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RESEARCH PAPER

Suppression of the Inflammatory Cascade is Implicated in Resveratrol Chemoprevention of Experimental Hepatocarcinogenesis

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ABSTRACT

Purpose Resveratrol, present in grapes and red wine, has been found to prevent diethylnitrosamine (DENA)-initiated rat liver tumorigenesis, though the chemopreventive mechanisms are not completely elucidated. The current study was designed to explore whether the antiinflammatory properties of resveratrol play a role in its antihepatocarcinogenic action.

Methods Liver samples were harvested from a 20-week chemopreventive study in which resveratrol (50, 100 and 300 mg/kg) was shown to inhibit DENA-induced hepatocyte nodules in Sprague-Dawley rats in a dose-responsive manner. Hepatic preneoplastic and inflammatory markers, namely heat shock protein (HSP70), cyclooxygenase-2 (COX-2) and nuclear factor-κB (NF-κB), were studied using immunohistochemical as well as Western blot techniques.

Results Resveratrol dose-dependently suppressed DENA-induced increased expressions of hepatic HSP70 and COX-2. Resveratrol also attenuated the DENA-mediated translocation of NF-κB p65 from the cytosol to the nucleus with stabilization of inhibitory κB.

Conclusion The present findings indicate that resveratrol exerts chemoprevention of hepatocarcinogenesis possibly through antiinflammatory effects during DENA-evoked rat liver carcinogenesis by suppressing elevated levels of HSP70, COX-2 as well as NF-κB. These beneficial effects combined with an excellent safety profile encourage the development of resveratrol for chemoprevention and intervention of human HCC that remains a devastating disease.

KEY WORDS chemoprevention · hepatocarcinogenesis · inflammation · NF-κB · resveratrol

ABBREVIATIONS

COX-2	cyclooxygenase-2
DENA	diethylnitrosamine
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HRP	horseradish peroxidase
HSP70	heat shock protein 70
IκB	inhibitor of κB
iNOS	inducible nitric oxide synthase
NF-κB	nuclear factor-kappa B
PB	phenobarbital
PBS	phosphate-buffered saline
PGs	prostaglandins
ROS	reactive oxygen species

INTRODUCTION

Hepatocellular carcinoma (HCC) represents the majority of all primary liver cancers. It is the sixth most common cancer and the third leading cause of cancer deaths worldwide (1). HCC has an extremely poor prognosis, with the number of deaths almost equal to the number of cases being diagnosed each year (more than 600,000), and the five-year survival rate is below 9% (1,2). The incidence of HCC has been steadily rising in several regions of the

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world, including southeast Asia, Japan, sub-Saharan Africa, northern and southern Europe as well as North America. In the United States, the incidence of HCC has increased by more than 70% in the past quarter century (3). According to the estimation of the American Cancer Society, there will be more than 22,000 new cases and nearly 18,000 deaths in the United States in 2009 due to liver cancer (4). The etiology of HCC includes infections caused by the hepatitis B and C virus (HBV and HCV), alcohol abuse, hemochromatosis, environmental pollutants as well as several dietary carcinogens, such as aflatoxins and nitrosamines (5-8). Although surgical resection is currently considered to be the optimal treatment modality, only 10–20% of patients qualify for this treatment due to tumor size, multifocality, vascular invasion, or hepatic decompensation. Moreover, the recurrence rates have been reported to be as high as 50% for those undergoing surgery within several years (9). While liver transplantation appears to be successful for the treatment of early-stage liver cancer patients, unfortunately only a small number of patients are candidates for this option. Again, potential of the surgical approach is severely limited due to acute organ shortage as well as frequent and rapid recurrence of the disease in the transplanted liver. There is only one drug (sorafenib) approved by the United States Food and Drug Administration for the treatment of unresectable HCC. Nevertheless, a recent report indicates severe adverse effects that include a significant risk of bleeding (10). Due to limited treatment options and grave prognosis of HCC, chemoprevention has been considered to be the best strategy in lowering the current morbidity and mortality associated with this disease (11,12). An insight into understanding the pathogenesis of HCC could hold the promise of finding an effective and novel strategy for the chemoprevention and treatment of liver cancer.

In recent years, compelling evidence has accumulated which provides an insight into the role of inflammation in initiation, promotion and progression of HCC. Hepatic inflammation, due to exposure to infectious agents including hepatotropic viruses as well as toxic compounds, may represent an early step in the development of malignancy with genetic and epigenetic events occurring as a later demonstration of a prolonged inflammatory process. Despite fundamental differences among etiological factors for HCC, a universal denominator of the genesis of malignancy happens to be the perpetuation of a wound-healing response triggered by parenchymal cell death as well as the ensuing inflammatory reaction (13). The process of chronic inflammation leads to a stressful condition, and ubiquitous molecules like heat shock proteins (HSPs) are induced in response to stressful stimuli, contributing to hepatocarcinogenesis (14). One of the prominent members of the HSP family is HSP70. Emerging evidence strongly supports the link between expression of HSP70 and the oncogenic potential of tumor cells, indicating HSP70 could be an attractive target for not only cancer therapy but also chemoprevention (15). Cyclooxygenase-2 (COX-2) catalyzes the production of inflammatory mediators prostaglandins (PGs) and is chronically overexpressed in chronic liver inflammation and cirrhosis as well as experimental and human HCC (reviewed in ref. (16)). Increasing evidence suggests that COX-2 signaling may play a key role in hepatocarcinogenesis (17,18), and blocking the COX-2 may prove effective in chemoprevention of HCC. One of the most ubiquitous eukaryotic transcription factors that regulate expression of genes involved in controlling cell proliferation, adhesion and inflammatory responses is nuclear factor-kappa B (NF-κB). Recently, a link between inflammation and cancer through the NF-κB pathway has been established (19). It has been shown that almost 90% of HCC cases have a natural history of unresolved inflammation and fibrosis or cirrhosis (20). Hence, the molecular regulator of HCC has been termed the "inflammation-fibrosis-cancer axis" (21), and NF-κB has been implicated as a potential "master orchestrator" of this axis (20). Accumulating evidence suggests that suppression of proinflammatory pathways regulated by NF-κB could lead to prevention and treatment of cancers linked to chronic inflammatory diseases (22).

Dietary agents, obtained from several fruits, vegetables, nuts and spices, have drawn tremendous attention due to their demonstrated ability to inhibit carcinogenesis in several animal models, with some of these substances able to partially prevent or delay cancer formation in several high-risk populations (23,24). A large number of bioactive food components have consistently been shown to modify molecular targets involved in inflammation and redox signaling (25,26), which are implicated in the development and progression of HCC. An impressive body of knowledge based on *in vitro* assays and experiments involving animal models support potential chemopreventive and chemotherapeutic effects of several phytochemicals in HCC (27). In this context, resveratrol, a naturally occurring antioxidant and antiinflammatory agent, has been considered as one of the promising molecules.

Resveratrol (3,4′,5-trihydroxy-*trans*-stilbene, $C_{14}H_{12}O_3$, molecular weight 228.2, Fig. 1) is a polyphenol found in

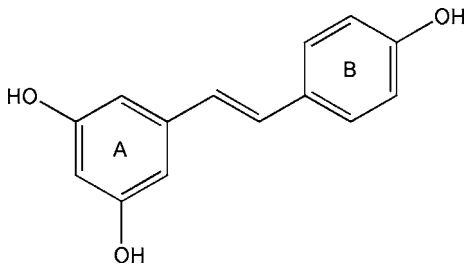

Fig. 1 Chemical structure of *trans*-resveratrol. **A**, ring A; **B**, ring B.

several dietary sources, such as grapes, berries, peanuts, and red wine. It is best known as the most likely dietary agent responsible for the French Paradox, a phenomenon in which consumption of red wine is linked to the reduced incidence of heart disease (28). An impressive number of experimental findings reveal multiple cellular targets of resveratrol affecting cellular proliferation and growth, apoptosis, inflammation, invasion, angiogenesis and metastasis (29,30). Resveratrol has been shown to suppress proliferation of a wide variety of human tumor cells *in vitro* (31,32), which has led to numerous preclinical animal studies to evaluate the cancer preventive and therapeutic potential of resveratrol (reviewed in ref. (33)). Although compelling evidence has shown that resveratrol exerts potent cytotoxic effects against various HCC cells *in vitro* and inhibits growth of transplanted hepatic tumor cells *in vivo* (reviewed in ref. (34)), the chemopreventive effects of resveratrol on liver cancer have not been systematically studied until recently.

Recently, we reported for the first time that resveratrol exerts chemopreventive effects against a two-stage rat model of hepatocarcinogenesis initiated with diethylnitrosamine (DENA) promoted by phenobarbital (PB) (35). According to this study, dietary resveratrol dose-dependently reduced the incidence, total number, and multiplicity of visible hepatocyte nodules, the precursors of HCC (36). Nevertheless, the mechanisms of resveratrol inhibition of hepatic tumorigenesis are not clearly understood. It is well known that resveratrol possesses strong anti-inflammatory properties (reviewed in ref. (37)), which might play an important role in protecting the liver against carcinogen-induced neoplasia. However, an experimental justification of this hypothesis is not found in current scientific literature. In view of the aforementioned caveat, the objective of the present study was to examine the hypothesis that anti-inflammatory mechanisms contribute to resveratrol-mediated chemoprevention of DENA-initiated rat liver carcinogenesis. The current study was designed to retrospectively measure several preneoplastic and inflammatory markers, namely HSP70, COX-2 and NF-κB, in livers from rats treated with DENA both in the presence as well as absence of dietary resveratrol. The liver tissues were obtained from our previously reported chemopreventive study in which rats exposed to dietary resveratrol (50–300 mg/kg) exhibited 22–60% hepatic tumor inhibition, respectively (35).

MATERIALS AND METHODS

Animals and Experimental Protocol

Liver samples for the present study were harvested from our previously reported chemopreventive study (35) following animal protocol approved by the Institutional Animal care and Use Committee of the Northeastern Ohio Universities Colleges of Medicine and Pharmacy. The animal protocol was based on the "Principles of Laboratory Animal Care" (NIH publication #85-23, revised in 1985). In short, 48 female Sprague-Dawley rats (Charles River Laboratory, Wilmington, MA), weighing between 65 and 85 g at the beginning of the study, were randomized into five groups of 6–13 animals each. While one group served as the vehicle control (normal group), hepatocarcinogenesis in all other four groups was induced by a single intraperitoneal (i.p.) injection of DENA (Sigma-Aldrich, St. Louis, MO) at a dose of 200 mg/kg, followed by promotion with PB (Sigma-Aldrich) at a concentration of 0.05% w/v in drinking water, which was started 2 weeks following the DENA injection. All rats had free access either to phytoestrogen-free pulverized standard food (LabDiet, St. Louis, MO) for normal as well as DENA control or the same food supplemented with various amounts of *trans*-resveratrol (~98% purity; Organic Herb, Inc., Changsha, Hunan, P.R. China), resulting in a daily dose of 50, 100 or 300 mg/kg body weight (for three resveratrol-treated groups). Resveratrol treatment was started 4 weeks before the initiation and continued for 20 successive weeks. At the termination of the study (20 weeks), the livers from all animals were perfused and subsequently excised under anesthesia. The livers were examined for morphological evaluation of visible hepatocyte nodules. The results on the incidence and size distribution of hepatic nodules have already been published (35). A portion of the non-nodular normal liver tissue (confirmed by histopathology) from various groups was collected and frozen immediately in liquid nitrogen and subsequently stored in a freezer at −80°C. Serial sections (~10-μm) of liver tissue were prepared from different groups and stored at −80°C freezer. These liver sections and liver tissues were used for immunohistochemical analysis and Western blotting, respectively, as described below.

Immunohistochemical Analysis

Immunohistochemical detection of HSP70, COX2, NF-κB p65 and inhibitor of κB (IκBα) in ~10-μm-thick liver sections was performed by standard immunohistochemical techniques. In short, the sections were hydrated in 1X phosphate-buffered saline (PBS) for 5 min followed by incubation for 10 min at 80°C in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval. The sections were cooled to room temperature for 20 min. All subsequent steps were carried out at room temperature. Following a 5-min wash with 1X PBS, the endogenous peroxidases were blocked by 1% hydrogen peroxide in PBS for 5 min. The sections were washed as before and blocked for 1 h in PBS containing 5% normal serum. The slides were then incubated overnight with primary antibodies at a dilution of 1:100 at 4°C in a humidified chamber. Rabbit polyclonal

anti-NF-κB p65, rabbit polyclonal anti-IκBα, goat polyclonal anti-COX2 antibody, mouse β-actin monoclonal antibody, and ABC staining systems were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse anti-HSP70 monoclonal antibody was the product of Stressgen (Victoria, Canada). After washing with PBS, the sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody (anti-rabbit and anti-mouse secondary antibodies from Invitrogen, Carlsbad, CA) at 1:200 dilution for 30 min at 37°C. The chromogenic reaction was developed with 3,3′-diaminobenzidine tetrahydrochloride solution. Negative control sections were processed simultaneously with the omission of the primary antibodies. All sections were dehydrated and mounted with cover slips and viewed under a light microscope. One-thousand hepatocytes from at least four separate fields were analyzed per animal, and four animals per group were used for immunohistochemical analysis. The results were expressed as percentage of positive cells.

Western Blot Analysis

Liver tissue samples were homogenized in ice-cold lysis buffer containing protease inhibitors and phenylmethylsulphonyl fluoride (PMSF). Homogenized liver tissue (10% w/v) was centrifuged at 14,000 *g* for 15 min at 4°C. The supernatant was used for quantification of HSP70 and COX-2. The pellet was subsequently used to prepare the nuclear protein extract for the NF-κB p65 assay. Nuclear proteins were isolated following the technique of Dignam *et al.* (38) with modifications. In short, the pellet was resuspended in ice-cold low-salt buffer (pH 7.9) containing 1.0 mM DTT, 0.2 mM EDTA, 25% (v/v) glycerol, 20 mM Hepes, 20 mM KCl, 1.5 mM $MgCl_2$ and 0.2 mM PMSF. The release of nuclear proteins was achieved by adding a high-salt buffer (pH 7.9) containing 1.0 mM DTT, 0.2 mM EDTA, 25% (v/v) glycerol, 20 mM Hepes, 1.2 M KCl, 1.5 mM $MgCl_2$ and 0.2 mM PMSF drop by drop to a final KCl concentration of 0.4 M (38). Following 30 min incubation on ice with smooth shaking, the soluble nuclear proteins were recovered by centrifugation at 25,000 *g* for 30 min at 4°C. The samples were stored at −80°C until assayed. Protein content was quantified using a Pierce BCA protein assay kit (Thermo Scientific, Rockford, IL). Approximately 60 μg protein sample was run on a 4–12% NuPage™ Bis-Tris gel (Invitrogen, Carlsbad, CA) at 200 V for 35 min. The samples were then transferred to Protran Whatman nitrocellulose membrane (Schleicher and Schuell, Dassal, Germany) at 20 V for 7 min on an iBlot™ Dry Blotting System (Invitrogen, Carlsbad, CA). The transferred protein was subjected to 1 h incubation in 2% blocking solution. A mouse anti-HSP70 monoclonal antibody (1:1000), goat anti-COX-2 polyclonal antibody (1:1000), or rabbit anti-NF-κB p65 polyclonal antibody (1:500) was then applied overnight at 4°C and followed by 1 h application of HRP-conjugated anti-mouse or anti-goat secondary antibody (1:1000). Each blot was then exposed to the ECL substrate (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and read on a Kodak Digital Science Image Station 440CF analyzer (NEN™ Life Science Products Inc, Boston, MA). The nitrocellulose membrane was then soaked in TBS with 1% Tween-20 overnight at 4°C followed by 1 h incubation in 2% blocking solution. A monoclonal anti-β-actin or anti-laminin antibody (1: 1000) was applied overnight at 4°C. This step was followed by 1 h application of HRP-conjugated secondary antibody (1:1000). Each blot was then developed and read on the Kodak analyzer.

Statistical Analysis

Data are presented as means ± SDs. One-way analysis of variance (ANOVA) was used to determine overall significance followed by post-hoc analysis using the Student-Neuman-Keul test. A probability level of 5% ($P<0.05$) was considered statistically significant. A commercially available software program (SigmaStat 3.1, Systat Software, Inc., San Jose, CA) was used for all statistical analyses.

RESULTS

Resveratrol Suppresses Hepatic HSP70 Induction During DENA-Mediated Hepatocarcinogenesis

HSP70 has an anti-apoptotic role and is found to be induced in response to a variety of stressful situations, including hepatocarcinogenesis. Our immunohistochemical results reveal no immunostaining or only faint expression of HSP70 in the livers of normal animals (Fig. 2A-a). On the other hand, a substantial increase in HSP70 immunoreactivity was noticed in both the nucleus as well as cytoplasm of the liver sections derived from DENA-initiated animals (Fig. 2A-b). Although resveratrol at 50 mg/kg did not modify HSP70 expression (figure not shown), a moderate reduction of immunoreactivity by 100 mg/kg (Fig. 2A-c) and a maximum inhibition by 300 mg/kg resveratrol (Fig. 2A-d) were observed. Fig. 2B shows percentage of hepatic HSP70-positive cells in various groups of animals. There was a significant ($P<0.001$) increase in HSP70-positive cells in DENA control group. Resveratrol treatment significantly attenuated the hepatic HSP70 protein expression in animals challenged with DENA at a dose of 100 mg/kg ($P<0.01$) or 300 mg/kg ($P<0.001$), respectively. To confirm our immunohistochemical results on HSP70, livers from various groups were subjected to Western blot

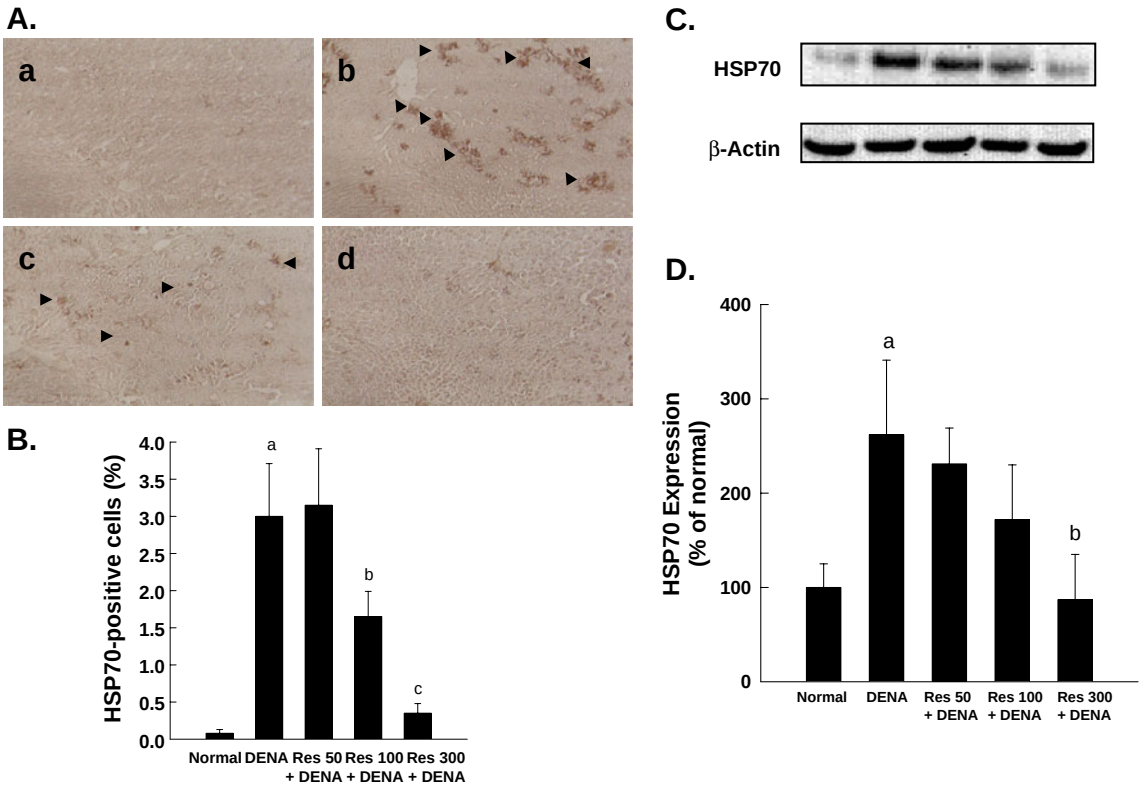

Fig. 2 Effects of resveratrol on hepatic HSP70 expression during DENA-initiated hepatocarcinogenesis in female Sprague-Dawley rats. (**A**) Representative immunohistochemical localization of HSP70 (magnification: 100x). Rats were sacrificed 20 weeks following the commencement of the study and immunohistochemistry was performed to detect HSP70. *Arrows* indicate immunohistochemical staining of HSP70. Near absence of immunopositivity in normal liver (*a*), intense immunoreactivity in DENA control liver (*b*), decreased HSP70 expression in 100 mg/kg resveratrol group (*c*), and near absence of HSP70 expression in 300 mg/kg resveratrol group (*d*) are noticed. (**B**) Quantification of HSP70-positive cells in rat livers of several experimental groups. One-thousand hepatocytes were counted per animal, and the results were based on 4 animals per group. Each *bar* represents the mean ± SD ($n = 4$ livers). $^{a}P < 0.001$ as compared to normal group; $^{b}P < 0.01$ and $^{c}P < 0.001$ as compared to DENA control. (**C**) Representative Western blot and (**D**) related densitometric analysis of hepatic HSP70 expression in various groups of rats. Total cellular protein was separated and blotted with anti-HSP70 antibody. Each *bar* represents the mean ± SD ($n = 4$–5). $^{a}P < 0.01$ as compared to normal group; $^{b}P < 0.01$ as compared to DENA control.

analysis. As depicted in Fig. 2C and D, there was a significant ($P<0.01$) up-regulation of HSP70 in DENA control animals compared to normal group. Again, DENA-induced overexpression of HSP70 was suppressed in rats fed resveratrol-supplemented diet. However, a statistically significant ($P<0.01$) result was obtained with the highest dose of resveratrol (300 mg/kg). Another interesting finding stemming from this study is that there is no difference in the levels of HSP70 protein expression between DENA-treated rats in the presence of resveratrol at 300 mg/kg compared to that of normal rats. Taken together, all these results indicate that resveratrol is able to completely reverse the high levels of HSP70 expression during DENA-initiated hepatocarcinogenesis in rats.

Resveratrol Inhibits Elevated Hepatic COX-2 Expression During DENA Hepatocarcinogenesis

As chronic inflammation plays a vital role in hepatocarcinogenesis, the ability of resveratrol to suppress the induction of inflammatory marker COX-2 has been investigated in the present study. Accordingly, we have determined the immunostaining for the COX-2 protein in the hepatic sections of rats injected with DENA and maintained on normal or resveratrol-supplemented diet. As Fig. 3A-a shows, a near absence of COX-2-positive hepatocytes was noticed in normal rat liver. On the contrary, a dramatic increase in hepatic COX2 expression was observed in both perinuclear region as well as cytoplasm of hepatocytes from DENA control animals (Fig. 3A-b). Hepatic Kupffer, sinusoidal as well as endothelial cells were found to be immunopositive for COX-2. Resveratrol at a dose of 50 mg/kg did not alter the extent of COX-2 immunopositivity compared to DENA alone (data not shown). On the other hand, a moderate and drastic suppression of COX-2 expression was noticed following resveratrol treatment at a dose of 100 mg/kg (Fig. 3A-c) as well as 300 mg/kg (Fig. 3A-d), respectively. Fig. 3B depicts percentage of COX-2-positive hepatocytes in all experimental groups. A 15-fold increase ($P<0.001$) in the percentage of COX-2-

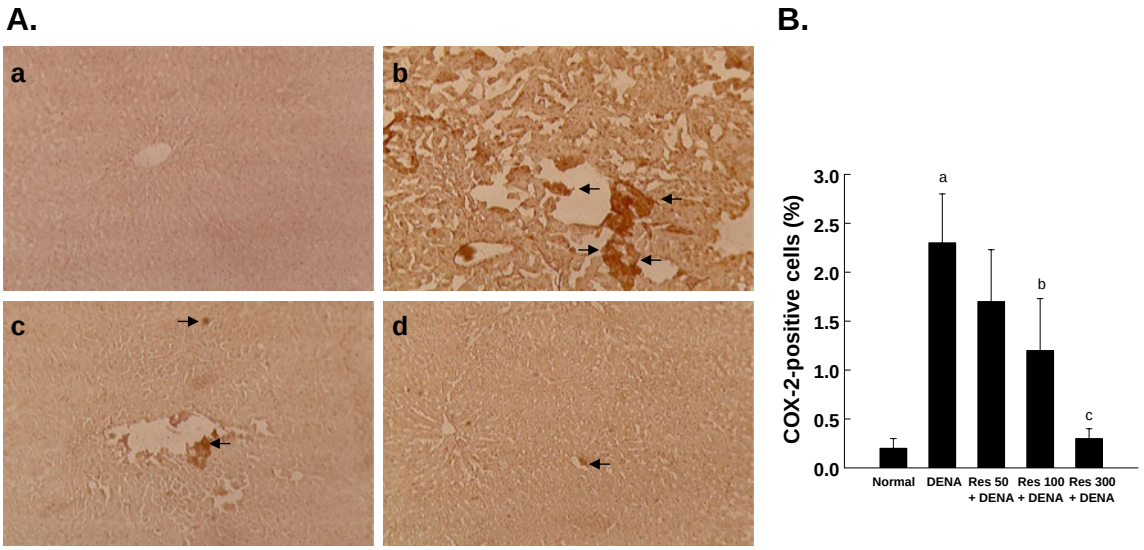

Fig. 3 Effects of resveratrol on hepatic COX-2 expression during DENA-induced hepatocarcinogenesis in female Sprague-Dawley rats. (**A**) Immunohistochemical localization of COX-2 (magnification: 100x). Rats were sacrificed and immunohistochemistry was performed 20 weeks following the commencement of the study to detect COX-2 immunopositivity. *Arrows* indicate immunohistochemical staining of COX-2. Absence of COX-2-containing protein in normal liver (*a*), intense COX-2 immunoreactivity in DENA control liver (*b*), decreased COX-2 expression in 100 mg/kg resveratrol group (*c*), and very limited expression of COX-2 in 300 mg/kg resveratrol group (*d*) are observed. (**B**) Quantification of COX-2-positive cells in rat livers of different experimental groups. One-thousand hepatocytes were counted per animal, and the results were based on 4 animals per group. Each *bar* represents the mean ± SD ($n = 4$). $^{a}P < 0.001$ as compared to normal group; $^{b}P < 0.01$ and $^{c}P < 0.001$ as compared to DENA control.

positive cells in DENA-treated rats compared to their normal counterparts was noticed. There was a significant inhibition of percentage of COX-2-positive cells in rats fed with either 100 ($P < 0.01$) or 300 mg/kg ($P < 0.001$) resveratrol compared to DENA control group. These results have been confirmed and validated by Western blot analysis. As depicted in Fig. 4A and B, a significantly ($P < 0.001$) elevated hepatic expression of COX-2 was observed in DENA control group compared to normal. Interestingly, resveratrol treatment registered a decline in COX-2 expression in all DENA-initiated animals as compared to DENA alone. Nevertheless, a statistically significant ($P < 0.001$) result was obtained in the group that received 100 or 300 mg/kg resveratrol.

Resveratrol Attenuates NF-κB Activation During DENA-Initiated Rat Liver Carcinogenesis

To elucidate the mechanism by which resveratrol modulates the *COX-2* gene expression, we investigated the influence of resveratrol on the activation of transcription factor NF-κB that regulates COX-2. Our immunostaining data show either absence or minimal expression of NF-κB p65 protein in nuclei of normal rat livers (Fig. 5-a), whereas a substantial amount of immunoreactivity was noticed in the cytoplasm (data not shown). A high expression of NF-κB p65 in nucleus (Fig. 5-b) and low expression of the same in cytoplasm was noticed in the livers of DENA-initiated animals, indicating activation and subsequent translocation

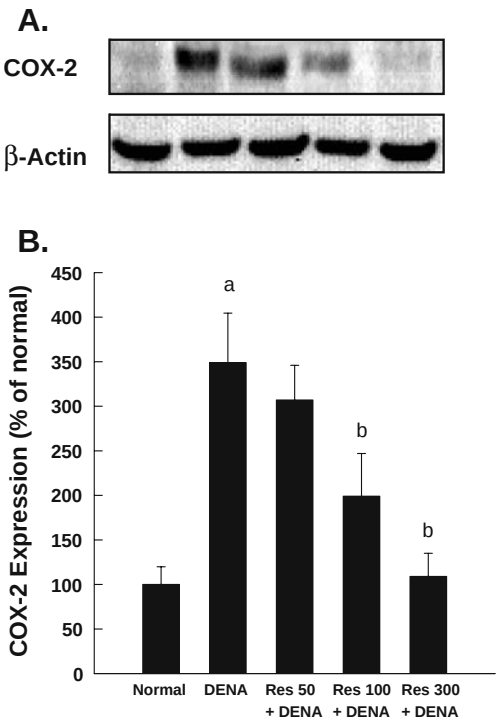

Fig. 4 Hepatic COX-2 protein expression in rats subjected to DENA hepatocarcinogenesis with or without resveratrol treatment. (**A**) Representative Western blot and (**B**) related densitometric analysis of hepatic COX-2 expression in various groups of rats. Total cellular protein was separated and blotted with anti-COX-2 antibody. Each *bar* represents the mean ± SD ($n = 4$). $^{a}P < 0.001$ as compared to normal group; $^{b}P < 0.001$ as compared to DENA control.

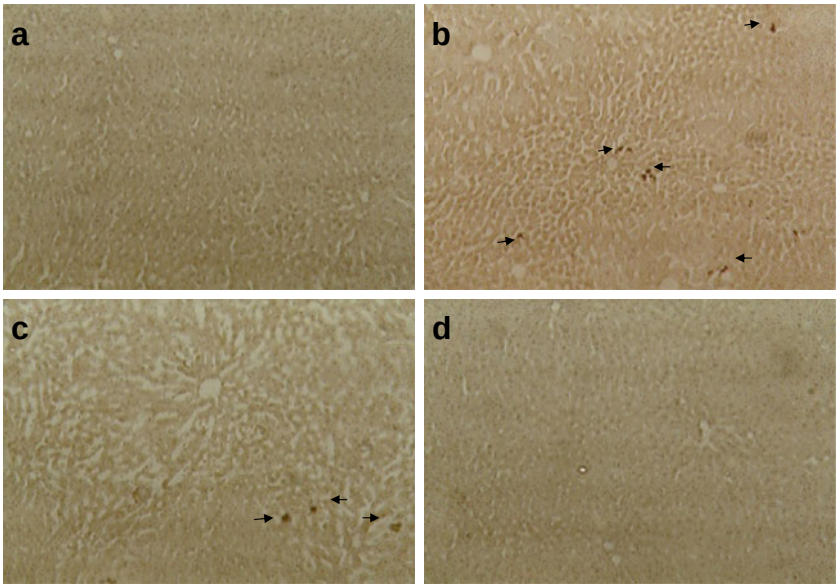

Fig. 5 Effects of resveratrol on hepatic NF-κB p65 expression during DENA-evoked hepatic neoplasia in female Sprague-Dawley rats. Representative immunohistochemical localization of NF-κB p65 in nucleus (magnification: 100x). Rats were sacrificed 20 weeks following the commencement of the study, and immunohistochemistry was performed to detect NF-κB p65. *Arrows* indicate immunohistochemical staining. Different experimental groups are (**a**) normal control; (**b**) DENA control; (**c**) resveratrol 100 mg/kg + DENA and (**d**) resveratrol 300 mg/kg + DENA.

of NF-κB p65 from the cytosol to the nucleus. Interestingly, upon treatment with resveratrol (100 or 300 mg/kg), the NF-κB p65 protein expressions were reversed compared to DENA control (Fig. 5-c,d). As depicted in Fig. 7, resveratrol at 300 mg/kg elicited a 12-fold ($P<0.001$) decrease in NF-κB p65 protein expression in the nucleus and nearly 9-fold ($P<0.001$) increase in the same subunit in the cytoplasm compared to DENA control. Resveratrol also suppressed DENA-induced degradation of IκBα protein in cytosol in a dose-responsive manner (Fig. 6-c,d), which may explain its observed effects on NF-κB p65. However, a statistically significant ($P<0.01$) result was obtained with the highest dose of resveratrol (i.e., 300 mg/kg) (Fig. 7). Data obtained by Western blot analysis further supports and confirms the

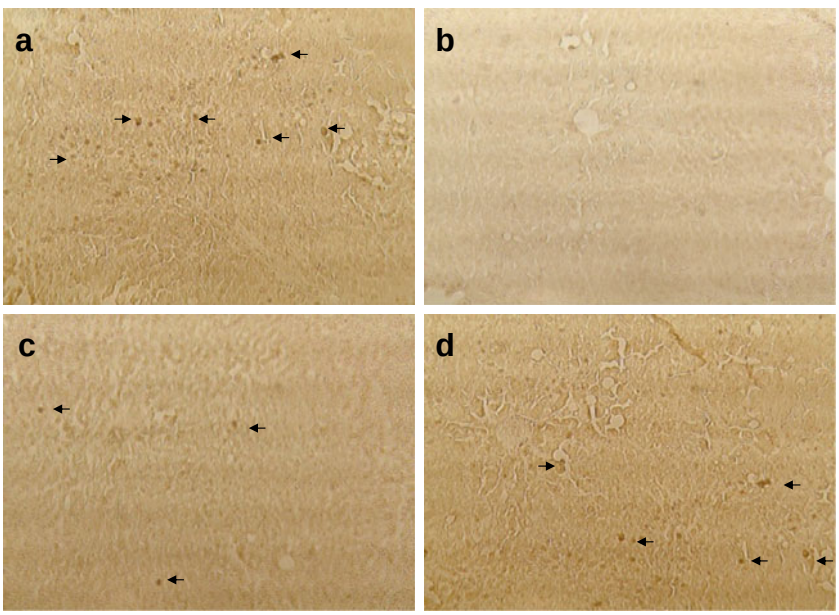

Fig. 6 Effects of resveratrol on hepatic IκBα expression during DENA-evoked hepatic neoplasia in female Sprague-Dawley rats. Representative immunohistochemical localization of IκBα in cytosol (magnification: 100x). Rats were sacrificed 20 weeks following the commencement of the study, and immunohistochemistry was performed to detect IκBα. *Arrows* indicate immunohistochemical staining. Different experimental groups are: (**a**) normal control; (**b**) DENA control; (**c**) resveratrol 100 mg/kg + DENA and (**d**) resveratrol 300 mg/kg + DENA.

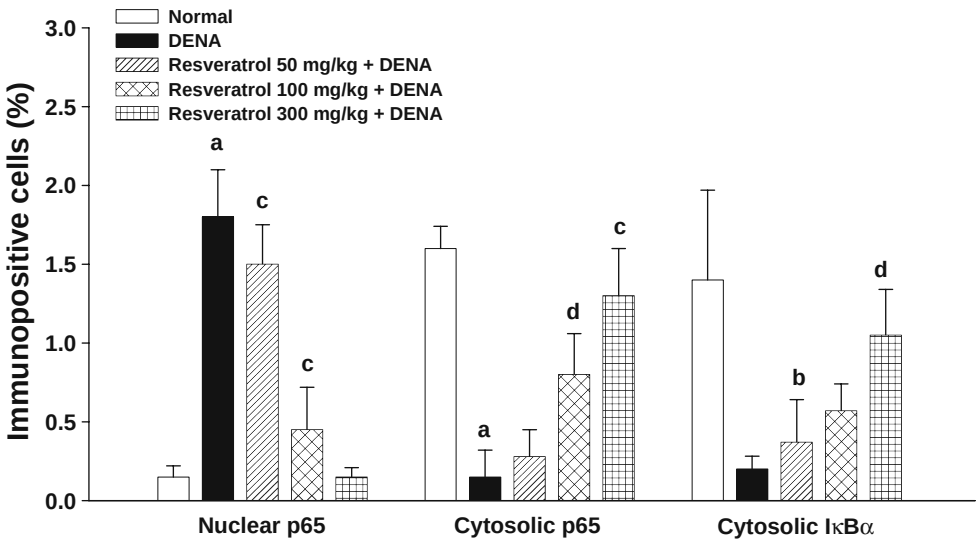

Fig. 7 Quantification of nuclear and cytosolic NF-κB p65- and cytosolic IκBα-immunopositive cells in rat livers of several experimental groups. One-thousand hepatocytes were counted per animal, and the results were based on 4 animals per group. Each *bar* represents the mean ± SD ($n = 4$). $^{a}P < 0.001$ and $^{b}P < 0.01$ as compared to normal group; $^{c}P < 0.001$ and $^{d}P < 0.01$ as compared to DENA control.

results with nuclear NF-κB p65 protein expression. A substantial elevation of NF-κB p65 subunit was observed in the nuclear protein extract from DENA control livers (Fig. 8A). There was a considerable decrease in this subunit in 50 mg/kg resveratrol group and near absence of the same in 100 or 300 mg/kg group. Accompanying densitometric analysis revealed a drastic elevation ($P < 0.001$) of NF-κB p65 in DENA control, which was significantly ($P < 0.001$) reduced in all resveratrol-treated animals (Fig. 8B).

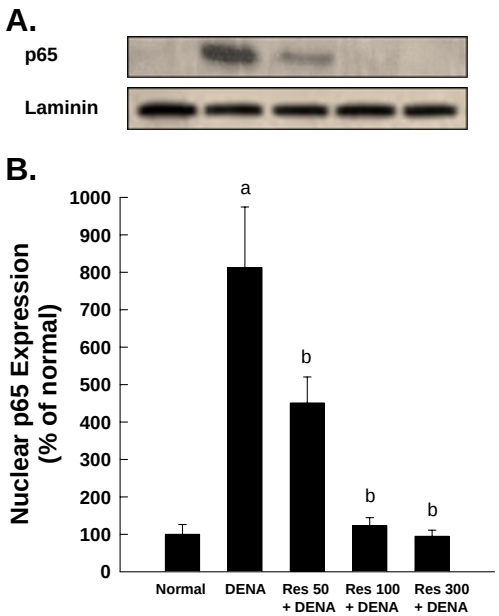

Fig. 8 Effects of resveratrol on hepatic nuclear NF-κB p65 expression in DENA-initiated hepatocarcinogenesis in female Sprague-Dawley rats. (**A**) Representative Western blot and (**B**) related densitometric analysis of NF-κB p65 expression in various groups of rats. Nuclear protein was separated and blotted with anti-p65 antibody. Each *bar* represents the mean ± SD ($n = 4$). $^{a}P < 0.001$ as compared to normal group; $^{b}P < 0.001$ as compared to DENA control.

These results indicate that resveratrol was able to inhibit the nuclear translocation of NF-κB p65 subunit.

DISCUSSION

Resveratrol, a naturally occurring polyphenol present in grapes and red wine, has been shown to possess a potent growth-inhibitory effect against various human cancer cells *in vitro* and chemopreventive effects in breast, colon, esophagus, lung and skin carcinogenesis *in vivo* (31-33). Nevertheless, mechanism-based chemopreventive actions of this dietary antioxidant and anti-inflammatory agent have not been investigated in detail against inflammation-associated hepatic malignancy. Recently, we reported the novel finding that dietary resveratrol significantly prevents DENA-initiated liver tumorigenesis in female Sprague-Dawley rats (35). In this study, long-term feeding of rats with resveratrol-supplemented diet dose-dependently inhibited the number, multiplicity and size distribution of hepatocyte nodules, the principal endpoint for evaluating the chemopreventive potential of a candidate agent. Nevertheless, the underlying mechanism(s) by which resveratrol exerts a chemopreventive action against experimental hepatocarcinogenesis remains to be clarified. Based upon our preliminary data and other studies, we hypothesize that anti-inflammatory mechanisms may contribute to resveratrol-mediated liver cancer chemoprevention. The results of the present study convincingly demonstrate that continuous dietary exposure of resveratrol to rats challenged with a potent hepatocarcinogen DENA combat DENA-initiated inflammatory response by reversing hepatic stress and inflammatory indices, including HSP70, COX-2 as well as NF-κB signaling.

The members of the HSP family are a group of highly conserved and functionally interactive chaperone proteins

with an important defense mechanism for protecting cells against diverse physiological damages inflicted by various adverse stimuli, including heat shock, hypoxia, reactive oxygen species (ROS) and viral transformations (39). HSPs have been classified in at least 6 major families according to their approximate molecular size, namely HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs including HSP27 (14). HSPs are known to regulate diverse cellular functions, including apoptosis, and hence can be broadly categorized into two groups: antiapoptotic and proapoptotic. HSP70 is an antiapoptotic protein, and its overexpression allows cells to survive under various adverse conditions, including carcinogenesis (40). Overexpression of HSP70 has been observed in human malignant tumors of the prostate, breast, lung, oral cavity as well as the uterus (14). The expression of HSP70 has been found to be up-regulated in HCC, and its expression pattern has been significantly associated with stepwise progression of HBV- and HCV-related hepatocarcinogenesis (41,42). HSP70 expression contributes to not only hepatocarcinogenesis but also tumor progression by promoting tumor cell proliferation in HCC (14). It has also been demonstrated that HSP70 could be a sensitive marker for the differential diagnosis of early HCC from preneoplastic lesions or non-cancerous liver tissue (43). It has been shown that DENA alone or in combination with heat shock induces HSP70 gene transcription in the liver of Fisher rats (44,45). Additionally, c-Met conditional knockout mice (more susceptible to DENA-induced hepatic tumorigenesis) exhibited elevated levels of HSP70 protein in the liver (46). In the present study, we have utilized both immunohistochemical as well as Western blot techniques to show an elevated level of hepatic HSP70 expression in DENA-treated animals, supporting all previous findings. A significantly elevated level of hepatic HSP70 in DENA control animals at the end of 20 weeks as observed in this study could be associated with antiapoptotic property of initiated hepatocytes and subsequent promotion of hepatic tumorigenesis as observed in our previous study (35). On the other hand, animals exposed to continuous dietary resveratrol suppressed DENA-induced overexpression of HSP70, indicating amelioration of carcinogenic stress. This observation confirms and extends our earlier finding that resveratrol suppresses cell proliferation with a concomitant induction of apoptosis to reverse DENA-induced hepatic neoplasia (35). It is plausible that resveratrol induces apoptosis in DENA-exposed rat liver to remove damaged hepatocytes by this process, which may eliminate hepatic stress that would otherwise trigger an induction of HSP70. It has been found that resveratrol induces apoptosis in human prostate cancer and leukemia cells by interfering with the expression of HSP70 (47,48). A recent study has shown that resveratrol supplementation inhibits chemically induced colon carcinogenesis in rats by limiting the expression of a number of apoptotic biomarkers, including HSP70 (49).

COX enzymes catalyze the synthesis of PGs, including PGE_2, from arachidonic acid. At least two isoforms of COX, namely COX-1 and COX-2, have been identified that have approximately 60% similarity in amino acids (50). COX-1 is constitutively expressed with homeostatic roles in various tissues. On the other hand, COX-2 is an inducible isoenzyme which has been found to be locally induced by proinflammatory mitogens, cytokines and growth factors during inflammation and carcinogenesis (51). COX-2 has been associated with cell proliferation, differentiation, apoptosis, angiogenesis, invasiveness and metastasis—all of which are involved in multi-stage tumorigenesis (52). It is now well established that COX-2 is chronically overexpressed in chronic liver inflammation and cirrhosis as well as experimental and human HCC (reviewed in ref. (16)). Overexpression of COX-2 in patients with HCC has been found to be higher in well-differentiated HCC compared to less-differentiated HCC or a histologically normal liver, suggesting the involvement of COX-2 in the early stages of hepatocarcinogenesis (17,53). Other investigators have also provided evidence that COX-2 expressions are independent of tumor mass and tumor stage, and the COX-2 system appears to be active both in the early as well as the late stages of hepatocarcinogenesis (18,54). Increasing evidence suggests that COX-2 signaling may play a key role in hepatocarcinogenesis. Consequently, blocking the COX-2-mediated PG pathway may prove effective in chemoprevention of HCC (55,56). In our study, a dramatic increase in the number of hepatic COX-2-expressing cells 20 weeks following DENA treatment indicates a state of severe hepatic inflammation. Previous studies have shown elevated expression and activity of COX-2 in the DENA model of hepatocarcinogenesis (57,58). It is noteworthy that we have noticed a similar upregulation of other inflammatory markers, including inducible nitric oxide synthase (iNOS) and 3-nitrotyrosine in the liver of DENA control animals (59). The present results also show that treatment with resveratrol dose-dependently decreased COX-2 expression, suggesting involvement of anti-inflammatory mechanisms. This finding is in concordance with a previous study that shows resveratrol suppresses COX activity in the livers of mice treated with DENA (60). Overexpression of antiapoptotic Bcl-2 has been associated with elevated COX-2 expression (61), and an upregulation of Bcl-2 in the livers of rats treated with DENA has been previously reported from our laboratory (35). We have also noticed an inhibitory effect of resveratrol on Bcl-2 under the same experimental conditions (35) which support and strengthen the present findings of resveratrol-mediated suppression of elevated COX-2 expression during hepatocarcinogenesis. The present results along with our previous report (35) are

the first, to our knowledge, to provide direct evidence that COX-2 is a potential target for chemopreventive action of resveratrol against DENA hepatocarcinogenesis. Recently, it has been shown that resveratrol binds with COX-2 directly to inhibit its enzyme activity, suppresses COX-2-mediated PGE_2 production and exerts antitumor effects on human colon adenocarcinoma cells *in vitro* and *ex vivo* (62). According to prior *in vivo* studies, tumor-inhibitory effects of resveratrol in breast, esophagus, intestine and skin have been associated with its ability to inhibit COX-2 (reviewed in ref. (33)). Although several COX-2 inhibitors are known to exert chemopreventive effects, not all of these agents are considered ideal candidates for chemoprevention due to the risk of adverse cardiovascular events (63). Accordingly, we have evaluated the effects of resveratrol on cardiac performance using transthoracic echocardiography during experimental hepatocarcinogenesis induced by DENA. Our results clearly demonstrate that chemopreventive doses of resveratrol do not exhibit any cardiotoxicity, but rather improve the cardiac function (64).

In order to better understand resveratrol-mediated inhibition of COX-2 expression during DENA-induced rat liver carcinogenesis, we have studied the expression of NF-κB, which is known to regulate COX-2. NF-κB, a eukaryotic transcription factor family, consists of dimers of the following proteins: NF-κB1 (p50 and p105), RelA (p65), NF-κB2 (p52 and p100), c-Rel, and RelB. It is normally found in the cytoplasm as an inactive dimer bound to an inhibitory subunit, IκB, which also has several family members, including IκBα, IκBβ, IκBγ, and IκBε (65). There are two main NF-κB pathways: the classical pathway, in which the p50:p65 heterodimer is predominate, and an alternative (non-classical) pathway, in which the p52:RelB dimer is activated (65). Activation of NF-κB leads to phosphorylation, ubiquitination and, subsequently, proteasome-mediated degradation of IκB. Following activation, NF-κB is then released from IκB and translocates to the nucleus, where it induces the transcription of specific genes including *COX-2* and *iNOS*, which are implicated in hepatocarcinogenesis (16,66,67). It has been confirmed that both COX-2 and iNOS promoter have binding sites for NF-κB (68,69). In this study, the expressions of p65 subunit of NF-κB in hepatic nuclear and cytosolic compartments and the major IκB protein IκBα in cytosol have been measured. We have found that DENA elicited the degradation of IκBα with a simultaneous translocation of p65 from cytoplasm to nucleus. It has been previously demonstrated that DENA-induced inflammation triggers ROS production, which activates NF-κB followed by release of pro-inflammatory cytokines, chemokines and iNOS—all these events leads to hepatocarcinogenesis (70). In the present study, we have also found that resveratrol has the ability to decrease the degradation of IκB and the nuclear translocation of p65, indicating that this dietary agent can suppress DENA-mediated NF-κB activation. In addition to inhibiting COX-2 as observed in the current study, resveratrol has also been previously found to suppress iNOS expression during DENA hepatocarcinogenesis (59). Studies from other laboratories show that resveratrol inhibits COX-2 and iNOS through its inhibitory effects on NF-κB both *in vitro* and *in vivo* (71-73). Resveratrol is also known to block experimental liver fibrosis in rats by preventing the translocation of NF-κB to the nucleus with down-regulation of profibrotic cytokine tumor growth factor-β (74). Collectively, all these studies indicate that resveratrol could counteract the early inflammatory cascades during hepatocarcinogenesis by inhibiting COX-2 and iNOS via modulation of NF-κB, and the anti-inflammatory action could represent, at least in part, the molecular mechanism of resveratrol chemoprevention of HCC. The precise mechanisms of resveratrol-mediated inhibition of NF-κB in our animal model are not completely understood at the present moment. Previous studies have provided evidence that resveratrol inhibits NF-κB by inhibiting IκB kinase, resulting in attenuation of NF-κB translocation into the nucleus (75) or via its sirtuin activity, which is known to deacetylate NF-κB (76). Confirmation of these possibilities in our experimental conditions requires further studies, which are being carried out in our laboratory.

In summary, the results of the present investigation clearly demonstrate that resveratrol suppresses the inflammatory cascade during rat liver carcinogenesis initiated with DENA by inhibiting overexpression of HSP70 and COX-2 as well as blocking nuclear translocation of NF-κB. Resveratrol has previously been found to exert striking chemopreventive effects against DENA-initiated rat liver tumorigenesis (35). It is possible that resveratrol prevents DENA hepatocarcinogenesis by inhibiting translocation of NF-κB from cytosol to nucleus, NF-κB-DNA binding and eventual trans-activation of genes regulated by NF-κB. The present findings underscore the possibility of targeting these events as a novel strategy in the prevention and treatment of HCC by dietary agent resveratrol. These attributes combined with an excellent safety profile encourage the development of resveratrol for chemoprevention and intervention of human HCC that remains a devastating disease.

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REFERENCES

1. Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. CA Cancer J Clin. 2005;55:74–105.
2. Sherman M. Hepatocellular carcinoma: epidemiology, risk factors, and screening. Semin Liver Dis. 2005;25:143–54.
3. El-Serag HB. Hepatocellular carcinoma: recent trends in the United States. Gastroenterology. 2004;127:S27–34.
4. American Cancer Society. Cancer Facts and Figures 2009. Atlanta, GA, 2009.
5. Wu L, Tang Z-Y, Li Y. Experimental models of hepatocellular carcinoma: developments and evolution. J Cancer Res Clin Oncol. 2009;135:969–81.
6. Schütte K, Bornschein J, Malfertheiner P. Hepatocellular carcinoma—epidemiological trends and risk factors. Dig Dis. 2009;27:80–92.
7. Bartsch H, Montesano R. Relevance of nitrosamines to human cancer. Carcinogenesis. 1984;5:1381–93.
8. Kensler TW, Egner PA, Wang JB, Zhu YR, Zhang BC, Lu PX *et al.* Chemoprevention of hepatocellular carcinoma in aflatoxin endemic areas. Gastroenterology. 2004;127:S310–8.
9. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. Lancet. 2003;362:1907–17.
10. Je Y, Schutz FAB, Choueiri TK. Risk of bleeding with vascular endothelial growth factor receptor tyrosine-kinase inhibitors sunitinib and sorafenib: a systematic review and mata-analysis of clinical trials. Lancet Oncol. 2009;10:967–74.
11. Okuno M, Kojima S, Moriwaki H. Chemoprevention of hepatocellular carcinoma: concept, progress and perspectives. J Gastroenterol Hepatol. 2001;16:1329–35.
12. Yates MS, Kensler TW. Keap1 eye on the target: chemoprevention of liver cancer. Acta Pharmacol Sin. 2007;28:1331–42.
13. Berasain C, Casillo J, Perugorria MJ, Latasa MU, Prieto J, Avila MA. Inflammation and liver cancer: new molecular links. Ann NY Acad Sci. 2009;1155:206–21.
14. Joo M, Chi JG, Hyucksang L. Expressions of HSP70 and HSP27 in hepatocellular carcinoma. J Korean Med Sci. 2005;20:829–34.
15. Murakami A, Ashida H, Terao J. Multitargeted cancer prevention by quercetin. Cancer Lett. 2008;269:315–25.
16. Wu T. Cyclooxygenase-2 in hepatocellular carcinoma. Cancer Treat Rev. 2006;32:28–44.
17. Giannitrapani L, Ingrao S, Soresi M, Maria Florena A, La Spada E, Sandonato L *et al.* Cyclooxygenase-2 expression in chronic liver diseases and hepatocellular carcinoma. Ann NY Acad Sci. 2009;1155:293–9.
18. Yildirim Y, Ozyilkan O, Bilezikci B, Akcali Z, Haberal M. Lack of influence of cyclooxygenase-2 expression in hepatocellular carcinomas on patient survival. Asian Pac J Cancer Prev. 2008;9:295–8.
19. Karin M. The IκB kinase-a bridge between inflammation and cancer. Cell Res. 2008;18:334–432.
20. Muriel P. NF-κB in liver diseases: a target for drug therapy. J Appl Toxicol. 2009;29:91–100.
21. Elsharkawy AM, Mann DA. Nuclear factor-κB and the hepatic inflammation-fibrosis-cancer axis. Hepatology. 2007;46:590–7.
22. Aggarwal BB, Vijayalekshmi RV, Sung B. Targeting inflammatory pathways for prevention and therapy of cancer: short-term friend, long-term foe. Clin Cancer Res. 2009;15:425–30.
23. Aggarwal BB, Shishodia S. Molecular targets of dietary agents for prevention and therapy of cancer. Biochem Pharmacol. 2006;71:1397–421.
24. Naithani R, Huma LC, Moriarty RM, McCormick DL, Mehta RG. Comprehensive review of cancer chemopreventive agents evaluated in experimental carcinogenesis models and clinical trials. Curr Med Chem. 2008;15:1044–71.
25. Rahman I, Biswas SK, Kirkham PA. Regulation of inflammation and redox signaling by dietary polyphenols. Biochem Pharmacol. 2005;72:1439–52.
26. Kim YS, Young MR, Bobe G, Colbum NH, Milner JA. Bioactive food components, inflammatory targets, and cancer prevention. Cancer Prev Res. 2009;2:200–8.
27. Mann CD, Neal CP, Garcea G, Manson MM, Dennison AR, Berry DP. Phytochemicals as potential chemopreventive and chemotherapeutic agents in hepatocarcinogenesis. Eur J Cancer Prev. 2009;18:13–25.
28. Vidavalur R, Otani H, Singal PK, Maulik N. Significance of wine and resveratrol in cardiovascular disease: French paradox revisited. Exp Clin Cardiol. 2006;11:217–25.
29. Shakibaei M, Harikumar KB, Aggarwal BB. Resveratrol addiction: to die or not to die. Mol Nutr Food Res. 2009;53:115–28.
30. Athar M, Back JH, Kopelovich L, Bickers DR, Kim AL. Multiple molecular targets of resveratrol: anti-carcinogenic mechanisms. Arch Biochem Biophys. 2009;486:95–102.
31. Aggarwal BB, Bhardwaj A, Aggarwal RS, Seeram NP, Shishodia S, Takada Y. Role of resveratrol in prevention and therapy of cancer: preclinical and clinical studies. Anticancer Res. 2004;24:2783–840.
32. Kundu JK, Surh Y-J. Cancer chemopreventive and therapeutic potential of resveratrol: mechanistic perspectives. Cancer Lett. 2008;269:243–61.
33. Bishayee A. Cancer prevention and treatment with resveratrol: from rodent studies to clinical trials. Cancer Prev Res. 2009;2:409–18.
34. Bishayee A, Politis T, Darvesh AS. Resveratrol in the chemoprevention and treatment of hepatocellular carcinoma. Cancer Treat Rev. 2010;36:43–53.
35. Bishayee A, Dhir N. Resveratrol-mediated chemoprevention of diethylnitrosamine-initiated hepatocarcinogenesis: inhibition of cell proliferation and induction of apoptosis. Chem-Biol Interact. 2009;179:131–44.
36. Farber E, Sarma DS. Hepatocarcinogenesis: a dynamic cellular perspective. Lab Invest. 1987;56:4–22.
37. Das S, Das DK. Anti-inflammatory responses of resveratrol. Inflamm Allergy Drug Target. 2007;6:168–73.
38. Dignam JD, Lebovitz RM, Roeder RG. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acid Res. 1983;11:1475–89.
39. Morimoto RI. Cells in stress: transcriptional activation of heat shock genes. Science. 1993;259:1409–10.
40. Jolly C, Morimoto RI. Role of the heat shock response and molecular chaperones in oncogenesis and cell death. J Natl Cancer Inst. 2000;92:1564–72.
41. Lim SO, Park SG, Yoo J-H, Park YM, Kim H-J, Jang K-T *et al.* Expression of heat shock proteins (HSP27, HSP60, HSP70, HSP90, GRP78, GRP94) in hepatitis B virus-related hepatocellular carcinomas and dysplastic nodules. World J Gastroenterol. 2005;11:2072–9.
42. Yoshida S, Hazama S, Tokuno K, Sakamoto K, Takashima M, Tamesa T *et al.* Concomitant overexpression of heat-shock protein 70 and HLA class-I in hepatitis C virus-related hepatocellular carcinoma. Anticancer Res. 2009;29:539–44.
43. Chuma M, Sakamoto M, Yamazaki K, Ohta T, Ohki M, Hirohashi S. Expression profiling in multistage hepatocarcinogenesis: identification of HSP70 as a molecular marker of early hepatocellular carcinoma. Hepatology. 2003;37:198–207.
44. Carr BI, Huang TH, Buzin CH, Itakura K. Induction of heat shock gene expression without heat shock by hepatocarcinogenesis. Cancer Res. 1986;46:5106–11.
45. Tacchini L, Schiaffonati L, Rappocciolo E, Cairo G, Bernelli-Zazzera A. Expression pattern of the genes for the different members of the heat-shock protein 70 family, ornithine decar-

boxylase, and c-Ha-*ras* during the early stages of hepatocarcinogenesis. Mol Carcinog. 1989;2:233–6.
46. Takami T, Kaposi-Novak P, Uchida K, Gomez-Quiroz LE, Conner EA, Factor VM *et al.* Loss of hepatocyte growth factor/c-Met signaling pathway accelerates early stages of *N*–nitrosodiethylamine-induced hepatocarcinogenesis. Cancer Res. 2007;67:9844–51.
47. Cardile V, Scifo C, Russo A, Falsaperla M, Morgia G, Motta M *et al.* Involvement of HSP70 in resveratrol-induced apoptosis of human prostate cancer. Anticancer Res. 2003;23:4921–6.
48. Chakraborty PK, Mustafi SB, Ganguly S, Chatterjee M, Raha S. Resveratrol induces apoptosis in K562 (chronic myelogenous leukemia) cells by targeting a key survival protein, heat shock protein 70. Cancer Sci. 2008;99:1109–16.
49. Sengottuvelan M, Deeptha K, Nalini N. Influences of dietary resveratrol on early and late molecular markers of 1, 2-dimethylhydrazine-induced colon carcinogenesis. Nutrition. 2009;25:1169–76.
50. Hla T, Nielson K. Human cyclooxygenase-2 cDNA. Proc Natl Acad Sci USA. 1992;89:7384–8.
51. Fosselin E. Biochemistry of cyclooxygenase (COX)-2 inhibitors and molecular pathology of COX-2 in neoplasia. Crit Rev Clin Lab Sci. 2000;37:431–502.
52. Prescott SM, Fitzpatrick FA. Cyclooxygenase-2 and carcinogenesis. Biochim Biophys Acta. 2000;1470:M69–78.
53. Koga H, Sakisaka S, Ohishi M, Kawaguchi T, Taniguchi E, Sasatomi K *et al.* Expression of cyclooxygenase-2 in human hepatocellular carcinoma: relevance to tumor dedifferentiation. Hepatology. 1999;29:688–96.
54. Sung YK, Hwang SY, Kim JO, Bae HI, Kim JC, Kim MK. The correlation between cyclooxygenase-2 expression and hepatocellular carcinogenesis. Mol Cells. 2004;17:35–8.
55. Hu KQ. Rationale and feasibility of chemoprevention of hepatocellular carcinoma by cyclooxygenase-2 inhibitors. J Lab Clin Med. 2002;139:234–43.
56. Koga H. Hepatocellular carcinoma: is there a potential for chemoprevention using cyclooxygenase-2 inhibitors? Cancer. 2003;98:661–7.
57. Ramakrishnan G, Elinos-Báez CM, Jagan S, Augustine TA, Kamaraj S, Anandakumar P *et al.* Silymarin downregulates COX-2 expression and attenuates hyperlipidemia during NDEA-induced rat hepatocellular carcinoma. Mol Cell Biochem. 2008;313:53–61.
58. Sivaramakrishnan V, Niranjali Devaraj S. Morin regulates the expression of NF-kappaB-p65, COX-2 and matrix metalloproteinases in diethylnitrosamine induced rat hepatocellular carcinoma. Chem-Biol Interact. 2009;180:353–9.
59. Bishayee A, Barnes KF, Bhatia D, Darvesh AS, Carroll RT. Resveratrol suppresses oxidative stress and inflammatory response in diethylnitrosamine-initiated rat hepatocarcinogenesis. Cancer Prev Res. 2010;in press.
60. Khanduja KL, Bhardwai A, Kaushik G. Resveratrol inhibits N-nitrosodiethylamine-induced ornithine decarboxylase and cyclooxygenase in mice. J Nutr Sci Vitaminol. 2004;50:61–5.
61. Tsujii M, DuBois RN. Alterations in cellular adhesion and apoptosis in epithelial cells overexpressing prostaglandin endoperoxide synthase 2. Cell. 1995;83:493–501.
62. Zycova TA, Zhu F, Zhai X, Ma W-Y, Ermakova SP, Lee KW *et al.* Resveratrol directly targets COX-2 to inhibit carcinogenesis. Mol Carcinog. 2008;47:797–805.
63. Cervello M, Montalto G. Cyclooxygenase in hepatocellular carcinoma. World J Gastroenterol. 2006;12:5113–21.
64. Luther DJ, Ohanyan V, Shamhart PE, Hodnichak CM, Sisakian H, Booth TD, *et al.* Chemopreventive doses of resveratrol do not affect cardiac function in a rodent model of hepatocellular carcinoma. Invest New Drugs. 2010; in press. doi: 10.1007/s10637-009-9332-7.
65. Karin M, Lin A. NF-kappaB at the crossroads of life and death. Nat Immunol. 2002;3:221–7.
66. Rahman MA, Dhar DK, Yamaguchi E, Maruyama S, Sato T, Hayashi H *et al.* Coexpression of inducible nitric oxide synthase and COX-2 in hepatocellular carcinoma and surrounding liver: possible involvement of COX-2 in the angiogenesis of hepatitis C virus-positive cases. Clin Cancer Res. 2001;7:1325–32.
67. Calvisi DF, Pinna F, Ladu S, Pellegrino R, Muroni MR, Simile MM *et al.* Aberrant iNOS signaling is under genetic control in rodent liver cancer and potentially prognostic for the human disease. Carcinogenesis. 2008;29:1639–47.
68. Tazawa R, Xu XM, Wu KK, Wang LH. Characterization of the genomic structure, chromosomal location and promoter of human prostaglandin H synthase-2 gene. Biochem Biophys Res Commun. 1994;203:190–9.
69. Xie QW, Kashiwabara Y, Nathan C. Role of transcription factor NF-B/Rel in induction of nitric oxide synthase. J Biol Chem. 1994;269:4705–8.
70. Ueno S, Aoki D, Kubo F, Hiwatashi K, Matsushita K, Oyama T *et al.* Roxithromyc inhibits constitutive activation of nuclear factor κB by diminishing oxidative stress in a rat model of hepatocellular carcinoma. Clin Cancer Res. 2005;11:5645–50.
71. Yang Z, Yang S, Misner BJ, Chiu R, Liu F, Meyskens FL. Nitric oxide initiates progression of human melanoma via a feedback loop mediated by apurinic/apyrimidinic endonuclease-1/redox factor-1, which is inhibited by resveratrol. Mol Cancer Ther. 2008;7:3751–60.
72. Kang O-H, Jang H-J, Chae H-S, Oh Y-C, Choi J-G, Lee Y-S *et al.* Anti-inflammatory mechanisms of resveratrol in activated HMC-1 cells: pivotal roles of NF-κB and MAPK. Pharmacol Res. 2009;59:330–7.
73. Cichocki M, Paluszczak J, Szaefer H, Piechowiak A, Rimando AM, Baer-Dubowska W. Pterostilbene is equally potent as resveratrol in inhibiting 12-*O*-tetradecanoylphorbol-13-acetate activated NFκB, AP-1, COX-2, and iNOS in mouse epidermis. Mol Nutr Food Res. 2008;52:S62–70.
74. Chávez E, Reyes-Gordillo K, Segovia J, Shibayama M, Tsutsumi V, Vergara P *et al.* Resveratrol prevents fibrosis, NF-κB activation and TGF-β increases by chronic CCl_4 treatment in rats. J Appl Toxicol. 2008;28:35–43.
75. Holmes-McNary M, Baldwin Jr AS. Chemopreventive properties of *trans*-resveratrol are associated with inhibition of activation of the IkappaB kinase. Cancer Res. 2000;60:3477–83.
76. Howitz KT, Bitterman KJ, Cohen HY, Lamming DW, Lavu S, Wood JG *et al.* Small molecule activators of sirtuins extend *Saccharomyces cerevisiae* lifespan. Nature. 2003;425:191–6.