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Quercetin supplementation suppresses the secretion of pro-inflammatory cytokines in the lungs of Mongolian gerbils and in A549 cells exposed to benzo[*a*]pyrene alone or in combination with β-carotene: in vivo and ex vivo studies

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Abstract

In vitro studies have shown that quercetin modulates the effects of β-carotene induced by stimulants. Whether these reactions happen in vivo, however, is unclear. Thus, we investigated whether quercetin supplementation suppresses the harmful effects of benzo[*a*]pyrene (BaP) alone or combined with β-carotene in the lungs of Mongolian gerbils. The gerbils were given quercetin (100 mg/kg body wt, 3 times/week), β-carotene (10 mg/kg body wt, 3 times/week), and BaP (8 mmol, 2 times/week) alone or in combination by gavage for 6 months. β-Carotene supplementation enhanced the pro-inflammatory effects of BaP in the lungs of gerbils. In contrast, quercetin supplementation significantly decreased the infiltration of inflammatory cells as well as the levels of TNF-α and IL-1β in the bronchoalveolar lavage fluid and plasma of gerbils exposed to BaP or BaP+β-carotene ($P<.05$). Such effects of quercetin supplementation were accompanied by a down-regulation of the expression of phospho-c-Jun and phospho-JNK induced by BaP or BaP+β-carotene in the lungs of gerbils. Furthermore, in the ex vivo study, we found that quercetin-metabolite-enriched plasma (QP) obtained from gerbils acted like a JNK inhibitor to significantly suppress the secretion of pro-inflammatory cytokines induced by BaP or BaP+β-carotene in A549 cells ($P<.05$). QP also suppressed the activation of the JNK pathway in the A549 cells. These results suggest that supplemental quercetin suppress the pro-inflammatory effect of β-carotene induced by BaP in vivo and ex vivo. The regulation of the JNK pathway by the metabolites of quercetin contributes, at least in part, to such effects of quercetin in vivo.

Keywords: Quercetin; β-Carotene; Benzo[*a*]pyrene; Mongolian gerbil; A549 cells

1. Introduction

Several studies have shown that diets rich in β-carotene, a phytochemical present in many fruits and vegetables (papayas, carrots, green leafy vegetables, etc) are beneficial to the health of humans and may prevent the development of inflammation-associated diseases, including cancers [1–3]. Such an effect has been attributed to the various bioactivities of β-carotene, including antioxidant activity [2,4]. In humans, β-carotene is mostly present in adipose tissue and the liver. Plasma levels of β-carotene consumed through diet have been found to be about 0.2-0.6 μM [5]; and a 12-fold increase in plasma levels of β-carotene was observed in those taking β-carotene supplements (30 mg/d) [6]. However, growing evidence suggests that high-dose β-carotene supplementation exerts harmful effects in the lungs of smokers [6,7] and animals exposed to cigarette smoke [8,9]. One of the possible reasons for such harmful effects is the production of oxidative metabolites of β-carotene induced by a free radical-rich environment, such as in lungs exposed to cigarette smoke [4]. It has been shown that the presence of other antioxidants, ascorbic acid and α-tocopherol, decreases the production of oxidative metabolites of β-carotene [9] and increases its safety and bioactivity [9,10]. However, except for ascorbic acid and α-tocopherol, limited data are available on whether other compounds existing in vegetables and fruit interact with β-carotene.

Quercetin, a flavonoid, is another phytochemical found in various vegetal foods, such as onions, apples, and green leafy vegetables, and has also been suggested to possess anti-oxidative and anti-inflammatory properties [11–13]. In our previous studies, we found that quercetin through its antioxidant property and inhibition of CYP450 enzyme expression may decrease the harmful effect of β-carotene in A549 cells induced by the cigarette-associated carcinogen 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone and benzo[*a*]pyrene (BaP) [14,15]. In addition, we found that quercetin alters the effects of β-carotene on the secretion of pro-inflammatory mediators

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in stimulated monocyte/macrophage-like cells [16]. However, all of the evidence for the regulation of quercetin on the effects of β-carotene is derived from in vitro studies with quercetin aglycone, which is almost entirely converted to various conjugated metabolites in human plasma due to its efficient phase II metabolism [17]. The sum of quercetin metabolites in plasma has been reported to be about 0.9 μM after the consumption of 270 g of fried onions [17], and the level could reach 1-5 μM after supplementation with high doses of quercetin [18]. In addition, an animal study has shown that quercetin and/or its metabolites are distributed to many tissues including the lungs in rats and pigs after consuming quercetin [19]. However, it is unclear whether quercetin metabolites have effects similar to doses of quercetin aglycone.

Thus, the aims of the present study were to investigate whether quercetin modulates the effect of β-carotene induced by BaP exposure in vivo and to determine the involvement of the regulation of the Jun N-terminal kinase (JNK) pathway, which has been shown to be involved in the development of several diseases induced by stimulants or chronic inflammation [9,20,21]. In the present study, Mongolian gerbils were supplied with β-carotene and quercetin alone or in combination by gavage and were additionally exposed to BaP for 3 or 6 months. BaP, present in cigarette smoke, air pollutants and foodstuffs, has been known to be a carcinogen which requires metabolic activation by cytochrome P450 in the intestine and liver in humans. The major carcinogenic metabolite of BaP, benzo[a]pyrene-7,8-diol-9,10-epoxide, attacks the bases of DNA and may lead to lung cancer in humans [22]. Several studies have also demonstrated that benzo[a]pyrene-7,8-diol- 9,10-epoxide is present in the forestomach, lungs and livers of mice administered BaP orally and induces tumor formation in these tissue [23–25]. In addition, BaP may act to regulate metabolic enzymes and signaling pathways, such as the JNK pathway [20], to express various toxic effects including pro-inflammatory effects [20,26,27]. For example, Uno et al. [28] found that oral exposure to BaP increases the immune cells in the bone marrow of mice with different gene types in different manners; Goulaouic et al. [27] found that BaP induces significant secretion of pro-inflammatory cytokines in macrophage-like cells. We used gerbils as an animal model, because they are well known, like humans, to absorb and accumulate intact β-carotene in the body [29–31] and are readily available. Furthermore, we also conducted an ex vivo study to confirm the effects of the metabolites of quercetin on modulating the pro-inflammatory effect of β-carotene induced by BaP in human lung cells. That is, we preincubated A549 human adenocarcinomic alveolar basal epithelial cells with quercetin-metabolite-enriched plasma, which was obtained from gerbils fed quercetin by gavage, alone or in combination with β-carotene followed by BaP exposure. Then, the secretion of pro-inflammatory cytokines and the phosphorylation of c-Jun and JNK in A549 cells were also determined.

2. Materials and methods

2.1. Reagents

All chemicals used were reagent grade or higher. BaP, β-carotene, quercetin, and tetrahydrofuran (THF) were purchased from Sigma Chemical Co. (St. Louis, Mo, USA). Dimethyl sulfoxide (DMSO) was purchased from Merck Co. (Darmstadt, Germany).

2.2. In vivo study and animals

Fifty male gerbils (weighing 50–60 g) were obtained from the laboratory animal center at Taichung Veterans General Hospital (Taichung, Taiwan). The animals were housed in hanging wire mesh cages in a room controlled for temperature (25±2°C), humidity (65±5%), and an alternating 12-hour light: dark cycle. After being acclimated for 1 week, the animals were randomly assigned to the following five groups (n=10/group) for 6 months: (1) control, (2) BaP-exposed, (3) BaP-exposed plus β-carotene supplementation, (4) BaP-exposed plus quercetin supplementation, and (5) BaP-exposed plus β-carotene in combination with quercetin supplementation. BaP, β-carotene, and quercetin were dissolved in refined lard (Weilih Food, Taipei) before being fed to the animals by gavage. We chose refined lard as the vehicle because the quercetin concentration in this oil was undetectable and was markedly lower than in soybean oil or in corn oil (about 0.075 and 0.009 μM, respectively). For BaP exposure, the animals were fed BaP in amounts of 8 μmol 2 times/week. According to a study by Hecht et al. [24], BaP at a total dose of 24 μmol administered by gavage induced lung adenoma in A/J mice after 26 weeks. For β-carotene supplementation, the animals were fed β-carotene at a pharmacological dose, that is, 10 mg/kg body weight 3 times/week. The weekly dose of β-carotene administered to the gerbils was approximately equivalent to a weekly intake of 30 mg β-carotene/day in a 70 kg person [6]. For quercetin supplementation, the animals were fed quercetin at a concentration of 100 mg/kg body weight 3 times/week, which was applied based on previous studies [18,19], our preliminary study and the dose of β-carotene we used in this study. In our preliminary study, we found that quercetin supplementation at a concentration of 100 mg/kg body weight (equivalent to an intake of 700 mg/d in a 70 kg person) but not 10 mg/kg body weight significantly increased the plasma levels of quercetin metabolites in gerbils (data not shown). The dose of quercetin was 10 fold the dose of β-carotene and the proportion of quercetin/β-carotene is possible in the human diet or with supplements [18]. The control group was given refined lard only. All animals were allowed free access to a standard rodent diet (Lab 5001, Purina Mills, St. Louis, MO) and water during the study.

During the 6-month experimental period, the gerbils' body weights were recorded weekly. However, there were no significant differences in body weight among the groups in the study (data not shown). Blood samples were collected from the retro–orbital plexus of the gerbils under deep isoflurane anesthesia. After the experiment, the animals were killed by CO_2 asphyxiation and a portion of the lungs was used to collect bronchoalveolar lavage fluid (BALF). The rest of the lungs were collected for protein analysis. All blood samples and tissues were stored at -80°C until analyzed.

To compare the levels of β-carotene in the tissues of the gerbils, we conducted an additional 3-month animal study. Twenty-four gerbils were randomly assigned to the following 4 groups (n=6/group): (1) control, (2) β-carotene supplementation, (3) BaP-exposed plus β-carotene supplementation, and (4) BaP-exposed plus β-carotene in combination with quercetin supplementation. After the experiment, the lungs and the livers of the gerbils were collected and stored according to the method mentioned above. Before determining the level of β-carotene, the tissues were homogenized in PBS-methanol (2:1, v/v; containing 1% ascorbic acid). Then, β-carotene was extracted in ethanol and hexane (1:2; v/v) and was determined at 450 nm by HPLC as described by Rundhaug et al. [32]. All study protocols were approved by the Institutional Animal Care and Use Committee at Chung Shan Medical University, and animal care followed the guidelines of the National Research Council [33].

2.3. Ex vivo study and cell culture

Using gerbils, we also conducted an ex vivo study. The gerbils received either refined lard alone (control group; 200 μL/kg body wt/week) or quercetin (100 mg/kg body wt/week) in refined lard by gavage weekly for 12 weeks. Blood samples were collected weekly from week 4 to week 12 from the retro-orbital plexus of the gerbils in a test tube containing heparin. After centrifugation (650×g, 20 min), the plasma samples were sterilized by filtration (0.22 μm filters, Millipore) and were stored at -80 °C. The plasma samples were analyzed and used within 1 week. The plasma samples obtained from the quercetin-fed group 2 h after feeding were referred to as QP. We measured concentrations of quercetin and its metabolites, quercetin-3-glucuronide (Q3G), quercetin-3′-sulfate (Q3'S), and isorhamnetin, in the plasma and homogenous lungs according to a method described previously [19,34]. The quercetin aglycone was undetectable while the total glucuronized and sulfated quercetin in the QP reached a maximum (5.6±0.8 μM; $P<.05$). In addition, the mean levels of three major metabolites of quercetin, quercetin-3-glucuronide, quercetin-3′-sulfate, and methylquercetin, were 3.5, 2.9, and 0.9 μM, respectively, in the QP. The plasma obtained from the control group at the same time was referred to as control plasma (CP). A549 cells were obtained from the Bioresource Collection and Research Center (Hsinchu, Taiwan) and were cultured as described previously [14,15]. An equal number (1×10^4/mL) of cells were incubated for 24 h before the various treatments. Before the experiment, the medium was removed and the cells were washed twice with PBS. Then, in the QP groups, a new medium containing 10% QP with or without 20 μM of β-carotene was added and the samples were incubated for 4 h before 20 μM of BaP exposure; in the 5 μM quercetin groups, the cells were incubated with the new medium containing 10% CP and 5 μM of quercetin (the final concentration in the medium) alone or in combination with β-carotene before BaP exposure; and in the control group the cells were incubated with 10% CP without quercetin. The doses of β-carotene and BaP used were according to our previous studies [15,16]. In some experiments the JNK inhibitor, SP600125, was added 1 h prior to the various treatments. Studies have demonstrated that SP600125 inhibits phosphorylation of JNK induced by various stimulants including BaP [35–37]. Quercetin was prepared in DMSO at a stock concentration of 50 mM and β-carotene was prepared in THF at 10 mM. These two compounds were dissolved in CP before use. After being washed twice with PBS, the cells were incubated with serum-free medium containing 20 μM BaP for the indicated time.

2.4. Immune cell counts and determination of concentrations of TNF-α and IL-1β in bronchoalveolar lavage fluid

We collected BALF using the method described by Miyabara et al. [38] and Lee et al. [39]. After centrifugation (300×g, 5 min) of the BALF, the supernatant was removed

and the cells were resuspended in 100 μL PBS. To count different immune cells, 10 μL of the cell suspension was added to a slide and was stained with the Diff Quick stain kit (IMEB Inc., San Marcos, California, USA), followed by microscopic examination with five fields per slide.

The concentrations of TNF-α and IL-1β in the BALF supernatant were determined using an enzyme-linked immunosorbent assay (ELISA) kit (eBioscience, San Diego, CA, USA). In addition, the concentrations of the two cytokines in the plasma and in the conditioned media from cell culture were also determined.

2.5. Western blotting

Total proteins were extracted from the lungs of gerbils and A549 cells. Expression levels of JNK, c-Jun, phospho-JNK, and phospho-c-Jun were determined by Western blotting. Briefly, lung tissue was gently homogenized with lysis buffer (with 50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 10% glycerol, 2 mM EDTA, and 2 mM EGTA) and the cells were lysed with 20% SDS containing 1 mM phenylmethylsulfonyl fluoride. The lysate was sonicated on ice for 1 min followed by centrifugation at 12,000×g for 30 min at 4 °C. An amount of protein (150 μg) from the supernatant was resolved by SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with Tris-buffered saline buffer (20 mM Tris-HCl, 150 mM NaCl, pH 7.4) containing 5% nonfat milk, the membrane was incubated with anti-JNK, anti-c-Jun, anti-phosphorylated-JNK, or anti-c-phosphorylated-Jun antibody (Santa Cruz, California USA) followed by horseradish peroxidase-conjugated second antibody and was then visualized using an ECL chemiluminescent detection kit (PerkinElmer Life Sciences, Inc., Waltham, MA, USA). The relative levels of the immunoreactive bands were quantified using a luminescent image analyzer (LSA-100, Fujifilm, Japan).

2.6. Statistical analysis

Values are expressed as means±SD. Using one-way factorial analysis of variance (ANOVA) followed by Duncan's multiple-range test we compared group means. Differences were considered statistically significant at $P<.05$. In addition, two-way ANOVA was performed to test the interaction of quercetin (or QP) and β-carotene in the regulation of the effects of BaP. No significant interactions were observed ($P>.05$ for F value).

3. Results

3.1. Cell profiles in the BALF

Changes in cell numbers in the BALF of gerbils exposed to BaP in combination with β-carotene or quercetin were investigated. As shown in Table 1, BaP significantly increased the number of total cells, macrophages, and lymphocytes by 52%, 175%, and 38 %, respectively, in BALF. β-Carotene significantly enhanced the stimulative effect of BaP on the numbers of total cells, lymphocytes, and neutrophils by 64%, 24%, and 360%, respectively, whereas quercetin significantly and completely suppressed such effects of BaP. In addition, quercetin also suppressed the combined effect of BaP and β-carotene on the number of total cells, macrophages and lymphocytes.

3.2. Levels of proinflammatory cytokines in the plasma and BALF

TNF-α and IL-1β are two well-known proinflammatory cytokines. As expected, BaP exposure significantly induced the expression of TNF-α in plasma and in BALF (Fig. 1). β-Carotene significantly enhanced the stimulative effect of BaP on the level of TNF-α in plasma and BALF by 124% and 217%, respectively. Quercetin significantly suppressed the effects of BaP and BaP plus β-carotene, respectively, on TNF-α expression in plasma and in BALF. For IL-1β, the trend was similar to that for TNF-α in plasma and in BALF (Fig. 2). That is, β-carotene enhanced the stimulative effect of BaP, whereas quercetin suppressed such an effect of β-carotene.

Table 1
The cell profile in bronchoalveolar lavage fluid

Cell numbers (1×10^3 / total BALF)				
Group	Total cells	Macrophages	Lymphocytes	Neutrophils
Control	4.41 ± 1.63^{a}	1.18 ± 0.01^{a}	3.81 ± 0.07^{b}	0.07 ± 0.02^{a}
BaP	6.72 ± 1.54^{b}	3.25 ± 1.14^{bc}	5.27 ± 0.15^{d}	0.06 ± 0.04^{a}
BC+BaP	11.02 ± 0.813^{c}	4.27 ± 1.75^{c}	6.51 ± 0.39^{e}	0.28 ± 0.07^{b}
Q+BaP	4.30 ± 1.23^{a}	1.03 ± 0.44^{a}	3.18 ± 0.60^{a}	0.03 ± 0.04^{a}
Q+BC+BaP	5.83 ± 1.47^{ab}	2.42 ± 0.04^{ab}	4.59 ± 0.27^{c}	0.21 ± 0.01^{b}

Gerbils were administered β-carotene (BC), quercetin (Q), and BaP alone or combined by gavage for 6 months. The control group was administered lard (the vehicle) only. Values (means±SD, n=9-10) in the same column not sharing a common letter are significantly different ($P<.05$).

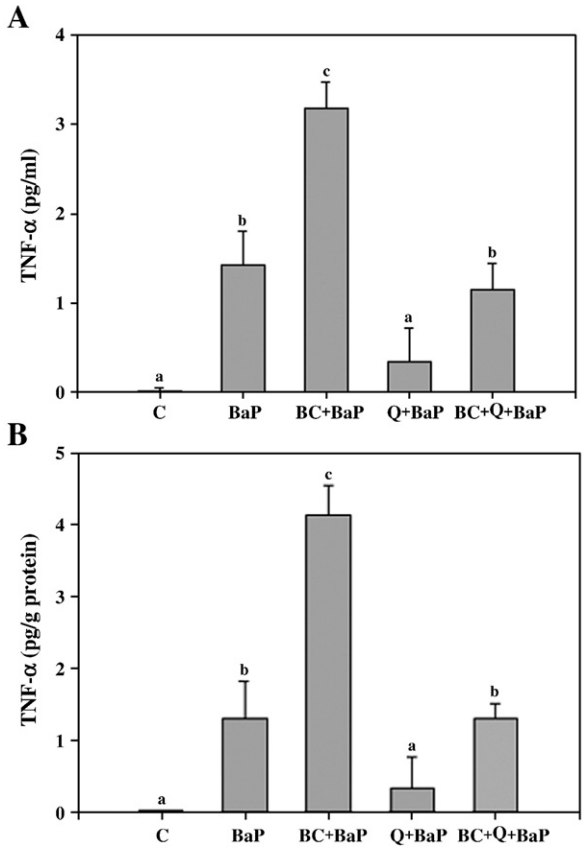

Fig. 1. The level of TNF-α in plasma (A) and in bronchoalveolar lavage fluid (B) in gerbils. Gerbils were administered β-carotene (BC), quercetin (Q), and BaP alone or combined by gavage for 6 months. The control group was administered lard (the vehicle) only. Values (means±SD, n=9-10) not sharing a common letter are significantly different ($P<.05$).

3.3. Phosphorylated c-Jun and JNK in the lungs of gerbils

BaP exposure significantly increased the levels of phospho-c-Jun and phospho-JNK in the lungs of gerbils by 1.9- and 3.6-fold, respectively, compared with the control group (Fig. 3). Supplementation with β-carotene significantly increased the levels of BaP-induced phospho-c-Jun and phospho-JNK by 16% and 72%, respectively. In contrast, quercetin treatment significantly suppressed the expression of the two phosphorylated proteins induced by BaP or BaP plus β-carotene to the control levels. There were no significant differences in total protein levels of either c-Jun or JNK among the groups.

3.4. β-Carotene levels in lungs and in livers of gerbils

Using HPLC with a UV-detector, we determined the concentration of β-carotene in the lungs and livers of gerbils after the various treatments for 3 months. As shown in Fig. 4, β-carotene supplementation significantly increased the accumulation of β-carotene in the lungs and livers of gerbils by 46% and 452%, respectively. BaP exposure decreased the levels of β-carotene in these two tissues, whereas quercetin supplementation suppressed the BaP-induced consumption of β-carotene in these tissues. We found that compared with the

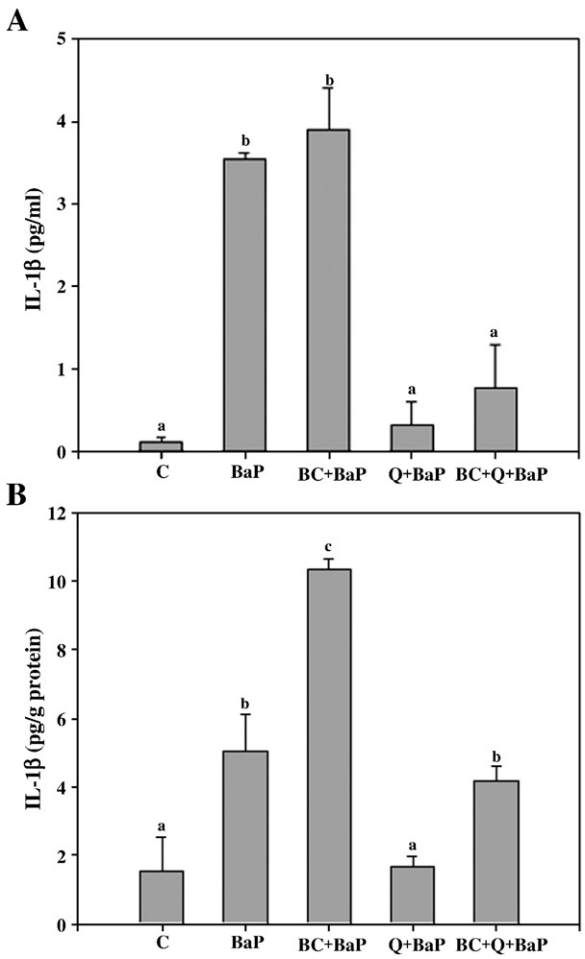

Fig. 2. The level of IL-1β in plasma (A) and in bronchoalveolar lavage fluid (B) in gerbils. Gerbils were administered β-carotene (BC), quercetin (Q), BaP alone or combined by gavage for 6 months. The control group was administered lard (the vehicle) only. Values (means±SD, n=9-10) not sharing a common letter are significantly different ($P<.05$).

control, quercetin supplementation significantly increased the level of the sum glucuronized and sulfated quercetin in the lungs and livers by 3.9±0.3 fold and 3.2±0.5 fold ($P<.05$), respectively.

3.5. Ex vivo study

To confirm the observation in gerbils, we incubated A549 cells with β-carotene in combination with QP before exposure to BaP. We also compared the effect of QP with that of quercetin aglycone. β-Carotene at 20 μM significantly increased the secretion of BaP-induced TNF-α and IL-1β (Fig. 5). QP and SP600125, a JNK inhibitor, significantly decreased the stimulative effect of BaP or BaP plus β-carotene on the secretion of proinflammatory cytokines. QP also suppressed the expression of phospho-c-Jun and phospho-JNK induced by BaP, although β-carotene had only a slight effect or no effect on BaP-induced phosphorylation of these two proteins (Fig. 6). The effects of QP were similar to those of 5 μM of quercetin in A549 cells (Figs. 5 and 6). However, neither QP nor β-carotene alone affected the secretion of proinflammatory cytokines nor the activation of the JNK pathway in A549 cells (data not shown).

4. Discussion

β-Carotene and quercetin are two phytochemicals that are ubiquitously present in vegetables and fruit. Our previous in vitro studies suggested that quercetin modulates the effects of β-carotene induced by stimulants [14–16]. However, whether quercetin exerts such an effect in vivo is unclear. In the present study, our data showed that supplementation with β-carotene alone in gerbils enhanced the stimulative effect of BaP on the increase in the number of inflammation-associated cells in BALF. This effect was particularly pronounced in neutrophils, which may play an important role in lung injury induced by smoking-associated carcinogens. Supplementation with β-carotene also enhanced the effect of BaP on the secretion of pro-inflammatory cytokines, although histopathologic examination of lung sections from gerbils exposed to BaP or BaP plus β-carotene did not show obvious inflammatory cell infiltration (data not shown). In contrast, quercetin significantly inhibited most of these β-carotene effects, indicating that quercetin suppressed the pro-inflammatory effects of β-carotene induced by BaP in vivo. Because conjugated metabolites of quercetin but not quercetin aglycone are mostly present in the plasma of gerbils, the effects of quercetin supplementation could be mainly attributed to the quercetin metabolites. Our ex vivo study provides supportive data, because QP also suppressed the enhancing effect of β-carotene on the secretion of proinflammatory cytokines induced by BaP in A549 cells.

Using ferrets, which also absorb and accumulate β-carotene similar to humans, as an in vivo model, Liu et al. [8] showed that supplementation with β-carotene in high doses enhances the proliferation of alveolar macrophages and keratinized squamous metaplasia lesions in the lungs of cigarette smoke-exposed animals. The harmful effects of high doses of β-carotene have been attributed to the oxidative metabolites of β-carotene that are induced by cigarette smoke [4,8]. The weekly dose of β-carotene administered to the gerbils in the current study was approximately equivalent to a weekly intake of 30 mg β-carotene/day in a 70 kg person [6], and we observed a similar effect of β-carotene supplementation on gerbils exposed to BaP. Furthermore, in agreement with previous studies [29,31], we also found that supplementation with β-carotene increased the accumulation of β-carotene in the livers and lungs of gerbils, whereas BaP exposure, which increased oxidative stress [13,15,40], diminished the levels of β-carotene in these two organs, suggesting the formation of oxidative metabolites of β-carotene. This provides an explanation for the proinflammatory effect of β-carotene supplementation in gerbils exposed to BaP.

The intake of quercetin has been suggested to be beneficial in the prevention of cigarette-smoke-induced disease in the human lungs [41,42]; however, the mechanisms underlying such effects remain unclear. A recent study suggested that quercetin possesses anti-inflammatory effects in vivo [43]; this study showed that quercetin reduced inflammatory pain by inhibiting oxidative stress and cytokine production in Swiss mice. In the present study, we also found that supplementation with quercetin and QP significantly suppressed the induction of pro-inflammatory cytokines by BaP or BaP plus β-carotene in vivo and ex vivo, respectively. These data indicated that the metabolites of quercetin possess anti-inflammatory properties. Although we do not know which metabolites contributed to such effects, it has been shown that quercetin-3′-sulfate, one of the major metabolites in the plasma of humans and gerbils, inhibits the production of pro-inflammatory cytokines in A549 cells induced by IL-1 [44]. Tribolo et al. [45] showed that quercetin-sulfate, quercetin glucuronides, and methylquercetin-3-glucuronide inhibit the expression of key molecules, such as vascular cell adhesion molecule-1, that regulate monocyte recruitment in atherosclerosis.

There may be various possible mechanisms underlying the anti-inflammatory effects of quercetin supplementation in gerbils, and the results of our study suggest that the down-regulation of the JNK

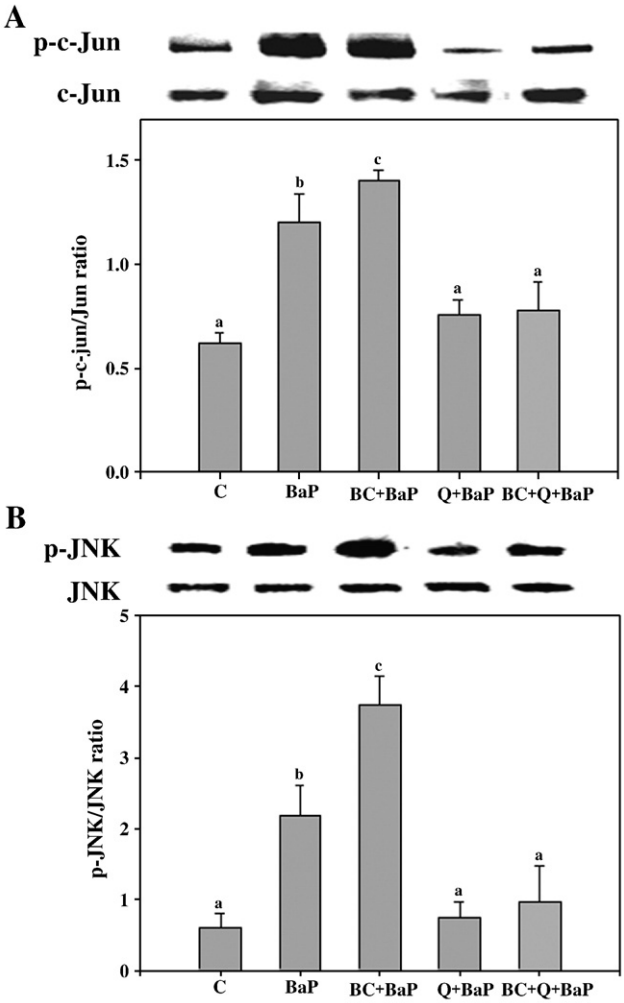

Fig. 3. The phospho-c-Jun(p-c-Jun)/total c-Jun (c-Jun) ratio (A) and phospho-JNK (pJNK)/total JNK (JNK) ratio (B) in lungs of gerbils. Gerbils were administered β-carotene (BC), quercetin (Q), and BaP alone or combined by gavage for 6 months. The control group was administered lard (the vehicle) only. Values (means±SD, n=9-10) not sharing a common letter are significantly different ($P<.05$).

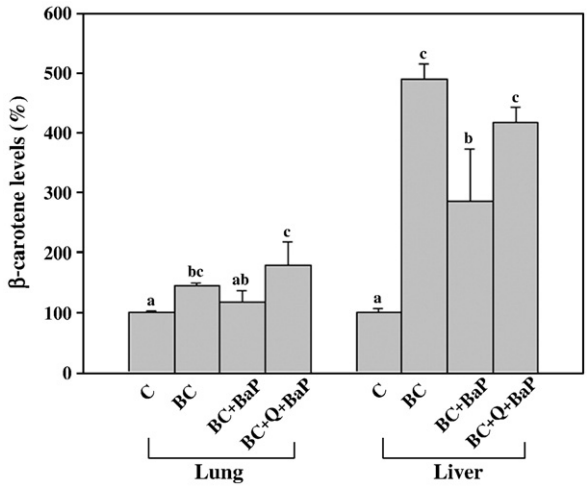

Fig. 4. The levels of β-carotene in lungs and livers of gerbils. Gerbils were administered β-carotene (BC), quercetin (Q), and BaP alone or combined by gavage for 3 months. The control group was administered lard (the vehicle) only. Values (means±SD, n=6) not sharing a common letter are significantly different ($P<.05$).

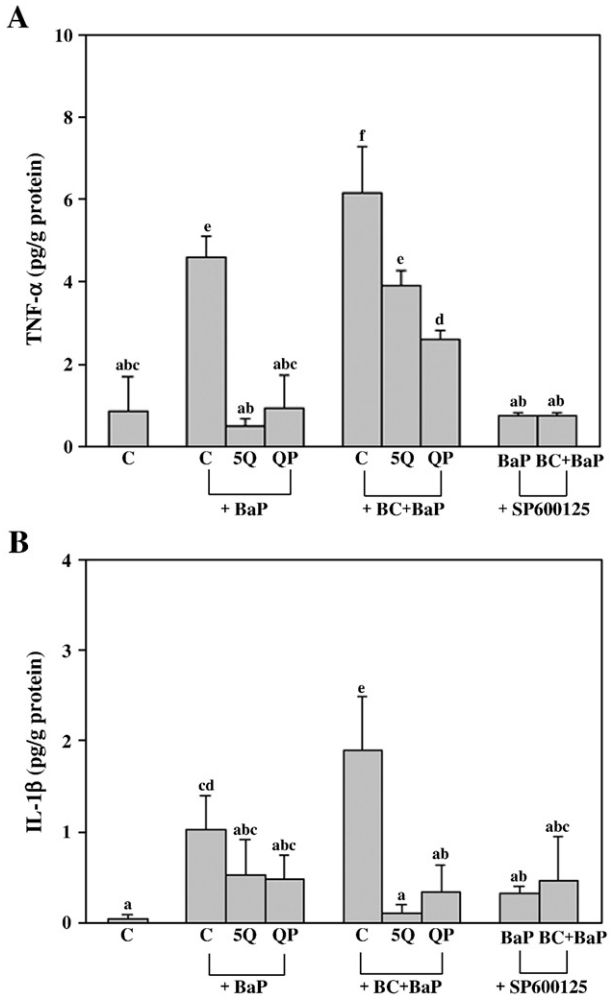

Fig. 5. The secretion of TNF-α (A) and IL-1β (B) in A549 cells. The cells were pre-incubated with 5 μM quercetin (5Q; dissolved in CP) or QP alone or in combination with 20 μM β-carotene (BC) for 4 h before BaP exposure for 24 h. The cells in the control group (C) were incubated with the medium containing CP without quercetin. QP was the plasma sample obtained from the gerbils fed quercetin, while CP was the plasma obtained from the gerbils fed the vehicle only. Values (means±SD, n=3) not sharing a common letter are significantly different ($P<.05$).

pathway is a possible mechanism. JNK, a stress-activated protein, is a mitogen-activated protein kinase and can phosphorylate c-Jun and increase AP-1 transcription activity [46] and, eventually, mediate cell proliferation and the production of inflammatory cytokines [47]. Our study showed that BaP- or BaP plus β-carotene-induced secretion of pro-inflammatory cytokines in gerbils and A549 cells accompanied the increase of the phosphorylation of c-Jun and its kinase, JNK. However, preincubation of A549 cells with the JNK inhibitor SP600125 completely suppressed the secretion of TNF-α and IL-1β, indicating the importance of the JNK pathway in such proinflammatory effects of BaP or BaP plus β-carotene in lung cells. Quercetin supplementation and QP also significantly inhibited the phosphorylation of c-Jun and JNK in vivo and in cell culture, respectively. These data also suggest that regulation of the JNK pathway contributes to the anti-inflammatory effect of quercetin metabolites. A recent study showed that quercetin sulfate/glucuronides may act by suppressing the JNK pathway to inhibit high-glucose-induced cell apoptosis [48], which supports our finding. In addition, we cannot rule out the regulation of other signaling pathways. For example, quercetin and its methylated metabolites have been shown to affect the survivability of primary cortical

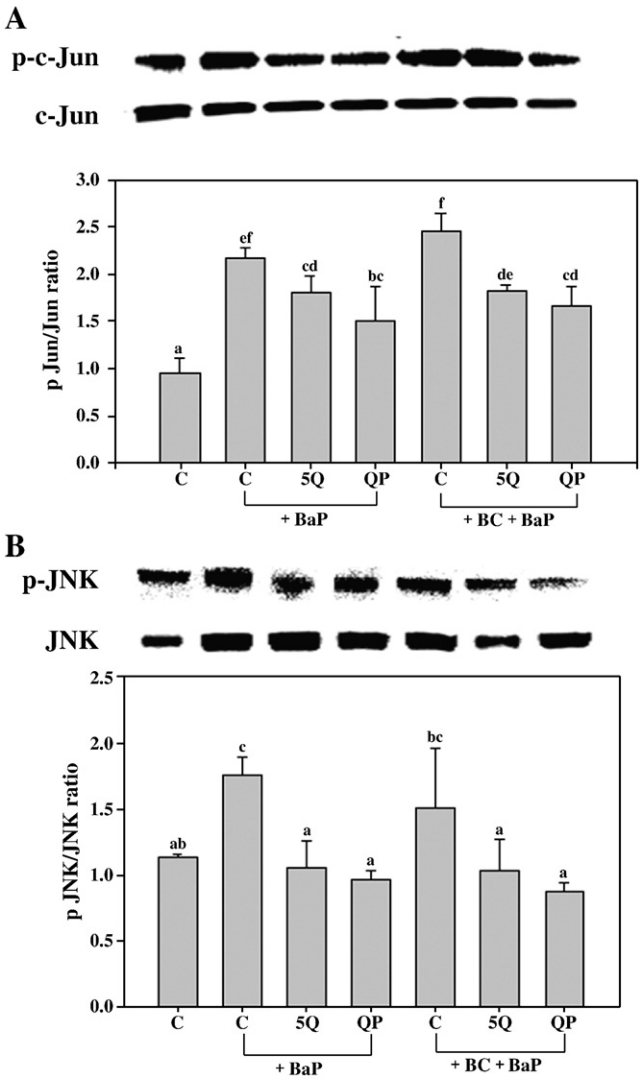

Fig. 6. The phospho-c-Jun (p-c-Jun)/total c-Jun (c-Jun) ratio (A) and phospho-JNK (pJNK)/total JNK (JNK) ratio (B) induced by BaP in A549 cells. The cells were pre-incubated with 5 μM quercetin (5Q; dissolved in CP) or QP alone or in combination with 20 μM β-carotene (BC) for 4 h before BaP exposure for 1 h. The cells in the control group (C) were incubated with the medium containing CP without quercetin. QP was the plasma sample obtained from the gerbils fed quercetin, while CP was the plasma obtained from the gerbils fed the vehicle only. Values (means±SD, n=3) not sharing a common letter are significantly different ($P<.05$).

neurons by inhibiting the Akt and ERK1/2 signaling cascades [49]; quercetin administered orally exerts an anti-inflammatory effect by suppressing the NF-κB activation in the lung of ovalbumin-sensitized and -challenged mice [50]. In addition, in our preliminary study, we found that quercetin-glucuronide suppressed the expression of cytochrome P (CYP) 1A1/2 induced by BaP (data not shown), indicating the possibility that the modulation of the array hydrocarbon receptor (AHR) pathway is involved in the beneficial effects of quercetin in vivo. However, further studies are warranted to investigate the possibility. The activation of the AHR pathway by BaP increases the expression of CYP enzyme and has been shown to play a crucial role in the harmful effects of BaP including the pro-inflammatory effect [51–53]. Furthermore, a decrease in oxidative stress by quercetin metabolites may also be involved in such anti-inflammatory effects of quercetin supplementation. Several studies have shown that quercetin metabolites may act as antioxidants [48,54,55], but further studies are needed to investigate the precise role of the individual quercetin metabolites in anti-inflammation.

Another question raised here is how quercetin influences the effect of β-carotene. Quercetin may inhibit β-carotene oxidative degradation directly or exert its effect by indirect mechanisms. The dose of quercetin used in our animal study was 10 fold the dose of β-carotene, and this proportion of quercetin/β-carotene is possible through the human diet or supplements [18]. We found that quercetin significantly inhibited the consumption of β-carotene induced by BaP in the lungs and livers of gerbils (Fig. 4), suggesting that quercetin metabolites (or quercetin) in vivo could act as an antioxidant or increase the activity of the antioxidative system. In addition, as we have mentioned above, quercetin metabolites could act as signal molecules by modulating the activation of c-Jun induced by stimulants or other pathways. Especially in our ex vivo study, quercetin metabolites could play the role of signal molecules rather than antioxidants because the final concentration of quercetin metabolites in the medium was only ~0.56 μM (because of 10 % of QP in the medium), while the concentration of β-carotene was 20 μM. The dose of quercetin metabolites in the medium was comparable to that in human plasma [17], however, the dose of β-carotene we used in the medium was higher than in human plasma. We used such a high dose of β-carotene because, according to our previous study, 20 μM of β-carotene increases the harmful effect of stimulants while 2 μM of β-carotene has a slightly suppressed effect [15,16].

In conclusion, the results of the current study demonstrate that β-carotene supplementation enhanced the proinflammatory effects of BaP in the lungs of gerbils. In contrast, quercetin supplementation suppressed this harmful effect of β-carotene and its consumption in vivo. Quercetin supplementation also down-regulated the activation of the JNK pathway induced by BaP or BaP plus β-carotene in the lungs of gerbils. Preincubation of A549 cells with QP, which was rich in quercetin metabolites but not quercetin aglycone, similarly suppressed the harmful effects of β-carotene induced by BaP. These data suggest that through inhibition of β-carotene degradation directly or indirect mechanisms, quercetin modulates the effects of β-carotene in vivo. This study may provide insight into why uptake of β-carotene from vegetables and fruit seems better in lung cancer prevention than pure supplementation.

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