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Short communication

The suppression of ornithine decarboxylase expression and cell proliferation at the promotion stage of lung tumorigenesis in mice by α-tocopheryloxybutyric acid

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Abstract

It is known that vitamin E inhibits tumor cell growth *in vitro* irrespective of its antioxidative effect. However, it is unclear whether the effect *in vitro* can be applied to the *in vivo* situation. In order to address this question, we estimated if α-tocopheryloxybutyric acid (TSE), a non-antioxidative vitamin E derivative *in vivo*, could inhibit cell proliferation during the tumorigenic process of lung in mice treated with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), the most potent carcinogen among tobacco-specific nitrosamines. TSE administration suppressed the labeling index of the proliferating cell nuclear antigen, a marker of cell proliferation at a promotion phase of NNK-induced lung tumorigenesis in mice. Similarly, TSE administration inhibited the elevation of ornithine decarboxylase (ODC) activity and its mRNA at the promotion phase. Of four transcription factors contributing to ODC induction, the change in the level of the c-Myc/Max–consensus oligonucleotide complex was only proportional to the change in ODC mRNA level. These results suggest that vitamin E can inhibit cell proliferation linked with ODC induction at the promotion phase of lung tumorigenesis irrespective of its antioxidative effect and that modulation of the transactivation of the c-Myc/Max complex for the *ODC* gene by TSE in part contributes to the suppression of ODC induction.

Keywords: α-Tocopheryloxybutyric acid; Cell proliferation; Lung tumorigensis; 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone; Myc; Ornithine decarboxylase

1. Introduction

The preventive effect of vitamin E on carcinogenesis in human and animals has not yet been established, because this vitamin has had contradictory effects on carcinogenicity in a variety of model systems [1–3]. For instance, vitamin E acted as a complete promoter in a liver carcinogenesis model [4], whereas it suppressed the development of lung tumors in mice [5]. These contradictions mean that vitamin E cannot be clinically used as a chemopreventive agent. The reason why vitamin E acts as a promoter in some animal models of carcinogenesis may depend on its prooxidant property [6]. In previous studies *in vitro*, a vitamin E ester derivative, α-tocopheryl succinate, had an antiproliferative effect against tumor cells without causing toxicity to normal cell lines, and this effect not related to the antioxidative effect of vitamin E [7]. If this beneficial effect *in vitro* could be applied to an *in vivo* system, it would seem to establish a new chemopreventive agent for cancer based on the non-antioxidative property of vitamin E.

We have previously reported that the vitamin E-dependent suppression of ODC, a rate-limiting enzyme of polyamine biosynthesis and subsequent cell proliferation at a

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Abbreviations: AP-1, activator protein-1; CREB, adenosine 3′,5′-cyclic monophosphate response element binding proteins; Dig, digoxigenin; NNK, 4-(methylnitrosoamino)-1-(3-pyridyl)-1-butanone; MSA, mobile shift assay; ODC, ornithine decarboxylase; PCNA, proliferating cell nuclear antigen; and TSE, α-tocopheryloxybutyric acid.

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promotion phase of lung carcinogenesis in mice treated with NNK, the most potent carcinogen among the tobacco-specific nitrosamines, finally led to the inhibition of carcinogenesis [8]. However, it is unclear whether the inhibitory effect of vitamin E is dependent on its antioxidative effect. Of the vitamin E derivatives, TSE has no antioxidative effect *in vivo*, because the ether bond in which TSE harbors could be non-hydrolyzable by enzymes *in vivo* [9]. With this in mind, we checked the inhibitory effect of TSE on the induction of ODC and cell proliferation at the promotion phase of lung tumorigenesis to establish the usefulness of the non-antioxidative effect of vitamin E on the inhibition of the development of lung tumors *in vivo*. Here, we present the first evidence that TSE suppresses cell proliferation linked with the induction of ODC and that one of the target points may be the transcriptional activity of the c-Myc/Max complex for expression of the *ODC* gene.

2. Materials and methods

Six-week-old A/J female mice (SLC) had *ad lib*. access to AIN-76A diet and tap water. NNK (99% pure, Chemsyn Science Laboratories) dissolved in saline was administered to mice i.p. at a dose of 100 mg/kg body weight. TSE was synthesized from α-tocopherol (99% pure, gift from Eisai Corp.) and 4-bromobutyronitrite (Aldrich Chemical Co., Inc.) according to an established method [9]. We also checked the structure and purity of synthesized TSE, using NMR, IR, and GC–MS. TSE was suspended in vitamin E-stripped corn oil and administered to mice orally at a dose of 100 mg/kg body weight. This dose of TSE was determined according to our preliminary result showing that it was sufficient to inhibit ODC induction without any influence on basal level (data not shown). On day 30 after NNK injection, all mice in each group were treated with TSE or vehicle, and the mice were killed by exsanguination of abdominal artery under pentobarbital anesthesia at 15 or 30 hr after the TSE treatment. At 30 hr after the final treatment, cell proliferation was estimated, and the other assays were performed at 15 hr. In our preliminary experiments, the maximum increase in ODC activity was observed on day 30 after NNK injection; we thus decided to check the effect of TSE on the same day. Additionally, at 15 and 30 hr after the single TSE treatment, maximum decreases in ODC activity and the PCNA labeling index, respectively, were shown in our preliminary experiments. Based on these observations, we selected these points to estimate the effect of TSE on ODC activity and the PCNA labeling index. Lungs were rapidly removed, frozen in liquid nitrogen, and then stored at $-80°$ until use except for MSA. Nuclear extracts for MSA were rapidly prepared from lung tissues after dissection and used immediately.

The lungs were fixed with 10% buffered formalin. The tissues were embedded in paraffin and sectioned. The deparaffinized sections of the tissues were stained with an avidin–biotin–peroxidase method with an anti-PCNA antibody (DAKO Labs). The sections were counterstained with methylgreen. The PCNA labeling index was determined by counting more than 500 nuclei in randomly selected microscopic fields of the alveolar region. A lung 20% homogenate in 0.05 M Tris–HCl–0.25 M sucrose (pH 7.5) was centrifuged at $100{,}000 \times g$ for 30 min, and the supernatant was used to estimate ODC activity by measurement of the amount of radioactive CO_2 liberated from L-[1-^{14}C]ornithine [10].

The total RNA of mouse lungs was prepared according to the guanidinium thiocyanate–phenol–choloroform extraction procedure [11] and subjected to Northern blot analysis as described [12]. Twenty micrograms of RNA/lane was subjected to formaldehyde–1% agarose gel electrophoresis, transferred to a nylon membrane, and fixed to the membrane by exposing to UV radiation. After prehybridization, blots were hybridized with ^{32}P-labeled cDNA probes. After washing at high stringency, the membranes were exposed at $-80°$ to Fuji x-ray film with an intensifying screen. Probe cDNA fragment was excised from mouse ODC cDNA plasmid with restriction enzymes [13]. Quantitative variabilities of isolation and transfer of RNAs were counted by reprobing the same filter with a cDNA probe for human 18S rRNA. Mouse lung nuclear extracts were prepared as described [14]. The extract was centrifuged at $13{,}000 \times g$ at 4° for 30 min, and the supernatant was used as a protein source for MSA. Ten micrograms of the nuclear extract protein was preincubated with binding buffer (20 mM Hepes, 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) and 1 μg of poly(dI-dC) (and 75 ng of unlabeled competitor or non-competitor oligonucleotide, where appropriate) for 2 min before 500 pg of labeled oligonucleotide was added. After the mixture had stood for 20 min at room temperature, it was loaded on a 6% non-denaturing polyacrylamide gel made and run in 0.25 X Tris/borate/EDTA buffer, transferred to the nylon membrane, and fixed to the membrane by exposing to UV radiation. Commercially supplied oligonucleotides for Myc, SP1, CREB, and AP-1 consensus sequences (Santa Cruz Biotechnology) were used for this MSA and labeled with Dig using the Dig Gel Shift Kit (Boehringer) as recommended by the supplier. The level of each transcription factor–consensus oligonucleotide complex was determined according to a chemiluminescent detection system for Dig (Boehringer).

Statistical analysis was performed by one-way analysis of variance followed by Duncan's multiple-range test. A P value of 0.05 or less was considered significant.

3. Results and discussion

We first checked the effect of TSE on cell proliferation at the promotion phase of NNK-induced lung tumorigenesis. The effect of TSE on the PCNA labeling index in lungs of

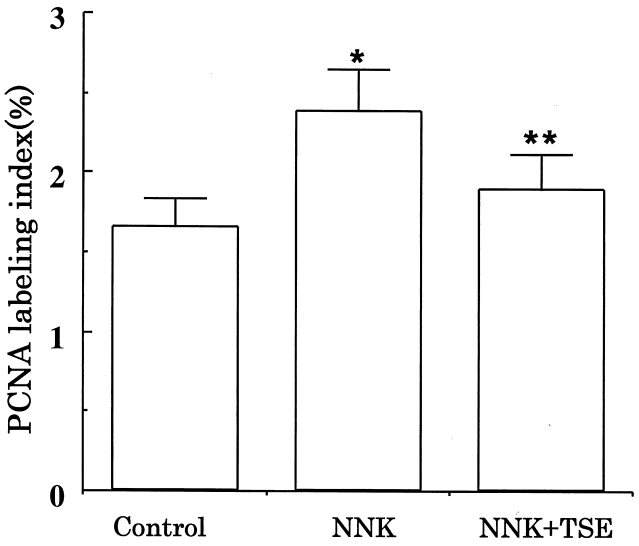

Fig. 1. The effect of TSE on the pulmonary PCNA labeling index at the promotion stage of NNK-induced lung tumorigenesis in mice. Values are expressed as means ± SEM from five mice. *Significantly different from the control group. **Significantly different from the NNK-treated group.

mice treated with NNK is shown in Fig. 1. The PCNA labeling index in the NNK-treated group showed an approximate 50% increase compared with that in the control group, this increase being significantly different. The elevation by NNK treatment was diminished by TSE treatment with a significant difference. Next, we estimated the suppressive effect of TSE on ODC induction at the promotion phase. As shown in Fig. 2, A and B, the ODC mRNA level as well as ODC activity in lungs of mice treated with NNK clearly increased compared with those in the control group. On the other hand, the mRNA level and activity of ODC were reduced by TSE treatment. In addition, we confirmed that vehicle treatment did not have any influence on cell proliferation and ODC induction at the promotion phase (data not shown).

Finally, we tried to determine a possible mechanism for the suppressive effect of TSE at the transcriptional level. Since it has been reported that the mouse *ODC* gene was a principal transcription target for c-Myc, SP1, CREB, and AP-1 [14], we investigated which transcription factor contributed to the TSE-dependent decrease in the ODC mRNA level. As judged from the result of MSA (Fig. 3A), NNK treatment elevated the binding of nuclear extracts to an oligonucleotide containing the Myc/Max consensus sequence, and TSE administration inhibited the elevation of the binding capacity. However, the administration of NNK and/or TSE did not affect the DNA-binding activities of three other transcription factors with oligonucleotide-containing consensus sequences. In addition, a competitive study confirmed the specificity of the binding complexes (Fig. 3B).

We have already reported that vitamin E acts as a useful chemopreventive agent against NNK-induced lung tumorigenesis through the suppression of the initiation and promotion stages [8,15]. On the other hand, as mentioned above, vitamin E acts as a promoter against some types of cancer, perhaps be due to its prooxidative effect [4,6]. In order to overcome the disadvantage of vitamin E as a chemopreventive agent for lung cancer, we attempted to clarify whether cell proliferation at the promotion stage of lung tumorigenesis can be induced based on the non-antioxidative effect of vitamin E. As the result, single TSE

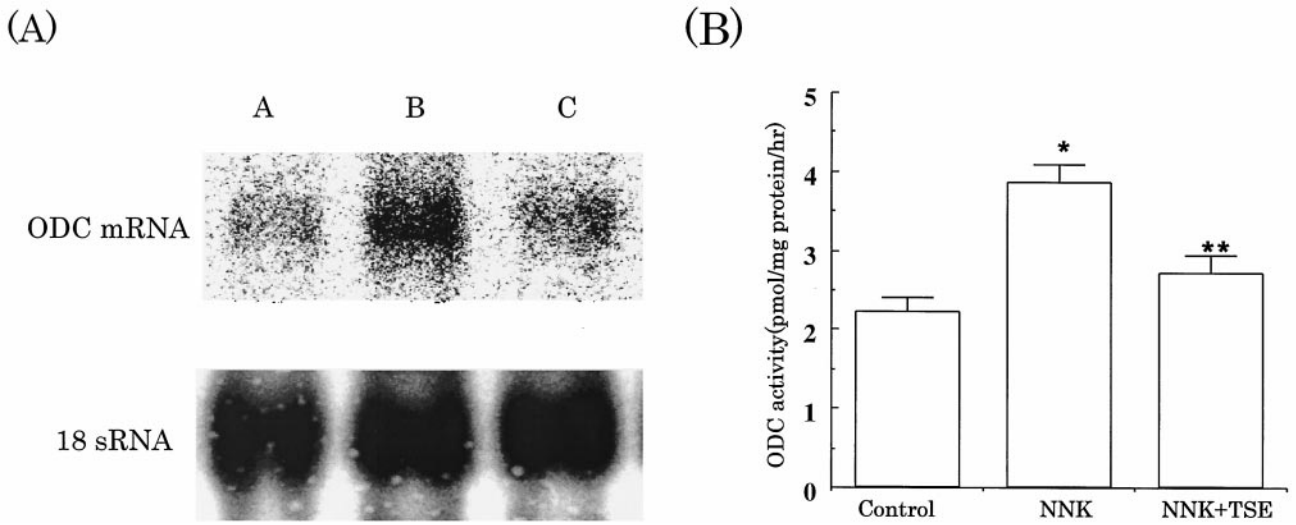

Fig. 2. The effects of TSE on the pulmonary ODC mRNA level and activity at the promotion stage of NNK-induced lung tumorigenesis in mice. (A) Northern blot analysis. The filters were hybridized with either ODC or 18S rRNA cDNA probe and the results shown in the upper and lower panels, respectively. Lane 1, control; lane 2, NNK-treated sample; lane 3, sample at 15 hr after TSE treatment in NNK-treated mouse lung. This figure is representative of one of three independent experiments. (B) ODC activity assay. Lane 1, control; lane 2, NNK-treated sample; lane 3, sample at 15 hr after TSE treatment in NNK-treated mouse lung. Values are expressed as means ± SEM from five mice. * Significantly different from the control group. ** Significantly different from the NNK-treated group.

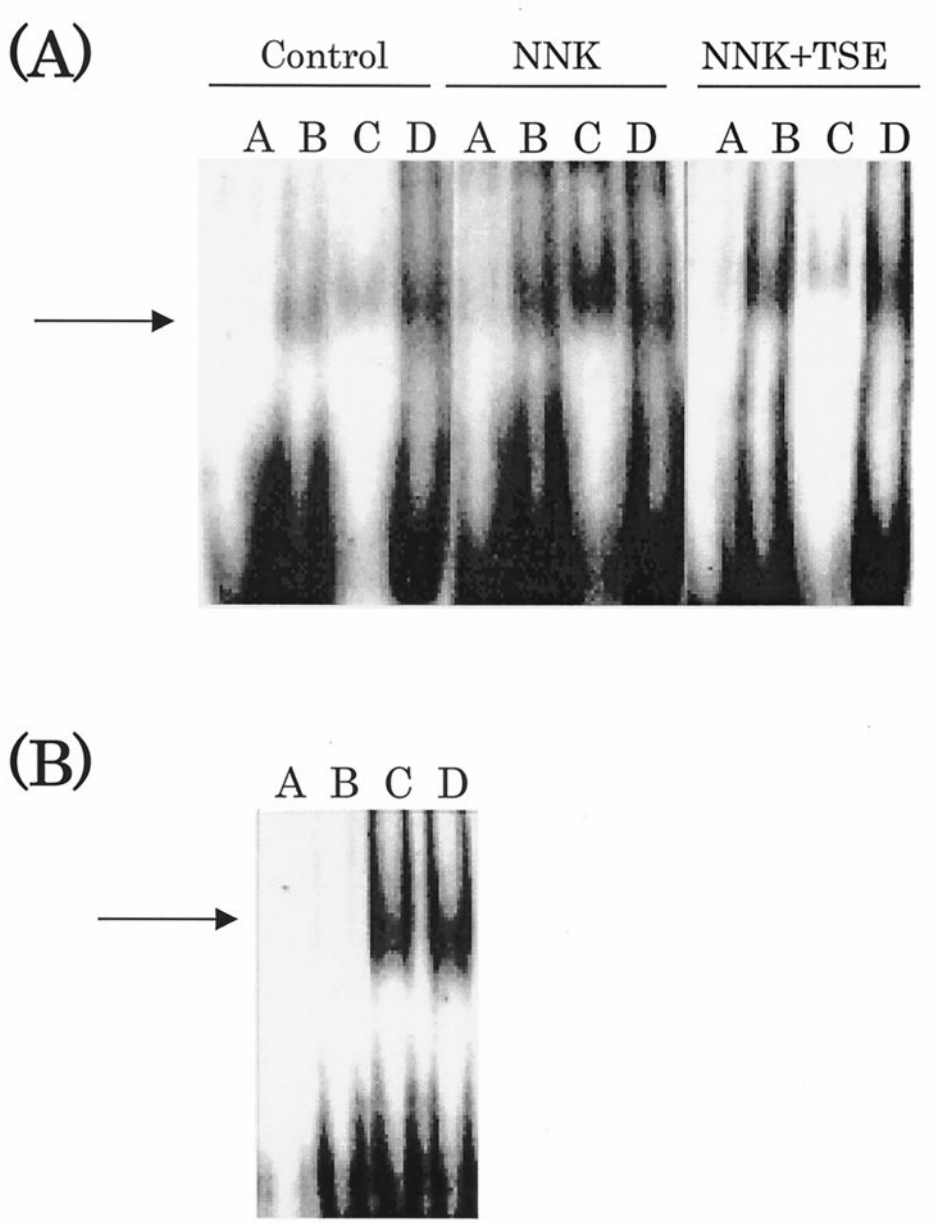

Fig. 3. The effect of TSE on the DNA-binding activity of four transcription factors (c-Myc/Max, SP1, CREB, and AP-1) related to the induction of ODC mRNA. (A) MSA using nuclear extracts from lung tissues and Dig-labeled double-stranded oligonucleotide containing each binding site of the transcription factor. Lane A, SP1; lane B, CREB; lane C, c-Myc/Max; lane D, AP-1. Control, control group; NNK, NNK-treated group; NNK + TSE, NNK-plus TSE-treated group. (B) Competitive assay using unlabeled oligonucleotide containing the c-Myc/Max binding site. Lane A, nuclear extract from control group; lane B, nuclear extract from NNK-treated group + unlabeled competing oligonucleotide; lane C, nuclear extract from NNK-treated group + unlabeled non-competing oligonucleotide; lane D, nuclear extract from NNK-treated group. These results were representative of three independent experiments.

treatment significantly reduced NNK-induced ODC expression as well as cell proliferation at the promotion stage. Furthermore, vitamin E had less inhibitory effect on cell proliferation and ODC induction in comparison with TSE under the same treatment condition[1] which signifies that ODC induction and cell proliferation during the lung tumorigenic process can be regulated through the non-antioxidative effect of vitamin E. Since it has been established that the inhibitory effect is closely linked with the suppression of lung tumorigenesis [8], these results lead us to further determine the role of non-antioxidative effect of vitamin E in preventing the development of lung tumors.

The *ODC* gene is now recognized as a proto-oncogene necessary for cell-cycle progression and cell transformation [16]. Additionally, the suppression of ODC induction during the carcinogenic process is an important and common event for the effective inhibition of tumor development [17]. Thus, clarifying a mechanism of TSE-dependent sup-

[1] Yano T, Ichikawa T. Unpublished data.

pression of ODC expression at the promotion stage of lung tumorigenesis may strongly support the establishment of new chemopreventive agents based on the non-antioxidative effect of vitamin E. ODC is subjected to a complex array of cell-specific regulatory mechanisms that govern transcription, mRNA stability, translation, and enzyme degradation [18]. In the present study, TSE-dependent inhibition of ODC mRNA expression appears to be a mechanism for the action on ODC expression, demonstrating an effect at the level of gene transcription. As mentioned above, *ODC* gene transcription can be regulated by at least four transcription factors [14], and, in particular, the c-Myc/Max complex can transactivate the mouse *ODC* gene through interaction with the E-box located in the first intron [19]. The present MSA data indicate that TSE inhibited the binding activity of nuclear extracts to the c-Myc/Max consensus sequence elevated by NNK treatment, while the binding activities of the other factors tested were not affected by TSE and NNK treatment. Taken together, it is likely that the present results are consistent with the involvement of the c-Myc/Max complex in TSE-induced inhibition of ODC expression by NNK treatment. In a previous report [20], the reduction in the c-Myc protein level was in parallel with the decrease in the binding activity of nuclear extracts to the oligonucleotide-containing c-Myc/Max consensus sequence and ODC mRNA expression. However, our preliminary experiment indicated that TSE treatment did not affect the level of c-Myc and Max proteins.[2] Thus, it seems that the reduction in the binding activity of c-Myc/Max complexes is due to a posttranslational modification of c-Myc and/or Max proteins. However, further study is needed to clarify this possible mechanism, and such a clarification may support the establishment of new chemopreventive agents for lung cancer based on the non-antioxidative property of vitamin E.

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