Pterostilbene Is More Potent than Resveratrol in Preventing Azoxymethane (AOM)-Induced Colon Tumorigenesis via Activation of the NF-E2-Related Factor 2 (Nrf2)-Mediated Antioxidant Signaling Pathway

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Ⓢ *Supporting Information*

ABSTRACT: Inflammatory bowel diseases have been a risk factor of colorectal cancer (CRC). The reactive oxygen species (ROS) generated by inflammatory cells create oxidative stress and contribute to neoplastic transformation, proliferation, and even metastasis. Previously, resveratrol (RS) and pterostilbene (PS) had been reported to prevent chemical-induced colon carcinogenesis by anti-inflammatory and pro-apoptotic properties. In this study, we investigated whether RS and PS could prevent the azoxymethane (AOM)-induced colon tumorigenesis via antioxidant action and to explore possible molecular mechanisms. Male BALB/c mice were injected with AOM (5 mg/kg of body weight) with or without RS or PS, and at the end of the protocol, all of the mice were euthanized and colons were analyzed. Administrations of PS can be more effective than RS in reducing AOM-induced formation of aberrant crypt foci (ACF), lymphoid nodules (LNs), and tumors. We also find that PS is functioning more effectively than RS to reduce nuclear factor-κB (NF-κB) activation by inhibiting the phosphorylation of protein kinase C-β2 (PKC-β2) and decreasing downstream target gene expression, including inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and aldose reductase (AR) in mouse colon stimulated by AOM. Moreover, administration of RS and PS for 6 weeks significantly enhanced expression of antioxidant enzymes, such as heme oxygenase-1 (HO-1) and glutathione reductase (GR), via activation of NF-E2-related factor 2 (Nrf2) signaling. When the above findings are taken together, they suggest that both stilbenes block cellular inflammation and oxidative stress through induction of HO-1 and GR, thereby preventing AOM-induced colon carcinogenesis. In comparison, PS was a more potent chemopreventive agent than RS for the prevention of colon cancer. This is also the first study to demonstrate that PS is a Nrf2 inducer and AR inhibitor in the AOM-treated colon carcinogenesis model.

KEYWORDS: Pterostilbene, azoxymethane, aldose reductase, heme oxygenase-1, Nrf2

■ INTRODUCTION

Colorectal cancer (CRC) is a major healthcare problem and the third most common cancer in the world.[1] Colon carcinogenesis is a multi-step process involving three distinct stages, initiation that mutates a normal cell, followed by promotion and progression that disrupt the homeostatic mechanisms controlling proliferation, inflammation, differentiation, and apoptosis.[2] Epidemiological and experimental studies suggest that colon cancer is usually mediated by dietary and environmental factors,[3] which can lead to inflammation and oxidative stress.[4] Some evidence indicated that inflammatory bowel diseases have been strongly linked to the increased risk of CRC.[5] In inflamed tissues, a wide variety of activated inflammatory cells create oxidative stress by generating reactive oxygen and nitrogen species (ROS/RNS). In addition, ROS and RNS can induce the formation of lipid peroxidation products, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal (4-HNE), which can cause DNA damage and thereby initiate carcinogenesis.[6]

Many experimental studies with rodents have demonstrated that azoxymethane (AOM) can induce colon aberrant crypt foci (ACF) and tumors by causing DNA damage, oxidative stress, and inflammation.[7,8] During AOM-induced colon tumorigenesis, increased amounts of lipid peroxidation products and decreased levels of glutathione (GSH) and associated antioxidant enzymes, glutathione reductase (GR) and glutathione peroxidase (GPx), were reported. Clinical studies also showed that the tissue and plasma levels of MDA and 4-HNE were significantly increased in colorectal cancer patients.[9,10] In addition, previous studies have displayed that aldose reductase (AR) could reduce glutathionyl-HNE to glutathionyl-dihydroxynonane (DHN), which in turn activates transcription factors, such as nuclear factor-κB (NF-κB)
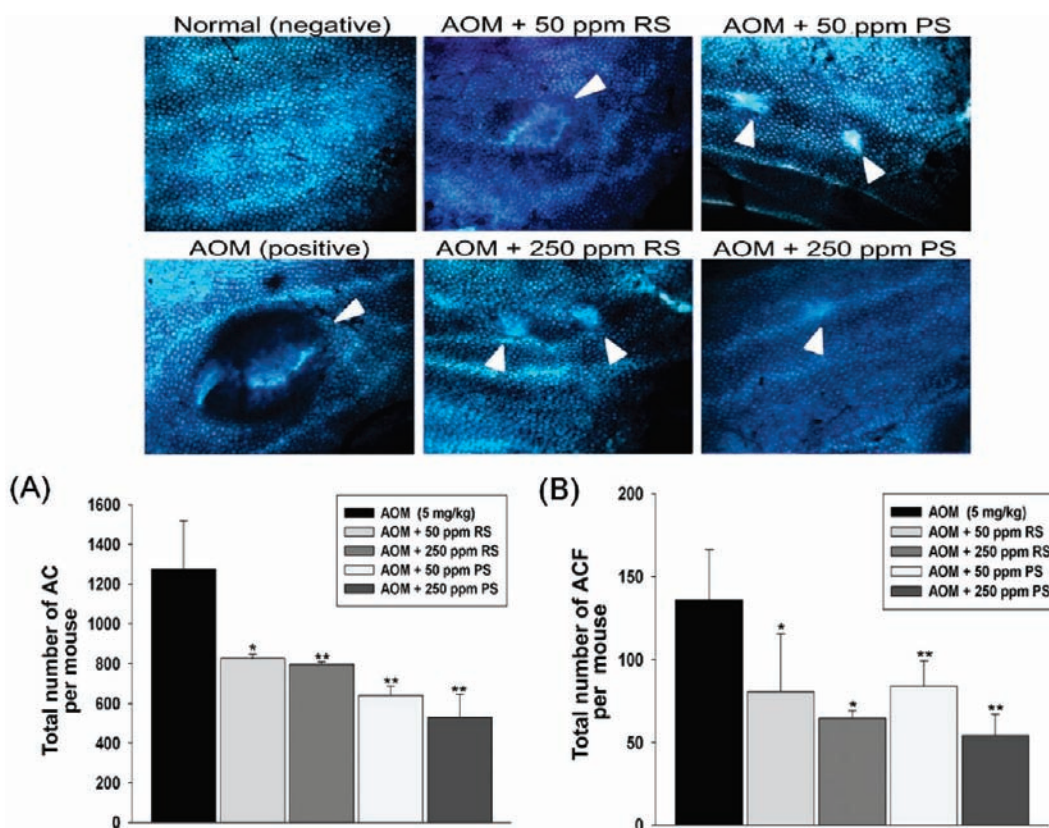
Figure 1. RS and PS prevented AOM-induced ACF formation in BALB/c mice. After 6 weeks of AOM treatment, mice were euthanized and colons were fixed in formalin and stained with 0.2% methylene blue for 30 min. ACF were identified under a light microscope ($40\times$ magnification), and multi-aberrant crypts with well-defined eye were identified and counted. Each bar represents the mean $\pm$ SD of the averages of six mice scored. Results were statistically analyzed with Student's t test ($*$, $p < 0.05$; $**$, $p < 0.01$, compared to the AOM-induced value).

and activating protein-1 (AP-1), to regulate expression of cyclo-oxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) by the protein kinase C-$\beta2$ (PKC-$\beta2$) signaling cascade.[11,12] In this model, oxidative-stress-modulated inflammation strongly promotes tumor development. Thus, heighten antioxidant ability may be an effective approach in preventing the progression of CRC.

Recent studies have exhibited that resveratrol (RS) and pterostilbene (PS) possess anti-inflammatory, antioxidant, and anticarcinogenic properties, which may be responsible for their cancer chemopreventive potency.[13,14] Our and other previous studies showed that RS and PS could significantly decreased AOM- and/or DSS-induced ACF and tumor formation by anti-inflammation and antioxidant mechanisms. Nevertheless, PS exerted its anti-tumor effect through downregulation of inflammatory iNOS and COX-2 gene expression and upregulation of apoptosis in AOM-induced colon tumorigenesis. Moreover, administration of PS significantly suppressed AOM-induced GSK3β phosphorylation and Wnt/β-catenin signaling.[15,16] Recent studies suggested that RS could attenuate oxidative stress and suppress inflammatory response through induction of anti-oxidant enzymes via NF-E2-related factor 2 (Nrf2) signaling.[17,18] Although the anti-inflammation and antioxidation of RS and PS have been widely investigated in AOM-induced colon carcinogenesis, the molecular mechanism is still unclear. In the present study, we examined the role of Nrf2 and AR in mediating oxidative-stress-induced iNOS and COX-2 expression and compared the effect of RS and PS on AOM-induced colon

carcinogenesis. We find that RS and PS could prevent AOM-induced colorectal carcinogenesis and inflammation response by mediating antioxidant enzyme expression (HO-1 and GR) through activation of the Nrf2 signaling pathway. The results indicated that, although both stilbenes affect the signaling pathways, PS might be a more potent chemopreventive agent than RS in colitis-associated colon cancer.

■ MATERIALS AND METHODS

Reagents and Antibodies. AOM was purchased from Sigma Chemical Co. (St. Louis, MO). PS and RS were obtained from Sabinsa Corp. (East Windsor, NJ). The purity of PS and RS was determined by high-performance liquid chromatography (HPLC) as higher than 99.2%. Antibodies against iNOS, Nrf2, AR, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-COX-2 monoclonal and anti-HO-1 antibodies were purchased from BD Transduction Laboratories (Lexington, KY) and Epitomics, Inc. (Burlingame, CA), respectively. Anti-p-p65 and p-PKC-$\beta2$ antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA).

Animal Treatment and Tissue Harvest. BALB/c mice at 5 weeks of age were purchased from the BioLASCO Experimental Animal Center (Taiwan Co., Ltd., Taipei, Taiwan). After 1 week of acclimation, animals were randomly distributed into control and experimental groups. All animals were housed in a controlled atmosphere (25 ± 1 °C at 50% relative humidity) and with a 12 h light/12 h dark cycle. Animals had free access to food and water at all times. Food cups were replenished with fresh diet every day. All experimental animal care and treatment followed the guidelines set up by the Institutional Animal Care and Use Committee
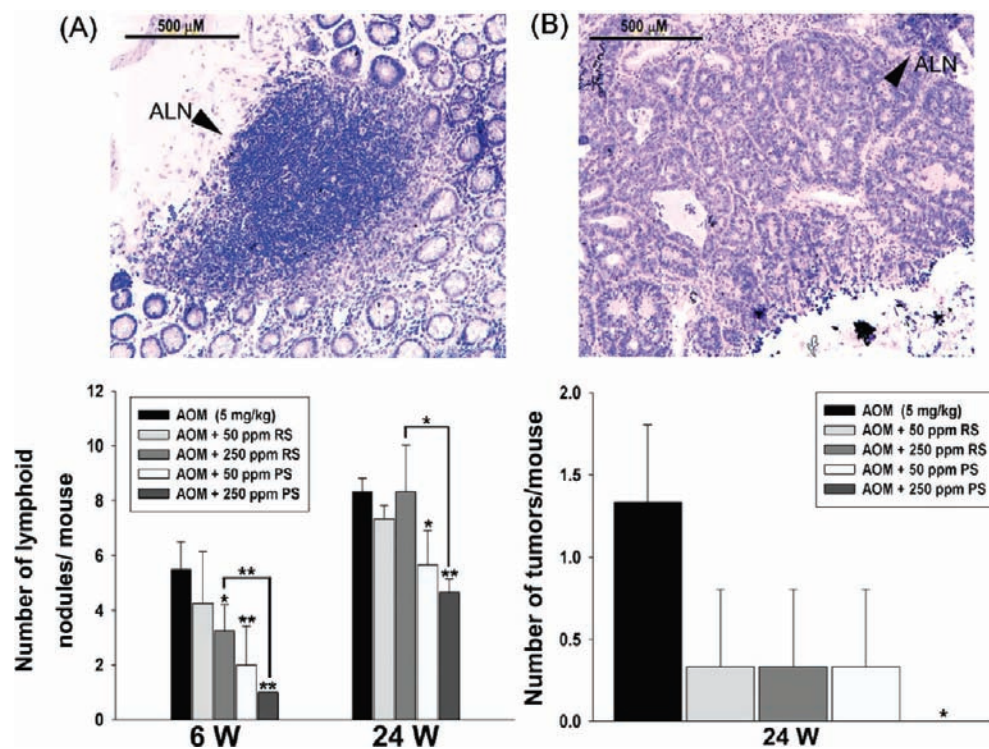
Figure 2. RS and PS decreased average LNs and tumor number. All mice were euthanized after 6 or 24 weeks of feeding. (Bottom panels) The colons were fixed with 10% buffered formalin for 24 h, and the (A) LN and (B) tumor numbers were counted. (Top panels) Representative H&E stained colon LNs and tumors (100× magnification). Each bar represents the mean ± SD of the averages of six mice scored. Results were statistically analyzed with Student's t test (*, $p < 0.05$; **, $p < 0.001$, compared to the AOM-induced value. Arrows in black indicate packed inflammatory cell infiltration and resulted in ALNs in AOM-treated mice.

of the National Kaohsiung Marine University (IACUC, NKMU). The experimental design is summarized in Figure S1 of the Supporting Information. Mice were sacrificed by euthanasia at 6 or 24 weeks, and colon, spleen, liver, and kidney were collected. All animal experimental protocols used in this study were approved by the IACUC, NKMU.

Identification of ACF and Adenomas. The entire colon was excised, cut longitudinally, rinsed with ice-cold phosphate-buffered saline (PBS), and fixed flat between sheets of filter paper with 3.7% neutral formalin overnight. The method by Bird and Good[19] was used to classify ACF. The total number of ACF and the number of aberrant crypts (ACs) in each focus were counted under a microscope (40×). Multiple formalin-fixed colonic sections were stained with hematoxylin and eosin (H&E) and examined for dysplasia and adjacent sections used for immunostaining. H&E stain sections were examined histologically and classified as ACF, aggregates of lymphoid nodules (ALNs), and tumors according to specific pathological criteria by a pathologist and referred to previous reports.[20]

Western Blot Analysis. For protein analyses, total scraped colon mucosa was homogenized on ice for 15 s with a Polytron tissue homogenizer and lysed in 0.5 mL of ice-cold lysis buffer [50 mM Tris-HCl at pH 7.4, 1 mM NaF, 150 mM NaCl, 1 mM ethylene glycol bis-(2-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1 mM phenyl-methanesulfonyl fluoride (PMSF), 1% NP-40, and 10 mg/mL leupeptin] on ice for 30 min, followed by centrifugation at 10000g for 30 min at 4 °C. The samples (50 μg of protein) were mixed with 5× sample buffer containing 0.3 M Tris-HCl (pH 6.8), 25% 2-mercaptoethanol, 12% sodium dodecyl sulfate (SDS), 25 mM ethylenediaminetetraacetic acid (EDTA), 20% glycerol, and 0.1% bromophenol blue. The mixtures were boiled at 100 °C for 5 min, subjected to stacking gel, then resolved by 12% SDS–polyacrylamide minigels at a constant current of 20 mA. Subsequently, electrophoresis was carried out on SDS–polyacrylamide

gels. For western blot analysis, proteins on the gel were electrotransferred onto the 45 μm immobile membrane [polyvinylidene difluoride (PVDF); Millipore Corp., Bedford, MA] with transfer buffer composed of 25 mM Tris-HCl (pH 8.9), 192 mM glycine, and 20% methanol. The membranes were blocked with blocking solution (20 mM Tris-HCl at pH 7.4, 0.2% Tween 20, 1% bovine serum albumin, and 0.1% sodium azide). The membrane was further incubated with respective specific antibodies at appropriate dilution (1:1000) using blocking solution with the primary antibodies, including iNOS, COX-2, p-PKC-β2, p-p65, and AR, overnight at 4 °C. The membranes were subsequently probed with anti-mouse or anti-rabbit immunoglobulin G (IgG) antibody conjugated to horseradish peroxidase (Transduction Laboratories, Lexington, KY) and visualized using enhanced chemiluminescence (ECL, Amersham, Piscataway, NJ). The densities of the bands were quantitated with a computer densitometer (AlphaImager 2200 System, Alpha Innotech Corporation, San Leandro, CA). All membranes were stripped and reprobed for β-actin (Sigma Chemical Co., St. Louis, MO) as loading control.

Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA were extracted from scraped colonic mucosa using TRIZOL reagent according to the protocol of the supplier. The template used was 4 μg of total cellular RNA in a 20 μL reaction solution with Super Script II RNase H-reverse transcriptase (Invitrogen, Renfrew-shire, U.K.). The cDNA (2 μL) was amplified by PCR with the following primers: iNOS, forward primer, 5′-CCCTTCCGAAGTTTCTGG-CAGCAGC-3′ (sense) and 5′-GGCTGTCAGAGAG CCTCGTGGC-TTTGG-3′ (anti-sense); COX-2, 5′-GGAGAGACTATCAAGATAG-TG ATC-3′ (sense) and 5′-ATGGTCAGTAGACTTTTACAGCTC-3′ (anti-sense); GR (540 bp), 5′-CTTCCTTCGACTACCTGG-3′ (sense) and 5′-ATGCCCGCGATCTCCACA-3′ (anti-sense); HO-1 (180 bp), 5′-GAGCAGAACCAGCCTGAACTA-3′ (sense) and

2727

dx.doi.org/10.1021/jf2000103 |*J. Agric. Food Chem.* 2011, 59, 2725–2733

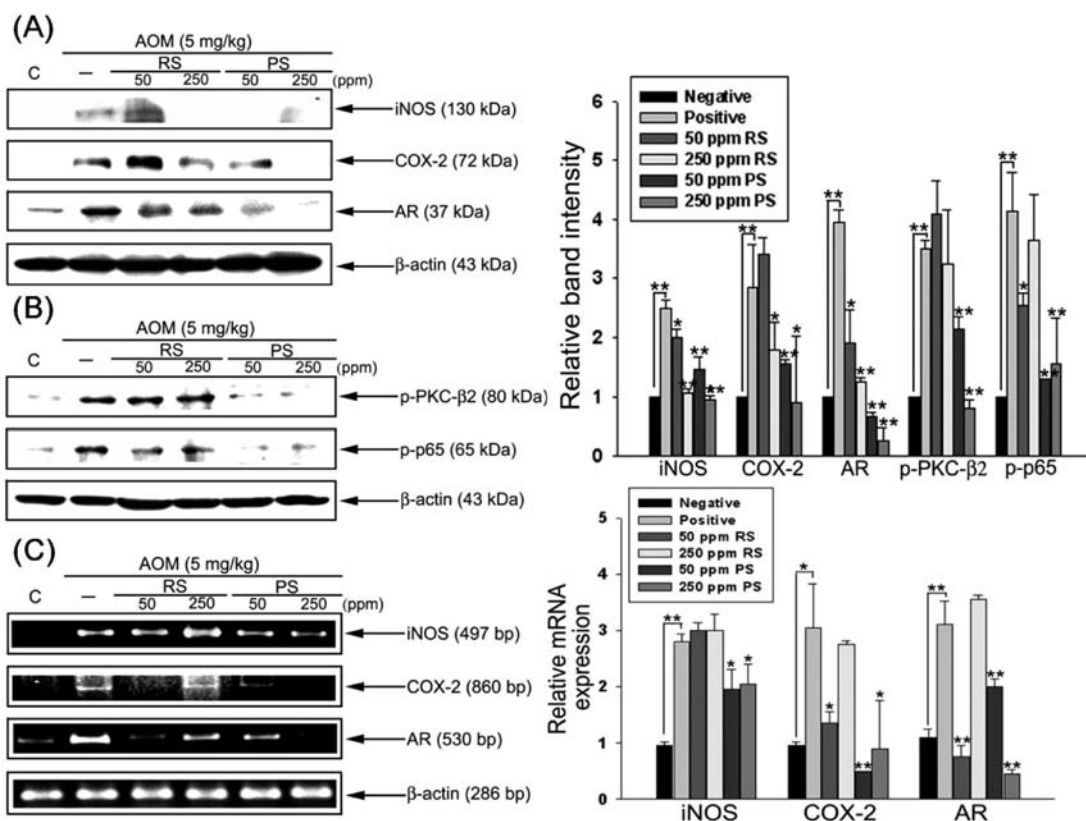**Figure 3.** RS and PS blocked inflammatory signaling in AOM-treated BALB/c mice. At 6 weeks (ACF group) after the first AOM injection, colonic mucosas were scraped and homogenized. (A and B) Equal amounts of cell lysates (50 μg of protein) were subjected to western blot analysis using antibodies against iNOS, COX-2, AR, p-PKC-β2, p-p65, and β-actin. (C) iNOS, COX-2, and AR primers were used for RT-PCR analysis. Each sample was normalized to β-actin. Quantification of protein and gene expression was normalized to β-actin using a densitometer. Results were statistically analyzed with Student's t test ($*$, $p < 0.05$; $**$, $p < 0.001$, compared to the AOM-induced value). Each bar represent the mean ± standard error (SE) ($n = 3$) of the averages of three independent experiments.

5'-GGTACAAGGAAGCCATCACCA-3' (anti-sense); AR (530 bp), 5'-CTCAGGGAACGTGATACCTA-3' (sense) and 5'-TGTTTGGC-ACAGCTCATCAAG-3' (anti-sense); and β-actin (286 bp), 5'-AAGA-GAGGCATCCTCACCCT-3' (sense) and 5'-TACATGGCTGGGG-TGTTGAA-3' (anti-sense). PCR (GeneAmp PCR System 9700, Applied Biosystems, CA) amplification was performed under the following conditions: 30 cycles at 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min, and followed by a final incubation at 72 °C for 10 min. PCR products were analyzed by 1% agarose gel and visualized by ethidium bromide staining.

Immunohistochemical Analysis. Three micrometer sections of colonic mucosa in ACF and tumor segments were deparaffinized, rehydrated, and treated with 0.3% hydrogen peroxide (H_2O_2) for 15 min to block endogenous peroxidase. Sections were pressure-cooked for 4 × 7 min in 10 mM citrate buffer at pH 6.0 (Immuno DNA retriever with citrate, BIO SB, Inc., Santa Barbara, CA) to unmask epitopes. Sections were incubated with primary antibody to AR and Nrf2 (1:100 dilution in PBS) for 1 h. Immunoreactivity was incubated with biotin-labeled secondary antibody and streptavidin–biotin–peroxidase for 30 min each. 3,3'-Diaminobenzidine tetrahydrochloride (DAB, 0.05%) was used as the substrate, and the positive signal was detected as a brown color under a light microscope. The detailed procedures for the stained tissue analysis method were reported previously.[21] Cytoplasm and nuclear staining was recorded with AR ectopic expression. For Nrf2, the criterion for positive expression showed nuclear staining. For the immunoreactive score (IRS), the scores for the percentage of positive cells and the staining intensity was multiplied.

Statistical Analysis. Relative expression values are given as the mean ± standard deviation (SD) for the indicated fold of expression in colon mucosa of mice. A one-way Student's t test was used to assess the statistical significance between the AOM-treated and PS or RS plus AOM-treated groups. A p value of <0.05 was considered statistically significant.

■ RESULTS

Effect of PS and RS on AOM-Induced ALNs, ACF, and Tumor Formation. During the experiment, all mice were monitored to investigate whether PS and RS feeding caused any adverse effects. As shown in Table 1 in the Supporting Information, the body weight in each group did not differ or show any unhealthy symptoms throughout the study. Furthermore, no significant difference of the mean weights of liver and spleen, and no pathologic alternations were found among the groups (data not shown). The results suggested no noticeable side effect or toxicity caused by dietary PS and RS treatment. Several investigations supported the use of the AOM model to study colonic carcinogenesis and chemoprevention.[22] AOM is a chemical carcinogen that induces colonic ACF, lymphoid nodules (LNs), and tumors in rodents, which exhibit many of the same clinical, histological, and molecular features identified in human colon cancer.[23] Using this model, we found that ACF, LNs, and tumors mostly occurred in the distal (rectum) colon. Representative photomicrographs are shown at the top of Figure 1,
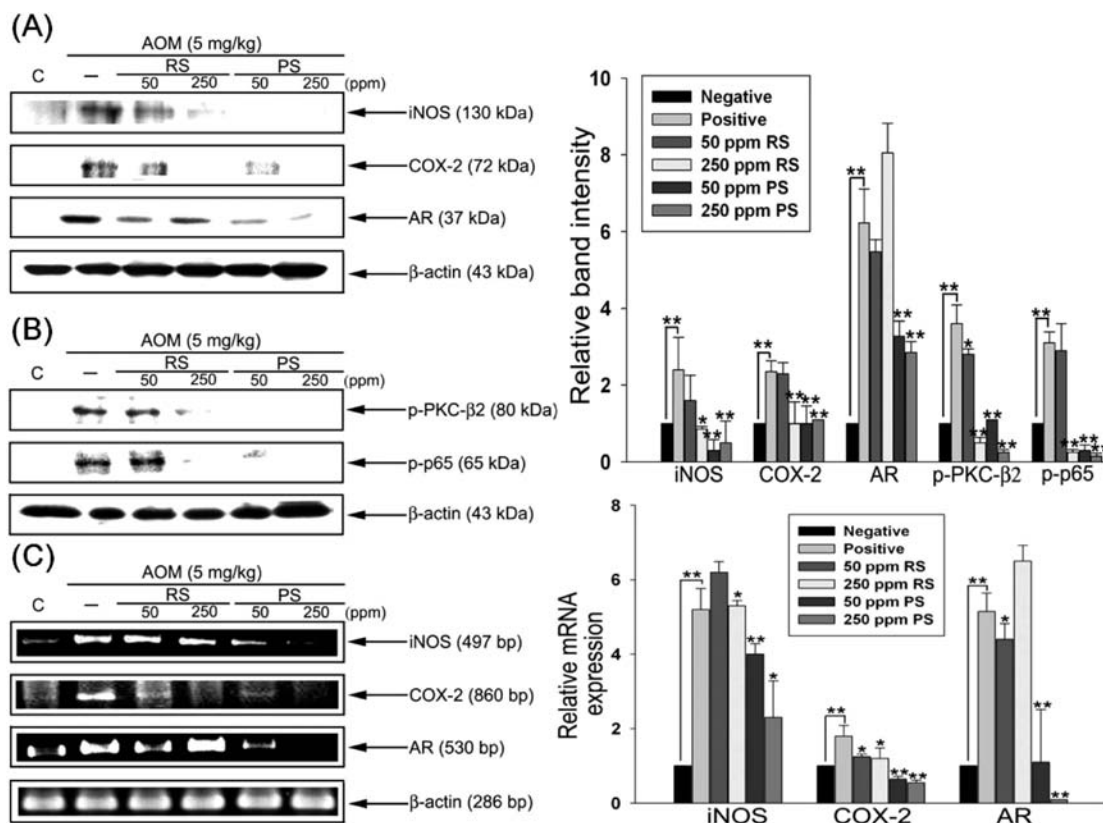
Figure 4. RS and PS blocked inflammatory signaling in AOM-treated BALB/c mice. At 24 weeks (tumor group) after the first AOM injection, colonic mucosas were scraped and homogenized. (A and B) Equal amounts of cell lysates (50 μg of protein) were subjected to western blot analysis using antibodies against iNOS, COX-2, AR, p-PKC-β2, p-p65, and β-actin. (C) iNOS, COX-2, and AR primers were used for RT-PCR analysis. Each sample was normalized to β-actin. Quantification of protein and gene expression was normalized to β-actin using a densitometer. Results were statistically analyzed with Student's t test ($\ast$, $p < 0.05$; $\ast\ast$, $p < 0.001$, compared to the AOM-induced value). Each bar represent the mean $\pm$ SE ($n = 3$) of the averages of three independent experiments.

and results of the total number of AC and ACF per mouse are summarized in panels A and B of Figure 1. In comparison to the control group with AOM treatment only, dietary PS-treated mice were lower in the total number of ACF and AC than RS-treated mice, especially by 250 ppm PS treatment. Colonic lymphatic nodules and tumors were characterized, and the results are shown in panels A and B of Figure 2. Previous studies indicated that the presence of LNs is promotional to colon carcinogenesis.[24] In this study, we observed that the AOM-treated group showed 100% LNs and tumor incidence, with an average number of 8.3 and 1.3, respectively. Furthermore, there was a significant positive linear progression relationship between the numerical distribution of LNs, ACF, and tumors of the colon in the AOM-treated group. Dietary supplementation of RS (250 ppm) and PS (50 and 250 ppm) reduced the LN number to 3.25 ($p < 0.05$), 2 ($p < 0.001$), and 1 ($p < 0.001$), respectively, in the colon 6 weeks after AOM treatment. PS treatment suppressed the formation of LNs more significantly than RS in the colon 24 weeks after AOM treatment. Administration of high-dose PS (250 ppm) to AOM-treated mice significantly decreased the tumor multiplicity ($p < 0.05$). The results suggested that dietary consumption of PS may be more effective than RS in preventing AOM-induced ACF and tumor formation.

Inhibitory Effect of Dietary PS and RS on AOM-Induced iNOS and COX-2 via Blocking AR Expression. Several reports suggested that AOM-initiated colon carcinogenesis by inducing

oxidative stress and causing lipid peroxidation.[25] During AOM-induced colon carcinogenesis, AR, an oxidative stress response protein would be evaluated.[26] Previous studies also displayed that AR could regulate iNOS and COX-2 expression via activation of the p-PKC-β2/NF-κB signaling cascade. We therefore examined the effect of inflammatory marker expression on the activation of AOM-induced AR in BALB/c mice. As shown in Figure 3, in comparison to RS, dietary PS resulted in a dramatic reduction of AR, iNOS, and COX-2 gene and protein levels in colonic mucosa at 6 weeks (panels A and C of Figure 3) and 24 weeks (panels A and C of Figure 4) after AOM injection. We next measured the phosphorylation of PKC-β2 and p65 in AOM-treated mice colons. Treatment of mice with PS significantly ($p < 0.001$) reduced the expression of p-PKC-β2 and p-p65 at 6 and 24 weeks (Figures 3B and 4B), whereas treatment of mice with RS showed a less effective response, suggesting that PS may be a more potent AR inhibitor than RS. Collectively, these results suggest that AR mediated AOM-induced ACF formation that was prevented by the AR inhibitor, PS.

Effect of Dietary PS and RS on AOM-Induced AR Expression and Activating Nrf2-Regulated Antioxidant Enzyme Expression. Because AOM-induced AR expression in the colonic epithelium is known to be associated with chronic inflammation and oxidative stress that could create a microenvironment contributing to the development of tumors in the colon. These results were further confirmed by immunohistochemistry (IHC)
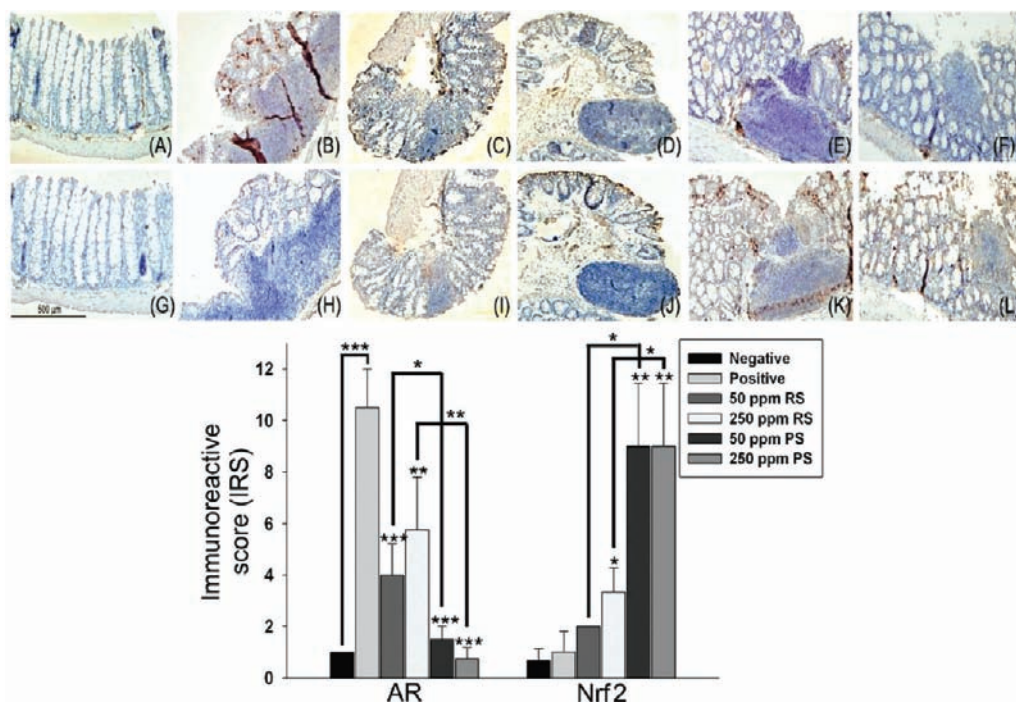
Figure 5. RS and PS increases expression and nuclear localization of Nrf2 in colonic ACF and inhibits expression of AR. At the end of week 6 (ACF group), the mice were sacrificed and detected by IHC methods. (Top panels) Staining and (bottom panels) quantification patterns of (A–F) AR and (G–L) Nrf2 expression in (A and G) normal mucosa of the negative control mice, (B and H) ACF of the AOM-treated positive mice, (C and I) ACF of AOM + 50 ppm RS-treated mice, (D and J) AOM + 250 ppm RS-treated mice, (E and K) ACF of AOM + 50 ppm PS-treated mice, and (F and L) AOM + 250 ppm PS-treated mice (100× magnification). Positive expressing cells are stained brown. Each bar represents the mean ± SD of the average of 12 mice scored. Results were statistically analyzed with Student's t test (∗, $p < 0.05$; ∗∗, $p < 0.01$; ∗∗∗, $p < 0.001$, compared to the AOM-induced value).

using anti-AR antibodies (panels A–F of Figure 5). Expression of the oxidative stress marker, AR, was significantly higher in AOM-treated mice compared to the untreated group. PS treatments significantly lower the level of AOM-induced expression of AR than the RS-treated group. Several studies have demonstrated that activation of Nrf2 signaling and the induction of its target genes could exert anti-inflammation and antioxidative effects.[27] Therefore, we further evaluate the role of Nrf2 in the AOM-induced colonic carcinogenesis mice model. To obtain further insight into molecular mechanisms underlying inhibition of AOM-induced AR expression by PS and RS, we next investigated if induction of Nrf2 could prevent AOM-induced AR expression in mice colons. Our results displayed that administration of RS (only in 250 ppm) and PS to AOM-treated mice significantly enhanced the activation of Nrf2 (panels G–L of Figure 5) and induction of Nrf2-mediated downstream antioxidant enzymes, heme oxygenase-1 (HO-1) and GR (Figure 6). Overall, it appears that PS may be a more potent Nrf2 inducer than RS. These results indicated that the dietary PS suppressed AOM-induced colonic tumorigenesis possibly through the induction of the antioxidant signaling pathway.

■ DISCUSSION

Several reports suggested that chemopreventive agents could prevent AOM-induced ACF formation in the initiation stage by increasing levels of antioxidant enzymes.[25] Our earlier and other studies showed that PS and RS could decrease expression of inflammatory markers, such as iNOS, COX-2, and p53, leading to reduced AOM- or DSS-induced ACF and tumor formation.[15,28] Previous studies indicated that RS could interfere with AOM-induced lipid peroxidation, inflammation, and oxidative stress signaling by elevating activities of enzymic antioxidants, including superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPX).[16] Although, RS and PS could abate inflammatory stress and oxidative damage, how to modulate oxidative stress signaling is unclear.

In this study, for the first time, we use the AOM-induced colon carcinogenesis model to compare the chemopreventive effect of RS and PS and elucidate their molecular mechanisms of anti-inflammation and antioxidation. We demonstrated that the chemopreventive effect of PS was more potent than RS and was associated with a decreased inflammation as well as modulation of the antioxidant signaling pathways in the colons of mice. These findings strongly suggested the chemopreventive potential of dietary administration of PS against colonic tumorigenesis. Colon carcinogenesis is a multi-step process, where ROS was found to enhance carcinogenesis at all stages: initiation, promotion, and progression. Numerous reports indicated that AOM could induce ROS generation by activation of growth factor and cytokine signaling, which could regulate inflammatory cell infiltration (ALN) and enhance cell proliferation of transformed colonic epithelial cells.[29] Previous reports have demonstrated the *in vivo* activities of RS and PS on the prevention of carcinogen-induced colon cancer via suppression of growth factor and cytokine signal transduction pathways.[15,16] In addition, several studies have suggested that ROS could cause oxidative stress and inflammation through AR. Various reports have also demonstrated that the inhibition of AR could prevent AOM-induced ACF and tumor formation.[26] In our study, we found that RS and PS could suppress AR expression in both colonic epithelias and LN cells
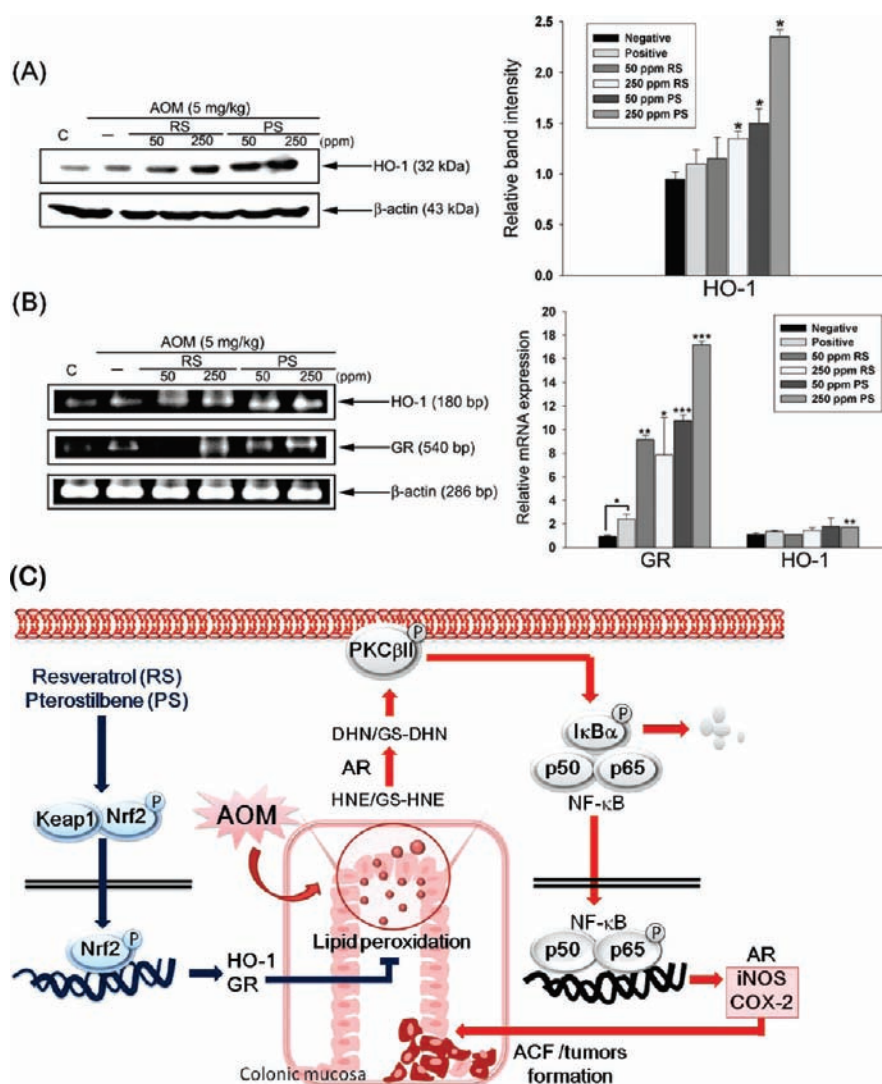
Figure 6. RS and PS induced Nrf-2-regulated antioxidant enzymes. At 6 weeks (ACF group) after the first AOM injection, colonic mucosas were scraped and homogenized. (A) Equal amounts of cell lysates (50 μg of protein) were subjected to western blot analysis using antibodies against HO-1 and β-actin. (B) HO-1 and GR primers were used for RT-PCR analysis. Each sample was normalized to β-actin. (C) Molecular mechanism by which PS and RS prevent ACF and tumor formation via increasing Nrf2-regulated HO-1 and GR expression. Quantification of protein and gene expression was normalized to β-actin using a densitometer. Results were statistically analyzed with Student's t test ($*$, $p < 0.05$; $**$, $p < 0.01$; $***$, $p < 0.001$, compared to the AOM-induced value). Each bar represents the mean $\pm$ SE ($n = 3$) of the averages of three independent experiments.

(panels A–F of Figure 5). Our results showed that PS might be significantly more potent than RS in lowing AOM-induced ACF and tumor numbers (Figures 1 and 2) by downregulating AR-dependent activation of PKC-β2 and NF-κB and expression of iNOS and COX-2 (Figures 3 and 4). Interestingly, RS was less effective in inhibiting PKC-β2 activity. The results of the present study together with the earlier data suggest that PS might be a chemopreventive agent in the inhibition of cancer initiation and provide novel mechanistic insight into the potential effects of PS on the suppression of colon carcinogenesis.

Many epidemiological and experimental studies have demonstrated that promotion of enzymic antioxidants, such as GR, NQO-1, and HO-1, have a chemopreventive effect on colon cancer by counteracting carcinogen-induced oxidative stress.[25,30,31] Furthermore, this evidence also showed that antioxidant enzymes could inhibit oxidative stress and inflammatory response by activation of Nrf2 signaling. Although most studies have

indicated that RS and PS are antioxidant and anti-inflammation compounds, few studies have investigated the role of AR and Nrf2 on colon carcinogenesis. AR is an oxidative-stress-response protein that has been proposed to be regulated by NF-κB and Nrf2.[32] Other findings suggest that many antioxidants derived from dietary and medicinal plants might effectively suppress the NF-κB-induced inflammatory marker expression via activation of Nrf2-ARE signaling, thereby preventing the initiation of carcinogenesis.[27] Our results suggested that AR expression could be regulated by the PKC/NFκB signaling pathway in AOM-treated mice (Figure 5). Moreover, our study demonstrated that PS and RS could suppress AR expression and induce Nrf2-regulated antioxidative enzymes, including HO-1 and GR (Figures 5 and 6). Although RS is considered a promising colon chemopreventive agent, its anticarcinogenic activity may be limited by its low bioavailability.[33] In contrast, PS may have improved bioavailability because of the substitution of two hydroxyl groups on RS

with two methoxy groups. According to these results, PS may be more effective than RS in preventing AOM-induced colon carcinogenesis. Moreover, we demonstrated for the first time that PS is not only an AR inhibitor but also a Nrf2 inductor. Our study displayed that PS was stronger in increasing the expression of HO-1 and GR than RS (Figure 6C). HO-1 and GR are believed to play an important role in cytoprotection and anti-inflammatory responses in a variety of pathological models. Previous studies have shown that Nrf2-deficient mice had markedly increased inflammation and oxidative damage in colonic mucosal, which was associated with decreased expression of phase-II detoxifying and antioxidant enzymes.[34] In addition to HO-1, current studies indicated that GR, which is involved in the production of reduced glutathione (GSH), is an important biomarker of oxidative stress and involved in healthy cells and tissues.[35] The augmented expression of GR in mice may raise the GSH concentration, which may protect cells and organs against carcinogen-induced toxicity.

In conclusion, we showed that PS might be more potent than RS in mediating the anti-tumorigenic effect by activation of Nrf2 signaling and the induction of its target genes, such as HO-1 and GR (Figure 6), as well as suppression of pro-inflammatory pathways regulated by NF-κB signaling in the initiation stage.

■ ASSOCIATED CONTENT

⑤ Supporting Information. Experimental design of AOM-induced colon carcinogenesis in BALB/c mice (Figure S1) and effect of AOM and PS or RS on the change in body weight and organ weight (Table 1). This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

RS, resveratrol; PS, pterostilbene; AOM, azoxymethane; ACF, aberrant crypt foci; iNOS, inducible nitric oxide synthase; COX-2, cyclooxygenase-2; PCR, polymerase chain reaction; Nrf2, NF-E2-related factor 2; HO-1, heme oxygenase-1; GR, glutathione reductase; PKC-β2, protein kinase C-β2; AR, aldose reductase

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