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Inhibitory Effects of Curcumin and Tetrahydrocurcuminoids on the Tumor Promoter-induced Reactive Oxygen Species Generation in Leukocytes *in vitro* and *in vivo*

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The inhibitory effects of curcumin and two tetrahydrocurcuminoids on tumor promoter-induced oxidative stress *in vitro* and *in vivo* were investigated. Curcumin, tetrahydrocurcumin (THC) and dihydroxytetrahydrocurcumin (DHTHC) exhibited significant inhibitory effects on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced O_2^- generation in differentiated HL-60 cells. The inhibitory activity of THC was weaker than that of curcumin. This tendency was the inverse of the results of previous studies on *in vitro* antioxidative activity against lipid peroxidation. The curcuminoids inhibited TPA-induced intracellular peroxide formation in differentiated HL-60 cells. THC exhibited much weaker inhibition of intracellular peroxide formation than curcumin, suggesting that this inhibition might be attributable to the inhibition of O_2^- generation. The inhibitory effects of curcuminoids on TPA-induced H_2O_2 formation in female ICR mouse skin were further examined using the double-TPA-application model. Each TPA application induces two distinct biochemical events, 1) recruitment of inflammatory cells to the inflammatory regions and 2) activation of oxidant-producing cells. Double pretreatment of mice with curcuminoids before each TPA treatment significantly suppressed double TPA application-induced H_2O_2 formation in the mouse skin. Coadministrations of curcumin with either first or second TPA treatment significantly inhibited H_2O_2 formation. In addition, THC tends to show weaker inhibitory activities than curcumin in bioassays related to tumor promotion, i.e., inhibition of tumor promoter-induced inflammation in mouse skin and Epstein-Barr virus activation. These tendencies were parallel to those in the tumor-suppressive potential of curcumin and THC in mouse skin, as previously reported. Thus, we concluded that curcuminoids significantly suppress TPA-induced oxidative stress via both interference with infiltration of leukocytes into the inflammatory regions and inhibition of their activation.

Key words: Curcumin — Tetrahydrocurcumin — Reactive oxygen species — Mouse skin — HL-60

Curcumin (diferuloylmethane), a major yellow pigment of turmeric occurring in the rhizomes of several tropical gingers such as *Curcuma longa*, is commonly used as a coloring condiment. A wide range of biological and pharmacological activities of curcumin have thus far been investigated.[1, 2)] Curcumin is a potent inhibitor of mutagenesis and chemically induced carcinogenesis.[3–10)] For example, topical application of commercial food grade curcumin containing minor amounts of derivatives or pure curcumin strongly inhibited 7,12-dimethylbenz-[*a*]anthracene-induced tumor initiation and 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced tumor promotion in mouse skin.[6–11)] Dietary curcumin also inhibited chemical carcinogenesis in some target organs of rats and mice.[3–5, 12)] Curcumin appears to have practical cancer-preventive potential in view of its relatively low toxicity to rodents.

The action mechanism(s) of curcumin for anti-tumor promoting activity is complicated.[7, 10, 13–16)] Among a wide range of biological and biochemical activities, its antioxidative property may be one of the essential actions for anti-tumor promotion. Previously, curcuminoids have been reported to exhibit antioxidative activities in some *in vitro* lipid peroxidation systems[17–19)] and to suppress TPA-induced hydrogen peroxide (H_2O_2) production and oxidized DNA formation in mouse epidermis.[10)] Curcumin is also an inhibitor of neutrophil responses[20)] and of superoxide (O_2^-) generation in macrophages.[21)]

Tumor promoter-induced reactive oxygen species (ROS) generation has been considered to play important roles in tumor promotion.[22)] In particular, TPA-type tumor promoters are reported to trigger O_2^- generation in epithe-

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lial cells and leukocytes through the xanthine/xanthine oxidase (XA/XOD) and NADPH oxidase systems, respectively. Yoon *et al.* advocated a close relationship between the generation of ROS, including O_2^-, by phagocytic cells in inflammatory processes and tumor promotion.[23] Kensler *et al.* have hypothesized that the first treatment of mouse skin with TPA causes a chemotactic action, i.e., recruitment of neutrophils responsible for ROS generation induced by the second TPA treatment.[24] In fact, double application of TPA is required for excessive ROS production in mouse skin.[25] Ji *et al.* concluded that each application triggers two distinct biochemical events, termed priming and activation.[26] The former event has been recognized mainly as recruitment of neutrophils, and the latter as the stage of ROS production in neutrophils, keratinocytes, etc. Moreover, ROS production by double or multiple TPA treatment is closely associated with the metabolic activation of proximate carcinogens[26, 27] and the increased levels of oxidized DNA bases.[25]

In recent studies, tetrahydrocurcumin (THC, Fig. 1), one of the major colorless metabolites of curcumin in the form of its glucuronide conjugate in bile, exhibited stronger antioxidative activities than curcumin in several *in vitro* systems.[18, 19] Thus, THC was thought to be one of the metabolites with higher physiological and pharmacological activities than curcumin in the intestine. THC has recently been reported to be a less effective chemopreventive agent in mouse skin than curcumin.[9] In contrast to the result in the case of skin carcinogenesis, feeding 0.5% THC in the diet significantly inhibited 1,2-dimethylhydrazine-induced mouse colon carcinogenesis, while the inhibitory effect of curcumin was not statistically significant.[28] These conflicting findings prompted us to determine how effectively curcumin, THC, and dihydroxytetrahydrocurcumin (DHTHC), having *ortho*-diphenol moieties which scavenge free radicals, inhibit tumor promoter-induced ROS generation *in vitro* and *in vivo*.

This paper describes the inhibitory effects of curcumin, THC, and DHTHC on TPA-induced ROS generation using a differentiated HL-60 cell system and the double-TPA-application model for H_2O_2 production in mouse skin. We also examined the inhibitory effects of these curcuminoids on tumor promoter-induced inflammation in mouse skin, and Epstein-Barr virus (EBV) activation to evaluate anti-tumor promoting activity.[29]

MATERIALS AND METHODS

Chemicals and cells Curcumin was purified by preparative silica gel TLC from commercial turmeric (Daiwa Kasei Co., Saitama). THC and DHTHC were prepared by hydrogenation with PtO_2 of curcumin or demethylation using BBr_3 as previously reported.[18, 30] Teleocidin B-4 was isolated from *Streptoverticillium blastomyceticum* NA 34-17 as previously reported.[31] Rosmarinic acid was purchased from Extrasynthese S.A., Genay, France. TPA was

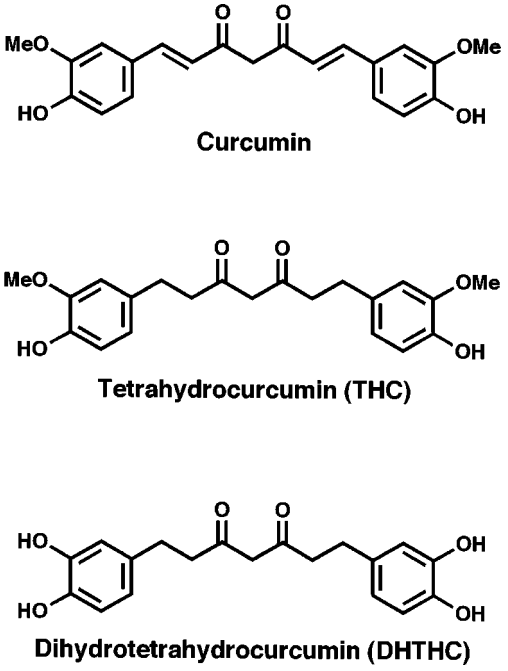

Fig. 1. Chemical structures of curcumin, tetrahydrocurcumin (THC), and dihydroxytetrahydrocurcumin (DHTHC).

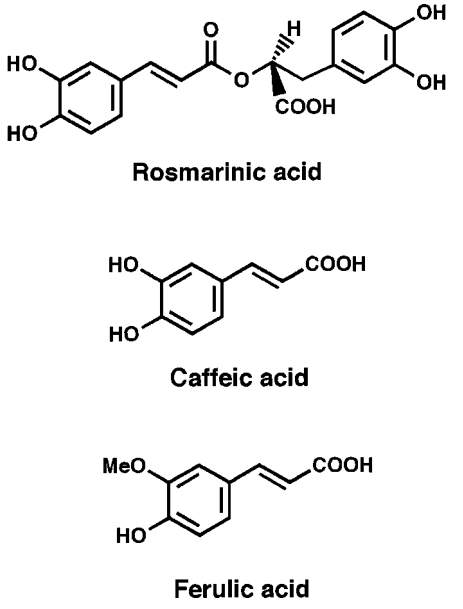

Fig. 2. Chemical structures of rosmarinic acid, caffeic acid, and ferulic acid.

obtained from Research Biochemicals International, MA. RPMI 1640 medium and fetal bovine serum were purchased from Gibco RBL, NY. 2′,7′-Dichlorofluorescein diacetate (DCFH-DA) was obtained from Molecular Probes, Inc., Leiden, The Netherlands. Cytochrome *c* and ferulic acid were obtained from Sigma, St. Lowis, MO. High-titer Epstein-Barr virus early antigen (EBV-EA)-positive sera from anaplastic nasopharyngeal carcinoma (NPC) patients were kind gifts from Prof. Dr. Ohsato (Health Sciences University of Hokkaido). FITC-labeled anti-human IgG was obtained from Dako, Glostrup, Denmark. All other chemicals were purchased from Wako Pure Chemical Industries, Osaka. Human B-lymphoblastoid Raji cells and human promyelocytic leukemia HL-60 cells were kind gifts from Prof. T. Ohsato and Prof. R. Sasaki (Kyoto University), respectively.

Inhibitory test of TPA-induced superoxide generation and intracellular peroxides formation in differentiated HL-60 cells Inhibition of TPA-induced O_2^- generation was assayed as previously reported.[32] Intracellular peroxides were detected by using DCFH-DA as an intracellular fluorescence probe as reported previously.[32] Experiments were repeated twice with similar results. The data are presented as one representative histogram.

Treatment of animals Female ICR mice (7 weeks old) were obtained from Japan SLC, Shizuoka. Mice used in each experiment were supplied with fresh tap water *ad libitum* and rodent pellets (MF, Oriental Yeast Co., Tokyo) which were changed twice a week. Animals were maintained in a room controlled at 24±2°C with a relative humidity of 60±5% and a 12-h light/dark cycle (06:00 to 18:00). The back of each mouse was shaved with surgical clippers two days before each experiment. All the test compounds (810 nmol/100 μl in acetone) were topically applied to the shaved area of dorsal skin 30 min before application of a TPA solution (8.1 nmol/100 μl in acetone). The minimal effective dose of curcumin against TPA-induced biological activities in mouse skin was reported to be 100-fold molar dose with respect to TPA.[7] In the single treatment protocol, one dose of TPA and one dose of test compounds were applied. In the double treatment protocol, two equal doses of TPA and test compounds were applied at an interval of 18 or 24 h.

Anti-inflammation test in mouse skin Two biomarkers of skin inflammation, skin edema formation and myeloperoxidase (MPO) activity, were determined by the method of Wei *et al.*[33] with slight modifications. Mice were killed by cervical dislocation 18 h after a single application of TPA. Mouse skin punches were obtained with an 8-mm-diameter cork borer and weighed on an analytical balance. The inhibitory effects (IE) were expressed as the ratio of relative increase of the weight of the treated punch to that of a control punch; IE (%) = ((TPA alone) − (test compound plus TPA)) / ((TPA) − (vehicle)) × 100. Statistical analysis was done by the use of Student's *t* test. For the determination of MPO activity, the skin punches were minced in 3 ml of 0.5% hexadecyltrimethyl ammonium bromide in 50 m*M* potassium phosphate buffer, pH 6.0 and homgenized at 4°C for 10 s, followed by centrifugation at 10,000*g* at 4°C for 20 min. To each 2-ml cuvette, 0.65 ml of 25 m*M* 4-aminoantipyrine-2% phenol solution and 0.75 ml of 2 m*M* H_2O_2 were added and the whole was allowed to equilibrate for 5 min. After the basal rate was established, a 100-μl sample of supernatant was added to the cuvette and quickly mixed. Increases in absorption at 510 nm for 1 min at 0.1-min intervals were recorded to determine MPO activity, which was calculated from the linear portion of the curve and expressed as units of MPO per skin punch. One unit of MPO activity is defined as that which degrades 1 μmol of H_2O_2 per min at 25°C.

Determination of H_2O_2 in mouse epidermis Mice exposed to the double-treatment protocol were killed 1 h after the last TPA treatment. To remove test compounds remaining on the skin surface, the skin was wiped with acetone-dipped gauze. A skin sample was removed and immersed in a 55°C water bath for 30 s, then the subcutis was scraped off. The mouse skin (epidermis and dermis) punches were obtained with a 8-mm-diameter cork borer and weighed on an analytical balance. The skin punches were minced in 3 ml of buffer containing 50 m*M* phosphate buffer (pH 7.4) and 5 m*M* sodium azide and then homogenized twice for 10 s at 4°C. The homogenate was centrifuged at 10,000*g* at 4°C for 20 min. The H_2O_2 content was determined by the phenol red-horseradish peroxidase (HRPO) method.[33] To each 1.5-ml cuvette, 0.5 ml of the supernatant and 0.5 ml of phenol red (200 μg/ml)-HRPO (100 μg/ml) solution were added and the mixture was incubated at 25°C for 10 min. One hundred microliters of 1 *M* NaOH was added to terminate the reaction, and the absorbance was determined spectrophotometrically at 610 nm. The final results were expressed as equivalents of nanomoles of H_2O_2 per skin punch, on the basis of the standard curve of HRPO-mediated oxidation of phenol red by H_2O_2.

Inhibitory test of EBV activation Human B-lymphoblastoid Raji cells were incubated in 1 ml of RPMI 1640 medium containing sodium *n*-butyrate (3 m*M*), teleocidin B-4 (50 n*M*), and the test compound at 37°C under a 5% CO_2 atmosphere for 48 h. EBV activation was estimated by detection of EBV-EA using the indirect immunofluorescence method as reported previously.[32]

RESULTS

Inhibitory activity of the curcuminoids against TPA-induced superoxide generation in differentiated HL-60 cells We examined the inhibitory activity of curcumin,

THC, and DHTHC against TPA-induced O_2^- generation because O_2^- is regarded as one of the initiators of ROS formation, which is possibly correlated with tumor promotion. The O_2^- generation was detected by a cytochrome *c* reduction method. As shown in Fig. 3, curcumin inhibited O_2^- generation by 85% at a concentration of 50 μM. THC and DHTHC at 50 μM also inhibited O_2^- generation by 50% and 81%, respectively. The inhibitory activity of these compounds was statistically significantly higher than those of rosmarinic acid, caffeic acid, and ferulic acid, which are all well known radical scavengers having an *ortho*-diphenol or *ortho*-methoxyphenol moiety (Fig. 2).

Inhibitory activity of the curcuminoids against TPA-induced intracellular peroxide formation The significant inhibitory activity of the curcuminoids toward O_2^- generation led us to address the inhibitory efficacy against intracellular peroxide formation by using DCFH-DA as an intracellular fluorescence probe. Fig. 4 shows the cytograms of differentiated HL-60 cells after treatment with or without 100 n*M* TPA. More than 80% of the cells (83%) were estimated to produce intracellular peroxides after stimulation with TPA alone. Curcumin at a concentration of 50 μM completely inhibited peroxide formation (IE >99%) and DHTHC exhibited weaker inhibitory activity than curcumin (IE=57%). THC showed little inhibitory activity (IE=12%).

Inhibitory activity of the curcuminoids against *in vivo* H_2O_2 production in mouse skin In order to discern whether or not curcumin, THC, and DHTHC inhibit H_2O_2 production in mouse skin, we used a double TPA treatment protocol according to Wei *et al*.[33] As shown in Fig. 5, double applications of 8.1 nmol of TPA at a 24-h interval increased the level of H_2O_2 by about 10-fold (7.66±0.68 nmol/skin punch versus 0.78±0.23, $P<0.001$) over that in the control mice treated only with acetone instead of TPA. A single dose application of TPA did not significantly enhance H_2O_2 production 1–24 h after treatment (data not shown). Curcumin (810 nmol) strongly inhibited H_2O_2 formation (3.18±1.36 nmol/skin punch, IE=58%, $P<0.001$). THC and DHTHC at the same dose also significantly reduced H_2O_2 formation (4.58±1.63 and 3.89±1.33 nmol/skin punch, IE=40% and 49%, respectively).

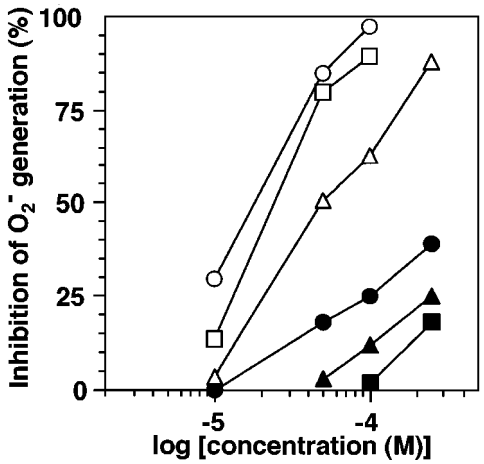

Fig. 3. Inhibitory effects of curcumin, THC, DHTHC, rosmarinic acid, caffeic acid, and ferulic acid on O_2^- generation in differentiated HL-60 cells. HL-60 cells were preincubated with 1.25% DMSO at 37°C for 6 days, causing them to differentiate them into granulocyte-like cells. Curcumin (○), THC (△), DHTHC (□), rosmarinic acid (●), caffeic acid (▲), or ferulic acid (■) solution was added to the cell suspension, and the mixture was incubated at 37°C for 15 min. Ninety seconds after stimulation with TPA (100 n*M*), cytochrome *c* solution was added to the reaction mixture. After incubation for another 15 min followed by centrifugation, visible absorption at 550 nm was measured. The maximal standard deviation for each experiment was 5%.

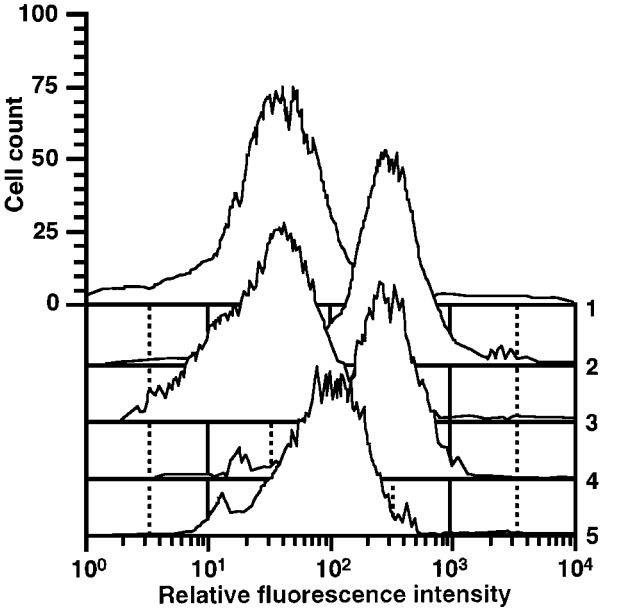

Fig. 4. DCF fluorescence distribution in DMSO-differentiated HL-60 cells. Cells were preincubated with 50 μM DCFH-DA at 37°C for 15 min. After having been treated with DMSO at 37°C for 15 min, the cells were treated with EtOH (unstimulated control, entry 1), or 100 n*M* TPA (positive control, entry 2). To determine the inhibitory effect of curcuminoids on TPA-induced intracellular peroxide formation, cells were preincubated with 50 μM DCFH-DA at 37°C for 15 min. After having been treated with 50 μM curcumin (entry 3), THC (entry 4), or DHTHC (entry 5) at 37°C for 15 min, the cells were treated with 100 n*M* TPA. The DCF fluorescence was monitored on a flow cytometer (CytoACE 150) with excitation and emission wavelengths of 488 nm and 600 nm, respectively.

Anti-inflammatory activities of the curcuminoids in mouse skin We examined the effects of curcumin, THC, and DHTHC on inflammatory responses induced by a single application of TPA, as measured in terms of skin edema formation and polymorphonuclear leukocyte (PMN) infiltration. As shown in Table I, single TPA application (8.1 nmol) resulted in edema formation (as measured by the weight of skin punch) amounting to 2.7-fold (96.3±6.0 versus 35.5±1.2 mg/skin punch, $P<0.001$) and increased PMN filtration measured in terms of MPO activity by 2.5-fold (6.72±1.60 versus 2.45±0.12 unit/skin punch, $P<0.001$) as compared with the control. Pretreatment with curcumin at 100-fold molar dose over TPA (810 nmol) reduced both skin edema formation and PMN infiltration (IE=38% and 56%, respectively). THC and DHTHC at 810 nmol also inhibited both biomarkers (THC; IE=20% and 30 %, DHTHC; 18% and 43%, respectively). The inhibitory effect of THC on edema formation was statistically significantly weaker than that of curcumin ($P<0.01$).

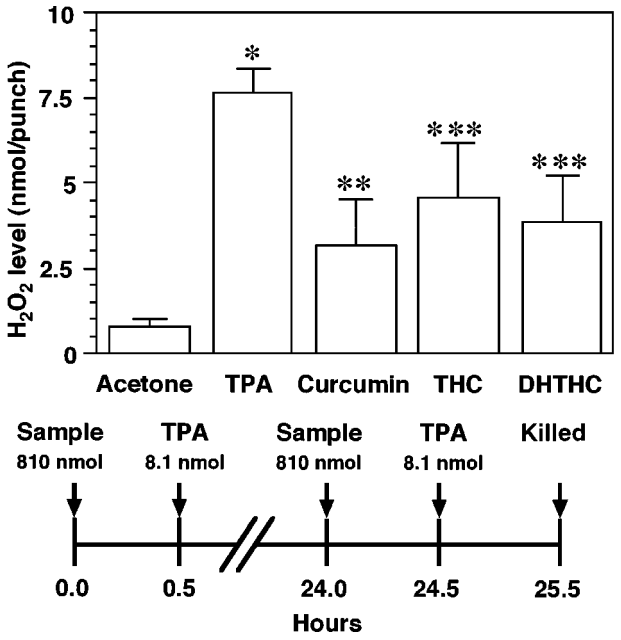

Fig. 5. Inhibitory effects of curcumin, THC, and DHTHC on H_2O_2 formation in mouse skin. ICR mice received the double treatment protocol as described here and in "Materials and Methods." Mouse skin was treated with curcumin, THC, or DHTHC (810 nmol) or acetone 30 min prior to each TPA treatment. The mice were killed 1 h after the second TPA application, and their skins were removed for H_2O_2 assays. Significance was determined by the use of Student's *t* test. * TPA versus acetone control, $P<0.001$. ** Inhibitor/TPA versus TPA, $P<0.001$. *** Inhibitor/TPA versus TPA, $P<0.01$.

Inhibitory effect of curcumin applied in the priming or activation phase on TPA-induced H_2O_2 generation in mouse skin To distinguish whether curcumin inhibits the priming or activation phase in the double TPA application model, curcumin was coadministered with either the first (priming) or second (activation) application of TPA. An interval of 18 h was chosen because the level of PMN infiltration was the highest at 18 h after the first TPA application (data not shown). Double applications of 8.1 nmol of TPA at an 18-h interval also increased the level of H_2O_2 by about 10-fold (5.00±1.35 nmol/skin punch versus 0.53±0.16, $P<0.001$) over that in the control mice. Fig. 6 shows the inhibitory effects of curcumin applied prior to either the first or second TPA treatment on H_2O_2 generation in mouse skin. A marked decrease in the H_2O_2 level was observed in both groups of mice to which curcumin was coadministered in the priming (2.38±1.11 nmol/skin punch, IE=52%) or activation phase (2.39±1.09 nmol/skin punch, IE=52%).

Inhibitory activity of the curcuminoids against EBV activation Inhibitory effects of curcumin, THC, and

Table I. Inhibitory Activities of Curcumin, THC, and DHTHC against Inflammation Induced by Single Dose of TPA in Mouse Skin

Treatment (nmol)	Edema (mg/punch)		MPO (units/punch)	
	Mean±SD[a)]	IE (%)	Mean±SD[a)]	IE (%)
Acetone/acetone	35.5±1.2	—	2.45±0.12	—
Acetone/TPA (8.1)	96.3±6.1*[b)]	—	6.72±1.60*[b)]	—
Curcumin (810)/TPA	73.5±4.1*[b, c)], **[d)]	36	4.35±1.29*[b)], ***[c)]	56
THC (810)/TPA	84.0±2.8*[b)], **[c)]	20	5.42±2.10*[b)]	30
DHTHC (810)/TPA	85.6±7.2*[b)], ***[c)]	18	4.90±0.62*[b)], ***[c)]	43

a) Data are the means of 5 experiments. Significance was determined by the use of Student's *t* test and is expressed as * $P<0.001$; ** $P<0.01$; *** $P<0.05$.
b) versus acetone/acetone control.
c) versus acetone/TPA.
d) versus THC.

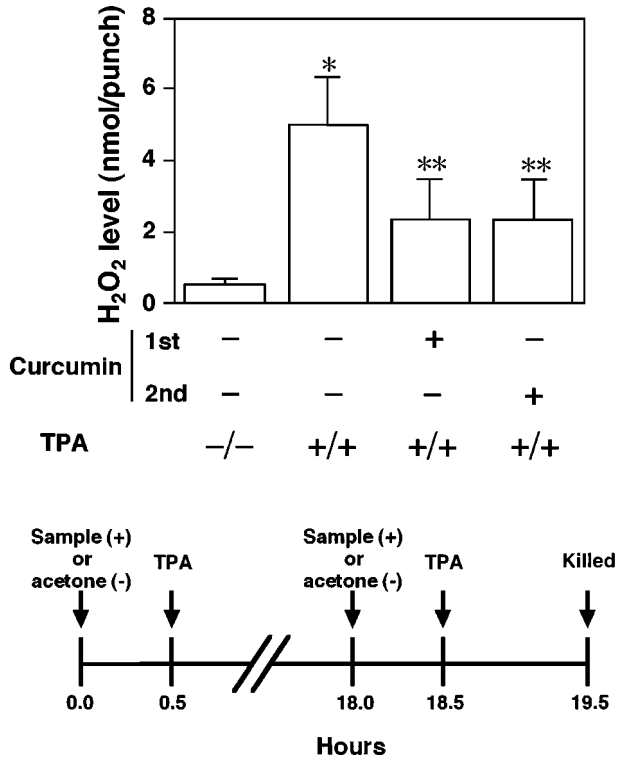

Fig. 6. Inhibitory effects of curcumin applied in the priming or activation phase on H_2O_2 formation in mouse skin. Mice were treated with curcumin (810 nmol) or acetone 30 min before either the 1st or 2nd TPA treatment. The mice were killed 1 h after the second TPA application. Statistical significance was determined by the use of Student's *t* test. * TPA versus acetone control, $P<0.001$. ** Inhibitor/TPA versus TPA, $P<0.05$.

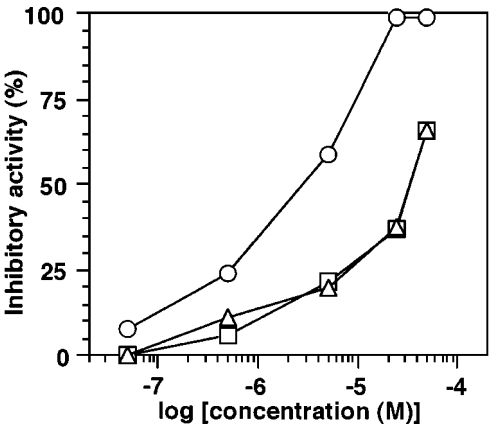

Fig. 7. Concentration-dependent inhibition of EBV activation by curcumin, THC, and DHTHC. Raji cells latently infected with EBV were incubated with *n*-butyrate (3 m*M*), teleocidin B-4 (50 n*M*), and curcumin (○), THC (△), or DHTHC (□) at 37°C for 48 h. The EBV-early antigen (EA) was detected by the indirect immunofluorescence method.

DHTHC on tumor promoter-induced EBV activation in Raji cells are shown in Fig. 7. THC and DHTHC exhibited significant inhibitory activities (IC_{50}=25 μM and 28 μM, respectively), comparable to that of β-carotene,[34] but markedly weaker than that of curcumin (3.1 μM). Curcumin exhibited cytotoxicity at 50 μM, while THC and DHTHC did not show any cytotoxicity at the same concentration.

DISCUSSION

We and others have recently reported that some natural chemopreventers inhibit O_2^- generation by leukocytes, suggesting that this inhibition to be at least one of the mechanisms of anti-tumor promotion.[32, 33, 35–38] We employed inhibitory assay of TPA-induced O_2^- generation in human promyelocytic leukemia HL-60 cells as a model of the NADPH oxidase system. Curcumin has been reported to be an inhibitor of neutrophil responses,[20] an inhibitor of O_2^- generation in macrophages[21] and a scavenger of active oxygen radicals.[39] The present study also demonstrated that curcumin significantly inhibited TPA-induced O_2^- generation in differentiated HL-60 cells (Fig. 3). In our system of O_2^- generation,[32] the extracellular O_2^- scavenging effect of a test compound can be ignored because the test compound is removed from the system by washing before TPA stimulation. NADPH oxidase is known to play a major role in O_2^- generation in leukocytes such as macrophages, neutrophils, or granulocytes.[40] The multicomponent NADPH oxidase system consists of heterodimeric cytochrome *b*, involving a β-subunit (gp91-*phox*) and α-subunit (p22-*phox*) associated with p47-*phox* and p67-*phox*.[41] A direct role of protein kinase C (PKC) or phospholipase A_2 in the activation of the assembled NADPH oxidase in neutrophils has been suggested.[42, 43] Thus, curcumin may inhibit TPA-induced assembly of this NADPH oxidase system or upstream signal transduction systems, possibly by inhibition of PKC activation.[16] Curcumin, THC, and DHTHC exhibited much stronger inhibition of O_2^- generation than phenolic radical scavengers such as rosmarinic acid, caffeic acid, and ferulic acid (Fig. 3). In addition, the IC_{50} values of curcumin, THC, and DHTHC against O_2^- generation are much lower than that of genistein (IC_{50}>100 μM),[33, 35] a well-known anti-tumor promoter from soybean.[38] On the other hand, the inhibitory effect of THC on O_2^- generation was weaker than that of curcumin, while DHTHC, having two *ortho*-diphenol moieties, significantly inhibited O_2^- generation (comparable to curcumin). These results suggested that the conjugated double bonds of the central seven-carbon

chain and the *ortho*-diphenol moieties are important for enhancing the inhibitory effect on O_2^- generation in differentiated HL-60 cells. Such trends in the structure-activity relationship of the curcuminoids are distinct from those in the lipid peroxidation tests as described above,[18, 19] but were parallel to the inhibition of *in vitro* lipoxygenase or tyrosinase activity (Osawa *et al.*, unpublished observation). It is likely that the conjugated double bonds of the central seven-carbon chain of curcumin may enhance the metal ion-chelating ability of the β-diketone moiety. These observations suggested that metal chelation by the curcuminoids may play a critical role in the inhibition of these metalloenzymes, but may not be sufficient to prevent lipid peroxidation.

It is well-known that O_2^- is converted to H_2O_2 nonenzymatically or by superoxide dismutase in biological systems. The hydroxyl radical (·OH), formed subsequently from H_2O_2, randomly reacts with biological components such as lipids or DNA bases within the cell. Takeuchi *et al.* reported that ·OH may directly induce formation of 8-hydroxydeoxyguanosine in DMSO-differentiated HL-60 cells.[44] Suppression of intracellular peroxide formation in differentiated HL-60 cells by curcumin (Fig. 4) could lead to the inhibition of O_2^- generation, since the greatest portion of peroxides is considered to originate from O_2^-. Furthermore, the close structure-activity relationships of the curcuminoids for the inhibition of O_2^- generation and reduction of intracellular peroxide levels (Figs. 2 and 3) support this assumption.

Double applications of phorbol esters trigger ROS production in mouse skin.[25] Reported data suggest that each application induces two distinct biochemical events, priming and activation.[26] Single application of TPA (8.1 nmol) significantly increased edema formation and MPO activity, a biomarker for infiltration of inflammatory cells (Table I), without significantly increasing the level of H_2O_2. Double applications of TPA (8.1 nmol) dramatically increased the H_2O_2 level in ICR mice (Fig. 5) as reported previously using outbred species of mice such as SENCAR or CD-1.[25, 33] The dose of TPA used in the present study falls within the tumor promotion range (1–10 nmol). The present results provide clear evidence for the suppression of tumor promoter-induced H_2O_2 formation by curcumin, THC, and DHTHC in mouse skin. It is likely that induction of lipoxygenase, which metabolizes arachidonic acid to hydroperoxy fatty acids, precursors of chemotactic leukotrienes, represents the early stage of the priming phase. The present results also demonstrate that the curcuminoids inhibit both TPA-induced edema formation and enhancement of MPO activity in mouse skin. Curcumin is a well-known inhibitor of arachidonic acid metabolism, via inhibition of the lipoxygenase and cyclooxygenase pathways.[13] Coadministration of curcumin with TPA in the priming phase significantly inhibited H_2O_2 formation (Fig. 6). These results strongly suggest that the anti-inflammatory effect of curcuminoids, which regulate the infiltration of ROS-producing leukocytes, is a possible mechanism of the inhibitory effects of TPA-induced H_2O_2 formation in mouse skin. On the other hand, this study demonstrated that the curcuminoid-suppressed O_2^- generation in turn resulted in reduced intracellular peroxide formation in differentiated HL-60 cells with DMSO. Furthermore, coadministration of curcumin with the second TPA treatment also significantly inhibited H_2O_2 formation to the same level as in the case of coadministration with the first TPA treatment (Fig. 6). These findings appear to be consistent with a mechanism by which the curcuminoids inhibit H_2O_2 formation in part via inhibition of leukocyte activation.

The NADPH oxidase system of neutrophils rather than the XOD system is implicated in the O_2^--generating system in double-TPA-treated mouse skin. A single TPA application was reported to induce enhancement of XOD activity.[45] However, it enhanced oxidized DNA base formation much less than double application of TPA,[46] suggestive of a critical role of the NADPH oxidase system in double application of TPA-induced oxidative stress. Conversely, a well-known XOD inhibitor, allopurinol also showed no inhibitory effect on double TPA application-induced H_2O_2 generation (Nakamura *et al.*, in preparation).

Evidence for the involvement of ROS production in carcinogenesis by double or multiple TPA treatments has been presented. Wei and Frenkel found increased levels of oxidized DNA bases in mice given double TPA treatment and multiple TPA treatment for 16 weeks.[25] Positive correlations between the formation of H_2O_2, oxidized DNA bases and first-stage tumor-promoting activity have been noted.[46] Wei and Frenkel also clearly demonstrated the relationship of the formation of H_2O_2 and DNA oxidation in SENCAR mice to the *in vivo* promoting potency of the phorbol ester-type tumor promoters.[37] H_2O_2 itself is known to be a first-stage tumor promoter. Thus, it is likely that the generation of H_2O_2 serves as a common link among many tumor promotion-related biochemical events. On the other hand, TPA-induced ROS and/or MPO bioactivates the proximate carcinogen 7,8-dihydroxy-7,8-dihydrobenzo[*a*]pyrene and enhances the formation of carcinogen-DNA adducts, through a pathway independent of cytochrome P-450.[24, 26, 27] These studies suggest that inhibition of H_2O_2 generation is one of the important pathways of anti-tumor promotion by curcuminoids *in vivo*.

The present study demonstrates, for the first time, inhibitory effects of THC and DHTHC on tumor promoter-induced EBV activation in Raji cells. As is already known, most EBV activation inhibitors have been proven to be effective inhibitors of TPA-induced tumor promotion

in mouse skin, and also of carcinogenesis of several other organs.[32, 34–36, 47] The IC_{50} value of curcumin (IC_{50}=3.1 μM) against EBV activation was ten times lower than that of β-carotene (IC_{50}=30 μM).[34] It is interesting to note that THC and DHTHC showed weaker inhibitory activity (IC_{50}=25 μM and 28 μM, respectively). In contrast to O_2^- generation inhibition, the conjugated double bonds of the central seven-carbon chain rather than the *ortho*-diphenol moiety are necessary for enhancing the inhibitory effect on EBV activation.

In conclusion, the curcuminoids significantly suppress TPA-induced oxidative stress through both suppression of leukocyte infiltration into the inflammatory regions and inhibition of the activation of leukocytes, including neutrophils. In particular, curcumin most effectively inhibits TPA-induced oxidative stress. Nevertheless, THC and DHTHC have some advantages for application as food additives because of their colorless character and easy preparation by usual hydrogenation of curcumin. Further investigation of these curcuminoids may lead to chemopreventive application in humans.

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