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Regular exercise reduces colon tumorigenesis associated with suppression of iNOS

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ABSTRACT

Several epidemiological studies have shown that regular exercise can prevent the onset of colon cancer, although the mechanism involved is unclear. Expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) is often elevated in an initial step of tumorigenesis and promotes colorectal cancer. We investigated the effect of exercise on colon tumorigenesis associated with iNOS and COX-2 in azoxymethan (AOM)-injected mice. Balb/c mice (8 weeks old) were divided into three groups of 20 animals each, consisting of a sedentary control group, an AOM group, and an exercise plus AOM group. Mice in the groups receiving AOM were injected intraperitoneally with AOM weekly for 2 weeks. Six weeks of regular exercise suppressed the generation of aberrant crypt foci (ACF) in the colon by AOM. Expression of iNOS was decreased by exercise compared with that in sedentary mice along with lower nitrotyrosine level while COX-2 was not changed by either AOM or exercise. Additionally, tumor necrosis factor α (TNFα) was decreased by exercise in the colon and plasma. There was no effect of exercise on the expression of antioxidant enzymes and chaperon protein in the colon. Our results suggest that regular exercise prevents colon tumorigenesis, at least partly via the suppression of iNOS expression associated with anti-inflammation.

1. Introduction

Cancer is one of the major causes of death in developed countries. In particular, colorectal cancer is the second most common cause of all cancer deaths and its incidence has been increasing more rapidly than any other type of cancer in recent years. Thus, it is vital to consider colorectal cancer prevention as an important part of any health care program. Several epidemiological studies performed in Europe, the United States, and Japan have shown that regular exercise reduces the incidence of colon cancer [1–4]. In addition, a report published by the World Cancer Research Fund (WCRF) and the American Institute for Cancer Research (AICR) has summarized the lifestyle factors related to cancer prevention that had been detected by epidemiological studies and indicated that physical activity is one of the lifestyle factors contributing to the reduction of colon cancer risk [5]. The antitumor effect of exercise has also been identified in experimental studies [6–8], in which some potential mechanisms such as activation of the immune system, metabolic improvement, and exercise-induced increase in gastrointestinal transit speed have been suggested. However, the exact mechanism involved is still unclear.

Colon tumorigenesis develops through a multi-step process, and the antitumor effect of exercise is supposed to result from prevention at an early stage. Since the expression of both inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) is often elevated in the initial phase of carcinogenesis [9], iNOS and COX-2 are considered key factors in the development of colon cancer. Upregulated iNOS produces excess nitric oxide (NO) and results in the generation of peroxynitrite by the reaction between NO and superoxide. Peroxynitrite can impair various proteins and DNA by nitration, nitrosylation, and oxidation, which can be a critical factor in tumor development in the colon [10]. Alternatively, a reaction product of COX-2, prostaglandin E_2 (PGE_2), has been shown to enhance colon tumorigenesis through induction of cell proliferation and reduction of apoptosis [11]. Based on this background, we hypothesized that exercise may suppress colon tumorigenesis in association with inhibition of iNOS and COX-2 in this tissue.

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Aberrant crypt foci (ACF) are putative precursor lesions of adenocarcinoma in the colon and can be detected during the early process of tumorigenesis induced in the colon by azoxymethan (AOM) [12]. Previous studies examining the influence of bioactive factors on tumorigenesis have shown that ACF are a useful marker for the evaluation of the preventive effect of certain interventions [13,14]. Thus, to test our hypothesis, we investigated the effect of regular exercise on the AOM-induced formation of ACF and analyzed the connection between these phenomena and the expression of iNOS, COX-2, and anti-inflammation-related factors such as antioxidants and chaperon proteins in mice. We report here that regular exercise can prevent the early process of colon tumorigenesis and suppress iNOS induction.

2. Materials and methods

2.1. Animals and experimental design

The present study complied with the principles and guidelines of the Japanese Council on Animal Care and was approved by the Committee for Animal Research of the Kyoto Prefectural University of Medicine (permission No. M21-4). Balb/c mice (6 weeks old) were obtained from Shimizu Laboratory Supplies Co., Ltd. (Kyoto, Japan) and were acclimatized to an air-conditioned (22 ± 2 °C) room with a 12-h light/dark cycle (lights on from 07:30 to 19:30 h) for 2 weeks. The mice were divided into three groups of 20 animals each, consisting of a sedentary control group (CON), an AOM treatment group (AOMC), and an AOM treatment with regular exercise group (AOMEx). Mice in the AOM treatment groups were injected i.p. with AOM (Sigma, St. Louise, MO) at a dose of 12.5 mg/kg body weight once a week for 2 weeks; and those in the sedentary control group were given two i.p. injections of saline only. Starting after the first injection of AOM, mice in the regular exercise group were subjected to a running exercise regimen on a motorized treadmill three times per week for 6 weeks. During the first 2 weeks, the level of exercise was gradually increased from running for 15 min with the treadmill set at a speed of 15 m/min to running for 30 min at 20 m/min; then, running speed was kept at 20 m/min for the following 4 weeks. At the same time of day, when AOMEx mice were subjected to running exercise, sedentary mice were placed on a stationary treadmill three times per week for the same duration of the exercise session. The diet was identical for the three groups of mice. Animals were euthanized 24 h after the last exercise session and the colon was removed. Colon samples obtained from 10 animals each group were used for counting the number of ACF, and samples from the remaining 10 animals each group were used for biochemical assays.

2.2. Counting the number of ACF

The ACF was determined according to the standard procedures described previously [15]. ACF are defined as single or multiple aberrant crypts (AC) that have altered luminal openings, exhibit thickened epithelia, and are larger than adjacent normal crypts. After the colons were fixed flat in 10% buffered formalin for 24 h, the mucosal surface of the colons were stained with methylene blue (0.5% in distilled water), and then the number of ACF and AC were counted under a light microscope.

2.3. Real-time PCR

Reverse transcription (RT) polymerase chain reaction (PCR) was performed using total RNA samples obtained from colon tissues with an ABI 7300 system (Applied Biosystems). Real-time PCR using the DNA-binding dye SYBR Green was employed for the detection of PCR products for RNA sample obtained from cells after synthesized cDNA. The following PCR primers (Sigma–Aldrich, Japan, Hokkaido, Japan) were used: iNOS, 5′-GGCAGCCTGTGA GACCTTTG (forward) and 5′-GCATTGGAAGTGAAGCGTTTC (reverse); COX-2, 5′-CTGAAGACGTCCTCCACTCATG (forward) and 5′-TGGTCGGTTTGATGTTACTGTTG (reverse); tumor necrosis factor-alpha (TNFα), 5′-ATCCGCGACGTGGAACTG (forward) and 5′-ACCG CCTGGAGTTCTGGAA (reverse) superoxide dismutase 1 (SOD1), 5′-TTCCATCATTGGCCGTACAA (forward) and 5′-AGCGGCTCCCAG CATTTG (reverse); superoxide dismutase 2 (SOD2), 5′-CACATT AACGCGCAGATCATG (forward) and 5′-GCCAGAGCCTCGTGGTACTT (reverse); glutathione peroxidase 1 (GPx1), 5′-(forward) and 5′-(reverse); glutathione peroxidase 2 (GPx2), 5′-(forward) and 5′-(reverse); catalase, 5′-CGACCAGGGCATCAAAAACT (forward) and 5′-CATTGGCGATGGCATTGAA (reverse); heat shock protein 70 (HSP70), 5′-CTGTAGGAAGGATTTGTACACTTTAAACTC (forward) and 5′-CCCTGGTCTGAGTCCCACACTCTCACC (reverse); 8-oxoguanine DNA-glycosylase 1 (OGG1), 5′-(forward) and 5′-(reverse); glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5′-TGTGT CCGTCGTGGATCTGA (forward) and 5′-CCTGCTTCACCACCTTCTTGA (reverse). The ratio of the other signals to that of GAPDH was calculated for every sample.

2.4. Protein measurements

Protein lysates obtained from colon tissue were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes. The blots were incubated with primary antibody against iNOS (Abcam, Cambridge, MA) and horseradish peroxidase (HRP)-conjugated secondary antibody (GE Healthcare Bio-Sciences, Buckinghamshire, UK) and the target protein was visualized by enhanced chemiluminescence (ECL plus, GE Healthcare Bio-Sciences). Band densities were measured with the Scion Image software (NIH, Research Service Branch).

Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to determine the levels of TNFα in the plasma (eBioscience; San Diego, CA) and nitrotyrosine in the colon (Cell Biolabs Inc., San Diego, CA). The absorbance was measured using a microplate reader and the concentrations of TNFα and nitrotyrosine were determined by comparison with calibration curves.

Citrate synthase activity was determined spectrophotometrically according to the method of Srere [16]. The enzymatic activity of citrate synthase was then normalized to the total protein content and was reported in micromoles per gram protein per minute.

2.5. Statistics

Results are reported as the mean ± SE. Differences between groups were evaluated by one-way ANOVA or Student's *t*-test. If ANOVA indicated a significance difference, Fisher's PLSD test was used to determine the significance of differences between mean values. In all analyses, $p < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Body weight, tissue mass, and citrate synthase activity

Body weight did not change in consequence of AOM injection, while it tended to decrease in exercise-trained mice (Table 1). Compared with the two sedentary groups of mice, epididymal fat mass was significantly decreased in the exercise group. The mass of the gastrocnemius muscle, however, was not significantly changed by either AOM injection or regular exercise. Skeletal muscle citrate synthase activity was significantly higher in the exercise group than in the sedentary groups.

Table 1
Body weight, tissue mass, and citrate synthase activity.

	CON	AOMC	AOMEx
Body weight (g)	31.6 ± 0.5	31.1 ± 0.3	30.1 ± 0.4
Gastrocnemius muscle (mg/g BW)	10.0 ± 0.1	9.9 ± 0.1	10.1 ± 0.1
Epididymal fat (mg/g BW)	9.9 ± 0.3	9.7 ± 0.3	8.8 ± 0.2[a,b]
Citrate synthase capacity (μmol/g prot./min)	341.5 ± 20.1	329.6 ± 23.1	388.4 ± 28.3[a,b]

Values are the mean ± SE. CON, sedentary normal control; AOMC, sedentary control with AOM; and AOMEx, exercise with AOM.
[a] Significant differences from CON at the level of $p < 0.05$.
[b] Significant differences from AOMC at the level of $p < 0.05$.

3.2. Number of ACF

ACF are common in humans and in animal models of colon cancer, and these preneoplastic lesions are thought to be a predictor of eventual tumor formation. Therefore, we examined ACF formation in the present mouse model to determine whether exercise could suppress colon carcinogenesis. ACF and AC developed in the colon of the mice injected with AOM, but not in the colon of the saline-treated mice (Fig. 1). Compared with the sedentary group, the exercise group showed a significant reduction in the number of both ACF and AC.

3.3. Expression of tumorigenesis factors

The expression of both iNOS and COX-2 increases during the initial period of tumorigenesis induced by AOM, and this event contributes to the formation of ACF [9]. Thus, we examined the effect of exercise on the expression of iNOS and COX-2 in the current study. Injection of AOM markedly elevated the expression of iNOS mRNA in the colon, while regular exercise suppressed this elevation (Fig. 2A). This response was also confirmed at the protein level (Fig. 2C). In addition, the level of nitrotyrosine in the colon was significantly reduced by regular exercise (Fig. 2D). However, COX-2 was not changed by either AOM injection or regular exercise (Fig. 2B).

Given that the expression of iNOS can be elevated through the inflammatory signaling cascade [17,18], we measured TNFα, one of the representative inflammatory cytokines. Compared with control mice, the expression of TNFα was increased in AOM-treated mice, while regular exercise significantly attenuated this increase (Fig. 3A). Furthermore, the concentration of TNFα in the plasma was reduced by exercise (Fig. 3B).

3.4. Expression of cancer protective factors

Regular exercise increases the expression of protective factors such as antioxidant enzymes and chaperon proteins in various organs, as suggested by previous studies [19,20]. Therefore, we analyzed the expression of both antioxidant enzymes and chaperon proteins in the colon tissue. We found that the expressions of SOD1/SOD2 and GPx1/GPx2 were not altered by either AOM injection or regular exercise (Table 2). The expressions of OGG1 and catalase were elevated by AOM, but not changed by exercise. In addition, the expression of HSP70 was markedly decreased by AOM, and a similar reduction was observed in the exercise group.

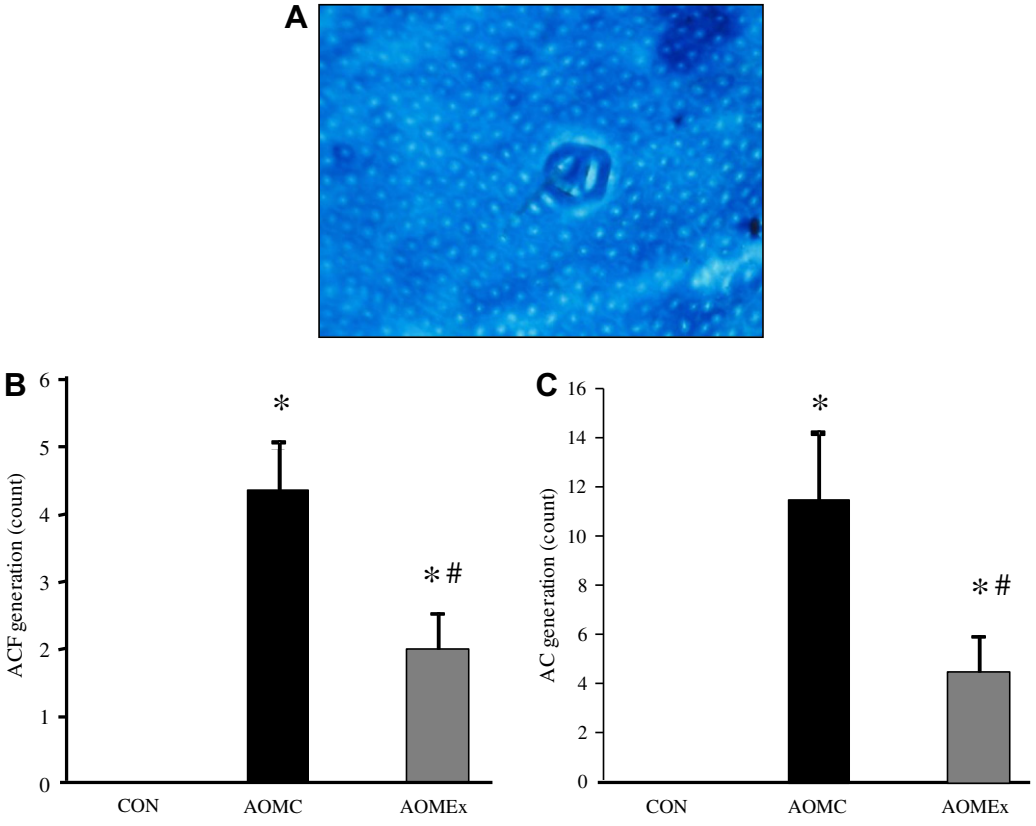

Fig. 1. AOM-induced formation of ACF and AC. The mucosal surface of the colons was stained with methylene blue (A), and then the number of ACF (B) and AC (C) were counted under a light microscope. Values are the mean ± SE. CON, sedentary normal control; AOMC, sedentary control with AOM; and AOMEx, exercise with AOM. ACF, aberrant crypt foci and AC, aberrant crypt. *Significant differences from CON at the level of $p < 0.05$. #Significant differences from AOMC at the level of $p < 0.05$.

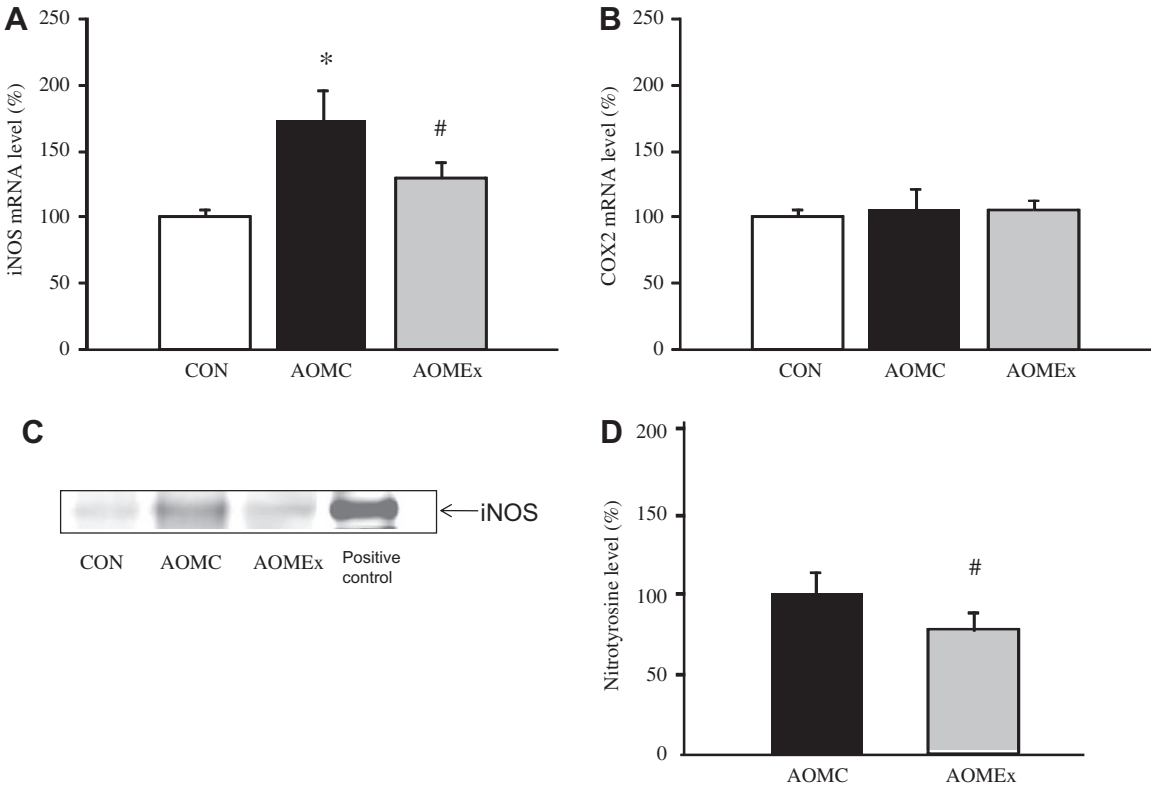

Fig. 2. mRNA levels of iNOS (A) and COX-2 (B), protein level of iNOS (C), and level of nitrotyrosine (D). The amount of the two mRNAs is expressed as a ratio to GAPDH gene expression. CON, sedentary normal control; AOMC, sedentary control with AOM; and AOMEx, exercise with AOM. *Significant differences from CON at the level of $p < 0.05$. #Significant differences from AOMC at the level of $p < 0.05$.

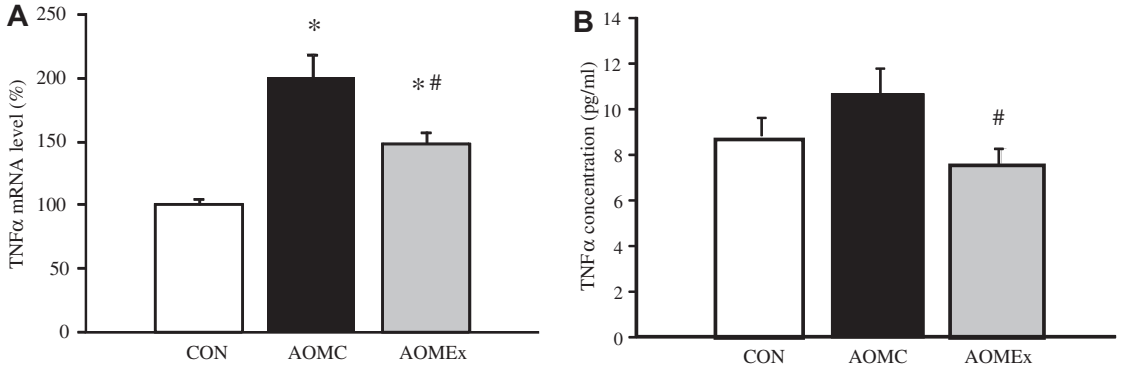

Fig. 3. Levels of TNFα in the colon (A) and circulation (B). The amount of mRNA is expressed as a ratio to GAPDH gene expression. CON, sedentary normal control; AOMC, sedentary control with AOM; and AOMEx, exercise with AOM. *Significant differences from CON at the level of $p < 0.05$. #Significant differences from AOMC at the level of $p < 0.05$.

4. Discussion

In summary, the present study revealed that regular exercise (1) decreased the AOM-induced formation of ACF in the colon, (2) suppressed the increase in iNOS and reduced nitrotyrosine concentrations in the colon, and (3) reduced the levels of TNFα in the colon and plasma. Although a few explanatory hypotheses have been proposed, the mechanism underlying the preventive effect of exercise on tumorigenesis remained unclear. Observations of the current study provide the first evidence that regular exercise prevents colonic cancer by, at least in part, suppressing iNOS. The expression of iNOS is hardly detectable in normal epithelial or stromal cells in the colon; however, increased expression of iNOS is frequently observed in epithelial cells when the process

Table 2
mRNA level of cancer protective factors.

	CON	AOME	AOMEx
SOD1	100 ± 6	117 ± 8	105 ± 7
SOD2	100 ± 4	97 ± 4	95 ± 4
GPx1	100 ± 4	97 ± 4	92 ± 5
GPx2	100 ± 9	117 ± 8	99 ± 4
Catalase	100 ± 6	135 ± 9[a]	126 ± 7[a]
OGG1	100 ± 6	133 ± 3[a]	130 ± 8[a]
HSP70	100 ± 12	55 ± 4[a]	58 ± 5[a]

Values are the mean ± SE. CON, sedentary normal control; AOMC, sedentary control with AOM; and AOMEx, exercise with AOM.
[a] Significant differences from CON at the level of $p < 0.05$.

of carcinogenesis has been induced by AOM. The progress of AOM-induced colorectal cancer involves multiple events including gene mutations of K-ras and β-catenin and upregulation of COX-2 and iNOS [9]. Especially, iNOS increases in the first step of the process that corresponds with ACF formation [9], indicating that iNOS associates with the initiation phase of colon carcinogenesis. Because the elevation of iNOS occurs earlier than that of COX-2, our results suggest that regular exercise suppressed the initial step of AOM-induced carcinogenesis. Therefore, the reduction of iNOS expression in association with the inhibition of ACF formation suggests that regular exercise is effective for primary prevention of carcinogenesis in the colon.

Exercise also decreased the production of nitrotyrosine, which is a marker of protein damage induced by excess NO. The results showed that nitrotyrosine levels are closely linked with changes in iNOS. Nitration of tyrosine residues can impair both the activity and transcription of the affected proteins. Indeed, some protein modifications induced by NO have been suggested to disturb apoptosis and proliferation of cells [21]. In addition, excess NO leads to the formation of 8-nitroguanosine, a DNA base modified by peroxynitrite, and causes a G-to-T transversion in the gene [22,23]. Thus, exercise may prevent the formation of ACF by iNOS suppression-derived inhibition of protein and DNA modification.

The expression of iNOS is upregulated by activation of the inflammatory signaling cascade [17,18]. In other words, TNFα is a major inflammatory cytokine that elevates the activity of inflammation-sensitive transcription factors such as nuclear factor kappa B, which, in its turn, regulates the expression of iNOS mRNA [18,24]. Accordingly, the inhibitory effect of exercise on TNFα abundance may lead to the reduction of ACF via suppression of iNOS. It has been reported that the sensitivity to inflammatory stimulation elevates in the initial phase of the carcinogenesis process induced by AOM [25], which would easily induce TNFα expression and further elevate iNOS expression. A potential mechanism related to the reduction of iNOS in the colon by exercise may be its anti-inflammatory effect in circulation. Regular exercise promotes the production of anti-inflammatory factors via induction of relevant enzymes [19,20] and also decreases the levels of inflammatory cytokines [26,27], and these effects have been suggested to involve not only organs but also the blood. In fact, we found that the circulating level of TNFα was reduced by regular exercise. Previous studies also showed that long-term exercise decreased the circulating levels of inflammatory cytokines and reduced the concentration of C-reactive protein [28,29]. Conversely, exercise can increase the concentrations of anti-inflammatory factors such as interleukin (IL)-10 and IL-1 receptor antagonist in the blood [30–32]. Therefore, such anti-inflammatory effect of exercise in circulation may lead to the reduction of iNOS via suppression of the inflammatory cascade in the colon. Because the adipose tissue secretes several circulating inflammatory cytokines including TNFα, decreased adipose tissue mass may represent one of the mechanisms that explain the anti-inflammatory effect of exercise. Indeed, it has been reported that the control of obesity by caloric restriction suppressed the AOM-induced formation of ACF [33]. In addition, a metabolic improvement-related adaptation in muscle may lead to the inhibition of ACF formation through reduction of other promoters of carcinogenesis such as insulin and insulin-like growth factor 1 [34,35].

Long-term exercise increases the levels of antioxidant and anti-inflammatory factors in the blood, internal organs, and skeletal muscle [19,20]. Thus, increases of these factors in colonic tissues could possibly lead to reductions in both ACF formation and iNOS expression via suppression of TNFα. However, regular exercise did not alter the expression of SODs, GPxs, catalase, and OGG1 in the colon, while catalase and OGG1 were increased in AOM-injected mice, probably as a result of the activation of a protective system against AOM stimulation. In addition, while the expression of the chaperon protein HSP70 was reduced by injection of AOM, it was not changed by regular exercise. HSP70 is a major protective protein against tumorigenesis, and it has been known that decreased amounts of HSP70 causes inhibition of apoptosis as well as anti-inflammatory responses. We hypothesize that reduced HSP70 in AOM-injected mice may be one of the mechanisms underlying initiation of tumorigenesis. Taking into account the fact that the levels of these protective factors in the colon were not changed by exercise, this result supports that the anti-tumorigenesis effect of regular exercise may be related to the levels of circulating factors, but not endogenous proteins in the colon.

In conclusion, we found that regular exercise decreased the AOM-induced formation of ACF in the colon. Exercise also attenuated the increase in iNOS and suppressed TNFα. The effect of exercise on colon tumorigenesis may derive from decreased levels of circulating TNFα associated with reduced adipose tissue mass. These observations demonstrate that regular exercise prevents colon tumorigenesis, at least in part through the suppression of iNOS expression associated with anti-inflammation.

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