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Dihydrolipoic acid inhibits skin tumor promotion through anti-inflammation and anti-oxidation

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ABSTRACT

α-Lipoic acid (LA) has been intensely investigated as a therapeutic agent for several diseases, including hepatic disorder and diabetic polyneuropathy. However, the effects of LA or its reduced form, dihydrolipoic acid (DHLA), on cancer chemoprevention has never been reported. In the present study, we examined the effects of DHLA/LA on the production of nitric oxide (NO) by inducible NO synthase (iNOS) and the formation of prostaglandin E2 (PGE_2) by cyclooxygenase-2 (COX-2), two important mediators associated with inflammation. DHLA/LA significantly inhibited lipopolysaccharide (LPS)-induced NO and PGE_2 formation in RAW 264.7 cells. Meanwhile, treatment with DHLA/LA suppressed the expression of iNOS protein but, unexpectedly, did not affect or increase the expression of COX-2 protein. The *in vivo* anti-inflammatory and antitumor-promoting activities were evaluated by a topical 12-O-tetradecanoylphorbol 13-acetate (TPA) application to mouse skin with measurement of edema formation, epidermal thickness and hydrogen peroxide production. DHLA significantly inhibited the priming and activation stages of skin inflammation induced by a double TPA application, by decreasing the inflammatory parameters. Furthermore, DHLA inhibited DMBA (0.3 μmol)/TPA (2.0 nmol)-induced skin tumor formation by reducing the tumor incidence and tumor multiplicity. When applied topically onto the shaven backs of mice prior to TPA, DHLA markedly inhibited the expression of iNOS protein. DHLA also strongly and directly inhibited COX-2 activity. These results suggest that DHLA can be a possible chemopreventive agent in inflammation-associated tumorigenesis.

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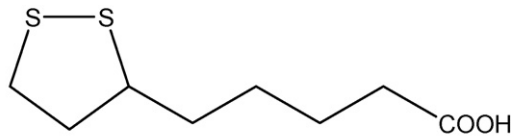
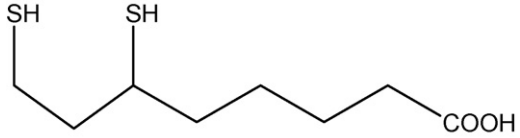
1. Introduction

Lipoic acid (LA) (Fig. 1) is a thiol-compound naturally occurring in plants and animals [1]. It is consumed in the daily diet, and taken up and transformed by cells and tissues into dihydrolipoic acid (DHLA) [2]. Both LA and DHLA serve as strong antioxidants through several mechanisms, including scavenging of free radicals, chelation of metal ions, and regeneration of endogenous and exogenous antioxidants, such as ubiquinone, glutathione, and ascorbic acid [3,4]. Therefore, it appears that LA could be a potential agent in the prevention of different diseases that may be related to an imbalance of the oxidoreductive cellular status. This occurs in cases of neurodegeneration, ischemia-reperfusion, polyneuropathy, diabetes, AIDS, and hepatic disorder status [5]. Nevertheless, little is known about the effects of LA/DHLA on cancer chemoprevention.

Reactive oxygen species (ROS) from both endogenous and exogenous sources can cause oxidative DNA damage and dysregulated cell signaling, which are involved in the multistage process of carcinogenesis, such as tumor initiation, promotion and progression. A number of structurally different anticarcinogenic agents inhibit inflammation and tumor promotion as they reduce ROS production and oxidative DNA damage [6]. Inflammatory processes are mediated by multiple molecular mechanisms. Two of the most prominent are the production of nitric oxide (NO) by inducible NO synthase (iNOS) and the formation of prostaglandins (PGs) by cyclooxygenase-2 (COX-2) [7,8]. Many cell types, especially macrophages, express iNOS upon stimulation, such as through exposure to bacterial LPS or cytokines. NO exerts a role in host defense due to its antibacterial and virustatic properties. However, if NO production gets out of control, damage to the host cells occurs due to the cytotoxic potential of NO [9]. NO has been reported to cause mutagenesis and deamination of DNA bases, and to form carcinogenic N-nitrosamines [10–12]. It is also involved in the production of VEGF, the overexpression of which induces angiogenesis, vascular hyperpermeability, and accelerated tumor development [13,14]. PGs are important for the initiation, promotion and progression of chemical carcinogenesis [15]. They can suppress the humoral and cellular immune action responsible for the killing of malignant cancer cells [15,16]. Direct interactions of PGs with their receptors through autocrine or paracrine pathways to enhance cellular survival or stimulate angiogenesis have been proposed as the molecular mechanisms underlying the pro-carcinogenic functions of COX-2 [17]. Topical application of TPA to mice led to edema and papilloma formation by enhancing COX-2 protein expression. Specific COX-2 inhibitors were able to counteract these biological events [18,19]. Collectively, suppression of enzyme induction and the activities of iNOS/COX-2 is a new paradigm for the prevention of carcinogenesis [20].



α-lipoic acid (LA)



dihydrolipoic acid (DHLA)

Fig. 1 – The chemical structures of α-lipoic acid and dihydrolipoic acid.

For more than 50 years, mouse skin has been used as a conventional model for studying the mechanisms of carcinogenesis and the modulation of sequential steps involved in this process [21,22]. Skin tumors can be induced by the sequential application of a sub-threshold dose of a carcinogen (initiation stage), followed by repetitive treatment with a non-carcinogenic promoter [21,23]. Topical application of the classical tumor promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) to mouse skin has been shown to result in a number of biochemical alterations, changes in cellular functions, and histological changes leading to skin tumor promotion [24,25]. Among these, the ones that correlate best with the skin tumor-promoting activity of TPA include skin edema, epidermal hyperplasia, inflammation, proliferation and oxidative stress [26,27]. All these changes in the skin have been defined as possible markers of skin tumor promotion, and were used to evaluate the potency of novel chemopreventive agents against tumor promotion [28,29].

In our previous study, we proved that two-stage skin tumorigenesis in the ICR mouse is a good model for determining the skin tumor-promoting potential of environmental toxicants [30]. Here, we applied the same model to examine the anti-tumor promotion effects of DHLA/LA on mouse skin. The in vivo anti-inflammatory activities were evaluated by a double application of TPA to mouse skin (priming and activation), with measurement of edema formation, epidermal thickness and H_2O_2 generation [20]. Due to the importance of iNOS and COX-2 in inflammatory skin damage, we first tested the hypothesis that the anti-tumor promotion effects of LA/DHLA might modulate the expression of these two crucial inflammatory mediators, by using a LPS-induced NO and PGE_2 generation test in mouse macrophage RAW 264.7 cells.

2. Materials and methods

2.1. Chemicals and animals

TPA, dimethylbenz(a)anthracene (DMBA), LA and DHLA were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals were obtained in the purest form available commercially. Six-week-old female CD-1 mice were acquired from the animal center of the National Cheng Kung University Medical College. The animals were housed five per cage at

24 ± 2 °C and 50 ± 10% relative humidity and subjected to a 12-h light/12-h dark cycle. They were acclimatized for 1 week before use, and fed with a Purina chow diet and water *ad libitum*. Prior to study, the dorsal side of the skin was shaved, and the tested compound that dissolved in 100 μl acetone was administered by micropipette. The test area was regularly shaved and the mice were observed daily.

2.2. Nitrite and PGE2 determination

RAW 264.7 cells, grown confluent in 2 ml of DMEM on a 60-mm dish, were treated with LPS (100 ng/ml), and LA/DHLA (0, 10, 50, 100, 250 or 500 μM) dissolved in DMSO. To determine whether LA/DHLA inhibits LPS-induced nitric oxide synthesis, the sum of stable nitric oxide metabolites, and nitrate and nitrite in culture media samples 20 h after exposure to LPS were measured. The culture media were harvested for analysis of nitrates using a colorimetric assay kit (Cayman Chemical, Ann Arbor, MI) that involves the Griess reaction. Nitrate concentrations were measured after reducing all nitrates into nitrite using nitrate reductase. The absorbance was measured at a wavelength of 570 nm. The values measured were compared with the standard curves. The concentrations of PGE_2 in the media were measured by the commercially available Prostaglandin E_2 Enzyme Immunoassay Kit (Assay Designs Inc, MI, USA). Each experiment was done in duplicate independently twice, and the data are expressed as mean ± S.D. values.

2.3. Western blotting

The female ICR mice were topically treated on their shaven backs with 2700 and 4050 nmol in 200 μl of acetone before 8.1 nmol TPA, and were killed by cervical dislocation at 4 h after TPA treatment. For the isolation of protein from mouse skin, the dorsal shaved skin was excised and was removed on ice, then immediately placed in liquid nitrogen. The epidermal protein was homogenized on ice for 15 s with a Polytron tissue homogenizer and lysed in 0.5 ml ice-cold lysis buffer [50 Mm, Tris–HCl, pH 7.4, 1 mM NaF, 150 mM NaCl, 1 mM EGTA, 1 mM dithiothreitol, 1 mM sodium orthovanadate 1% NP-40 containing 1 mM of phenyl methyl sulfonyl fluoride (PMSF), 10 μg/ml of aprotinin, and 5 μg/ml of leupeptin] on ice for 30 min, followed by centrifugation at 10,000 × *g* for 30 min at 4 °C. Afterward, the stimulated murine macrophage cell line RAW 264.7 cells were washed with PBS and lysed in an ice-cold RIPA buffer (Tris–HCl pH 7.2, 25 mM; SDS 0.1%; Triton X-100 1%; sodium deoxycholate 1%; NaCl 0.15 M; EDTA 1 mM) containing 1 mM of phenyl methyl sulfonyl fluoride (PMSF), 10 μg/ml of aprotinin, 1 mM of sodium orthovanadate and 5 μg/ml of leupeptin. Protein concentrations were determined using the BCA method (Pierce, Rockford, IL, USA). Protein (50 μg) was resolved by 12.5% polyacrylamide gel electrophoresis and blotted onto nitrocellulose sheets using the semidry blot system (TE 70; Hoefer Scientific Instruments, San Francisco, CA) at 2 mA/cm^2 for 60 min in 25 mM Tris–HCl, pH 8.3; 192 mM glycine; and 20% methanol. The membrane was blocked overnight at room temperature with a blocking reagent (20 mM Tris, pH 7.4; 125 mM NaCl; 0.2% Tween 20; 4% non-fat dry milk; and 0.1% sodium azide). Then it was incubated for 1 h with anti-iNOS mAb (Transduction Laboratories) and anti-COX-2 mAb (Santa Cruz Biotechnology). The proteins were washed three times, and incubated with alkaline phosphatase-conjugated rabbit anti-mouse in PBS and 0.5% Tween 20 for another 45 min with gentle shaking. After three final washes, the proteins were made visible by the Bio-Rad NBT-BCIP color development system.

2.4. Double TPA treatment of mouse skin

The double TPA treatment experiment was performed as reported by Nakamura et al. [31]. Briefly, the back of each mouse was shaved 2 days before each experiment; each experimental group consisted of 5 mice. DHLA (4050 nmol in 100 μl of acetone) was applied topically to the shaved area of the dorsal skin 30 min before application of a TPA solution (8.1 nmol in 100 μl of acetone). In the double-treatment protocol, the same doses of TPA and DHLA or acetone were applied twice at an interval of 24 h. We divided the mice into five groups as follows: group 1 (acetone × 2/acetone × 2); group 2 (acetone-TPA/acetone-TPA); group 3 [DHLA (priming)-TPA/acetone-TPA]; group 4 [acetone-TPA/DHLA (activation)-TPA]; and group 5 (DHLA-TPA/DHLA-TPA).

2.5. Epidermal hyperplasia study

For the epidermal hyperplasia study, skin samples from different treatment groups were fixed in 10% formalin and embedded in paraffin. Vertical sections were cut, mounted on a glass slide, and stained with H&E. Epidermal hyperplasia was determined by measuring the mean vertical epidermal thickness and the mean number of vertical epidermal cell layers through microscopic examinations of each treated skin tissue section. For each section of the skin, the thickness of the epidermis from the basal layer to the stratum corneum was measured at several equal distance interfollicular sites, using a Nikon light microscope (Japan) equipped with an ocular micrometer. For each individual value, a mean of 25 vertical epidermal measurements, equidistant from each other at 2 mm, in 50 mm (linear) of skin section, was determined.

2.6. Measurement of H_2O_2 and edema formation in mouse skin

Measurement of the levels of H_2O_2 and edema formation was done as reported by Nakamura et al. [31]. In brief, mice treated by the double-treatment protocol were sacrificed 1 h after the second TPA treatment. Skin punches (epidermis and dermis) were obtained with an 8-mm-diameter cork borer and were weighed with an analytical balance. The skin punches were minced in 3 ml of 50 mM phosphate buffer (pH 7.4) containing 5 mM sodium azide, and then homogenized at 4 °C for 30 s twice. The homogenate was centrifuged at 10,000 × *g* for 20 min at 4 °C. The H_2O_2 content was determined by the phenol red-horseradish peroxidase method.

2.7. Two-stage carcinogenesis experiment in mouse skin

The antitumor promoting activity of DHLA was examined by a standard initiation–promotion protocol with DMBA and TPA, as reported previously [30]. The mice were randomly divided

into six groups (groups I–VI) of 10 animals each, and a single 300 nmol dose of DMBA in 100 μl of acetone as a tumor initiator was applied topically on the dorsal shaved skin of each mouse in all groups. One week later, group I was treated with 100 μl of acetone topically as negative controls for monitoring any spontaneous tumor induction, and group II was treated with 2.0 nmol of TPA in 100 μl of acetone topically as positive controls. Groups III, IV, and V were treated with DHLA (300, 900, and 2700 nmol/100 μl acetone) 30 min before each TPA treatment. Group VI was treated the same as group II, but with a 0.2% LA diet supplement. Treatments of DHLA and TPA were repeated two times per week up to the termination of the experiment at 20 weeks from the treatment of DMBA. The antitumor-promoting activity was evaluated by both the ratio of tumor-bearing mice and the number of tumors that measured more than 1 mm in diameter, per mouse.

2.8. COX-2 enzyme activity assay

The purified COX-2 enzyme activity assay used a specific chemiluminescent substrate (Correlate-Enzyme™ assay) to detect the peroxidative activity of cyclooxygenase. The COX-2 activity was measured using a commercial assay kit (Assay Designs, Inc. Ann Arbor, MI, USA) according to the manufacturer's instructions.

2.9. Statistical analysis

The statistical significance of differences between the means was evaluated using the Student's *t*-test. A difference with a *P*-value less than 0.05 was considered statistically significant. All statistical analyses were carried out using the SAS package.

3. Results

3.1. DHLA/LA reduced LPS-induced nitrite accumulation in RAW 264.7 cells

Stimulation of RAW 264.7 cells with LPS for 20 h resulted in NO generation and then nitrite and nitrate (NOx) accumulation in the media (Fig. 2). DHLA, at a concentration range of 100–500 μM, concentration-dependently suppressed NOx production by 21.2–59.8% (Fig. 2A). A similar result was found in LA with mild inhibitory effects. LA, at a concentration range of 100–500 μM, suppressed NOx production by 13.8–43.0% (Fig. 2B). iNOS protein expression was then detected using Western blotting. As shown in Fig. 2, under control conditions, RAW 264.7 cells do not express iNOS protein, but LPS induces a remarkable increase in this enzyme. Treatment with DHLA or LA suppressed the expression of iNOS significantly when compared with LPS treatment alone. The reduced expression of iNOS protein was consistent with reductions in total nitrates (NOx) in culture media.

3.2. DHLA/LA reduced LPS-induced PGE2 production, but not COX-2 protein expression in RAW 264.7 cells

Stimulation of RAW cells with LPS for 20 h led to PGE_2 production in the media (about 6 ng/ml; Fig. 3). DHLA or LA

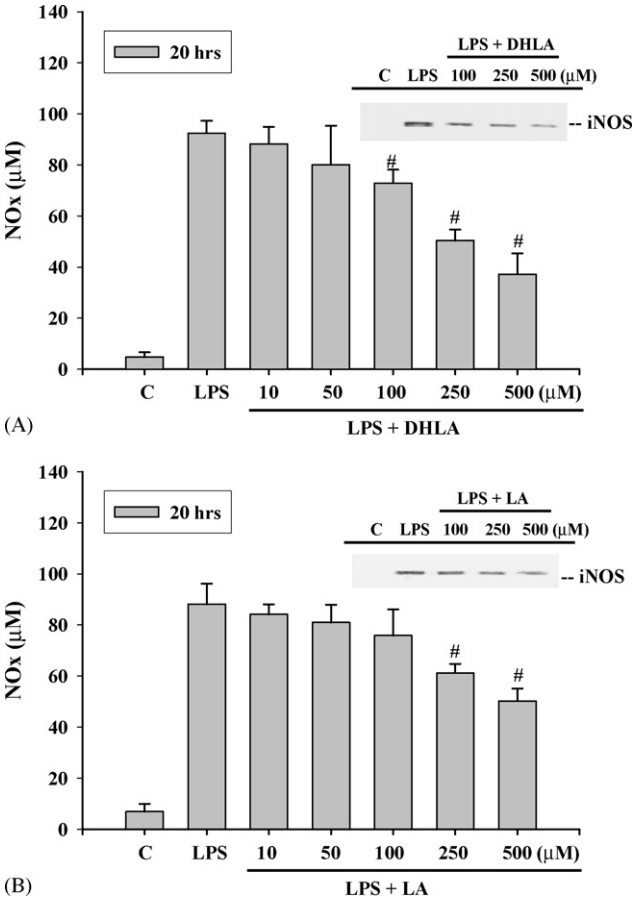


Fig. 2 – Effects of DHLA and LA on the LPS-induced iNOS protein level and nitrite production in RAW 264.7 cells. The cells were treated with 100 ng/ml of LPS only, or with different concentrations of (A) DHLA and (B) LA for 20 h. At the end of the incubation period, 100 μl of the culture medium was collected for nitrite assay. The expression of iNOS protein was detected by Western blot using a specific antibody. The values are expressed as means ± S.E. of triplicate tests. $^{\#}P < 0.05$ indicates statistically significant differences from the LPS-treated group.

suppressed PGE_2 production at the concentrations of 10–500 μM. Both DHLA and LA reversed completely the increased PGE_2 production stimulated by LPS at concentrations higher than 50 μM. Stimulation of RAW 264.7 cells also led to a marked up-regulation of COX-2 protein expression, which was much less expressed in the control. Interestingly, treatment with DHLA or LA did not suppress the expression of COX-2 protein when compared with LPS treatment alone (Fig. 3). Thus, the unchanged or even increased expression of COX-2 protein was inconsistent with reductions in PGE_2 production in culture media. We further analyzed the effects of DHLA or LA alone on the expression of COX-2 in RAW 264.7 cells, and found that both DHLA and LA can induce COX-2 protein expression significantly (Fig. 4A). Notable cytotoxicity was not observed in any of the above experimental conditions (Fig. 4B).

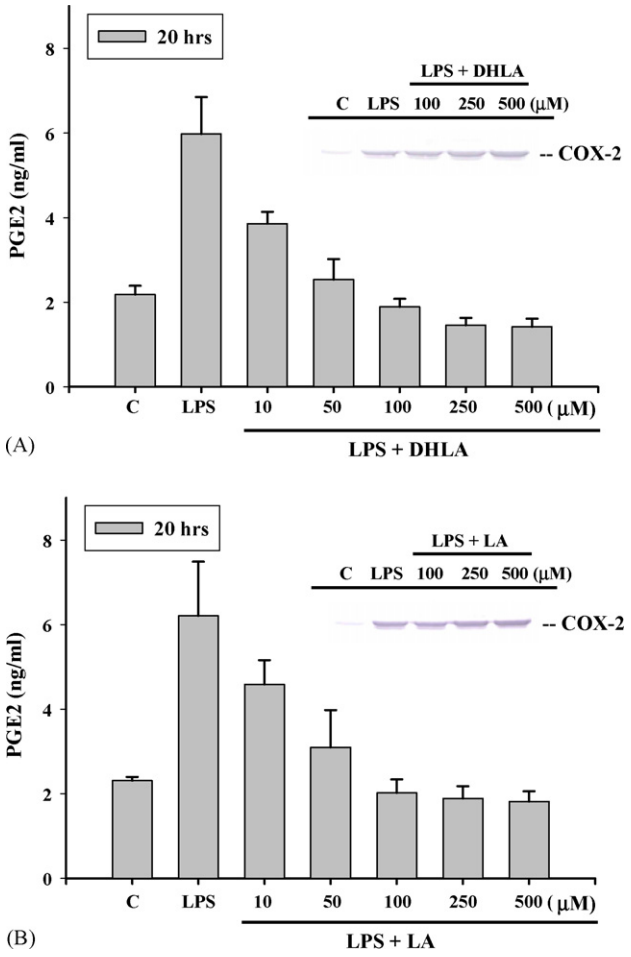


Fig. 3 – Effects of DHLA and LA on LPS-induced COX-2 protein levels and PGE_2 production in RAW 264.7 cells. The cells were treated with (A) DHLA and (B) LA for 20 h. At the end of the incubation period, 100 μl of the culture medium was collected for PGE_2 assay. Equal amounts of total proteins (50 μg) were subjected to 10% SDS-PAGE. The expression of COX-2 protein was detected by Western blot using a specific antibody. The values are expressed as means ± S.E. of triplicate tests.

3.3. *Anti-inflammatory and antioxidative activities of DHLA in mouse skin*

We examined whether DHLA inhibits the priming and/or activation stages using the double-TPA-application model. As shown in Table 1, double TPA applications, at a dose of 8.1 nmol each with a 24-h interval, led to marked edema formation (76.9 ± 9.7 mg/punch in group 2) as compared with the control (44.3 ± 3.6 mg/punch in group 1; $P < 0.05$ *versus* group 2). Pretreatment in the priming phase (group 3) was found to be more effective for edema suppression than in the activation phase (group 4; $P < 0.05$). Double pretreatment with DHLA 30 min prior to each TPA application suppressed edema formation completely (group 5; $P < 0.05$). In accordance with the TPA-induced enhancement of edema formation, an increase in epidermal thickness was observed with double TPA applications (Table 1 and Fig. 4A and B; 18.6 ± 3.8 μm in group 1 *versus* 31.0 ± 4.9 μm in group 2; $P < 0.05$). Pretreatment with DHLA in the priming stage was also more suppressive than that in the activation stage (Table 1 and Fig. 4). Double DHLA pretreatment showed the highest inhibition. In addition, the double TPA application also dramatically increased the level of H_2O_2 4.15-fold in the mouse epidermis and dermis (Table 1; 1.17 ± 0.24 nmol/punch in group 1 *versus* 4.86 ± 0.32 nmol/punch in group 2; $P < 0.05$). A higher inhibition by DHLA use was again observed in the priming stage (IR, 50.9% in group 3) than in the activation phase (IR 32.8% in group 4). Double pretreatment with DHLA showed the highest inhibition of H_2O_2 production among the three inhibitory groups (IR, 65.6% in group 3).

3.4. *Antitumor-promoting activity of DHLA/LA in mouse skin*

We then examined the inhibitory effects of a topical application of DHLA, at a dose range of 300–4050 nmol, on tumor formation in DMBA (0.3 μmol)-initiated and TPA (2.0 nmol)-promoted mouse skin. As shown in Table 2, with DMBA and TPA treatment, the tumor incidence in this positive control group was 70% 20 weeks after promotion. In contrast, administration of DMBA followed by repeated applications of acetone produced no tumors. When DHLA (4050 nmol) was pretreated before the initiator DMBA, the tumor incidence was completely inhibited. In the other three DHLA-pretreated

Table 1 – Inhibitory effects of DHLA on TPA-induced edema, epidermal hyperplasia and H_2O_2 in mouse skin

Group	Edema (mg/punch)	Inhibitory Rate (%)	Hyperplasia (μm)	Inhibitory Rate (%)	H_2O_2 (nmol/punch)	Inhibitory Rate (%)
1. Ac/Ac → Ac/Ac[a]	44.3 ± 3.6		18.6 ± 3.8		1.17 ± 0.24	
2. Ac/TPA → Ac/TPA[b]	76.7 ± 9.7[#]		31.0 ± 4.9[#]		4.86 ± 0.32[#]	
3. DHLA/TPA → Ac/TPA	54.6 ± 8.2[*]	68.2	24.0 ± 3.4[*]	56.5	2.98 ± 0.32[*]	50.9
4. Ac/TPA → DHLA/TPA	67.8 ± 12.0	27.2	26.1 ± 5.3[*]	3.65	3.65 ± 0.28[*]	32.8
5. DHLA/TPA → DHLA/TPA	45.4 ± 0.9[*]	96.7	22.8 ± 4.0[*]	66.1	2.44 ± 0.41[*]	65.6

Results are expressed as the Mean ± S.E. for three independent experiments.
[a] Solvent control (Acetone).
[b] Positive control (TPA).
[*] $P < 0.05$ versus positive control (TPA alone) in the Student's *t* test.
[#] $P < 0.05$ versus solvent control.

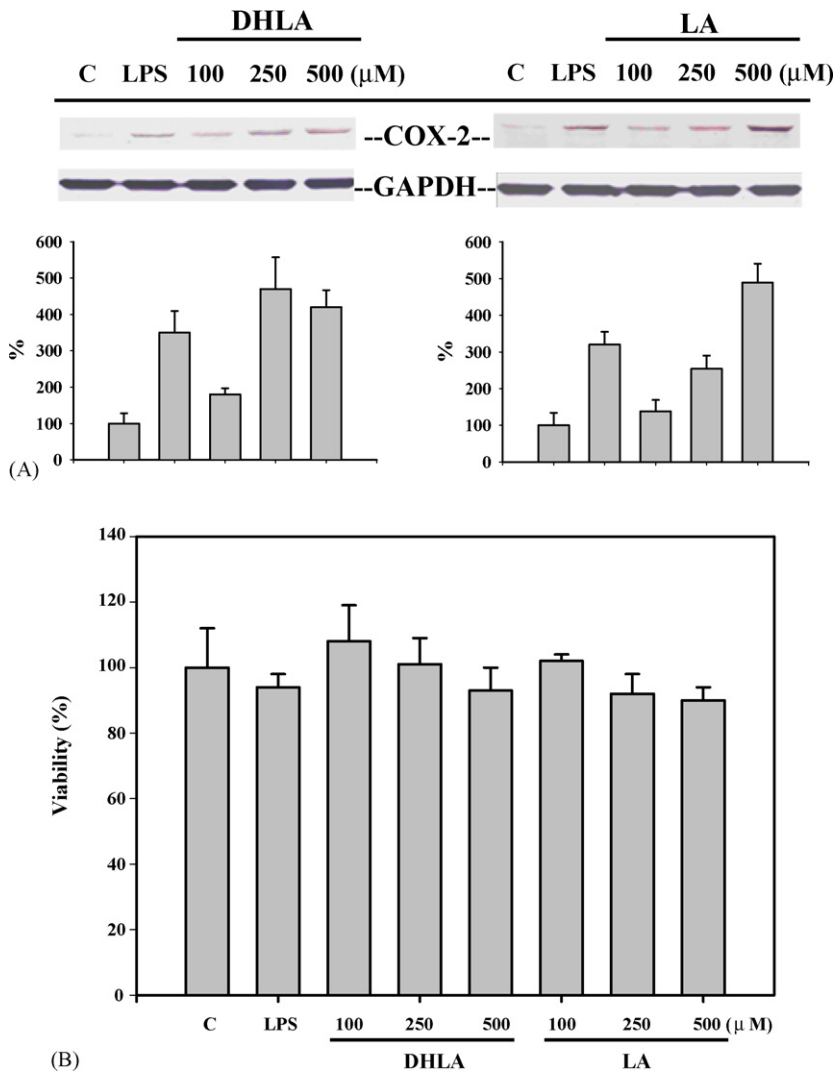


Fig. 4 – Effects of DHLA and LA on COX-2 protein expression in RAW 264.7 cells. (A) The cells were treated with various concentrations of DHLA and LA for 20 h. Equal amounts of total proteins (50 μg) were subjected to 10% SDS-PAGE. The expression of COX-2 and GAPDH protein was detected by Western blot using specific antibodies. Quantification of COX-2 protein expression was performed by densitometric analysis of the immunoblot. These experiments were repeated three times with similar results. (B) Cytotoxic effects of DHLA and LA in RAW 264.7 cells. Cytotoxicity was estimated by trypan blue exclusion using a hemocytometer chamber. The values are expressed as means ± S.E. of triplicate tests.

Table 2 – Anti-tumor promotion potency of DHLA/LA in DMBA-initiated TPA-promoted ICR mice

Group	Number of mice	% of mice with tumors	Tumors per mouse
Acetone[a]	10	0	0
TPA[b]	10	70[#]	3.7 ± 0.32[#]
DHLA (4050)/DMBA	10	0	0
DHLA (300)/TPA	10	60[*]	2.1 ± 0.15[*]
DHLA (900)/TPA	10	50[*]	1.4 ± 0.08[*]
DHLA (2700)/TPA	10	10[*]	0.3 ± 0.01[*]
LA (0.2%, diet)/TPA	10	80	4.4 ± 1.47

Tumor formation in all mice was initiated with DMBA (300 nmol) and promoted with TPA (2.0 nmol) twice weekly, starting 1 week after initiation.

Results are expressed as the Mean ± S.E. for the indicated number of independently performed experiments.

[a] Solvent control (Acetone).

[b] Positive control (TPA).

[*] $P < 0.05$ versus positive control (TPA alone) in the Student's t test.

[#] $P < 0.05$ versus solvent control.

groups (30 min before TPA), the incidence was reduced by 10–60%. The average number of tumors per mouse in the control was 3.7, and pretreatment with DHLA at 300–2700 nmol, dose-dependently, reduced the number of tumors by 43.2, 62.2 and 91.9%, respectively. Unfortunately, we did not observe any LA antitumor-promoting activities in this model, when supplemented by 0.2% in the diet.

3.5. *Inhibitory effects of DHLA on TPA-induced iNOS and COX-2 expression in mouse skin*

In order to examine whether DHLA can inhibit iNOS and COX-2 expression in TPA-treated mouse skin, we topically applied the DHLA 30 min prior to TPA treatment. Upon treatment with TPA for 4 h, the expression of iNOS was markedly suppressed by DHLA pretreatment (Fig. 6A). Interestingly, DHLA weakly suppressed the expression of COX-2 protein induced by TPA-induced COX expression. To verify whether DHLA caused a concentration-dependent reduction of PGE_2 levels not accompanied with the reduction of COX-2 expression in LPS-induced RAW 264.7 cells, the effect of DHLA/LA on the activity of purified of COX-2 enzyme was directly assessed. As shown in Fig. 6B, DHLA (2.7 μM) and LA exhibited a significant inhibitory effect on COX-2 activity. The data indicated that DHLA was markedly more active than LA.

4. Discussion

In the present study, we demonstrated that both DHLA and LA treatment inhibited the expression of iNOS and NO synthesis in mouse macrophages (RAW 264.7 cells) activated with LPS. LA has recently been suggested as a regulator of iNOS in RAW 264.7 macrophages [32,33]. The inhibitory effects on iNOS expression and NO synthesis are mediated, in part, through the NF-κB signaling pathway [32]. In addition, previous work using RAW 264.7 cells has identified NF-κB enhancer elements in the iNOS promoter that permit iNOS induction by cytokines and LPS [34]. Thus, LA interacts with a crucial mediator in inflammation and cancer [35]. This potency is most likely linked to the antioxidative properties of LA. The activation of NF-κB is commonly seen as an indicator for oxidative stress,

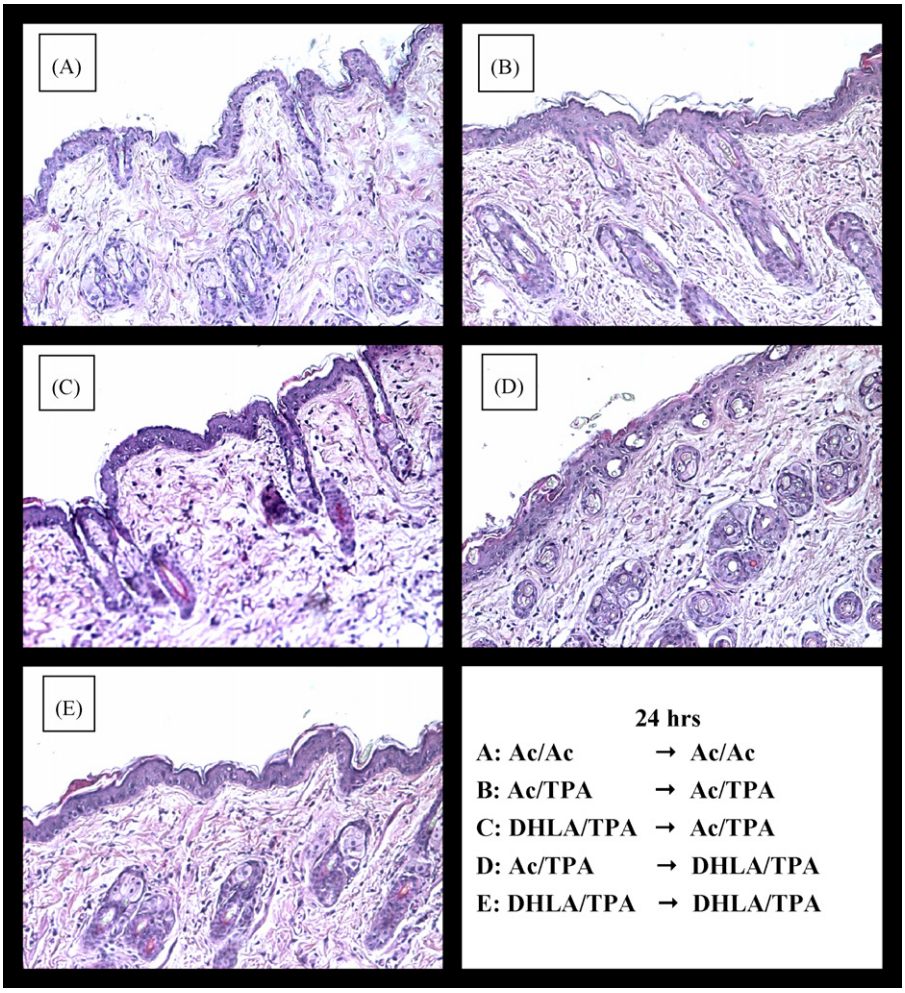


Fig. 5 – Suppression by DHLA of TPA-induced skin morphological changes, observed by hematoxylin and eosin staining. DHLA (4050 nmol in 100 μl acetone) was topically applied to the shaved area of the dorsal skin 30 min before application of a TPA solution (8.1 nmol in 100 μl acetone). After 24 h, the same doses of DHLA or acetone were applied 30 min prior to a second TPA application. Methods for histological staining are described in Section 2. Ac: acetone. Original magnification: (A–E) 100×.

and antioxidants are well-described inhibitors of NF-κB activation [5,36,37]. DHLA is a more potent antioxidant than LA. We demonstrated, for the first time, the higher inhibitory effect of DHLA than LA on the induction of iNOS and NO in RAW 264.7 cells stimulated by LPS; this has never been reported before. It is important to note that LA not only attenuates NO production *in vitro* in cell cultures but also *in vivo*. LA was demonstrated to protect from hyperdynamic circulation in biliary cirrhosis, which was related to an LA-mediated decrease in bile duct ligation-induced NO formation [38].

COX-2 overexpression has been reported to be associated with carcinogenesis, tumor growth, and metastasis. The contribution of COX-2 to these processes has been attributed to the COX-2-mediated production of PGs, with the subsequent conversion of procarcinogens to carcinogens, inhibition of apoptosis, promotion of angiogenesis, modulation of the inflammation and immune function, and increased tumor cell invasiveness [39]. Therefore, PGE_2 production caused by the increased expression or activity of COX-2 in macrophages after treatment with LPS or proinflammatory cytokines might increase the risk of tumorigenesis. LPS and proinflammatory cytokines promote PGE_2 production by increasing COX-2 expression in a manner dependent on NF-κB activation [40,41]. NF-κB has been proposed to be a redox-sensitive transcription factor [42], and LA inhibited LPS-induced NF-κB by inhibiting the activities of IκB kinase [32]. However, in this study, DHLA and LA (10–500 μM) inhibited PGE_2 synthesis by LPS without affecting the protein expression level of COX-2 in RAW 264.7cells (Fig. 3). Furthermore, treatment of RAW 264.7 cells with high doses of DHLA/LA (250–500 μM) alone increased the COX-2 protein expression (Fig. 4). Thus, it is unlikely that DHLA/LA affects the LPS-induced signaling pathways, including NF-κB activation, required for the increases in enzymes involved in PGE_2 biosynthesis.

As summarized in Table 1, DHLA inhibited double-TPA-application-induced biological and histological parameters relating to oxidative damage and inflammation. DHLA significantly suppressed all of the parameters in both the priming and the activation stages. The inhibitory effects of the double DHLA pretreatment appear to be caused by the additive effects of each application in both stages, because the double pretreatments were most suppressive. TPA has recently been reported to produce NO and VEGF in human polymorphonuclear leukocytes [14,43,44]. Overexpressed VEGF leads to the induction of vascular hyperpermeability [13]. On the other hand, PGE_2 is well known to increase vascular permeability. DHLA inhibited the release of PGE_2 from RAW 264.7 cells (Fig. 3). Although we have not examined the effects of DHLA on vascular permeability, the inhibition by DHLA of edema formation and the reduction of epidermal thickness (Table 1 and Fig. 5) may be partly attributable to the suppression of NO, VEGF, and PGE_2 production. Double-TPA-application-induced H_2O_2 production *in vivo* was markedly inhibited by the pretreatment(s) of DHLA. Decreased levels of H_2O_2 may be attributable to the inhibition of O_2^- generation because H_2O_2 is mostly derived from O_2^- as a function of O_2^- dismutase or nonenzymatically. DHLA, a dual inhibitor of both O_2^- and NO radical generation, can be recognized as a potent, naturally occurring anti-inflammatory agent, because peroxynitrite, a coupling product of O_2^- and NO, enhances COX-2 activity [45], involving inflammatory processes and thereby leading to carcinogenesis. Besides, iNOS and COX-2 have also been reported to contribute to tumor growth. Co-expression of iNOS and COX-2 has been observed in malignancies [46]. In our study, inhibition of iNOS by DHLA attenuated TPA-induced tumor promotion, but weakly inhibited COX-2 expression in mouse skin. To our knowledge this is the first report in which DHLA strongly and directly inhibited COX-2 activity more than the non-steroidal anti-inflammatory drug (NSAID), ibuprofen (Fig. 6). We suggest that the anti-tumor promotional effect of DHLA may have resulted from the suppression of iNOS expression, the reduced NO production, and the directly inhibited COX-2 enzyme activity.

Early studies of the skin during chemically induced carcinogenesis have already pointed to the important role of the interaction between inflammatory cells and epithelia cells in tumor initiation and progression [47]. During the early stages of skin tumor development, mast cells, macrophages and neutrophils are recruited to the activated stroma. This inflammatory cell recruitment is highly similar to the initial inflammatory reaction during wound healing. With malignant

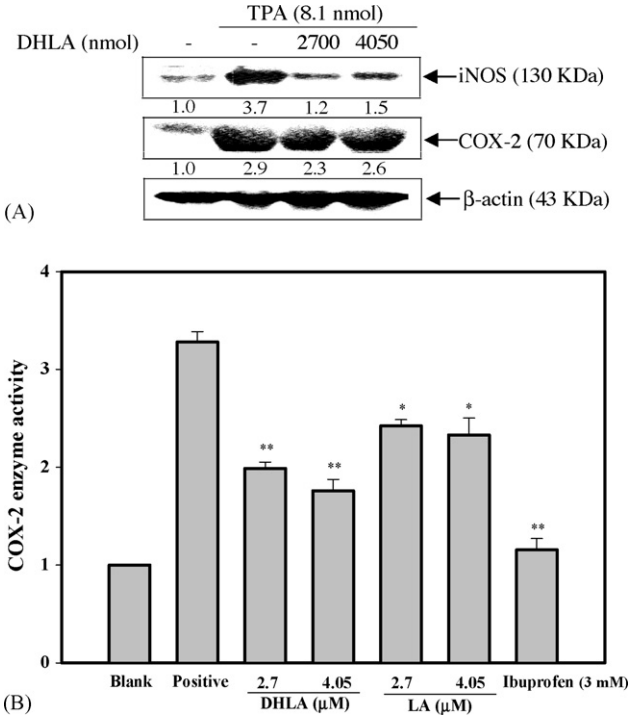


Fig. 6 – Inhibitory effects of DHLA on TPA-induced iNOS and COX-2 expression (A) and the effects of DHLA/LA on the activity of purified COX-2 enzyme (B). Female ICR mice were treated topically with 100 μl acetone or DHLA in the same volume of acetone 30 min prior to 8.1 nmol TPA, and the animals were killed 4 h after TPA treatment. Protein was analyzed for iNOS and COX-2 by immunoblotting. The Western blot was representative of two independent experiments. Quantification of iNOS and COX-2 expression was normalized to actin using a densitometer. (B) The values are expressed as means ± S.E. of triplicate tests, indicating statistically significant differences from the positive group ($^*P < 0.05$ and $^{**}P < 0.01$; Student's *t*-test). Ibuprofen is a COX inhibitor.

progression induced by the expression of granulocyte–monocyte stimulating factor (GM-CSF) in the tumor cells, macrophage recruitment is earlier and enhanced, and the macrophages then invade the tumour epithelium. Neutrophils might contribute to a tumor-promoting inflammatory infiltrate by modulating the phenotype of the macrophages in the tumor vicinity via the secretion of cytokines or growth factors. Therefore, the studies suggest that the tumor-promoting micro-environment is indispensable for tumor formation and progression, and that mast cells might have an important role in the initial stages of tumor development, while the interaction of granulocytes and macrophages might be necessary for progression to a malignant tumor phenotype [48]. We suggest that DHLA can block the activation and infiltration of macrophages into the skin tumor.

Most clinical tumors have a long history of pathological development during which they pass through several preneoplastic and pre-malignant stages before becoming malignant. This situation offers the opportunity to interrupt or reverse tumor development at a still harmless stage, for instance by chemoprevention, i.e., by taking medicines acting on the distinct molecular processes of tumorigenesis [49]. In recent years, considerable emphasis has been placed on identifying new cancer chemopreventive agents which may be useful in human populations [50]. As predicted by the suppressive efficacies of biochemical markers relating to oxidative stress and inflammation, topical application of DHLA at doses of 300–2700 nmol inhibited the incidence and multiplicity of skin tumors in a dose-dependent manner (Table 2). We also found a completely inhibitory effect of DHLA on tumor incidence when applied before the initiator DMBA in this two-stage mouse skin tumorigenesis model. In a previous study, LA was consumed in the daily diet, taken up and transformed into DHLA in cells and tissues. To examine the possibility, we compared the effect of 0.2% LA in a dietary supplement and DHLA topically applied to a shaved area of the dorsal skin. Unfortunately, we did not observe any antitumor-promoting activities of LA in this model, when supplemented in the diet (Table 2). We suggested that the local concentration of DHLA, transformed from 0.2% LA in the diet, is lower than DHLA directly applied to skin. However, the detailed mechanisms are still unclear and need to be further investigated. In any case, this is the first report demonstrating the chemopreventive ability of DHLA in an animal model.

In summary, the results presented here demonstrate that LPS-induced NO and PGE_2 generation in macrophages can be inhibited by DHLA and LA. Searching for NO and PGE_2 inhibitors in this model may be a promising strategy for discovering effective chemopreventive agents. DHLA/LA was found to be a functionally novel antitumor promoter by working in both the priming and activation stages in mouse skin. Our results also indicate the possibility that DHLA/LA could have beneficial effects on preventing several other diseases mediated by NO and PGE_2 overproduction.

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