Low Dose β-Carotene Supplementation of Ferrets Attenuates Smoke-Induced Lung Phosphorylation of JNK, p38 MAPK, and p53 Proteins

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ABSTRACT We demonstrated previously that smoke exposure and/or high-dose β-carotene supplementation decreases levels of retinoic acid and retinoic acid receptor β (RARβ) protein, but increase levels of c-Jun and proliferating cellular nuclear antigen protein in the lungs of ferrets. In contrast, low-dose β-carotene can prevent the smoke-induced lung lesions. In the present study, we investigated whether smoke exposure and/or β-carotene supplementation could affect Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and p53 in the lungs of ferrets. Ferrets were subjected to cigarette smoke exposure and either a high or low dose of β-carotene (2 × 3 factorial design) for 6 mo. There were greater protein levels of phosphorylated JNK, p38, and c-Jun, but lower levels of MAPK phosphatase-1 (MKP-1) in groups exposed to smoke and/or high dose β-carotene. Both phosphorylated-p53 and total p53 were substantially increased in the lungs of these groups. In contrast, low-dose β-carotene greatly attenuated the smoke-induced phosphorylation of JNK, p38, c-Jun, p53, and total p53, accompanied by upregulated MKP-1. Smoke exposure increased MAPK kinase-4 (MKK4) phosphorylation regardless of β-carotene supplementation. These data indicate that restoration of retinoic acid and MKP-1 by low-dose β-carotene in the lungs of ferrets may prevent the smoke-induced activation of the JNK-dependent signaling pathway, p38 MAPK, and the associated phosphorylation of p53, thereby lowering the risk of the smoke-related lung lesions. These data provide supportive evidence that the beneficial vs. detrimental effects of β-carotene supplementation are related to the dosage of β-carotene administered.


KEY WORDS: β-carotene • smoke • signal transduction pathway

Retinoic acid can modulate cell proliferation and differentiation in epithelial cells and suppress carcinogenesis in certain epithelial tissues (1). Our previous studies in ferrets showed that high-dose β-carotene supplementation (HBC) and/or cigarette smoke exposure decreases levels of retinoic acid and retinoic acid receptor β (RARβ) protein but increases levels of c-Jun and cyclin D1 proteins, and cell proliferation in lung tissue (2,3). In contrast, low-dose β-carotene supplementation (LBC) does not lower levels of retinoic acid and RARβ and has weak protective effects against cigarette smoke-induced damage in ferrets in vivo (3) and against benz[a]pyrene-reduced RARβ expression in normal human bronchial epithelial cells in vitro (4). Recently, we demonstrated further that diminished levels of retinoic acid in the lungs of ferrets after either smoke exposure, HBC, or both are caused in part by enhancement of retinoic acid catabolism via induction of cytochrome P450 (5), which provides a possible explanation for enhanced lung carcinogenesis with pharmacologic dose β-carotene supplementation in cigarette smokers (6).

Jun N-terminal kinase (JNK) and p38, members of the mitogen-activated protein kinase (MAPK) family, mediate cellular responses to cytokines and environmental stress and may play an important role in carcinogenesis (7,8). JNK and p38 are activated by dual phosphorylation on Thr and Tyr by members of the MAPK kinases (MKKs) [MKK 4 and 7 for JNK (9,10) and MKK 3, 4, and 6 for p38 (11–15)]. JNK further regulates activator protein-1 transcriptional activity by phosphorylation of c-Jun on sites Ser-63 and Ser-73 (16,17), whereas phosphorylated-p38 activates several targets including activating transcription factor (18). In addition, JNK and p38 mediate phosphorylation of the p53 tumor suppressor gene (19–24), which in turn modulates cell proliferation, differentiation, and apoptosis (25), and plays a critical role in suppressing lung carcinogenesis (26,27). It was suggested that p53 phosphorylation is a critical event in the upregulation and functional activation of p53 during cellular stress (28).

MAPK phosphatases (MKPs), a family of dual-specificity protein phosphatases that play an important role in regulating the activity of MAPK, can dephosphorylate both phosphothreonine and phospho-tyrosine residues to inactivate JNK and p38 both in vitro and in vivo (29–33). It was shown that
phosphorylated-JNK and phosphorylated-p38 are preferred substrates for MKP-1 among isomers of MKPs (30,33,34). Previous studies found that retinoic acid suppresses JNK activity by inhibiting JNK phosphorylation and inducing MKPs in cell models (35,36). Inhibition of RAR was also shown to activate the p38 MAPK in primary cultures of mouse limb mesenchyme (18). Although we showed that smoke exposure, β-carotene supplementation, or their combination affects lung retinoic acid levels using in vivo ferret models (3), it is unknown whether these different retinoic acid levels would affect the JNK signaling pathway and p38 phosphorylation, and if so, whether these changes are due to regulating MKP-1, thereby influencing the phosphorylation of their downstream genes of p53 and c-Jun. In addition, JNK and p38 can be activated by MKKs; therefore, it is possible that the treatments with smoke exposure and/or β-carotene supplementation may also affect the JNK signaling pathway and p38 phosphorylation through MKKs. In the present study, we addressed these questions using the lung tissues collected from normal ferrets and from ferrets exposed to smoke exposure and/or supplemented with different doses of β-carotene.

**MATERIALS AND METHODS**

**Animals, diet, and study groups.** The maintenance and husbandry of ferrets was described previously (3). Male ferrets (n = 36; 1.0–1.2 kg) from Marshall Farms were randomly assigned to 6 groups (2 × 3 factorial design; n = 6/group) for 6 mo as follows: 1) control; 2) smoke exposed; 3) LBC (per unit body weight, 0.43 mg/kg · d); 4) HBC (per unit body weight, 2.4 mg/kg · d); 5) smoke-exposed + LBC; 6) HBC alone. Ferrets had free access to both water and diet (Ferret diet #5280, Purina Mills, LLC). During the 6-mo experimental period, the ferret body weights were recorded weekly. At the end of the 6-mo experimental period, all ferrets were killed by exsanguination from heart under deep isoflurane anesthesia. Lung tissues collected from these ferrets were used in the current study. All experimental procedures were approved by the Animal Care and Use Committee at the Human Nutrition Research Center on Aging at Tufts University.

**Cigarette smoke exposure.** The administration of cigarette smoke exposure in ferrets was described previously (3). The amount of smoke exposure in the ferret was equivalent to ~1.5 packages of cigarettes/d in humans based upon the concentrations of urinary cotinine equivalents (3).

**β-Carotene supplementation.** The procedure of β-carotene supplementation in ferrets was described previously (2,3). The β-carotene intake in the LBC group per unit body weight was 0.43 mg/(kg · d), which is ~6 mg of β-carotene/d intake in a 70-kg person. This amount of β-carotene can be easily obtained in humans by eating a diet rich in fruits and vegetables (6). The β-carotene intake in the HBC group per unit body weight was 2.4 mg/(kg · d), which is ~30 mg of β-carotene/d intake in a 70-kg person, a dose used in smokers of the human intervention trial (37).

**Immunoprecipitation and Western blot analysis.** Immunoprecipitation and Western blot for JNK, p38, MKP-1, MKK4, phosphorylated-JNK, phosphorylated-p38, and phosphorylated-MKK4 were conducted using the whole-tissue lysates of lung from 6 ferrets in each group, which were prepared as described previously (38). Western blot analysis for phosphorylated-c-Jun, p53, and phosphorylated-p53 at serine 15 was conducted using nuclear protein extracts from the lungs of the ferrets in each of the 6 groups, which were prepared as previously described (2). Antibodies against JNK, phosphorylated-JNK, p38, phosphorylated-p38, phosphorylated-c-Jun, and MKP-1 were purchased from Santa Cruz Biotechnology. Antibodies against p53, MKK4, and phosphorylated-MKK4 were purchased from Calbiochem. Antibodies against phosphorylated-p53 at serine 15 were purchased from Cell Signaling. The intensity of the protein signal was quantified by Densitometry (GS-710 calibrated imaging densitometer, Bio-Rad) and expressed by the relative values. The relative values were defined as the intensity of signal of each sample of 5 treatment groups divided by the intensity of signal of each control sample in each run.

**Statistical analysis.** Results are expressed as means ± SD and data were analyzed by 2-way ANOVA followed by Tukey’s honestly significant difference test. Differences were considered significant at P < 0.05.

**RESULTS**

**Levels of total JNK, phosphorylated-JNK, phosphorylated-c-Jun, total p38, and phosphorylated-p38 in the lung tissue of ferrets.** There was no difference in protein levels of total JNK among the 5 treatment groups compared with the control group (Fig. 1A). Compared with the control group, phosphorylated-JNK protein levels were markedly increased in the smoke-exposed + HBC (~5-fold), smoke-exposed (~3-fold), and HBC alone (~2-fold) groups (Fig. 1A). Phosphorylated-JNK protein levels did not differ among the LBC alone group, the smoke-exposed + LBC group, and the control group. In addition, c-Jun phosphorylation (Fig. 1B) was up-regulated in the smoke-exposed + HBC (~4-fold), smoke-exposed alone (~3-fold), and HBC alone (~2-fold) groups. c-Jun phosphorylation did not differ among the LBC alone group, the smoke-exposed + LBC group, and the control group. Furthermore, protein levels of total p38 did not differ among the 5 treatment groups compared with the control group (Fig. 1C). Similarly, phosphorylated-p38 protein levels were markedly increased in the smoke-exposed + HBC (~4-fold), smoke-exposed (~3-fold), and HBC (~2-fold) groups (Fig. 1C). Also, phosphorylated-p38 protein levels did not differ among the LBC alone group, the smoke-exposed + LBC group, and the control group.

**Levels of MKP-1, MKK4, and phosphorylated-MKK4 in the lungs of ferrets.** To further investigate the mechanism of JNK activation, we analyzed the levels of MKP-1 protein, which dephosphorylates and thus inactivates JNK, and the levels of MKK4, a major upstream kinase for JNK activation. Compared with the control group, MKP-1 protein levels were downregulated in the groups of smoke-exposed + HBC (by 80%), HBC alone (by 51%), and smoke-exposed alone (by 68%) (Fig. 2A). However, MKP-1 protein levels in ferrets supplemented with LBC alone and smoke-exposed + LBC did not differ from those in the control group. Total MKK4 did not differ among all groups (Fig. 2B). Phosphorylated-MKK4 protein levels were doubled in the groups of smoke-exposed, smoke-exposed + LBC, and smoke-exposed + HBC. There were no changes in phosphorylated-MKK4 in the groups of control, LBC alone, or HBC alone (Fig. 2B).

**Levels of total p53 and phosphorylated-p53 in the lungs of ferrets.** There was an approximate 2-fold increase in total p53 protein levels in the groups of smoke-exposed + HBC, smoke-exposed, and HBC alone (Fig. 3A). Total p53 protein levels did not differ among the LBC alone group, smoke-exposed + LBC group, and the control group. Because p53 phosphorylation is mediated by MAPK such as JNK and p38, we then examined p53 phosphorylation. Compared with the control group, p53 protein phosphorylation was substantially increased in the groups of smoke-exposed + HBC (~5-fold), smoke-exposed (~4-fold), and HBC alone (~2-fold). Phosphorylated-p53 protein levels did not differ among the LBC alone group, the smoke-exposed + LBC group, and the control group (Fig. 3B).

**DISCUSSION**

Components of smoke exposure or smoke exposure itself were shown to increase the phosphorylation of JNK (39–41)
and p38 (41,42) in cell models. However, little is known concerning whether long-term smoke exposure affects JNK and p38 activity in animal models. In addition, data on the effects of β-carotene supplementation on the JNK signaling pathway and p38 MAPK in animal models are lacking. One study reported that administration of antioxidant vitamins in combination (β-carotene, 20 mg, vitamin C, 200 mg, and α-tocopherol, 200 mg) to rabbits with pacing-induced congestive heart failure reduced the phosphorylation of JNK but not p38 (43). However, it is unclear whether β-carotene itself affected the phosphorylation of JNK and p38 in that study (43). In the present study, using the lung tissues collected from ferrets exposed to smoke and/or supplemented with β-carotene chronically at 2 doses, we demonstrated that smoke exposure, HBC, and their combination activated the phosphorylation of JNK and p38 MAPK. In contrast, LBC alone did not increase JNK and p38 phosphorylation and, instead, attenuated smoke-induced JNK and p38 phosphorylation. The amounts of total JNK and p38 proteins were similar in all treatment groups, indicating that smoke exposure and β-carotene affect JNK and p38 phosphorylation.

FIGURE 1 Effects of β-carotene supplementation and smoke exposure on protein levels of total JNK, phosphorylated-JNK, phosphorylated-c-Jun, total p38 MAPK, and phosphorylated-p38 MAPK in the lung tissue of ferrets. The graphs show the intensity of the protein signal of phosphorylated-JNK (panel A), phosphorylated-c-Jun (panel B), and phosphorylated-p38 (panel C). Values are means ± SD, n = 6. Means without a common letter differ, P < 0.05. Upper portions of each panel are representative Western blots in the same order as in the graph. The sizes were 46 kDa for JNK and phosphorylated-JNK, 39 kDa for phosphorylated-c-Jun, and 38 kDa for p38.

FIGURE 2 Effects of β-carotene supplementation and smoke exposure on the protein levels of MKP-1, total MKK4, and phosphorylated-MKK4 in the lung tissue of ferrets. The graphs show the intensity of the protein signal of MKP-1 (panel A) and phosphorylated-MKK4 (panel B). Values are means ± SD, n = 6. Means without a common letter differ, P < 0.05. Upper portions of each panel are representative Western blots in the same order as in the graph. The sizes were 41 kDa for MKP-1 and ~42 kDa for both MKK4 and phosphorylated-MKK4.
MKK4 activates both JNK and p38 MAPK (7,8,44), and both can be inactivated by MKP-1 (29–31), we hypothesized that smoke exposure, β-carotene supplementation, and their combination may modulate the phosphorylation of JNK and p38 through the regulation of MKP-1 and/or MKK4. Indeed, smoke exposure, HBC, and their combination significantly reduced lung MKP-1 protein levels in the present study. In contrast, LBC prevented downregulation of MKP-1 due to smoke exposure. Moreover, phosphorylated-MKK4 protein was increased at similar levels in the groups of smoke exposed, smoke-exposed + LBC, and smoke-exposed + HBC, whereas the control, LBC alone, and HBC alone groups were not affected. These data suggest that both downregulation of MKP-1 and upregulation of phosphorylated-MKK4 contribute to JNK and p38 activation in the groups exposed to smoke (smoke exposed, smoke exposed + LBC, and smoke exposed + HBC), and that downregulation of MKP-1 contributes to the activation of JNK and p38 in the group of HBC alone. However, the possibility that others MKKs and/or molecular pathways contribute to the activation of JNK and p38 cannot be excluded. Smoke exposure and HBC-induced activation of the JNK-dependent signaling cascades was further demonstrated by increased levels of phosphorylated-c-Jun in the present study. Increased levels of phosphorylated-c-Jun in the lungs of ferrets were correlated with elevated levels of total c-Jun of the same ferrets that were described earlier (3), suggesting that the changes in levels of total c-Jun in response to smoke exposure and β-carotene supplementation may be due to phosphorylation of c-Jun. These data provide a potential biological explanation for our previous observations that the overexpression of total c-Jun induced by smoke exposure, HBC, or their combination increased cyclin D1 and proliferating cell nuclear antigen levels in ferrets after 6 mo of treatment (3). The p38 MAPK pathway is crucial to inflammatory cytokine production and signaling (45). Activation of the p38 MAPK pathway was also shown to block T cell-mediated immune responses in vivo (8). Recent evidence suggests the involvement of inflammation in tumor development and progression (46). Therefore, p38 activation caused by smoke exposure, HBC, and their combination may also contribute to adverse pathologic changes in the lungs of ferrets.

Previous studies found that retinoic acid upregulates MKP-1 activity and blocks both serum- or H2O2-induced JNK phosphorylation in cell models (35,36) and alcohol-induced JNK phosphorylation in a rat model (47). Although there is no direct evidence that retinoic acid blocks p38 phosphorylation in normal cell models, one study in primary cultures of mouse limb mesenchyme showed that the p38 MAPK activity is activated in response to RAR inhibition (18), which suggests the retinoic acid may inactive the p38 MAPK pathway. Our previous study in ferrets demonstrated that smoke exposure, HBC, and their combination substantially lowered the levels of retinoic acid and RARβ protein in lung tissue (3). In contrast, LBC partially restored the decreased levels of retinoic acid in the lungs caused by smoke exposure, thus affording protection against smoke-induced lung cell proliferation and squamous metaplasia (3). Using the same groups of ferrets, we found that lower levels of retinoic acid and RARβ protein in the lungs caused by smoke exposure, HBC, and their combination correspond to upregulation of the phosphorylation of JNK, p38, and c-Jun proteins, and downregulation of MKP-1 protein in the present study. Moreover, LBC reversed the changes in the phosphorylation of JNK and p38 induced by smoke exposure. These results thus provide evidence that altered retinoic acid levels in the lung tissues may affect the
JNK signaling pathway and the p38 MAPK through their influences on MKP-1 activity. The study also indicates that discrepancies in the effects on MKP-1 between LBC and HBC observed in the present study may be due to their influences on retinoic acid levels.

It was reported that HBC did not act as a tumor promoter; rather, it inhibited the conversion of skin papillomas to carcinomas in a 2-stage model of mouse skin carcinogenesis (48). The levels of β-carotene in the skin of mice after higher pharmacologic dose β-carotene (600 mg/kg diet) was 0.9 nmol/g skin tissue (49), which was lower than the 2.8 nmol/g lung tissue in the low dose β-carotene feeding group in ferrets (3). Therefore, the protective effect of β-carotene on skin carcinogenesis is consistent with the present findings in ferrets fed LBC, which showed the alleviation of smoke-induced lung phosphorylation of JNK, p38, and p3.

The p53 tumor suppressor protein plays a critical role in the cellular response to various cellular stresses (25). P3 phosphorylation at serine 15 is an early cellular response to various genotoxic stresses and facilitates both accumulation and functional activation of p53 (21,28,50). Previous studies showed that p38 MAPK activation can phosphorylate p53 at serine 15 in cells exposed to radiation, UV, or chemicals that produce reactive oxygen species (19–21). Recent studies also demonstrated that JNK activation can phosphorylate p53 at serine 15 in cells exposed to drugs (23) and in selenium-deficient mouse liver under moderate oxidative stress (24). However, no studies evaluated chronic smoke exposure and/or β-carotene supplementation in relation to p53 phosphorylation at serine 15. We found that smoke exposure and HBC substantially increased p53 phosphorylation at serine 15. In contrast, LBC alleviated the smoke-induced p53 phosphorylation. These findings are consistent with the changes in JNK and p38 MAPK protein levels in these groups. In addition, parallel changes in total p53 protein levels in these groups of ferrets suggest the accumulation and functional activation of p53 by its phosphorylation, indicating that p53 accumulation induced by smoke exposure and HBC may be due in part to its phosphorylation by JNK and p38 MAPK. These data are supported by previous studies that showed JNK and p38 activation of phosphorylated-p53 at serine 15, which caused an accumulation of p53 by stabilization of the protein in cell models (22,23).

In summary, data from the present study suggest that smoke exposure and its combination with HBC enhance lung phosphorylation of JNK and p38, and their downstream genes of p53 and c-Jun, and that these changes are associated with both increasing phosphorylation of MKK4 and decreasing MKP-1. The present study also indicates that partial restoration of retinoic acid by LBC may prevent the smoke-induced activation of the JNK-dependent signaling pathway, p38 MAPK, and the associated phosphorylation of p53, thereby lowering the risk of smoke-related lung lesions. These data provide supportive evidence that the beneficial vs. detrimental effects of β-carotene supplementation are related to the dosage of β-carotene administered.

LITERATURE CITED