Biochemical and Biophysical Research Communications 410 (2011) 108–113

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Renin–angiotensin system inhibitors suppress azoxymethane-induced colonic preneoplastic lesions in C57BL/KsJ-*db/db* obese mice

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ARTICLE INFO

*Article history:*
Received 17 May 2011
Available online 26 May 2011

*Keywords:*
Obesity
Colorectal cancer
Chemoprevention
Renin–angiotensin system
Inflammation
Oxidative stress

ABSTRACT

Obesity-related metabolic abnormalities, including chronic inflammation and oxidative stress, increase the risk of colorectal cancer. Dysregulation of the renin–angiotensin system (RAS) also plays a critical role in obesity-related metabolic disorders and in several types of carcinogenesis. In the present study, we examined the effects of an angiotensin-converting enzyme (ACE) inhibitor and angiotensin-II type 1 receptor blocker (ARB), both of which inhibit the RAS, on the development of azoxymethane (AOM)-initiated colonic premalignant lesions in C57BL/KsJ-*db/db* (*db/db*) obese mice. Male *db/db* mice were given 4 weekly subcutaneous injections of AOM (15 mg/kg body weight), and then, they received drinking water containing captopril (ACE inhibitor, 5 mg/kg/day) or telmisartan (ARB, 5 mg/kg/day) for 7 weeks. At sacrifice, administration of either captopril or telmisartan significantly reduced the total number of colonic premalignant lesions, i.e., aberrant crypt foci and β-catenin accumulated crypts, compared to that observed in the control group. The expression levels of TNF-α mRNA in the colonic mucosa of AOM-treated *db/db* mice were decreased by captopril and telmisartan. Captopril lowered the expression levels of TNF-α, IL-1β, IL-6, and PAI-1 mRNAs, while telmisartan lowered the expression levels of COX-2, IL-1β, IL-6, and PAI-1 mRNAs in the white adipose tissues of these mice. In addition, these agents significantly reduced the levels of urinary 8-OHdG, a surrogate marker of oxidative damage to DNA, in the experimental mice. These findings suggested that both ACE inhibitor and ARB suppress chemically-induced colon carcinogenesis by attenuating chronic inflammation and reducing oxidative stress in obese mice. Therefore, targeting dysregulation of the RAS might be an effective strategy for chemoprevention of colorectal carcinogenesis in obese individuals.



## 1. Introduction

Mounting evidence indicates that obesity, a result of a positive energy balance, and its related metabolic abnormalities raise the risk of colorectal cancer (CRC) [1,2]. Obesity is regarded as a state of chronic inflammation, which is closely associated with colorectal carcinogenesis [3]. Increased levels of adipose tissue lead to the expression of a variety of pro-inflammatory cytokines, including tumor necrosis factor-α (TNF-α) [4], which stimulates tumor promotion and progression of carcinogenesis [5]. Oxidative stress, which is induced by increased energy availability [6], has also been suggested to play an important role in the development of CRC [1,2]. Thus, these findings suggest that targeting inflammation and oxidative stress may be an effective strategy for preventing the development of CRC, especially in overweight individuals. For instance, a recent study shows that administration of pitavastatin, a hypolipidemic drug, prevents obesity-related colorectal tumorigenesis by attenuating chronic inflammation [7].

Hyperactivity of the renin–angiotensin system (RAS), an endocrine system with critical roles in cardiovascular function, has been implicated in the etiology of high blood pressure, obesity, and metabolic syndrome [8]. In addition, there is strong evidence that the RAS is frequently dysregulated in human malignancies, which correlates with poor patient outcomes. Abnormalities in the RAS influences cancer cell migration, invasion, and metastasis, all of which are closely associated with chronic inflammation and angiogenesis [9,10]. In cancer tissues, the RAS is upregulated through systemic

*Abbreviations:* ACE, angiotensin converting enzyme; ACF, aberrant crypt foci; AOM, azoxymethane; ARB, angiotensin-II type-1 receptor blocker; BCAC, β-catenin accumulated crypt; COX-2, cyclooxygenase-2; CRC, colorectal cancer; *db/db* mice, C57BL/KsJ-*db/db* mice; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin and eosin; IL, interleukin; 8-OHdG, 8-hydroxy-2′-deoxyguanosine; PAI-1, plasminogen activator inhibitor-1; RAS, renin–angiotensin system; RT-PCR, reverse transcription-PCR; TNF-α, tumor necrosis factor-α.

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doi:10.1016/j.bbrc.2011.05.115

oxidative stress and hypoxia mechanisms, which triggers chronic inflammatory processes to remodel the surrounding environment [11].

Drugs that reduce the synthesis (angiotensin-converting enzyme [ACE] inhibitors) or action (angiotensin-II type-1 receptor blockers [ARBs]) of angiotensin-II, the active product of RAS, are widely used for the treatment of hypertension. These agents have also been expected to exert beneficial effects that improve the symptoms of metabolic disorders [12,13]. In addition, retrospective studies have shown that patients taking ACE inhibitors or ARBs had decreased risk of developing some types of cancers, including CRC [14–16]. The expression levels of ACE are higher in colorectal adenomas and CRC epithelial cells than in the corresponding non-neoplastic crypt and surface epithelia [17]. In a mouse model of CRC liver metastasis, administration of an ACE inhibitor and ARB significantly reduced tumor volume by blocking the RAS activity [18]. These reports suggest that the RAS might be a critical target for the treatment and/or prevention of certain types of human malignancies, including CRC. However, the possibility of CRC chemoprevention by targeting the RAS is yet to be considered.

The C57BL/KsJ-*db/db* (*db/db*) mouse is one of the most widely used models of type 2 diabetes. The development of diabetes in *db/db* mice results in the activation of RAS and induction of oxidative stress, which promotes progressive inflammation [19]. In the present study, we used male *db/db* mice injected with azoxymethane (AOM) to examine the effects of captopril (ACE inhibitor) and telmisartan (ARB) on the development of aberrant crypt foci (ACF) and β-catenin accumulated crypts (BCAC), both of which are putative precursor lesions for colonic adenocarcinoma [20,21], by focusing on the attenuation of inflammation and reduction of oxidative stress. This preclinical animal model is useful for investigating specific agents for their ability to prevent inflammation-related colorectal carcinogenesis caused by obesity [7].

## 2. Materials and methods

### 2.1. Animals, chemicals, and diet

Male homozygous *db/db* mice aged 4 weeks (Japan SLC, Inc., Shizuoka, Japan) were maintained at the Gifu University Life Science Research Center in accordance with the Institutional Animal Care Guidelines. AOM, captopril, and telmisartan were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

### 2.2. Experimental procedure

The animal experiment, as described previously [7,22,23], was approved by the Committee of the Institutional Animal Experiments of Gifu University. A total of 45 male *db/db* mice were divided into six groups. To induce colonic preneoplastic lesions, at 5 weeks of age, the mice in Groups 4 (10 mice), 5 (10 mice), and 6 (10 mice) were given 4 weekly subcutaneous injections of AOM (15 mg/kg body weight). The mice in Groups 1 (5 mice), 2 (5 mice), and 3 (5 mice) were subcutaneously injected with saline once a week for 4 weeks. Groups 2 and 5 received drinking water containing captopril (5 mg/kg/day) for 7 weeks, starting 1 week after the last injection of AOM. Similarly, the mice in Groups 3 and 6 were given drinking water containing telmisartan (5 mg/kg/day). Captopril and telmisartan intake was maintained by adjusting the concentration of these agents in drinking water, whose volume was measured three times a week. Groups 1 and 4 were given tap water throughout the experiment. At the end of the study (16 weeks of age), all the mice were sacrificed by $CO_2$ asphyxiation for colon resection. The third portion of excised colons (cecum side) was used to extract RNA, and the remaining part was used to determine the numbers of colonic ACF and BCAC.

### 2.3. Counting the number of ACF and BCAC

The frequency of ACF and BCAC was determined according to the standard procedures [7,22,23]. The colon samples fixed with 10% buffered formalin were stained with methylene blue (0.5% in distilled water), and the number of ACF was counted under a light microscope. To identify BCAC intramucosal lesions, the distal part (1 cm from the anus) of the colon (mean area: 0.7 $cm^2$/colon) was embedded in paraffin, and 20 serial sections (4-μm thick) per mouse were created by an *en face* preparation. The sections were then subjected to H&E staining for histopathology and β-catenin immunohistochemistry to count the number of BCAC. The anti-β-catenin primary antibody was purchased from BD Transduction Laboratories (San Jose, CA, USA), and immunohistochemical staining was performed using a labeled streptavidin–biotin method (DAKO, Glostrup, Denmark). β-Catenin-stained BCACs were counted and the values were expressed as per $cm^2$ of mucosa [7,22,23].

### 2.4. RNA extraction and quantitative real-time reverse transcription-PCR

The expression levels of TNF-α and interleukin (IL)-6 genes in the colonic mucosa and those of TNF-α, cyclooxygenase (COX)-2, IL-1β, IL-6, and plasminogen activator inhibitor-1 (PAI-1) genes in the white adipose tissues of AOM-treated *db/db* mice were determined by quantitative real-time reverse transcription-PCR (RT-PCR) analysis [7,24]. Total RNA was isolated using the RNAqueous-4PCR Kit (Applied Biosystems, Austin, TX, USA). cDNA was synthesized from 0.2 μg of total RNA by using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, CA, USA). The specific primers used for the amplification of TNF-α, COX-2, IL-1β, IL-6, and β-actin genes were as previously described [24]. The specific primers used for amplification of the PAI-1 gene were as follows: sense 5′-TTC AGC CCT TGC TTG CCT C-3′ and antisense 5′-ACA CTT TTA CTC CGA AGT CGG T-3′. Real-time RT-PCR was performed using a LightCycler (Roche Diagnostics GmbH, Mannheim, Germany) with the SYBR Premix Ex Taq (TaKaRa Bio Inc., Shiga, Japan). The expression level of each gene was normalized to that of the β-actin gene by using the standard curve method.

### 2.5. Measurement of urinary 8-OHdG levels

Urine samples were collected at the time of sacrifice, and the levels of 8-hydroxy-2′-deoxyguanosine (8-OHdG) were determined by using an enzyme-linked immunosorbent assay (ELISA) kit (NIKKEN SEIL, Shizuoka, Japan) according to the manufacturer's protocol.

### 2.6. Statistical analyses

The statistical analyses were performed using the JMP 8 software program (SAS Institute, Cary, NC, USA), and the results are presented as mean (SD). Statistical significance was evaluated using Dunnet's *t*-test for multiple comparisons. Differences were considered statistically significant when the two-tailed *p*-value was less than 0.05.

## 3. Results

### 3.1. General observations

As listed in Table 1, the average body weight and relative liver weight of the AOM-injected groups (Groups 4–6) at the end of the experiment were significantly ($p < 0.01$) lower than those of

**Table 1**
Body, liver, and kidney weights of the experimental mice.

| Group No. | Treatment | No. of mice | Body weight (g) | Relative weight (g/100 g body weight) of: | |
|---|---|---|---|---|---|
| | | | | Liver | Kidney |
| 1 | Saline | 5 | 54.6 ± 8.8[a] | 5.95 ± 0.92 | 0.90 ± 0.26 |
| 2 | Saline + captopril | 5 | 58.2 ± 1.8 | 6.28 ± 0.44 | 0.79 ± 0.07 |
| 3 | Saline + telmisartan | 5 | 62.0 ± 3.6 | 7.65 ± 1.05 | 0.76 ± 0.10 |
| 4 | AOM alone | 10 | 40.9 ± 5.5[b] | 4.65 ± 0.60[b] | 0.93 ± 0.27 |
| 5 | AOM + captopril | 10 | 37.4 ± 8.4[b] | 4.53 ± 0.64[b] | 1.05 ± 0.22 |
| 6 | AOM + telmisartan | 10 | 42.2 ± 8.8[b] | 4.49 ± 0.55[b] | 0.96 ± 0.15 |

[a] Mean ± SD.
[b] Significantly different from Group 1 ($p < 0.01$).

the saline-injected group (Group 1). This might be caused by the toxicity of AOM, as observed in previous experiments [7,22,23]. No significant differences were observed in the mean relative weight of the kidney among the groups. No histopathological findings suggesting toxicity of captopril or telmisartan in the liver, kidney, and spleen of the mice were obtained (data not shown).

### 3.2. Effects of captopril and telmisartan on AOM-induced ACF and BCAC in db/db mice

Table 2 summarizes the total number of ACF (Fig. 1A and B) and BCAC (Fig. 1C and D) in the mice from all groups. Both ACF and BCAC developed in the colons of all mice that received AOM (Groups 4–6), but not in those without AOM treatment (Groups 1–3). When compared with Group 4 (AOM alone), administration of either captopril or telmisartan in drinking water significantly reduced ACF frequency; the inhibition rates were 43% in Group 5 (AOM + captopril, $p < 0.01$) and 39% in Group 6 (AOM + telmisartan, $p < 0.01$). Similarly, both captopril- (76% reduction, $p < 0.01$) and telmisartan- (71% reduction, $p < 0.01$) treatment groups had significantly decreased numbers of BCAC than the AOM alone-treated group.

### 3.3. Effects of captopril and telmisartan on the expression levels of TNF-α and IL-6 mRNA in the colonic mucosa of AOM-treated db/db mice

TNF-α is an important tumor promoter involved in obesity, inflammation, and carcinogenesis [3–5]. As shown in Fig. 2A, quantitative real-time RT-PCR analyses showed that both captopril and telmisartan significantly decreased the expression levels of TNF-α mRNA in the colonic mucosa of AOM-treated mice ($p < 0.05$). On the other hand, the expression levels of IL-6 mRNA in the colonic mucosa (Fig. 2B), which also are possibly involved in obesity- and inflammation-related colorectal carcinogenesis [3,25], were not significantly lowered by treatment with these agents.

### 3.4. Effects of captopril and telmisartan on the expression levels of TNF-α, COX-2, IL-1β, IL-6, and PAI-1 mRNA in the white adipose tissues of AOM-treated db/db mice

In the white adipose tissues of AOM-treated *db/db* mice, the expression levels of TNF-α (Fig. 3A), IL-1β (Fig. 3C), IL-6 (Fig. 3D), and PAI-1 (Fig. 3E) mRNAs were significantly inhibited by captopril administration compared to the control mice ($p < 0.05$ for each). Drinking telmisartan also caused a decrease in the expression levels of COX-2 (Fig. 3B), IL-1β (Fig. 3C), IL-6 (Fig. 3D), and PAI-1 (Fig. 3E) mRNAs in the white adipose tissues of AOM-treated mice ($p < 0.05$). These findings (Figs. 2 and 3) indicated that administration of these agents attenuates the inflammatory response in the colonic mucosa and in the white adipose tissues of obese mice.

### 3.5. Effects of captopril and telmisartan on the urinary levels of 8-OHdG in AOM-treated db/db mice

Urinary 8-OHdG levels in AOM-treated *db/db* mice were determined using ELISA method (Fig. 4). The mice treated with either captopril (6.5 ± 2.0 ng/mL) or telmisartan (7.3 ± 2.2 ng/mL) showed a significant decrease in the urinary levels of 8-OHdG compared to the untreated mice (17.9 ± 3.5 ng/mL; $p < 0.01$ for each comparison). These findings indicated that captopril and telmisartan suppresses obesity-related systemic oxidative stress.

## 4. Discussion

There is accumulating evidence to indicate that abnormalities in the RAS play a critical role in several types of carcinogenesis; therefore, agents targeting the RAS might augment cancer therapies [9,10]. The results of the present study clearly indicated that the RAS inhibitors captopril and telmisartan effectively suppress the development of colonic preneoplastic lesions, ACF and BCAC, in male *db/db* obese mice. This is the first report that shows the preventive effect of an ACE inhibitor and ARB on the development of chemically-induced colorectal carcinogenesis in any mouse

**Table 2**
Effects of captopril and telmisartan on AOM-induced ACF and BCAC formation in the experimental mice.

| Group No. | Treatment | No. of mice | Length of colon (cm) | Total No. of ACFs/colon | Total No. of BCACs/cm$^2$ |
|---|---|---|---|---|---|
| 1 | Saline | 5 | 11.1 ± 0.8 [a] | 0 | 0 |
| 2 | Saline + captopril | 5 | 11.9 ± 0.9 | 0 | 0 |
| 3 | Saline + telmisartan | 5 | 11.7 ± 0.8 | 0 | 0 |
| 4 | AOM alone | 10 | 10.6 ± 0.8 | 134.0 ± 24.5 | 3.4 ± 1.8 |
| 5 | AOM + captopril | 10 | 10.6 ± 1.3 | 76.9 ± 24.3[b] | 0.8 ± 0.9[b] |
| 6 | AOM + telmisartan | 10 | 10.8 ± 0.7 | 81.8 ± 14.0[b] | 1.0 ± 1.0[b] |

[a] Mean ± SD.
[b] Significantly different from Group 4 ($p < 0.01$).

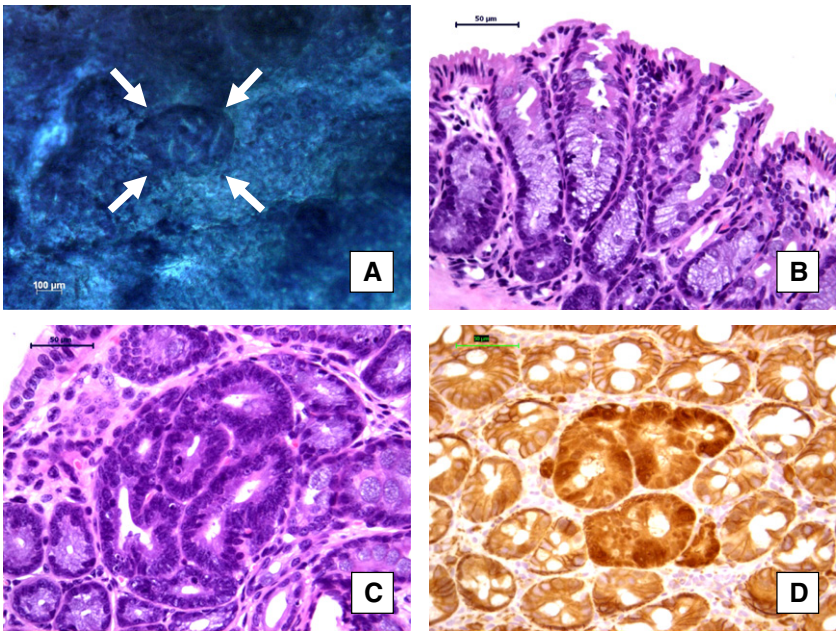


**Fig. 1.** Histopathology and β-catenin-immunohistochemistry of ACF and BCAC in AOM-exposed *db/db* mice (Group 4). Arrows indicate ACF (A) stained by methylene blue on the colonic mucosa. Representative photographs of ACF (B) and BCAC (C) stained with H&E. Basophilic cytoplasm and hyperchromatic nuclei are observed in the atypical cryptal cells in BCAC (C). Immunohistochemistry of β-catenin protein in BCAC (D). The localization of the accumulated β-catenin protein is apparent in the cytoplasm and nucleus of atypical cryptal cells. Scale bars, 100 μm (A) and 50 μm (B–D).

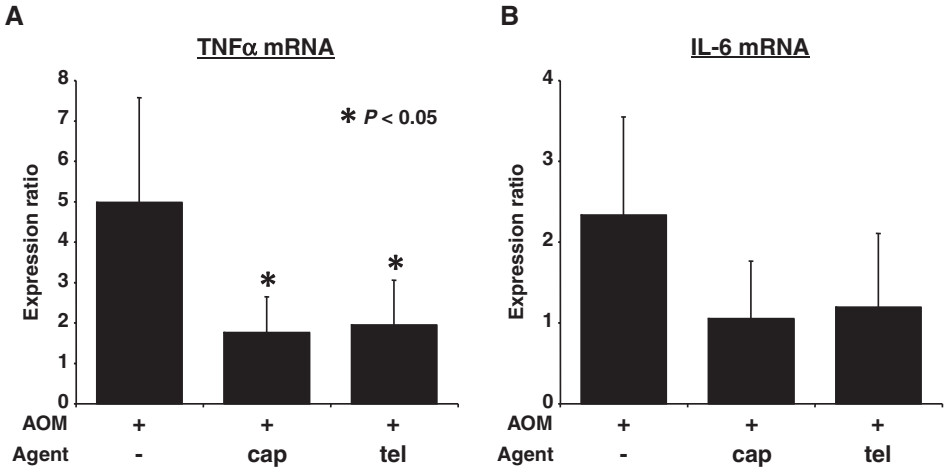


**Fig. 2.** The effects of captopril and telmisartan on the expression levels of TNF-α and IL-6 mRNAs in the colonic mucosa of AOM-treated *db/db* mice. cDNA was synthesized from scraped colonic mucosa, and real-time RT-PCR was performed using specific primers for TNF-α (A) and IL-6 (B). The expression levels of these genes were normalized to that of the β-actin gene. Data represent mean ± SD ($n = 8$). $^{*}p < 0.05$ vs. AOM-treated control group.

model. The finding seemed to be significant in clinical medicine because these drugs are widely used for patients with hypertension who frequently are obese. Furthermore, high blood pressure is involved in the increased risk of development of CRC and colonic adenomas [26–28], thus indicating that obese and hypertensive patients might be regarded as a high-risk group for CRC development. On the other hand, a recent retrospective study shows that use of an ACE inhibitor is significantly associated with reduction in the incidence and size of colorectal adenomas, the precancerous lesions for CRC [29]. This report [29] along with the results of the present study suggests that inhibition of RAS might be an effective strategy for the prevention of colorectal tumorigenesis, especially in obese individuals.

A key feature of obesity is increased inflammation in the adipose tissue, which might be involved in cancer promotion and progression [3]. Angiotensin-II is considered a pro-inflammatory mediator because activation of its receptor induces a number of molecules that participate in inflammatory responses [8–10]. For instance, mice with elevated adipocyte angiotensinogen expression have increased the expression of TNF-α, IL-6, and IL-1β in the adipose tissue [30]. Treatment with ARB decreased plasma levels of TNF-α and IL-6 in patients with congestive heart failure [31]. In the present study, either captopril or telmisartan decreased the mRNA levels of TNF-α, COX-2, IL-1β, IL-6, and PAI-1 in the white adipose tissue of AOM-treated *db/db* mice. Therefore, the chemopreventive effect of an ACE inhibitor and ARB on obesity-related colorectal carcinogenesis is most likely associated with the attenuation of systemic inflammation. In addition, the inhibition of the expression levels of TNF-α mRNA in the colonic mucosa might also contribute to this beneficial effect because this cytokine promotes

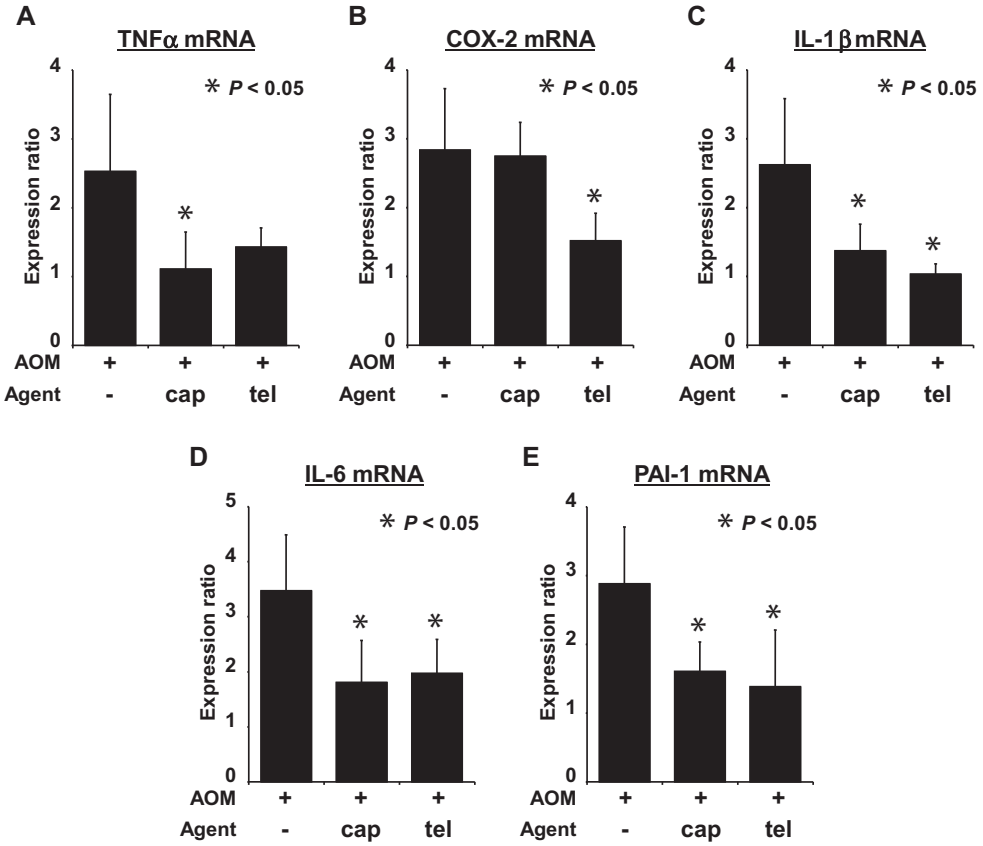


**Fig. 3.** The effects of captopril and telmisartan on the expression levels of TNF-α, COX-2, IL-1β, IL-6, and PAI-1 mRNAs in the white adipose tissues of AOM-treated *db/db* mice. cDNA was synthesized from the white adipose tissues of the retroperitoneum, and real-time RT-PCR was performed using specific primers for TNF-α (A), COX-2 (B), IL-1β (C), IL-6 (D), and PAI-1 (E). The expression levels of these genes were normalized to that of the β-actin gene. Data represent mean ± SD ($n = 8$). $^*p < 0.05$ vs. AOM-treated control group.

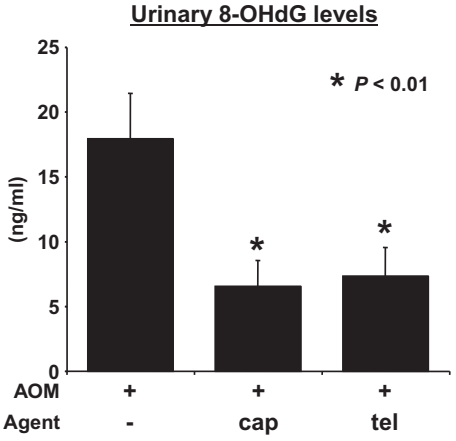


**Fig. 4.** The effects of captopril and telmisartan on urinary 8-OHdG levels in AOM-treated *db/db* mice. At sacrifice, urine samples were collected from the experimental mice, and the levels of urinary 8-OHdG were measured by ELISA. Data represent mean ± SD ($n = 8$). $^*p < 0.01$ vs. AOM-treated control group.

inflammation-related colorectal carcinogenesis, and thus, are critical targets for CRC chemoprevention [5,7,32].

Increased oxidative stress, which is associated with obesity due to metabolic and inflammatory changes [6], promotes damage to cell structures including DNA; this plays a key role in cancer development [33]. Certain types of chemopreventive agents, such as polyphenolic compounds, inhibit colorectal carcinogenesis by exerting anti-oxidant effects [34,35]. Activation of the RAS by enhanced levels of angiotensin-II leads to an increase in oxidative stress [36], but this is significantly reduced by treatment with RAS inhibitors [37,38]. In prostate cancer cells, candesartan, an ARB, also significantly reduces angiotensin-II-upregulated oxidative stress [39]. In the present study, both captopril and telmisartan decreased the levels of urinary 8-OHdG, which is a useful marker of DNA damage induced by oxidative stress, and this might be associated with inhibition of colorectal carcinogenesis. These findings, together with the results of recent studies [36–39], suggest that increased oxidative stress might be a critical target of RAS inhibitors for suppression of CRC.

Recent studies have revealed that insulin resistance and hyperinsulinemia, which are closely related to obesity, are some of the key factors in the development of obesity-related CRC, and thus, may be critical targets for the prevention of this malignancy [1,2,22,23]. In addition, activation of the RAS has been implicated in the etiology of obesity and insulin resistance [8,40]. Therefore, in the present study, it was expected that captopril and telmisartan might improve insulin resistance. However, contrary to our expectations, there was no clear evidence indicating an improvement in insulin resistance by these agents (data not shown). Therefore, at least in the present study, insulin resistance might not be a critical target of ACE inhibitors and ARBs to prevent colorectal tumorigenesis in obese mice.

The present experimental study was performed using *db/db* obese mice, which exhibit increased RAS activation and oxidative stress [19]. These mice are also highly susceptible to the colonic carcinogen AOM compared to the wild (+/+) and heterozygous

*db*/+ mice, neither of which exhibit obesity [41,42]. However, a question whether RAS inhibitors can prevent CRC development under non-obese condition has not yet been determined. Therefore, further studies that can clarify the effects of RAS inhibitors on the development of CRC under physiological RAS condition are required to confirm the possibility that these agents can be widely used as chemopreventive drugs for CRC.

In summary, prevention of CRC by targeting chronic inflammation and oxidative stress, which is caused by obesity and is related to RAS activation, might be a promising chemopreventive strategy for obese people, who are at an increased risk of developing CRC. Therefore, the agents targeting RAS, including ACE inhibitors and ARBs, appear to be potentially effective candidates for this purpose because these drugs attenuate inflammation while reducing oxidative stress.

## Conflicts of interest

The authors declare that no conflicts of interest exist.

## Acknowledgments

This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan (No. 22790638 to M.S. and No. 21590838 to H.M.) and by a Grant-in-Aid for the 3rd Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labor and Welfare of Japan.

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