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Lycopene Supplementation Inhibits Lung Squamous Metaplasia and Induces Apoptosis via Up-Regulating Insulin-like Growth Factor-binding Protein 3 in Cigarette Smoke-exposed Ferrets[1]

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ABSTRACT

Higher intake of lycopene is related to a lower risk of lung cancer in human studies. Lung cancer risk is associated with higher plasma levels of insulin-like growth factor I (IGF-I) and/or lower levels of IGF-binding protein 3 (IGFBP-3). However, little is known regarding whether lycopene can inhibit cigarette smoke-induced lung carcinogenesis through modulation of IGF-I/IGFBP-3, cell proliferation, and apoptosis. We investigated the effects of lycopene supplementation at a low dose (1.1 mg/kg/day, which is equivalent to an intake of 15 mg/day in humans) and a high dose (4.3 mg/kg/day, which is equivalent to 60 mg/day in humans) on plasma IGF-I/IGFBP-3 levels, histopathological changes, proliferating cellular nuclear antigen (PCNA) expression, BAD phosphorylation, and apoptosis (caspase 3 assay) in lungs of ferrets with or without cigarette smoke exposure for 9 weeks. We found that ferrets supplemented with lycopene and exposed to smoke had significantly higher plasma IGFBP-3 levels ($P < 0.01$) and a lower IGF-I/IGFBP-3 ratio ($P < 0.01$) than ferrets exposed to smoke alone. Both low- and high-dose lycopene supplementations substantially inhibited smoke-induced squamous metaplasia and PCNA expression in the lungs of ferrets. No squamous metaplasia or PCNA overexpression were found in the lungs of control ferrets or those supplemented with lycopene alone. Furthermore, cigarette smoke exposure greatly increased BAD phosphorylation at both Ser^{136} and Ser^{112} and significantly decreased cleaved caspase 3 in the lungs of ferrets, as compared with controls. The elevated phosphorylation of BAD and down-regulated apoptosis induced by cigarette smoke in the lungs of ferrets was prevented by both low- and high-dose lycopene supplementations. Lycopene levels were increased in a dose-dependent manner in both plasma and lungs of ferrets supplemented with lycopene alone. However, lycopene levels were markedly lower in both plasma and lungs of ferrets supplemented with lycopene and exposed to smoke. Furthermore, smoke exposure increased *cis* isomers (26% for 13-*cis* and 22% for 9-*cis*) of lycopene in the lungs of ferrets, compared with that of ferrets supplemented with lycopene alone (20% for 13-*cis* and 14% for 9-*cis*). In conclusion, lycopene may mediate its protective effects against smoke-induced lung carcinogenesis in ferrets through up-regulating IGFBP-3 and down-regulating phosphorylation of BAD, which promote apoptosis and inhibit cell proliferation.

INTRODUCTION

Beneficial effects of carotenoid-rich fruits and vegetables on lung cancer risk have been found in numerous observational studies (1–4). In contrast, three large randomized clinical trials conducted to assess the chemopreventive effect of β-carotene supplementation against lung cancer found either no beneficial effect among healthy men (5) or a possible harmful effect among smokers or asbestos workers (6–8). One potential explanation for these findings is that other carotenoids or phytonutrients rather than β-carotene may account for the protective effects of fruits and vegetables on lung cancer risk observed in observational studies.

Recent epidemiological studies provide supportive evidence that lycopene may have chemopreventive effect against a broad range of epithelial cancers including lung cancer (9–13). Lycopene reduces the incidence of lung adenocarcinomas in mice treated with chemical carcinogens (14). Lycopene is an antioxidant that exhibits a physical quenching rate constant for singlet oxygen almost twice as high as that of β-carotene (15, 16). Lycopene may also enhance gap junction communication (17, 18), suppress growth factor-induced cell proliferation (19, 20), and inhibit neoplastic transformation of normal human and mouse cells (21).

The IGFs[3] are mitogens that play a pivotal role in regulating cell proliferation, differentiation, and apoptosis (22). Several lines of evidence implicate IGF-I and its receptor, IGF-IR, in lung cancer and other malignancies (22, 23). The ability of IGF-I to increase cell survival is mediated by phosphorylating BAD (a member of the BH3-only subfamily of Bcl-2) on serine residues (Ser^{136}, Ser^{112}, and Ser^{155}; Refs. 24, 25). Ser^{136} of BAD is preferentially phosphorylated by one of the downstream pathways of the IGF-I receptor PI3K/Akt/protein kinase B (26, 27). Cytokines that activate the MAPK pathway phosphorylate BAD on Ser^{112} and Ser^{155} (28, 29). In the presence of phosphorylation, BAD is found in the cytosol, bound to 14-3-3 proteins rather than Bcl-xL, and does not induce cell death, whereas nonphosphoralyted BAD is localized to the mitochondria, bound to Bcl-xL, which promotes its proapoptotic effect (24).

IGFBP-3, one of the six members of the IGFBP family and a major circulating protein in human plasma (23, 30), regulates bioactivity of IGF-I by sequestering IGF-I away from its receptor in the extracellular milieu, thereby inhibiting the mitogenic and antiapoptotic action of IGF-I. IGFBP-3 also inhibits cell growth and induces apoptosis independent of IGF-I and its receptors (31, 32). IGFBP-3 is a potent inhibitor of both PI3K/Akt/PKB and MAPK signaling pathways (33). Furthermore, the results from studies in lung cancer cell lines (20, 34) and human populations studies (35–37) strongly support an important role of high levels of IGF-I and low levels of IGFBP-3 in lung cancer. A strong negative correlation between plasma IGFBP-3 and lung cancer risk has been observed in a number investigations (35–37). Furthermore, a recent study suggests the important role of IGFBP-3 as a tumor suppressor against lung carcinogenesis (38). Finally, serum levels of IGFBP-3 are also negatively correlated with the number of cigarettes smoked/day or pack-year history of smoking (39).

Recent studies have shown that higher intake of cooked tomatoes or lycopene is significantly associated with lower circulating levels of IGF-I (40) and higher levels of IGFBP-3 (41). Lycopene treatment markedly reduced the IGF-I stimulation of activator protein 1 binding

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[3] The abbreviations used are: IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; PI3K, phosphatidylinositol 3′-kinase; MAPK, mitogen-activated protein kinase; HPLC, high performance liquid chromatography; PCNA, proliferating cellular nuclear antigen; RAR, retinoic acid receptor.

and was associated with suppression of IGF-stimulated cell cycle progression in mammary cancer cells (20). However, little is known regarding whether lycopene can inhibit smoke-induced lung carcinogenesis through modulation of IGF-I/IGFBP-3 levels, cell proliferation, and apoptosis.

The ferret is an excellent animal model for studying lycopene's effect on lung carcinogenesis because of the similarities between ferret and human in absorption and accumulation of intact carotenoids (42, 43), lung architecture, and cigarette smoke-induced lung pathology (44–47). In this study, we evaluated the effects of lycopene supplementation at both a low dose and a high dose on blood and tissue IGF-I/IGFBP-3 concentrations, lung histopathological changes, phosphorylation of BAD, cell proliferation, and apoptosis in ferrets with or without cigarette smoke-exposure for 9 weeks. Ferrets in the low-dose lycopene group were supplemented with 1.1 mg/kg/day of lycopene, which is equivalent to an intake of 15 mg/day in humans. This dose of lycopene is higher than the average intake of lycopene (9.4 ± 0.3 mg/day) in the United States men and women (Dietary Reference Intakes: Vitamin A, Vitamin K and Micronutrients. IOM Report). Ferrets in the high-dose lycopene group were supplemented with 4.3 mg/kg/day of lycopene, which is equivalent to 60 mg/day in humans and achievable in a diet enriched by tomato products or supplements.

MATERIALS AND METHODS

Animals, Diet, and Study Groups. Male adult ferrets (1.0–1.2 kg) from Marshall Farms (North Rose, NY) were housed in an Association for Assessment and Accreditation of Laboratory Animal Care international accredited animal facility at the Human Nutrition Research Center on Aging at Tufts University, and fed a semipurified ferret diet (Research Diets, Inc., New Brunswick, NJ) that contains no lycopene, as determined by HPLC analysis in our laboratory and water *ad libitum*. The ferrets were maintained individually in suspended stainless steel cages measuring 24 × 24 × 18 inches. Before the experiment began, all ferrets were quarantined for a minimum of 2 weeks to confirm health status. Thirty-six male ferrets were randomly assigned to six groups ($n = 6$ in each group) for 9 weeks as follows: (*a*) control; (*b*) smoke exposed; (*c*) low-dose lycopene (1.1 mg/kg body weight/day); (*d*) high-dose lycopene (4.3 mg/kg body weight/day); (*e*) smoke-exposed plus low-dose lycopene; and (*f*) smoke-exposed plus high-dose lycopene. During the 9-week experimental period, ferret body weights were recorded weekly. All ferrets were terminally exsanguinated under deep isoflurane anesthesia after the 9 weeks of the experimental period. Tissues and plasma were collected and stored at −80°C until analyzed. All experimental procedures were approved by the Animal Care and Use Committee at the Human Nutrition Research Center on Aging and conducted under the supervision of the Animal Care and Use Committee.

Cigarette Smoke Exposure. The procedure of cigarette smoke exposure in ferrets was as described elsewhere with minor changes (44). Briefly, ferrets were placed in a chamber connected with a smoking device. Cigarette smoke was drawn out of the cigarettes (Standard Research Cigarettes, Type 1R4F; Tobacco and Health Research Institute, University of Kentucky, Lexington, KY) with a vacuum and then exhausted into the chamber. During the first 2 weeks of study, the number of cigarettes was gradually increased to a rate of 10 cigarettes/30 min twice in the morning and twice in the afternoon and then maintained for the rest of the 9-week experimental period. In our previous study, we demonstrated that this amount of smoke exposure in the ferret is similar to that found in humans smoking one and a half packages of cigarettes/day based upon the concentration of urinary cotinine equivalents (44). In this study, we exposed ferrets to smoke from nonfiltered cigarettes to ensure that the animals would develop squamous metaplasia in the lungs. The concentration of urinary cotinine equivalents (~18 μg/ml urine) of this amount of smoke exposure to the ferret is similar to that found in humans smoking two packages of cigarettes/day. The ferrets had free access to food and water. The nonsmoke-exposed ferrets were housed in a separate room and underwent the exact same procedures as the smoke-exposed animals, except that they received no smoke exposure.

Lycopene Supplementation. Because the total absorption of β-carotene by the ferret is about five times less than humans (43, 44) and similarity in chemical structure of lycopene with β-carotene, the calculation for lycopene dosage was based upon the assumption that the ferret absorbs lycopene five times less efficiently than humans. Lycopene 10% dry power (Lycovit 10%; BASF, Edison, NJ) was dissolved into 0.5 ml of corn oil and fed p.o. to ferrets at either a low dose or a high dose (1.1 *versus* 4.3 mg/kg body weight/day in the ferret, which is equivalent to 15 *versus* 60 mg/day in a 70-kg human) every morning for 9 weeks. Ferrets in the control group received corn oil only (0.5 ml/kg body weight/day) every morning for 9 weeks.

Measurement of IGF-I and IGFBP-3. Ferret plasma levels of IGF-I and IGFBP-3 were measured by ELISA using kits from Diagnostic Systems Laboratory, Inc. (Webster, TX). Briefly, after pretreatment with sample buffer to separate IGF-I from its binding proteins, 20 μl of 1:100 diluted sample and 100 μl of assay buffer were incubated in microtitration wells coated with anti-IGF-I antibody at room temperature for 2 h. Samples were then aspirated, and the wells were washed five times with a wash solution. The enzyme-conjugated anti-IGF-I antibody was added to the wells and incubated at room temperature for 30 min. After washing, the 3,3′,5,5′-tetramethylbenzidine substrate buffer was added. When color developed, the reaction was stopped, and the absorbance of the solution in the wells was read at 450 nm using a microplate reader. The level of serum IGF-I was calculated based on human IGF-I standards. For measurement of IGFBP-3, ferret serum samples were diluted to 1:50 with a sample dilution buffer. The IGFBP-3 levels were measured using a similar method to IGF-I, except that anti-IGFBP-3 antibody was used instead of anti-IGF-I antibody.

Histopathology and Immunohistochemistry. The histopathological procedure that was used has been described elsewhere (48). Briefly, the right upper lobe of each ferret lung was inflated and fixed by an intratracheal instillation of 10% formalin. The samples were then embedded in paraffin. Five-μm sections were made using a microtome and stained with H&E for histopathological examination. The sections were examined by two blinded independent investigators by light microscopy to determine the presence or absence of lung squamous metaplasia. Because the squamous metaplasia lesions are spotty and localized, we treated a ferret as positive if any squamous metaplasia lesion was observed. Such histological changes were confirmed by immunohistochemistry with anti-keratin antibody. Otherwise, the animal was considered negative.

Tissue Extraction and HPLC Analyses. A total of 0.2 g of lung tissue (wet weight) was homogenized with 5 ml of CHCl3/CH3OH (2:1, v/v) and 50 μl of internal standard (Echinenone). After the addition of 500 μl of normal saline, the mixture was vortexed and centrifuged for 10 min at 800 × *g* at 4°C. Hexane (3 ml) was added after the lower layer had been collected. The chloroform and hexane layers were evaporated under N_2, and a 50-μl aliquot of the extract reconstituted with ethanol was injected onto the HPLC. The HPLC system consisted of a series 410 LC pump (Perkin-Elmer, Norwalk, CT), a Waters 717 plus autosampler (Millipore, Milford, MA), a C30 carotenoid column (3 μm, 150 × 4.6 mm; YMC, Wilmington, NC), a HPLC column temperature controller (model 7950, column heater/chiller; Jones Chromatography, Lakewood, CO), a Waters 994 programmable photodiode array detector, and a Waters 840 digital 350 data station. The Waters 994 programmable photodiode array detector was set at 450 nm for lycopene. The HPLC mobile phase was methanol/methyl-tert-butyl ether/water (83:15:2, v/v/v, and 15 g/liter ammonium acetate in the water, solvent A) and methanol/methyl-tert-butyl ether/water (8:90:2, v/v/v, and 10 g/liter ammonium acetate in the water, solvent B). The gradient procedure, at a flow rate of 1 ml/min (16°C), was as follows: (*a*) 100% solvent A was used for 3 min followed by a 7-min linear gradient to 70% solvent A; (*b*) a 5-min hold followed by a 9-min linear gradient to 45% solvent A; (*c*) a 2-min hold, then a 7-min linear gradient to 5% solvent A; and (*d*) a 6-min hold, then a 1-min linear gradient back to 100% solvent A. In this HPLC system, 13-*cis*-lycopene, 9-*cis*-lycopene and all-*trans*-lycopene eluted at 30.7, 34.3, and 37.1 min, respectively. Lycopene isomers were quantified relative to the internal standard by determining peak areas calibrated against known amounts of standards.

Immunoprecipitation and Western Blot Analysis. Immunoprecipitation and Western blot for total BAD, phospho-BAD-Ser^{136}, phospho-BAD-Ser^{112}, and BcL-xL were carried out using the whole tissue lysates of lung from 6 ferrets in each group. Briefly, lung tissues were incubated in extraction buffer (25 mM HEPES, 300 mM NaCl, 1.5 $MgCl_2$, 0.2 mM EDTA, 0.05% Triton

Table 1 *Plasma concentrations of lycopene isomers in six groups of ferrets after 9 weeks of treatment*

Treatment	All-*trans*-lycopene (nmol/liter)	13-*cis*-lycopene (nmol/liter)	9-*cis*-lycopene (nmol/liter)	Total lycopene (nmol/liter)
Control (sham exposure)	ND	ND	ND	ND
Low-dose lycopene	128 ± 28^{a}	$48 \pm 5^{a,d}$	52 ± 8^{a}	226 ± 35^{a}
High-dose lycopene	208 ± 38^{b}	83 ± 14^{b}	83 ± 11^{b}	373 ± 60^{b}
Smoke	ND	ND	ND	ND
Smoke plus low-dose lycopene	77 ± 17^{c}	$31 \pm 15^{a,c}$	35 ± 9^{c}	142 ± 36^{c}
Smoke plus high-dose lycopene	110 ± 21^{a}	53 ± 7^{d}	$65 \pm 14^{a,b}$	228 ± 33^{a}

^{a,b,c,d} For a given column, data not sharing a common superscript letter are statistically significantly different at $P < 0.05$.
Values are expressed as mean ± SD. $n = 6$ ferrets in each group.
ND, not detected.

X-100, and 20 mM β-glycerophosphate and a mixture of protease inhibitors) with agitation at 4°C for 30 min. The mixture was then centrifuged, and supernatants were collected. The resulting lysates were precleared by incubation with protein A agarose (1:10). Antibodies against BAD and BcL-xL (1:50) were then added and incubated with gentle rocking overnight at 4°C. Protein A agarose (1:10) was added; the mixture was rocked gently for 3 h at 4°C before the beads were washed five times with ice-cold cell lysis buffer. Protein concentrations were determined using a Commassie Plus protein assay kit (Pierce, Rockford, IL). Thirty μg of protein were added to a solution containing 50 mM Tris-HCl (pH 6.8), 200 mM 2-mercaptoethanol, 2% SDS, 0.1% bromphenol blue, 10% glycerol, and boiled for 6 min before being run on 12% SDS-PAGE. The protein was transferred to a polyvinylidene difluoride membrane by using a semi-dry transfer system. The membrane was incubated with 5% nonfat milk in Tris-buffered saline containing 0.05% Tween 20 in Tris-buffered saline (TBS-T) for 60 min. The membrane was incubated with primary antibodies (BAD, Phospho-BAD-Ser^{136}, Phospho-BAD-Ser^{112}, and BcL-xL) diluted in TBS-T (1:1000) overnight at 4°C. The blots were washed and incubated with a horseradish peroxidase-labeled secondary antibody for 60 min and then washed for 15 min. The blots were developed using the enhanced chemiluminescence Western Blotting system (Amersham, Piscataway, NJ), and analyzed by using a densitometer (GS-710 calibrated imaging densitometer; Bio-Rad, Hercules, CA). Antibodies against total BAD, phospho-BAD-Ser^{136,} phospho-BAD-Ser^{112}, Bcl-xL, and cleaved caspase 3 were purchased from Cell Signaling (Beverly, MA). Western blot for cleaved caspase 3 was carried out by using the whole tissue lysate of lung from 6 ferrets in each group. Western blot analysis for PCNA and RARα was carried out by using nuclear protein extracts from the lungs of the ferrets in each of the six groups, which were prepared as described previously (44). Monoclonal antibodies against PCNA and polyclonal antibodies against RARα were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Statistical Analysis. Concentrations of lycopene isomers, IGF-I, IGFBP-3, and IGF-I/IGFBP-3, and expressions of PCNA and cleaved caspase 3, total BAD, phospho-BAD, and Bcl-xL were expressed as means ± SD, and significant differences were compared using ANOVA followed by Tukey's Honest test at $P < 0.05$. Fisher's exact test was used for pathological changes to compare the difference among groups at $P < 0.05$.

RESULTS

Concentrations of Lycopene Isomers in Plasma and Lung Tissue of Ferrets after 9 Weeks of Treatment. Lycopene supplementation at both a low dose and a high dose for 9 weeks significantly increased the concentrations of lycopene in both plasma (Table 1) and lung tissue (Table 2) of the ferrets. Because the semipurified ferret diet used in this study did not contain lycopene, we did not detect lycopene in plasma and lung tissue in the control and smoke exposure alone groups of ferrets. Lycopene concentrations in both plasma (Table 1) and lung tissue (Table 2) in the high-dose lycopene groups with or without smoke exposure were significantly higher than those in the low-dose lycopene groups with or without smoke exposure. Smoke exposure decreased plasma and lung lycopene concentrations in both the low-dose and high-dose lycopene groups; the percentages of decrease in lycopene concentrations were ~40% in plasma for both the low-dose and high-dose lycopene groups (Table 1) and 90% in lung tissue for both the low-dose and high-dose lycopene groups (Table 2). In the lung tissue of ferrets supplemented with lycopene, all-*trans*-lycopene was the predominant isomer (64–70%), and 13-*cis*- and 9-*cis*-lycopene accounted for 18–20% and 12–16%, respectively. Smoke exposure decreased the percentage of the all-*trans*-isomer of lycopene, whereas *cis* isomers (24–28% for 13-*cis* and 20–23% for 9-*cis*) of lycopene were proportionally increased in the lungs of ferrets (Table 2). In plasma, all-*trans*-lycopene accounted for an average of 54% of total lycopene and 13-*cis*- and 9-*cis*-lycopene accounted for 22 and 24%, respectively (Table 1). There were no changes in the proportions of lycopene isomers in either the low-dose and high-dose lycopene groups with or without smoke exposure (Table 1).

Plasma Concentrations of IGF-I and IGFBP-3 in Six Groups of Ferrets after 9 Weeks of Treatment. Ferrets supplemented with either a low dose or a high dose of lycopene with or without smoke exposure had significantly higher plasma IGFBP-3 concentrations than control ferrets (Table 3). Compared with controls, ferrets exposed to smoke exposure alone had significantly lower plasma IGFBP-3 concentrations. However, ferrets exposed to smoke and supplemented with lycopene had similar plasma IGFBP-3 concentrations to those supplemented with lycopene alone. There were no significant differences in plasma IGF-I concentrations in the six groups of ferrets. Ratio of IGF-I/IGFBP-3 was lower in ferrets supplemented with either a low dose or a high dose of lycopene alone than control ferrets, although this reduction reached a significant level

Table 2 *Lung concentrations of lycopene isomers in six groups of ferrets after 9 weeks of treatment*

Treatment	All-*trans*-lycopene (nmol/kg)	13-*cis*-lycopene (nmol/kg)	9-*cis*-lycopene (nmol/kg)	Total lycopene (nmol/kg)
Control (sham exposure)	ND	ND	ND	ND
Low-dose lycopene	240.2 ± 35.2^{a} (70%)	61.8 ± 10.4^{a} (18%)	40.2 ± 9.7^{a} (12%)	342.2 ± 42.3^{a} (100%)
High-dose lycopene	735.7 ± 123.7^{b} (64%)	236.2 ± 49.4^{b} (20%)	187.2 ± 36.2^{b} (16%)	1159.2 ± 145^{b} (100%)
Smoke	ND	ND	ND	ND
Smoke plus low-dose lycopene	15.6 ± 3.8^{c} (53%)	6.8 ± 1.9^{c} (24%)	6.6 ± 1.7^{c} (23%)	29.2 ± 6.8^{c} (100%)
Smoke plus high-dose lycopene	51.7 ± 8.7^{d} (52%)	27.2 ± 4.8^{d} (28%)	21.4 ± 4.2^{d} (20%)	100.3 ± 16.5^{d} (100%)

^{a,b,c,d} For a given column, data not sharing a common superscript letter are statistically significantly different at $P < 0.05$.
Values are expressed as mean ± SD in nmol/kg wet weight. $n = 6$ ferrets in each group.
ND, not detected.

only for the high dose of lycopene. In contrast, ratio of IGF-I/IGFBP-3 was significantly higher in ferrets exposed to smoke alone than the control ferrets. However, the ratio of IGF-I/IGFBP-3 was significantly lower in ferrets supplemented with either a low dose or a high dose of lycopene and exposed to smoke than in those exposed to smoke alone.

Histopathological Changes and PCNA Protein Expressions in the Lung Tissue of Ferrets after 9 Weeks of Treatment. Normal ferret lung histology and lung squamous metaplastic lesions are illustrated in Fig. 1. Squamous metaplastic lesions were observed in the lung tissue of 6 of 6 ferrets exposed to smoke alone but only in 2 of 6 ferrets exposed to smoke and supplemented with a low-dose lycopene (Table 4). No squamous metaplasia was observed in the lung tissue of ferrets in the control group, the low-dose lycopene group, the high-dose lycopene group, and the high-dose plus smoke exposure group after 9 weeks of intervention (Table 4). PCNA expression was increased by 3-fold in the smoke exposed group (Fig. 2). There were no significant differences in PCNA protein expression in ferrets supplemented with either a low dose or a high dose of lycopene alone as compared with controls. Furthermore, PCNA expression did not differ between ferrets supplemented with either a low dose or a high dose of lycopene and exposed to smoke and control ferrets.

Table 3 *Plasma concentrations of IGF-I and IGFBP-3 in six groups of ferrets after 9 weeks of treatment*

Treatment	IGF-I (ng/ml)	IGFBP-3 (ng/ml)	IGF-I/IGFBP-3 ratio
Control (sham exposure)	754.07 ± 208.30[a]	2.33 ± 0.83[a]	360.01 ± 134.57[a]
Low-dose lycopene	1022.88 ± 121.83[a]	3.68 ± 0.63[b]	282.08 ± 41.98[a,b]
High-dose lycopene	928.36 ± 333.93[a]	3.85 ± 1.31[b]	238.27 ± 46.55[b]
Smoke	745.02 ± 141.36[a]	1.55 ± 1.15[c]	706.98 ± 40.65[c]
Smoke plus low-dose lycopene	929.04 ± 127.71[a]	3.34 ± 0.69[b]	281.89 ± 30.20[a,b]
Smoke plus high-dose lycopene	993.30 ± 80.25[a]	3.79 ± 0.86[b]	275.93 ± 76.58[a,b]

[a,b,c] For a given column, data not sharing a common superscript letter are statistically significantly different at $P < 0.05$.

Values are expressed as mean ± SD.

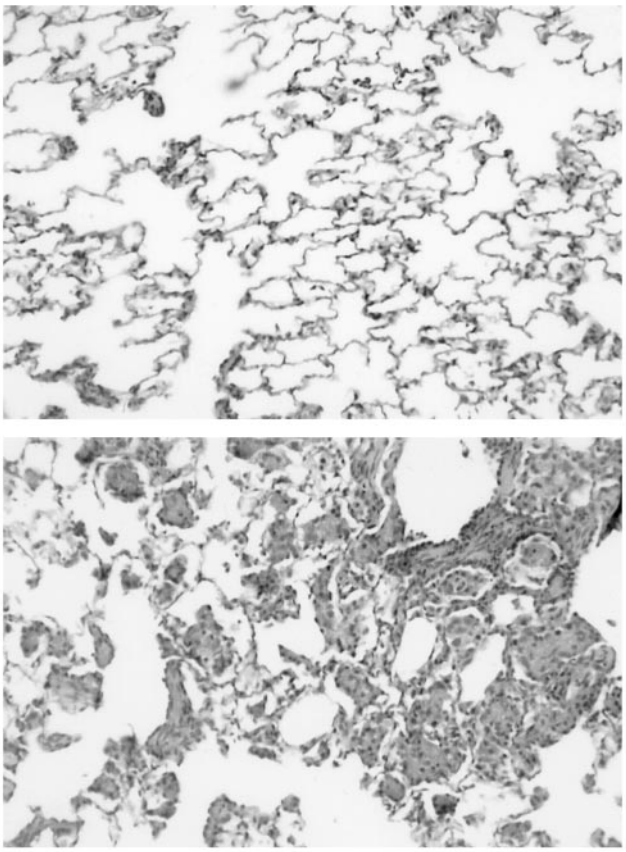

Fig. 1. Histopathology of the ferret lungs under light microscopy. Lung tissue sections were stained with H&E. Panels are at magnification of ×100. *Top panel:* normal lung; *bottom panel:* lung squamous metaplastic lesions induced by tobacco smoke exposure.

Table 4 *The incidence of lung squamous metaplasia in ferrets after 9 weeks of cigarette smoke exposure with or without lycopene supplementation*

Treatment	No. of ferrets with lung squamous metaplasia/the total number of ferrets in each group
Control (sham exposure)	0/6[a]
Low-dose lycopene	0/6[a]
High-dose lycopene	0/6[a]
Smoke	6/6[b]
Smoke plus low-dose lycopene	2/6[a,c]
Smoke plus high-dose lycopene	0/6[a]

[a,b,c] Data not sharing a common superscript letters for a given value indicate that the incidence of lung squamous metaplasia statistically significantly different from each other ($P < 0.05$, Fisher's exact test).

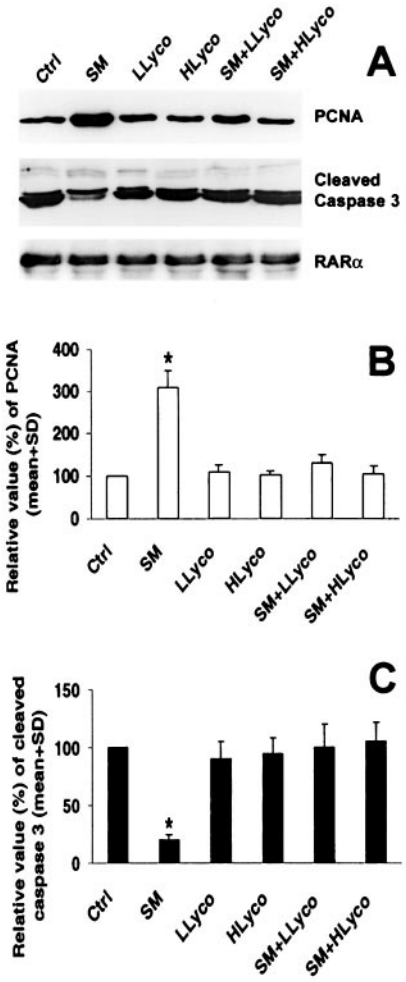

Fig. 2. Expressions of PCNA, cleaved caspase 3, and RARα protein levels in lung tissue from six groups of ferrets (Ctrl, control; SM, smoke-exposed; LLyco, low-dose lycopene supplemented; HLyco, high-dose lycopene supplemented; SM + LLyco, smoke-exposed plus low-dose lycopene supplemented, and SM + HLyco, smoke-exposed plus high-dose lycopene supplemented). *A*, representative Western blot analysis for PCNA, cleaved caspase 3, and RARα protein levels. The size of the detected PCNA was M_r 34,000. The sizes of the detected cleaved Caspase 3 were M_r 17,000–19,000. The size of the detected RARα was M_r 53,000. *B*, the intensity of the protein signal of PCNA determined by densitometry ($n = 6$ ferrets in each group) and expressed by the relative values (means ± SD). *C*, the intensity of the protein signal of cleaved caspase 3 determined by densitometry ($n = 6$ ferrets in each group) and expressed by the relative values (means ± SD). *Asterisk* (*) indicates that this value is significantly different from others ($P < 0.05$).

Cleaved Caspase 3, BAD, Phosphorylation BAD, and Bcl-xL Protein Expression in the Lung Tissue of Ferrets after 9 Weeks of Treatment. To investigate whether lung cell apoptosis can be regulated by either smoke exposure or lycopene supplementation, we assessed both the cleaved caspase 3 and the BAD phosphorylation, relative to controls, in the lung tissue of the ferrets. Smoke exposure in ferrets for 9-week period significantly decreased cleaved caspase 3 (74% reduction, Fig. 2), as compared with controls. However, there were no differences in cleaved caspase 3 levels in ferrets supplemented with either a low dose or a high dose of lycopene with or without smoke exposure as compared with control, suggesting that lycopene supplementation restored to normal the reduced apoptosis because of smoke exposure. The levels of total BAD and Bcl-xL did not change among six treatment groups (Fig. 3). However, smoke exposure alone substantially increased BAD phosphorylation at both Ser^{112} (~4 fold) and Ser^{136} (~3.4 fold), compared with controls (Fig. 3); whereas, lycopene supplementation at either a low dose or a high dose prevented the smoke-induced BAD phosphorylation at both Ser^{112} and Ser^{136}. Either low dose or high dose of lycopene supplementation alone did not alter status of BAD phosphorylation (Fig. 3) or levels of cleaved caspase 3 protein (Fig. 2).

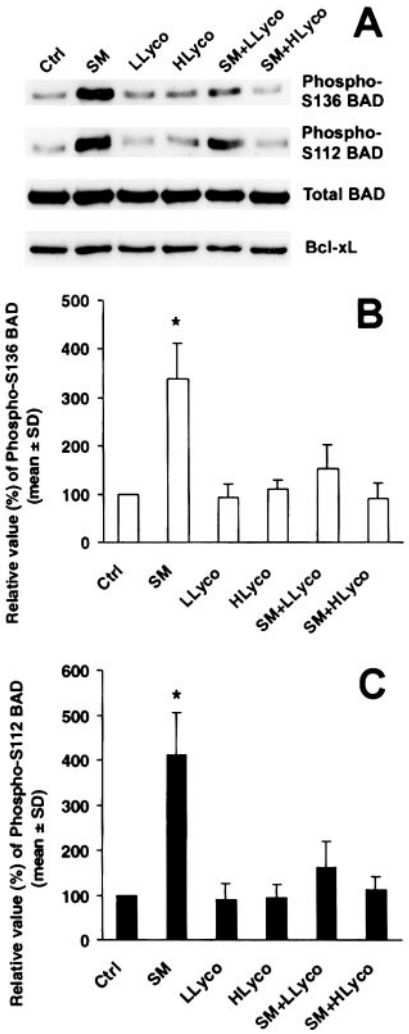

Fig. 3. Expressions of phosphorylation of BAD (Ser^{136} and Ser^{112}), total BAD, and Bcl-xL protein levels in lung tissue from six groups of ferrets (Ctrl, control; SM, smoke-exposed; LLyco, low-dose lycopene supplemented; HLyco, high-dose lycopene supplemented; SM + LLyco, smoke exposed plus low-dose lycopene supplemented; and SM + HLyco, smoke exposed plus high-dose lycopene supplemented). *A*, representative Western blot analysis for phosphorylated BAD-Ser^{136}, phosphorylated BAD-Ser^{112}, total BAD, and Bcl-xL protein levels. The sizes of the detected BAD and phosphorylated BAD were M_r 23,000. The size of the detected Bcl-xL was M_r 30,000. *B*, the intensity of the protein signal of phosphorylated BAD-Ser^{136}, determined by densitometry ($n = 6$ ferrets in each group) and expressed by the relative values (means ± SD). *C*, the intensity of the protein signal of phosphorylated BAD-Ser^{112}, determined by densitometry ($n = 6$ ferrets in each group) and expressed by the relative values (means ± SD). *Asterisk* (*) indicates that this value is significantly different from others ($P < 0.05$).

DISCUSSION

Low blood IGFBP-3 concentrations and high IGF-I concentrations and IGF-I/IGFBP-3 ratios have been implicated in a variety of cancers, including lung cancer (22, 23, 36). Cigarette smoke exposure, a strong risk factor for lung cancer, may promote genomic instability and neoplasia by affecting normal IGF-I signaling, cell proliferation, and apoptosis. In this study, we have shown that cigarette smoke-induced lesions [*e.g.*, squamous metaplasia (Table 4), PCNA overexpression, and diminished apoptosis (Fig. 2)] in the lungs of ferrets were associated with reduced plasma IGFBP-3 concentrations and increased IGF-I/IGFBP-3 ratios (Table 3). We additionally demonstrated that these smoke-induced changes were partially or completely prevented by either a low dose or a high dose of lycopene supplementation, which suggests that lycopene may inhibit smoke-induced lung carcinogenesis through modulation of circulating IGFBP-3 levels and IGF-I/IGFBP-3 ratios. Our results are consistent with the findings from numerous epidemiological studies that have shown a reduced risk of lung cancer associated with high intakes of tomato or lycopene (49–59) and from a recent study that had shown a positive correlation between intake of cooked tomatoes or lycopene and plasma IGFBP-3 concentrations (41).

IGFBP-3 has both IGF-dependent and IGF-independent antiproliferative and proapoptotic effects as shown by the finding that IGFBP-3 sequesters IGF-I away from its receptor and that IGFBP-3 suppresses the growth of IGF-I receptor null fibroblasts (60–62). In the current study, we observed no significant differences in plasma IGF-I concentrations in ferrets exposed to smoke alone and those exposed to smoke with or without lycopene supplementation. However, cigarette smoke exposure resulted in lowered plasma IGFBP-3 concentrations and elevated IGF-I/IGFBP-3 ratios in the ferrets. These changes in IGF-I/IGFBP-3 ratios in the plasma of the ferrets significantly affected the status of apoptosis and cell proliferation in the lungs of ferrets. Smoke exposure alone significantly decreased cleaved caspase 3 protein expression by 74% and increased PCNA by 4-fold, relative to controls, in the lungs of ferrets (Fig. 2). In contrast, lycopene supplementation at either a low dose or a high dose reversed the reduction in plasma IGFBP-3 and lung cellular apoptosis, as well as hyperproliferation induced by smoke exposure, which is illustrated by no differences in plasma IGFBP-3 (Table 3), cleaved caspase 3 protein, and PCNA protein among the groups supplemented with lycopene with or without smoke exposure (Fig. 2). Furthermore, we found that smoke exposure suppressed BAD-mediated apoptosis by inducing the phosphorylation of BAD at both Ser^{136} and Ser^{112}. It appears that multiple signaling pathways are involved in this process because the PI3K appears to mediate survival factor-induced phosphorylation of BAD Ser^{136}, whereas MAPK are thought to mediate survival factor-induced phosphorylation of BAD Ser^{112}. Our data suggest that the phosphorylation of BAD at both Ser^{136} and Ser^{112} induced by smoke exposure can be blocked by lycopene supplementation. A recent study reported that IGFBP-3 can inhibit both PI3K/Akt/PKB and MAPK, mediators of IGF-induced signaling pathways (33). It is possible that lycopene blocks the induction of the phosphorylation of BAD at both Ser^{136} and Ser^{112} by its ability to up-regulate IGFBP-3 in this *in vivo* animal study. Our study also supports the concept that BAD has the potential to be chemopreventive or therapeutic target because it has a central position between multiple growth

factor signaling pathways and apoptosis (29). Studies regarding the dependence of growth inhibition/apoptosis on lycopene induction of IGFBP-3 and the exact mechanism(s) as to how lycopene induces IGFBP-3 are being undertaken in this laboratory using a cell culture model.

In this study, we found that smoke exposure decreased the elevated lycopene concentrations in plasma and lung tissue of ferrets supplemented with lycopene, which is consistent with the data from National Health and Nutrition Examination Survey III that has found that smokers had lower serum level of lycopene compared with nonsmokers (63). The concentration of plasma lycopene (range from 226 to 373 nmol/liter) in the ferret after lycopene supplementation was similar to the lycopene concentration (range, 290–350 nmol/liter) reported in humans (64, 65). In this study, we supplemented ferrets with lycopene using a 3.9-fold difference in doses (either 1.1 or 4.3 mg/kg/day), which resulted in an ~1.6-fold difference in plasma lycopene levels (mean values, 226 nmol/liter for 1.1 mg/kg/day and 373 nmol/liter for 4.3 mg/kg/day). This magnitude of change in plasma levels of lycopene is comparable with the variability seen in humans in whom a 4.1-fold difference in doses of lycopene resulted in 1.7-fold increase in plasma levels of lycopene (66). Furthermore, the lycopene concentrations in the lungs of ferrets that were given a low dose of lycopene (equivalent to 15 mg/day in humans) reached 342 nmol/kg, which is within the range of lung lycopene concentration in normal humans (100–500 nmol/kg; Ref. 67). These data indicate a similarity between humans and ferrets with respect to lycopene absorption and accumulation. We also observed that lycopene concentration in ferrets supplemented with a high dose of lycopene (equivalent to 60 mg/day in humans) increased 3.4-fold in lung tissue and 1.6-fold in plasma, compared with those in ferrets supplemented with a low dose of lycopene (equivalent to 15 mg/day in humans). The fact that a higher increase in lycopene concentrations occurred in lung tissue than in plasma after lycopene supplementation should be considered in the design of future studies of lycopene supplementation. In addition, we demonstrated that although all-*trans*-lycopene is the predominant isomer in plasma and lung tissues of ferrets supplemented with lycopene (followed by 13-*cis*-lycopene and 9-*cis*-lycopene), smoke exposure appears to increase the *cis*-isomers and decrease the *trans*-isomers of lycopene in the lungs of ferrets. Furthermore, we detected at least three metabolites of lycopene by HPLC analysis in the lungs of ferrets with lycopene supplementation, and cigarette smoke exposure increased the formation of these lycopene metabolites in the lungs of ferrets supplemented with lycopene, which indicate that cigarette smoke can enhance lycopene catabolism *in vivo* (68). The significance of these smoke-induced isomerization and degradation of lycopene warrants additional investigation.

This study has important implications for future studies regarding chemopreventive effects of carotenoids against cancer, particularly for lung cancers. Using the ferret model, we have demonstrated that the potential mechanisms for the harmful effects of high-dose β-carotene supplementation in smokers observed in human clinical trials may be associated with the production of undesirable oxidative metabolites of β-carotene in the lung tissue (44, 48, 69). The formation of oxidative byproducts from β-carotene can induce cytochrome p450 enzymes and interfere with retinoic acid metabolism (69, 70), as well as down-regulate RARβ (44, 48, 69). In the ferrets supplemented with β-carotene at a dose of 30 mg/day, the concentration of β-carotene in the lungs of ferrets was 26 μmol/kg lung tissue, which was associated with an enhanced development of lung squamous metaplasia induced by cigarette smoke exposure (44). In this study, the concentration of lycopene in the lungs was only 1.2 μmol/kg lung tissue in ferrets supplemented with lycopene at a dose of 60 mg/day, which caused no harmful effect but rather prevented the development of lung squamous metaplasia and cell proliferation induced by smoke exposure. The different outcome between the lycopene and β-carotene studies in ferrets may be attributable to the differences in the levels of carotenoids that accumulated in lung tissue.

In summary, our present study demonstrates that lycopene supplementation at either low dose or high dose prevents the development of lung squamous metaplasia, cell proliferation, the reduction of apoptosis, and elevated phosphorylation of BAD induced by smoke exposure in ferrets. Both low-dose and high-dose lycopene supplementation reversed decreased plasma IGFBP-3 concentrations and increased the IGF-I/IGFBP-3 ratio induced by smoke exposure. Lycopene supplementation at either a low dose or a high dose increased plasma and lung tissue levels of lycopene, but the elevated levels of lycopene in both the plasma and lungs of ferrets supplemented with lycopene were lowered by smoke exposure. In conclusion, the findings from this study suggest that lycopene may mediate its protective effects against smoke-induced lung carcinogenesis in ferrets through up-regulating IGFBP-3 and down-regulating phosphorylation of BAD, which promote apoptosis and inhibit cell proliferation.

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