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碩士學位論文

Anti-inflammatory Effects of Kahweol and Cafestol

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List of Abbreviations

AP-1	Activator protein-1
CA	Cafestol acetate
COX	Cyclooxygenase
DMSO	Dimethylsulfoxide
EMSA	Electrophoretic mobility shift analysis
FBS	Fetal bovine serum
IκB	Inhibitor κB
iNOS	Inducible nitric oxide synthase

KA	Kahweol acetate
LPS	Lipopolysaccharide
MTT	3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NF-κB	Nuclear factor κB
NO	Nitric oxide
PGE_2	Prostaglandin E_2
PGs	Prostaglandins
RT-PCR	Reverse transcriptase polymerase chain reaction

ABSTRACT

Anti-inflammatory effects of Kahweol and Cafestol

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Excessive nitric oxide (NO) production by inducible nitric oxide synthase (iNOS) in stimulated inflammatory cells is thought to be a causative factor of cellular injury in cases of inflammation. Inducible cyclooxygenase-2 (COX-2) has been implicated in the processes of inflammation and carcinogenesis. In recent studies, these have been shown that kahweol and cafestol, coffee-specific diterpenes, exhibit chemoprotective effects. In this study, the effects of kahweol and cafestol on the production of NO, the expression of inducible nitric oxide synthase (iNOS) and COX-2 were investigated in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages. When kahweol and cafestol were treated with LPS, the NO production induced by LPS was markedly reduced in a dose-dependent manner. Kahweol and cafestol also directly inhibited the activity of iNOS. In addition, kahweol and cafestol suppressed the expression of iNOS protein and iNOS mRNA. Since iNOS transcription has been shown to be under the control of the

transcription factor, NF-κB, the effects of kahweol on NF-κB activation were examined. Transient transfection experiments showed that kahweol and cafestol inhibited NF-κB-dependent transcriptional activity. Moreover, electrophoretic mobility shift assay experiments indicated that kahweol blocked the LPS-induced activation of NF-κB. The results of these studies suggest that the suppression of the transcriptional activation of iNOS by kahweol might be mediated through the inhibition of NF-κB activation. Kahweol and cafestol significantly suppressed the LPS-induced production of prostaglandin E_2 (PGE_2), expression of COX-2 protein as well as the mRNA, and COX-2 promoter activity in a dose-dependent manner. Furthermore, kahweol blocked the LPS-induced activation of NF-κB through the prevention of IκB degradation and the inhibition of IκB kinase activity. In the mouse carrageenan air pouch model of inflammation, kahweol and cafestol significantly suppressed PGE_2 production and COX-2 expression in the pouch. Taken together, the results of this study provide evidence that kahweol and cafestol possess the anti-carcinogenesis property or anti-inflammatory potential which constitutes a previously unrecognized biologic activity, and may provide new insights to explain some of the chemopreventive properties observed in mouse macrophage studies and in animal experiments.

Ⅰ. Introduction

Nitric oxide (NO) is produced from L-arginine by nitric oxide synthase (NOS), a family of ubiquitous enzymes. NOS plays a major role in regulating vascular tone, neurotransmission, the killing of microorganisms and tumor cells and other homeostatic mechanisms [Mayer and Hemmens, 1997]. Molecular cloning and sequencing analyses have revealed the existence of at least three main types of NOS isoforms. Both neuronal NOS and endothelial NOS are constitutively expressed [Yun *et al.*, 1996], whereas inducible NOS (iNOS) is expressed in response to interferon-γ, lipopolysaccharide (LPS) and a variety of pro-inflammatory cytokines [MacMicking *et al.*, 1997]. Following exposure to LPS or cytokines, iNOS can be induced in various cells, such as macrophages, Kupffer cells, smooth muscle cells, and hepatocytes. iNOS activation catalyzes the formation of a large amount of NO, which plays a key role in a variety of pathophysiological processes including various forms of circulatory shock, inflammation and carcinogenesis [Lala and Chakraborty, 2001; MacMicking *et al.*, 1997; Maeda and Akaike, 1998]. Therefore, the amount of NO produced by iNOS may be a reflection of the degree of inflammation, and therefore provide a means of assessing the effect of drugs on the inflammatory process. Because cells cannot sequester and regulate the local concentration of NO, the regulation of NO synthesis is the key to elicit its biological activity. NO production by iNOS is mainly regulated at the transcriptional level [MacMicking *et al.*, 1997]. In macrophages, LPS activates the transcription factor nuclear factor-κB (NF-κB), which leads to the induction of expression of many immediate early genes [Baeuerle and Henkel, 1994]. The presence of the cis-acting NF-κB element has been demonstrated in the 5'-flanking regions of the iNOS genes [Baeuerle and Henkel, 1994]. The activation of NF-κB by LPS is induced by a cascade of

events which ultimately lead to the activation of inhibitor κB (IκB) kinase, which phosphorylates IκB, resulting in its degradation and the translocation of NF-κB to the nucleus [Griseavage *et al*., 1996]. Thus, NF-κB translocates to the nucleus and induces gene transcription through the cis-acting κB element. NF-κB is, therefore, an obvious target for new types of anti-inflammatory treatment [Baeuerle and Baichwal, 1997].

Cyclooxygenase (COX) catalyzes the synthesis of prostaglandins (PGs) from arachidonic acid. Two isozymes, designated COX-1 and COX-2, have been identified but are encoded by separate genes. The COX-1 isozyme is believed to be a housekeeping protein in most tissues and appears to catalyze the synthesis of prostaglandins for normal physiological functions. In contrast, COX-2 is not present under normal physiological conditions but is rapidly induced by the tumor promoters, growth factors, cytokines and mitogens in various cell types [Prescott and Fitzpatrick, 2000; Simon, 1999]. Many cell types associated with inflammation, such as macrophages, endothelial cells and fibroblasts, express the COX-2 gene upon induction [Simon, 1999]. It is well established that COX-2 is important in carcinogenesis. COX-2 is over expressed in transformed cells and in various forms of cancer [Prescott and Fitzpatrick, 2000; Simon, 1999]. Because targeted inhibition of COX-2 is a promising approach to inhibit inflammation and carcinogenesis and to prevent cancer, chemopreventive strategies have focused on inhibitors of the COX-2 enzyme activity. An equally important strategy may be to identify compounds that suppress the signaling path that regulate COX-2 expression [Chinery *et al*., 1998; Subbaramaiah *et al*., 1998].

COX-2 is an early gene expressed in response to many cytokines. Its transcriptional regulation is, at least in part, under the control of transcription factor nuclear factor-κB (NF-κB) [D'Acquisto *et al*., 1997]. In macrophages, LPS activates NF-κB, which leads to the induction of the expression of many immediate

early genes [D'Acquisto *et al*., 1997]. The presence of a cis-acting NF-κB element has been demonstrated in the 5'-flanking regions of the COX-2 gene [D'Acquisto *et al*., 1997; Tak and Firestein, 2001]. NF-κB activation is induced by a cascade of events leading to the activation of the inhibitor κB (IκB) kinase (IKK), which phosphorylates IκB, leading to its degradation and the translocation of NF-κB to the nucleus [Chen *et al*., 2002; Tak and Firestein, 2001]. Because NF-κB plays a key role in regulating the genes involved in the initiation of the immune, acute phase, and inflammatory responses, there is growing interest in modulating its activity. Therefore, the pathways leading to NF-κB activation are frequent targets for a variety of anti-inflammatory drugs [Tak and Firestein, 2001].

It is increasingly being acknowledged that foods contain non-nutritional constituents, which may possess biological activities compatible with beneficial health effects, such as anti-inflammatory and anti-carcinogenic properties [Bellisle *et al*., 1998]. Kahweol and cafestol (Fig. 1) are two diterpenes that are present in considerable quantities in coffee beans, as well as in the final, unfiltered beverage, e.g. in Turkish or Scandinavian style coffees [Gross *et al*., 1997]. These compounds can be considered as interesting examples of such biologically active food components. They have been shown to exhibit both adverse and chemoprotective properties [Cavin *et al*., 2002; De Roos *et al*., 1999]. It is well documented that kahweol and cafestol increase blood cholesterol level in both human and animal models [De Roos *et al*., 1999]. However, animal studies have shown that kahweol and cafestol afford protection against the action of well-known carcinogens, such as nitrosamine, 7,12-dimethylbenz[a]anthracene, aflatoxin B_1, and 2-amino-l-b]-pyridine (PhIP) [Cavin *et al*., 2001; Huber *et al*., 1997; Miller *et al*., 1991]. In line with these observations, there is epidemiological evidence in humans that the consumption of coffee with a high amount of kahweol and cafestol is associated with a lower rate of colon cancer, one of the most frequent cancers in

the western world [Giovannucci, 1998]. The chemoprotective effects of kahweol and cafestol have thus far been primarily related to the beneficial modifications of the xenobiotic metabolism. Such effects include the reduced activation of mutagens/carcinogens e.g. via the inhibition of cytochrome P450 enzymes [Cavin *et al*., 2001], as well as their enhanced detoxification, e.g. via the induction of carcinogen-detoxifying enzyme systems such as glutathione S-transferase and UDP-glucuronosyl transferase [Cavin *et al*., 2002; Huber *et al*., 2002].

Excessive NO production by iNOS in stimulated inflammatory cells is thought to be a causative factor of cellular injury in cases of inflammatory disease. Thus, compounds inhibiting iNOS activity or its transcriptional activity in inflammatory cells are potentially anti-inflammatory. However, there has been no report designed to investigate the anti-inflammatory properties of kahweol and cafestol. Excessive prostaglandin E_2 (PGE_2) production by COX-2 in stimulated inflammatory cells is believed to be a causative factor of cellular injury in inflammatory disease. Therefore, compounds inhibiting COX-2 activity or its transcriptional activity are potentially anti-inflammatory or cancer chemopreventive. In the present study, therefore, we investigated the effects of kahweol and cafestol on iNOS expression, the activity of the iNOS enzyme itself, and COX-2 expression, in order to further elucidate the chemopreventive and anti-inflammatory mechanisms of kahweol and cafestol in murine macrophages. Herein, we show for the first time that kahweol suppresses the activation of iNOS gene expression that both kahweol and cafestol also directly inhibit the activity of iNOS, and that kahweol and cafestol suppress the activation of COX-2 gene expression through NF-κB inhibition by targeting the IKK complex. In addition, this report shows that decreased PGE_2 production and COX-2 expression were observed in the mouse carrageenan air pouch model of inflammation. These data provide a mechanistic basis for the chemopreventive and anti-inflammatory properties of kahweol and cafestol.

Ⅱ. MATERIALS & METHODS

A. Materials

Chemicals and cell culture materials were obtained from the following sources: Cafestol acetate, Kahweol acetate, Escherichia coli 0111:B4 lipopolysaccharide (LPS), and LDH diagnostic kits from Sigma Co.; MTT colorimetric assay kit from Roche Co.; Prostaglandin E_2 (PGE_2) and enzyme immunoassay reagents for the PGE_2 assays from Cayman Co.; LipofectAMINE Plus, RPMI 1640 medium, fetal bovine serum (FBS), and penicillin-streptomycin solution from Life Technologies, Inc. NF-κB consensus oligonucleotides, pGL3-4κB-Luc, and the luciferase assay system from Promega; pCMV-β-gal from Clonetech; Antibodies to iNOS, COX-2, COX-1, β-actin, IKKβ, IκBα, and the phosphorylated form of IκBα (Ser 32) from Santa Cruz Biotechnology, Inc. Western blotting detection reagents (ECL) from Amersham Pharmacia Biotech. Other chemicals were of the highest commercial grade available.

B. Cell Culture

RAW 264.7 cells, of the mouse macrophage cell line, were obtained from the American Type Culture Collection (Bethesda, MD), and grown in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 g/ml streptomycin at 37^{o}C in a 5% CO_2 humidified incubator. Cafestol acetate, and kahweol acetate were dissolved in dimethylsulfoxide (DMSO) and added directly to the culture media. Control cells were treated only with solvents, the final concentration of which never exceeded 0.1%, and this concentration did not have any noticeable effect on the assay systems.

C. Cell viability

Cell viability was assessed by measuring the release of LDH and by means of the MTT assay. The levels of LDH release were measured in the supernatants. After the supernatant was removed for LDH determination, the cells were used for the MTT assay. The LDH and MTT assays were performed according to the manufacturer's instructions.

D. Nitrite assay

The cells (5 X 10^5 cells/ml) were cultured in 48-well plates. After incubating for 24 h, NO synthesis was determined by assaying the culture supernatants for nitrite, which is the stable reaction product of NO with molecular oxygen, using Griess reagent, as described previously [Jeong and Kim, 2002].

E. LPS-induced iNOS enzyme activity

For the iNOS enzyme activity assay, the cells were plated in 100-mm tissue culture dishes (4 X 10^6 cells) and incubated with LPS (0.5 μg/ml) for 12 h. Then, the cells were washed twice with PBS, harvested and plated into a 24-well plate and cultured in the absence or presence of the compounds to be tested for a further 12 h without LPS being present in the medium. The supernatants were then removed, and the Griess reaction was performed as above. For the assay in cell lysates, the cells were washed three times with PBS, scraped into cold PBS, and centrifuged at 500 X g for 10 min at 4°C. The cell pellet was resuspended in 0.5 ml of 40 mM Tris buffer (pH 8.0) containing 5 μg/ml of pepstatin A, 1 μg/ml of chymostatin, 5 μg/ml of aprotinin, and 100 μM phenylmethylsulfonyl fluoride, and lysed by means of three freeze-thaw cycles. Aliquots of the lysate were used for the Bradford protein assay [Bradford *et al.*, 1976]. iNOS enzyme activity was

measured as described previously in the literature [Vodovotz *et al*., 1993]. Briefly, 200 μg of cell lysate protein was incubated in 20 mM Tris-HCI (pH 7.9) containing 4 μM FAD, 4 μM tetrahydrobiopterin, 3 mM dithiothreitol, and 2 mM each of L-arginine and NADPH. The reaction was carried out for 180 min at 37°C. Residual NADPH was oxidized enzymatically and the Griess reaction was performed as described above.

F. Determination of PGE_2 production

The cells were incubated with chemicals and/or LPS (0.5 μg/ml). After incubating for 24 h, the culture medium was collected and the level of PGE_2 released into culture media were quantified using a specific enzyme immunoassay according to the manufacturer's instructions.

G. Western Blotting

The cells were cultured with the chemicals for 24 h and then cell lysates were prepared by treating the cells with lysis buffer (150 mM NaCl, 100 mM Tris, pH 8.0, 1% Tween 20, 50 mM diethyldithiocarbamate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml trypsin inhibitor, and 10 μg/ml leupeptin). The lysates were sonicated for 20 s on ice and centrifuged at 10,000 X g for 10 min to sediment the particulate material. The protein concentration of the supernatant was measured by the method of Bradford (Bradford, 1976). SDS-PAGE was performed under reducing conditions on 10% polyacrylamide gels. The resolved proteins were transferred onto polyvinylidene difluoride membranes. After blocking, the membranes were incubated with iNOS polyclonal antiserum, COX-2 polyclonal antiserum, COX-1 polyclonal antiserum, or monoclonal anti-β-actin. The secondary antibody to IgG conjugated to

horseradish peroxidase was used. The blots were probed with the ECL Western blot detection system according to the manufacturer's instructions.

H. Reverse transcription-polymerase chain reaction (RT-PCR)

The cells were cultured with chemicals and/or LPS (0.5 μg/ml) for 6 h. Total cellular RNA was isolated by the acidic phenol extraction procedure of Chomczynski and Sacchi [Chomczynski,, and Sacchi, 1987]. cDNA synthesis, semiquantitative RT-PCR for iNOS, COX-1, COX-2, and β-actin mRNA, and the analysis of the results were all performed as described previously [Jeong., and Kim., 2002]. PCR reactions were electrophoresed through a 2.5% agarose gel and visualized by ethidium bromide staining and UV irradiation. Prior to analysis the PCR product band intensities were checked to ensure that they had not reached the saturation intensity.

I. Transient Transfection and luciferase and β-galactosidase assays

The cells (5 X 10^5 cells/ml) were plated in each well of a 12-well plate, and 12 h later transiently co-transfected with the plasmids pGL3-4κB-Luc and pCMV-β-gal, or with the plasmids (the COX-2 promoter construct (-1432/+59), which was generously provided by Dr. Tadashi Tanabe and Chieko Yokoyama (National Cardiovascular Center Research Institute, Osaka, Japan), pGL3-4κB-Luc, and pCMV-β-gal) using LipofectAMINE Plus according to the manufacturer's protocol. Briefly, the transfection mixture containing 0.5 μg of pGL3-4B-Luc and 0.2 μg of pCMV-β-gal was mixed with the LipofectAMINE Plus reagent and added to the cells. After 18 h, the cells were treated with LPS and/or the test compound for 12 h, and then lysed. Luciferase and β-galactosidase activity were determined as described previously [Jeong and Kim, 2002]. Luciferase activity was normalized

with respect to β-galactosidase activity and was expressed relative to the activity of the LPS group.

J. Electrophoretic mobility shift assay (EMSA)

The cells were harvested and nuclear extracts were prepared as described previously [Jeon *et al.*, 1999]. Two double-stranded deoxyoligonucleotides containing the NF-κB binding site (5'-GGGGACTTTCC-3') were end-labeled with [γ-^{32}P]dATP. Nuclear extracts (5 μg) were incubated with 2 μg of poly (dI-dC) and the ^{32}P-labeled DNA probe in binding buffer (100 mM NaCl, 30 mM HEPES, 1.5 mM $MgCl_2$, 0.3 mM EDTA, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml concentration each of aprotinin and leupeptin) for 10 min on ice. The specificity of binding was examined by competition with the unlabeled oligonucleotide. The DNA protein complex was separated on 6% non-denaturing acrylamide gels. Following electrophoresis, the gel was dried and autoradiographed.

K. IκBα degradation and IKK assay

The cytoplasmic extracts were prepared from the cells pretreated with the chemicals for 30 min, and then treated with LPS (0.5 μg/ml) for 30 min. The extracts were then resolved on 10% SDS-PAGE and analyzed by western blotting using the antibody against IκBα as described above. For the IKK assay, the cells were treated with chemicals for 15 min and equal amounts of the total cellular protein (200 μg) were immunoprecipitated with IKKβ antibody and protein A/G-PLUS agarose for 12 h at 4°C. The kinase assay was carried out in the kinase buffer containing 5 mM cold ATP, 10 Ci [γ-^{32}P]ATP (5000 Ci/mmol) and 1 μg of the GST-IκBα fusion protein as a substrate, and incubated for 20 min at 25°C. The

reaction was stopped by adding the Laemmli buffer, and the material was subjected to 10% SDS-PAGE. The phosphorylated GST-IκBα was visualized by autoradiography

L. Air pouch model of inflammation

Male Balb/c mice (20-25 g) were used. The air cavities were produced by s.c. injection of 4 ml of sterile air into the interscapular area of the back. Every 2 days, 2 ml of air was again injected into the cavity to keep the space open. Seven days after the first injection, 1 ml of a 1% solution of carrageenan in saline was injected directly into the pouch to produce an inflammatory response. Kahweol or cafestol (2-10 mg/kg) and indomethacin (2 mg/kg) were dissolved in polyethylene glycol (2:1) and intraperitoneally injected to the fasted animals 2 h before being carrageenan. The animals were sacrificed 24 h later after administering the carrageenan, and the pouch exudates were collected. The cells were pelleted by centrifugation at 1200 g for 5 min at 4°C, and PGE_2 was determined in the supernatant by enzyme immunoassay. The cells were used for COX-2 immunoblotting.

M. Histology

The excised pouch tissues were fixed in 10% formalin in 0.01 M phosphate buffer (pH 7.4) and embedded into paraffin wax blocks. Sections were stained with haematoxylin and eosin.

N. Statistical Analysis

All experiments were repeated at least three times. Data are presented as means ± SE for at least three different sets of plates and treatment groups. The Student's t-

test was used to assess the statistical significance of the differences. A confidence level of < 0.01 was considered to be statistically significance. Differences were considered statistically significant when $p < 0.01$.

Ⅲ. RESULTS

A. Kahweol and cafestol inhibit nitrite production in LPS-activated macrophages

To investigate their anti-inflammatory effects, kahweol and cafestol (Fig. 1) were tested with regard to their effect on NO production in LPS-activated RAW 264.7 macrophages. The amount of nitrite accumulated in the culture medium was estimated by the Griess reaction as an index for NO production originating from the cells. Kahweol and cafestol, at a concentration of 20 μM, did not interfere with the reaction between nitrite and the Griess reagents (data not shown). As shown in Fig. 2 and 3, after treatment with LPS (0.5 μg/ml) for 24 h, the NO concentration in the medium increased remarkably, i.e. about 12-fold. When the cells were treated with various concentrations of kahweol and cafestol, the NO production induced by LPS was significantly inhibited in a dose-dependent manner (Fig. 2, 3). The stimulation of NO production by LPS was inhibited completely by 5 μM kahweol. The data indicated that kahweol was much more effective than cafestol at inhibiting NO production. Cell viability was assessed by measuring the release of LDH (data not shown) and by means of the MTT assay (Fig. 4). An examination of the cytotoxicity of kahweol and cafestol in RAW 264.7 macrophages, performed by measuring the release of LDH and by means of the MTT assay, indicated that these compounds did not decrease cell viability in the cells (> 90% cell viability, Fig. 4). Therefore, the inhibition of LPS-induced nitrite production by kahweol and cafestol was not the result of their cytotoxicity against the cells.

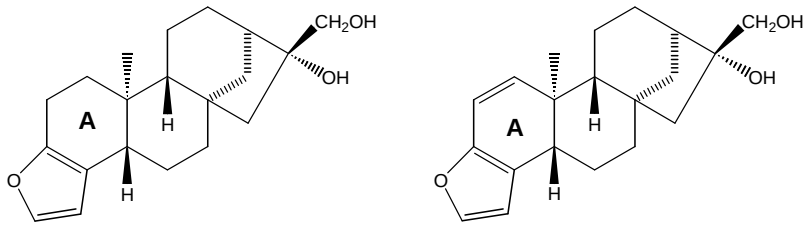

Fig. 1. Chemical structures of kahweol and cafestol. The arrows indicate the chemical differences between the two compounds; a double bond in kahweol is lacking in cafestol.

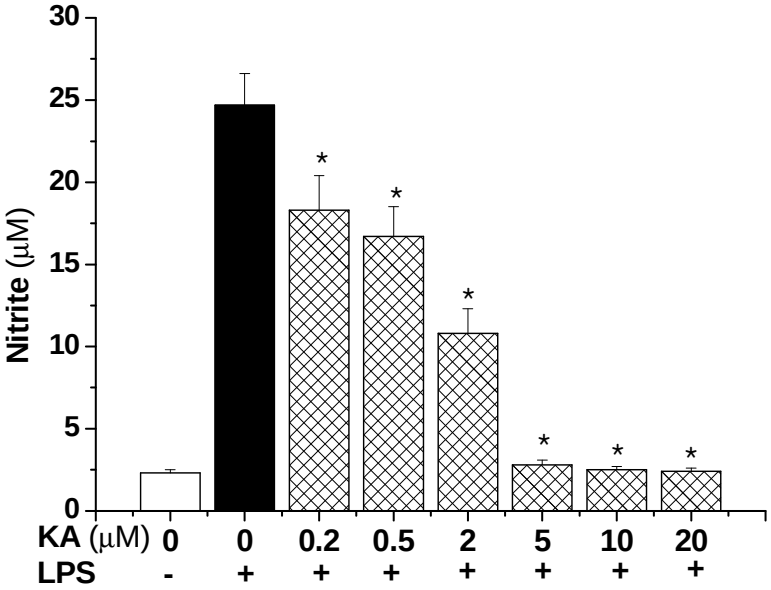

Fig. 2. Effect of kahweol on NO production. RAW 264.7 cells were treated with kahweol in the presence of LPS (0.5 μg/ml). The supernatants were harvested 24 h later and assayed for NO production. Values are expressed as mean ± SD of three individual experiments, performed in triplicate. *P < 0.01, significantly different from the LPS.

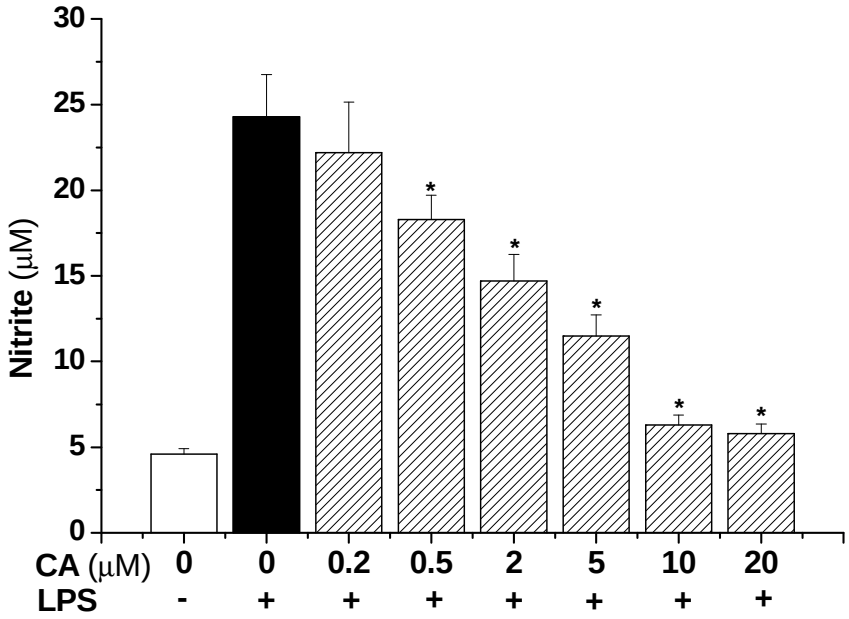

Fig. 3. Effect of cafestol on NO production. RAW 264.7 cells were treated with cafestol in the presence of LPS (0.5 μg/ml). The supernatants were harvested 24 h later and assayed for NO production. Values are expressed as mean ± SD of three individual experiments, performed in triplicate. *P < 0.01, significantly different from the LPS.

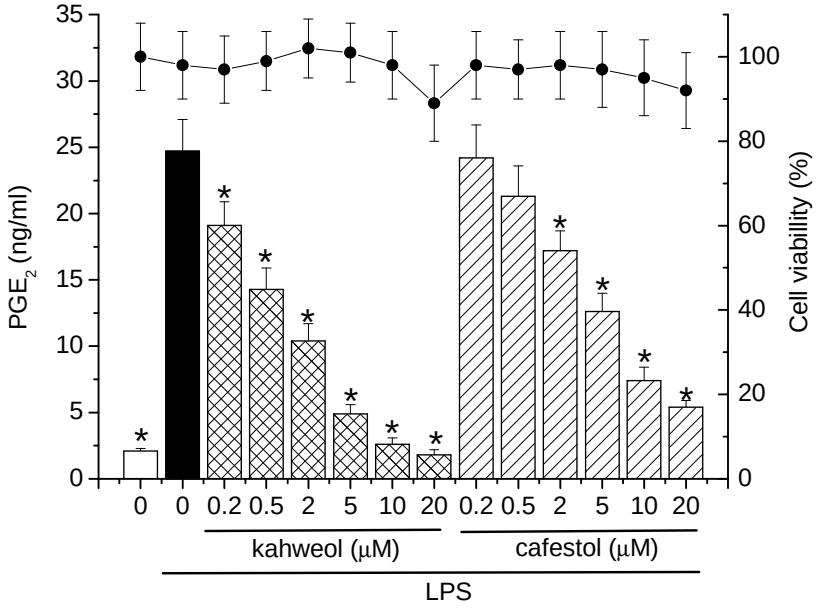

Fig. 4. Effects of kahweol and cafestol on PGE_2 production. The cells were treated with kahweol or cafestol in the presence of LPS (0.5 μg/ml). The supernatants were harvested 24 h later and assayed for PGE_2 production. The cell viability was evaluated with the MTT assay (solid line connecting solid circles). The results are presented as a percentage of the control value obtained from non-treated cells. The values are expressed as a mean ± SD of three individual experiments, performed in triplicate. *$P < 0.01$, significantly different from the LPS.

B. Kahweol decreases iNOS expression in LPS-activated macrophages

To determine whether the above effects on NO production were related to differences in the levels of iNOS, western blotting of cell lysate protein was carried out. Fig. 5A shows that LPS induced varying levels of iNOS protein in the macrophages. Treatment with kahweol caused a dose-dependent decrease in the LPS-activated induction of iNOS with the maximal effect being observed at 5 μM. The amount of β-actin protein, which was present as an internal control, remained unchanged. In contrast, cafestol, at a high concentration of 10 μM did not affect levels of LPS-activated iNOS protein. The observed changes in the amount of iNOS protein might be a reflection of altered protein synthesis or degradation. To further elucidate the mechanism responsible for the changes in the amount of iNOS protein, we determined the levels of iNOS mRNA by RT-PCR analysis. As shown in Fig. 5B, when the cells were treated with kahweol, the iNOS mRNA levels induced by LPS (0.5 μg/ml) were markedly decreased in a dose-dependent manner. These results suggest that kahweol suppresses iNOS expression at the transcriptional level, and thus contributes to decreasing the production of iNOS protein and NO.

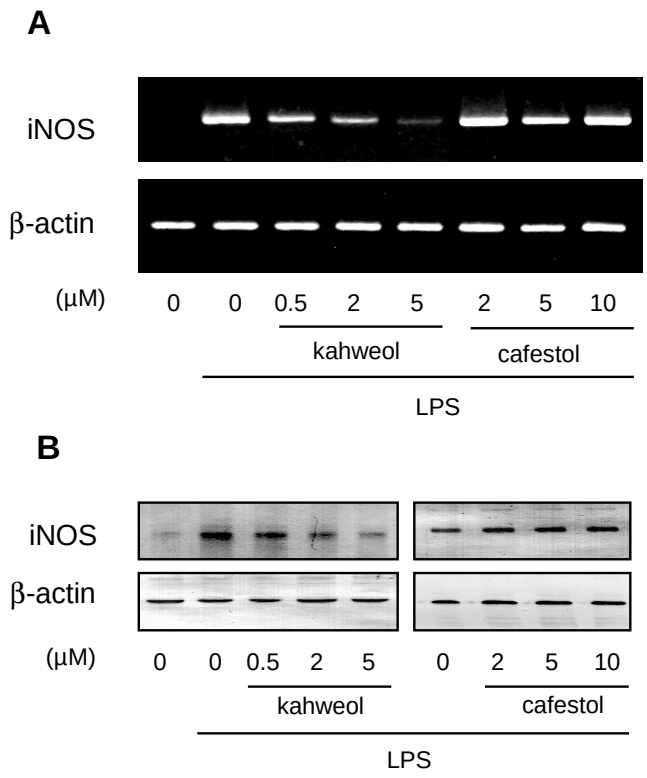

Fig. 5. Effects of kahweol and cafestol on LPS-induced expression of iNOS protein and mRNA. RAW 264.7 cells were treated with kahweol or cafestol in the presence of LPS (0.5 μg/ml). (A) Immunoblot analysis. After 24 h of incubation, the cell lysates (30 μg protein) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and blotted with an anti-iNOS or β-actin antibody. (B) RT-PCR analysis. After 6 h of incubation, total RNA was prepared and RT-PCR was performed as described in the Materials and Methods section. The PCR products were separated on a 2.5% agarose gel and stained with ethidium bromide. These blots (A, B) are a representative of each of three independent experiments and β-actin (A, B) was used as an internal control.

C. Kahweol and cafestol inhibitor iNOS enzyme activity

The effects of kahweol and cafestol on iNOS activity have not previously been evaluated. In this study, we found that kahweol suppressed both NO production and iNOS expression in LPS-activated macrophages, whereas cafestol only suppressed NO production and did not affect iNOS expression. Therefore, in order to determine whether the inhibitory effects on inducible NO production were due to a direct effect produced by these compounds on the intrinsic enzyme activities of iNOS, we investigated the effects of kahweol and cafestol on iNOS activities, by measuring NO production in intact cells, in which iNOS was induced by LPS. In this experiment, the cells were treated with LPS for 12 h. Fresh medium containing either kahweol or cafestol was added and then, 6 h later, the levels of NO in the medium were measured. N-nitro-L-arginine methyl ester (L-NAME), which is known to inhibit iNOS enzyme activity, was used as positive control. As shown in Table 1, both kahweol and cafestol inhibited the LPS-stimulated production of NO in a dose-dependent manner. In the same part of the experiment, the NOS inhibitor L-NAME, which was used as positive control, effectively inhibited NO production in the medium. These data indicate that kahweol (IC_{50} = 7.3 μM) and cafestol (IC_{50} = 12.6 μM) directly inhibited iNOS activity in intact cells. At the maximal concentration of 20 μM, kahweol and cafestol inhibited the production of NO by 92% and 76%, respectively. An examination of the cytotoxicity of kahweol and cafestol in the cells by means of the MTT assay indicated that these compounds, even at a concentration of 20 μM, did not decrease cell viability (data not shown). Therefore, the inhibition of iNOS activity by kahweol and cafestol in LPS-induced intact cells was not the result of their cytotoxicity against the cells. The evidence for there being direct enzyme inhibition by kahweol and cafestol was further supported by the additional findings, which indicated that kahweol (IC_{50} = 10.2 μM) and cafestol (IC_{50} = 14.3 μM) treatment significantly inhibited NO production

resulting from iNOS enzyme activity in the cell lysates, as determined by direct NOS enzyme activity assays *in vitro* (Table 1). Although L-NAME showed a more potent inhibitory effect on iNOS enzyme activity than either kahweol or cafestol, both of these diterpenes could directly block the reaction catalyzed by iNOS.

Table 1. Effects of kahweol and cafestol on iNOS activity in the intact macrophages and macrophages lysates.

LPS pretreatment of cells	Addition to LPS-treated cells or cell lysate	NO in medium [a] (μM)	iNOS specific activity: NO formation [b] (μM)
None	**DMSO control**	**1.2 ± 0.4**	**1.5 ± 0.3**
LPS, 6 h	**Control**	**14.5 ± 1.6**	**10.8 ± 1.3**
	kahweol 5 μM	**9.8 ± 1.2***	**8.7 ± 1.1**
	kahweol 10 μM	**5.4 ± 0.8***	**6.1 ± 0.8***
	kahweol 20 μM	**2.3 ± 0.5***	**4.1 ± 0.6***
	cafestol 5 μM	**11.6 ± 1.4***	**9.5 ± 1.2**
	cafestol 10μM	**9.2 ± 1.2***	**6.9 ± 0.9***
	cafestol 20 μM	**5.3 ± 0.7***	**5.2 ± 0.7***
	L-NAME 3 mM	**1.8 ± 0.3***	**2.2 ± 0.4***

RAW 264.7 macrophages were stimulated with LPS (0.5 μg/ml) for 12 h, and then the cells were washed twice with PBS to remove the LPS.

[a] The cells were cultured with the indicated compounds for an additional 6 h. The amount of NO accumulated in the medium were measured by indirect NOS enzyme assay as described in the Materials and Methods section.

[b] Cell lysate preparation and each indicated compound was added to the lysates (200 μg) from LPS-treated macrophages, and direct iNOS activity was measured as described in the Materials and Methods section.

Values are expressed as mean ± SD of three individual experiments, performed in triplicate. *$P < 0.01$, significantly different from the LPS.

D. Effects of kahweol and cafestol on PGE_2 production in LPS-activated macrophages

In order to investigate their anti-inflammatory effects, kahweol and cafestol (Fig. 1) were tested with regard to their effect on PGE_2 production in LPS-activated RAW 264.7 macrophages. When the cells were treated with the various concentrations of kahweol and cafestol, the PGE_2 production induced by LPS was significantly inhibited in a dose-dependent manner (Fig. 4) and kahweol is much more effective at inhibiting PGE_2 production than cafestol. The cell viability was assessed by a MTT assay. An examination of the cytotoxicity of kahweol and cafestol in the RAW 264.7 macrophages indicated that these compounds did not decrease the cell viability in the cells (> 90% cell viability, Fig. 4). Therefore, the inhibition of LPS-induced PGE_2 production by kahweol and cafestol was not the result of their cytotoxicity against the cells.

E. Effects of kahweol and cafestol on the COX-2 expression in LPS-activated macrophages

Western blotting of cell lysate protein was carried out in order to determine whether or not the above effects on PGE_2 production are related to differences in COX levels. LPS induced COX-2 in the macrophages, and a treatment with kahweol and cafestol caused a dose-dependent decrease in the LPS-mediated induction of COX-2 (Fig. 6A). Kahweol is much more effective at inhibiting COX-2 expression than cafestol. Neither LPS nor kahweol and cafestol affected the COX-1 level (data not shown).

The observed changes in the COX-2 protein level might be a reflection of an alteration in protein synthesis or degradation. The COX-2 mRNA levels were measured by RT-PCR analysis in order to further elucidate the mechanism responsible for the changes in the amount of COX-2 protein. Kahweol and cafestol markedly decreased the COX-2 mRNA levels induced by LPS (Fig. 6B). This suggests that kahweol and cafestol suppress COX-2 expression at the transcriptional level, thereby contributing to decreasing the production of the COX-2 protein and PGE_2.

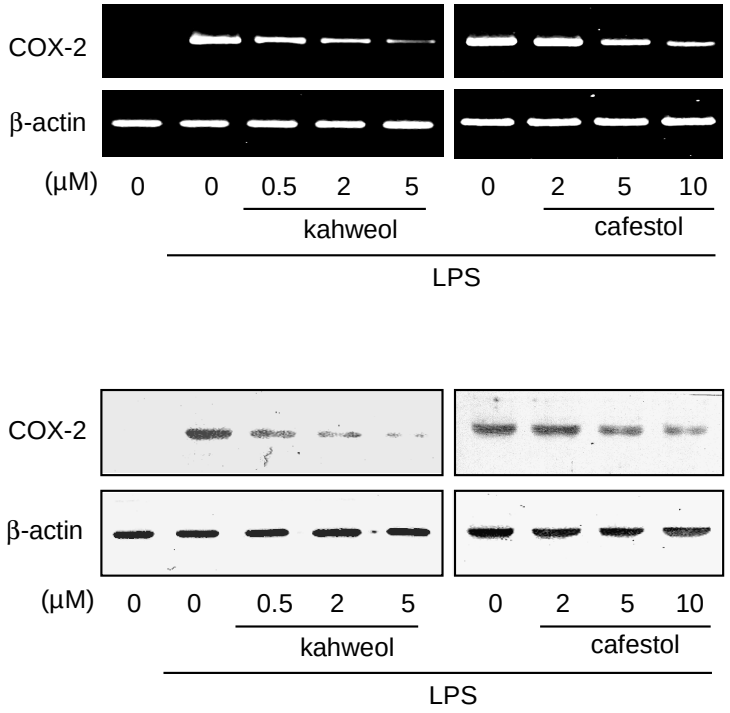

Fig. 6. Effect of kahweol and cafestol on LPS-induced expression of the COX-2 protein and mRNA. The cells were treated with either kahweol or cafestol in the presence of LPS (0.5 μg/ml). (A) Immunoblot analysis. After 24 h of incubation, the cell lysates (30 μg protein) were separated by SDS-PAGE, transferred to a nitrocellulose membrane and blotted with an anti-COX-2 or β-actin antibody. (B) RT-PCR analysis. After 3 h of incubation, the total RNA was prepared and RT-PCR was performed as described in the Materials and Methods section. The PCR products were separated on a 2.5% agarose gel and stained with ethidium bromide. These blots (A, B) are a representative of each of three independent experiments.

F. Effects of kahweol on the activation of NF-κB in LPS-activated macrophages

Because the activation of NF-κB is vital for the induction of COX-2 by LPS or other inflammatory cytokines [D'Acquisto. *et al*., 1997] and kahweol is much more effective at inhibiting PGE_2 production and COX-2 expression by LPS than cafestol, this study determined if kahweol could suppress NF-κB activation in the LPS-activated macrophages using an electrophoretic mobility shift assay. The induction of the NF-κB binding activity by LPS was markedly inhibited by kahweol in a dose-dependent manner (Fig. 7). The addition of an excessive quantity of an unlabeled wild type probe completely prevented the NF-κB binding, demonstrating the binding specificity of the NF-κB complex. In order to further investigate the importance of LPS and kahweol in modulating the expression of COX-2 and NF-κB activity in the LPS-activated macrophages, transient transfections were performed using the COX-2 luciferase promoter construct and the NF-κB-dependent luciferase reporter plasmid. Kahweol inhibited the LPS-activated COX-2 promoter activity and NF-κB transcriptional activity (Fig. 8). These results suggest that the suppression of COX-2 expression by kahweol occurred via the prevention of NF-κB activation. Since it has been well documented that NF-κB activation correlates with the rapid proteolytic degradation of IκB, the prevention of IκB degradation was also examined as an indication of the inhibition of NF-κB activation by kahweol. LPS induced a transient degradation of IκBα in the cells, whereas kahweol prevented the degradation of IκBα (Fig. 9). Finally, kahweol was examined to determine if it could inhibit IKK activity. Kahweol significantly inhibited the IKK activity induced by LPS (Fig. 10). However, kahweol had no effect on the level of IKK protein level (data not shown).

This suggests that the inhibition of COX-2 expression by kahweol occurred via the suppression of IKK activity resulting in the prevention of NF-κB activation.

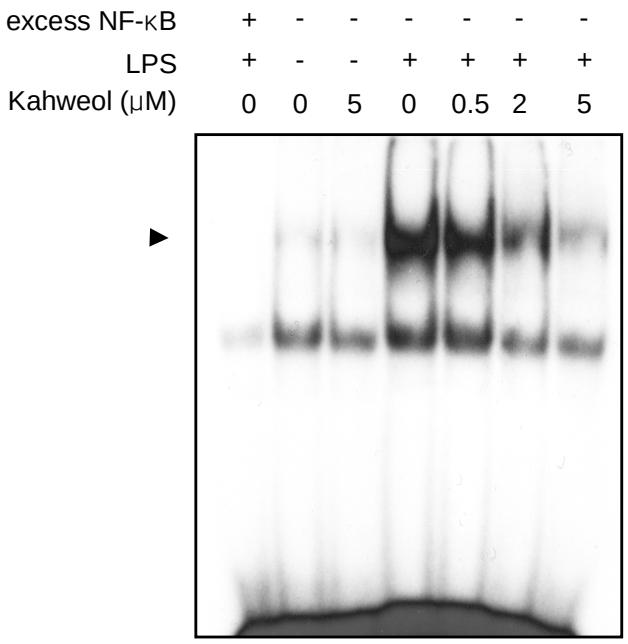

Fig. 7. Effect of kahweol on LPS-induced NF-κB activity determined by EMSA. The cells were treated with kahweol and LPS (0.5 μg/ml) for 30 min. The nuclear extracts were prepared and EMSA was carried out. The arrow indicates the NF-κB binding complex. Excess NF-κB; 200-fold molar excess of non-labeled NF-κB probe. This blot was a representative of each of three independent experiments.

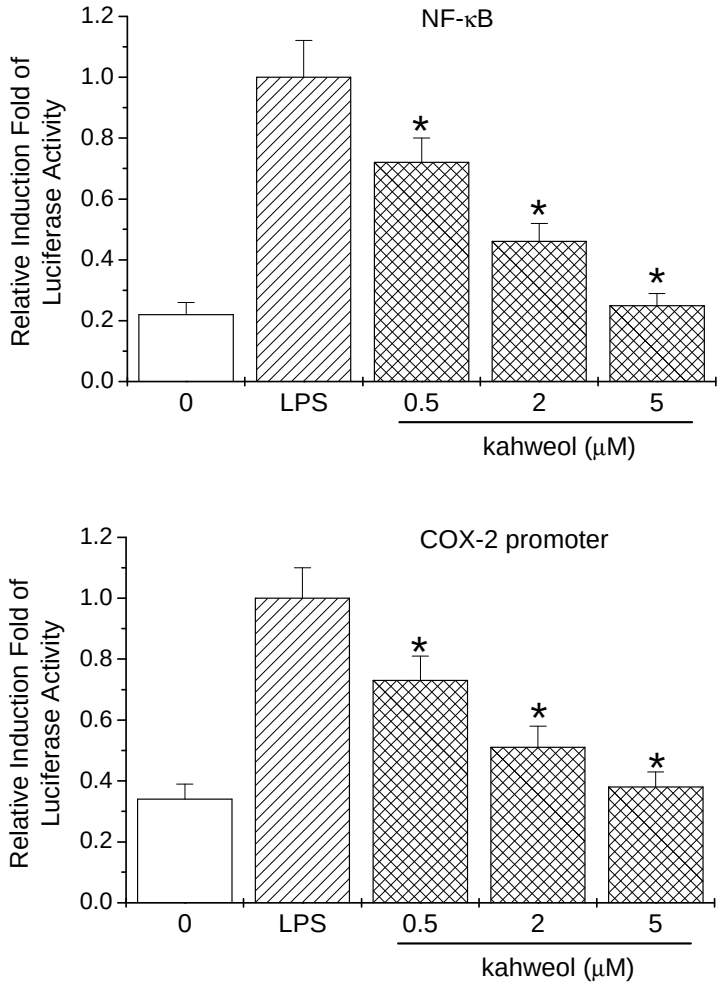

Fig. 8. Effect of kahweol on pNF-κB-Luc and COX-2 promoter-Luc reporter activities. The cells were transiently co-transfected with pGL3-4κB-Luc or COX-2 promoter-Luc and pCMV-β-gal. After 18 h, the cells were treated with kahweol in the presence of LPS (0.5 μg/ml) for 12 h, harvested and their luciferase and β-galactosidase activities were determined. Luciferase activities were expressed relative to the LPS. The values are expressed a mean ± SD of three individual experiments, performed in triplicate. *P < 0.01, significantly different from the LPS.

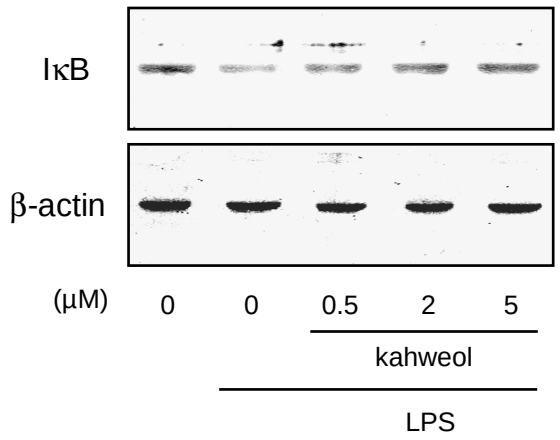

Fig. 9. Effect of kahweol on IκB degradation. The cells were pretreated with kahweol for 30 min and then treated with LPS (0.5 μg/ml) for 30 min. Total cellular protein (50 μg) was separated on 10% SDS-polyacrylamide gels and blotted with antibody specific for IκB. This blot was a representative of each of three independent experiments.

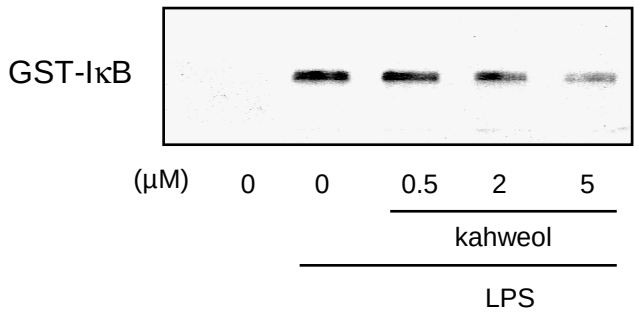

Fig. 10. Effect of kahweol on LPS-induced IκB kinase activity. The cells were treated with 10 M kahweol and LPS (0.5 μg/ml) for 15 min. The total cell lysates (200 μg) were used for immunoprecipitation. IκB kinase activities were assayed with GST-IκB as substrate. This blot was a representative of each of three independent experiments.

H. Effect of kahweol and cafestol dose on the inflammatory response

Various doses (10, and 20 mg/kg) of kahweol, cafestol or saline were locally administered 1 h before being challenged with 1% carrageenan into the five groups of mice with five in each group. All animals were sacrificed at 72 h after the carrageenan administration. The anti-inflammatory indices, such as the volume, protein amounts and cell counts, in the exudates did differ significantly with the two doses (Fig. 11).

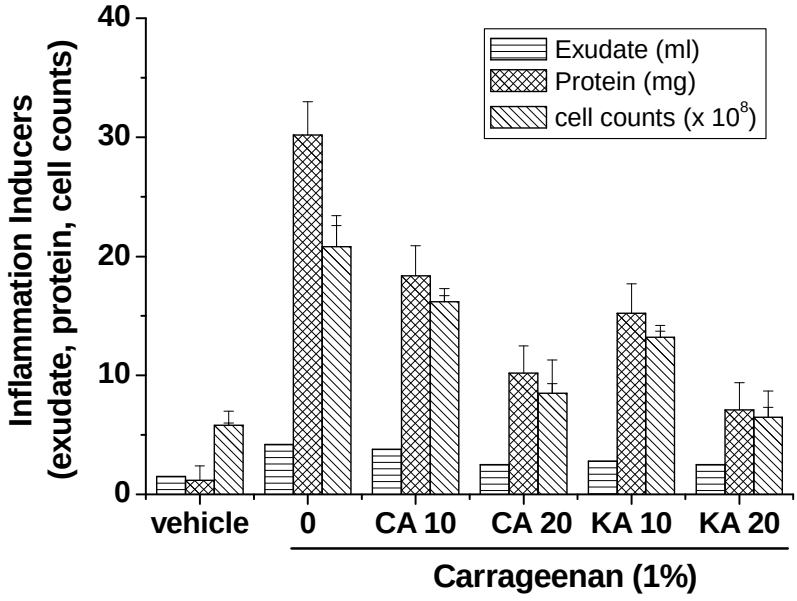

Fig. 11. Effects of cafestol and kahweol on the volume, protein amount and cell counts in the exudates of the air pouch. Randomly chosen mice were treated with vehicle, cafestol and kahweol at 1 h before carrageenan challenge. The animals were sacrificed 72 h later and the exudates in the air pouches were collected. The exudation was assessed for inflammatory cell counts and the concentration of protein. Data are expressed as mean ± SE of three independent experiments, performed in triplicate. *$P < 0.01$, significantly different from the control.

I. Effect of treatment with kahweol and cafestol on COX-2 mRNA expression

To examine whether or not the decrease of PGE_2 in the kahweol- or cafestol-treated animals was caused by inhibition of upregulation of COX-2, expression of COX-2 mRNA within the exudate cells from the mice treated with or without kahweol or cafestol (10, 20 mg/kg) were analyzed by RT-PCR. Five mice were used in each group. Results from the experiments indicated that COX-2 mRNA expressed by the exudate cells in kahweol- or cafestol-treated animals was significantly less than in vehicle-treated animals (Fig. 12).

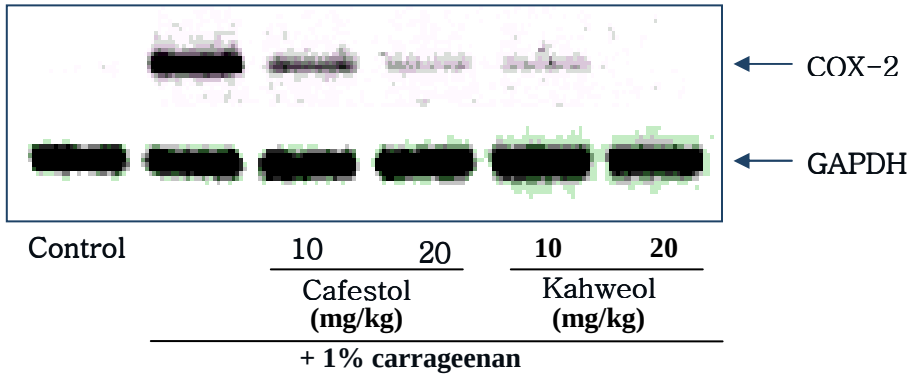

Fig. 12. Effects of cafestol or kahweol on COX-2 mRNA in the exudates of carrageenan-treated air pouch. The total RNA was prepared within the exudate cells from the mice treated with or without kahweol or cafestol (10, 20 mg/kg) were analyzed by RT-PCR. The PCR products were separated on a 2.5% agarose gel and stained with ethidium bromide.

J. Effects of kahweol or cafestol on the PGE_2 production in the mouse air pouch model of inflammation

The carrageenan air pouch model of inflammation was used to determine the capacity of kahweol and cafestol to inhibit PGE_2 production *in vivo*. Carrageenan induced an outstanding PGE_2 release into the exudate fluid when it was administered into the intrascapular area of the control mice. Vasodilatation, edema, and a significant increase in the cellular in were also observed after a carrageenan injection. The administration of kahweol or cafestol (10-20 mg/kg, i.p.) to the mice inhibited the COX-2 protein expression in the cells present in the exudate and caused a decrease in the PGE_2 levels (Fig. 13).

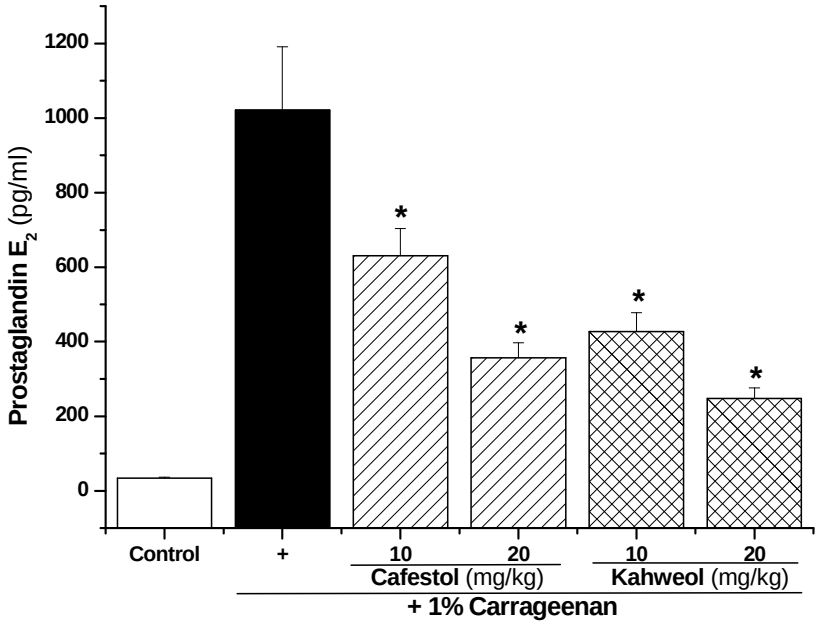

Fig. 13. Effects of kahweol and cafestol on PGE_2 production in carrageenan air pouch model. Kahweol or cafestol (10-20 mg/kg, i.p.) was administered 2 h before the 1% carrageenan injection. The animals were sacrificed 24 h later, and the exudate in the air pouch was collected. PGE_2 in the pouch exudate supernatants was determined by specific enzyme immunoassay. The values are expressed as a mean ± SE of three individual experiments (n=5). *P < 0.01, significantly different from the carrageenan.

L. Histochemical analysis of inflammatory air pouch

The inflammatory response in the pouch tissues were also examined histologically. In the vehicle-treated animals the pouch wall appeared to be very oedematous. There were many inflammatory cells, such as neutrophils and macrophages, and a small number of mast cells were evident, suggesting that the acute inflammatory response was induced in the pouch wall. Recruitment of some neutrophils to the arteriole and the accumulation of mononuclear cells around the vessels were observed. By contrast, the pouch wall of kahweol-, or cafestol-treated animals had little inflammatory cell infiltration and the pouch wall was very thin. These histological findings suggested that the acute inflammatory response in the pouch wall was significantly suppressed by the kahweol, or cafestol treatment (Fig.14).

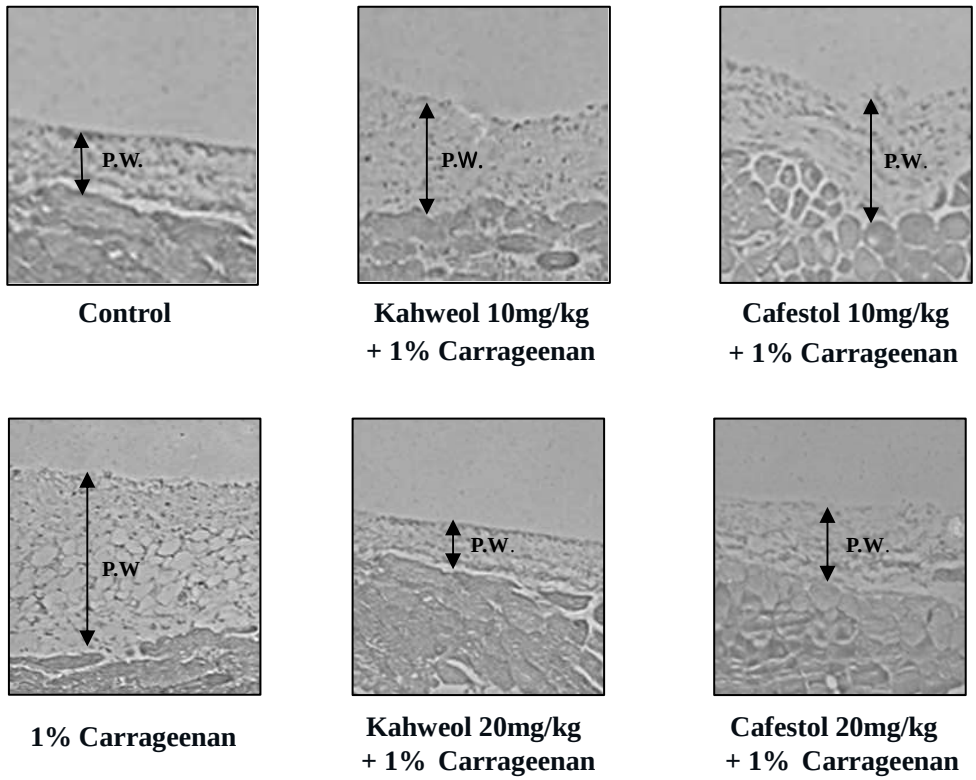

Fig. 14. Histological change in the pouch tissues of kahweol- or cafestol-treated mice in carrageenan air pouch model. Air pouches were removed from the mice 72 h after the challenge, fixed with 10% formalin and embedded in paraffin. Sections were stained with hematoxylin-eosin. (P.W.: Pouch wall)

Ⅳ. Discussion

NO has been implicated in the processes of inflammation and carcinogenesis. There is growing evidence to suggest that inhibitors of iNOS activity or expression are useful for treating inflammation and for the prevention or treatment of cancer [Lala and Chakraborty *et al*., 2001; MacMicking *et al*., 1997; Maeda and Akaike, 1998; Rao *et al*., 2002]. Therefore, agents that interfere with the signaling mechanisms governing the transcription of iNOS would also be expected to inhibit inflammation and tumorigenesis. In this respect, natural product-derived compounds including curcumin, epigallocatechin gallate and other polyphenols were also shown to potentially inhibit iNOS activity and expression [Chan *et al*., 1997; Chan *et al*., 1998; Rao *et al*., 2002]. The major focus of this study was to investigate the anti-inflammatory efficacy of kahweol and cafestol, in relation to their ability to act as an inhibitor of iNOS expression, using the macrophages model. The present work shows that kahweol and cafestol, which are both coffee diterpenes, dose-dependently inhibit the production of NO in LPS-stimulated RAW264.7 macrophage cells, and that kahweol was markedly more active than cafestol in terms of its effect on NO production (Fig. 2, 3). These results indicate the possible inhibition of iNOS induction by these compounds. To obtain a better understanding of the inhibitory mechanism of NO production, the effect of kahweol and cafestol on iNOS expression was investigated in detail. It was found that the simultaneous treatment of kahweol with LPS significantly inhibited iNOS mRNA and protein expression (Fig. 5). However, cafestol did not affect iNOS mRNA or protein expression (Date not shown.).

It has been reported that some compounds, such as epigallocatechin gallate and aspirin, suppress iNOS gene expression, as well as directly inhibiting the catalytic activity of iNOS [Amin *et al*., 1995; Carnovale *et al*., 2001; Chan *et al*., 1997]. By

measuring iNOS enzyme activity in intact cells and cell lysates (Table 1), in which iNOS was induced by LPS, kahweol and cafestol were shown to effectively inhibit iNOS activity at relatively high concentrations of 10-20 μM. As in the case of NO production, kahweol showed a more potent inhibitory effect on iNOS enzyme activity than cafestol. Thus, these results suggest that the inhibition of NO production in LPS-activated RAW264.7 cells by kahweol might be due to both the suppression of iNOS expression at lower concentrations (0.2-5 μM) and the direct inhibition of iNOS enzyme activity at higher concentrations (5-20 μM) however, the inhibition of NO production by cafestol at higher concentrations (5-20 μM) might be due to direct inhibition of iNOS enzyme activity. These phenomena might depend on the structures of these two diterpenes (Fig. 1). The absences of one double bond (C1-C2) on the A ring of kahweol causes its suppressive potency on iNOS expression to differ from that of cafestol. The presence of this one double bone within A ring of cafestol seems to be insufficient to inhibit iNOS expression. Future experiments are needed to determine the relationship between the structures of these two diterpenes and their different functions.

Animal studies have demonstrated that kahweol and cafestol exhibit chemoprotective properties (anti-mutagenic and anti-carcinogenic effects) [Cavin *et al*., 2001; Cavin *et al*., 2002; Huber *et al*., 1997; Miller *et al*., 1991]. These activities have been explained, in part, by the induction of several beneficial modification the xenobiotic metabolism, which, depending on the individual compound, may involve both the reduced activity and enhanced detoxification of mutagens/carcinogen [Cavin *et al*., 2001; Cavin *et al*., 2002; Huber *et al*., 1997; Huber *et al*., 2002]. Epidemiological studies have revealed a protective association between coffee consumption and the risk of certain cancers [Giovannucci, 1998; Inoue *et al*., 1998]. The epidemiology of colorectal cancer provides the most supportive evidence for the existence of a potential coffee dependent protection. In

case-control epidemiology studies, it has been consistently observed that the consumption of coffee with a high amount of kahweol and cafestol was associated with a lower risk of colorectal cancer [Giovannucci, 1998]. Meanwhile, there is increasing evidence to suggest that inhibitors of iNOS activity can be effective anti-inflammatory agents, as well as being beneficial in the prevention and treatment of colon cancer, because NO is a mediator of inflammation, and chronic inflammation predisposes the organism to carcinogenesis [Lala and Chakraborty, 2001; Maeda and Akaike, 1998; Rao *et al*., 2002]. Although numerous agents have been synthesized that effectively inhibit NO by acting as substrate analogs for iNOS, an alternative approach might be to determine whether agents that suppress both the transcription and activity of iNOS are more effective than agents that suppress only one of these effects. In this regard, to the best of our knowledge, this is the first time that it has been suggested that the inhibition of iNOS activity and expression by kahweol and cafestol might contribute to both its anti-inflammatory and chemopreventive activity.

The murine iNOS promoter contains various transcription-factor binding sites, including NF-κB and activator protein-1 (AP-1) sites, which are involved in the induction of other genes by cytokines or LPS [MacMicking *et al*., 1997; Xie *et al*., 1994]. An analysis of the transcriptional activity of the iNOS promoter using deletional mutants revealed the essential role of two NF-κB binding motifs in the control of iNOS expression [MacMicking *et al*., 1997; Xie *et al*., 1994]. Bacterial LPS and cytokines are key mediators in the inflammatory response and have been shown to activate NF-κB, which is a critical component in the inducible expression of multiple genes involved in inflammation [Baeuerle and Baichwal, 1997; MacMicking *et al*., 1997]. Therefore, the analysis of NF-κB activation is a prerequisite for understanding the control mechanism of iNOS gene transcription by kahweol. The transient transfection assay with a NF-κB-dependent luciferase

reporter plasmid showed that kahweol inhibited LPS-induced NF-κB transcriptional activity in a dose-dependent manner (Fig. 10). The present results also showed that kahweol suppressed LPS-induced NF-κB binding activity (Fig. 7). Therefore, it can be concluded that kahweol suppresses iNOS gene transcription through the inhibition of NF-κB activation in the iNOS promoter.

Although we demonstrated the down-regulatory ability of kahweol on iNOS expression by showing the inhibition of NF-κB activation in LPS-stimulated macrophages, the precise mechanism by which kahweol suppresses iNOS expression in macrophages and exerts anti-inflammatory effects is still largely unknown. The activation of p38 or ERK-1/2 has been shown to be involved in the stimulation of NF-κB activity and the subsequent expression of iNOS in murine macrophages [Ajizian *et al*., 1999; Chen and Wang, 1999]. Kahweol might also inhibit these kinase activities, leading to NF-κB activation at or before the phosphorylation step of IκB. The suppression of activation of the NF-κB by kahweol may partially account for this, because there are known to be response elements on the promoters of iNOS genes [Baeuerle and Baichwal, 1997; MacMicking *et al*., 1997]. However, kahweol might also inhibit other transcription factors induced by LPS and suppress the gene transcription of iNOS. Additional studies are needed to answer these questions and to elucidate the mechanisms involved. The current study suggests that kahweol and cafestol might act as anti-inflammatory agents. There is limited information available concerning the anti-inflammatory effects of kahweol and cafestol in humans, so that further *in vivo* studies are necessary to confirm whether kahweol and cafestol do indeed have these effects. In order to investigate the overall anti-inflammatory properties of kahweol and cafestol, a study of the effects of kahweol and cafestol on the *in vivo* suppression of iNOS and TNF- gene expression and the production of immunomodulatory cytokines in mice is underway in our laboratory.

In recent studies, it was shown that kahweol and cafestol, coffee-specific diterpenes, have anti-carcinogenic effects. The present data links these effects of kahweol and cafestol to the inhibition of PGE_2 production. With regard to PGE_2 production, it was shown that kahweol and cafestol inhibit the LPS-activated induction of COX-2 in macrophages. The suppressive effects of kahweol and cafestol were also observed *in vivo*.

The chemopreventive or anti-carcinogenic properties of kahweol and cafestol can be understood, at the least in part, from the induction of several beneficial modification the xenobiotic metabolism, which, depending on the individual compound, may involve both the reduced activation and enhanced detoxification of mutagens/car [Cavin *et al*., 2001; Cavin *et al*., 2002; Huber *et al*., 1997; Huber *et al*., 2002]. Epidemiological studies have revealed a protective association between coffee consumption and the risk of certain types of cancer [Giovannucci, 1998; Inoue *et al*., 1998]. The epidemiology of colorectal cancer provides the most supportive evidence for the existence of a potential coffee-dependent protection. In case-control epidemiology studies, it has been consistently observed that the consumption of coffee with a high amount of kahweol and cafestol is associated with a lower risk of colorectal cancer [Giovannucci, 1998]. Meanwhile, COX-2 has been implicated in the carcinogenic processes [Prescott and Fitzpatrick, 2000], and its over-expression by malignant cells has been shown to enhance cellular invasion, induce angiogenesis, regulate the anti-apoptotic cellular defenses and augment the immunological resistance via PGE_2 production [Hirschowitz *et al*., 2002]. In addition, it has been demonstrated that COX-2 is over-expressed in colon cancer patients [Tsujii *et al*., 1997].

There is growing evidence to suggest that inhibitors of COX-2 activity can be effective anti-inflammatory agents, as well as being beneficial in the prevention and treatment of colon cancer [Chinery *et al*., 1998; Oshima *et al*., 1996].

Therefore, agents that interfere with the signaling mechanisms governing the transcription of COX-2 should also inhibit the inflammation and tumorigenesis [Chinery *et al*., 1998; Subbaramaiah *et al*., 1998]. The major focus of this study was to investigate the effects of kahweol and cafestol on COX-2 expression using a macrophage model. This study showed that kahweol and cafestol, which are both coffee diterpenes, inhibit PGE_2 production in LPS-stimulated macro dose-dependently, and that kahweol had a greater effect on PGE_2 production than cafestol (Fig. 4). These results indicate the possible suppression of COX-2 induction by these compounds. In order to obtain a better understanding of the inhibitory mechanism of PGE_2 production, the effect of kahweol and cafestol on COX-2 expression was investigated in detail. It was found that the simultaneous treatment of kahweol or cafestol with LPS significantly inhibited COX-2 mRNA and protein expression (Fig. 6). Kahweol showed a more potent inhibitory effect on PGE_2 production and the COX-2 expression than cafestol. These phenomena might depend on the structures of these two diterpenes (Fig. 1). The absences of one double bond on the A ring of kahweol causes its suppressive potency on COX-2 expression to differ from that of cafestol. The presence of this one double bone within A ring of cafestol seems to be more insufficient to inhibit COX-2 expression than kahweol. Future experiments are needed to determine the relationship between the structures of these two diterpenes and their different efficacy. This study also investigated whether or not the inhibition of COX-2 expression could be observed in an *in vivo* model of inflammation after treatment with kahweol and cafestol. The murine air pouch model was used because it is characterized by the release of prostaglandins due to the rapid induction of the COX-2 mRNA and protein in the exudate cells [Masferrer *et al*., 1994]. The administration of kahweol and cafestol (2-10 mg/kg, i.p) blocked the COX-2 expression in the cells present in the exudate and produced a dose-dependent decrease in PGE_2 production.

Animal studies have demonstrated that kahweol and cafestol have chemoprotective properties (anti-carcinogenic effects) against carcinogens, such as nitrosamines, 7,12-dimethylbenz[a]anthracene, aflatoxin B_1, and 2-aminol(PhIP) [Cavin *et al*., 2002; Cavin *et al*., 2001; Huber *et al*., 1997]. Carcinogenesis typically involves a cellular transformation, hyperproliferation, invasion, angiogenesis, and metastasis. These processes are activated by various carcinogens, inflammatory agents, and tumor promoters. Carcinogenic agents, such as nitrosamines, 7,12-dimethylbenz[a]anthracene, aflatoxin B_1, which have been used to examine the anti-carcinogenic effects of kahweol and cafestol in animal studies, have been shown to activate NF-κB [Kim *et al*., 2000; Banerjee *et al*., 2002]. Furthermore, NF-κB has been shown to regulate the expression of a number of genes whose products are involved in carcinogenesis/tumorigenesis [Garg and Aggarwal, 2002; Pahl, 1999]. These include COX-2, the anti-apoptosis genes, matrix metalloprotease-9, adhesion molecules, chemokines, inflammatory cytokines, and inducible NO synthase. Because suppression of NF-κB has been implicated in chemoprevention, it is also possible that the anti-carcinogenic effects of kahweol are mediated via the suppression of NF-κB-dependent gene expression.

This study demonstrated that kahweol and cafestol suppress the LPS-activated expression of COX-2, which has NF-κB binding sites in its promoter, and regulates its transcription in macrophages. The inhibitory activity by kahweol correlated with the inhibition of LPS-induced IKK activation, IκBα phosphorylation and degradation, as well as NF-κB-dependent reporter gene transcription. There are various ways that kahweol might inhibit NF-κB activation. In response to diverse stimuli, including LPS, TNF-α, and PMA, NF-κB activation requires the sequential phosphorylation at the serines 32 and 36 of IκBα. In addition, IκBα undergoes phosphorylation by activating IKK, which leads to its ubiquitination, and eventual degradation. Therefore, kahweol must act at a step upstream of IκBα

phosphorylation. In this study, it was found that kahweol inhibited the LPS-induced activation of IKK, IκBα phosphorylation and degradation. The phosphorylation of IκBα is regulated by IKK, which in turn is regulated by many upstream kinases, including NIK, Akt, and mitogen-activated protein kinase kinase kinase 1 [Chen *et al*., 2002; Pahl, 1999; Tak and Firestein, 2001]. This study found that kahweol did not directly affect the activity of IKK (data not shown) which suggesting that kahweol inhibits the LPS-induced IKK activity by an indirect mechanism. Thus, it is possible that kahweol inhibit IKK activation by inhibiting one or many of the upstream kinases responsible for IKK activation.

Although the down-regulatory ability of kahweol on COX-2 expression was demonstrated by showing the inhibition of NF-κB activation in the LPS-stimulated macrophages, the precise mechanism by which kahweol suppresses COX-2 expression in the macrophages and exerts anti-inflammatory effects is still largely unknown. The activation of the MAPK members, ERK and p38 MAPK, has been shown to be involved in the stimulation of NF-κB activity and the subsequent expression of COX-2 in the LPS-activated macrophages [Feng *et al*., 1999]. Kahweol might also inhibit the activity of these kinases, leading to NF-κB activation at or before the IκB phosphorylation step. The suppression of NF-κB activation by kahweol may partially account for this, because there are responsive elements on the promoters of the COX-2 genes. However, kahweol might also inhibit other transcription factors, such as AP-1 and C/EBP, induced by LPS and suppress the COX-2 gene transcription. Additional studies will be needed to answer these questions and elucidate the mechanisms involved. This study suggests that kahweol and cafestol might act as anti-inflammatory agents.

In conclusion, we demonstrated in this study that the coffee-specific diterpenes, kahweol and cafestol, inhibit NO production, PGE_2 production and COX-2 expression in macrophages *in vitro* via the inhibition of NF-κB activation and

mouse inflammatory cells *in vivo*. At least two independent mechanisms contribute to the protective effect produced by kahweol, the suppression of iNOS gene transcription via the inhibition of NF-κB activation and the direct inhibition of the catalytic activity of iNOS. These novel findings may help us to elucidate additional ways in which anti-inflammatory activities can be elicited and provide new insights into the mechanism by which the previously unrecognized biologic activity of kahweol and cafestol might be mediated. Caution has to be exercised with regard to the hypercholesterolemic effect that was previously reported in consumers of coffees with a high kahweol and cafestol content [De Roos *et al.*, 1999], nevertheless these promising data trigger the need for further work, in order to clarify the overall significance of the biological effects of kahweol and cafestol on human health.

Ⅴ. REFERENCES

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국문초록

카와웰 (Kahweol)과 카페스톨(Cafestol)의 항염증효과

정 경 식

염증성 세포가 자극되면 iNOS 에 의하여 일산화산소 증가가 되어 세포에 손상을 가하는데 이것 과정이 염증의 주요한 요인으로 사료된다. 증가된 COX-2 역시 염증 및 암 유발에 관련이 있다. 최근 연구에서 커피원두 함유물질인 카와웰과 카페스톨이 화학적 암억제 효과가 있음이 알려지고 있다. 본 논문에서는 일산화산소의 생성과 iNOS 와 COX-2 의 발현에서 카와웰과 카페스톨 효과를 LPS 처리 후, 활성화된 RAW 264.7 대식세포로부터 조사하였다. 그 결과, LPS 와 함께 처리한 카와웰과 카페스톨에서는 LPS 에 의해 증가된 일산화산소 생성물이 농도의존적으로 감소시켰고, iNOS 활성 및 iNOS 단백질과 mRNA 발현을 억제하였다. iNOS 전사가 NF-κB 에 의해서 조절되기 때문에 NF-κB 상에서 카와웰의 효과를 조사하였다. 카와웰과 카페스톨은 NF-κB 에 의존적으로 전사활성을 억제시킴을 밝혔다. 이들 중 카와웰이 LPS-증식된 NF-κB 의 활성을 억제함을 EMSA 를 통해서 확인하였다. 이런 결과는 카와웰과 카페스톨에 의해 iNOS 의 전사활성 억제가 NF-κB 활성의 억제를 통해서 조절되는 것으로 사료된다.

또 다른 결과에서 카와웰과 카페스톨은 농도 의존적으로 LPS 에 의해 증가된 PGE_2 생성물, COX-2 단백질 발현 및 COX-2 전사부위 활성을 억제하였다.

카와웰은 또한 IκB 분해 및 IκB kinase 활성 억제를 통해 LPS 로 증가된 NF-κB 활성을 차단하였다. 마우스 카라기난(carrageenan) 염증 유발 모델에서도 카와웰과 카페스톨은 염증 유발 모델의 공기낭 내 PGE_2 생성물과 COX-2 발현을 억제하였다.

결론적으로 본 논문 결과는 카와웰과 카페스톨이 이전까지 알려져 있지 않는 생물학적 활성에 기여함으로 암생성 억제 및 항염증 특성을 가지고 있으므로, 실험동물의 연구결과로 함께 화학적 암치료등 새로운 개념에 기여할 바가 클 것으로 사료된다.

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Jesus looked at them intently and said,
"Humanly speaking, it is impossible. But with GOD everything is possible."
Matthew 19:26
But those who wait on the LORD will find new strength.
They will fly high on wings like eagles.
They will fun and not grow weary.
They will walk and not faint.
Isaiah 40:31

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