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Research Article

Cancer Prevention Research

Resveratrol Suppresses Oxidative Stress and Inflammatory Response in Diethylnitrosamine-Initiated Rat Hepatocarcinogenesis

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Abstract

Hepatocellular carcinoma (HCC), one of the most frequent and deadliest cancers, has been increasing considerably in the United States. In the absence of a proven effective therapy for HCC, novel chemopreventive strategies are urgently needed to lower the current morbidity and mortality of HCC. Recently, we have reported that resveratrol, a compound present in grapes and red wine, significantly prevents diethylnitrosamine (DENA)–induced liver tumorigenesis in rats, although the mechanism of action is not completely understood. In the present study, we have examined the underlying mechanisms of resveratrol chemoprevention of hepatocarcinogenesis by investigating the effects of resveratrol on oxidative damage and inflammatory markers during DENA-initiated rat liver carcinogenesis. There was a significant increase in hepatic lipid peroxidation and protein oxidation in carcinogen control animals compared with their normal counterparts at the end of the study (20 weeks). Elevated expressions of inducible nitric oxide synthase and 3-nitrotyrosine were noticed in the livers of the same animals. Dietary resveratrol (50-300 mg/kg) administered throughout the study reversed all the aforementioned markers in a dose-responsive fashion in rats challenged with DENA. Resveratrol also elevated the protein and mRNA expression of hepatic nuclear factor E2–related factor 2 (Nrf2). Results of the present investigation provide evidence that attenuation of oxidative stress and suppression of inflammatory response mediated by Nrf2 could be implicated, at least in part, in the chemopreventive effects of this dietary agent against chemically induced hepatic tumorigenesis in rats. The outcome of this study may benefit the development of resveratrol in the prevention and intervention of human HCC. *Cancer Prev Res; 3(6); 753–63. ©2010 AACR.*

Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third leading cause of cancer mortality worldwide (1). The incidence of HCC in the United States has dramatically increased by more than 70% in the past 25 years (2). The American Cancer Society has estimated that more than 22,000 new cases and ~18,000 deaths will occur in the United States in 2009 due to liver cancer (3). The majority of HCC cases are attributable to underlying infections caused by hepatitis B and C viruses (4). However, several other risk factors, including alcohol consumption, obesity, iron overload, environmental pollutants, as well as several dietary carcinogens, such as aflatoxins and nitrosamines, have been shown to be involved in its etiology (5–7). Currently, there is no proven effective systemic chemotherapy for HCC. Considering the limited treatment and grave prognosis of liver cancer, chemoprevention has been considered to be the best strategy in lowering the current morbidity and mortality associated with this disease (8).

Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene) is a phytochemical found in several dietary sources, such as grapes, berries, peanuts, and red wine. It is best known as the compound widely considered to be the dietary agent responsible for the "French paradox," a phenomenon in which consumption of red wine is thought to reduce the incidence of heart disease (9). Subsequent studies have shown that resveratrol can prevent or slow the progression of a wide variety of illnesses, including cancer, neurodegenerative diseases, cardiovascular ailments, ischemic injury, and viral infections, as well as enhance stress resistance and extend the life span of various organisms (10, 11). An impressive body of experimental findings reveals multiple cellular targets of resveratrol affecting cellular proliferation and growth, apoptosis, inflammation, invasion, angiogenesis, and metastasis (12). Resveratrol has been shown to suppress proliferation of a wide variety of human tumor cells *in vitro* (13), which have led to numerous preclinical animal studies to evaluate the cancer chemopreventive and chemotherapeutic potential of this compound (reviewed in

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ref. 14). However, the chemopreventive effect of resveratrol has not been investigated against carcinogen-initiated hepatic neoplasia *in vivo* until very recently (15). We reported for the first time that resveratrol significantly prevents diethylnitrosamine (DENA)–initiated and phenobarbital–promoted hepatic tumorigenesis in rats (16). In this study, dietary resveratrol dose-dependently reduced the incidence, total number, and multiplicity of visible hepatocyte nodules, the precursors of HCC. Nevertheless, the mechanisms of the inhibitory effects of this dietary polyphenol against rat liver carcinogenesis are not clearly understood.

Oxidative stress, through generation of reactive oxygen species (ROS) and reactive nitrogen species, acts as an important predisposing factor to hepatocarcinogenesis and is a common and major driving force of HCC in chronic liver diseases (17). It is well known that inflammation is one of the biological responses driven by oxidative stress. Modulation of oxidative damage as well as inflammation is believed to be an important means of protecting against hepatocarcinogenesis and considered to be of advantage for cancer prevention. It has been shown that resveratrol has potent antioxidant (18) and anti-inflammatory (19) properties, which might play an important role in protecting the liver against carcinogen-induced neoplasia. However, an experimental validation of this premise has not been presented to date according to the best of our knowledge and belief. Accordingly, in the present study, we have extended our previous work to examine the underlying mechanisms of resveratrol chemoprevention of hepatocarcinogenesis by investigating the effects of resveratrol on oxidative damage and inflammatory insult during DENA-initiated rat liver carcinogenesis. As the nuclear factor E2-related factor 2 (Nrf2), a redox-sensitive member of the cap 'n' collar basic leucine-zipper family, is known to play vital role in the reduction of oxidative and electrophilic stress as well as suppression of inflammation (20, 21), we have investigated Nrf2 signaling as a possible target of resveratrol-mediated inhibition of hepatocellular carcinogenesis.

Materials and Methods

Materials

Trans-resveratrol (~98% purity) was obtained from Organic Herb, Inc. DENA, phenobarbital, 2-thiobarbituric acid (TBA), and trichloroacetic acid were purchased from Sigma-Aldrich. Rabbit polyclonal inducible nitric oxide synthase (iNOS, also known as NOS2) antibody, rabbit polyclonal Nrf2 antibody, mouse β-actin monoclonal antibody, and ABC staining systems were procured from Santa Cruz Biotechnology. Rabbit polyclonal antibody for 3-nitrotyrosine (3-NT) was a product of Millipore. Horseradish peroxidase (HRP)–conjugated anti-rabbit and anti-mouse secondary antibodies were purchased from Invitrogen. OxyBlot Protein Oxidation Detection kit was obtained from Chemicon. Protein assay kits were purchased from Bio-Rad Laboratories and Thermo Scientific. Amersham blocking solution and enhanced chemiluminescence (ECL) substrate were obtained from GE Healthcare.

Animals and treatment

Hepatic samples for the present investigation were harvested from our previous chemopreventive study (16) following animal protocol approved by the Institutional Animal Care and Use Committee. Briefly, 48 female Sprague-Dawley rats (Charles River Laboratory), weighing 65 to 85 g at the beginning of the study, were randomized into five different groups of 6 to 13 animals each. Although one normal group served as the vehicle control, initiation of hepatocarcinogenesis in all other four groups was done by a single i.p. injection of DENA (200 mg/kg), followed by promotion with phenobarbital (0.05% w/v) in drinking water, which was started 2 weeks following DENA injection. The rats had free access either to a pulverized standard food (for normal and DENA control) or food supplemented with resveratrol equivalent to 50, 100, or 300 mg/kg body weight/d (for three resveratrol groups). The doses of resveratrol have been calculated based on dietary concentrations of resveratrol, food intakes, and body weights of animals at various time points of the study as previously reported (16). Resveratrol treatment was started 4 weeks before the initiation and continued for 20 weeks. At the end of the study (20 weeks), the livers from all animals were perfused and subsequently excised under anesthesia. The livers were subjected to morphologic analysis of visible hepatocyte nodules. Data on incidence and size distribution of nodules have been published, which showed 22% to 60% inhibition of hepatic nodules in rats exposed to dietary resveratrol (50-300 mg/kg; ref. 16). A portion of non-nodular liver tissue from different groups was collected and frozen immediately in liquid nitrogen and subsequently transferred to and stored in a −80°C freezer. Serial sections (~10 μm) of liver tissue were prepared from different groups and stored at −80°C freezer. Due to the lack of sufficient number of nodules in 300 mg/kg resveratrol-treated animals, non-nodular liver areas across the groups were used in the present study to have a uniform comparison of various end-point biomarkers. Histopathologic examination revealed the presence of hepatic foci in some liver sections. Four to six livers per group were analyzed for various parameters.

Lipid peroxidation

Hepatic lipid peroxidation was determined by the modified method of Ohkawa et al. (22) based on the reaction between TBA and malondialdehyde formed from peroxidation of lipids. In short, the liver was homogenized in lysis buffer and then centrifuged at 2,000 × *g* for 5 minutes to remove debris. Two hundred microliters of 0.38% TBA–15% trichloroacetic acid–0.25 N HCl were added to 100 μL supernatant, and the mixture was heated in a boiling water bath for 15 minutes. After cooling at room temperature, the sample was centrifuged at 1,000 × *g* for 10 minutes. The aliquot of the supernatant was collected, and the

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absorbance at 532 nm was measured. The extent of lipid peroxidation was expressed as micromole of thiobarbituric acid reactive substances (TBARS) per milligram of protein using the molar extinction coefficient, $E = 1.56 \times 10^5$ $(mol/L)^{-1}$ cm^{-1}. Protein concentration was estimated using the Bio-Rad bovine serum albumin protein assay kit, following the manufacturer's protocol.

Slot-immunoblot measurement of protein carbonyls

Liver samples were homogenized in 5% SDS in TBS on ice. The liver homogenate was centrifuged at 800 × *g* for 5 minutes. Protein concentration in the supernatant solution was quantified using the Pierce BCA protein assay kit, following the instructions provided by the manufacturer. Samples were processed using the OxyBlot kit. Protein (5 μg) was transferred onto a 0.45 μm nitrocellulose membrane using a vacuum slot-blot apparatus (Schleicher & Schuell). Each slot was washed with 200 μL TBS, and the membrane was dried. The membrane was treated with 2% blocking solution for 1 hour. Transferred protein was incubated with the rabbit polyclonal anti-dinitrophenyl hydrazine antibody (present in OxyBlot kit) overnight at 4°C for the detection of protein carbonyl levels. This step was followed by 1 hour application of the secondary antibody obtained from the OxyBlot kit. Each blot was exposed to the ECL substrate and read on a Kodak Digital Science Image Station 440CF analyzer (NEN Life Science Products, Inc.).

Immunohistochemistry

Immunohistochemical detection of iNOS, 3-NT, and Nrf2 in ~10-μm-thick liver sections was done by standard immunohistochemical techniques. Briefly, the sections were incubated for 10 minutes at 80°C in 10 mmol/L sodium citrate buffer (pH 6.0) for antigen retrieval. Following a 5-minute wash with PBS, the endogenous peroxidases were blocked by 1% H_2O_2 in PBS for 5 minutes. The sections were washed as before and blocked for 1 hour in PBS containing 5% normal goat serum. The slides were washed and then incubated overnight with primary antibodies (1:100 for iNOS and Nrf2 and 1:50 for 3-NT) at 4°C in a humidified chamber. After washing with PBS, the sections were incubated with HRP-conjugated secondary antibody (goat anti-rabbit, 1:200 dilution) for 30 minutes at 37°C. The chromogenic reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride solution. Negative control sections were processed similarly with the omission of the primary antibodies. All sections were viewed under a light microscope; 1,000 hepatocytes were analyzed per animal; and results were expressed as percentage of positive cells.

Western blot analysis

Liver tissue samples were homogenized in ice-cold lysis buffer, and the homogenized liver tissue (10% w/v) was centrifuged at 14,000 × *g* for 15 minutes at 4°C. Protein in the supernatant was quantified using the Pierce BCA protein assay kit. Approximately 100 μg protein sample per lane were run on a 4% to 12% NuPage Bis-Tris gel (Invitrogen), and the transferred protein was subjected to 1 hour incubation in 2% blocking solution. A rabbit polyclonal anti-iNOS antibody (1:50) or a rabbit polyclonal anti–3-NT antibody (1:500) was then applied overnight at 4°C and followed by 1 hour application of HRP-conjugated anti-rabbit secondary antibody (1:1,000). Each blot was then exposed to the ECL substrate and read on a Kodak Digital Science Image Station 440CF analyzer. The nitrocellulose membrane was then soaked in TBS with 1% Tween 20 overnight at 4°C followed by 1 hour incubation in 2% blocking solution. A mouse monoclonal anti–β-actin antibody (1:1,000) was applied overnight at 4°C. This step was followed by 1 hour application of HRP-conjugated anti-mouse secondary antibody (1:1,000). Each blot was then developed and read on the Kodak analyzer.

RNA extraction and quantification of mRNA by reverse transcriptase-PCR

Total RNA was extracted from 20 mg of liver tissue using Qiagen RNeasy plus mini kit according to the instructions provided with the kit. cDNA was synthesized from 100 ng of total RNA using Superscript II cDNA synthesis kit (Invitrogen). PCR was done using specific primers for rat Nrf2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as follows: Nrf-2 sense 5′-CCTAAAGCACAGCCAACACA-3′, antisense 5′-ACAGTTCTGAGCGGCAACTT-3′; GAPDH sense 5′-AGACAGCCGCATCTTCTTGT-3′, antisense 5′-TACTCAGCACCAGCATCACC-3′. PCR products were analyzed by agarose gel electrophoresis and visualized by ethidium bromide staining.

Statistical analysis

Data are expressed as mean ± SD. One-way ANOVA was used to the estimate overall significance followed by post hoc analysis using the Student-Neuman-Keuls test. A probability level of 5% ($P < 0.05$) was considered significant. A commercial software program (SigmaStat 3.1, Systat Software, Inc.) was used for all statistical analyses.

Results

Resveratrol exerts antioxidant effects during hepatocarcinogenesis

The extent of lipid peroxidation in hepatic tissue was determined by measuring TBARS. DENA treatment exhibited an 11-fold increase ($P < 0.05$) in the generation of TBARS in rat liver (Fig. 1A). Resveratrol dose-dependently inhibited oxidative damage during DENA hepatocarcinogenesis as evidenced from its ability to prevent DENA-induced hepatic lipid peroxidation in rats. A statistically significant ($P < 0.05$) result was achieved with resveratrol at a dose of 100 or 300 mg/kg. At 300 mg/kg, resveratrol not only suppressed DENA-induced lipid peroxidation but also brought the levels back to normal. To explore the effects of resveratrol feeding on the oxidative modifications of hepatic proteins during DENA hepatocarcinogenesis, we measured the carbonyl contents of proteins in several

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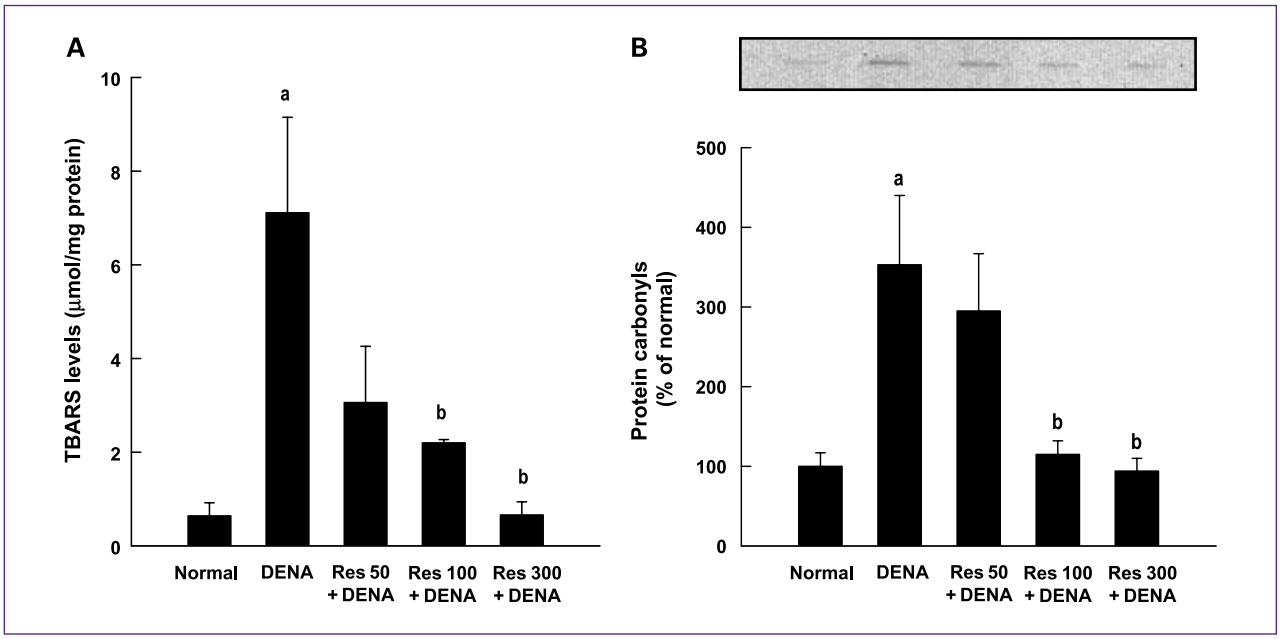

Fig. 1. Antioxidant effects of resveratrol during DENA-initiated hepatocarcinogenesis in female Sprague-Dawley rats. A, extent of lipid peroxidation as measured by estimating TBARS in the livers of various groups of rats. Rats were sacrificed 20 wk following the commencement of the study, and estimations were done to measure TBARS. Each column represents mean ± SD (n = 4-6 livers). a, $P < 0.05$ compared with normal group. b, $P < 0.05$ compared with DENA control. B, effects of resveratrol on hepatic protein carbonyl formation in rats subjected to DENA hepatocarcinogenesis. Rats were sacrificed 20 wk following the commencement of the study, and estimations were done by the slot-blotting technique. Each column represents mean ± SEM (n = 4-6 livers). a, $P < 0.01$ compared with normal group. b, $P < 0.01$ compared with DENA control.

experimental groups. As shown in Fig. 1B, DENA administration produced a significant ($P < 0.01$) increase in protein carbonyls as measured by the immunoblotting technique, compared with normal animals. Treatment with resveratrol elicited a reduction in DENA-induced increment in protein carbonyl content in a dose-responsive fashion. Resveratrol at a dose of 100 or 300 mg/kg produced a statistically significant ($P < 0.01$) result. Interestingly, the level of protein carbonyl in the 300 mg/kg resveratrol group was found to be slightly less than that of the normal group.

Resveratrol abrogates DENA-mediated hepatic iNOS induction during hepatocarcinogenesis

As chronic inflammation plays a crucial role in hepatocarcinogenesis (23, 24), the ability of resveratrol to suppress the induction of inflammatory marker iNOS expression has been investigated. Although a minimal hepatic iNOS expression was observed in normal animals (Fig. 2A-a), a significant increase in iNOS-positive brown-stained hepatic foci and proliferating oval cells was noticed in the liver sections of DENA-initiated animals (Fig. 2A-b). Although resveratrol at 50 mg/kg did not change iNOS expression (figure not shown), a moderate improvement by 100 mg/kg (Fig. 2A-c) and a maximum reduction by 300 mg/kg resveratrol (Fig. 2A-d) were observed. Figure 2B shows the percentage of hepatic iNOS-positive cells in various groups of animals. There was a significant ($P < 0.01$) increment in iNOS-positive cells in the DENA control group. Resveratrol treatment reduced the hepatic iNOS protein expression in animals challenged with DENA; however, results reached statistical significance at a dose of 100 mg/kg ($P < 0.05$) or 300 mg/kg ($P < 0.01$). To confirm the immunohistochemical data on iNOS, livers from various groups were subjected to Western blot analysis. As shown in Fig. 2C, there was a significant ($P < 0.05$) upregulation of this protein in DENA control animals compared with the normal group. Further, DENA-induced overexpression of iNOS was suppressed from 2.4-fold in rats fed normal diet to 2.2- to 1.1-fold ($P < 0.05$) in rats fed resveratrol-supplemented diet (100 and 300 mg/kg, respectively). Taken together, all these results indicate that resveratrol was able to reverse the high levels of iNOS during DENA-initiated rat liver carcinogenesis.

Resveratrol suppresses 3-NT expression during DENA hepatocarcinogenesis

As NO produced by iNOS is an important free radical involved in inflammation-driven hepatocarcinogenesis (25), the aforementioned data undermine the importance of studying related and additional markers of inflammation as potential targets of resveratrol chemoprevention. Accordingly, we have investigated the immunohistochemical localization of 3-NT in the livers of rats treated with DENA and fed with normal or resveratrol-supplemented diet. As Fig. 3A-a reveals, 3-NT was rarely detected in normal rat liver. On the other hand, 3-NT–containing protein was frequently localized in the foci of altered hepatocyte

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belonging to DENA control (Fig. 3A-b). Resveratrol at 50 mg/kg did not alter the extent of 3-NT immunopositivity compared with DENA alone (data not shown). In contrast, a moderate and drastic suppression of 3-NT expression was noticed following resveratrol treatment at a dose of 100 mg/kg (Fig. 3A-c) or 300 mg/kg (Fig. 3A-d), respectively. Figure 3B depicts percentage of 3-NT–positive cells in the livers from all experimental groups. A dramatic increase ($P < 0.001$) in the percentage of 3-NT–positive cells in DENA-exposed rats compared with their normal counterparts was noticed. There was a significant ($P < 0.001$) inhibition in the percentage of 3-NT–positive cells in rats fed with either 100 or 300 mg/kg resveratrol compared with the DENA alone group. The aforementioned effects of resveratrol on DENA-induced 3-NT expression were also confirmed by Western blot analysis. As shown in Fig. 3C, DENA markedly increased 3-NT expression in the liver samples. This effect was reversed by resveratrol (100 or 300 mg/kg) treatment.

Resveratrol induces Nrf2 expression during DENA hepatocarcinogenesis

Because Nrf2 signaling has been postulated to play an important role in the reduction of oxidative stress and suppression of inflammation (20, 21), we next sought to determine the role of Nrf2 in the observed antioxidant and anti-inflammatory responses of resveratrol during DENA hepatocarcinogenesis. Our immunohistochemical data revealed very limited expression of Nrf2 in the liver sections of normal as well as DENA-challenged animals (Fig. 4A-a,b).

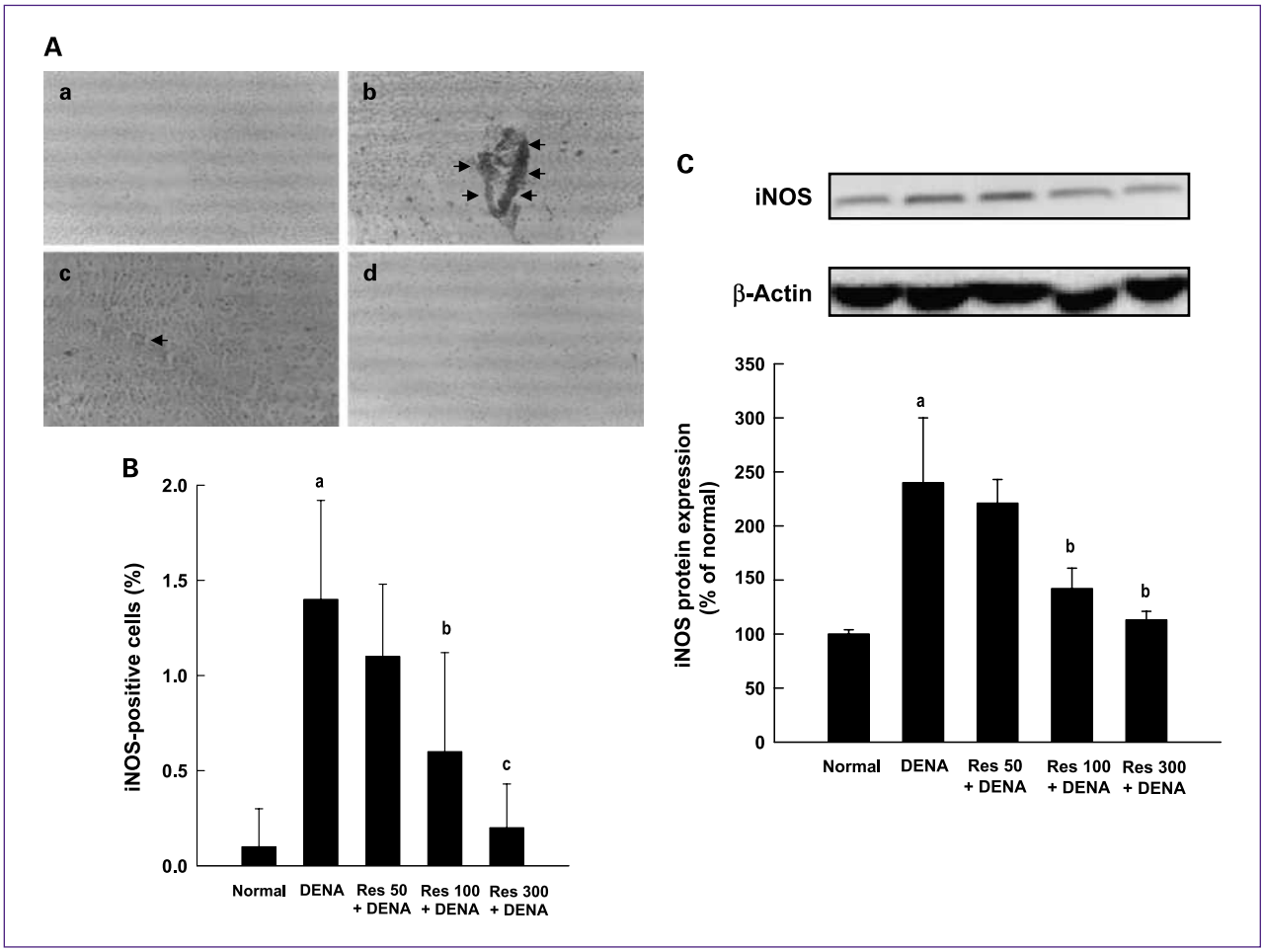

Fig. 2. Effects of resveratrol on hepatic iNOS expression during DENA-initiated hepatocarcinogenesis in female Sprague-Dawley rats. A, representative immunohistochemical localization of iNOS (magnification, 100×). Rats were sacrificed 20 wk following the commencement of the study, and immunohistochemistry was done to detect iNOS. Arrows indicate immunohistochemical staining of iNOS. Absence of immunostaining in normal liver (a), intense immunoreactivity in DENA control liver (b), decreased iNOS expression in the 100 mg/kg resveratrol group (c), and almost normal–appearing liver of the 300 mg/kg resveratrol group (d) were noticed. B, quantification of iNOS-positive cells in rat livers of several experimental groups. One thousand hepatocytes were counted per animal, and the results were based on four animals per group. Each column represents mean ± SD ($n = 4$ livers). a, $P < 0.01$ compared with the normal group; b, $P < 0.05$ and c, $P < 0.01$ compared with DENA control. C, representative Western blot and densitometric analysis of hepatic iNOS expression in various groups of rats. Rats were sacrificed 20 wk following the commencement of the study. Total cellular protein was separated and blotted with anti-iNOS antibody. Each column represents mean ± SEM ($n = 4$-6 livers). a, $P < 0.05$ compared with normal group; b, $P < 0.05$ compared with DENA control.

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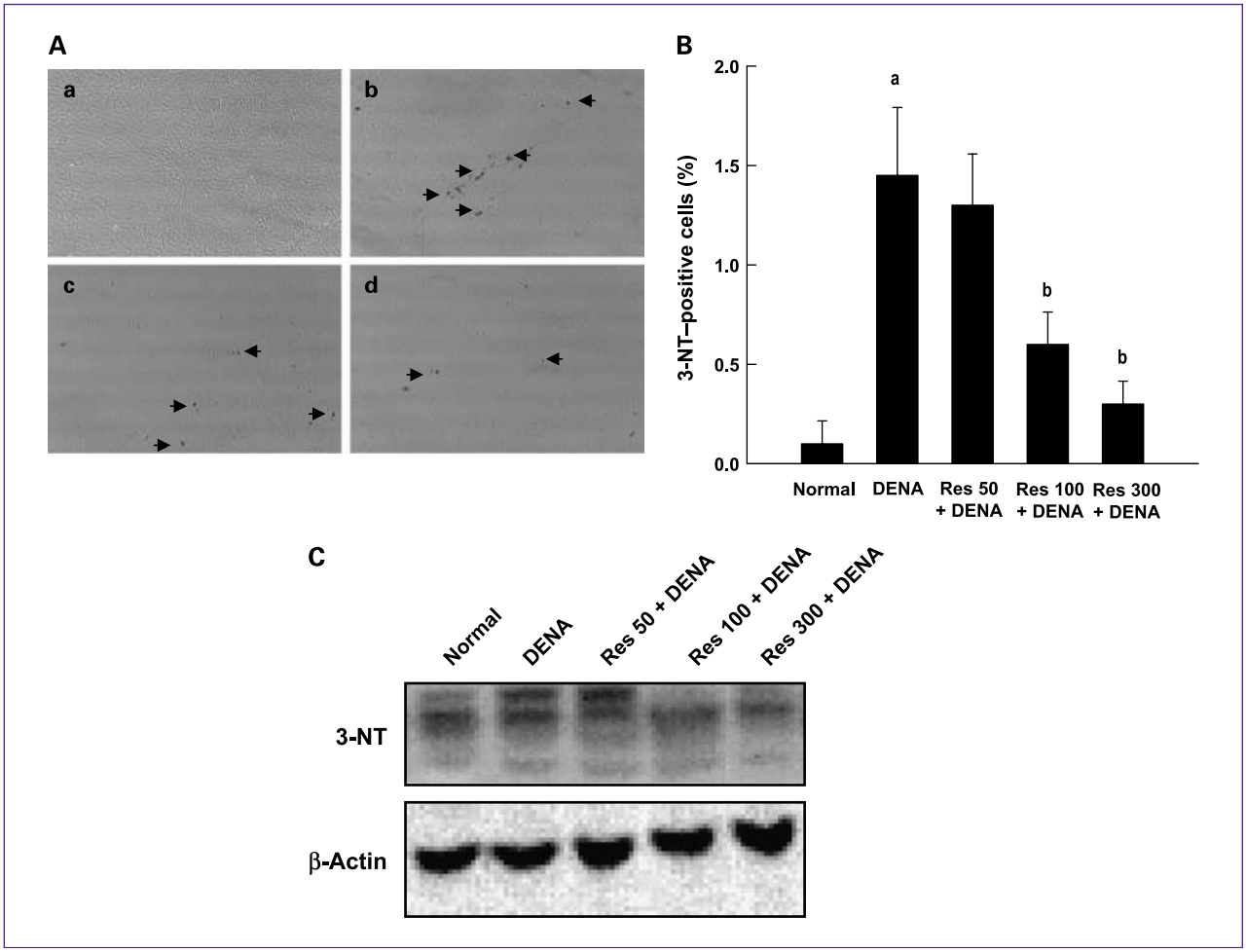

Fig. 3. Effects of resveratrol on hepatic 3-NT expression during DENA-induced hepatocarcinogenesis in female Sprague-Dawley rats. A, immunohistochemical localization of 3-NT (magnification, 100×). Rats were sacrificed, and immunohistochemistry was done 20 wk following the commencement of the study to detect 3-NT. Arrows indicate immunohistochemical staining of 3-NT. Absence of 3-NT–containing proteins in normal liver (a), intense 3-NT immunoreactivity in DENA control liver (b), decreased 3-NT expression in 100 mg/kg resveratrol group (c), and very limited expression of 3-NT in 300 mg/kg resveratrol group (d) were observed. B, quantification of 3-NT–positive cells in rat livers from several experimental groups. One thousand hepatocytes were counted per animal, and the results were based on four animals per group. Each column represents mean ± SD (n = 4 livers). a, $P < 0.001$ compared with normal group; b, $P < 0.001$ compared with DENA control. C, representative Western blot analysis of hepatic 3-NT expression in various groups of rats. Rats were sacrificed 20 wk following the commencement of the study. Total cellular protein was separated and blotted with anti–3-NT antibody.

There was a slight increase in the expression of Nrf2 in hepatic cells of resveratrol (50 mg/kg) plus DENA group (data not shown). In contrast, a large quantity of Nrf2-positive cells was identified in the other two resveratrol-treated groups; that is, 100 and 300 mg/kg (Fig. 4A-c,d). Most of the immunoreactivity for Nrf2 in these two resveratrol-treated groups was observed in the nucleus, which indicates the activation of Nrf2 and subsequent nuclear translocation. The quantitative evaluation of hepatic Nrf2-positive cells showed only a limited presence of such cells in normal as well as DENA control group (Fig. 4B). There was a significant increase ($P < 0.001$) in Nrf2 positivity in resveratrol-supplemented groups at 100 and 300 mg/kg compared with DENA control. We have also studied Nrf2 gene expression by measuring mRNA levels of Nrf2 in hepatic tissues of various experimental animals. It has been observed that dietary resveratrol at a dose of 100 or 300 mg/kg elicited a substantial increase in Nrf2 mRNA expression in the liver of DENA-challenged animals compared with carcinogen control. These data provide valuable information about the involvement of Nrf2 in the chemopreventive action of resveratrol.

Discussion

In our previous study (16) using a two-stage model of rat liver carcinogenesis initiated with DENA and promoted by phenobarbital, resveratrol dose-dependently suppressed hepatic tumor multiplicity, the principal end point for evaluating the chemopreventive potential of a candidate agent. Nevertheless, the precise mechanism(s) by which

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resveratrol exerts a chemopreventive action against liver tumor development in rats subjected to potent hepatocarcinogenic insult has not been completely elucidated. The present study, having capitalized on the availability of liver tissues collected from our previous experiments, probed into the possible mechanisms underlying the chemopreventive action of resveratrol against hepatic neoplasia.

ROS, capable of producing lipid peroxidation and oxidation of DNA and other cellular macromolecules, has a role in the initiation, promotion, and progression of liver cancer. DENA confers its hepatocarcinogenicity through the metabolic activation in the hepatic microsomes, resulting in the release of ethylcarbonium ions that bind to the DNA, producing adducts and generating superoxide radicals through lipid peroxidation of phospholipid membrane fatty acids (26). Malondialdehyde, a product of lipid peroxidation of polyunsaturated fatty acid metabolism and degradation, has been established as a mutagenic and carcinogenic entity (27). Together with DENA, the tumor-promoting agent phenobarbital is known to cause oxidative damage to rat liver (28). Continuous administration of phenobarbital has been reported to elevate the formation of ROS in DENA-induced neoplastic hepatic nodules in rats (29). Our present data show that a single injection of DENA (200 mg/kg) followed by phenobarbital (0.05%) administration through drinking water for 14 successive weeks elicited a dramatic induction of hepatic lipid peroxidation in rats, as evidenced from 11-fold increase in malondialdehyde levels, indicating severe oxidative stress due to excessive generation of free radicals. Our previous studies (30) as well as those reported from other laboratories (31–33) have also shown

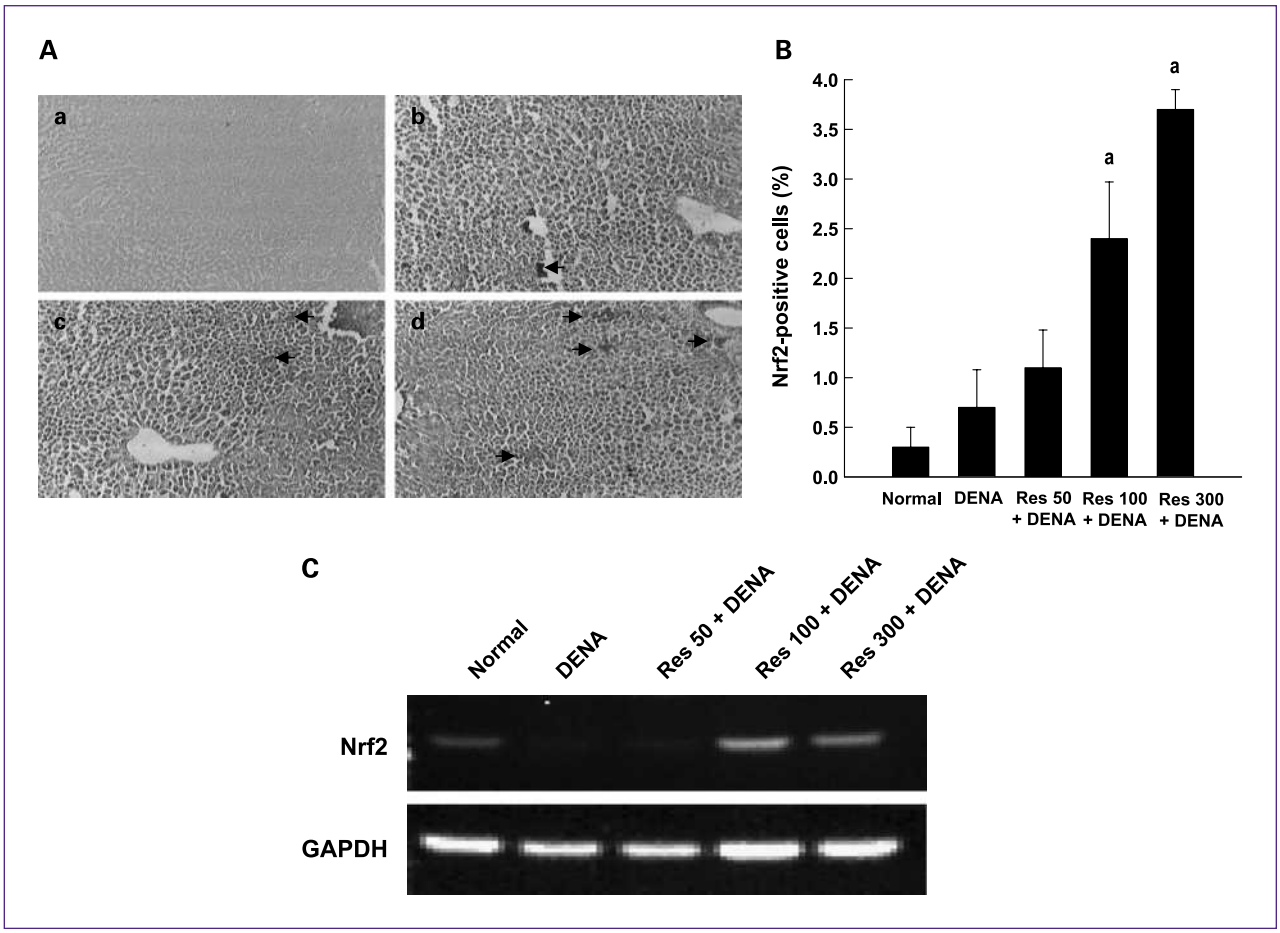

Fig. 4. Effects of resveratrol on hepatic Nrf2 expression during DENA-initiated hepatocellular carcinogenesis in female Sprague-Dawley rats. A, immunohistochemical staining of Nrf2 (magnification, 100×). Rats were sacrificed, and immunohistochemistry was done 20 wk following the commencement of the study to detect Nrf2. Arrowheads indicate immunohistochemical staining of Nrf2. Representative observation of Nrf2 immunoreactivity in different groups: very limited expression in normal (a) and DENA liver (b), and resveratrol-mediated induction in the 100 mg/kg (c) and 300 mg/kg group (d). B, immunochemical quantification of Nrf2 in livers of various groups. One thousand hepatocytes were counted per animal, and the results were based on four animals per group. Each column represents mean ± SD (n = 4 livers). a, $P < 0.001$ compared with DENA control. C, effects of resveratrol on hepatic gene expression of Nrf2. Total RNA was isolated from liver and subjected to reverse transcription. The resulting cDNA was subjected to PCR using specific primer sequences for Nrf2. Representative reverse transcription-PCR gel pictures are shown with *GAPDH* as the housekeeping gene.

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a similar induction of hepatic lipid peroxidation following DENA-phenobarbital treatment in rats. Our data also show that resveratrol reverses the DENA-phenobarbital–induced lipid peroxidation in a dose-responsive fashion, suggesting the ability of resveratrol in scavenging free radicals produced by these xenobiotics. In conjunction with our previous study (16), the present findings indicate that inhibition of lipid peroxidation is involved in the observed chemopreventive action of resveratrol. Resveratrol has potent antioxidant properties and scavenges lipid peroxyl radicals (reviewed in ref. 34). Recent studies have shown that resveratrol pretreatment enhances the antioxidant status of primary hepatocytes isolated from Sprague-Dawley rats (35) and effectively protects these cells from oxidative damage (18). In recent years, an overwhelming number of studies provide convincing evidence that inhibition of lipid peroxidation and oxidative damage plays a substantial role in the hepatoprotective effects of resveratrol against chemically induced liver damage in rodents (36–38). However, our present results provide the first experimental evidence that the antioxidant potential of this polyphenol plays a valuable role in the inhibition of hepatocellular carcinogenesis in rats.

It has been established that ROS could modify the chemical structure of proteins with formation of protein carbonyls due to oxidative cleavage of the main peptide backbone or by oxidation of amino acids, including arginine, lysine, proline, and threonine (39). Protein carbonyl content has been the most commonly used marker of protein oxidation. Recent study shows that oxidative stress elevates the protein carbonyl content in plasma of HCC patients (40). As a hallmark of protein oxidation, total protein carbonyl content was measured in our present study in the livers of DENA-exposed animals. In line with previous observations (31, 41), we have observed an elevated hepatic level of protein carbonyl formation in DENA-treated animals, indicating oxidative protein damage. We have also noticed that dietary resveratrol (100 or 300 mg/kg) completely abrogated DENA-induced enhanced protein carbonyl formation, which implicates the ability of this dietary agent in attenuating oxidative stress in the liver. Our results are reminiscent of a recent clinical study that shows a distinct decrease of oxidative stress–induced carbonyl group formation in human plasma proteins by resveratrol (42).

During recent years, compelling evidence strongly implicates the role of inflammation in initiation, promotion, and progression of HCC (43). One of the major contributors of chronic inflammatory reactions is NO, produced by hepatic parenchymal as well as nonparenchymal cells from L-arginine by iNOS. Oxidative stress is known to increase iNOS gene transcription and promoter activity in hepatocytes (44). Mounting evidence underlines the important role that iNOS plays in the development and progression of HCC as this enzyme has been found to be overexpressed in rodent as well as human HCC (45, 46). Recent studies by Calvisi et al. (46) have shown that suppression of iNOS by aminoguanidine, a selective iNOS inhibitor, leads to suppression of HCC growth, suggesting that iNOS signaling could be an important target for prevention and treatment of human HCC. In the present study, we have observed an elevated level of hepatic iNOS expression in DENA-treated animals, confirming the results of prior studies (45, 47). Additionally, we have shown that resveratrol is able to reverse the elevated iNOS expression and thereby the subsequent NO production in the livers of experimental animals during DENA-induced hepatocarcinogenesis. As the generation of NO is a consequence of iNOS induction in inflammatory processes, our results substantiate a clear anti-inflammatory effect of resveratrol through iNOS downregulation in our experimental conditions. Another important aspect of the present finding is that iNOS could represent a potential target of resveratrol-mediated chemoprevention of hepatocellular carcinogenesis in rats. This is in line with a recent observation that the iNOS inhibitory effects of resveratrol are related to its antitumor responses against human neoplastic cells (48).

Uncontrolled and chronic iNOS-induced endogenous NO production triggers liver damage, inflammation, and subsequently development of tumors (49). Deleterious effects of NO may be ascribed to its nonenzymatic reaction with superoxide anion (O_2^-), which yields peroxynitrite ($ONOO^-$), often referred to as a reactive nitrogen species. $ONOO^-$ can react with susceptible amino acids, including arginine, cysteine, histidine, and lysine, and cause protein oxidation (carbonylation). Additionally, $ONOO^-$ is able to oxidize nuclear DNA and cause nitration of tyrosine and other aromatic amino acids to generate 3-NT. Because 3-NT is stable under physiologic conditions, its presence has been considered to be an important footprint and biomarker of oxidative damage and inflammation inflicted by NO-derived $ONOO^-$ (50). A large increase in protein nitration as detected by a 15-fold increase in 3-NT immunoreactivity in the livers of DENA control animals suggests an increase in the nitration of tyrosine moieties. This also indicates that a major portion of oxidative damage was induced by $ONOO^-$ during rat liver carcinogenesis. Our present data are in line with a previous study that reported the presence of 3-NT–containing proteins in DENA-induced preneoplastic as well as neoplastic rat liver tissues (45). It is possible that resveratrol-mediated suppression of iNOS as observed in this study could reduce $ONOO^-$ generation by limiting NO production. Although no other study describing the effects of resveratrol on 3-NT during carcinogenesis is available in the literature, several lines of experimental evidence have shown that resveratrol attenuates elevated 3-NT in various physiologic conditions of oxidative stress and inflammation (42, 51).

Oxidative and inflammatory insults are intimately connected with each other in multistage carcinogenesis. Hence, it is expected that an agent with anti-inflammatory property will inhibit oxidative stress and vice versa (52). The transcription factor Nrf2, a member of the basic leucine-zipper family, plays an essential role in the antioxidant response element–mediated expression of many antioxidant and phase 2 detoxifying enzymes (53, 54).

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Accumulating evidence has shown that Nrf2 signaling pathway plays an essential role in the protection of the host against inflammation and inflammatory damage (55). Nrf2 is normally sequestered in the cytosol by Kelch-like ECH-associated protein 1 (Keap1). Upon oxidative or electrophilic stress or stimulation by compounds that possess the ability to oxidize or covalently modify thiol groups of Keap1 (56), Nrf2 dissociates from Keap1 and translocates to the nucleus, where it heterodimerizes with small Maf protein and binds to the antioxidant response element or electrophilic response element (57). This leads to the synthesis of a number of antioxidant and detoxifying enzymes, which efficiently protects mammalian cells from various forms of stress and consequently reduce the propensity of tissues to develop malignancy (58). Recent studies with Nrf2-deficient mice show that Nrf2 plays a role in protecting the liver from xenobiotic-initiated hepatocarcinogenesis (59). All these studies indicate that Nrf2 is a key target of chemoprevention of HCC. In the present study, we have observed for the first time an increased hepatic Nrf2 protein with enhanced nuclear translocation in rats pretreated with chemopreventive doses of resveratrol and subsequently exposed to the potent hepatocarcinogen DENA. This has been further supported by a parallel increase in the transcription of Nrf2 gene by resveratrol during rat liver carcinogenesis. Recent studies from other laboratories have shown that resveratrol activates Nrf2 with the consequent induction of antioxidant and phase 2 enzymes in various mammalian cells, including primary hepatocytes (18, 60–62). In view of these observations, the inhibitory effects of resveratrol against DENA-induced lipid peroxidation and protein oxidation as seen here could be achieved by the induction of antioxidant and phase 2 conjugating enzymes through modulation of Nrf2.

It is possible that both anti-initiation and antipromotion response of resveratrol could be linked to the antihepatocarcinogenic effects of this dietary agent. Resveratrol may act by an Nrf2-regulated induction of cytoprotective enzymes, which may result in enhanced excretion of an electrophilic carcinogen, leading to reduced formation of free radicals and consequently initiation of hepatocarcinogenesis. On the other hand, the anti-inflammatory response by inhibition of iNOS induction as observed in the current study, coupled with antiproliferative and proapoptotic effects of resveratrol reported in our previous communication (16), may suggest a possible involvement of antipromotional effects. The effects of resveratrol on mediators of oxidative stress and inflammatory response as presented here need to be explained by further investigation of detailed mechanisms at early preneoplastic stages of DENA-initiated hepatocarcinogenesis. As iNOS overexpression during hepatocarcinogenesis could be a consequence of NF-κB activation (46), the inhibitory effects of resveratrol on iNOS expression could be related to its ability to modulate NF-κB signaling. However, this does not exclude the possibility that resveratrol could regulate iNOS expression by other mechanisms, including proinflammatory cytokines. Active research is under way in our laboratory to explore these and other possibilities to understand the full spectrum of resveratrol action in the chemoprevention of hepatocellular carcinogenesis.

In conclusion, the results presented in this study clearly show that resveratrol combats oxidative stress and suppresses inflammatory cascade in a dose-responsive fashion during DENA-induced rat liver carcinogenesis. The present study also indicates that attenuation of oxidative and nitrosative stress as well as alleviation of the inflammatory response could be mediated through transcriptional and translational regulation of Nrf2 signaling. All these could be implicated, at least in part, in the previously reported chemopreventive effects of resveratrol against chemically induced hepatic tumorigenesis in rats. Because both oxidative stress and inflammation play crucial roles in the development and progression of human liver cancer, the present findings underscore the potential of targeting these processes as a strategy for achieving liver cancer chemoprevention and intervention by the dietary agent resveratrol.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cancer Prevention Research

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