BJP British Journal of Pharmacology

DOI:10.1111/bph.13137
www.brjpharmacol.org

RESEARCH PAPER

Effective suppression of pro-inflammatory molecules by DHCA via IKK-NF-κB pathway, *in vitro* and *in vivo*

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Received
4 September 2014
Revised
27 January 2015
Accepted
27 February 2015

BACKGROUND AND PURPOSE

Dehydrodiconiferyl alcohol (DHCA), a lignan compound isolated from *Cucurbita moschata*, has previously been shown to contain anti-adipogenic and antilipogenic effects on 3T3-L1 cells and mouse embryonic fibroblasts. As some of phytochemicals derived from natural plants show anti-inflammatory or antioxidative activities, we determined whether DHCA affects the production of pro-inflammatory mediators and also investigated its underlying mechanisms.

EXPERIMENTAL APPROACH

Raw264.7, a murine macrophage cell line, and primary murine macrophages derived from bone marrow cells were treated with LPS in the presence of DHCA. Furthermore, cells were treated with LPS and palmitate in the presence of DHCA to examine its effect on inflammasomes. The production of various pro-inflammatory mediators was examined and the underlying mechanisms investigated using a variety of molecular biological techniques. To test whether DHCA exhibits anti-inflammatory effects *in vivo*, mouse dextran sodium sulfate (DSS)-induced colitis model was used.

KEY RESULTS

DHCA reduced the production of pro-inflammatory cytokines (TNF-α, IL-6, IL-1β and CCL2) and mediators (iNOS, COX-2 and ROS) by down-regulating the activity of I-κB kinase and, subsequently, the DNA binding activity of NF-κB. Moreover, DHCA effectively suppressed the palmitate-mediated activation of inflammasomes, which resulted in decreased production of IL-1β. DHCA also showed therapeutic effects in the mouse DSS-induced colitis model by suppressing the production of TNF-α and IL-1β and thus preventing weight loss and colon shrinkage.

CONCLUSIONS AND IMPLICATIONS

Our data suggest that DHCA is a novel phytochemical that by regulating key molecules involved in inflammation and oxidative stress might exert a broad range of anti-inflammatory activities.

Abbreviations

DHCA, dehydrodiconiferyl alcohol; DSS, dextran sodium sulfate; IKK, I-κB kinase; ROS, reactive oxygen species

Tables of Links

TARGETS	
Catalytic receptors[a]	**Enzymes**[b]
CD11b (integrin, alphaM subunit)	Caspase-1
Nod-like receptors (NLRs)	COX-2
NLRP3	ERK
Toll-like receptors (TLRs)	IKKα
TLR4	IKKβ
Other protein targets	Inducible NOS (iNOS)
CREB	JNK
IL-1β	MEKK1
TNF-α	NIK
	p38
	TAK1

LIGANDS
ATP
CCL2
IL-6
LPS
NO
Palmitate
PGE_2

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 ([a,b]Alexander *et al.*, 2013a,b).

Introduction

Inflammation is one of the primary defence mechanisms involved in innate immunity, playing a key role(s) in pathogen clearance and wound healing (Medzhitov and Horng, 2009; Chen and Nunez, 2010). The invasion of pathogens causes a series of cellular immune responses, in which macrophages are critically involved. Macrophages directly recognize pathogens through pattern recognition receptors, such as toll-like receptors (TLRs) or Nod-like receptors (NLRs), and ingest pathogens by phagocytosis. They also produce various pro-inflammatory mediators including TNF-α, IL-6, IL-1β, CCL2, NO and PGE_2. These mediators play an essential role in not only strengthening the innate immune response but also in activating other immune cells, such as dendritic cells, lymphocytes or granulocytes to promote a secondary immune response (Chen and Nunez, 2010).

LPS is a component of the cell walls of gram-negative bacteria that activates macrophages via TLR4 to initiate various pro-inflammatory responses. When recognized by TLR4, signal transduction pathways such as MAPK or I-κB kinase (IKK) activate pro-inflammatory transcription factors including NF-κB, activator protein 1 (AP-1) and cAMP response element-binding protein (Akira and Takeda, 2004; Oeckinghaus *et al.*, 2011). These transcription factors are critically involved in the expression of various pro-inflammatory genes such as TNF-α, IL-6, IL-1β, CCL2, inducible NOS (iNOS) and COX-2 (Caivano and Cohen, 2000; Kleinert *et al.*, 2004).

The production of pro-inflammatory mediators needs to be tightly regulated as excessive amounts of these molecules result in a chronic inflammatory state, which severely damages tissue and leads to the initiation of various inflammatory diseases, including inflammatory bowel diseases, metabolic diseases and arthritis (Feldmann *et al.*, 1996; Papadakis and Targan, 2000; Osborn and Olefsky, 2012). Thus, agents that are effective at controlling these pro-inflammatory mediators are considered to be promising therapeutics for these diseases (Pizarro and Cominelli, 2007). *Cucurbita moschata* is one of the most widely cultivated vegetables worldwide and extracts from its seeds and fruits have been shown to evoke various biological effects, such as hepatocyte protection (Makni *et al.*, 2008), glucose-lowering effects (Jiang and Du, 2011), anticancer activity (Zhang *et al.*, 2012) and have the ability to ameliorate fatigue (Wang *et al.*, 2012).

Dehydrodiconiferyl alcohol (DHCA), a single chemical compound isolated from the stem parts of *C. moschata* (MW : 358) has recently been identified as a lignan that has anti-adipogenic and antilipogenic effects (Lee *et al.*, 2012). A variety of phytochemicals including polyphenols or lignans have previously been reported to contain anti-inflammatory activity, largely mediated by inhibiting the DNA binding activity of NF-κB (Jobin *et al.*, 1999; Manna *et al.*, 2000). In this study, the effect of DHCA on the production of various pro-inflammatory mediators was investigated using LPS-stimulated Raw264.7 cells. Our data suggest that DHCA reduces the LPS-evoked expression of pro-inflammatory genes by lowering the activity of IKK, subsequently the DNA binding activity of NF-κB. Moreover, DHCA efficiently decreased the palmitate-mediated secretion of IL-1β by suppressing the transcription of IL-1β and the activation of inflammasome.

Consistent with these *in vitro* data, in the mouse dextran sodium sulfate (DSS)-induced colitis model, DHCA reduced the production of pro-inflammatory cytokines, protecting animals from weight loss and colon shrinkage. Taken together, our data suggest that DHCA might induce anti-inflammatory effects by controlling the activities of key factors involved in inflammation and, consequently, down-regulating the expression of pro-inflammatory molecules.

Methods

Cell culture and reagents

The synthetic DHCA was obtained from Biochemnet (Seoul, Korea) by previously described methods (Hu and Jeong, 2006). Other experimental reagents purchased were as follows: Raw264.7 cell line from the American Type Culture Collection (Manassas, VA, USA); LPS (*Escherichia coli*, 0111:B4), palmitate and BSA from Sigma (St. Louis, MO, USA); macrophage colony-stimulating factor (M-CSF) from Prospec (East Brunswick, NJ, USA) and DSS salt (MW 36 000–50 000) from MPBiomedicals (Santa Ana, CA, USA). Cells were cultured in DMEM containing 5% FBS (Hyclone, Logan, UT, USA), 100 U·mL^{-1} penicillin and 100 μg·mL^{-1} streptomycin.

Experimental animals

All animal protocols were performed in compliance with the guidelines set by the Institutional Animal Care and Use Committee of Seoul National University. Male 7-week-old C57BL/6 mice were purchased from Orientbio Inc. (Seongnam, Korea) and housed in an air-conditioned facility at Seoul National University with a fixed 12 h light/dark cycle. The total number of animals used in this study was 117; 9 for the *in vitro* BMDM culture and 108 for the DSS-induced mouse model. All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Preparation and differentiation of primary bone marrow-derived macrophages (BMDMs)

Bone marrow cells were prepared and differentiated into macrophages using M-CSF as described previously (Zhang *et al.*, 2008). Briefly, femurs from male C57BL/J mice were prepared and the bone marrow cavity of each femur was flushed out using a 25 g syringe with 5 mL of Dulbecco's PBS (DPBS) without calcium and magnesium. Cells were collected by centrifugation at 453× *g* for 3 min and red blood cells were removed using red blood cell lysis buffer (Sigma). Cells were then washed twice with DPBS and cultured in sterile plastic Petri dishes in DMEM/F12 (Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Hyclone), penicillin-streptomycin-glutamin (Gibco, Carlsbad, CA, USA) and 20 ng·mL^{-1} of M-CSF. After 3 days, 5 mL of fresh culture media were added. On day 7, fully differentiated macrophages were washed twice with DPBS and used for experiments. The purity of BMDM was 97 to 99% as determined by FACS analysis using an antibody against CD11b (eBioscience, San Diego, CA, USA).

Measurement of cytokines, NO, PGE_2 and reactive oxygen species (ROS)

Measurement of cytokines, NO and PGE_2 was performed as previously described (Choi *et al.*, 2012). Briefly, Raw264.7 cells and BMDMs were plated 2.5 × 10^5 cells·mL^{-1} per well in 24-well culture plates. Twenty-four hours later, cells were treated with 100 ng·mL^{-1} (for TNF-α, IL-6, CCL2 and PGE_2) or 1 μg·mL^{-1} (for IL-1β) of LPS and various concentrations of DHCA for 24 or 48 h respectively. The supernatant was collected and the amount of secreted cytokines and PGE_2 was measured by specific ELISA kits (TNF-α, IL-6 and CCL2, Thermo Fisher Scientific, Woburn, MA, USA; IL-1β and PGE_2, R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions. The level of NO in the culture supernatant was measured using a modified method, as described previously (Choi *et al.*, 2012). Briefly, the level of nitrite was measured by adding 100 μL of Griess reagent to 50 μL of culture supernatant. The OD at 550 nm was measured with a microplate reader and then determined from a sodium nitrite standard curve.

To determine the production level of intracellular ROS, Raw264.7 cells treated with 100 ng·mL^{-1} LPS and various concentrations of DHCA were incubated with PBS containing 5 μM DCF-DA (Roche, Mannheim, Germany) dye for 30 min. After this incubation, the total amount of ROS was analysed by FACS, which measures the intensity of fluorescence produced by the conversion of DCF-DA to DCF.

MTT assay

The cytotoxicity of DHCA was evaluated using the Cell Proliferation Kit I (Roche, Basel, Switzerland) according to the manufacturer's instructions. Briefly, Raw264.7 cells or primary BMDMs were treated with various concentrations of DHCA for 24 h. Cells were then incubated with an MTT labelling reagent for 4 h followed by the addition of solubilization solution. After 24 h, cytotoxicity was determined by measuring the OD at 550 nm using an ELISA microplate reader.

Western blot analysis

Western blot analysis was performed as described previously (Choi *et al.*, 2012). Briefly, Raw264.7 cells were plated, 2.5 × 10^6 cells per dish, in 60 mm culture dishes. Twenty-four hours later, cells were treated with 100 ng·mL^{-1} LPS and various concentrations of DHCA. After an appropriate time, cells were washed with cold PBS and scraped into PBS. The cell pellets were collected by centrifugation at 800× *g* for 5 min and lysed using phosphosafe extraction buffer (Novagen, Madison, WI, USA). Total lysates were obtained by centrifugation at 12000× *g* for 10 min. Total protein contents in the supernatant were determined by the Bradford assay and 20 μg of proteins were subjected to SDS-PAGE. Proteins separated by SDS-PAGE were then electrophoretically transferred to a nitrocellulose membrane. Membranes were incubated with Tris-buffered saline with tween 20 containing specific antibodies at 4°C overnight as shown in Table 1. Membranes were then incubated with HRP-conjugated anti-mouse or anti-rabbit IgG (1:100 000, Sigma) and visualized in films using ECL solution (Millipore, Billerica, MA, USA).

Northern blot hybridization

Raw264.7 cells were plated, 2.5 × 10^6 cells per dish, in 60 mm culture dishes. Twenty-four hours later, cells were treated with 100 ng·mL^{-1} LPS and various concentrations of DHCA for 6 h. Total RNAs were isolated using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Twenty micrograms of RNA were resolved by electrophoresis in a 1% agarose-formaldehyde gel and transferred to a nylon membrane by overnight capillary blotting. Membranes were then hybridized with the specific ^{32}P-labelled probes overnight at

Table 1
List of antibodies used in Western blot analysis

Target protein	Reaction condition	Company
iNOS	1:4000	BD Bioscience, Franklin Lakes, NJ, USA
COX-2	1:200	BD Bioscience, Franklin Lakes, NJ, USA
Phosphorylated IKK	1:500	Cell Signaling, Beverly, MA, USA
IKK	1:500	Santa Cruz Biotechnology, Santa Cruz, CA, USA
I-κB	1:500	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Phosphorylated p38	1:1000	Cell Signaling, Beverly, MA, USA
p38	1:500	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Phosphorylated ERK	1:1000	Cell Signaling, Beverly, MA, USA
ERK	1:500	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Phosphorylated JNK	1:500	Santa Cruz Biotechnology, Santa Cruz, CA, USA
JNK	1:500	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Caspase-1	1:200	Santa Cruz Biotechnology, Santa Cruz, CA, USA
β-actin	1:100 000	Sigma, St. Louis, MO, USA

68°C, using Expresshyb solution (Clontech, Mountain View, CA, USA), and developed using autoradiography films or phosphoimager BAS-1500 (Fujifilm, Tokyo, Japan).

The same membrane was used to analyse the RNA level of TNF-α, IL-1β, IL-6, CCL2, iNOS, COX-2 and GAPDH (results shown in Figures 1B and 2C). Specific cDNA probes for mouse TNF-α, IL-1β, IL-6, CCL2, iNOS, COX-2 and GAPDH were labelled using random primer kit (Stratagene, La Jolla, CA, USA) and [α-^{32}P]-dCTP (Perkin Elmer, Norwalk, CT, USA) under the manufacturer's instruction. Primer sequences used to generate these cDNA probes were shown in Table 2.

Promoter luciferase assay

Raw264.7 cells were plated, 2.5×10^5 cells·mL^{-1} per well, in a 24-well culture plate. After 24 h, transfection was performed using Attractene reagent (Qiagen, Frederick, MD, USA) according to the manufacturer's instructions. Briefly, cells were transfected with 0.4 μg per well of plasmid DNA in the presence of 2 μL per well Attractene reagent in the serum-free condition. Three hours later, DMEM containing 10% FBS was added and incubated for another 21 h. After transfection, cells were treated with LPS and DHCA for 24 h. Total proteins were obtained using reporter lysis buffer (Promega, Madison, WI, USA) and 100 μL of luciferin were added to an aliquot of 20 μg proteins. The activity of luciferase was measured using a microplate luminometer (Berthold Technologies, Bad Wildbad, Germany) and normalized by use of a negative control.

EMSA

Raw264.7 (7.5×10^6 cells per dish) cells were plated in 100 mm culture dishes. Twenty-four hours later, cells were treated with 100 ng·mL^{-1} LPS and various concentrations of DHCA for 1 h. Cells were washed with cold PBS and harvested by centrifugation at 800× *g* for 5 min. Cell pellets were resuspended with 200 μL of lysis buffer A [10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA and 1 mM dithiothreitol) and incubated on ice for 20 min. Twenty microlitres of lysis buffer B (lysis buffer A containing 0.5% Nonidet-P40) were added for another 20 min to disrupt cell membranes, followed by centrifugation at 5000× *g* for 5 min. Crude nuclei were resuspended in 20 μL of lysis buffer C [10 mM HEPES (pH7.9), 400 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol] and incubated on ice for 1 h. The nuclear proteins were obtained by centrifugation at 16 000× *g* for 10 min. An aliquot of 10 μg of nuclear proteins was mixed with 0.01 unit poly(dI-dC), 2 μg salmon sperm DNA and 0.05 pmol ^{32}P-labelled DNA probe in EMSA binding buffer (Novagen) and incubated for 30 min on ice. The DNA-protein complex was resolved in a non-denaturing 7% polyacrylamide gel with 0.5X Tris-Borate-EDTA buffer. Gels were dried and radioactive bands were detected using autoradiography film. An oligonucleotide for NF-κB (Promega) was radiolabelled with [γ-^{32}P]-ATP (Perkin Elmer) by incubating with T4 polynucleotide kinase (Takara, Tokyo, Japan) for 30 min at 37°C and inactivated at 65°C. Specificity of the retarded complexes was confirmed by competition with 100-fold excess amount of unlabelled wild-type or mutant oligonucleotide.

Preparation and treatment of palmitate

Palmitate was purchased from Sigma and prepared as previously described (Wen *et al.*, 2011). Briefly, 20 mg of palmitate were dissolved in 830 μL of 95% ethanol overnight at 65°C. Six hundred microlitres of palmitate solution were then mixed with 3 mL of culture media containing 0.108 g of fatty acid-free BSA to yield a final concentration of 15 mM palmitate and 3% BSA.

Mouse DSS-induced colitis model

Colitis was induced in 7-week-old male C57BL/6 mice by the administration of 3.5% DSS dissolved in drinking water. The

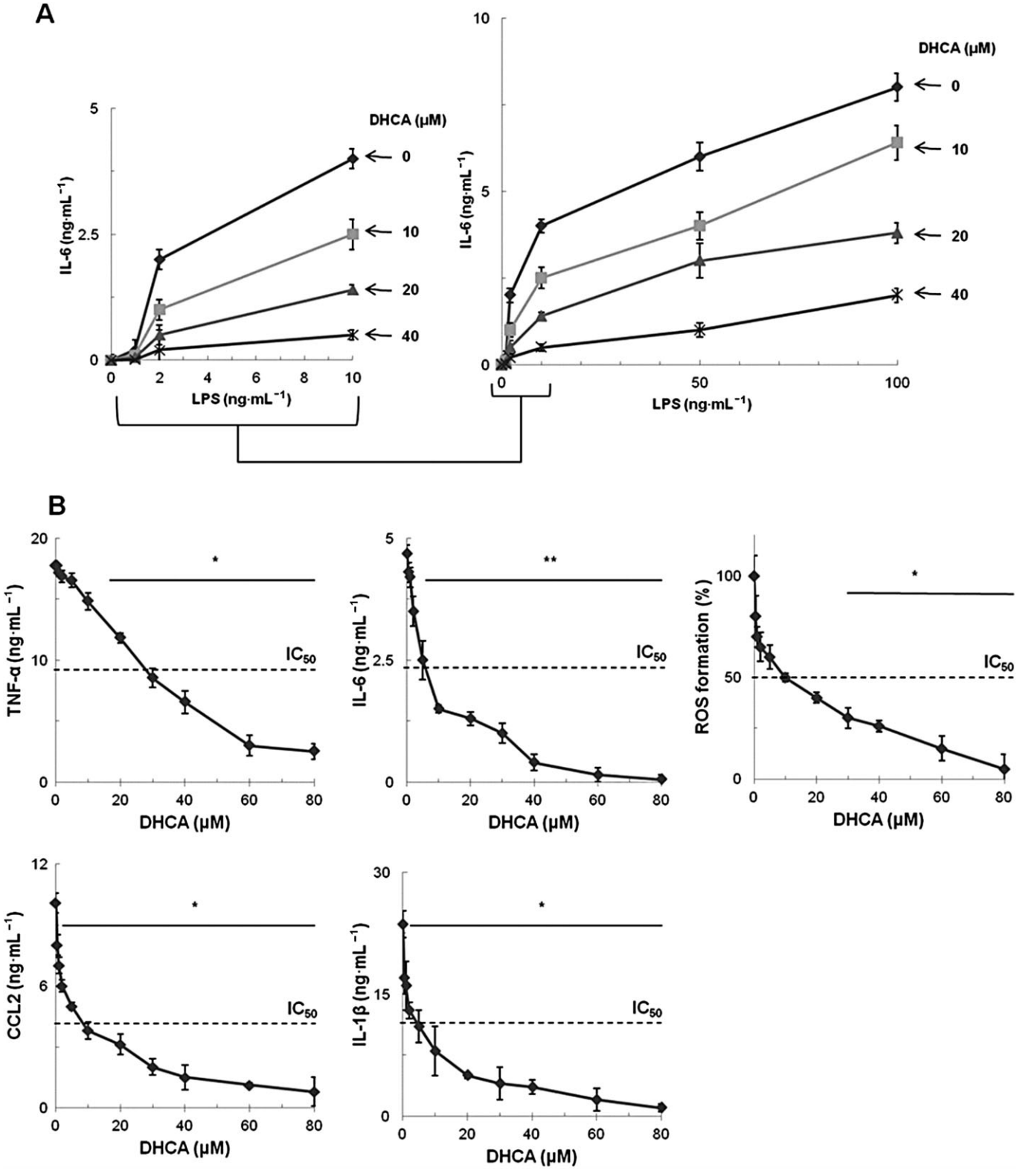

Figure 1

Effects of DHCA on the production of pro-inflammatory cytokines and ROS in Raw264.7 cells. (A) Effect of LPS concentration on the DHCA-mediated inhibition of IL-6. Raw264.7 cells were treated with various concentrations of LPS in the presence of 10, 20 and 40 μM DHCA respectively. The culture supernatants were collected followed by IL-6-specific ELISA. Values represent the mean ± SD of triplicate samples. (B) Effect of DHCA on the protein level of cytokines. To measure the protein level of various cytokines, Raw264.7 cells were treated as described in the Methods section. The culture supernatants were collected followed by a respective ELISA. Values represent the mean ± SD of triplicate samples.

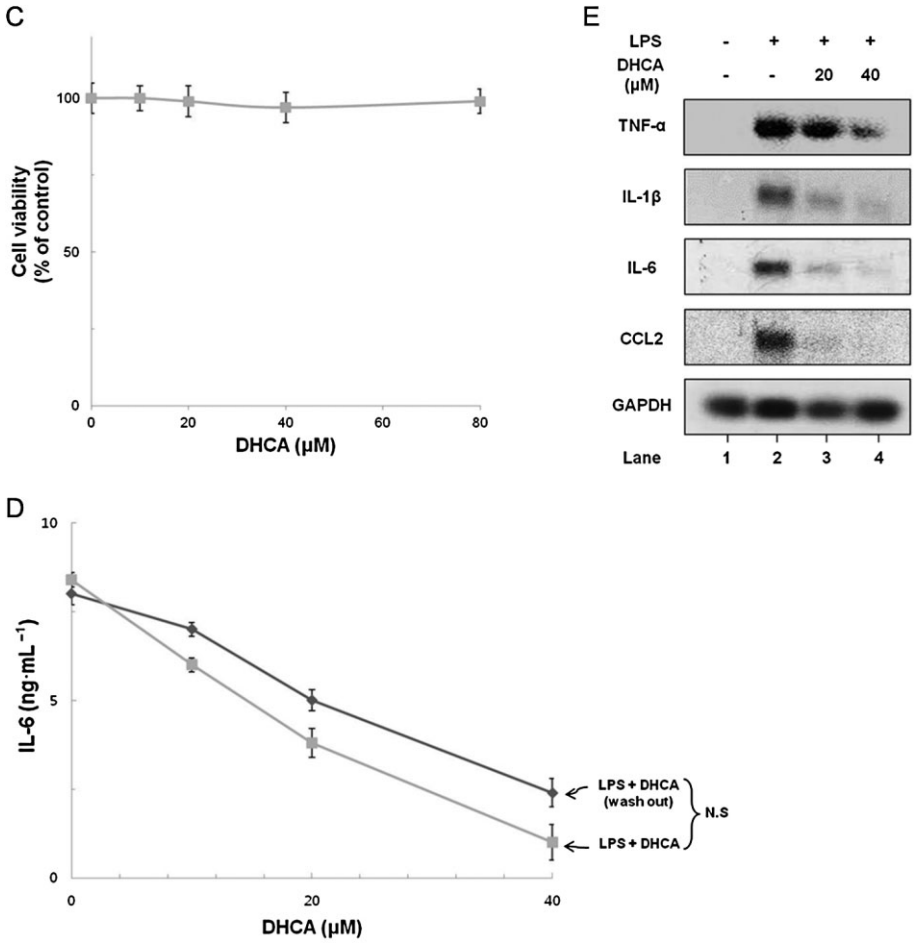

Figure 1

Continued

(C) Cytotoxic effect of DHCA. Raw264.7 cells were treated with various concentrations of DHCA for 24 h. Cells were then subjected to MTT assay as described in the Methods section. (D) Effect of DHCA on the production of IL-6. Raw264.7 cells were treated with DHCA in two different manners. Various concentrations of DHCA were added after 6 h of LPS stimulation and wash out (LPS + DHCA wash out) or simultaneously (LPS + DHCA). The protein level of IL-6 in the culture supernatant was measured using an ELISA. (E) Effect of DHCA on the RNA level. Total RNAs isolated from Raw264.7 cells treated with LPS and DHCA were subjected to Northern blot hybridization using ^{32}P-labelled gene specific probes. After hybridization, specific bands were visualized using BAS-1500 (for TNF-α and CCL2) or on films (for IL-1β, IL-6 and GAPDH). GAPDH mRNA was measured as a loading control and three independent experiments were performed. For Figure 1A–E, values represent the mean ± SD of triplicate samples. *$P < 0.05$, **$P < 0.01$.

experimental groups were divided into four groups (Naïve, 3.5% DSS-Vehicle, 3.5% DSS-100 mg·kg^{-1} DHCA and 3.5% DSS-300 mg·kg^{-1} DHCA). DHCA was injected daily, i.p., into mice at a dosage of 100 mg·kg^{-1} and 300 mg·kg^{-1}, respectively, and the body weight of the mice was measured once a day. After 7 days, the mice were killed and the colon was excised between the ileocaecal junction and the proximal rectum. Colons were then placed on a glass plate, pictures of representative colons were taken and the colon length was measured.

To examine the production level of TNF-α and IL-1β, colons were washed with cold PBS and their weight was measured. Colons were then homogenized with the RIPA buffer containing protease inhibitor cocktail (Roche, Mannheim, Germany) followed by centrifugation at 12 000× *g* for 15 min. The supernatant of tissue homogenates was collected and subjected to ELISAs to measure TNF-α (Thermo Fisher Scientific) or IL-1β (R&D System). The mouse DSS-induced colitis model experiment and subsequent analyses were performed more than twice.

Haematoxylin and eosin (H&E) staining and histological score assessment

DSS-induced mice were killed on day 7. For H&E staining, mouse colons were isolated and fixed in 10% neutral buffered formalin solution. Fixed tissues were embedded in paraffin, sectioned into 4 μm thick slices and stained with H&E. Histological score assessment was performed as described previously (Laroui *et al.*, 2012). The severity of inflammation, crypt damage and ulceration were scored according to the criteria shown in Table 3.

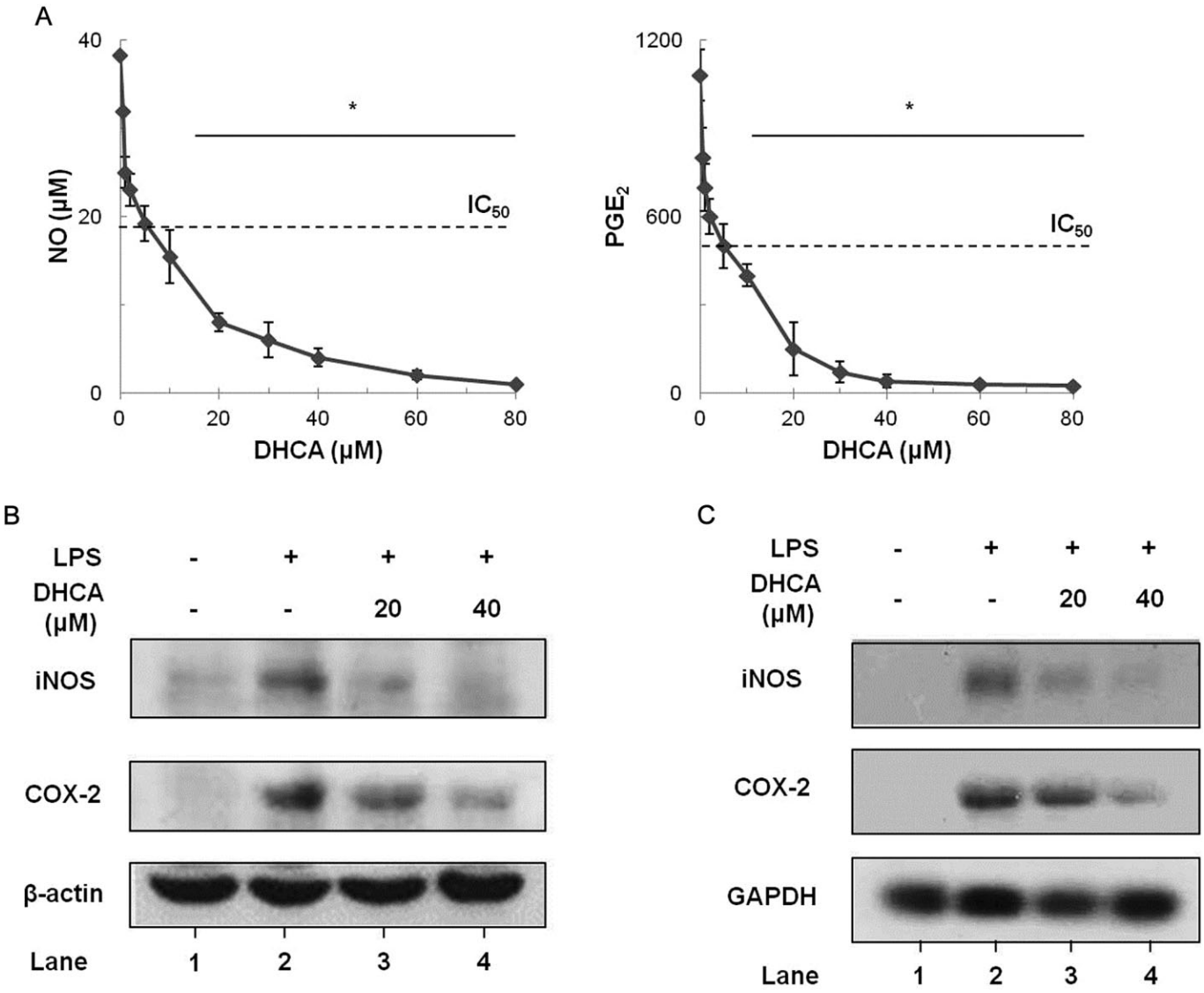

Figure 2

Effects of DHCA on the production of NO, PGE_2, iNOS and COX-2. (A) Effect of DHCA on NO and PGE_2. To measure the level of NO and PGE_2, the culture supernatant used in Figure 1A was analysed using the Griess assay and ELISA respectively. Values represent the mean ± SD of triplicate samples. *$P < 0.05$. (B) Effect of DHCA on the protein levels of iNOS and COX-2. An aliquot of total cell lysate prepared from Raw264.7 cells treated with LPS and DHCA for 24 h was subjected to Western blot using specific antibodies against iNOS, COX-2 and β-actin. β-actin protein was used as a loading control. (C) Effect of DHCA on the RNA levels of iNOS and COX-2. The membrane used to examine the RNA levels of cytokines in Figure 1B was hybridized with a specific ^{32}P-labelled probe for iNOS or COX-2 and specific bands were visualized by exposing the blot on films. The RNA level of GAPDH measured in Figure 1B was used as a loading control. Three independent experiments were performed for Figure 2B and C.

Statistical analysis

All individual experiments were repeated at least three times. All values are presented as the mean ± SD of triplicate samples and differences between two groups were statistically analysed using Student's *t*-test. *P*-values were calculated and when less than 0.05 were considered to be statistically significant. In animal studies, values are presented as the mean ± SEM and differences among the experimental groups were analysed using a two-way ANOVA with Bonferroni *post hoc* test. *P*-values were calculated and when less than 0.05 were considered to be statistically significant.

Results

Effects of DHCA on the production of pro-inflammatory cytokines and ROS

DHCA, a lignan isolated from *C. moschata*, was previously shown to have anti-adipogenic and antilipogenic activities in 3T3-L1 cells and primary mouse embryonic fibroblasts (Lee *et al.*, 2012). Because some lignan compounds were reported to contain anti-inflammatory or antioxidative activities (Jobin *et al.*, 1999; Manna *et al.*, 2000; Leonard *et al.*, 2003),

Table 2

List of primer sequences used in RT-qPCR experiment

Target gene	Primer sequence Forward	Reverse
TNF-α	5′- TGG CCT CCC TCT CAT CAG TTC TAT G – 3′	5′ – GTC TAA GTA CTT GGG CAG ATT GAC C – 3′
IL-1β	5′ – AAA TCT CGC AGC AGC ACA TCA A – 3′	5′ – TCT TCT TCT TTG GGT ATT GCT T – 3′
IL-6	5′ – CAT GTT CTC TGG GAA ATC GTG G – 3′	5′ – AAC GCA CTA GGT TTG CCG AGT A – 3′
MCP-1 (CCL2)	5′ – CAG CCA GAT GCA GTT AAC G – 3′	5′ – GAA GTG CTT GAG GTG GTT GTG – 3′
iNOS	5′ – ACC CGT CCA CAG TAT GT – 3′	5′ – TGT TGG TGG CAT AAA GTA TGT – 3′
COX-2	5′ – CCC GGA CTG GAT TCT ATG – 3′	5′ – AAC CCA GGT CCT CGC TTA TGA – 3′
GAPDH	5′ – ACG GCA AAT TCA ACG GCA CAG – 3′	5′ – GGC GGC ACG TCA GAT CC – 3′

Table 3

Histological assessment criteria for dextran sodium sulfate (DSS)-induced colitis model

Assessment criteria	Score	Description
Severity of inflammation	0	Rare inflammatory cells
	1	Increased infiltration of granulocyte
	2	Confluence of inflammatory cells infiltrate into the submucosa
	3	Transmural extension of the inflammatory cell infiltration
Crypt damage	0	Norma crypt
	1	Loss of the basal one-third
	2	Loss of the basal two-thirds
	3	Loss of the entire crypt
	4	Change of epithelial surface with erosion
	5	Extensive erosion
Ulceration	0	No ulcer
	1	1–2 ulcer foci
	2	3–4 ulcer foci
	3	Confluent ulceration

we investigated whether DHCA affected the production of relevant molecules using Raw264.7 cells, a murine macrophage cell line.

For this, the effect of DHCA on the production of IL-6, as one representative inflammatory cytokine, was initially tested in cells stimulated with various concentrations of LPS. Raw264.7 cells were treated with LPS, at concentrations ranging from 1 to 100 ng·mL^{-1}, in the presence of 10, 20 and 40 μM DHCA. The culture supernatants were collected and subjected to IL-6-specific ELISA. As shown in Figure 1A, DHCA decreased the protein level of IL-6 in a dose-dependent manner at all LPS concentrations used in this study, indicating that a broad range of LPS concentrations markedly increased the protein level of IL-6 and that DHCA effectively suppressed LPS-mediated increased production of IL-6.

To determine the IC_{50} value, Raw264.7 cells were treated with 100 ng·mL^{-1} LPS and various concentrations of DHCA for 24 h and the levels of TNF-α, IL-6, IL-1β, CCL2 and ROS were measured by ELISA (for the first four) or DCF-DA assay (for the last). Background levels of these molecules in naïve-untreated cells were very low or undetectable, but the presence of LPS dramatically increased their level. Treatment with DHCA decreased the level of inflammatory factors in a dose-dependent manner and the estimated IC_{50} values for TNF-α, IL-6, IL-1β, CCL2 and ROS were 37.3, 19.3, 11.8, 12.7 and 14.7 μM respectively (Figure 1B). When cells were treated with DHCA alone in the absence of LPS, the production level of these inflammatory mediators was nearly undetectable (data now shown). Consistent with previous data (Lee *et al.*, 2012; Lee and Kim, 2014), DHCA did not have any significant cytotoxic effect on Raw264.7 cells at the concentrations used in this study, as determined by MTT assay (Figure 1C).

To test if DHCA might induce such inhibitory effects by directly interacting with LPS in the extracellular space, Raw264.7 cells were stimulated with LPS and then washed with fresh culture media three times, followed by treatment with various concentrations of DHCA. Similar to the earlier experiments, LPS treatment markedly increased the protein level of IL-6 (Figure 1D). DHCA treatment after LPS wash out still suppressed the LPS-induced production of IL-6 in a dose-dependent manner and the reduction level was comparable with that of the simultaneously treated cells (Figure 1D). These results suggested that DHCA did not directly interact with LPS in the extracellular space.

Pro-inflammatory cytokines induced by LPS are known to be regulated mainly at the transcriptional level (Akira and Takeda, 2004; Medzhitov and Horng, 2009). To test whether DHCA affected the production of these proteins at the RNA level, total RNAs were prepared and subjected to Northern blot hybridization. The RNA levels of all pro-inflammatory genes measured in this study were significantly increased by LPS stimulation, but significantly decreased by DHCA treatment in a dose-dependent manner, consistent with the protein data (Figure 1E). Forty micromole of DHCA reduced the RNA levels of IL-6, IL-1β and CCL2 to almost

undetectable amounts. These data suggest that DHCA effectively down-regulates the production of pro-inflammatory cytokines by controlling their mRNA levels.

Effects of DHCA on the production of NO, PGE_2, iNOS and COX-2

NO and PGE_2 are important inflammatory mediators mainly produced by macrophages (Bogdan, 2001). To examine the effect of DHCA on these mediators, Raw264.7 cells were treated with LPS and various concentrations of DHCA for 24 h. The level of NO and PGE_2 in the culture supernatant was measured using the Griess assay and ELISA respectively. The levels of both mediators were very low or undetectable under normal conditions, but were dramatically increased by LPS stimulation. DHCA treatment significantly lowered the level of NO and PGE_2 in a dose-dependent manner and the estimated IC_{50} values for NO and PGE_2 were estimated to be 13.4 and 7.6 μM respectively (Figure 2A).

It has been reported that iNOS and COX-2 are the key enzymes involved in the production of NO and PGE_2 respectively (Kleinert *et al.*, 2004; Park *et al.*, 2006). To investigate whether DHCA affects the expression of these enzymes, Raw264.7 cells were treated with LPS and various concentrations of DHCA for 24 h. Total cell lysates were prepared followed by Western blot analysis using specific antibodies to iNOS and COX-2. In the presence of LPS, the protein levels of iNOS and COX-2 were significantly increased, but DHCA treatment lowered their levels in a dose-dependent manner (Figure 2B).

Similar to the pro-inflammatory cytokines, iNOS and COX-2 are also known to be regulated at the transcriptional level (Kleinert *et al.*, 2004; Park *et al.*, 2006). Hence, their RNA levels were analysed by Northern blot hybridization. LPS stimulation dramatically increased the RNA levels of iNOS and COX-2, but DHCA reduced their levels in a dose-dependent manner (Figure 2C). These data indicate that DHCA also down-regulates the production of NO and PGE_2 by controlling the RNA and protein levels of iNOS and COX-2.

Effects of DHCA on MAPKs and NF-κB pathways

To elucidate the molecular mechanism underlying the inhibitory effects of DHCA, the activities of NF-κB and AP-1 were analysed by use of the luciferase promoter assay as they are key transcription factors involved in the expression of pro-inflammatory proteins (Akira and Takeda, 2004). Raw264.7 cells were transfected with reporter plasmids in which luciferase expression is under the control of DNA binding sequences for respective transcription factors; they were then treated with LPS and various concentrations of DHCA. After 24 h, total cell lysates were prepared and the relative activity of luciferase was examined by luminometer. As shown in Figure 3A, treatment with DHCA lowered the LPS-induced activity of NF-κB in a dose-dependent manner, but not that of AP-1.

To confirm this result, EMSA was performed using the NF-κB binding oligonucleotide sequences. Raw264.7 cells were treated with LPS and various concentrations of DHCA for 1 h. Nuclear proteins were prepared and subjected to EMSA using a probe specific for NF-κB. The basal level of retarded DNA-protein complex was undetectable (Figure 3B, lane 1). LPS stimulation increased the level of DNA-protein complex, but DHCA treatment decreased the amount of DNA-protein complex (Figure 3B, compare lanes 2, 3 and 4). This DNA-protein complex was specific because it was effectively inhibited by the unlabelled wild-type oligonucleotide, but not by respective mutant sequence (Figure 3B, compare lanes 5 and 6).

To elucidate how NF-κB is affected by DHCA, the protein levels of upstream signalling molecules, such as IKKα/β, and I-κB were measured. Under normal conditions, I-κB binds to NF-κB, preventing its translocation to the nucleus. When stimulated by LPS, however, IKKα/β becomes activated and phosphorylates I-κB, resulting in its degradation and translocation of NF-κB (Hacker and Karin, 2006). Raw264.7 cells were treated with LPS and various concentrations of DHCA for 30 min. Total cell lysates were analysed by Western blot using antibodies to p-IKKα/β, IKKα/β and I-κB. As shown in Figure 3C, the level of total IKKα/β was high in all conditions and the basal level of p-IKKα/β was very low. LPS stimulation markedly increased the level of p-IKKα/β, but DHCA treatment down-regulated the level of p-IKKα/β in a dose-dependent manner (Figure 3C). As expected from this result, the protein level of I-κB was decreased by LPS stimulation, but 40 μM of DHCA treatment increased the level of I-κB, comparable with that of negative control (Figure 3C, compare lanes 1 and 4).

The activity of NF-κB is also regulated by MAPKs such as p38, ERK and JNK (Hayden and Ghosh, 2004). To examine whether DHCA has an effect on these kinases, Raw264.7 cells were treated with LPS in the presence or absence of 40 μM DHCA for 30 min. Total cell lysates were isolated and subjected to Western blot analysis using specific antibodies for unphosphorylated or phosphorylated forms of the respective kinases. The phosphorylated forms of these kinases were low or undetectable in normal conditions, whereas LPS stimulation upregulated their levels. However, none of these kinases were influenced by DHCA (Figure 3D). Taken together, these data suggest that DHCA down-regulates the production of various pro-inflammatory mediators specifically by lowering the level of p-IKKα/β, and consequently inhibiting the NF-κB binding to its nucleotide sequences.

Effects of DHCA on the production of IL-1β

Inflammasomes are protein complexes which convert pro-IL-1β to its mature form and palmitate, a saturated fatty acid, has recently been reported to activate NLRP3 inflammasomes by inducing the production of cellular ROS (Tschopp and Schroder, 2010; Davis *et al.*, 2011; Wen *et al.*, 2011). Because DHCA affects the levels of IL-1β and ROS, its effect on the palmitate-mediated production of IL-1β was tested. Raw264.7 cells were stimulated with LPS for 3 h and treated with palmitate-BSA and various concentrations of DHCA for another 21 h. The culture supernatants were collected and the level of IL-1β was measured by ELISA. Treatment with palmitate-BSA following LPS stimulation markedly increased the level of IL-1β as compared with those cells treated with LPS or palmitate-BSA alone. However, DHCA decreased this palmitate-mediated induction of IL-1β in a dose-dependent

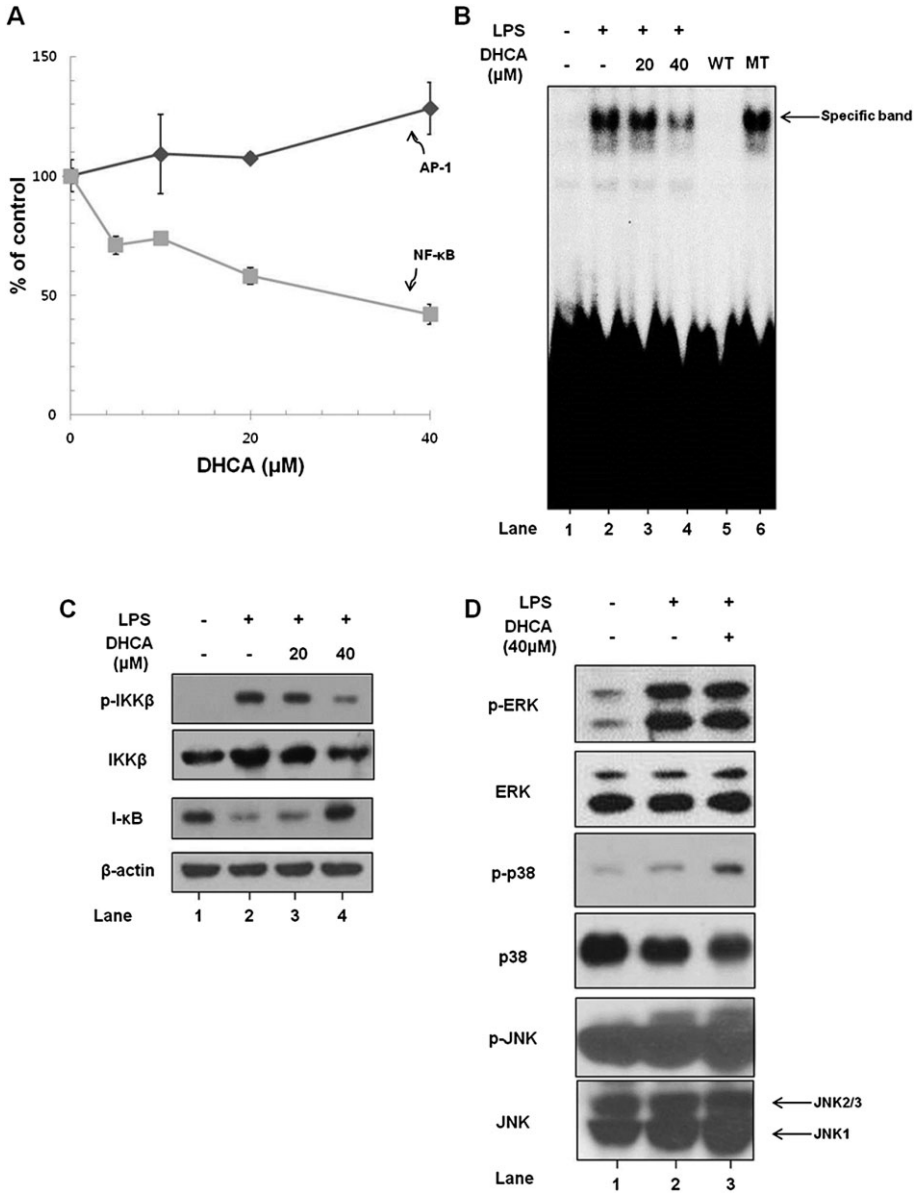

Figure 3

Effects of DHCA on the DNA binding activity of NF-κB and related signalling pathways. (A) Effect of DHCA on the promoter activity of AP-1 and NF-κB. Raw264.7 cells were transfected with plasmid containing the luciferase gene whose expression is under the control of the DNA binding sequences of AP-1 or NF-κB respectively. Twenty-four hours later, cells were treated with LPS and various concentrations of DHCA followed by luciferase assay. One hundred microlitres of luciferin was added to an aliquot of 20 μg proteins and the activity of luciferase was analysed using a luminometer. (B) Effect of DHCA on the DNA binding activity of NF-κB. Raw264.7 cells were treated with LPS and various concentrations of DHCA for 1 h. Nuclear proteins were prepared followed by EMSA using ^{32}P-labelled oligonucleotide targeting NF-κB and visualized by using films. The specificity of the DNA-protein complex was determined by competition analysis using an excessive amount of unlabelled wild-type (WT) or mutant (MT) oligonucleotides. (C) Effect of DHCA on the protein level of IKK and I-κB. Total cell lysates were isolated from Raw264.7 cells and subjected to Western blot analysis using specific antibodies to p-IKKα/β, IKKα/β, I-κB and β-actin. The protein level of IKKα/β and β-actin was measured as a loading control for p-IKKα/β and I-κB respectively. (D) Effect of DHCA on the protein levels of MAPKs. Total cell lysates were prepared from Raw264.7 cells and analysed by Western blot assay using specific antibodies to phosphorylated or unphosphorylated forms of the MAPKs. The protein level of the unphosphorylated forms of the MAPKs were used as controls. The blots shown are each representative of three independent experiments.

manner and the IC_{50} value was estimated to be 2.8 μM (Figure 4A).

To elucidate the underlying mechanisms, the RNA level of IL-1β was examined. Raw264.7 cells were stimulated with LPS for 3 h and then treated with palmitate-BSA and DHCA for 6 h. Total RNAs were analysed by Northern blot hybridization. Consistent with the data from Figure 1B, untreated normal cells produced an undetectable RNA level of IL-1β, but LPS

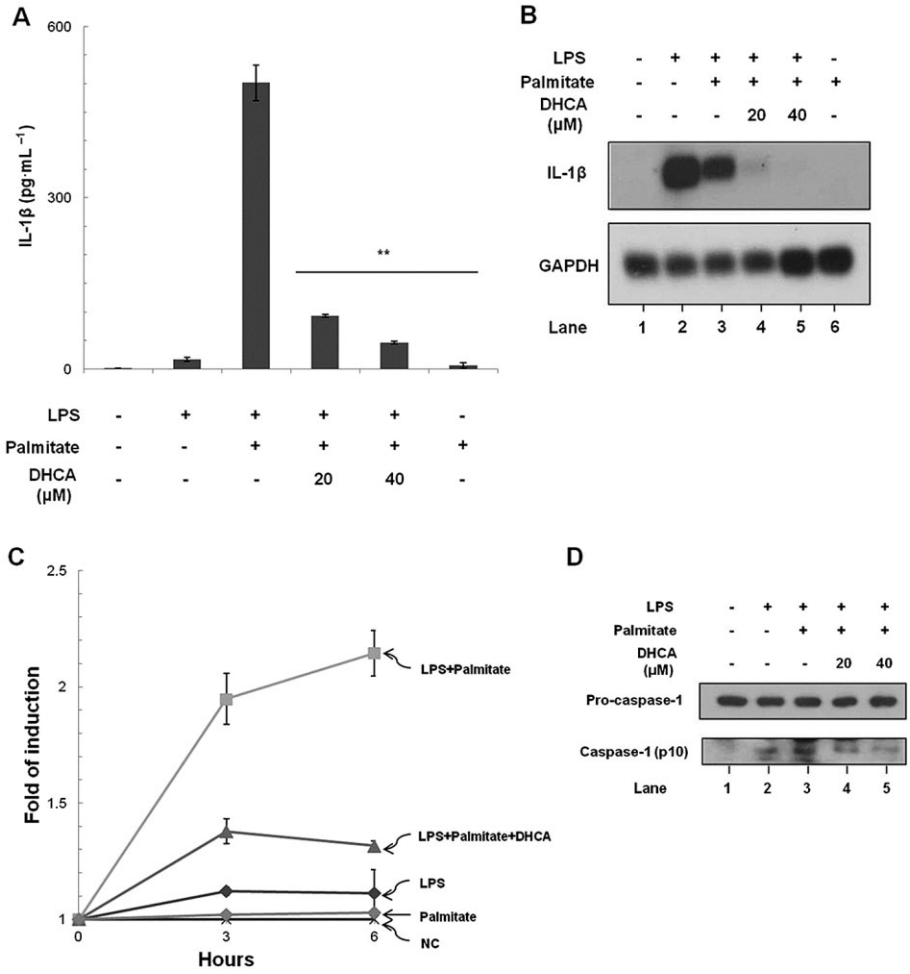

Figure 4

Effects of DHCA on the palmitate-mediated production of IL-1β. (A) Effect of DHCA on the protein level of palmitate-mediated IL-1β. Raw264.7 cells were treated as described in the Methods section. The culture supernatants were collected and the protein level of IL-1β was analysed using ELISA. Values represent the mean ± SD of triplicate samples. $^{**}P < 0.01$. (B) Effect of DHCA on the RNA level of IL-1β. Total RNAs, prepared from Raw264.7 cells treated with LPS for 3 h following palmitate-BSA and DHCA for another 6 h, were subjected to Northern blot hybridization using ^{32}P-labelled cDNA probes for IL-1β and GAPDH and visualized by exposing the blot on films. GAPDH was measured as a loading control and three independent experiments were performed. (C) Effect of DHCA on the production of ROS. Raw264.7 cells were treated with LPS for 3 h and then stimulated with palmitate-BSA in the presence or absence of 20 μM DHCA. After 3 or 6 h, the DCF-DA assay was performed and the level of ROS was determined using FACS. Values represent the mean ± SD of triplicate samples. (D) Effect of DHCA on the protein level of caspase-1. Total cell lysates were isolated from Raw264.7 cells treated under the same conditions as described in (C), followed by Western blot analysis using a specific antibody to caspase-1. The pro-form of caspase-1 was measured as a loading control. Three independent experiments were performed.

stimulation increased its level (Figure 4B compare lanes 1 and 2). Treatment of LPS-stimulated cells with palmitate slightly decreased the level of IL-1β (Figure 4B compare lanes 2 and 3). When LPS- and palmitate-stimulated cells were treated with DHCA, the RNA level of IL-1β was decreased to a point comparable with that of untreated negative control cells (Figure 4B). These data indicate that DHCA effectively down-regulates the production of IL-1β by controlling its RNA level.

Next, we tested whether DHCA affects the palmitate-mediated increase in the production of cellular ROS and the cleavage of caspase-1. Raw264.7 cells were stimulated with LPS for 3 h and then treated with palmitate-BSA in the absence or presence of 40 μM of DHCA for 3 or 6 h followed by the DCF-DA assay to determine the level of cellular ROS. The basal level of ROS was very low. When stimulated with LPS or palmitate-BSA alone, its level was slightly increased. When cells were primed with LPS and then treated with palmitate-BSA, the level of ROS was greatly increased, but DHCA inhibited its level at each time point by up to 70% (Figure 4C).

The cellular ROS is a major upstream signal generated by palmitate to trigger the cleavage of pro-caspase-1 to its active form (Martinon, 2010; Tschopp and Schroder, 2010). To test the effect of DHCA on caspase-1, Raw264.7 cells were stimulated with LPS for 3 h followed by treatment with palmitate-BSA and various concentrations of DHCA for 21 h. Total cell

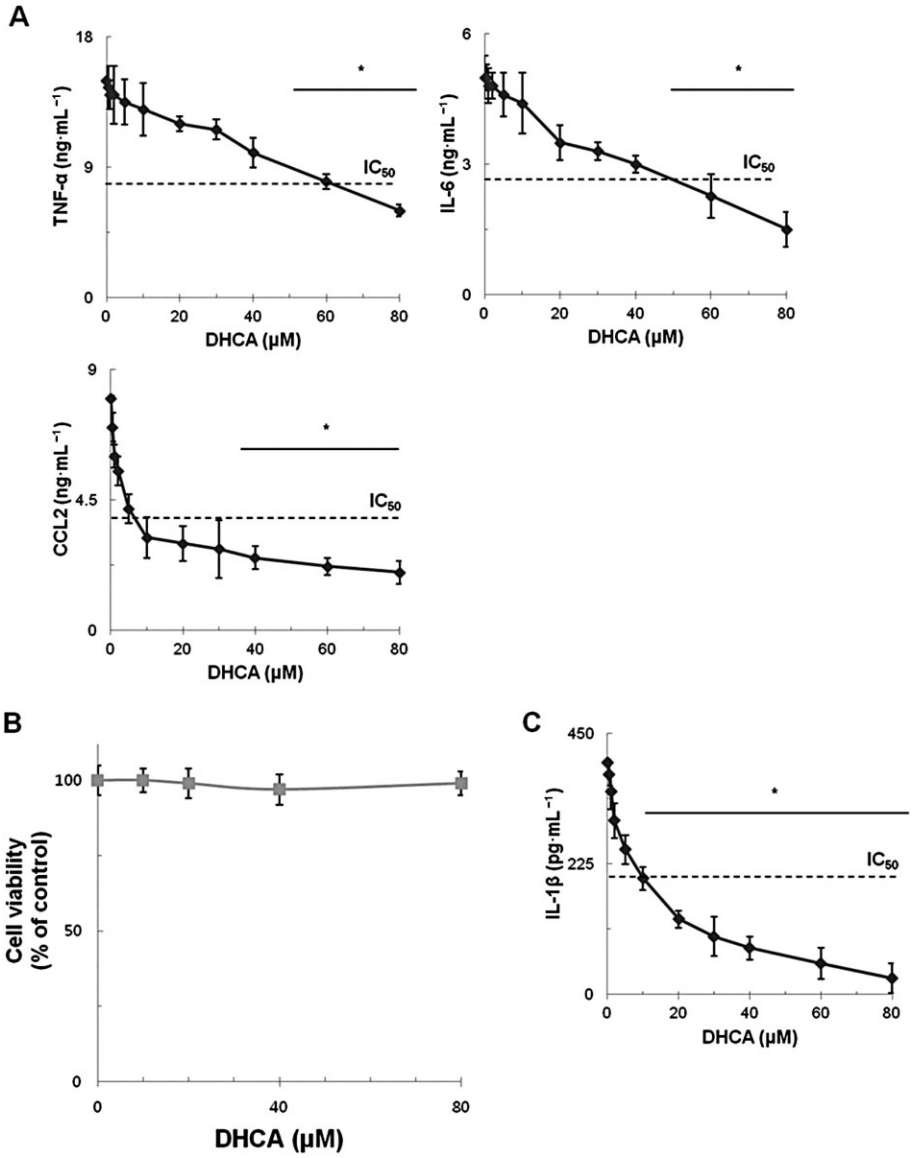

Figure 5

Effects of DHCA on the production of pro-inflammatory cytokines in primary BMDMs. (A) Effect of DHCA on the expression of cytokines in primary BMDMs. BMDMs were prepared as described in the Methods section and treated with LPS and various concentrations of DHCA. The culture supernatant was collected and analysed using respective ELISA. (B) Cytotoxic effect of DHCA. Primary BMDMs were treated with various concentrations of DHCA for 24 h followed by MTT assay as described in the Methods section. (C) Effect of DHCA on the palmitate-mediated IL-1β secretion in BMDMs. BMDMs were treated under the same conditions as described in Figure 4A. The level of IL-1β in the supernatant was measured by ELISA. Values represent the mean ± SD of triplicate samples. *$P < 0.05$.

lysates were subjected to Western blot analysis. Consistent with the data from the DCF-DA assay, in LPS-stimulated cells, palmitate-BSA treatment increased the amount of cleaved form of caspase-1, but DHCA reduced it to a basal level at 40 μM (Figure 4D). Taken together, these data suggest that DHCA inhibits the production of cellular ROS and the active form of caspase-1.

Effects of DHCA in primary BMDMs

To test that the earlier observations were not restricted to one particular cell line, the effects of DHCA were also tested using primary BMDMs. BMDMs were prepared from the femurs of mice and differentiated into macrophages with the media containing 20 ng·mL^{-1} of M-CSF. After 7 days, differentiated macrophages were treated with LPS and various concentrations of DHCA for 24 h. The culture supernatants were collected and the levels of TNF-α, IL-6 and CCL2 were measured by ELISA. LPS increased the protein levels of these cytokines and DHCA treatment decreased the levels of respective cytokines in a dose-dependent manner (Figure 5A) without affecting the cell viability (Figure 5B). The estimated IC_{50} values for IL-6 and CCL2 were 54 and 22.2 μM respectively.

Overall, the responses of BMDMs to DHCA appears to be less sensitive, as the IC_{50} value was increased, with TNF-α being the least affected cytokine consistent with data from Raw264.7 cells.

The effect of DHCA on the palmitate-mediated IL-1β secretion was also examined. BMDMs were stimulated with LPS for 3 h and then treated with palmitate-BSA and various concentration of DHCA for another 21 h. When cells were treated with palmitate-BSA following LPS stimulation, the level of IL-1β was dramatically increased, but this was effectively decreased by DHCA in a dose-dependent manner. The IC_{50} value of this inhibitory effect was calculated to be 21 μM (Figure 5C). These data indicate that DHCA down-regulates the expression of various cytokines in primary macrophages.

Effects of DHCA in the mouse DSS-induced colitis model

Before testing the effects of DHCA in the animal disease model, it was first examined whether this lignan molecule has any effect on naïve mice. Mice were treated with 100 or 300 mg·kg^{-1} DHCA by i.p. injection on a daily basis for 7 days. The weight of the body and other internal organs were measured. These parameters remained unchanged in mice treated with DHCA when compared with either vehicle-treated or naïve mice, suggesting that DHCA has little effect in naïve mice (Table 4).

It was further tested whether DHCA could control inflammatory responses in the mouse DSS-induced colitis model. To induce colitis, 3.5% DSS was dissolved in the drinking water and administered to 6-week-old C57BL/6 mice for 7 days. Various doses of DHCA, dissolved in vehicle solution (DMSO : PBS = 1:1), were injected i.p. once a day. For the control mice, a vehicle solution containing no DHCA was used. When the mice received 3.5% DSS-containing water, their body weight gradually decreased starting on day 4 and was reduced by 30% at day 7 compared with normal mice. When animals were treated with DHCA, such weight loss was inhibited in a dose-dependent manner (Figure 6A) and the difference in body weight between the control and 300 mg·kg^{-1} DHCA-treated mice was statistically significant ($P < 0.01$).

When the length of the colon between the ileocaecal junction and the proximal rectum was measured at day 7, significant colon shrinkage was observed in colitis-induced mice (Figure 6B). DHCA treatment alleviated the DSS-mediated reduction in colon length in a dose-dependent manner (Figure 6B).

The effects of DHCA on the DSS-induced mouse colons were also examined at the histological level. DSS-induced mice were killed at day 7 and the colons were prepared followed by fixation in 10% neutral buffered formalin solution. Fixed tissues were embedded, sectioned and stained with H&E. The histological score was assessed as described in the Methods section. As shown in Figure 6C, the histological score of colons from DSS-treated mice increased markedly when compared to naïve mice, indicating that DSS induced severe inflammation and histological damage in the mouse colon. DHCA treatment, however, reduced this colon damage in a dose-dependent manner (Figure 6C).

To determine whether DHCA had an effect on the production of inflammatory cytokines at the actual site, the colons used in Figure 6B were washed with cold PBS and the total proteins were extracted using RIPA buffer. After centrifugation, the supernatant of tissue homogenates was obtained followed by ELISAs for TNF-α and IL-1β. When colitis was induced by DSS, the production level of both TNF-α and IL-1β was markedly increased in the colitis-induced mice compared with that of naïve mice. However, DHCA treatment decreased DSS-mediated induction of both cytokines in a dose-dependent manner (Figure 6D). Taken together, these data suggest that DHCA ameliorates the severity of colitis probably by decreasing the inflammatory response in the mouse colon.

Discussions

DHCA, a member of the lignan family isolated from *C. moschata*, was previously shown to contain anti-adipogenic and antilipogenic activities in 3T3-L1 and primary mouse embryonic fibroblasts (Lee *et al.*, 2012). Our data suggest that DHCA also decreases the levels of various pro-inflammatory molecules such as TNF-α, IL-6, IL-1β, CCL2, NO, PGE_2 and ROS in

Table 4

Effect of dehydrodiconiferyl alcohol (DHCA) on naïve mice

		DHCA (mg·kg^{-1})		
	Naïve	0 (Vehicle)	100	300
Body weight (g)	23.4 ± 0.04	23.2 ± 0.04	23.5 ± 0.03	23.1 ± 0.03
Heart (g)	0.62 ± 0.02	0.61 ± 0.03	0.65 ± 0.01	0.64 ± 0.02
Liver (g)	4.85 ± 0.03	4.85 ± 0.03	4.91 ± 0.03	4.88 ± 0.04
Brain (g)	1.31 ± 0.02	1.29 ± 0.03	1.26 ± 0.02	1.32 ± 0.03
Kidney (g)	0.70 ± 0.02	0.69 ± 0.03	0.68 ± 0.02	0.69 ± 0.05
Lung (g)	0.57 ± 0.01	0.59 ± 0.03	0.57 ± 0.01	0.59 ± 0.02
Spleen (g)	0.34 ± 0.03	0.33 ± 0.03	0.32 ± 0.02	0.31 ± 0.01

Mice were divided into four groups (naïve, vehicle, 100 or 300 mg·kg^{-1} DHCA) and treated with vehicle solution, 100 or 300 mg·kg^{-1} DHCA, respectively, via i.p. injection on a daily basis. After 7 days, the weights of the body and other organs were measured.

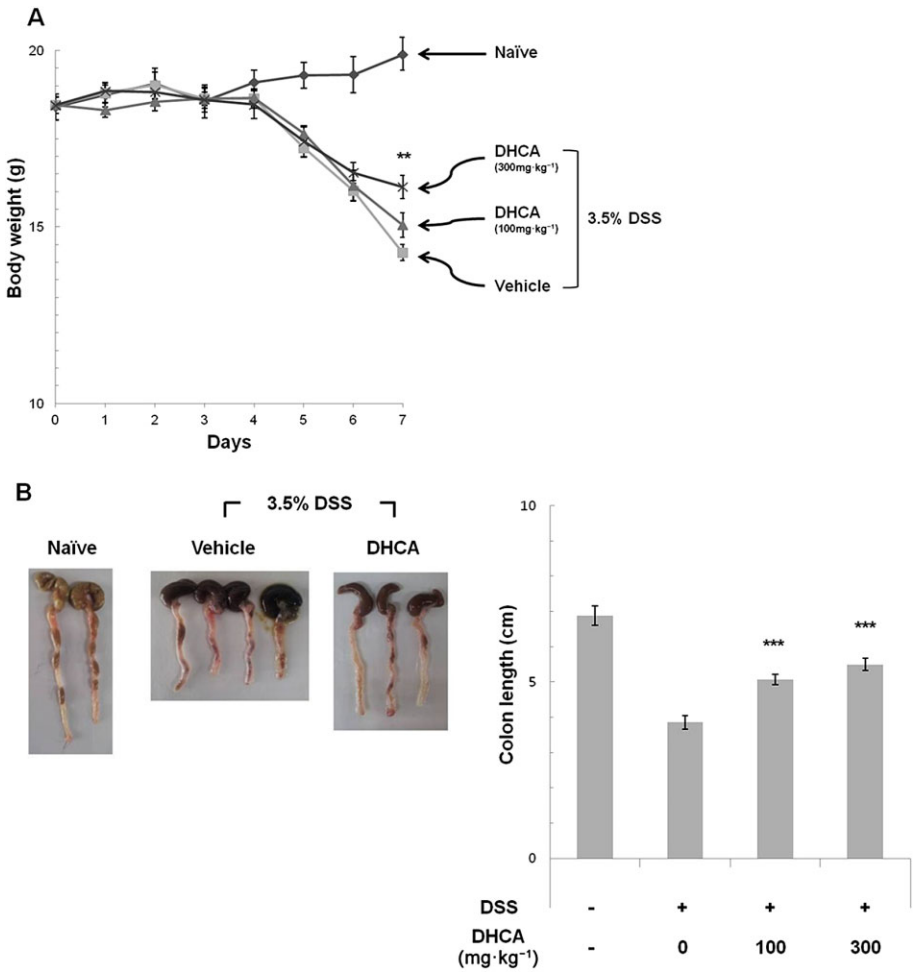

Figure 6

Effects of DHCA in the mouse DSS-induced colitis model. (A) Effect of DHCA on the body weight of DSS-induced mice. Colitis was induced by the administration of 3.5% DSS in drinking water for 7 days. Mice were divided into three groups, treated with vehicle ($n = 10$), 100 mg·kg^{-1} ($n = 10$) or 300 mg·kg^{-1} DHCA ($n = 10$), respectively, via i.p. injection. The body weight of mice was measured once a day. Values represent the mean ± SEM. $^{**}P < 0.01$. (B) Effect of DHCA on colon length. Mice were killed at day 7 and the colon was prepared. The macroscopic appearance and length of colons were analysed. Values represent the mean ± SEM. $^{***}P < 0.001$. (C). Effect of DHCA on the DSS-induced colitis at the histological level. To analyse the effect of DHCA on the DSS-mediated histological damage, colons were prepared at day 7 and fixed in 10% neutral buffered formalin solution. Tissues were then embedded in paraffin followed by H&E staining. The histological score was evaluated as described in the Methods section. Values represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. (D) Effect of DHCA on the production of pro-inflammatory cytokines in the mouse colon. The colons used in part (B) were washed with cold PBS and total proteins were extracted using RIPA buffer containing protease inhibitor cocktail. Tissue homogenates were prepared and an ELISA specific to TNF-α and IL-1β was performed. The level of cytokines in naïve mice is shown as a white diamond on the y-axis. Values represent the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$.

Raw264.7 cells. The IC_{50} value of DHCA for almost all inflammatory molecules, except for TNF-α, was 10–20 μM. DHCA appears to regulate the expression of various proteins by affecting the phosphorylation status of IKKα/β and eventually NF-κB activity. In the case of IL-1β, DHCA seems to exert an additional level of control through inflammasomes. Consistent with these *in vitro* data, DHCA ameliorated the severity of DSS-induced colitis probably by reducing the production of inflammatory mediators in the colon.

NF-κB and AP-1 are two pivotal transcription factors that modulate LPS-mediated inflammatory responses by triggering the expression of a variety of pro-inflammatory mediators (Whitmarsh and Davis, 1996; Hayden and Ghosh, 2004). LPS stimulates various signal transduction pathways, involving IKK and MAPKs (p38, ERK and JNK) which eventually activates NF-κB and AP-1. The IKK complex controls NF-κB, while MAPKs regulate both NF-κB and AP-1. Our data suggest that DHCA inhibits the activity of IKK and NF-κB, but not that of MAPKs and AP-1 indicating that this lignan compound might target specific proteins involved in the IKK signalling cascade.

The activity of IKKα/β has been reported to be controlled by several upstream kinases such as MAPK/ERK kinase kinase (MEKK1), NF-κB-inducing kinase (NIK) or TGF-β-activated kinase 1 (TAK1) (Lee *et al.*, 1997; Ling *et al.*, 1998; Wang *et al.*,

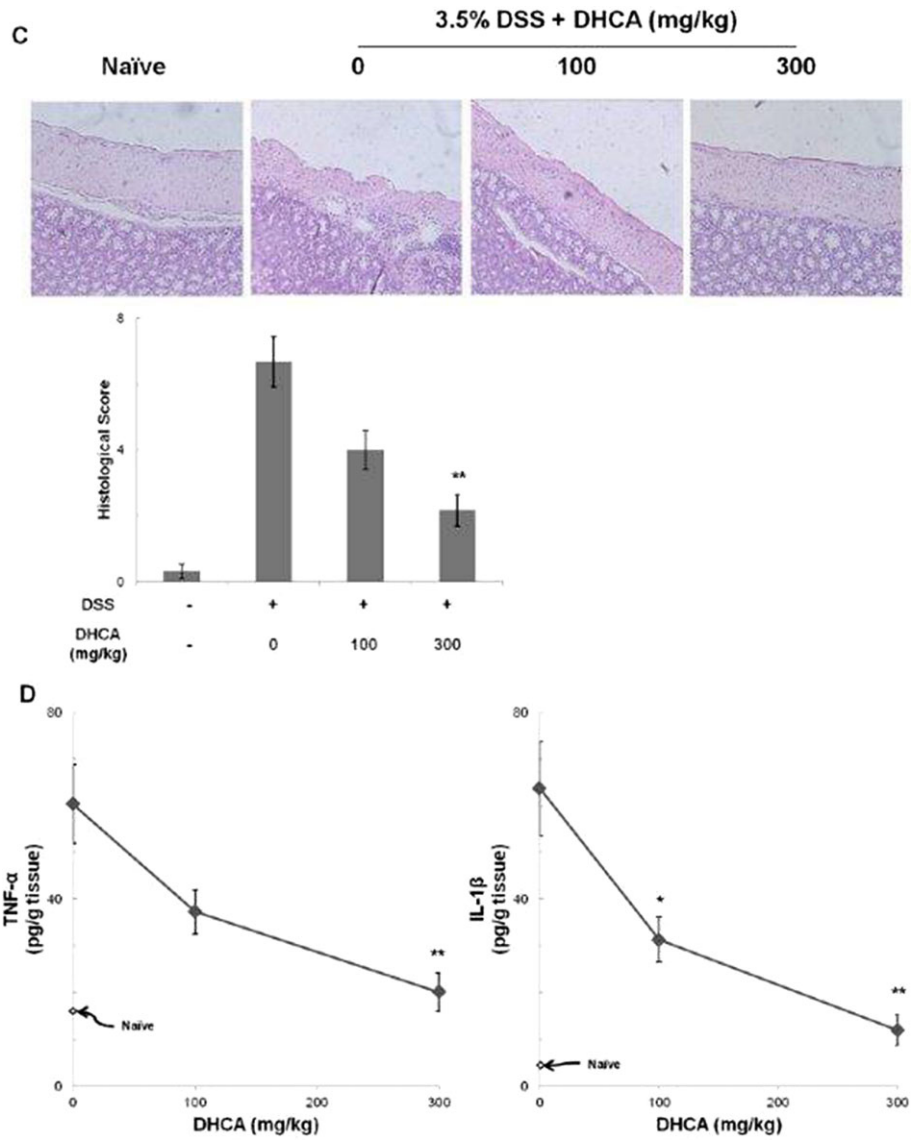

Figure 6
Continued

2001). MEKK1 was originally identified as an upstream kinase of JNK, but it has also been shown to phosphorylate IKKα/β in a direct manner (Lee *et al.*, 1997). Because DHCA had no influence on JNK or AP-1, it is thought that MEKK1 might not be a target of DHCA. NIK and TAK1 have also been reported to activate IKKα/β. The former is a kinase responsible for the activation of IKK, but has no role in that of JNK (Ling *et al.*, 1998). TAK1 is an ubiquitin-dependent kinase known to trigger JNK or IKK signalling by binding to their activating proteins TAK1-binding protein (TAB) 1 or 2 respectively (Wang *et al.*, 2001; Adhikari *et al.*, 2007). It was previously reported that the formation of a TAK1-TAB2 complex is necessary for the activation of IKK, while the TAK1-TAB1 complex could activate both the JNK and IKK signalling pathways (Adhikari *et al.*, 2007). Taken together, the inhibitory effect of DHCA on NF-κB might have resulted from its suppression of NIK or interference of the interaction between TAK1 and TAB2. The endeavour to elucidate the detailed molecular mechanism is currently underway.

TNF-α appears to be the pro-inflammatory cytokine least affected by DHCA among the various molecules tested in this study. The IC_{50} value for the protein level of TNF-α was almost fivefold higher than that of PGE_2, while the RNA level of TNF-α was decreased by twofold when that of other proteins was almost undetectable at the same concentration. DHCA has previously been shown to inhibit the phosphorylation of CCAAT/enhancer-binding protein (C/EBP)β and consequently down-regulate its DNA binding activity (Lee *et al.*, 2012). C/EBPβ has been reported to interact with NF-κB and produce synergistic effects on the production of pro-inflammatory genes such as IL-6, IL-1β, CCL2, iNOS or COX-2, but not on that of TNF-α (Matsusaka *et al.*, 1993;

Stein *et al*., 1993; Sakitani *et al*., 1998; Sorli *et al*., 1998; Dlaska and Weiss, 1999; Hu *et al*., 2000; Liu *et al*., 2000; Gorgoni *et al*., 2001). Therefore, it is possible that DHCA affects IL-6, IL-1β, CCL2, iNOS and COX-2 to a greater extent than TNF-α by inhibiting two transcription factors, instead of one.

DHCA might exert a broad range of anti-inflammatory effects by controlling the activities of key transcription factors. It is not yet clear whether these seemingly pleiotropic effects of DHCA are the result of its control of several individual proteins such as NF-κB and C/EBPβ or that of the one 'master' upstream factor. Further investigations are warranted not only to unravel the molecular mechanism underlying the anti-inflammatory activities of DHCA, but also to sort out the complex relationship between the large number of signalling molecules and transcription factors involved in the regulation of inflammation and oxidative stress.

Acknowledgements

This work was supported by three research grants: (i) 2012008018 (National Research Foundation of Korea); (ii) 2012K001130 (Brain Research Center of 21st Century Frontier Research Program); and (iii) industry/academy cooperation research grant from ViroMed Co. Ltd. The first two are administered by the Ministry of Education, Science and Technology, Korea.

Author contributions

J. L., J. C. performed the research. J. L. and S. K. designed the research study. J. L. and J. C. contributed essential reagents. J. L., J. C. and S. K. analysed the data. J. L. and S. K. wrote the paper.

Conflict of interest

None.

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