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Fenofibrate exhibits a high potential to suppress the formation of squamous cell carcinoma in an oral-specific 4-nitroquinoline 1-oxide/arecoline mouse model

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ABSTRACT

The excessive use of areca nut and/or tobacco may induce the production of free radicals and reactive oxygen species, which affect the lipid contents of the cell membrane and are possibly involved in tumorigenic processes in the oral cavity. The aim of this study was to investigate the therapeutic efficacy of fenofibrate (0.1% or 0.3%, w/w), a ligand of the peroxisome proliferator-activated receptor alpha (PPARα), in a 4-nitroquinoline 1-oxide (4-NQO)/arecoline-induced oral cancer mouse model. The carcinogen, 4-NQO/arecoline, was administrated to C57BL/6JNarl mice for 8 weeks followed by fenofibrate treatment for 12 or 20 weeks. After 28 weeks, changes in serum lipids, the multiplicity of tumor lesions, and tumor sizes were determined together with changes in the immunohistochemical expressions of PPARα, acetyl-coenzyme A carboxylase (ACC), the epidermal growth factor receptor (EGFR), and cyclooxygenase-2 (COX2). The results showed that when compared to the 4-NQO/arecoline only group, 0.3% fenofibrate treatment increased serum total cholesterol, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) levels. 0.3% fenofibrate treatment suppressed the incidence rate of tongue lesions, reduced the multiplicity of squamous cell carcinoma (SCC), decreased the tumor size, and increased the immunoreactivity of EGFR and COX2 in oral dysplasia but decreased EGFR and COX2 expressions in SCC. These findings indicated that fenofibrate reduced the tumor incidence rate and suppressed the tumor progression into SCC and that these molecular events might be linked to the EGFR and COX2 regulatory pathways. We suggest that fenofibrate provides a new strategy for preventing oral tumor progression.

1. Introduction

Smoking, betel quid (BQ) chewing, and the consumption of alcohol and coffee are common habits related to the incidence of oral cancer [1]. Most patients with oral cancer are not diagnosed until the advanced stages of the disease and often fail to respond to available therapies. Therefore, the 5-year survival rate of oral cancer patients is relatively low when compared to other types of cancer [2]. Thus, understanding whether precancerous lesions and their progression to cancer can be prevented will aid in the development of novel molecular strategies for oral cancer prevention and treatment.

The excessive use of areca nut and/or tobacco induces the production of free radicals and reactive oxygen species, which are responsible for the high rate of oxidation/peroxidation of polyunsaturated fatty acids [3]. Lipid peroxidation affects essential constituents of the cell membrane and may be involved in tumorigenic processes in the oral cavity [4,5]. Lipids are the major cell membrane components essential for various biological functions including cell growth and the division of normal and malignant tissues. An inverse association was found between a lower plasma lipid profile and head and neck malignancies and an oral precancerous condition [6,7]. The lower levels of plasma lipids in oral cancer patients might be due to their increased utilization by neoplastic cells for biosynthesis of new

Abbreviations: ACC, acetyl-coenzyme A carboxylase; BQ, betel quid; COX2, cyclooxygenase-2; EGFR, epidermal growth factor receptor; FAS, fatty acid synthase; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; 4-NQO, 4-nitroquinoline 1-oxide; PPARα, peroxisome proliferator-activated receptor alpha; SCC, squamous cell carcinoma; TC, total cholesterol; VLDL, very low-density lipoprotein

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membrane [7]. Therefore, the control of lipid homeostasis is crucial for developing a new therapeutic strategy for the treatment of oral cancer. This hypolipidemic strategy would be beneficial in preventing the development of oral tumorigenesis.

The peroxisome proliferator-activated receptor alpha (PPARα), a member of the nuclear receptor superfamily of ligand-activated transcription factors, is a key regulator of lipolysis, triglyceride-rich lipoprotein metabolism, and inflammatory/vascular pathways [8–11]. Fenofibrate, a ligand of PPARα, is a widely used hypolipidemic drug with anti-inflammatory and anti-atherosclerotic effects in the vessel wall [8,10]. Fenofibrate exerts a wide spectrum of lipid-modifying actions that influence atherogenic dyslipidemia, such as a reduction in levels of triglycerides, very low-density lipoproteins (VLDL), VLDL remnants, intermediate-density lipoproteins, an increase in plasma levels of high-density lipoprotein (HDL) cholesterol, and a decrease in the proportion of small, dense low-density lipoprotein (LDL) particles in the LDL density profile [12,13].

To explore whether oral tumorigenesis can be prevented by fenofibrate, animal models that faithfully simulate the human oral cancer condition are critical. 4-nitroquinoline 1-oxide (4-NQO) is one of the chemical carcinogens used for inducing oral carcinogenesis and served as a surrogate for tobacco exposure [14]. Arecoline, the major alkaloid in areca nut extracts and BQ, promoted 4-NQO carcinogenesis in mice [15]. Our previous studies showed that 4-NQO plus arecoline-induced oral lesions manifested as epithelial hyperplasia, dysplasia, and invasive squamous cell carcinoma (SCC), which mimicked the pathogenesis, environment, and subsequent molecular events in SCC patients in Southeast Asia and Taiwan [15–17]. In this model, the tongue tumor incidence rate was 100% in mice treated concomitantly with 200 μg/ml 4-NQO and 500 μg/ml arecoline for 8 weeks, and many tongue lesions evolved spontaneously into highly malignant invasive SCC over the following 20 weeks. Therefore, the present study was aimed to investigate the therapeutic efficacy of fenofibrate in a 4-NQO/arecoline-induced oral tumorigenesis mouse model. Changes in plasma lipids and tumor size were determined along with variations in the immunohistochemical expressions of PPARα, fatty acid synthase (FAS), acetyl-coenzyme A carboxylase (ACC), epidermal growth factor receptor (EGFR), and cyclooxygenase-2 (COX2), which are involved in lipid metabolism and inflammatory/tumor progression pathways [8,10,16,18].

2. Material and methods

2.1. Administration of 4-NQO and arecoline

Sixty 6-week-old male C57BL/6JNarl mice were purchased from the National Laboratory Animal Center. The mice were handled in accordance with the Animal Care and Use Guidelines of the China Medical University, and the study protocol was approved by the Institutional Animal Care Use Committee. Mice were randomized into one of six groups (Table 1). The experiments were carried out under controlled conditions with a 12-h light/dark cycle. Mice were fed with standard mouse chow (Prolab® RMH 2500 PMI Nutrition International, LLC, MO, USA). The carcinogens, 200 μg/ml 4-NQO (Sigma-Aldrich, St. Louis, MO, USA) and 500 μg/ml arecoline hydrobromide (Fluka, Buchs, China), were dissolved in the drinking water; the water was replaced once a week. Ten mice per group were allowed to access the drinking water and chow diet ad libitum during the treatment. All mice were weighed every 4 weeks.

2.2. Fenofibrate treatment

For the analysis of the effects of fenofibrate on tumor development, mice were exposed to 4-NQO/arecoline for 8 weeks and then randomly divided into six treatment groups for the following 20 weeks, as shown in Table 1. Mice received fenofibrate orally either at a low dose of 0.1% diet/mouse/day (about 200 mg/kg body weight/day) or at a high dose of 0.3% diet/mouse/day (about 500 mg/kg body weight/day). Chow diet was ground into powder, and the powdered diet was used as a basal diet during the experiment. The experimental diet was prepared by mixing fenofibrate (0.1% or 0.3%, w/w) with the powdered diet. All mice were monitored, and diets were replaced daily. To maintain the diet stability, all diets were stored at 4 °C before use. Precancerous and cancerous lesions of the tongue were diagnosed and photographed bi-weekly. According to our previous study [15,16], a therapeutic window between week 14 and week 20 was observed in this mouse model. In this study protocol, administration of fenofibrate from week 9 to week 20 (a total 12 weeks) was designed to explore whether a lowering of serum lipids would affect preneoplastic lesion formation. In addition, administration of fenofibrate from week 9 to week 28 (a total 20 weeks) was intended to investigate the effects of long-term fenofibrate treatment on tumorigenesis. At the end of 28 weeks, all mice were sacrificed after a 12-h overnight fast for blood analysis. The tongues were then excised and fixed in 10% formaldehyde for pathological and immunohistochemical analysis.

2.3. Serum lipids analysis

At the end of 28 weeks, blood samples were obtained from each mouse after a 12-h overnight fast. The serum concentrations of the total cholesterol (TC), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), and triglyceride were measured by a Roche/Hitachi COBAS MIRA PLUS analyzer.

2.4. Tongue lesion and pathological examination

Paraffin-embedded tongue specimens were stained with hematoxylin and eosin for histopathological examination. The lesions observed were classified as epithelial hyperplasia, dysplasia, or

Table 1
Body weights in each group during the 28-week treatment.

Group treatment (10 mice in each group)				Body weight (g)			
Group number	4-NQO/arecoline-induced period (weeks)	Dose of fenofibrate (g/100 g diet)	Period of fenofibrate treatment (weeks)	0 week	8 weeks	20 weeks	28 weeks
1	0	0	0	23.2 ± 1.7	28.7 ± 2.1	33.3 ± 3.0	33.6 ± 3.3
2	8	0	0	22.8 ± 1.6	25.8 ± 1.7[a]	29.4 ± 2.8[a]	25.7 ± 4.1[a]
3	8	0.1	12	22.6 ± 1.1	25.8 ± 1.2[a]	27.6 ± 1.9[a]	27.3 ± 3.3[a]
4	8	0.3	12	22.5 ± 1.2	24.8 ± 2.0[a]	23.5 ± 3.1[a,b,c]	26.2 ± 4.2[a]
5	8	0.1	20	22.2 ± 1.7	24.9 ± 1.9[a]	27.0 ± 1.9[a,d]	25.5 ± 3.2[a]
6	8	0.3	20	22.9 ± 1.3	25.3 ± 1.1[a]	24.4 ± 1.2[a,b]	22.8 ± 1.5[a]

[a] Significantly different from group 1 by One way ANOVA ($p<0.005$).
[b] Significantly different from group 2 by One way ANOVA ($p<0.001$).
[c] Significantly different from group 3 by One way ANOVA ($p<0.005$).
[d] Significantly different from group 4 by One way ANOVA ($p<0.05$).

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invasive SCC. The histopathological diagnoses were established as previously described [15]. The multiplicity from all tumor lesions in each tongue was evaluated as previously described [19]. Tongue lesions attained from 4-NQO/arecoline-induced mice after the 28-week time point were more widespread, thus the number of every macroscopic tumor lesions was counted. The tumor sizes of visible lesions in each tongue were measured individually in width (mm) and only the largest lesion is presented in statistics (Fig. 1C). For example, in a case with four lesions with the widths of 3 mm, <1 mm, 1.5 mm, and 1 mm, the tumor size in this animal was recorded as 3 mm as shown in Fig. 1B.

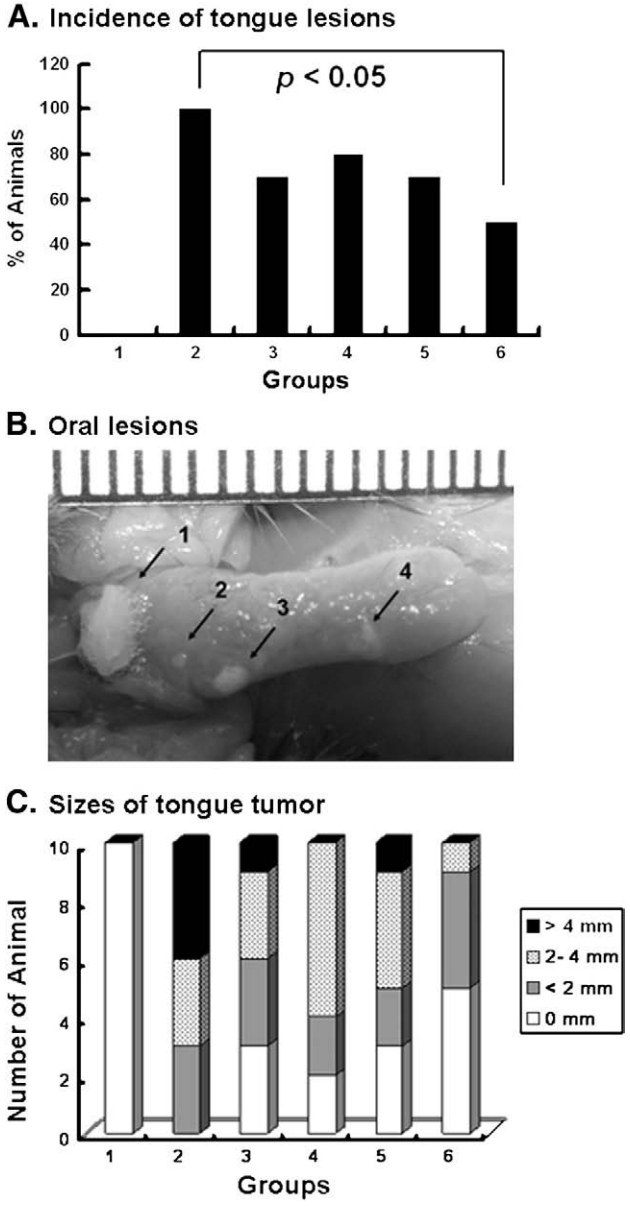

Fig. 1. Incidence of tongue lesions and tumor size in each group after a 28-week treatment. Ten mice in each group were examined. (A) The incidence of tongue lesions was presented as the percentage of mice exhibiting tongue lesions after 28 weeks. Lesions included hyperplasia, dysplasia, and invasive squamous cell carcinoma. (B) The tongue lesions in one representative mouse are shown by arrows. The tumor sizes were measured as 3 mm, <1 mm, 1.5 mm, and 1 mm, respectively. (C) The tumor sizes in each group are presented as indicated. Group treatments are described as in Table 1. Mice treated concomitantly with fenofibrate (0.3%, w/w) and 4-NQO/arecoline (group 6) had a significant decrease in the incidence of tongue lesions ($p<0.05$, 1A) and tumor size ($p<0.05$, 1 C) when compared with the 4-NQO/arecoline treated mice (group 2) using Fisher's exact test.

2.5. Immunohistochemical analysis

Four primary antibodies from Abcam, Cambridge, UK were used at the following concentrations: PPARα (ab2779) mouse monoclonal antibody, 1:200; FAS (ab22759) rabbit polyclonal antibody, 1:100; ACC (ab45174) rabbit monoclonal antibody, 1:30; and EGFR (ab2430) rabbit polyclonal antibody, 1:50. A rabbit monoclonal antibody against COX-2 (Cat. no. 4212-1, Epitomics, California, USA) was used at a 1:100 dilution. During immunostaining, samples were first treated with a target retrieval solution (Dako, Denmark) and then incubated with the diluted primary antibody for 60 min at 25 °C. Next, samples were thoroughly washed three times with PBS, treated with diaminobenzidine as the chromogen, and incubated with EnVision-Plus (Dako, Carpinteria, CA, USA) for 30 min. The skeletal muscle derived from murine tongue tissue was used as an internal positive control for the staining quality of these five antibodies. The staining intensities were graded as negative (−), weak positive in less than 10% of the area (+/−), weak positive (1+), moderate positive (2+), and strong positive (3+) [19]; the staining intensities were scored as 0, 1, 2, 3, and 4, respectively. All lesions in each tongue were scored. For example, in a case with two lesions, the results of histopathological diagnosis were hyperplasia and SCC. The staining intensities of weak positive staining in hyperplasia and of moderate positive staining in SCC would be assigned a score of 2 and 3, respectively.

2.6. Statistical analysis

All data were analyzed using SPSS 15.0 for Windows. Significant differences in body weight, total cholesterol, triglyceride, LDL-C, and HDL-C between groups were analyzed using the one way analysis of variance (ANOVA) followed by multiple comparison tests. Statistical analyses of the tumor size and incidence of tongue lesions were performed using Fisher's exact probability test. Immunostaining intensities, which included PPARα, FAS, ACC, EGFR, and COX2, were compared using the one way ANOVA. A p-value of less than 0.05 was considered statistically significant. All data are shown as mean ± S.D.

3. Results

3.1. Body weight changes during the 28-week treatment

Mice administered 4-NQO/arecoline and fenofibrate did not display any clinical signs of poor health or a low survival rate. There were no significant differences in the average food intake of the 4-NQO/arecoline groups fed with or without fenofibrate (data not shown). The body weight changes in each group over the 28-week time course are shown in Table 1. At the end of 8 weeks, the body weight was significantly lower in mice fed with 4-NQO/arecoline than in mice fed with tap water (group 1; $p<0.005$, Table 1). After week 8, 4-NQO/arecoline administration was discontinued, and mice were treated with high (0.3%) or low (0.1%) doses of fenofibrate. At the end of 20 weeks, the body weight was lower in mice fed with 0.3% fenofibrate (group 4, $p<0.001$; group 6, $p<0.001$) than that in mice fed the basal diet (Group 2). When fenofibrate administration was continued until 28 weeks, the average body weight slightly decreased, but no significant difference was found between groups 5 and 6. At the end of 28 weeks, the body weight was the highest in group 1.

3.2. Effects of fenofibrate on serum lipid levels

After the 28-week treatment, mice treated with 4-NQO/arecoline (group 2) showed a significant increase in serum LDL-C when compared with control mice (group 1) ($p<0.05$, Table 2). Administration of 0.3% fenofibrate to 4-NQO/arecoline-induced mice for 20 weeks (group 6) showed significant increases in serum total cholesterol ($p<0.001$), LDL-C ($p<0.001$), and HDL-C ($p<0.001$) levels

Table 2
Serum lipids concentration in each group after 28 weeks.

Group number	Group treatment (10 mice in each group)			Serum lipids			
	4-NQO/arecoline-induced period (weeks)	Dose of fenofibrate (g/100 g diet)	Period of fenofibrate treatment (weeks)	Total cholesterol (mg/dl)	LDL-C (mg/dl)	HDL-C (mg/dl)	Triglyceride (mg/dl)
1	0	0	0	65.2 ± 5.6	6.9 ± 1.2[a]	46.9 ± 3.6	41.4 ± 4.5
2	8	0	0	64.7 ± 8.5	12.8 ± 5.6	43.8 ± 4.9	48.6 ± 11.9
3	8	0.1	12	65.9 ± 9.4	9.3 ± 2.6	45.7 ± 3.7	33.5 ± 15.6
4	8	0.3	12	68.5 ± 8.4	11.4 ± 2.5	50.0 ± 5.7[a]	41.7 ± 20.1
5	8	0.1	20	70.4 ± 3.8	14.0 ± 2.3	51.0 ± 2.0[a]	57.3 ± 5.5[c]
6	8	0.3	20	92.7 ± 5.1[b,e]	24.4 ± 5.7 [b,c,e]	65.9 ± 3.8[b,c,e]	61.5 ± 4.2 [d]

LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol.
[a] Significantly different from group 2 by One way ANOVA ($p<0.05$).
[b] Significantly different from group 2 by One way ANOVA ($p<0.001$).
[c] Significantly different from group 3 by One way ANOVA (c, $p<0.005$).
[d] Significantly different from group 4 by One way ANOVA ($p<0.01$).
[e] Significantly different from group 4 by One way ANOVA ($p<0.001$).

compared with mice in the 4-NQO/arecoline group. Notably, 0.3% fenofibrate treatment for 12 weeks (group 4) did not display the above hypercholesterolemic effects of the 20-week fenofibrate treatment. In addition, no significant differences in triglyceride levels were observed between the 4-NQO/arecoline only group and the fenofibrate-treated groups (Table 2). When both 0.1% and 0.3% fenofibrate administration in groups 5 and 6 was extended from 12 weeks to 20 weeks, the serum triglyceride levels significantly increased (group 5 vs. group 3, $p<0.005$; group 6 vs. group 4, $p<0.01$).

3.3. Effects of fenofibrate on tumor incidence and tumor formation

To carefully characterize the development of tumors in this model, lesions of the tongue were examined and photographed bi-weekly. As found in our previous report [15], no tongue lesion was found in the 4-NQO/arecoline-induced groups at week 8. However, the incidence of tongue lesions increased spontaneously over the following 20 weeks. After 28 weeks, the incidence rate of tongue lesions in the 0.3% fenofibrate-treated mice (group 6) was lower than that in the 4-NQO/arecoline only group ($p<0.05$, Fig. 1A). We compared the incidence rates between different fenofibrate-treated durations. The results indicated that the administration of 0.3% fenofibrate did not show a significant difference ($p=0.35$) in the incidence rates between the 12-week (80%, group 4) and the 20-week (50%, group 6) treatment groups (Fig. 1A).

Since all mice exposed to 4-NQO/arecoline developed one or more lesions, the largest lesion per mouse from each group was compared at the end of 28 weeks. The administration of 0.3% fenofibrate (group 6) significantly reduced the tumor size compared with tumors in the mice treated with 4-NQO/arecoline only (group 2, $p<0.05$, Fig. 1C) after the 28-week observation. In group 2, we found three mice with a small lesion (<2 mm), three mice with a medium lesion (2–4 mm), and four mice with a large lesion (>4 mm). As for the fenofibrate treatment (group 6), five mice were lesion free, four mice had small lesions, and one mouse with a medium lesion. In addition, no significant difference in tumor size was found between group 4, which was treated with 0.3% fenofibrate for 12 weeks, and group 2 ($p=0.071$) or between groups 4 and 6 ($p=0.106$). These results indicated that 0.3% fenofibrate suppressed the tumor incidence and formation after continuing administration for 20 weeks.

3.4. Multiplicity of tongue lesions in mice treated with fenofibrate

Based on group 6 having the lowest incidence of tongue tumors after 0.3% fenofibrate treatment, we conducted a detailed histological analysis of the tongue lesions among groups 1, 2, and 6. The multiplicity of the tongue was evaluated from all lesions in each tongue because tongue lesions obtained from 4-NQO/arecoline mice after the 28-week time point were more widespread. The tongue lesions were classified into five categories of hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia, and SCC. The multiplicity of SCC in group 6 was significantly lower than that in group 2 ($p<0.05$, Table 3). This finding indicated that fenofibrate suppressed tumor progression into SCC.

3.5. Expressions of PPARα, ACC, EGFR, FAS, and COX2 during the histological progression of oral tumorigenesis in fenofibrate-treated mice

Immunohistochemical staining of PPARα, ACC, EGFR, and COX2 was examined from all lesions in each tongue in groups 2 and 6. The results of the immunohistochemical expressions among histological grades in the tongue lesions are shown in Fig. 2. The staining intensity of PPARα, ACC, EGFR, and COX2 in normal epithelium, squamous hyperplasia, dysplasia, and SCC was increased through the progression of the lesions from hyperplasia to SCC. PPARα, a ligand-activated transcription factor, which is a key regulator of lipid metabolism and inflammatory/vascular pathways, was expressed in the nucleus (Fig. 2A). Expression of ACC, a rate-limiting enzyme that catalyzes the formation of malonyl CoA (precursor for long chain fatty acyl CoA) from acetyl CoA, was found in the cytoplasm of immuno-positive cells (Fig. 2B). Expression of EGFR, a prototype member of the type 1 receptor tyrosine kinases, was found in the cell membrane (Fig. 2C). The EGFR and its downstream effectors signal diverse cellular functions, including cell proliferation, differentiation, motility, survival, and tissue development [16]. Expression of COX2 was found in the cytoplasm (Fig. 2D). The elevated levels of COX2 expression are involved in the possible contribution of proinflammatory pathways [18]. A diffuse immunoreactivity of COX2 was present in the epithelial dysplasia and SCC (Fig. 2D). Also, minimal lymphocytic infiltration was noted (Supplementary Fig. 1).

In addition, the results of the semi-quantitative scoring of the immunoreactivity in the tongue lesions of group 2 (4-NQO/arecoline treatment only) and group 6 (0.3% fenofibrate treatment) are shown in Fig. 3. Administration of 0.3% fenofibrate significantly increased the

Table 3
Multiplicity of tongue lesions in mice after 28-week treatment.

Group number	1	2	6
Group treatment			
4-NQO/arecoline-induced period (weeks)	0	8	8
Dose of fenofibrate (g/100 g diet)	0	0	0.3
Period of fenofibrate treatment (weeks)	0	0	20
No. of mice	10	10	10
Multiplicity (no. of tongue lesions/mouse, mean ± SD)			
Hyperplasia	0	1.00 ± 0.00	1.00 ± 0.00
Mild dysplasia	0	0.40 ± 0.52	0.20 ± 0.42
Moderate dysplasia	0	0.40 ± 0.52	0.60 ± 0.52
Severe dysplasia	0	0.40 ± 0.52	0.30 ± 0.48
SCC	0	0.80 ± 0.42	0.30 ± 0.48[a]

[a] Significantly different from group 2 by One way ANOVA ($p<0.05$).

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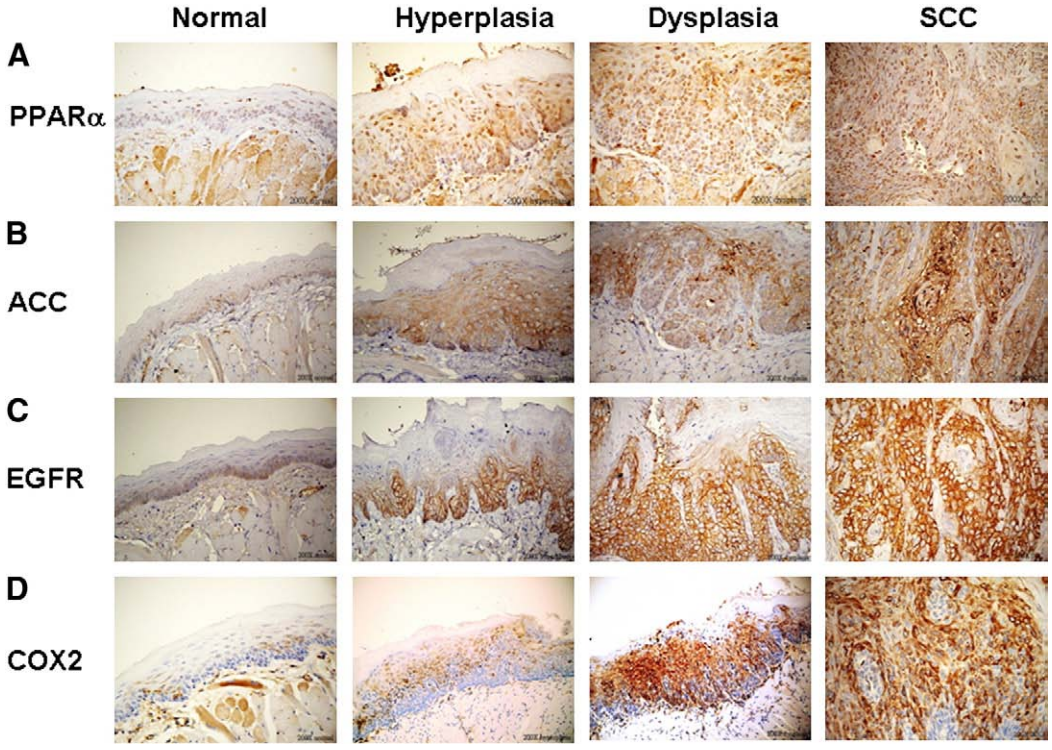

Fig. 2. Immunohistochemical staining of PPARα, ACC, EGFR, and COX2 in the 4-NQO/arecoline mouse model of oral cancer. Tissue sections containing areas of normal, hyperplasia, dysplasia, and squamous cell carcinoma. The staining intensity of PPARα (A), ACC (B), EGFR (C), and COX2 (D) in normal epithelium, squamous hyperplasia, dysplasia, and SCC increases progressively (Magnification, 200×). The skeletal muscle derived from murine tongue tissue was used as an internal positive control for the staining quality of these four antibodies. PPARα, peroxisome proliferator-activated receptor alpha; ACC, acetyl-coenzyme A carboxylase; EGFR, epidermal growth factor receptor; COX2, cyclooxygenase-2.

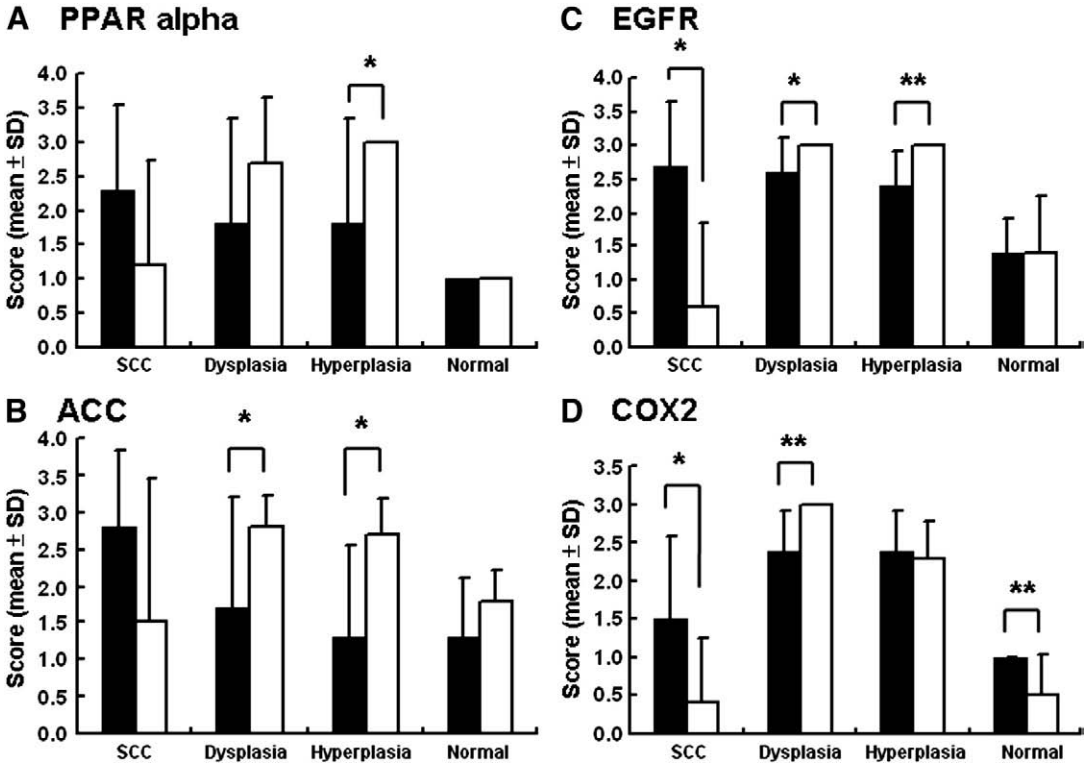

Fig. 3. Expression of PPARα, ACC, EGFR, and COX2 in the 4-NQO/arecoline-induced oral tumorigenesis mouse model. Ten mice in each group were examined. The immunohistochemical staining intensity of each protein in the tongue lesions was determined using a scoring system with five grades from negative (0), weak positive in less than 10% of the area (1), weak positive (2), moderate positive (3), and strong positive immunoreactivity (4). The open bars represent the mean immunoreactivity score of group 6 (0.3% fenofibrate treatment). The close bars represent the mean immunoreactivity score of group 2 (4-NQO/arecoline only). The error bars represent SD. *$p<0.05$; **$p<0.01$.

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occurrence of hyperplasia with strong immunoreactivity of PPARα when compared with group 2 (Fig. 3A, $p<0.05$), but no significant difference was found in lesions showing dysplasia or SCC. This finding indicated that PPARα activation might play an important role in hyperplastic lesions. Because PPARα is a key regulator of lipolysis, we next asked whether the suppression of the tumor incidence and formation by fenofibrate was not limited to the lipolysis pathway. The central enzymes in *de novo* lipogenesis, such as ACC and FAS, were also examined. The immunoreactivity of ACC was increased with dietary exposure to fenofibrate in both dysplasia (Fig. 3B, $p<0.05$) and hyperplasia ($p<0.05$) tissues. However, no significant differences in the expression of FAS were found between the 0.3% fenofibrate-treated and 4-NQO/arecoline only groups (data not shown). These results indicated that there is another signal between PPARα activation and tumor progression.

Because EGFR signaling was required for cell survival in the EGFR-amplified/overexpressing oral SCC [16] and activation of EGFR signaling has been reported to drive COX2 transcription [18], we examined the expressions of EGFR and COX2 after fenofibrate treatment. We found that administration of 0.3% fenofibrate (group 6) significantly increased EGFR expression levels in lesions with hyperplasia (Fig. 3C, $p<0.05$) and dysplasia ($p<0.05$), and significantly decreased the expression of EGFR in SCC lesions ($p<0.01$) when compared with group 2. The immunoreactivity of COX2 was increased with dietary exposure to fenofibrate in dysplasia lesions (Fig. 3D, $p<0.01$) and was decreased in SCC lesions ($p<0.05$). These findings indicated that fenofibrate suppressed tumor progression into SCC and suggested that this might be related to the lower expression levels of EGFR and COX2 in SCC lesions.

4. Discussion

Activation of PPARα by fenofibrate results in lipid-modifying actions that influence atherogenic dyslipidemia [20]. In this study, we found that the mice treated with the carcinogen 4-NQO/arecoline (group 2) showed an increase in serum LDL-C and no changes in total cholesterol and HDL-C levels when compared to control mice (group 1) at the end of 28 weeks. After withdrawal of the carcinogen, administration of 0.3% fenofibrate for the following 20 weeks increased serum total cholesterol, LDL-C, and HDL-C levels when compared to group 2. HDL-C levels were increased in both the 0.1% and 0.3% fenofibrate feeding groups. PPARα agonists were found to enhance components of the HDL synthetic pathways and to increase reverse cholesterol transport [10]. Fenofibrate, a PPARα agonist, was shown to increase serum HDL-C levels by increasing expression of the genes for both ApoA-I and ApoA-II, decreasing the cholesteryl ester transfer protein-mediated transfer of cholesterol from HDL to VLDL. This transfer enhances the cholesterol efflux of the cell by induction of ATP-binding cassette transporter A1 (ABCA1) expression and decreasing scavenger receptor-class B type 1 (SR-B1) in the liver [12,21]. Therefore, the increase in total cholesterol levels in the 0.3% fenofibrate-treated group might be due to increases in the HDL-C and LDL-C levels, which were induced by fenofibrate and 4-NQO/arecoline, respectively. Interestingly, no significant differences in triglyceride levels were observed between the 4-NQO/arecoline only group and the 4-NQO/arecoline groups treated with fenofibrate. Administration of fenofibrate did not show the triglyceride lowering effects on tumor progression after a complete 28-week observation. In contrast, both 0.1% and 0.3% fenofibrate-treated mice showed a significant increase in triglyceride levels after 20 weeks of treatment when compared to mice after 12 weeks of treatment. At the end of 28 weeks, we found that 50% of the 0.3% fenofibrate-treated mice (group 6) were tumor free, 40% had a tumor size less than 2 mm, and 10% had a tumor size of 2 to 4 mm; however, the corresponding percentages in 0.1% fenofibrate-treated mice (group 4) were 20%, 20%, and 60%, respectively. These findings indicated that the higher levels of serum triglyceride in the 0.3% fenofibrate-treated mice might be due to their decreased utilization by neoplastic cells for the biosynthesis of new membrane as compared with the 0.1% fenofibrate-treated mice. The lower rate of tumor incidence and smaller tumor sizes were evident in the 0.3% fenofibrate-treated mice (Fig. 1). An inverse association between a lower plasma lipid profile and head and neck cancer was also found in human studies [6,7].

Long-term (20 weeks) administration of 0.3% fenofibrate reduced the severity and extent of tongue lesions, which was evident from the decreased tumor incidence and size as well as the tumor histological examination. With respect to the incidence of tongue lesions, a 50% reduction in the 0.3% fenofibrate-treated group was observed when compared to the 4-NQO/arecoline only group (Fig. 1A). Also, an average reduction rate of 62.5% was observed in the multiplicity of SCC (Table 3). However, fenofibrate at a low dose (0.1%/mouse/day) lacked anti-tumor activity in this study. This result is consistent with the previous observation that fenofibrate at a low daily dose (0.1%–0.25%) lacked anti-tumor activity in primary hamster melanoma [22] and murine endometrial cancer [23]. Recently, animal studies showed that activation of PPARα might be attributable to the inhibition of angiogenesis [24].

Using the 4-NQO/arecoline mouse model, we identified a tumor suppressive effect of fenofibrate and suggested that this lipid-lowering agent might exert some indirect actions on pathways known to be relevant to oral tumorigenesis. There were two possibilities that could explain the effect of fenofibrate on tumorigenesis. First, there was crosstalk between PPARα activation and EGFR expression in our study. Fenofibrate increased the EGFR expression levels in hyperplastic and dysplastic lesions and decreased the EGFR expression in SCC (Fig. 3C). Notably, EGFR signaling was required for cell survival in the EGFR-amplified/overexpression oral SCC [16]. Secondly, fenofibrate-induced COX2 expression was linked to the development of oral tumorigenesis (Fig. 3D). Previous reports indicated that activation of the EGFR signaling pathway can drive COX2 transcription and prostaglandin E2 synthesis, which could stimulate cell proliferation, induce angiogenesis, inhibit apoptosis, and suppress immune surveillance [18,25–27]. Moraitis et al. reported that tobacco smoke stimulated EGFR tyrosine kinase activity leading to enhanced transcription of COX2 [18]. In our study, 4-NQO, a surrogate of tobacco exposure, treated concomitantly with arecoline-induced the expression of EGFR and COX2 in tumor progressive grades (hyperplasia, dysplasia, and SCC) when compared to normal tissue. Increases in the expression of EGFR and COX2 were observed in the preneoplastic lesions of fenofibrate-treated mice versus untreated mice, and, most importantly, there was a decrease in the expression of EGFR and COX2 in the neoplastic cells. Fenofibrate exhibited a high potential to inhibit the formation of SCC. Therefore, these results provide insight into fenofibrate as a suppressor of the progression of oral tumorigenesis.

In conclusion, the activation of PPARα, which can be linked to the expression of EGFR and COX2, shows the beneficial effects of fenofibrate in this oral-specific 4-NQO/arecoline mouse model. The cascade of molecular events causes the reduction in the incidence and size of squamous carcinoma and suppresses the progression of the preneoplastic lesion into SCC. Therefore, we suggest that fenofibrate should be considered as a strategy for preventing oral tumor progression.

Supplementary materials related to this article can be found online at doi:10.1016/j.bbadis.2010.11.002.

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