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Influence of dietary resveratrol on early and late molecular markers of 1,2-dimethylhydrazine–induced colon carcinogenesis

Murugan Sengottuvelan, Ph.D., Kumaraswamy Deeptha, M.Phil., and Namasivayam Nalini, Ph.D.*

Department of Biochemistry and Biotechnology, Annamalai University, Annamalainagar, Tamilnadu, India

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Abstract

Objective: Colon cancer is an exceptionally aggressive disease, and the use of natural or synthetic substances to prevent or decrease cancer risk without adverse effects remains a major challenge. In this study, the mechanistic basis for the chemopreventive effect of resveratrol (Res) on 1,2-dimethylhydrazine–induced colon carcinogenesis in an rat model was evaluated.

Methods: Rats were randomized into six groups. Group 1 were control rats, group 2 were control rats that received Res (8 mg/kg of body weight orally every day), and rats in groups 3–6 were treated once per week with 1,2-dimethylhydrazine (20 mg/kg of body weight, subcutaenously, 15 times). In addition, groups 4–6 received Res (as in group 2) in three dietary regimens: initiation, postinitiation, and entire period. All rats were sacrificed after 30 wk and the degree of inflammation, cell proliferation, apoptosis, and mucosal integrity was evaluated.

Results: Res supplementation during the entire period significantly amended the expression of inflammatory, cell proliferative, and apoptotic biomarkers such as cyclo-oxygenase-2, ornithine decarboxylase, caspase-3, and heat shock proteins 70 and 27. Moreover, supplementing Res for the entire study period modulated the colonic mucosal protein mucin 1 and 2 expression.

Conclusion: The results clearly indicate that chronic Res supplementation inhibited the colon cancer development through modulating the early and late events of carcinogenesis and helped to maintain the colonic mucosal integrity. Thus our study demonstrates that the chemopreventive efficacy of Res could be attributed to its action on multiple direct targets of carcinogenesis.

Keywords: Apoptosis; Cell proliferation; Chemoprevention; 1,2-Dimethylhydrazine; Resveratrol

Introduction

Colorectal cancer is the third most common cause of cancer deaths in developed countries [1]. Several ecological, case-control, cohort, and experimental studies on the etiology of colon cancer have been extensively reviewed [2]. Early carcinogenesis studies have identified several colon carcinogens including 1,2-dimethylhydrazine (DMH), which produce their effects through a series of early steps involving inflammation, increased cell proliferation in colon crypts, epigenetic and genetic alterations, and late events such as decreased sensitivity to apoptosis induction resulting in the development of cancer [3]. A DMH-induced rodent colon carcinogenesis model provides a reproducible system, primarily for investigating the role of activators/inhibitors during tumorigenesis and chemoprevention trials [4].

The link between inflammation and carcinogenesis is well established. Cyclo-oxygenase (COX) enzymes catalyze the conversion of arachidonic acid in prostaglandins and related eicosanoids. COX-1 is constitutively expressed in many tissues and cell types, whereas COX-2 is an inducible form, frequently upregulated by mitogens, cytokines, and tumor promoters [5]. In general, COX-2 overexpression has also been considered to be an early event in colon cancer development, but how early it is in relation to other early events is undetermined. It has been hypothesized that loss of both

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*Corresponding author. Tel.: +91-4144-238-343; fax: +91-4144-239-141.

E-mail address: nalininum@yahoo.com (N. Nalini).

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adenomatous polyposis coli alleles at the early stages of the cancer process will induce COX-2 expression, leading to self-promotion of tumorigenesis [6]. This notion supports the view that COX-2 inhibitors provide additional means that can delay or prevent the development of colorectal carcinogenesis in rodents [7].

Abnormal cellular proliferation is an important mechanism in carcinogenesis. Moreover, a study [8] has demonstrated that ornithine decarboxylase (ODC) overexpressing cells undergo spontaneous malignant transformation. Recent studies in animal models have indicated that ODC inhibition might be an effective approach to cancer prevention and treatment [9]. During carcinogenesis the reduction in the activities of caspase-3, caspase-7, and caspase-9 generally results in resistance to apoptosis [10]. Another mode of apoptosis regulation is performed by heat shock proteins (HSPs). HSP-70 and HSP-27 overexpressions in malignant cells have been reported to rescue cells from apoptosis induced by effector caspases and can delay the tumor cell death process [11]. In the colon, the mucus is mainly composed of the secreted mucin called MUC-2; in addition, other membrane-bound mucins were expressed, mainly MUC-1, MUC-3, and MUC-4 [12]. Modulation in the expression of mucins in premalignant and malignant conditions might alter the tumorigenic pathogenesis of some adenocarcinomas [13].

Chemoprevention with natural and synthetic agents show promise in preventing, arresting, and reversing cancer development [14]. Resveratrol (Res), a polyphenolic phytoalexin, has been found in 72 plant species distributed in 31 genera and 12 families [15]. Besides diverse biological actions in preclinical models, Res was found to block the development of preneoplastic/neoplastic lesions in carcinogen-treated mouse mammary glands and in mouse skin cancer model [16,17].

Our previous finding showed that chronic Res supplementation suppressed free radical–mediated oxidative stress, the aberrant crypt foci incidence, tumor development, and cell proliferation (AgNOR count) in DMH-induced colon carcinogenesis when supplemented throughout the experimental period [18,19]. To understand the mechanisms underlying the potential chemopreventive effect of Res, in the present study we monitored the impact of Res on a panel of inflammation, cell proliferation, and apoptotic markers. We also observed the effect of Res on mucosal integrity by analyzing the expression of mucins in DMH-induced rat colon carcinogenesis. Thus, the detection of inflammation, proliferation, and apoptosis-associated protein expression would help to understand the role of Res against carcinogen-mediated colon cancer.

Materials and methods

Animals and diet

Male albino Wistar rats (5 wk old) were obtained from the Central Animal House, Rajah Muthiah Institute of Health Sciences, Annamalai University. The animals were cared for in compliance with the principles and guidelines of the ethical committee for animal care of Annamalai University in accordance with the Indian National Law on Animal Care and Use (Regulation 160/2004/CPCSEA). At the beginning of the study, rats were fed ad libitum sterilized tap water and a standard rat chow composed of 19.5% protein, 6.8% fat, 53.1% carbohydrates, 4.5% fiber, and 1.7% minerals. After a 1-wk acclimatization period of consuming standard rat chow, rats were stratified by body weight and assigned to the experimental diets (Hindustan Lever Ltd., Mumbai, India) ad libitum for 30 wk. The experimental diet consisted of 22% protein, 20% fat, and 56.5% carbohydrates by weight. Other ingredients were at the same levels for all groups and the total caloric intake by the rats in all the groups was adjusted to be the same. The animals were housed four per cage in polypropylene cages with a wire mesh top and a hygienic bed of husk in a specific pathogen-free animal room-under controlled conditions of 12-h light/dark cycles, with temperature of $24 \pm 2\,^{\circ}C$, and relative humidity of $50 \pm 10\%$ until the end of the experimental period of 30 wk.

Chemicals and antibodies

The DMH and trans-Res were purchased from Sigma Chemical Company (St. Louis, MO, USA). Antibodies were purchased as follows: rabbit polyclonal anti–COX-2, goat polyclonal anti-ODC, mouse monoclonal anti–HSP-70, goat polyclonal anti–HSP-27, rabbit polyclonal anti–caspase-3, goat polyclonal anti–MUC-1, and rabbit polyclonal anti–MUC-2 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). All other reagents used were of analytical grade or the highest grade available.

Experimental design

Rats were assorted into six groups (eight per group) and the initial body weights of all the animals in this study protocol were ensured to be 120–130 g. Rats in group 1 received no treatment and served as the untreated control. Group 2 animals received Res by intragastric intubation daily at the dose of 8 mg/kg of body weight suspended in 0.1% carboxymethylcellulose. Rats in groups 3–6 received DMH injections (20 mg/kg of body weight in 1 mM ethylenediaminetetraacetic acid, pH adjusted to 6.5 with 1 mM NaOH, and used immediately). Injections were given once a week subcutaneously for the first 15 wk. In addition to DMH, group 4 (initiation) rats received Res as in group 2 starting 1 wk before DMH injections and continued until 1 wk after the final exposure of DMH (DMH + RES [I]). Group 5 (postinitiation) rats received Res as in group 2 starting 2 d after the cessation of DMH injections and continued until the end of 30 wk (DMH + RES [PI]). Group 6 (entire period) animals received resveratrol as in group 2 starting on the day of DMH injection and continued until the end of the experimental period (DMH + RES [EP]). Food intake was measured daily and body weight was determined weekly throughout the experimental period and before sacrifice.

Animal sacrifice and tissue procurement

At the end of 30 wk, all the animals were alive and were sacrificed under anesthesia (ketamine hydrochloride, 30 mg/kg of body weight, intraperitoneally) by cervical dislocation between 08:00 and 10:00 after an overnight fast. After removal of the colon, sections (3-μm wide strips) were prepared for immunohistochemical analysis and the remaining colonic sections were gently scraped with a microscope slide and the mucosa was used for determining protein expression.

Protein extraction and immunoblotting

Rat colonic mucosa from different treatment groups were homogenized in 5 vol of homogenization buffer (50 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 2 mM ethylenediaminetetra-acetic acid, 1 mM ethyleneglycol bis[2-aminoetyl ether]-N,N,N′,N′-tetra-acetic acid, 25 μg/mL each of leupeptin, pepstatin, and aprotinin, 1 μg/mL of soybean trypsin inhibitor, 50 μM sodium fluoride, 50 μg/mL of 4-[2-aminoethyl]-benzene sulfonyl fluoride, and 10 mM 2-mercaptoethanol) using 10 strokes of a Teflon-in-glass homogenizer. The protein concentrations of the samples were determined by the method described by Bradford [20] and the protein concentrations were adjusted to 0.2 mg/mL. Colonic mucosa homogenates were then subjected to polyacrylamide gel electrophoresis in 10% precast mini-gels (Novel Experimental Technology, San Diego, CA, USA) according to the method of Laemmeli [21]. After electrophoresis, gels were soaked for 15 min in transfer buffer and electroblotted onto polyvinyldifluoride membranes using the Trans Blot Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA) at 200 mA for 3 h as described previously [22]. After staining with Ponceau-S (0.5% Ponceau-S in 5% v/v glacial acetic acid solution), the presoaked membranes were blocked in 5% non-fat dry milk in Tris buffered saline for 2–4 h at room temperature and incubated with specific primary antibodies according to the manufacturer's instruction. Alkaline phosphatase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA) was incubated for 1 h at room temperature, followed by colorimetric development with nitro-blue tetrazolium chloride/5-bromo-4-chloro-3′-indolylphosphate p-toluidine salt stable mix (Gibco BRL-Life Technologies, Grand Island, NY, USA) to visualize protein bands. β-Actin levels were also determined as an internal standard for loading. Blots were scanned using a ScanJet 3c flat bed scanner and their backgrounds were clarified using Image-Pro Plus 4.5 software (Media Cybernetics, Inc., Silver Spring, MD, USA). The density of the immunologically stained bands was analyzed by scanning densitometry and expressed as band-relative intensities (optical density $\times$ band area).

Immunohistochemistry

Immunohistochemical staining of MUC-2 was performed by the avidin-biotin complex method (Vecstain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA). Tissue sections were deparaffinized with xylene, dehydrated through a graded ethanol series, immersed in 0.3% H_2O_2 in absolute methanol for 30 min at room temperature to block endogenous peroxidase activity, and then washed with phosphate buffered saline (pH 7.2). After incubation with normal rabbit serum at room temperature for 10 min to block background staining, the sections were incubated with anti–MUC-2 antibody for 12 h in a humid chamber at room temperature. The slides were incubated with biotinylated secondary antibody and then with avidin–biotinylated peroxidase complex for 30 min at room temperature (Vector Laboratories) and then reacted with 3′-diamino benzamine and lightly counterstained with Harris hematoxylin. For each slide, three to five different fields were scored for the precipitate staining pattern. The images were photographed randomly using a Leica microscope (Leica, Inc., Heidelberg, Germany), and digital images were collected using a Kodak DC290 ZOOM digital camera (Kodak, Rochester, NY, USA). The staining intensity was scored as weak reactivity, moderate reactivity, and strong reactivity.

Statistical analysis

Data were analyzed by one-way analysis of variance and significant differences among treatment groups were evaluated by Duncan's multiple range test. The results were considered statistically significant at $P < 0.05$. All statistical analyses were made using SPSS 11.0 (SPSS, Inc., Tokyo, Japan).

Results

General effects

Our previous study [19] showed that there were no adverse effects on body weight gain during chronic Res supplementation compared with control rats. Moreover, there was no evidence of mortality or toxicity in any of the groups (data not shown).

Effect of Res on expression of inflammatory markers

After 30 wk, western blot analysis showed that the COX-2 protein expression by the colonic mucosa cells of DMH-alone treated animals was significantly ($P < 0.05$) greater compared with control animals (groups 1 and 2). Res supplementation for the EP (group 6) suppressed overexpression of the COX-2 protein ($P < 0.01$). Res supplementation during the I and PI periods of treatment had no significant effect on COX-2 expression (Fig. 1A,B) compared with DMH-alone exposed rats.

Inhibitory effect of Res on expression of cell proliferation marker

Immunoblotting results showed that the enzyme ODC was upregulated in DMH-alone treated rats (group 3) compared with the control rats (groups 1 and 2). Further, the results

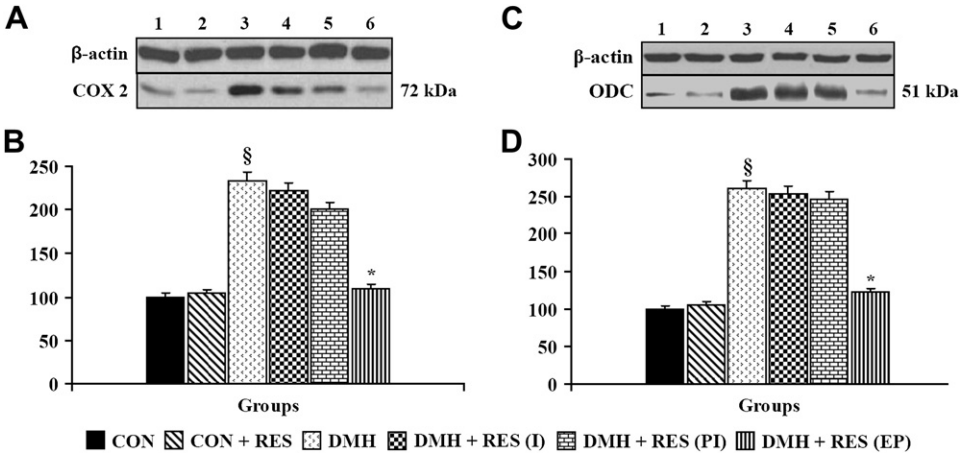

Fig. 1. Expression of COX-2 and ODC in the colon of control and experimental rats. Western blots of (A) COX-2 and (C) ODC expressions in the colonic mucosa of six groups (lane 1: CON; lane 2: CON + RES; lane 3: DMH alone; lane 4: DMH + RES [I]; lane 5: DMH + RES [PI]; lane 6: DMH + RES [EP]). (B, D) Each lane was analyzed by densitometry and the expression in the control group was considered 100%. β-Actin was used as a loading control. Column heights are the means ± SDs of eight determinants. SDs are shown as bars. *$P < 0.05$ compared with DMH and CON groups. §$P < 0.01$ compared with CON groups (groups 1 and 2). CON, control; COX-2, cyclo-oxygenase-2; DMH, 1,2-dimethylhydrazine; EP, entire period; I, initiaton; ODC, ornithine decarboxylase; PI, postinitiation; RES, resveratrol.

(Fig. 1C,D) indicate that Res supplementation for the entire period (group 6) significantly ($P < 0.01$) inhibited the overexpression of ODC, whereas no significant difference in ODC expression was observed in the other treatment groups (groups 4 and 5).

Modulatory effect of Res on expression of apoptotic markers

In DMH-alone treated rats, prominent immunoreactive bands of HSP-70 (Fig. 2A,B) and HSP-27 (Fig. 2C,D) were observed. The intensity of these protein expressions were reversed on Res supplementation during the PI and EP (groups 5 and 6) treatment regimens. Maximum ($P < 0.01$) suppression of HSP-70 and HSP-27 expressions was observed only in the group supplemented with Res for the entire study period (group 6).

At the end of the experimental period, western blot analysis of DMH-treated colonic mucosa revealed significantly ($P < 0.05$) lowered caspase-3 protein expression (Fig. 3A,B) compared with control (groups 1 and 2). We observed that supplementation with Res significantly ($P < 0.01$) elevated the expression of caspase-3 in the EP group (group 6), which implies that chronic Res supplementation is very effective in inducing apoptosis. Res supplementation during the I (group 4) and PI (group 5) periods showed very minimal effect on caspase-3 expression (Fig. 3A,B) compared with DMH-alone treated rats.

Modulatory effect of Res on MUC-1 and MUC-2 protein expression

Immunoblotting analysis revealed that overexpression of MUC-1 (Fig. 4A,B) in carcinogen-alone (group 3) treated rats was significantly inhibited on treatment with Res for the entire period (group 6). In contrast, immunohistochemical analysis revealed that MUC-2 protein expression was lower in the colonic epithelial cells of DMH-alone (group 3) administered rats (Fig. 5B) compared with normal expression in control (Res alone) rats (Fig. 5A). Intensity of MUC-2 expression was upregulated typically on Res supplementation during EP (group 6, $P < 0.05$) treatment regimen (Fig. 5C,D), indicating that Res helps to maintain mucosal integrity by upregulating mucin secretion in carcinogen-treated animals.

Discussion

Natural polyphenols are considered chemopreventive agents that are able to prevent tumor initiation, promotion, and cancer growth by activating multitude of pathways including apoptosis [23]. In the present study, the COX-2 expression pattern was elevated in DMH-alone treated rats. Evidences from in vitro and animal model studies have suggested that the COX-2 inhibition may suppress carcinogenesis by affecting/promoting a number of pathways such as angiogenesis, tumor invasion, and apoptosis [24]. Our results revealed that Res supplementation (entire period) inhibits the overexpression of COX-2 in DMH-treated rats. Moreover, a recent study [25] clearly demonstrated that pterostilbene, a polyphenol structurally similar to Res, supplementation suppressed azoxymethane-induced colon carcinogenesis by suppressing the proliferation of malignant colonocytes and abnormal expression of inducible nitric oxide synthase in the colonic crypts.

Animal studies have shown that ODC activity increases not only during the early stages of tumor cell proliferation, but also during the promotion stage of carcinogenesis [26]. In our study, DMH exposure to rats resulted in increased expression of colonic ODC compared with the control group. Immunoblot analysis demonstrated that compared with the DMH-alone exposed animals, EP Res supplementation apparently attenuated ODC overexpression. This could be,

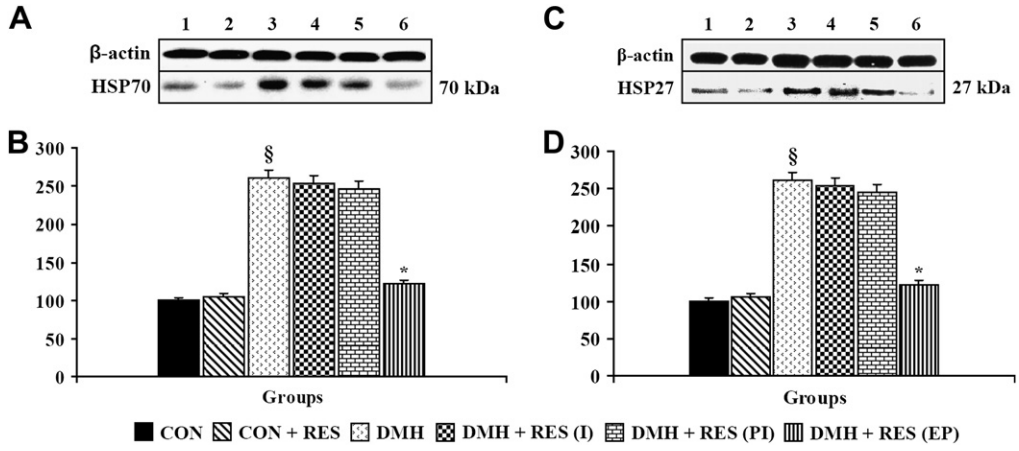

Fig. 2. Expression of HSP-70 and HSP-27 in the colon of control and experimental rats. Western blots of (A) HSP-70 and (C) HSP-27 expressions in the colonic mucosa of six groups (lane 1: CON; lane 2: CON + RES; lane 3: DMH-alone; lane 4: DMH + RES [I]; lane 5: DMH + RES [PI]; lane 6: DMH + RES [EP]). (B, D) Each lane was analyzed by densitometry and the expression in the control group was considered 100%. β-Actin was used as a loading control. Column heights are means ± SDs of eight determinants. SDs are shown as bars. $^{*}P < 0.05$ as compared to DMH and control groups. $^{\S}P < 0.01$ as compared to control groups (groups 1 and 2). CON, control; DMH, 1,2-dimethylhydrazine; EP, entire period; HSP, heat shock protein; I, initiaton; PI, postinitiation; RES, resveratrol.

at least in part, related to the accumulation of cells at the S/G2 phase transition and suppression of the activity of the nuclear transition factors that are involved in a number of different signaling pathways associated with proliferation, differentiation, and cell death. In addition, Res downregulates the expression of cyclins D1 and D2, which are directly involved in cell cycle progression [27]. They are generally stimulated during malignancy [28] and repressed by anticancerous phytochemicals [29]. Moreover, cell cycle arresting properties of Res on several cancer cell lines are well documented [30]. Res is also known to reduce significantly the expression of transcription factors, including the dimerization partner 1

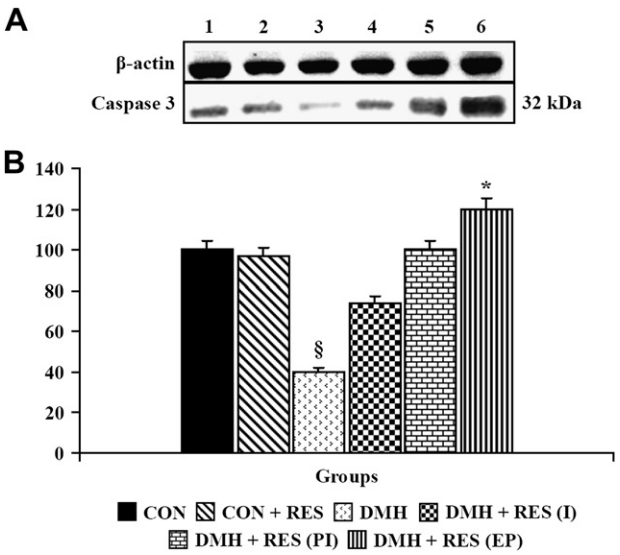

Fig. 3. Expression of caspase-3 protein in the colon of control and experimental rats. (A) Immunoblot analysis of caspase-3 expression in control and experimental groups was determined in the colonic mucosa of six groups (lane 1: CON; lane 2: CON + RES; lane 3: DMH-alone; lane 4: DMH + RES [I]; lane 5: DMH + RES [PI]; lane 6: DMH + RES [EP]). (B) Each lane was analyzed by densitometry and the expression in the control group was considered 100%. β-Actin was used as a loading control. Column heights are means ± SDs of eight determinants. SDs are shown as bars. $^{*}P < 0.05$ compared with DMH and CON groups. $^{\S}P < 0.01$ compared with CON groups (groups 1 and 2). CON, control; DMH, 1,2-dimethylhydrazine; EP, entire period; I, initiaton; PI, postinitiation; RES, resveratrol.

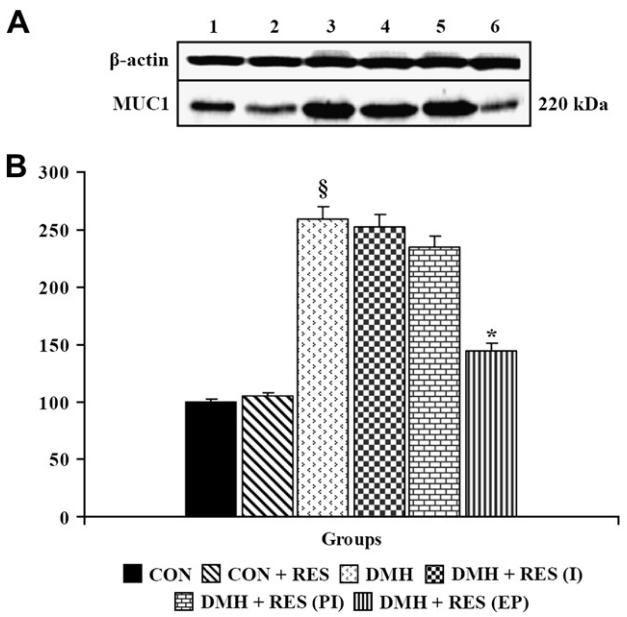

Fig. 4. Differential expressions of MUC-1 proteins in the colon of control and experimental rats. (A) Western blot of MUC-1 expression in the colonic mucosa of six groups (lane 1: CON; lane 2: CON + RES; lane 3: DMH-alone; lane 4: DMH + RES [I]; lane 5: DMH + RES [PI]; lane 6: DMH + RES [EP]). (B) Each lane was analyzed by densitometry and the expression in the control group was considered 100%. β-Actin was used as a loading control. Column heights are means ± SDs of eight determinants. SDs are shown as bars. $^{*}P < 0.05$ compared with DMH and CON groups. $^{\S}P < 0.01$ compared with CON groups (groups 1 and 2). CON, control; DMH, 1,2-dimethylhydrazine; EP, entire period; I, initiaton; MUC, mucin; PI, postinitiation; RES, resveratrol.

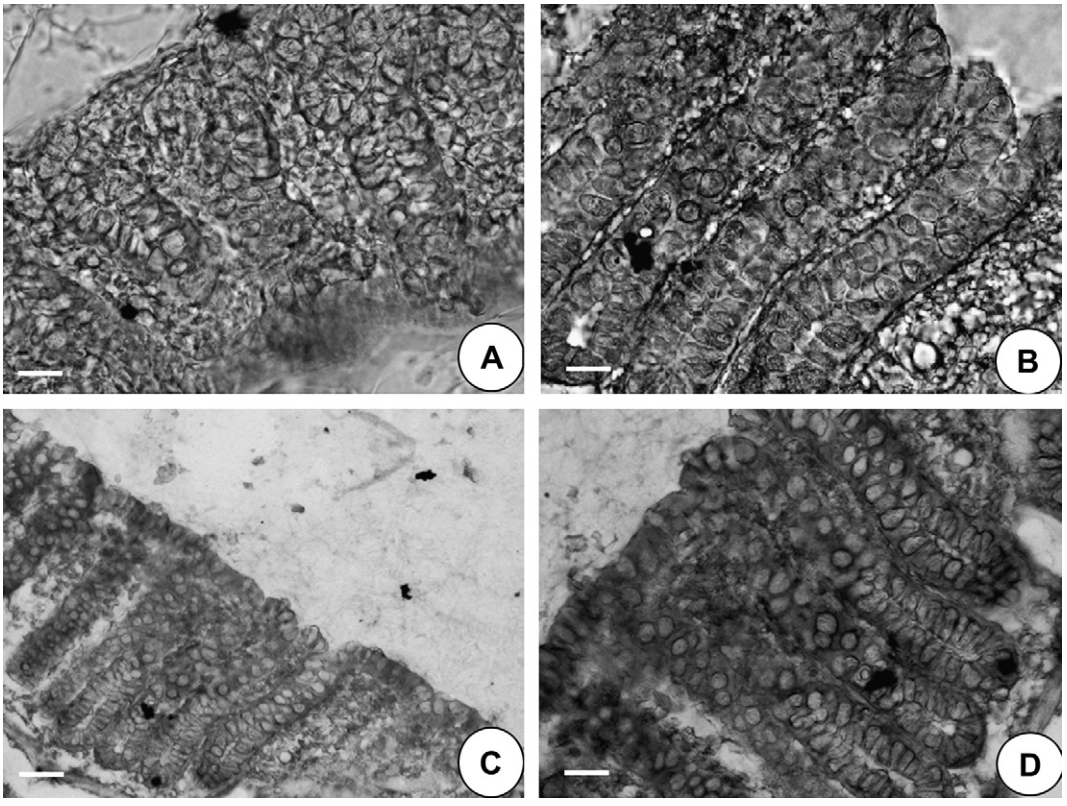

Fig. 5. Immunohistochemistry of mucin-2 protein in the colon of control and experimental rats. (A) Control (Res alone) colonic crypts with normal mucin expression (40×). (B) On 1,2-dimethylhydrazine treatment, the mucin expression was very minimal in the colonic crypts (20×). (C) Entire-period Res supplementation showed increased mucin-2 protein expression (20×). (D) Higher magnification view of entire-period Res-supplemented rat colon revealed the aberrant expression (intense staining) of mucin-2 protein (40×). Scale bars = 40 μm.

transcription factor, involved in the control of cell proliferation [31]. Furthermore, Res can inhibit the activities of several enzymes associated with cell proliferation and DNA replication [32]; these cumulative effects could be the plausible reason for the modulation of ODC expression, thereby inhibiting the cell proliferation.

Changes in the rate of colonocyte proliferation (dysregulated), apoptosis, or both are involved in colon tumorigenesis [33]. Apoptosis is a mode of cell death usually mediated through the activation of caspases [34]. Our results showed that Res supplementation for the entire period activated the expression of caspase-3 compared with carcinogen-alone treated rats. The antitumor effect observed on Res supplementation against colonic tumor formation may also be associated with marked activation of caspase-3 and the shift of malignant cells toward apoptosis. In addition, emerging evidence indicates that Res induces cell death in various colon cancer cell lines through the activation of various apoptotic pathways [35–39]. Therefore, it is reasonable to expect that culmination of various signaling pathways within the cell can potentially modulate the growth kinetics of cancer cells.

We previously showed that tumor tissue cholesterol levels increased and tissue phospholipids were decreased during colon carcinogenesis [40]. Moreover, the inhibition of cholesterol formation/accumulation by Res in DMH-induced tumor cells, as evident by the lower cholesterol content (unpublished data), may alter the integrity of membranes (cell/mitochondrial) of tumor cells, thereby leading to activation of proapoptotic effector molecules including caspase-3. The precise mechanism behind the induction of caspase-3 by Res is not known.

Several HSPs are normally expressed in cells and are differentially expressed and/or regulated during the cell cycle and at various stages of development and differentiation [41]. We found elevated expression of HSP-70 and HSP-27 in the colonic mucosa of DMH-alone exposed rats at the end of 30 wk. Animals supplemented with Res suppressed the overexpression of HSP-70 and HSP-27. Overexpression of HSP-27 may increase cell tumorigenicity in the syngeneic host, possibly as a result of a drastic decrease in tumor cell ability to undergo apoptosis. Moreover, overexpression of HSP-70 with low levels of caspase-3 activity is known to play an antiapoptotic role in malignant human tumors of various origins. It has been shown that HSP-70 overproduction can ameliorate apoptotic cell death and inhibit caspase-3 activation leading to reduced apoptosis [42]. Induction and activation of some HSPs in tumor cells may be controlled by many regulatory factors, including oncogenes such as *c-myc*, *ras*, and tumor suppressor genes (p53) [43]. The capability of inducing apoptosis on cancer cells can be linked to

its antitumor activities, thus highlighting the chemotherapeutic potential of Res.

Mucins are widely and differentially expressed in the gastrointestinal tract. The specific functions of the various mucins are still unclear, but they appear to have other functions than only gel-forming. For example, it has been shown that MUC-2 deficiency leads to inflammation of the colon and contributes to the onset and perpetuation of experimental colitis [44]. In most carcinomas, MUC-1 is overexpressed and displays an altered glycosylation, making the MUC-1 mucin a promising tumor antigen with diagnostic and therapeutic potential in the treatment of cancer [45]. In our study, immunoblot analyses showed upregulated MUC-1 expression in colonic mucosa of DMH-alone administered rats. The overexpression of MUC-1 in carcinogen-treated rats was less severe in the EP Res supplemented group. Moreover, colonic MUC-2 expression decreased significantly on DMH treatment, which is in agreement with a recent study [25]. MUC-2 protein expression was reversed on Res supplementation for the entire period to DMH-treated animals and maintained the normal integrity of the colon, and thereby protected the colonic mucus layer. This effect may be due to modulation in the expression of the enzymes that initiate *O*-glycosylation of mucin by Res.

Conclusion

Collectively, this study emphasizes the protective effect of Res against the carcinogen-induced colon carcinogenesis through modulation of inflammation, cell proliferation, and apoptotic-related factors. This study also demonstrates that chronic (EP) administration of Res holds greater potential, provides pathomechanistic insights, and encourages the long-term benefits of Res consumption.

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