as mean and standard deviation (S.D.) values for at least three replications for each prepared sample.

2.5. Western blotting

Confluent cells were pretreated with CPE at the indicated concentrations for 30 min before being exposed to 100 μM H_2O_2 for different periods. The harvested cells were disrupted, and the supernatant fractions were boiled for 5 min. The protein concentration was determined using a dye-binding protein assay kit (Bio-Rad Laboratories) as described in the product manual. Lysate protein (20 μg) was subjected to 10% SDS–polyacrylamide gel electrophoresis and electrophoretically transferred to a polyvinylidene fluoride membrane. After blotting, the membrane was incubated with the phosphorylated MAPK/ERK kinase (MEK) (Ser217/221), phosphorylated ERK (Tyr202/Tyr204), phosphorylated p38 (Thr180/ Tyr182), total MEK, total ERK, total p38 or Cx43 specific primary antibody at 4°C overnight. Protein bands were visualized by a chemiluminescence detection kit (Amersham Pharmacia Biotech) after hybridization with the horseradish peroxidase-conjugated secondary antibody. The relative amounts of proteins associated with specific antibodies were quantified using Scion Image (NIH, Bethesda, MD, USA).

2.6. Immunohistochemistry

Localization of Cx43 in the cells was assessed by immunofluorescent staining as described previously [14]. RLE cells were grown to confluence on coverslips in 3-cm-diameter plastic dishes, briefly washed in PBS and kept in serum-free medium overnight before being treated with the respective agent or DMSO (vehicle control). After treatment, cells were washed twice with cold PBS and fixed with 5 ml of methanol per dish for 15 min at 20°C. Fixed cells were then thoroughly washed with ice-cold PBS, followed by blocking with 5% (v/v) normal goat serum (GIBCO BRL) in PBS containing 0.3% (v/v) Triton X-100 for 90 min at room temperature. For detection of phosphorylated Cx43, cells were incubated at 4°C overnight under slight agitation with rabbit polyclonal anti-phospho-Cx43 antibody (sc-12900-R) diluted 1:1500 in PBS containing 1% (v/v) goat serum. Cells were then washed with PBS and incubated with an Alexa-488-coupled goat antirabbit IgG (H+L) antibody (Molecular Probes) for 1 h at 37°C. Nuclei were stained after thorough washing with PBS by the addition of 4′,6-diamidino-2-phenylindole (0.2 μg/ml final concentration) in PBS for 15 min, followed again by thorough washing before embedding. Fluorescent staining of Cx43 was viewed and photographed using a fluorescence microscope.

2.7. Fluorescent measurement of intracellular ROS generation

The ROS-sensitive fluorescent probe DCF-DA was used to assess the generation of intracellular ROS. Cells in monolayers were preincubated with PBS supplemented with 50 μM DCF-DA for 30 min at 37°C. The supernatant was replaced with fresh unsupplemented PBS prior to stimulation with PBS supplemented with chemicals that were added from stock solutions for 15 min at 37°C. Time series of the relative fluorescence intensity data and fluorescent images was obtained using argon laser microscopy with excitation and emission wavelengths of 485 and 530 nm, respectively.

2.8. Ex vivo MEK1 immunoprecipitation and kinase assay

Confluent RLE cells were either treated or not treated with CPE at the indicated concentrations for 30 min, then exposed to 100 μM H_2O_2 for 30 min, disrupted with lysis buffer [20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1 mM EGTA, 1% Triton X-100, 1 mM β-glycerophosphate, 1 mg/ml leupeptin, 1 mM sodium orthovanadate (Na_3VO_4) and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and finally centrifuged at 20,000×*g* for 10 min in a microcentrifuge. The lysates containing 500 μg of protein were used for immunoprecipitation with an antibody against MEK1 and then incubated at 4°C overnight. After adding Protein A/G Plus agarose beads, the mixture was continuously rotated for 3 h at 4°C. The beads were washed three times with kinase buffer [20 mM MOPS (3-(*N*-morpholino)propanesulfonic acid) (pH 7.2), 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM Na_3VO_4 and 1 mM dithiothreitol (DTT)] and then resuspended in 20 μl of 1× kinase buffer supplemented with 1 μg of inactive ERK2 and incubated for an additional 30 min at 30°C. Then 20 μg of myelin basic protein and 10 μl of diluted [γ-^{32}P]ATP solution were added, and the mixture was incubated for 10 min at 30°C. A 20-μl aliquot was transferred onto p81 filter paper and washed three times with 0.75% phosphoric acid for 5 min per wash followed by once with acetone for 2 min. The radioactive incorporation was determined using a scintillation counter. Each experiment was performed three times.

2.9. Ex vivo pull-down assays

In brief, a RLE cellular supernatant fraction (500 μg) was incubated with CPE-Sepharose 4B (or Sepharose 4B alone as a control) beads (100 μl, 50% slurry) in a reaction buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40, 2 μg/ml bovine serum albumin, 0.02 mM PMSF and 1× protease inhibitor mixture]. After incubation with gentle rocking overnight at 4°C, the beads were washed five times with buffer [50 mM Tris-HCl (pH 7.5), 5 mM EDTA, 150 mM NaCl, 1 mM DTT, 0.01% Nonidet P-40 and 0.02 mM PMSF] and proteins bound to the beads were analyzed by immunoblotting.

2.10. Statistical analysis

When applicable, data are expressed as mean and S.D. values, and the Student's *t* test was used for single statistical comparisons. A probability value of $P<.01$ was used as the criterion for statistical significance.

3. Results

3.1. CPE protects against H_2O_2-induced inhibition of GJIC in RLE cells

Since the inhibition of GJIC is associated with the tumor promotion stage of carcinogenesis, identifying agents that can recover the down-regulation of GJIC is an important strategy for cancer chemoprevention. We used the SL/DT assay to examine the protective effect of CPE on H_2O_2-induced inhibition of GJIC in nontumorigenic RLE cells. Exposure of RLE cells to 100 μM H_2O_2 significantly inhibited GJIC up to 41%, but this inhibitory effect was strongly prevented by CPE in a dose-dependent manner (Fig. 1).

3.2. CPE blocks H_2O_2-induced phosphorylation of Cx43 in RLE cells

Cx43 plays a key role in the modulation of GJIC. Several previous studies have shown that H_2O_2 induced the hyperphosphorylation of Cx43 in RLE cells which is responsible for the inhibition of GJIC [9]. We performed Western blotting with antibodies specific to Cx43 to detect the changes in phosphorylation resulting from treating RLE cells with CPE. Four major bands (P0, P1, P2 and P3) were detected in untreated RLE cells, and mobility shifts from band P0 or P1 to higher-molecular-weight band P2 or P3 indicated the phosphorylation of Cx43. Cells exposed to H_2O_2 exhibited band shifts to band P2 or P3 (Fig. 2). Treating cells exposed to H_2O_2 with CPE decreased the phosphorylation ratio (P3/P0) of Cx43 compared to that for H_2O_2 exposure only (Fig. 2).

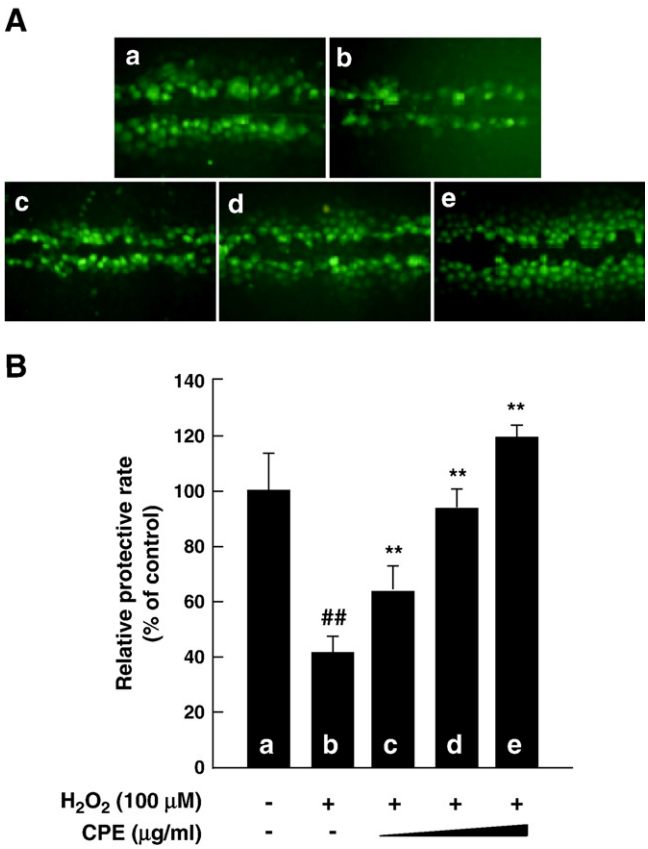

Fig. 1. CPE protected against the inhibition of GJIC by H_2O_2 in RLE cells. Confluent cells were pretreated with CPE at the indicated concentrations for 30 min before being exposed to 100 μM H_2O_2 for 1 h. GJIC was assessed using the SL/DT method. The results of one of three independent experiments are shown. (A) a, Untreated control; b, H_2O_2 (100 μM) only; c, H_2O_2 (100 μM) and CPE (10 μg/ml); d, H_2O_2 (100 μM) and CPE (50 μg/ml); and e, H_2O_2 (100 μM) and CPE (100 μg/ml). (B) The relative recovery rate was determined by counting the number of communicating cells as indicated by the dye under an inverted fluorescence microscope. Data are mean and S.D. values for at least three independent experiments. The sharp signs indicate a significant inhibition of the number of communicating cells in the group treated with H_2O_2 alone compared to control ($^{\#\#}P<.01$). The asterisks indicate a significant increase in the number of communicating cells in cells exposed to H_2O_2 together with CPE compared to cells exposed to H_2O_2 only ($^{**}P<.01$).

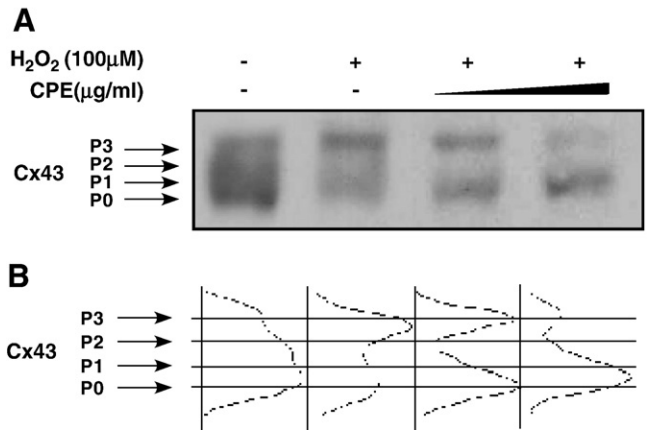

Fig. 2. CPE blocked the H_2O_2-induced phosphorylation of Cx43 in RLE cells. Lane 1, Untreated control; Lane 2, H_2O_2 (100 μM) only; Lane 3, H_2O_2 (100 μM) and CPE (10 μg/ml); and Lane 4, H_2O_2 (100 μM) and CPE (50 μg/ml). (A) Cells were pretreated with CPE at the indicated concentrations for 30 min before being exposed to 100 μM H_2O_2 for 30 min. The phosphorylation status of Cx43 was analyzed by Western blot analysis as described in Materials and methods. Cell lysates were immunoblotted with Cx43 antibody. Data are representative of three independent experiments. P0 and P1 are the phosphorylation patterns of Cx43 in untreated cells, and P2 and P3 are the hyperphosphorylation patterns of Cx43 in cells exposed to H_2O_2. (B) Diagram showing the intensity of each Cx43 band as quantified using an image-analysis program.

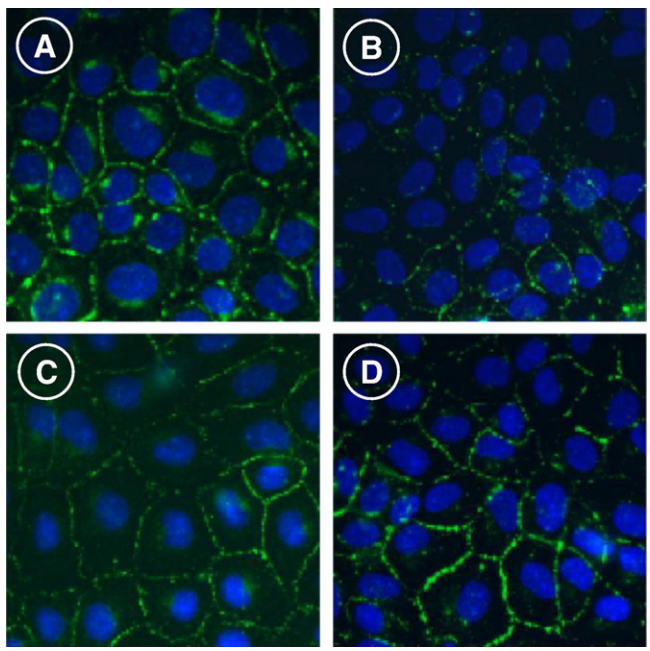

Fig. 3. CPE blocked the H_2O_2-induced internalization of Cx43 in RLE cells. The results of one of three independent experiments are shown: (A) untreated control; (B) H_2O_2 (100 μM) only; (C) H_2O_2 (100 μM) and CPE (10 μg/ml); and (D) H_2O_2 (100 μM) and CPE (50 μg/ml). Cells were exposed to 100 μM H_2O_2 for 1 h in the absence or presence of CPE at different concentrations. Cx43 localization was assessed using immunohistochemistry as described in Materials and methods.

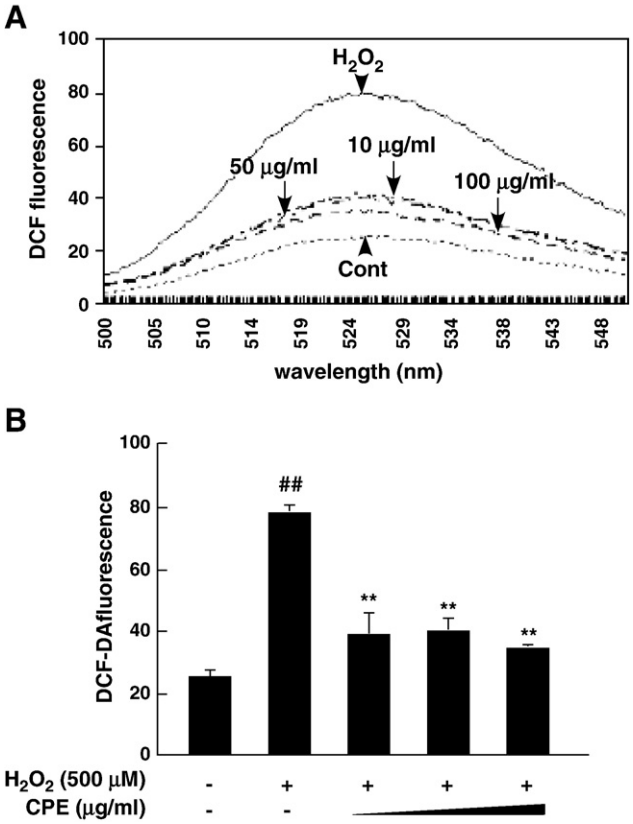

Fig. 4. CPE attenuated the H_2O_2-induced accumulation of intracellular ROS in RLE cells. (A) Intracellular ROS levels were determined by DCF-DA assay. Cells were pretreated with CPE at the indicated concentrations for 30 min before being exposed to 500 μM H_2O_2 for 15 min. Data are mean and S.D. values (n=3). (B) The quantity of intracellular ROS generated by H_2O_2 with or without CPE was measured in three independent experiments, as quantified by the absorbance. The sharp signs indicate a significant increase in the generation of intracellular ROS in cells treated with H_2O_2 alone compared to control ($^{\#\#}P<.01$). The asterisks indicate a significant difference in the generation of intracellular ROS between cells treated with CPE and H_2O_2 together and cells exposed to H_2O_2 only ($^{**}P<.01$).

3.3. CPE inhibits H_2O_2-induced internalization of Cx43 in RLE cells

In addition to blocking the hyperphosphorylation of Cx43, GJIC might also be restored by up-regulating Cx43 expression in the plasma membrane. Several studies have suggested that a decrease in GJIC is correlated with a loss of gap junctions from the cell plasma membrane. Tumor promoter-induced phosphorylation can trigger the internalization of Cx43 [8]. Therefore, we next examined the effect of CPE on H_2O_2-induced localization of Cx43 in RLE cells. We found that the Cx43 distribution was modified by exposure to H_2O_2 alone, while the treatment with CPE resulted in Cx43 stain reappearing as bright fluorescent spots on plasma membranes comparable to the control state (Fig. 3).

3.4. CPE attenuates H_2O_2-induced accumulation of intracellular ROS in RLE cells

Excessive oxidative stress induces inhibition of GJIC, which is a prominent factor contributing to carcinogenesis in normal cells. We next examined whether CPE reduces the H_2O_2-induced accumulation of intracellular ROS level in RLE cells using the DCF-DA assay. The results revealed that exposure to 500 μM H_2O_2 significantly increased the intracellular ROS level in RLE cells, and this was markedly attenuated by CPE (Fig. 4). This result indicates that the protective effect of CPE on H_2O_2-induced GJIC inhibition is partially mediated by attenuation of intracellular ROS.

3.5. CPE blocks H_2O_2-induced phosphorylation of ERK, but not that of p38 MAPK, in RLE cells

A previous study has shown that phosphorylation of Cx43 via activation of the ERK and p38 MAPK signaling pathway is strongly involved in the H_2O_2-induced inhibition of GJIC [9]. We next investigated the effects of CPE on H_2O_2-induced ERK and p38 MARK phosphorylation in RLE cells and found that treatment of RLE cells with 50 μg/ml CPE completely suppressed the H_2O_2-induced phosphorylation of ERK in RLE cells (Fig. 5A,B). However, CPE did not inhibit the H_2O_2-induced phosphorylation of p38 MAPK under the same conditions (Fig. 5C,D). These results suggest that the protective effects of CPE against the H_2O_2-induced inhibition of GJIC in RLE cells are attributable to its inhibition of ERK activation, but not of p38 MAPK.

3.6. CPE inhibits MEK1 activity in RLE cells

Although CPE significantly inhibited the H_2O_2-induced phosphorylation of ERK in RLE cells, it had no effect on the H_2O_2-induced phosphorylation of MEK1, which is an upstream kinase of ERK (Fig. 6A,B). We next examined whether MEK1 might be a molecular target of CPE for the protection of H_2O_2-induced GJIC inhibition. Results from an *ex vivo* kinase assay indicated that CPE strongly inhibited MEK1 activity in H_2O_2-stimulated RLE cell lysates (Fig. 6C). We performed an *ex vivo* CPE pull-down assay to further confirm whether CPE can directly bind with MEK1. We observed *ex vivo* binding between CPE and MEK1 in H_2O_2-stimulated RLE cell lysates (Fig. 6D). These results indicated that the prevention of GJIC inhibition by CPE involves the direct inhibition of MEK1 activity and subsequent inhibition of its downstream signaling pathway. Collectively, these results suggest that MEK1 is an important target molecule for protection against the H_2O_2-induced GJIC inhibition by CPE.

4. Discussion

There is currently considerable interest in the beneficial health effects of dietary polyphenols, because these compounds may exhibit antioxidative, anti-inflammatory and anticarcinogenic activities. Cocoa is a major source of antioxidants due to the considerable amount of polyphenol contents. HPLC analysis data revealed that polyphenol compounds comprise 12–18% of total cocoa bean weight [15]. Previous study reported that consumption of cocoa beverage provides the highest amount of major polyphenol compounds among six polyphenol-rich beverages including instant coffee, green tea, orange juice and so on [16]. We previously showed that cocoa has higher total phenolic contents than teas and wine [3] and that CPE contains 468 mg/g gallic acid-equivalent phenolics and 413 mg/g epicatechin-equivalent flavonoids, therefore exerting antioxidant and anti-inflammatory activity [13]. Major polyphenol compounds found in CPE are the epicatechin oligomer class, namely, procyanidins. It was reported that procyanidins comprise about 88% of ACTICOA powder, a cocoa polyphenolic extract [17]. Several recent studies have shown that CPE suppressed the proliferation of cancer cells and various biomarkers of tumorigenesis in both cell culture and animal models [4,5,18]. Our previous study also showed that cocoa polyphenols blocked oxidative stress and inflammation in the HL-60 cell line and a mouse skin model, respectively [13]. However, the potent antitumor-promoting effects of cocoa and their underlying molecular mechanisms still remain unclear. The present study has clearly shown that CPE exerts strong protective effects against the H_2O_2-induced GJIC in

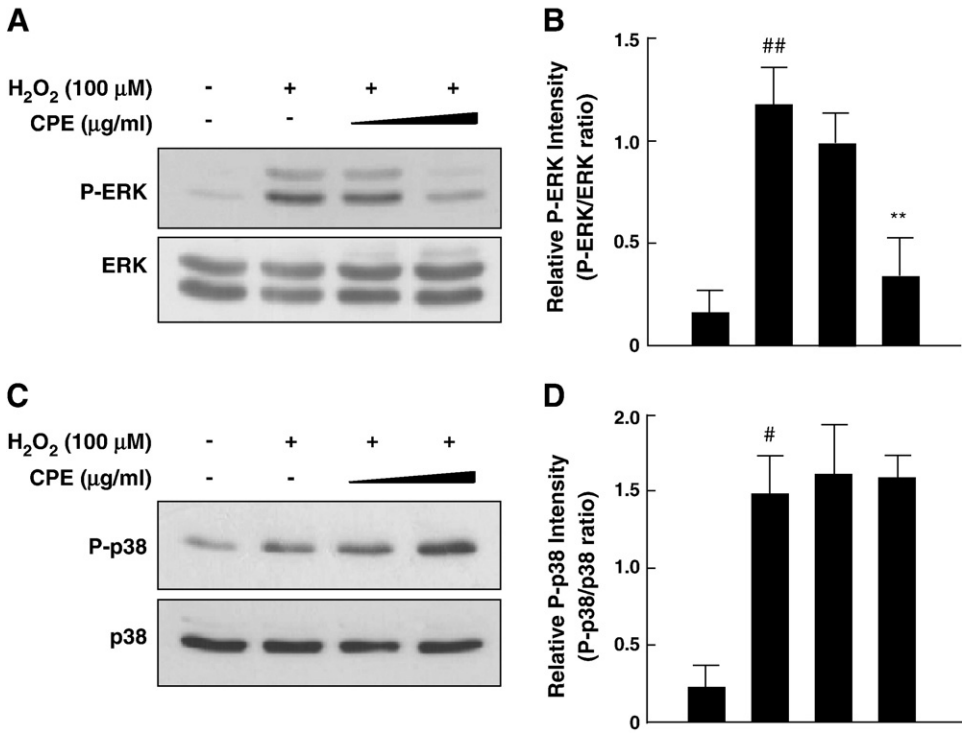

Fig. 5. CPE inhibited the H_2O_2-induced phosphorylation of ERK but not of p38 MAPK in RLE cells. Lane 1, Untreated control; Lane 2, H_2O_2 (100 μM) only; Lane 3, H_2O_2 (100 μM) and CPE (10 μg/ml); and Lane 4, H_2O_2 (100 μM) and CPE (50 μg/ml). (A) CPE inhibited the H_2O_2-induced phosphorylation of ERK in RLE cells. The cells were pretreated with the indicated concentration of CPE for 30 min before they were exposed to 100 μM H_2O_2 for 30 min. The levels of phosphorylated ERK (P-ERK) and total ERK were analyzed by Western blot analysis as described in Materials and methods. Cell lysates were immunoblotted with P-ERK or ERK antibodies. Data are representative of three independent experiments. (B) The bar graphs represent the mean of the P-ERK/ERK ratio measured using an image-analysis program and S.D. values for three independent experiments. (C) CPE had no effect on H_2O_2-induced phosphorylation of p38 MAPK in RLE cells. The cells were pretreated with the indicated concentration of CPE for 30 min before they were exposed to 100 μM H_2O_2 for 30 min. The levels of phosphorylated p38 (P-p38) and total p38 MAPK were analyzed by Western blot analysis as described in Materials and methods. Cell lysates were immunoblotted with P-p38 or p38 MAPK antibodies. Data are representative of three independent experiments. (D) The bar graphs represent the mean of the P-p38/p38 MAPK ratio measured using an image-analysis program and S.D. values for three independent experiments. The sharp signs indicate a significant increase in the p-ERK/ERK or p-p38/p38 ratio in cells treated with H_2O_2 alone compared to control ($^{\#\#}P<.01$, $^{\#}P<.05$). The asterisks indicate a significant difference between cells treated with H_2O_2 and CPE and cells exposed to H_2O_2 only ($^{**}P<.01$).

the nontumorigenic RLE cell line. These results therefore support the chemopreventive potential of CPE.

The inhibition of GJIC was attributed to phosphorylation of Cx43 or loss of Cx43 in the plasma membrane at points of contact between cells. There is increasing evidence that dysfunctional GJIC is strongly associated with tumor cell development, with aberrant communication being observed in tumor cells. Epidemiological studies and animal experiments have shown that the expression of connexin is dramatically decreased (with aberrant localization of connexin) in cancer tissues, including that of the skin [19], breast [20] and prostate [21]. However, retransfection of tumor cells with connexin genes restores normal growth control in cells. The present study demonstrated that CPE inhibited the H_2O_2-induced phosphorylation and internalization of Cx43, which is a regulating protein of GJIC, in RLE cells.

ROS can be produced from both endogenous and exogenous substances. Primary endogenous sources include mitochondria, peroxisomes, cytochrome P450 metabolism and inflammatory reaction [22]. In particular, H_2O_2 is a ubiquitous ROS that can function as a signaling molecule and damage cell components such as nucleic acid, protein or lipid [23]. Additionally, H_2O_2 can activate other intracellular ROS. Recent studies have reported that H_2O_2 induced superoxide anion production by stimulation of NAD(P)H oxidase activity in nonphagocytic cell [24] and smooth muscle cells [25]. On the other hand, under stress conditions, an excess of superoxide releases free iron from iron-containing molecules. H_2O_2 can react with the released Fe(II) and participate in the Fenton reaction, generating a highly reactive hydroxyl radical ($Fe(II)+H_2O_2 \rightarrow Fe(III)+{\bullet}OH+OH-$) [26].

Our previous studies have shown that certain antioxidants, such as ascorbic acid and quercetin, protect against the H_2O_2-induced inhibition of GJIC [27,28]. In contrast, gallic acid, Trolox, BHA [2(3)-*tert*-butyl-4-hydroxyanisole] and BHT [2,6-bis(1,1-dimethylethyl)-4-methylphenol] did not protect against the H_2O_2-induced inhibition of GJIC [29]. Indeed, gallic acid actually inhibited GJIC dose dependently in RLE cells [30]. Phenolic phytochemicals are generally recognized as major antioxidants, but they can also exhibit prooxidant activities. In fact, most free-radical scavengers act in oxidation–reduction reactions that are reversible, and some (such as phenolic phytochemicals) can act both as antioxidants and prooxidants depending on their structure and the reaction conditions. Thus, the effect of dietary phenolic phytochemicals on carcinogenesis varies with the structures of the individual compounds and their dosages. Here we have shown that CPE attenuated intracellular ROS and protected against the H_2O_2-induced inhibition of GJIC in RLE cells.

The MAPK signaling pathways are important for the phosphorylation of Cx43 in response to various extracellular stimuli, including phorbol ester, growth factors, and hormones. Protein of the ERK family, which was the first to be characterized, is especially critical for phorbol ester- and epidermal growth factor (EGF)-induced inhibition of GJIC. Phorbol ester phosphorylates connexin as well as inhibits GJIC through activation of ERK [31]. EGF has also been previously shown to induce phosphorylation of Cx43 through activation of ERK, which is responsible for the down-regulation of GJIC by EGF [32]. Oxidative stress also activates signal transduction pathways. In particular, exogenous and endogenous H_2O_2 can also trigger the induction of MAPKs and subsequently result in the inhibition of GJIC in RLE cells.

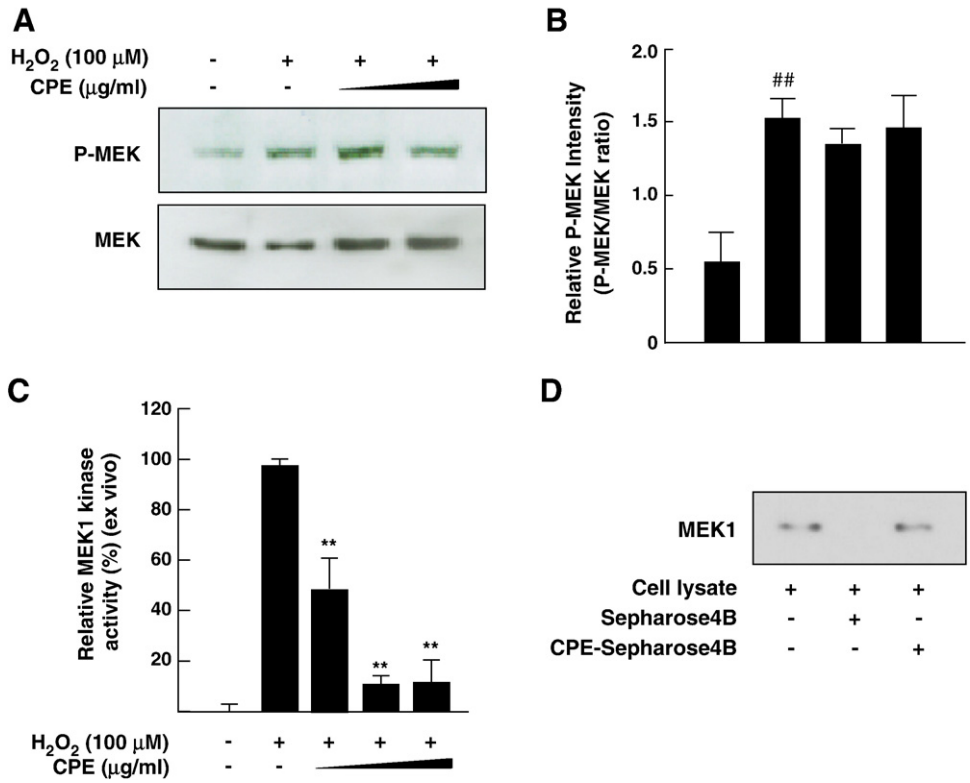

Fig. 6. CPE inhibited MEK1 activity and directly bound with MEK1 in RLE cells. (A) CPE did not inhibit H_2O_2-induced phosphorylation of MEK in RLE cells. Cells were pretreated with CPE at the indicated concentrations for 30 min before being exposed to 100 μM H_2O_2 for 30 min. The levels of phosphorylated MEK (P-MEK) and total MEK proteins were then determined by Western blot analysis. Lane 1, Untreated control; Lane 2, H_2O_2 (100 μM) only; Lane 3, H_2O_2 (100 μM) and CPE (10 μg/ml); and Lane 4, H_2O_2 (100 μM) and CPE (50 μg/ml). (B) The bar graphs represent the mean of the P-MEK/MEK ratio measured using an image-analysis program and S.D. values for three independent experiments. The sharp signs indicate a significant increase in the p-MEK/MEK ratio in cells treated with H_2O_2 alone compared to control ($^{\#\#}P<.01$). (C) CPE inhibited MEK1 activity in RLE cells. Confluent cells were pretreated with CPE at the indicated concentrations (10, 25 or 50 μg/ml) for 30 min before being exposed to 100 μM H_2O_2 for 30 min. Cells were harvested, and immunoprecipitation and MEK1 activity assays were performed as described in Materials and methods. Data are mean and S.D. values for three independent experiments. The asterisks indicate a significant difference between cells treated with H_2O_2 and CPE and cells exposed to H_2O_2 only ($^{**}P<.01$). (D) CPE directly bound with MEK1 in RLE cells. Lane 1 (input control), Whole-cell lysate from RLE cells; Lane 2 (control), a lysate of RLE cells precipitated with Sepharose 4B beads; and Lane 3, whole-cell lysate from RLE cells precipitated with CPE-Sepharose 4B beads. The presence of MEK1–CPE binding *ex vivo* was confirmed by immunoblotting using an antibody against MEK1 as described in Materials and methods.

Therefore, the ERK signaling pathway represents a promising target for chemopreventive strategies against carcinogenesis. To investigate the molecular basis of protective mechanisms against the H_2O_2-induced inhibition of GJIC, we hypothesized that this could be modified by upstream kinases involving the ERK signaling pathway. Our present data showed that CPE suppresses the H_2O_2-induced phosphorylation of ERK in RLE cells, with this being related to the protective effect of CPE against the inhibition of GJIC. In addition, previous studies have shown that phosphorylation of p38 MAPK is associated with the H_2O_2-induced inhibition of GJIC in RLE cells [9]. The present study showed that CPE does not block H_2O_2-induced p38 MAPK phosphorylation in RLE cells.

Polyphenols are the most abundant antioxidants, but recent research has focused on their capability to regulate cellular signaling proteins, thereby altering signal transduction. MEK is capable of phosphorylating both serine and threonine residues and a tyrosine residue of their only known substrates, ERK. There is accumulating evidence that ERK is commonly activated in tumor cell lines, which has prompted many researchers to develop MEK inhibitors. There is already substantial evidence that MEK plays a key role in the transformation of cells and development of tumors. Furthermore, a mutant H-*ras* gene perpetually activates the MEK/ERK signaling pathway and drives cells to develop a more aggressive cancer-like phenotype, such as anchorage-independent growth. The constitutive activation of MEK1 results in cellular transformation, while a small-molecule inhibitor of MEK successively inhibits transformation and tumor growth in both cell culture and mouse models [33,34]. The present study clearly demonstrated that CPE strongly inhibited MEK1 activity through binding with MEK1.

In summary, CPE protected against H_2O_2-induced inhibition of GJIC in RLE cells by attenuating intracellular ROS and blocking the ERK/Cx43 signaling pathway, but not the p38 MAPK signaling pathway. We suggest that CPE is not only a natural dietary antioxidant but also a potent direct inhibitor of MEK, which may contribute to its chemopreventive effects.

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