Cocoa Polyphenols Inhibit Phorbol Ester-Induced Superoxide Anion Formation in Cultured HL-60 Cells and Expression of Cyclooxygenase-2 and Activation of NF-κB and MAPKs in Mouse Skin In Vivo1,2

Ki Won Lee,*3 Joydeb Kumar Kundu,† Sue Ok Kim,‡ Kyung-Soo Chun,‡ Hyong Joo Lee,*4 and Young-Joon Surh*4

*Department of Food Science and Technology, School of Agricultural Biotechnology, and †College of Pharmacy, Seoul National University, Seoul 151-742, Republic of Korea

ABSTRACT We investigated the antioxidant and antiinflammatory activities of a flavonoid-rich polyphenolic fraction of cocoa. Cocoa polyphenol (CP) was fractionated from commercial cocoa powder and contained 468 mg/g of gallic acid–equivalent phenolics and 413 mg/g epicatechin-equivalent flavonoids. CP exhibited a dose-dependent free radical–scavenging activity as determined by both 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) and 2,2'-diphenyl-1-picrylhydrazyl radical scavenging assays. CP also dose-dependently inhibited xanthine oxidase activity and 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced superoxide-anion generation in cultured human promyelocytic leukemia HL-60 cells. Oral administering of CP (4, 20, 40, and 200 mg/kg body weight) to ICR mice 1 h prior to TPA (10 nmol) inhibited ear edema at 5 h in a dose-dependent manner. The levels of COX-2 expression induced in mouse skin after 4-h treatment with topical TPA (10 nmol) was also diminished significantly by pretreating CP (40 or 200 mg/kg) for 30 min. CP at the same doses inhibited TPA-induced nuclear translocation of NF-κB at 1 h by blocking the degradation of IκBα in mouse skin. Moreover, phosphorylation of p38 mitogen-activated protein kinase in ICR mouse skin, measured 4 h after TPA treatment, was suppressed by oral pretreatment of CP (40 or 200 mg/kg). Although extracellular signal–regulated protein kinase 1/2 phosphorylation was unaffected, CP inhibited the catalytic activity of extracellular signal–regulated protein kinase 1/2 in TPA-stimulated mouse skin. Since cellular proinflammatory and prooxidant states are closely linked to tumor promotion, the antioxidant and antiinflammatory properties of CP may constitute the basis of possible antitumor promoting effects of this phytochemical. J. Nutr. 136: 1150–1155, 2006.

KEY WORDS: • cocoa polyphenols • antiinflammation • cyclooxygenase-2 • nuclear factor-κB
• mouse skin

In recent years, there has been substantial progress in identifying a variety of chemopreventive phytochemicals from our daily diet (1,2). However, little attention has been given to the chemopreventive potential of cocoa, which is the main ingredient of widely consumed chocolates and cocoa beverages (3). High concentrations of flavonoids, predominantly as flavonol oligomers of monomeric base units known as procyanidins, are present in cocoa (4). Flavonols and procyanidins of cocoa have been shown to inhibit the growth and biosynthesis of polyamines in human colon cancer (Caco-2) cells (5). Our recent study has revealed that cocoa contains more phenolic phytochemicals and a higher antioxidant capacity than tea and red wine (6). Consuming chocolate has been reported to increase the total antioxidant capacity of human blood plasma in vivo (7). Previous studies indicate that cocoa powder extract and polyphenols prolong the lag time of LDL oxidation (8,9). Cocoa flavonoids inhibit both the dioxygenase and 5,6-leukotriene A4 synthase activities of human 5-lipoxygenase (10). Moreover, orally administered cocoa powder or cocoa liquor inhibits chemically induced carcinogenesis in experimental animals (11,12).

Oxidative stress and inflammation are implicated in multistage carcinogenesis (13–15). Reactive oxygen species (ROS)5, produced as typical by-products of eicosanoid metabolism during the inflammatory tissue damage, can alter the course of normal biochemical processes, leading to preneoplastic transformation of cells (16,17). The generation of the superoxide anion is elevated during oxidative burst or inflammation. Xanthine oxidase

5 Abbreviations used: ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid); COX-2, cyclooxygenase-2; CP, cocoa polyphenol; DPPH, 2,2-diphenyl-1-picrylhydrazyl; EKR, extracellular signal–regulated protein kinase; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-kappa B; ROS, reactive oxygen species; SDS, sodium dodecylsulfate; TPA, 12-O-tetradecanoylphorbol-13-acetate.
activity is elevated during the promotional phase of tumorigenesis (18). Tumor promoters, in particular, phorbol ester, generate superoxide anion radicals in epithelial cells and leukocytes. One of the key enzymes that mediates inflammatory response is cyclooxygenase-2 (COX-2), a rate-limiting enzyme in prostaglandin biosynthesis. Evidence suggests that inappropriate induction of COX-2 plays a pivotal role in tumor promotion and progression (19–21). Therefore, antioxidant and antiinflammatory phytochemicals that target COX-2 are potential antitumor promoting agents.

The redox-sensitive eukaryotic transcription factor, nuclear factor-κB (NF-κB), has been known to regulate COX-2 expression and is a critical target for chemoprevention with antiinflammatory substances (22,23). In response to oxidative and proinflammatory stimuli, NF-κB is activated, at least in part, by a series of upstream kinases, including those belonging to the mitogen-activated protein kinase (MAPK) family. The activated form of MAPK-family proteins, such as extracellular signal–regulated protein kinase (ERK), c-Jun NH2-terminal kinase, and p38 MAPK, can phosphorylate and activate transcription factors, thereby altering the expression of COX-2 (15,24). A typical tumor promoter, 12-O-tetradecanoylphorbol-13-acetate (TPA), has been shown to be a potent stimulator of COX-2 expression in various cell lines as well as in mouse skin in vivo (25,26). Previous studies from this laboratory revealed that topical application of TPA on mouse skin resulted in the activation of MAPK and aforementioned transcription factors that regulate COX-2 expression (22,26). In the present work, we attempted to evaluate the antioxidant and antiinflammatory activities of a flavonoid-rich polyphenolic fraction, prepared from commercially available cocoa.

### MATERIALS AND METHODS

**Chemicals.** Gallic acid, epicatechin, vitamin C, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as diammonium salt, 2,2-diphenyl-1-picrylhydrazyl (DPPH), and Folin and Ciocalteu’s phenol reagent were obtained from Sigma Chemical. Trolox was purchased from Aldrich Chemical. 2,2'-Azobis(2-amidinopropane)dihydrochloride was obtained from Wako Chemicals. 12-O-Tetradecanoylphorbol-13-acetate was obtained from Alexis Biochemicals. All other chemicals used were in the purest form available commercially.

**Sample preparation.** Commercial cocoa powder (50 g) was extracted with 500 mL of 50% (v/v) aqueous ethanol under reflux for 6 h. After extraction, the solution was filtered off to collect the extract, with this process repeated twice. The collected cocoa extract was loaded onto a styrene-based adsorption resin column (60 mm × 450 mm, HP-20, Mitsubishi), washed with 20% (v/v) aqueous ethanol, and then eluted with 60% (v/v) aqueous ethanol. The eluted cocoa polyphenol fraction was concentrated at 40 or 50° C under reduced pressure and used to determine antioxidant and antiinflammatory activity and the polyphenol content.

**Measurement of the total phenolic content.** The total phenolic content of cocoa polyphenol (CP) was measured by following Folin-Ciocalteu’s method. The assay was replaced 5 times. The total phenolic content in CP was determined as mg/g of gallic acid equivalent.

**Determination of the total flavonoid content.** The total flavonoid content was measured by a colorimetric assay method developed by Zhishen et al. (27). The assay was repeated 5 times. The total flavonoid content is expressed as mg/g of epicatechin equivalent.

**Assessment of ABTS radical-scavenging activity.** The method developed by van den Berg et al. (28,29) was used with slight modifications for assessing the ABTS radical-scavenging activity of CP as described previously.

**Assessment of DPPH radical-scavenging activity.** The DPPH radical-scavenging activity of CP was measured by the method described by Brand-Williams et al. (28,30) with minor modifications as described previously.
Antioxidant activities of CP. CP extracted from commercial cocoa powder contained 468 mg/g gallic acid-equivalent phenolics and 413 mg/g epicatechin-equivalent flavonoids. Similar to our previous observations with pure phenolic phytochemicals (28), CP exhibited a dose-dependent free radical-scavenging activity as determined by both ABTS and DPPH radical-scavenging assays (Fig. 1A). The elevation of xanthine oxidase activity and generation of the superoxide anion during the promotional phase of tumorigenesis have been reported previously (18). CP dose-dependently inhibited xanthine oxidase activity (Fig. 1B). Treatment with CP also suppressed the TPA-induced generation of superoxide anion in cultured HL-60 cells (Fig. 1B) without affecting cell viability (data not shown).

CP inhibits TPA-induced ear edema and COX-2 expression in mouse skin. Intragastric administering of CP significantly decreased TPA-induced ear edema by 58 and 87% at doses of 40 and 200 mg/kg body weight, respectively (P < 0.01, Table 1). Oral administering of CP at 0.5 h prior to topical application of TPA on the shaved back of female ICR mice resulted in a dose-dependent reduction in TPA-induced COX-2 expression (Fig. 2).

Inhibitory effects of CP on TPA-induced DNA binding activity of NF-κB, nuclear translocation of p65, and degradation of IkBα. Because NF-κB plays a major role in regulating the COX-2 gene expression, we examined the effects of CP on the activation of this transcription factor in mouse skin stimulated with TPA. Pretreatment with CP exhibited an inhibitory effect on NF-κB DNA binding (Fig. 3A) in TPA-treated mouse skin. To elucidate a possible mechanism underlying the inhibition of TPA-induced DNA binding of NF-κB by CP in mouse skin, we examined the effect of CP on the expression of IkBα and p65. Consistent with the inhibition of NF-κB DNA binding, pretreatment with CP inhibited TPA-induced nuclear localization of p65 (Fig. 3B) as well as degradation of IkBα (Fig. 3C).

Effects of CP on TPA-induced activation of ERK and p38 MAPK. ERK and p38 MAPK are involved in upregulation of COX-2 expression via NF-κB signaling in TPA-stimulated mouse skin (26,34), and we investigated whether CP could suppress the TPA-induced activation of these MAPKs through phosphorylation. Pretreatment with CP suppressed the TPA-stimulated phosphorylation of p38 MAPK (Fig. 4A). Although pretreatment with CP did not affect TPA-induced phosphorylation of ERK1/2 (Fig. 4B), CP attenuated the catalytic activity of ERK1/2 as revealed by a reduced expression of phosphorylated Elk-1, which is a substrate of ERK1/2 (Fig. 4C).

DISCUSSION

Regular consumption of fruits and vegetables, particularly those rich in flavonoids, can reduce the risk of cancer (1,2). Cocoa extracted from the fruits of Theobroma cacao is one of the most widely consumed beverages. Flavonoids in cocoa are mostly procyanidin oligomers that exist as complex forms of flavan-3-ol monomer. Procyanidin oligomers derived from choc-
The inflammatory response is causally linked to tumor promotion, and several studies have demonstrated that the activation of the arachidonic acid cascade leads to the production of inflammatory mediators such as prostaglandins, prostacyclins, and leukotrienes. These mediators are produced by the catabolism of arachidonic acid, an essential fatty acid that is present in cell membranes. The production of these inflammatory mediators is regulated by the cyclooxygenase (COX) enzymes, which catalyze the conversion of arachidonic acid to prostaglandins. COX-1 and COX-2 are the two isoforms of the COX enzyme, and they play different roles in inflammation and cancer.

Recent studies have shown that COX-2 is upregulated in many types of cancer, and its expression is associated with tumor promotion and progression. COX-2 expression is regulated by transcription factors such as nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), which are activated by various stimuli including tumor promoters.

Cocoa is a source of polyphenols, which are antioxidants that have been shown to have anti-inflammatory effects. A previous study demonstrated that cocoa polyphenols inhibited the expression of COX-2 in mouse skin (26). Enhanced DNA binding of NF-κB depends on the release of this transcription factor from its cytosolic repressor inhibitory protein IκBα, which undergoes extensive degradation upon exposure of cells or tissues to oxidative stimuli/tumor promoters. Our study demonstrates that CP strongly suppresses TPA-induced degradation of IκBα, which may represent the molecular mechanism responsible for the inhibitory effect of CP on the activation of NF-κB.

The molecular signaling mechanisms involved in the induction of COX-2, as well as activation of NF-κB in response to various external stimuli, have not been thoroughly clarified. The MAPK pathway is one of the most extensively investigated intracellular signaling cascades involved in proinflammatory responses. MAPKs regulate NF-κB activation via multiple pathways, including the extracellular signal-regulated kinase (ERK) and p38 MAPK pathways. The regulation of MAPK activity is complex and involves both upstream kinases and downstream targets. The importance of these pathways in the regulation of COX-2 expression and NF-κB activation is not fully understood.
mechanisms. Accumulating evidence suggests that enzymes of the MAPK family play a role in cox-2 gene expression (24). Although the MAPK-signaling pathways have been extensively investigated in cultured cell lines, much less is known about the specificity of MAPKs and the extent to which they are activated during tumor promotion in mouse skin in vivo. Our previous studies (22,34) demonstrate that treating the dorsal skin of female ICR mice with TPA significantly enhances both the catalytic activities and phosphorylation of p38 MAPK and ERK1/2. In the present study, we found that CP inhibited TPA-induced phosphorylation of p38 MAPK. Our results indicate that CP has no inhibitory effect on the phosphorylation of ERK1/2, but it elicits strong inhibitory effects on the catalytic activity of ERK1/2 when stimulated by TPA. A similar effect has also been observed with curcumin, a well-known chemopreventive phytochemical, which inhibits the kinase activity of ERK without affecting its phosphorylation state in TPA-stimulated mouse skin (26). However, the exact molecular mechanism underlying this phenomenon is not clear.

In conclusion, our study demonstrates that CP prepared from cocoa possesses free radical–scavenging, antioxidant, and antiinflammatory properties. As a mechanistic basis of its antiinflammatory effects, CP inhibits the induction of COX-2 expression, the activation of MAPKs, and NF-κB signaling in TPA-treated mouse skin, which indicates the role of CP as a potential cancer chemopreventive agent.

LITERATURE CITED